THE ROLE OF MALONYL-CoA IN ISOPRENOID BIOSYNTHESIS

A Thesis submitted for

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- by -

MALCOLM JOHN PETER HIGGINS

Department of Biochemistry
The University
Edgbaston
Birmingham 15

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Synopsis

The observation that malonyl-CoA is involved in the synthesis of HMG-CoA by a pigeon liver fatty acid synthesising system, has led to uncertainty concerning the origin of the acetoacetyl intermediate for isoprenoid synthesis. A study was undertaken of the utilisation of malonyl-CoA for this purpose.

Initial experiments with crude preparations of rat liver, yeast and the latex of *Hevea brasiliensis* indicated that malonyl-CoA was incorporated into isoprenoid compounds, including mevalonic acid. However, when avidin was added to incubations, fatty acid synthesis from acetate or acetyl-CoA was inhibited while the incorporation into isoprenoids was not affected. This indicated that a biotin dependent carboxylation of acetyl-CoA is not involved in the synthesis of isoprenoid compounds, and that malonyl-CoA was decarboxylated before the incorporation of acetyl-CoA as such. Experiments in which the incorporation of [2-^{14}C] malonyl-CoA was compared to the incorporation of [1,3-^{14}C] malonyl-CoA did not resolve this question,

but degradation of HMG-CoA and Ergosterol indicated that the incorporation of radioactivity was that to be expected from acetyl-CoA. In accord with these observations, the malonyl-CoA decarboxylase activity could account for the incorporation of malonyl-CoA observed. When this was reduced by isolation conditions in which mitochondrial integrity was preserved, the incorporation into mevalonate was reduced.

An examination of the purification of the pigeon liver fatty acid synthetase showed that HMG-CoA synthesising activity did not copurify with fatty acid synthesising activity.

It was concluded that, in the systems studied, the acetoacetyl intermediate for isoprenoid synthesis arises by the condensation of two molecules of acetyl-CoA without the intervention of malonyl-CoA.

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CONTENTS

ABBREVIATIONS

Abbreviations used in this work are:

ACP	Acyl Carrier Protein
APL	Apeizon L grease
CoA, Acyl-Coa	Coenzyme and its acyl derivatives
DEAE-, CM-,	Diethylaminoethyl-, and Carboxymethyl-, cellulose derivatives.
FA	Fatty acid fraction
GLC	Gas Liquid Chromatography
HMG	β-hydroxy-β-methyl glutaric acid
MVA	Mevalonic acid
PEGA	Poly Ethylene Glycol Adipate
POPOP	1,4-di-2-(5-phenyl-oxasole) benzene
PPO	2,5-diphenyl oxazole
TLC	Thin Layer Chromatography
TAL	Tri-acetic acid lactone
US	Unsaponifiable fraction
TCA	Trichloracetic acid

Other abbreviations are as recommended in the Biochemical Journal.

INTRODUCTION

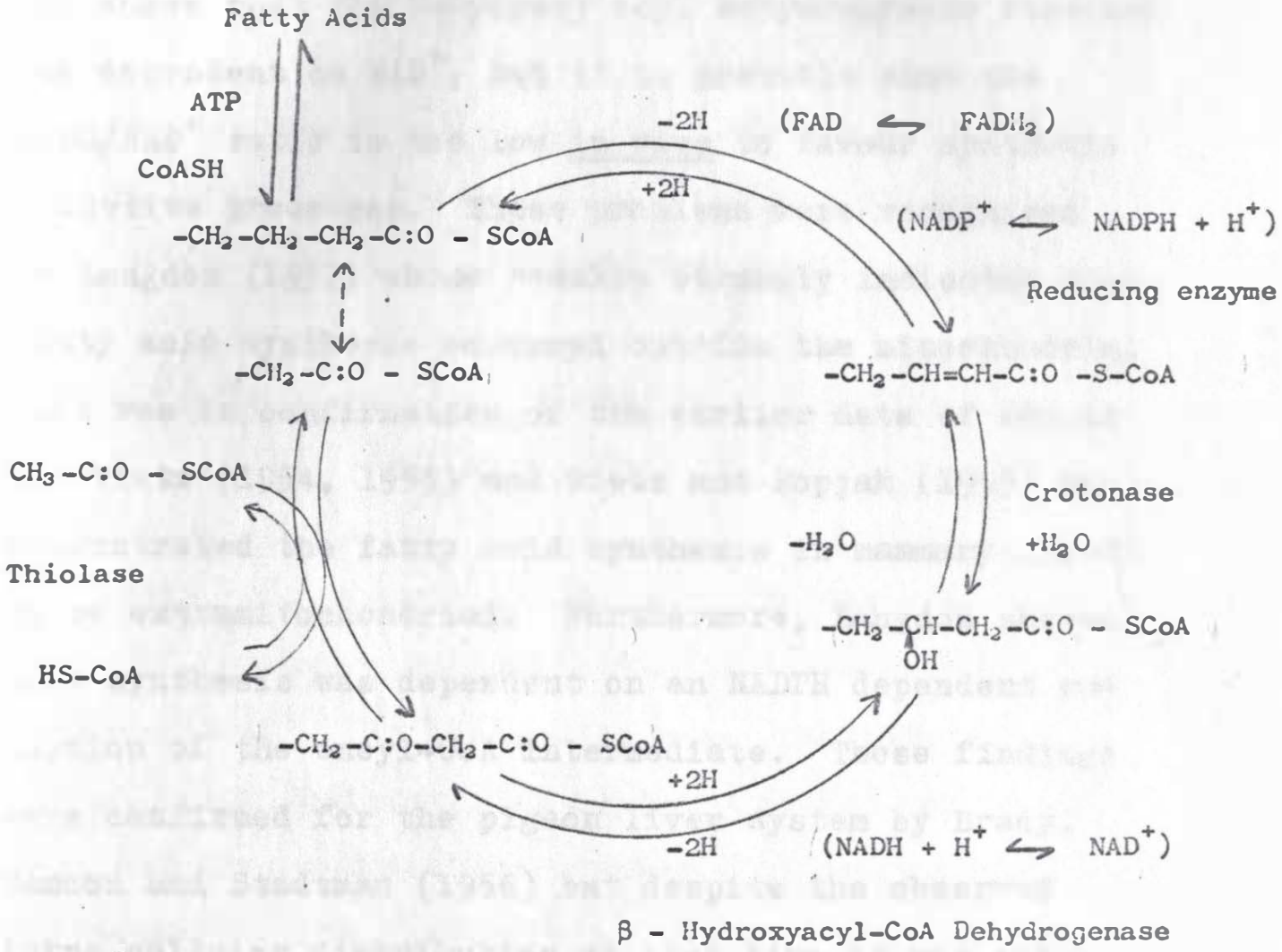
Some Early Problems in the Understanding of Fatty Acid Synthesis

Lynen and Ochoa (1953) showed that each enzyme on the β-oxidation pathway of fatty acid degradation was reversible, and the conclusion was drawn that the same sequence of reactions functioned in the synthetic direction (for reviews see Lynen, 1952-53 and Green, 1954). Lynen envisaged a fatty acid cycle such as shown in scheme I. It was also assumed that synthesis and degradation both occurred in the mitochondrion and the belief in this was strengthened by the results of Brady and Gurin, (1952), Van Baalen and Gurin, (1953) and Dituri and Gurin (1953), which indicated that an extract of lysed mitochondria, in addition to the soluble fraction of the cell, was necessary for fatty acid synthesis.

There were, however, several difficulties with such a mechanism. It was not possible to demonstrate conversion of acetyl-CoA to butyryl-CoA by recombination of the purified oxidative enzymes from mitochondria unless a leuco dye was added. This provided sufficient reducing power for the conversion of crotonyl-CoA to butyryl-

Scheme 1

THE FATTY ACID CYCLE AS ENVISAGED IN 1952 - 1953 (Lynen)



CoA (Seubert and Lynen, 1953; Stansly and Beinert, 1953). Secondly; Wakil, Green, Mii and Mahler (1954) had shown that the β-hydroxy acyl dehydrogenase reaction was dependent on NAD^+, but it is probable that the $NADH/NAD^+$ ratio is too low <u>in vivo</u> to favour synthetic reductive processes. These problems were recognised by Langdon (1957) whose results strongly indicated that fatty acid synthesis occurred outside the mitochondria. This was in confirmation of the earlier data of Popjak and Tietz (1954, 1955) and Tietz and Popjak (1955) who demonstrated the fatty acid synthesis in mammary gland to be extramitochondrial. Furthermore, Langdon showed that synthesis was dependent on an NADPH dependent reduction of the enoyl-CoA intermediate. These findings were confirmed for the pigeon liver system by Brady, Mamoon and Stadtman (1956) but despite the observed intra cellular distribution at that time it was still assumed that the synthesis of fatty acids was achieved by reversal of the β-oxidation enzymes. Seubert, Gruell and Lynen (1957) found that addition of the

NADPH dependent enoyl-CoA reductase to the reconstituted β-oxidation system promoted the synthesis of short chain fatty acids.

The initial reaction in this scheme of synthesis was thought to be the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA.

The Synthesis of Acetoacetyl-CoA by the Reversal of β-ketothiolase

Soodak and Lipmann (1948) were the first to observe the CoA dependent synthesis of acetoacetate from acetate and ATP. In 1951, Stadtman, Doodorff and Lipmann found that both the acetyl moieties had to be activated for acetoacetate synthesis to occur. With the discovery that the activated form of acetate was acetyl-CoA (Lynen and Reichert, 1951) the reaction was written as:

$$2\ CH_3 - C{:}O - S - CoA \rightarrow CH_3 - C{:}O - CH_2 - CO - S - CoA + CoASH$$

The equilibrium constant for this reaction was calculated by Lynen, Wessely, Wieland and Rueff (1952) and by Stern, Coon and Del Campillo (1953) as

$$K_{eq} = \frac{[\text{Acetoacetyl-CoA}][\text{CoASH}]}{[\text{Acetyl-CoA}]^2}$$

$$= 2 \times 10^{-5} \text{ at pH } 8.1$$

It is interesting that the apparent equilibrium constant varies with pH even though there is no H^+ term in the equation. This is due to the keto-enol tautomerism of the acetoacetic ester. Acetoacetyl-CoA has an apparent pK of 8.5 so in alkaline conditions enolisation alters the equilibrium position to favour carbon-carbon bond formation. At pH 6.0 the apparent equilibrium constant is 1.48×10^{-5} and at pH 9.0, 6.62×10^{-5} (Goldman, 1954; Lynen and Decker, 1957). However, these differences are probably not significant for even at an unphysiological alkaline pH the equilibrium of the reaction is very much in favour of the

thioclastic splitting of the acetoacetyl-CoA molecule.

Lynen and Ochoa (1953) were able to demonstrate the formation of acetoacetyl-CoA from acetyl-CoA when the reaction was coupled with an excess of the enzyme β-keto reductase - the enzyme that catalyses the subsequent reaction in the reversed β-oxidation cycle.

Although the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA is a reversible reaction, the equilibrium is very much in favour of reduction. The equilibrium constant of the reaction

$$K_{eq} = \frac{[\text{Acetoacetyl-CoA}]\,[\text{NADPH}]\,[H^+]}{[\text{L-}\beta\text{-hydroxybutyryl-CoA}]\,[\text{NAD}^+]}$$

was given as 6.3×10^{-11} by Wakil (1963) and 1.9×10^{-10} by Jaenicke and Lynen (1960). Acetoacetyl-CoA was therefore trapped as β-hydroxybutyryl-CoA and the thiolase reaction never allowed to reach equilibrium.

The Role of Malonyl-CoA in Fatty Acid Biosynthesis

It followed from these experiments that if synthesis

of acetoacetyl-CoA was to be significant, the product of the reaction had to be removed as soon as it was formed; and the concentration of acetyl-CoA had to be maintained at a high level.

The full cofactor requirements of the pigeon liver fatty acid synthesising system had been determined (Wakil, Porter and Gibson, 1957; Porter, Wakil, Tietz, Jacob and Gibson, 1957): and Gibson, Titchener and Wakil (1958) observed an absolute requirement for carbon dioxide, but there was no incorporation of carbon dioxide into fatty acids. Brady and Gurin (1950) had already observed the stimulatory effect of carbon dioxide on fatty acid synthesis by rat liver slices, and Klein (1957) had observed that carbon dioxide stimulated the incorporation of acetate into fatty acids by a yeast system, but was not itself incorporated. These observations were amplified by Wakil, Titchener and Gibson (1958). A pigeon liver enzyme system was resolved into two fractions each of which was necessary for fatty acid synthesis from acetate. One fraction was rich in biotin, and since the enzymic activity was abolished by avidin, there was good reason to presume that the biotin was involved in

the synthetic reaction.

All these observations led Lynen in 1958 to propose that acetyl-CoA was first carboxylated to malonyl-CoA; he considered that the subsequent decarboxylation occurring at the same time as the condensation with acetyl-CoA would provide a sufficient change in free energy to drive the sequence of reactions in the synthetic direction.

Lynen's proposal was shown to be fact, and the role of both carbon dioxide and avidin were explained when Wakil (1958) demonstrated that malonyl-CoA was a precursor of long chain fatty acids. This was soon confirmed by other reports: Brady, (1958); Wakil and Ganguly (1959); Formica and Brady, (1959). The biotin containing protein was shown to be acetyl-CoA carboxylase which catalyses the reaction:

$$CH_3\text{-}CO\text{-}S\text{-}CoA + ATP + CO_2 \rightarrow \underset{\displaystyle COOH}{\overset{\displaystyle CH_2}{|}} - C\text{-}O\text{-}S\text{-}CoA + ADP + Pi$$

The chemistry of the synthesis of fatty acids was worked out in Lynen's laboratory (1961) using the highly purified yeast enzyme and model substrates. The purification of this enzyme resulted in the isolation of a homogenous particle with a molecular weight of about 2.3×10^6. All the reactions involved in the condensation of successive two carbon units took place through intermediates that were bound to the enzyme. The first reactions involve the transfer of the acetyl and malonyl moieties to the protein:

Acetyl-CoA + Enzyme-H → Acetyl-Enzyme + CoASH

Malonyl-CoA + Enzyme-H → Malonyl-Enzyme + CoASH

followed by condensation to form enzyme bound acetoacetate:

Acetyl-Enzyme + Malonyl-Enzyme + H^+ → Acetoacetyl-Enzyme-H + CO_2

The equilibrium constant for the overall reaction:

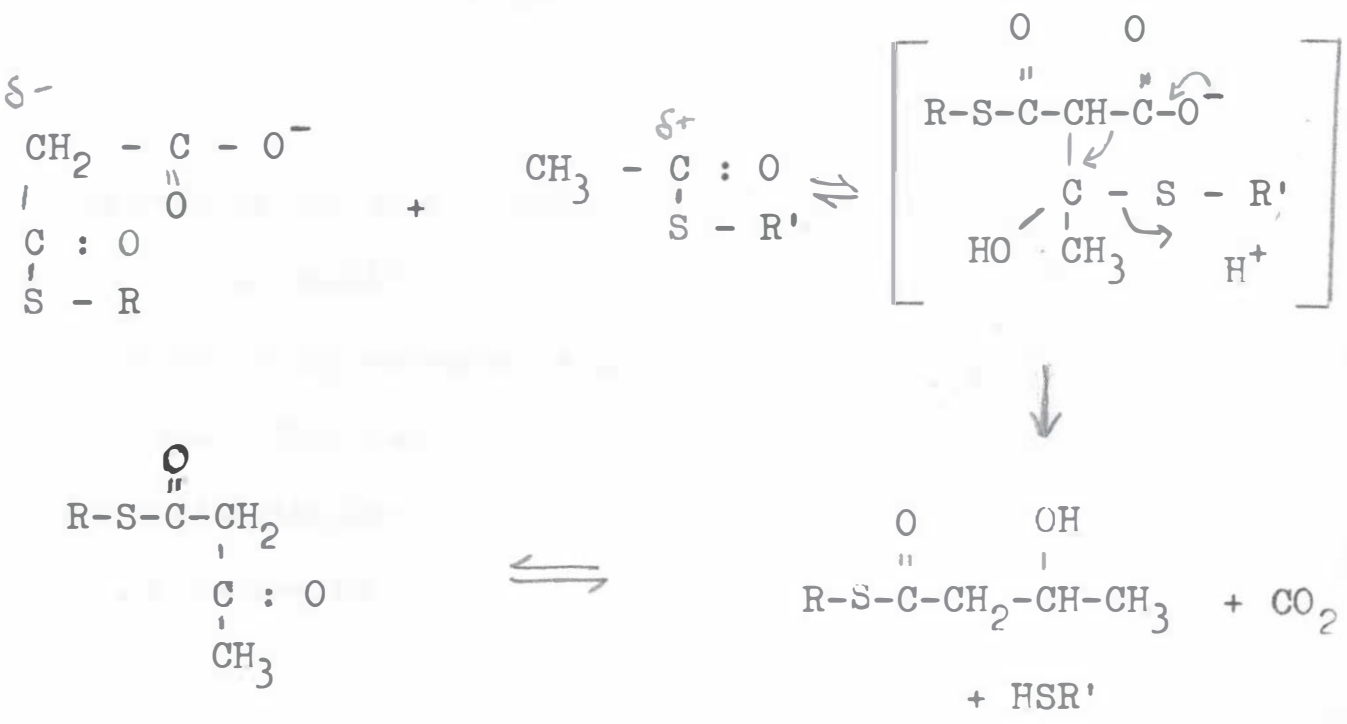
Acetyl-CoA + Malonyl-CoA + Enzyme + H^+ → Acetoacetyl-Enzyme-H + 2CoASH + CO_2

$$K_{eq} = \frac{[\text{Acetoacetyl-Enzyme}][\text{CoASH}]^2[CO_2]}{[\text{Acetyl-CoA}][\text{Malonyl-CoA}][\text{Enzyme}][H^+]}$$

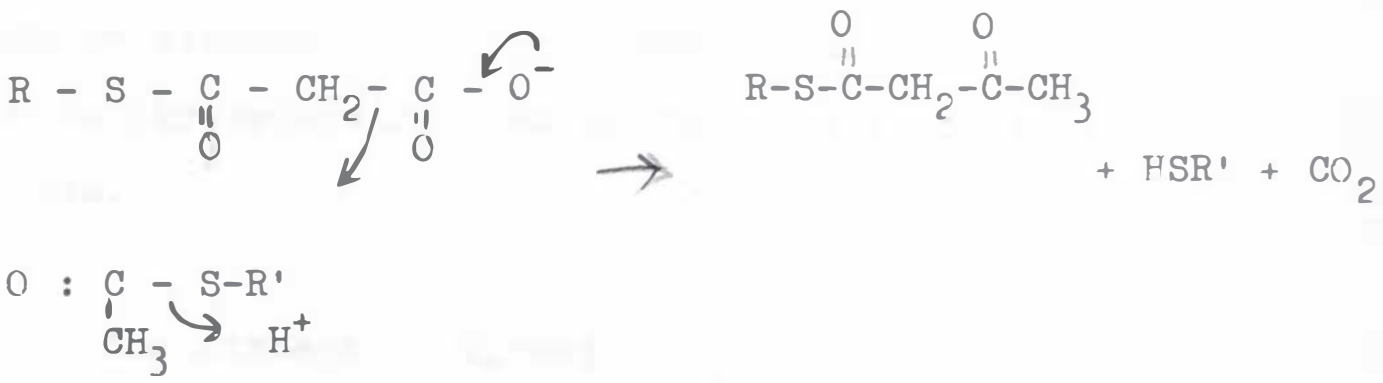
2×10^5 at $0^\circ C$ and pH 7.0 (Lynen, 1965)

When this value is compared with the equilibrium constant for the thiolase reaction of 1.56×10^{-5}, the thermodynamic advantage obtained by the decarboxylation is apparent. The energy, of course, was ultimately derived from the ATP used in the acetyl-CoA carboxylase reaction.

Lynen (1967) described the condensation of the malonyl-thioester with the acetyl-ester in chemical terms as an acylation of a malonic ester. The methylene group of the malonic ester is more nucleophilic than that of an acetyl thioester, and adds to the electrophilic carbonyl-carbon of the sulphur bound carboxylic acid.



An alternative concerted mechanism is:



The acetoacetyl derivative produced in this way

is converted to the β-hydroxy-butyryl enzyme with the oxidation of NADPH. This is followed by dehydration to give crotonyl-enzyme and then another NADPH linked reduction. The saturated butyryl enzyme complex condenses with another malonyl unit and the synthetic cycle is repeated.

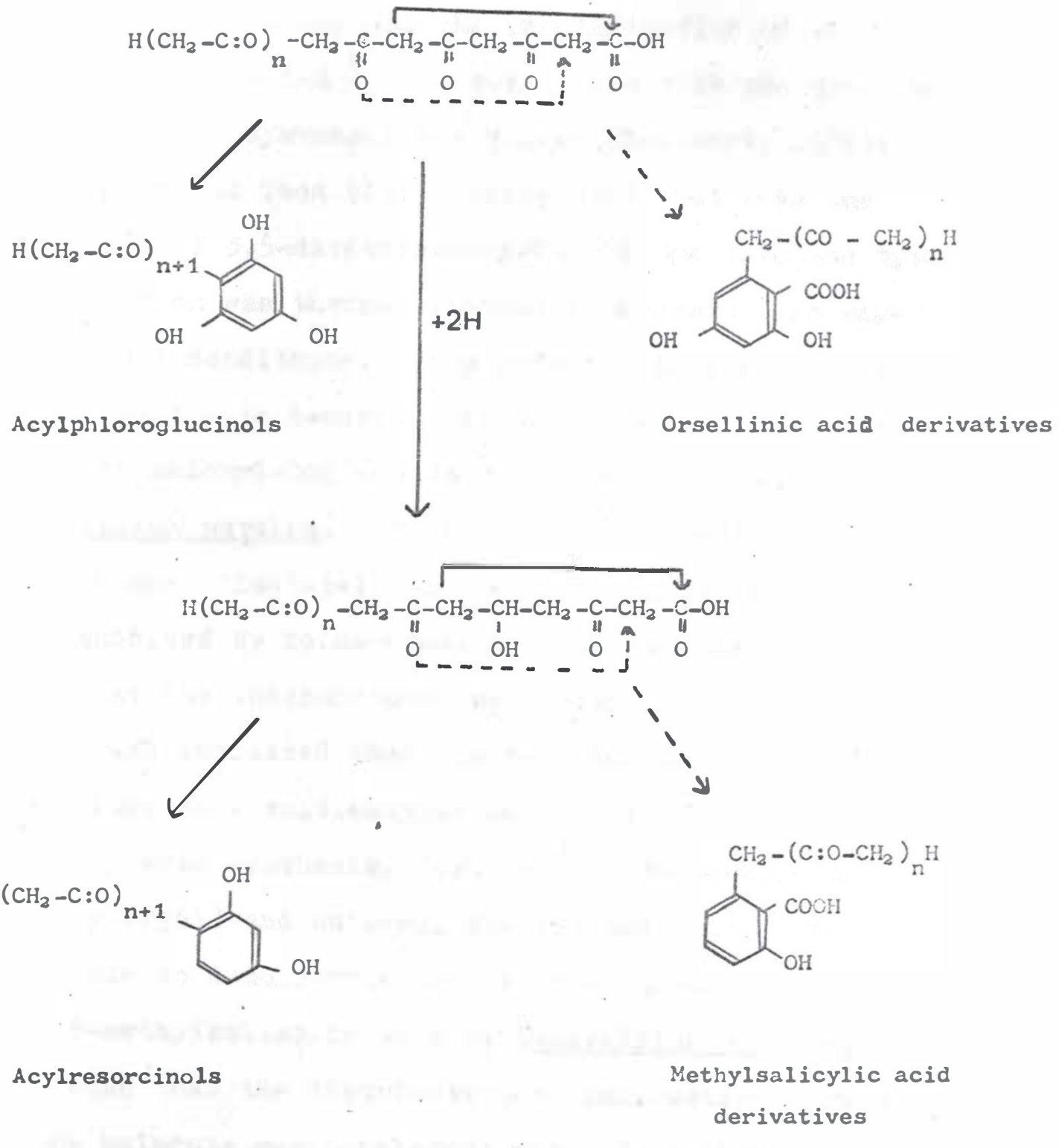
Biosynthesis of Aromatic polyketide compounds from Malonyl-CoA

Polyketide compounds are those whose structures can be derived from β-polyketone chains, thus fatty acids (by reduction) can be included in this biogenetic class.

The concept of direct head to tail condensation of acetate was proposed by Collie (1907) and was developed by Birch (1956). Based on extensive isotope incorporation studies he proposed schemes for the origin of aromatic polyketides, such pathways are shown in Scheme 2.

Scheme 2

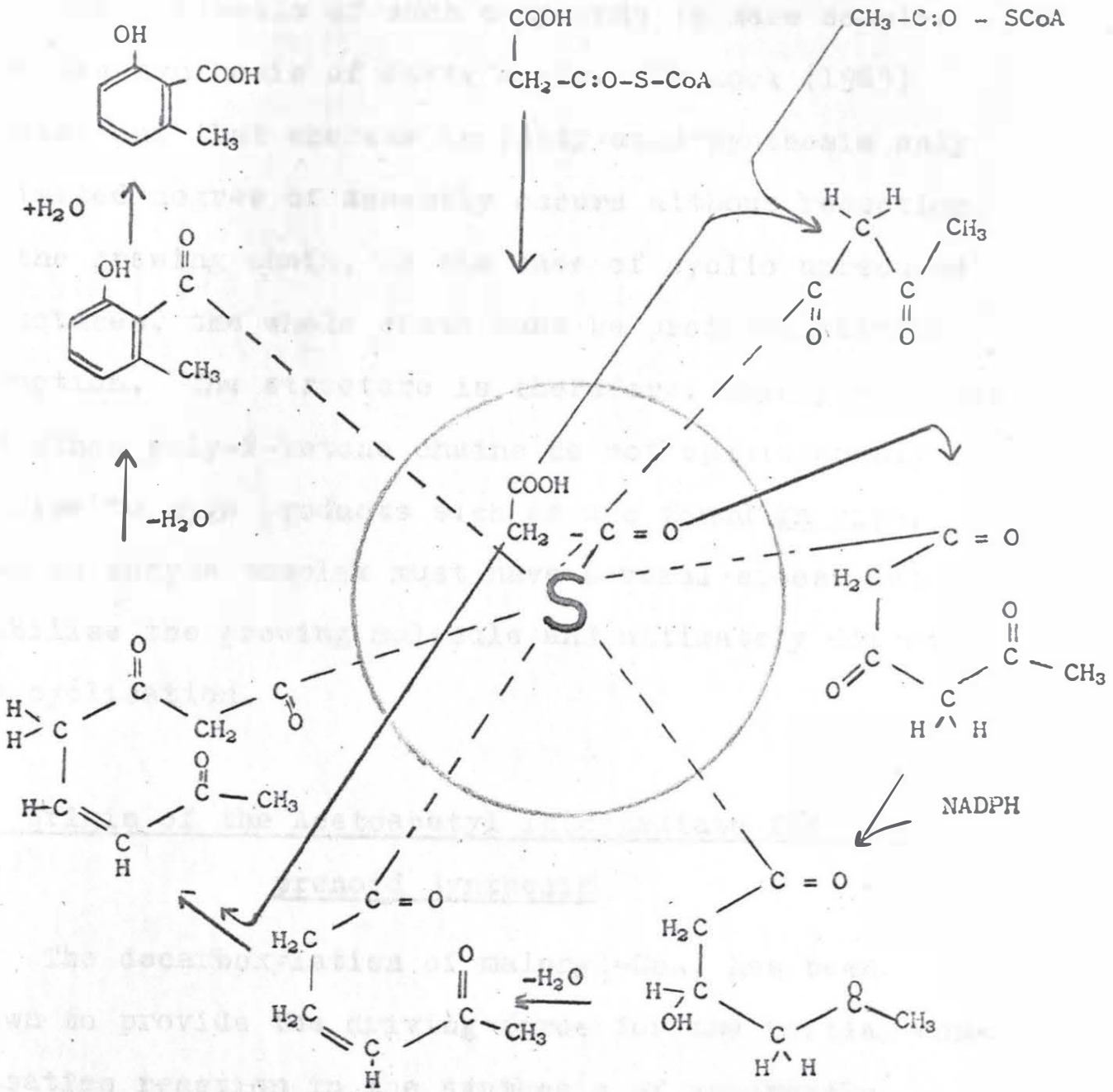
THE ORIGIN OF THE AROMATIC POLYKETIDES (After Birch, 1956)



As in the field of fatty acid synthesis, the discovery of acetyl-CoA and the identification of the thiolase enzyme led to the conclusion that the problem of polyketide synthesis was solved (Woodward, 1957); but, Lynen and Tada (1961) calculated that even the synthesis of 3,5-diketohexanoyl-CoA by the thiolase type condensation was thermodynamically impossible in physiological conditions. They reported that acetyl-CoA was converted to 6-methylsalicyclic acid in the presence of malonyl-CoA and NADPH, by soluble extracts of Penicillium patulum. It was found that cysteine and glutathione stimulated incorporation while synthesis was inhibited by iodoacetamide. This suggested to them that the intermediates were bound to sulphydryl groups and indicated that the reaction sequence might take place on a multienzymic complex in a similar way to fatty acid synthesis, (scheme 3). Bu'Lock, and Smalley (1961) and Bu'Lock, Smalley and Smith (1962) were able to demonstrate the incorporation of malonate into 6-methylsalicylic acid by Penicillium urticae, and found that the distribution of radioactive carbon in the molecule was consistent with the scheme proposed

Scheme 3

HYPOTHETICAL REACTION SCHEME OF 6-METHYLSALICYLIC ACID SYNTHESIS ON A MULTIENZYME COMPLEX (Lynen & Tada, 1961)



by Lynen and Tada.

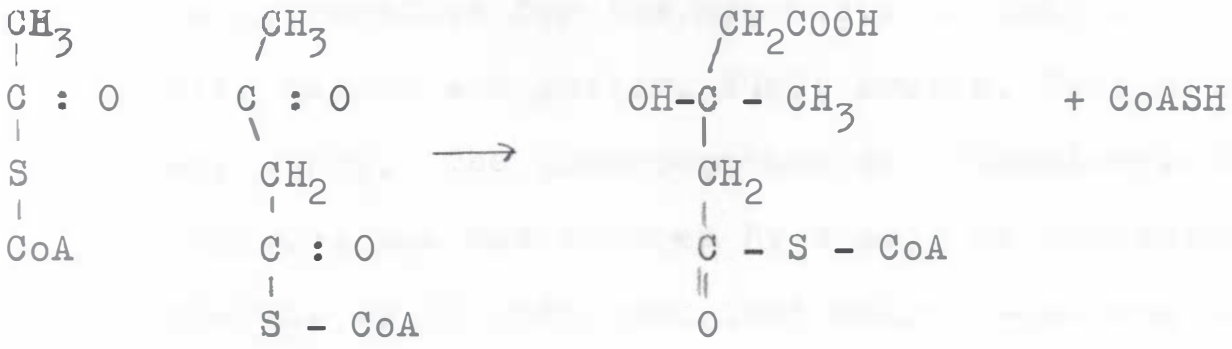
The synthesis of such compounds is more complex than the synthesis of fatty acids. Bu'Lock (1965) pointed out that whereas in fatty acid synthesis only a limited degree of assembly occurs without reduction of the growing chain, in the case of cyclic unreduced structures, the whole chain must be produced without reduction. The structure is therefore, highly reactive and since poly-β-ketone chains do not spontaneously cyclise to give products such as are found in vivo, such an enzyme complex must have several sites that stabilise the growing molecule and ultimately direct its cyclisation.

The Origin of the Acetoacetyl Intermediate for Isoprenoid Synthesis

The decarboxylation of malonyl-CoA, has been shown to provide the driving force for the initial condensation reaction in the synthesis of apparently quite different products.

Just as the condensation of two molecules of acetyl-CoA was thought to be the route for the formation of acetoacetyl-CoA in de novo long chain fatty acid synthesis, this reaction was thought to provide the acetoacetyl-CoA needed for the synthesis of isoprenoids.

Acetoacetyl-CoA condenses with acetyl-CoA in a reaction catalysed by the HMG-CoA condensing enzyme (E.C.4.1.3.5.) which was partially purified from yeast by Lynen, Henning, Bublitz, Sorbo and Kröplin-Rueff (1958) and by Rudney and Ferguson, (1959), Ferguson and Rudney (1959).



Acetyl-CoA β-hydroxy, β-methyl glutaryl CoA

Acetoacetyl-CoA

The formation of acetoacetyl-CoA and then HMG-CoA

is thermodynamically feasible because the condensation of acetoacetyl-CoA with acetyl-CoA to give HMG-CoA was shown not to be reversible (Rudney and Ferguson, 1959). However, a malonyl-CoA condensation as the initial reaction of isoprenoid synthesis would be a more favourable reaction for synthesis and an enzyme bound sequence of intermediates would confer the kinetic advantages enjoyed by fatty acid synthesising systems from animals and yeast.

Porter and his coworkers observed the malonyl-CoA dependent incorporation of acetyl-CoA into HMG-CoA and mevalonic acid by a pigeon liver system that had been partially purified for the synthesis of fatty acids (Brodie, Wasson and Porter, 1962; Brodie, Wasson and Porter, 1963). The incorporation of ^{14}C-malonyl-CoA into HMG-CoA was not reduced by a pool of unlabelled acetyl-CoA, which indicated that malonyl-CoA was not decarboxylated to acetyl-CoA before incorporation. Furthermore, the incorporation of ^{14}C-malonyl-CoA into mevalonic acid was not significantly affected by added acetoacetyl-CoA, HMG-CoA or mevaldic acid.

The new pathway of isoprenoid synthesis and its relationship to fatty acid synthesis is shown in scheme 4.

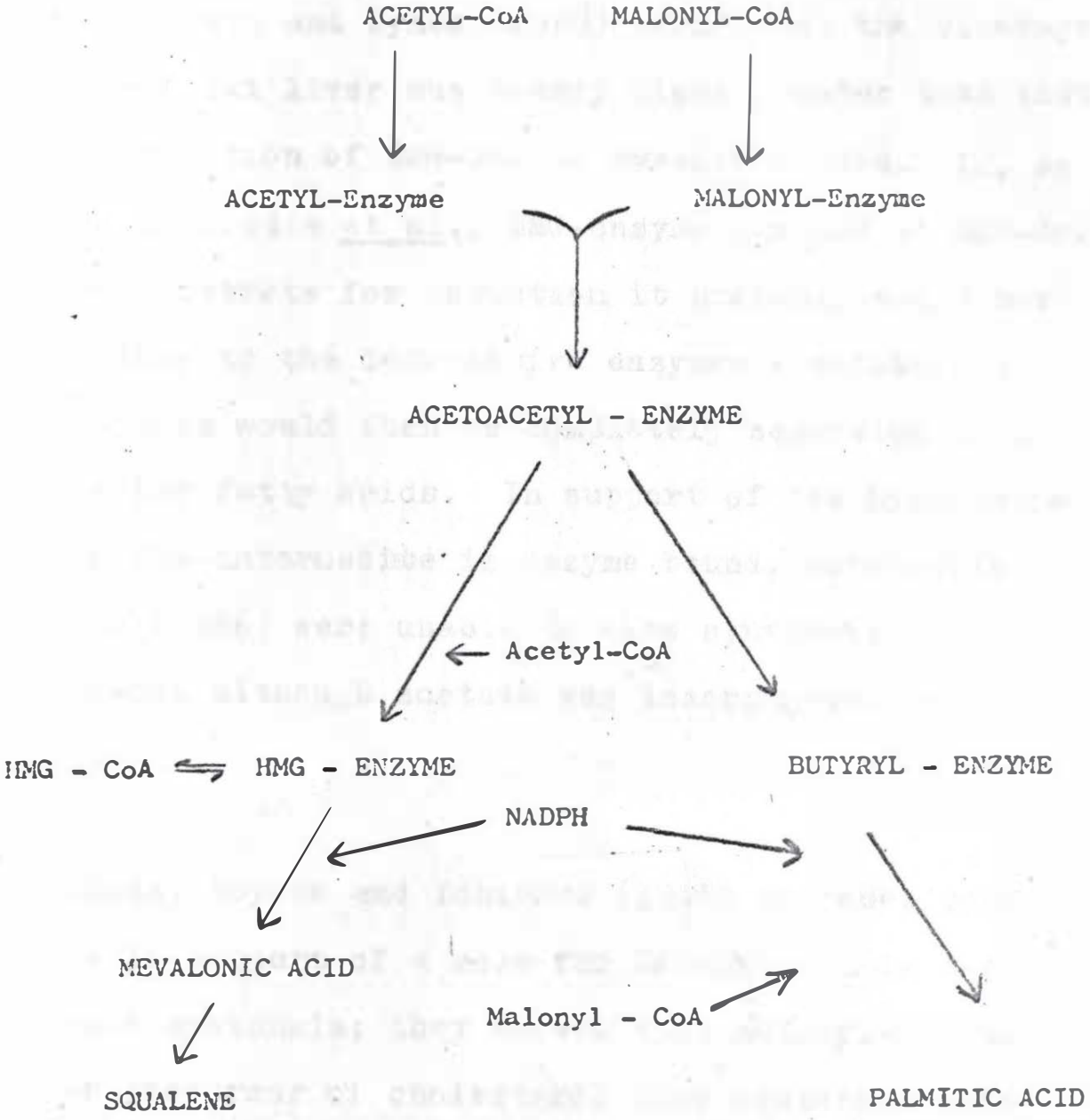
The role of an HMG-Enzyme intermediate was doubted by Stewart and Rudney (1966b) who showed that the thioester bond in HMG-CoA synthesised by a condensing enzyme system from yeast was derived from acetoacetyl-CoA. This did not, however, exclude the condensation of acetyl-CoA with an acetoacetyl-enzyme derivative. This is discussed in more detail in a later section.

Porter, Guchhast and Vadlamindi (1964) extended their hypothesis to the rat liver. They reported that the distribution of radioactivity in the unsaponifiable lipids extracted from incubations of a liver homogenate was similar whether the radioactivity was derived from malonyl-CoA or acetyl-CoA.

Observations that have supported this postulated pathway have emerged from other laboratories. Bachhawat, Robinson and Coon (1955) and Stegink and Coon (1968)

Scheme 4

THE RELATIONSHIP BETWEEN FATTY ACID AND ISOPRENOID SYNTHESIS AS ENVISAGED BY BRODIE, WASSON & PORTER (1963, 1964)



described enzymes that cleave HMG-CoA to acetyl-CoA and acetoacetate-CoA; Dekker, Schlesinger and Coon (1958) described an enzyme active in the deacylation of HMG-CoA. Bucher, Overath and Lynen (1960) found that the cleavage activity of rat liver was twenty times greater than that for the reduction of HMG-CoA to mevalonic acid. If, as proposed by Brodie et al., HMG-enzyme instead of HMG-CoA were the substrate for reduction it probably would not be available to the degradative enzymes - catabolism and anabolism would then be completely separated as is the case for fatty acids. In support of the hypothesis that the HMG-intermediate is enzyme bound, Siperstein and Fagan (1966) were unable to show synthesis of MVA from HMG-CoA although acetate was incorporated satisfactorily.

Takeda, Koyama and Ichihara (1965) provided more evidence in support of a role for malonyl-CoA in mevalonic acid synthesis; they showed that malonyl-CoA was a better precursor of cholesterol than acetate in dispersed rat liver cells. The results from this investigation were interpreted as showing that both the "classi-

cal" and the malonyl-CoA pathways were operative, but the former only assumes importance in conditions where the carboxylation of acetyl-CoA is disturbed (e.g. starvation, or diabetes).

Foster and Bloom (1963) reporting a reciprocal relationship between fatty acid synthesis and cholesterol synthesis in rat liver, observed that the synthesis from acetate of both classes was inhibited by avidin. On the other hand, although fatty acid synthesis was stimulated up to 60 fold by citrate (presumably by activating acetyl-CoA carboxylase) cholesterol synthesis was reduced by a similar amount. Other results obtained from studies of the relationship between sterol and fatty acid synthesis have indicated that the reciprocal relationship was dependent on the activity of acetyl-CoA carboxylase. Sterol synthesis from acetate was depressed while fatty acid synthesis was stimulated when yeast preparations were incubated in the presence of carbon dioxide (Klein, 1957); when biotin deficient mutant yeast was incubated with biotin (Bloomfield and Bloch, 1960) and when a Lactobacillus culture was incubated

with biotin (Thorne and Kodicek, 1962)

Jacobsohn and Corley (1957) studied the formation of cholesterol in biotin deficient rats and showed that the incorporation of [2-^{14}C]acetate into cholesterol was increased while the incorporation of [1-^{14}C]dimethylacrylate was decreased with respect to normal animals. The latter observation provided a useful control to the experiment, since the conversion of ββ - di-methyl-acrylate (as the CoA ester) requires a biotin dependent carboxylation. In yet another investigation using avidin and biotin, Abraham, Matthes and Chaikoff (1961) considered the question of the necessity of the intermediary formation of malonyl-CoA for the condensation of acetyl-CoA with acyl-CoA derivatives. Fatty acid synthesis was completely inhibited by the addition of avidin to homogenate preparations of both mammary gland and liver of the rat, while in the liver, acetoacetate production was significantly increased. Acetoacetate production in the mammary gland was not affected. It was concluded that the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA does not require a

carboxylation reaction. Fletcher and Myant (1961) arrived at the same conclusion when they found that cholesterol synthesis in rat liver was unaffected by avidin.

Bu'Lock, Smalley and Smith attacked the problem in a different way. They cultured P. urticae in media containing [2-^{14}C] diethyl malonate and followed the incorporation of label into fatty acids, 6-methyl salicylic acid and ergosterol. The partial degradation of 6-methyl salicyclic and fatty acids indicated that they were synthesised in the typical fatty acid pattern, i.e. successive condensations of two-carbon units from malonyl-CoA with a priming two-carbon unit from acetyl-CoA. The rate of labelling of ergosterol, however, was similar to the rate of incorporation of label into the priming acetyl of the other compounds. The distribution of radioactive carbon in ergosterol was that to be expected from the use of acetyl-CoA as such.

The evidence for the postulated malonyl-CoA path-

way in tissues other than pigeon liver is not, therefore, conclusive. Consequently direct evidence for the involvement of malonyl-CoA in isoprenoid synthesis in rat liver was sought by Fimognari (1965) and Fimognari and Rodwell (1964). They found that both acetyl-CoA and malonyl-CoA were good precursors of mevalonate in a rat liver homogenate, but malonyl-CoA was not significantly incorporated by a 14,000g supernatant. Furthermore, bicarbonate stimulated the synthesis of fatty acids from acetyl-CoA but it did not stimulate the incorporation of this substrate into mevalonate. These workers concluded that in rat liver both the classical and the malonyl-CoA pathways operate, but that the former pathway was of major quantitative importance.

The Biosynthesis of Mevalonic Acid from Acetoacetyl-CoA

The formation of mevalonic acid from HMG-CoA was demonstrated by Ferguson, Durr and Rudney (1959) and the reaction was shown to be catalysed by the NADPH dependent enzyme, HMG-CoA reductase; (Knappe, Ringelmann and Lynen, 1959). HMG-CoA itself is formed by the con-

densation of acetoacetyl-CoA with another molecule of acetyl-CoA. The enzyme that accomplishes this condensation was partially purified from yeast (Lynen, Hemming, Bublitz, Sørbo, and Kröplin-Rueff, 1958; Ferguson and Rudney, 1959; Rudney and Ferguson, 1959) and identified in rat liver microsomes (Rudney and Ferguson, 1957; Ferguson and Rudney, 1959). Detailed mechanistic studies of this reaction have been hindered by the contamination of the condensing enzyme preparations with β-ketothiolase activity: Stewart and Rudney (1966a) were unable to separate β-keto thiolase from condensing enzyme by physical techniques and discussed the possibility that both activities may lie in the same enzyme protein. Despite this contamination, it was possible to study the HMG-CoA condensing enzyme activity in isolation by treating the preparation with iodoacetamide, trypsin or chymotrypsin which preferentially inactivated the β-keto thiolase activity. It has been shown subsequently that the enzymes do have separate identities (Rudney, personnal communication), and Middleton (1967) has reported the preparation of an HMG-CoA condensing enzyme from yeast that was free of β-keto thiolase and acetoacetyl -CoA deacylase

activity. The results from his studies were in agreement with those of Stewart and Rudney (1966a) in that an acetyl-enzyme derivative could be isolated and that ~~acetoacetyl-CoA but not derivative could be isolated and that~~ acetoacetyl-CoA but not acetyl-CoA is inhibitory at high concentrations. The hydrolysis of the acetyl-enzyme complex accelerated in the presence of acetyl-CoA or other CoA derivative. Middleton considered that this phenomenon might be due to a conformational change in the molecule and be a model of the final step of the reaction sequence:-

i) Acetyl-CoA + Enzyme ⟶ Acetyl-Enzyme + CoASH

ii) Acetyl-Enzyme + Acetoacetyl-CoA ⟶ Enzyme-HMG-CoA

iii) Enzyme-HMG-CoA + H_2O ⟶ Enzyme + HMG-CoA

The demonstration that the thioester bond in HMG-CoA was derived from acetoacetyl-CoA (Stewart and Rudney, 1966b), referred to earlier, is of considerable importance in the light of the alternative pathway of isoprenoid synthesis postulated by Brodie et al. (1964)

When yeast HMG-CoA condensing enzyme was incubated with acetoacetyl-CoA and acetyl-$[^{14}C]$ CoA no radioactivity was detected in HMG-CoA. On the other hand, when the substrates were acetyl-CoA and acetoacetyl- $[^{3}H]$ CoA the HMG-CoA retained the tritium label. This could not have been the case if all the reactants were enzyme bound and HMG-CoA was formed as the last stage of the sequence:-

Acetyl-CoA + Enzyme $\longrightarrow$ Acetyl-Enzyme + CoASH

Acetyl-Enzyme + Acetoacetyl-Enzyme $\longrightarrow$ HMG-Enzyme

HMG-Enzyme + CoASH $\longrightarrow$ HMG-CoA + Enzyme - SH

However, Rudney, Stewart, Majerus and Vagelos (1966) showed that when acetoacetyl-Acyl Carrier Protein (ACP) was substituted for acetoacetyl-CoA, protein (ACP) bound HMG was formed as the product of the condensation reaction. Here HMG-ACP was analogous to the enzyme bound HMG proposed by Brodie et al. That acetoacetyl-ACP was not such a good substrate as acetoacetyl-CoA in the yeast system might be explained by the fact that the ACP used was isolated from Escherichia coli.

Perhaps the most important property of this enzyme in the context of synthesis is its irreversibility. Rudney and Ferguson (1959) incubated HMG-CoA with citrate condensing enzyme, HMG-CoA condensing enzyme preparation, malate, malate dehydrogenase, NAD^+ and CoASH. Any acetyl-CoA formed by the reverse of the condensing enzyme reaction would have been trapped as citrate and the oxidation of malate in these circumstances would have led to a reduction of NAD^+. This did not occur. The irreversibility of this reaction is in contrast to the β-hydroxy-butyryl-CoA dehydrogenase reaction (the next stage in the synthesis of fatty acids, by the β-oxidation reversal mechanism). This was demonstrated by Rudney and Ferguson (1959) who incubated crystalline β-hydroxy-butyryl-CoA dehydrogenase with a system containing HMG-CoA, HMG-CoA condensing enzyme, reduced CoA and NADH. Any acetoacetyl-CoA formed by the reversal of the condensing enzyme reaction would have been reduced to β-hydroxybutyryl-CoA and NADH would have been oxidised. In fact, the reverse occurred. On the addition of acetyl-CoA, β-hydroxybutyryl-CoA and NAD^+, NAD^+ was reduced.

The reduction of HMG-CoA to mevalonic acid is catalysed by HMG-CoA reductase (E.C.1.1.1.34), a reaction that was also shown to be irreversible. (Durr and Rudney, 1960; Knappe et al., 1959). The enzyme has been partially purified from yeast (Kirtley and Rudney, 1967) but the status of the enzyme in animal systems has been in some doubt. In 1960 Bucher, Overath and Lynen demonstrated the reduction of HMG-CoA by a microsomal preparation from rat liver, but until Linn (1967a and 1967b) substantiated this observation and succeeded in solubilising the enzyme from rat liver microsomes there was not another report of an investigation into HMG-CoA reductase from an animal source. Indeed, the significance of the reduction of HMG-CoA as such had been doubted by Brodie et al. Siperstein and Fagan (1966) were not able to demonstrate mevalonic acid synthesis from HMG-CoA in a rat liver system although acetate was an active precursor.

There is now strong evidence that the activity of HMG-CoA reductase is an important control on the synthesis of cholesterol. The feeding of cholesterol to rats

strongly depresses the rate of synthesis of this sterol in liver (Gould, 1951; Tomkins, Sheppard and Chaikoff, 1953; Langdon and Bloch, 1953a; Frantz, Schneider and Hinkelman, 1954). Siperstein and Guest (1960) showed that the convertion of squalene to cholesterol was not effected by cholesterol feeding and similarly the synthesis of squalene from mevalonic acid was not affected. It was therefore concluded that the site of control was prior to the formation of mevalonic acid. The same conclusion was reached by Gould and Popjak (1957).

HMG-CoA is probably a major source of the "ketone bodies" particularly acetoacetic acid (Caldwell and Drummond, 1963). Siperstein and Guest observed that cholesterol feeding had no effect on the formation of acetoacetate or β-hydroxybutyrate which led them to the tentative conclusion that the negative feedback reaction controlling cholesterol synthesis was at the HMG-CoA reductase stage. Direct measurement of the formation of HMG-CoA and mevalonate confirmed this conclusion (Siperstein and Fagan, 1964).

In 1962 Siperstein and Fagan introduced a gas - liquid chromatographic assay for mevalonic acid. This has led to many more studies of the formation of mevalonate from HMG-CoA in liver systems. Linn (1967b) demonstrated a quantitative correlation between the microsomal conversion of HMG-CoA to mevalonate, the synthesis of cholesterol and the nutritional state of the animal.

Bortz (1968) demonstrated a noradrenalin induced increase in hepatic cholesterol synthesis and the blocking of this increase by puromycin. Since noradrenaline did not affect the formation of ketone bodies, it was suggested that the effect was on HMG-CoA reductase; furthermore, the effect of puromycin coupled with a lag period before the noradrenaline took effect led to the conclusion that the increase in activity was due to the synthesis of new protein. Similar observations were made by Kandutsh and Saucier (1969) and Guder, Nolte and Wieland (1968). The former showed that the increase in the rate of sterol synthesis in mice injected with triton was compatible with an increase in HMG-CoA reductase activity, which could be prevented by puromycin.

Guder et al. found that thyroidectomy lowered cholesterol synthesis in rat liver by 50% Injection of 3'3'5'-triiodothyronine into the hypothyroid rats brought the activity up to the range of normal animals in 30 hours. The authors proposed the analogy between this effect, ascribed to the induction of HMG-CoA reductase, and the induction by similar means of another microsomal enzyme - NADPH cytochrome C reductase (Tata, 1967).

Mevalonic acid is produced, therefore, by the sequential condensation of two molecules of acetyl-CoA with acetoacetyl CoA. The origin, and nature of the acetoacetyl intermediate, however, is in doubt.

The purification of HMG-CoA reductase from an animal source should solve many of the problems of cholesterol synthesis.

Summary and Aims of the Work

It has been demonstrated that fatty acid synthesis and the synthesis of other polyketide derived compounds utilise the favourable equilibrium of the malonyl-acetyl

condensation.

Isoprenoid synthesis also requires an initial condensation to form an acetoacetyl intermediate, but in this case the initial condensation is followed by two additions of two-carbon units catalysed by reactions which have been shown to be irreversible.

Malonyl-CoA has been implicated in the biosynthesis of isoprenoid compounds in a pigeon liver system that had been purified for the synthesis of fatty acids. Other evidence for this pathway is largely circumstantial and much of it is conflicting.

The object of this work at the outset was:

a) To determine whether malonyl-CoA *per se* is a precursor of the acetoacetyl unit used for isoprenoid synthesis in different systems.

b) If this pathway were operative, to determine the relative importance of this mechanism to the previously

accepted pathway of the direct condensation of acetyl-CoA molecule with acetyl-CoA molecule, and to investigate the nature of the protein bound intermediates involved.

EXPERIMENTAL

I METHODS

A General Methods

Materials

Radioactive Materials

All radioactive compounds were obtained from the Radiochemical Centre, Amersham, Bucks.

Reagent Enzymes

Malate dehydrogenase (E.C.4.1.3.7.) sp.act. 120μmole/min/mg.protein and citrate synthase (E.C.4.1.3.7.) sp. act. 170μmole/min./mg.protein were obtained from Sigma (London) Ltd.

Freeze dried serum of Hevea brasiliensis

Samples of freeze dried latex serum were obtained from the Natural Rubber producers Research Association, Welwyn Garden City, and had been prepared at the Rubber Research Institute of Malaya, Kuala Lumpur according to the method of Archer and Sekhar (1955).

Other Biochemicals

ATP	Sigma (London) Ltd.
Avidin	Nutritional Biochemicals Corp.

	Cleveland, Ohio, U.S.A.
Biotin	Sigma (London) Ltd.
Coenzyme A	Sigma (London) Ltd. C.F. Boehringer und Söhne, GmbH. or prepared as described in the text.
Di (5-carboxy-4-nitrophenyl) disulphide	Aldwich Chemical Co.
Diketene	Sigma (London) Ltd.
a,p-dibromoacetophenone	R. N. Emanuel Ltd. Alperton, Middlesex.
Fatty acids for G.L.C. standards	Sigma (London) Ltd.
β-hydroxy-β-methyl glutaric acid	Koch-Light Ltd, Colnbrook, Bucks.
Hyamine hydroxide (1M, in methanol)	Nuclear Enterprises Ltd, Sighthill, Edinburgh.
Ion-Exchange cellulose materials	Prepared by W.R. Balston, Ltd. Obtained from H. Reeve Angel and Co. Ltd.
N.-Methyl-p-toluene sulphonyl nitrosamide	May and Baker Ltd.

POP (2,5,diphenyl oxazole)	Nuclear Enterprises Ltd. Sighthill, Edinburgh.
POPOP (1,4-di-2-(5-phenyl-oxazole) benzene)	
Sephadex materials	Pharmacia Ltd. London
Silica Gel G	Prepared by E. Merck AG, Darmstadt. Supplied by Anderman and Co. Ltd. London.

Other chemicals were obtained from Fisons Ltd., Loughborough, Hopkin and Williams Ltd. B.D.H. Ltd. and were A.R. grade unless otherwise stated.

1 The Measurement of Radioactivity

a) Liquid Scintillation Counting

The scintillator solutions used were:- Toluene-phosphor: 3.0g./l.P.P.O. (2,5-diphenyl oxazole) and 0.3g./l. P.O.P.O.P. (1,4-di-2-(5-phenyl-oxazole)-benzene) in A.R. toluene. Bray's scintillant (1960): Naphthalene (60g.), P.P.O. (4.0g.), P.O.P.O.P. (0.2g.), 100ml. methanol and 1,4-dioxane to give a volume of 1 l.

Counting equipment was a Nuclear Chicago model 720 or Unilux II liquid scintillation counter. Individual samples were often counted using single photomultiplier tube Panax equipment.

i) Samples soluble in light petroleum

Radioactive samples dissolved in 5 ml. light petroleum were added to 5ml. toluene phosphor in a scintillation vial. The count rate was determined to within 2% statistical variation, (Wang and Willis, 1965a).

ii) Aqueous samples

a) Up to 1ml. of a radioactive solution was added to

10ml. of a scintillant consisting of toluene-phosphor: Triton X-100, (2 : 1 v/v); (Patterson and Green, 1965). Occasionally the solution to be assayed contained buffer salts which precipitated in the toluene solution. Such precipitates could usually be dissolved by shaking after the addition of single drops of water.

b) Alternatively, the toluene phosphor was replaced by Bray's scintillant which is capable of absorbing large amounts of water. In general, the efficiencies obtained using the Triton method were more reproducible.

iii) Radioactivity on thin layer chromatograms

The distribution of radioactivity on silica gel-G thin layer chromatograms was determined by one of two methods.

a) Direct Counting: The band of silica gel containing the radioactive components was scraped from the glass plate and introduced directly into the scintillation vial. 5.0ml. of light petroleum was added, followed by 5.0ml. of phosphor toluene. In this way a histogram of the radioactive distribution could be obtained. If the components were to be recovered or if the adsorbent

contained Rhodamine-6G dye, the following procedure was used.

b) Prior elution: Small columns (approximately 1cm. x 10cm.) were prepared containing about 1.0cm. dry silica gel retained by a glass wool plug. Silica gel was scraped from the thin layer plate, added to a column, and eluted by the careful addition of an appropriate solvent. A fraction of the eluate was evaporated to dryness in a scintillation vial. The thin film on the walls of the vial could be dissolved in the light petroleum-phosphor toluene mixture.

This procedure facilitated the radioactive assay of a known proportion of an isolated component and the retention of the remainder, samples that had been chromatographed on plates containing Rhodamine as a fluorescent indicator could be recovered uncontaminated by rhodamine when chloroform or dichloromethane was used as the eluting solvent.

iv) Paper chromatograms

Radioactivity on paper chromatograms was determined

without elution by directly inserting strips of paper into the scintillation vial. (Wang and Jones, 1959; Loftfield and Eigner, 1960)

v) ^{14}C-carbon dioxide

^{14}C-carbon dioxide was collected in 0.2ml. of 1.0M-hyamine hydroxide in methanol in the centre well of Warburg vessels, (Snyder and Godfrey, 1961)., to terminate the incubation. Strong acid (0.2ml. of 60% perchloric, or 6N sulphuric acid) was added to the main compartment, by tipping from a side arm or from a syringe through a tightly fitting rubber serum cap. After agitating gently to mix the contents, the flask was left at room temperature for two hours. The hyamine hydroxide, containing hyamine carbonate, was quantitatively transferred to a scintillation vial with the aid of 5ml. of toluene phosphor. Although significant fluorescence quenching has been observed when hyamine hydroxide was used in a liquid scintillant (Wang and Willis, 1965b) this effect was not observed here. The batches of hyamine hydroxide used gave almost quantitative recoveries of $^{14}CO_2$ from ^{14}C-$NaHCO_3$.

Table 1

THE TRAPPING OF $^{14}CO_2$ BY HYAMINE HYDROXIDE IN WARBURG VESSELS

Carbon dioxide was absorbed by 0.2ml. 1.0M-hyamine hydroxide in methanol in the centre well of Warburg flasks.

a) 50 mμmole of ^{14}C-$NaHCO_3$

Collection time (min.)	15	30	45	60	90	120
% recovery	12.5	49.7	69.5	84.2	96.5	98.2

b) 120 min. collection time

$NaHCO_3$ (mμmole)	5	10	20	50	100	250
% recovery	62.5	87.1	92.5	98.2	97	73

The results are the mean of 5 determinations. Total volume 1.0ml.

Determination of Efficiency

i) Single Phase Systems

The channels ratio method was used with the automatic liquid scintillation systems. Samples of known radioactivity (^{14}C-hexadecane) were washed into scintiallation vials. Quencher (chloroform), or light petroleum was added in increasing amounts to each vial. The count rate and the channels ratio (B/C) for each sample were determined. A curve for each machine was plotted of the channels ratio against counting efficiency. Counting efficiencies determined by the channels ratio method were periodically checked by the use of an internal standard.

The internal standard method of determining the counting efficiency was used when determinations were made with the single channel Panax equipment. After determining the count rate of the sample, an approximately equal known amount of radioactivity in the form of ^{14}C-hexadecane was added to the vial from an "Agla" micrometer syringe (Burroughs-Wellcome Ltd).

The total counting rate was obtained, and the degree of quenching thereby determined.

ii) Two Phase Systems

Paper strips inserted in vials. A standard curve was constructed when a quantitative assessment of a radioactive paper chromatogram was required. Aliquots of known radioactivity, if possible of the compound of interest (e.g. mevalonate) were dried on strips of chromatography paper, which was subsequently counted in a scintillation vial. The efficiency of counting by this method was usually 55-60%.

b) Gas Flow Counting

Gas flow counting was used to determine the position of radioactive peaks in some column eluates. This technique was later replaced by the use of Triton X-100 in the liquid scintillant.

Aliquots of column eluate fractions were transferred to aluminium planchets, dried and the count rate determined with a Nuclear Chicago gas flow detector

(D47), fitted with a micromil window, linked with a Nuclear Chicago automatic sample changer (186a). The samples were assumed to be infinitely thin and so the count rates obtained were directly comparable. The efficiency of the method was approximately 20%.

c) End Window Geiger Counting

End window geiger tubes were used to assay the radioactivity of some rubber samples. The sample, dissolved in chloroform, was applied to a 2cm. aluminium planchet which had been fitted with a lens tissue to ensure even deposition of the film of rubber. The chloroform was allowed to evaporate and the weighed planchets were counted beneath a Mullard geiger tube mounted in a Panax universal lead castle.

The count rate for each sample was graphically corrected to the count rate at infinite thickness (Yankwich, Norris and Huston, 1947). The specific activity of the sample was then calculated by a comparison of the count rate at infinite thickness with

the count rate of an infinitely thick standard source. (^{14}C Perspex, CFP 2, The Radiochemical Centre)

2 Estimation of Protein

a) Biuret Method

The biuret method was the procedure used routinely to estimate protein concentration. The reagent, which was prepared according to Weichselbaum (1947), consisted of 45g.-sodium potassium tartrate, 15g.-cupric sulphate ($5H_2^O$), and 5g. potassium iodide. The tartrate was dissolved in about 400ml. of 0.2N-sodium hydroxide. The sulphate was added and completely dissolved when the potassium iodide was dissolved in this solution. The solution was made up to 500ml. with 0.2N-sodium hydroxide. Before use the concentrated reagent was diluted with an equal volume of 0.2N-sodium hydroxide.

Procedure 1

1.5ml of protein solution containing 0.5 to 5.0mg. protein was mixed with 1.5ml. of diluted biuret reagent, and incubated at $37^{O}C$ for 15 minutes. The optical density was read at 555mμ or 540mμ in a Unicam SP.500 or

Beckman DB spectrophotometer. Standard curves were prepared simultaneously by incubating appropriate amounts of bovine serum albumen with the reagent.

Procedure 2

A second procedure was employed when the protein content of particulate fractions was to be determined. The suspension in 1.0ml. of water or buffer in a tapered centrifuge tube was mixed with 0.5ml. 15%-trichloracetic acid. The solution was left at room temperature for 30 minutes. The precipitated protein was centrifuged (using a bench machine), the supernatant solution removed by decantation and the precipitate drained by invertion of the tube. The precipitate was suspended in 0.5ml. water, 3ml. of biuret reagent added and the mixture incubated for 60 minutes at 37°C. The tubes were cooled to room temperature and each mixed thoroughly with 3.0ml. diethyl ether. This procedure removed any cloudiness due to suspended lipid. Standard tubes containing BSA were treated in an identical manner.

b) Protein estimation by ultra violet absorption

Rapid, albeit approximate, measurements of protein concentration were made using the ultra violet absorption of tryptophane and tyrosine residues. This procedure was useful when ammonium sulphate was present, and when only small quantities of protein were available.

The procedure is described by Layne (1957) and is based on the data of Warburg and Christian (1941) and a suggestion of Lowry.

$$\text{Protein (mg./ml.)} = 1.55\ OD_{280m\mu} - 0.76\ OD_{260m\mu}$$

c) Estimation of protein by the Kjeldahl method

Proteins from solution were precipitated by making the TCA concentration 10% by adding an equal volume of 20% TCA. After standing at room temperature for 30 minutes, the supernatant was removed by centrifuging, and the protein precipitate washed by another aliquot of TCA. Samples that contained up to 1.0mg. nitrogen in 1.0ml. were incinerated in a 30ml. digestion flask with 2ml. of concentrated sulphuric acid (nitrogen free),

and 200mg. of catalyst ($CuSO_4.5H_2O$;K_2SO_4;Se: 20/80/0.3) for eighteen hours.

The digest was quantitatively transferred into a Markham still (Markham 1942) and the ammonia released by 10ml. 40%-sodium hydroxide removed by steam distillation. The distillate was collected in a saturated solution of boric acid, containing one drop of an achromic indicator (0.2%-methyl red and 0.1%-methylene blue). The ammonium borate was titrated with 0.02N-hydrochloric acid.

Table 2 Comparison of the biuret and Kjeldahl methods using bovine albumen and a partially purified yeast fatty acid synthetase preparation

Nominal dry wt. (mg.) air equilibrium		1	2	3	4	5
BSA.	TCA precipitate					
	Biuret method	0.82	1.74	2.35	3.19	4.17
	Kjeldahl method	0.87	1.76	2.71	3.42	4.38
FAS	TCA precipitate					
	Biuret method	0.65	1.32	2.64		
	Kjeldahl method	0.71	1.43	2.85		

The nominal weight was dissolved in water and precipitated by T.C.A.

3 Column fractionation techniques

a) Sephadex

Sephadex column chromatography was used in the preparation of acetyl-CoA synthetase (G-100), pigeon liver fatty acid synthetase (G-100), and in the purification of CoA thioesters. (G-10).

The dry Sephadex was weighed, and stirred with the appropriate buffer. At cold room temperature, G-100 was allowed to swell for three days, G-25 and G-10 for one day. At the end of this time, the particles were allowed to settle and the supernatant decanted. The gel was then suspended in sufficient liquid to allow air bubbles to be released.

Buffer was introduced into the column, and the Sephadex added in one pour: if necessary a funnel extension having been fitted. The column was stabilised by washing with the buffer overnight at the hydrostatic pressure required.

b) Substituted celluloses

DEAE-cellulose was used extensively for purification

of coenzyme-A and its thioesters, and for the preparation of the pigeon liver fatty acid synthetase. CM-cellulose was used for the purification of Avidin. The substituted cellulose (Whatman grade DE-11, later replaced by DE-22; or grade CM-22) were precycled according to the manufacturers instructions.

The DEAE cellulose, which was to be used in the chloride form for CoA or CoA-ester purification, was equilibrated with a large volume of 0.2N-HCl. After filtration on a Buchner funnel, the cellulose was suspended on 0.02N-HCl and again filtered. When it had been washed with a large volume of 0.003N-HCl the cellulose was suspended in the initial salt solution for gradient elution.

Columns were prepared in one pass and were washed overnight with the starting buffer or salt solution at the desired flow rate to stabilise the column packing.

c) Celite column chromatography

Isolation of mevalonate

The procedure was described by Rudney (1957) and

was used by Brodie et al. (1964). Mevalonic acid was extracted from acidified incubations saturated with sodium sulphate by the method described by Lynen and Grassl (1959). The ether was evaporated and the dry residue taken up in 0.3ml. of 12N-sulphuric acid.

10g. of celite was ground in a mortar with 5ml. of 0.2N-sulphuric acid, then thoroughly mixed with chloroform which had previously been equilibrated with 0.2N-sulphuric acid. The slurry was poured to form a column (1.8 cm diameter), which was washed with 100ml. of acid equilibrated chloroform.

The mevalonate - lactone was quantitatively applied and the column was developed and eluted with acid equilibrated chloroform. Mevalonolactone appeared at about 45ml, and was completely eluted by 80ml. of solvent. The radioactive material delactonised with KOH, chromatographed as a single band on paper using two solvent systems: Ethanol, Ammonia, Water - 7/2/1; (Chesterton, 1966) and n-Butanol, Formic acid, Water - 77/11/12 (Tchen, 1958)

Isolation of Acetate

This method was used to isolate acetate produced by the hydrolysis of the acetyl-CoA formed by the HMG-CoA cleavage enzyme. When acetyl-CoA had been completely hydrolysed with KOH, the mixture was acidified to pH 2 with 6N-sulphuric acid and mixed thoroughly with 10g. of dry celite. The moist celite was transferred to a column and the organic acids eluted with 300ml. of diethyl ether, (Swim and Utter, 1957). 1N-KOH in methanol was added until the ether was alkaline when the solvents were removed by a stream of nitrogen. The mixture was taken up in 1.0ml. 0.2N-sulphuric acid, mixed with 2.0g. of dry celite and applied to the column prepared as for the isolation of mevalonate. 50ml. of chloroform was passed through the column, followed by a chloroform - n-butanol mixture (95:5v/v), which eluted acetic acid in 100ml. The solution was made strongly alkaline and evaporated to dryness.

4 Protein Fractionation by Gel Adsorption

a) Alumina Gel

Alumina gel adsorption was used in the isolation of yeast fatty acid synthetase and Acetyl-CoA synthetase.

The gel was prepared by the method described by Dawson and Magee (1955) which was based on Willstäter's method (1923).

34g. of $Al_2(SO_4)_3 . 18H_2O$ was dissolved in 400ml. of water at 60°C in a five litre flask. 400ml. of warm water containing 10g. $(NH_4)_2SO_4$ was added. While the flask was being swirled, 30ml. of concentrated ammonia was added to produce a heavy precipitate of $Al(OH)_3$. The flask was filled with hot tap water and the precipitate allowed to settle. When NH_4OH had been added until no more precipitation occurred, the clear solution was removed with the aid of a siphon. The vesselwas refilled with fresh water, the suspension mixed, allowed to settle and the supernatant siphoned off again. This was repeated until the pH was below 8.0. Final washings with distilled water brought the

pH to 7.0.

When the suspension had been made up to 400ml. with distilled water, it contained 10.4mg. dry wt./ml.

The use of the gel is described with its particular application. In general, preliminary tests were performed, to ascertain the quantity required to bind the required proportion of protein.

b) Calcium Phosphate gel

This adsorbent was used in the preparation of the fatty acid synthetases from yeast and pigeon liver and in the preparation of HMG-CoA condensing enzyme from yeast. The method of preparation followed that described by Keilin and Hartree (1938).

150ml. of a solution of $CaCl_2.6H_2O$ (132 g/l.) was diluted to 1500ml. 150ml. of a solution of $Na_3PO_4.12\ H_2O$ (152 g/l.) was added and mixed. After bringing to pH 7.4 with acetic acid the gel was washed four times by the decantation of large volumes of

tap water and finally with distilled water using a centrifuge.

Again, preliminary tests for each application were performed in order to determine the amount required.

5 Ammonium Sulphate Fractionation of Proteins

1. Using solid ammonium sulphate

The calculated amount of solid ammonium sulphate was added to the protein solution slowly and with stirring. The solution was stirred for thirty minutes after the last addition of salt had dissolved. The quantity was calculated from the data given by Green and Hughes (1955).

2. Using saturated ammonium sulphate solution

A saturated solution of ammonium sulphate (the temperature and pH are stated in the text) was added slowly with stirring, until the required amount had been added. The quantity was calculated from the equation:-

$A = b(S-s) / 1-s$ (Knauss, Porter and Wasson, 1959)

A = volume of saturated solution required

b = initial volume of solution

S = desired % saturation

s = Initial % saturation

6 Extraction of Lipids

a) Latex of Hevea brasiliensis

(i) The procedure summarised below was used for earlier experiments and is based on that described in detail by Weeks (1966). Incubations were terminated by adding 2ml. of chloroform-methanol (4:1 v/v) in which the latex sample was dissolved. The solution, which had been transferred to round bottomed flasks, was reduced to a thin film by evaporation of the solvent in a slowly rotating flask on a rotary evaporator. An alcoholic extract was prepared by heating the contents of the flask under a reflux condenser with 50 ml. 2N-KOH in 95% methanol for 18 hours. The saponification mixture contained approximately 0.5%-sodium acetate or malonate to reduce the specific activity of the labelled precursor. The alcoholic extracts were carefully decanted to separate flasks, leaving the coagulated rubber sample

adhering to the walls of the saponification vessels. This was washed with three separate aliquots of methanol and the washings were combined with the extract. After removal of most of the methanol by rotary evaporation at 30°C, water was added to a volume of approximately 100ml. The extract was shaken four times with 25ml. aliquots of light petroleum (b.p. 30°-40°C "low in aromatic hydrocarbons"), to remove unsaponifiable lipids. Contaminating labelled substrate was almost completely removed by three re-extractions with large volumes of water. The remaining aqueous extract, combined with these washings was reduced in volume by rotary evaporation at 40°C, and acidified to pH 2 with concentrated hydrochloric acid. The acid solution was extracted three times with 25ml. aliquots of light petroleum to extract fatty acids. After re-extraction with large volumes of water the petroleum solution was dried with anhydrous sodium sulphate. The solution was filtered, and the salt washed with petrol. The relatively large volume of petroleum solution resulting from these manipulations was reduced to approximately 25ml. by rotary evaporation, and to about 3ml. under

a stream of nitrogen. Fatty acids and contaminating acetate were methylated by an excess of diazomethane, in ether solution (James, 1960). The products were dissolved in 20ml. methyl acetate and evaporated to dryness in vacuo. This was repeated a further four times, to obtain a fatty acid fraction of constant radioactivity, (Rowe, 1964).

The rubber coagulum was dissolved in 50ml. chloroform, andcontaminating substrate removed by vigorous shaking, on a mechanical shaker, with large volumes of water. This washing was continued, the water being decanted from the chloroform twice daily, until the radioactivity in zero time incubations had been reduced to insignificant levels and that in other incubations had been reduced to a constant value.

Weeks (1966) showed that this procedure allowed reproducible extraction of lipid components from latex incubations.

B. A second procedure used a liquid - liquid continuous extraction apparatus. The saponification

was performed and the methanol removed as described above. The aqueous extracts were introduced into the extractors which were filled with light petroleum. The apparatus was connected to a 250ml. round bottomed flask containing boiling light petroleum. Extraction was allowed to continue for at least 72 hours.

After the first period of extraction, the flasks containing the lipid extract were removed to be replaced by others containing fresh solvent. The aqueous extracts were acidified by carefully pouring 10ml. of concentrated hydrochloric acid down the centre tube. Fatty acids were extracted for at least 72 hours.

The solutions of unsaponifiable lipids and saponifiable lipids were washed with distilled water in the same manner as described for the first procedure. The fatty acid components were purified and counted as their methyl esters.

The latter method of extraction was therefore considerably less tedious than the manual procedure using separating funnels. However, to ensure good

replication of duplicate samples it was necessary to continue extraction for at least 72 hours.

b) Lipids from incubations of yeast, rat liver and pigeon liver preparations

1) Earlier experiments with yeast systems used a procedure essentially as described for latex. These techniques were superseded by a chloroform-methanol method described below, followed, when required, by the saponification stage and light petroleum extractions performed manually, or with the aid of the continuous extractors.

The advantages of this technique are:-

i. The washing procedure is sufficient to remove all contaminating substrates. It is therefore not necessary to purify fatty acids as their methyl esters before assaying radioactivity.

ii. All incubations may be worked up simultaneously in 50ml. glass centrifuge tubes, and the procedure itself is rapid.

iii. The extraction of lipids is very efficient, because all components (except for the precipitated

protein) are initially in a single phase.

iv The procedure has been shown to be reliable for several systems, e.g. Galliard, Michell and Hawthorne (1965); Chesterton (1968); Garbus, Deluca, Loomans and Strong (1963).

From a study of the chloroform - methanol - water phase diagram, Bligh and Dyer (1959) introduced the efficient and rapid extraction procedure which was dependent on maintaining the volumes of chloroform, methanol and water in the proportions 1:2:0.8 initially, and in the proportions 2:2:1.8 after diluting to produce the bi-phasic system. Garbus et al. (1963) modified the procedure by increasing the ionic strength of the aqueous solution to ensure recovery of the phospholipids. The procedure described below was that used by Galliard et al. (1965).

3.75 volumes of chloroform-methanol (1/2, v/v) were added to incubation mixtures (i.e. one volume), in 50ml. pyrex glass centrifuge tubes fitted with ground glass stoppers, and thoroughly mixed. The

monophasic solvent system, containing precipitated protein, was allowed to stand at room temperature for 60 minutes. This mixture was diluted by adding 1.25 volumes of chloroform and 1.25 volumes of 2M-potassium chloride containing 0.5M potassium phosphate buffer (pH 7.5) to produce a two phase system. After thorough mixing, the mixture was centrifuged at 2,000g for 20 minutes to cleanly separate the layers and to give a packed layer of protein at the interphase. The upper aqueous phase, which contained only non-lipid material, was carefully removed by Pasteur-pipette or by aspiration at a filter pump. The chloroform layer was transferred to another tube using a Pasteur-pipette and the protein layer remaining was rinsed with 2ml. of chloroform and then discarded. The total lipid extract was washed twice with 4.75 volumes of "upper phase" from a similarly constituted mixture of solvents, an equivalent volume of water being substituted for the incubation. Phosphate, and potassium chloride were ommitted from the first and second washes respectively. The phospholipid that precipitated at the interphase during the last wash was taken with the lower layer. The chloroform solution was taken to dryness by rotary evaporation at 30°C, the last

traces of water being removed with the aid of methanol. Lipids were redissolved in chloroform and stored at -15°C.

92% of contaminating substrates were retained in the first methanol-water layer. After the two washes, the lipid extract was uncontaminated by substrates or salts.

7. Lipid Analysis

a) Gas-liquid chromatography

All gas liquid chromatographic analyses were performed using the Pye-Argon apparatus, and all columns were 4ft. in length, 0.5cm. diameter. The procedures followed those described by James (1960).

i. Preparation of the columns

Celite (obtained from Hopkin and Williams, Ltd, labelled for gas chromatography) was suspended in 2N-hydrochloric acid. After 30 minutes, the cloudy, slightly yellow supernatant was decanted to be replaced by more hydrochloric acid. After another 30 minutes the hydrochloric acid was replaced by distilled water, which was removed by filtration on a Buchner funnel. The acid washed celite was washed with water until the effluent was neutral. After a rinse with acetone, the celite was dried in an oven at 110°C.

The weighed quantity of stationary phase (PEGA, or APL grease), was dissolved in the appropriate solvent, (Acetone for PEGA, chloroform for APL). The correct

weight of celite was wetted with an excess of solvent and mixed thoroughly with the solution of stationary phase in a large beaker. The container was placed in a water bath, or on an electric hot plate, and the solvent was evaporated while the mixture was continually stirred. When most of the solvent had been removed, the almost dry column packing was left overnight in an oven at 110°C.

The dry packing material was slowly added to a clean column with continuous and vigorous vertical tapping. When the column had been filled to within approximately 10cm. of the top, a coil of glass yarn was placed onto the top of the packing and pushed well down with a glass rod.

Before use, the column was purged of volatile contaminants by the passage of Argon for 18 hours at a temperature 10°C higher than that to be used.

ii. Preparation of fatty-acid methyl esters

The fatty acid solution in light petroleum was

dried with anhydrous sodium sulphate, or by rotary evaporation to complete dryness. The volume was reduced to less than 1ml. or the solvent was removed completely, by a stream of oxygen free nitrogen. Excess diazomethane, prepared from N-methyl-p-toluene-sulphonyl-nitrosamide by the method described by Vogel (1956) was added and the yellow mixture was allowed to stand at room temperature for 60 minutes. Solvent and excess diazomethane were removed with nitrogen; the methyl esters were redissolved in light petroleum and stored under nitrogen at -15°C.

iii Chromatography

Method 1

The solvent free methyl esters were applied to the top of a hot column using a Pye micropipette (0,025 or 0.05 µl.) The esters were allowed to volatilise for 15 seconds before the argon supply was restarted. All analyses were performed using an argon pressure of 15 pounds/sq.ins. Retention times were measured from the centre of the air peak to the centre of the mass peaks.

Method 2

The solvent free methyl esters were dissolved in

a known volume of light petroleum. An aliquot of this solution applied to the column using a fine glass pasteur pipette which had been graduated with water to hold, for example, 0.7μl.; or using a syringe graduated in microlitres. This procedure was used when radioactive samples were to be chromatographed since it allowed the analysis of a known proportion of the whole sample.

iv. Identification of Peaks

A mixture of authentic samples of fatty acid methyl esters was periodically chromatographed. The retention time for a particular compound was found to be very reproducible providing that the column, oven temperature and gas pressure were not varied. The ratio of the retention time of each methyl ester to the retention time of methyl palmitate was calculated and a graph of the $\log_{10}$ of this value: carbon number enabled the identity of the common fatty acids to be established (James, 1960).

v. Collection of Radioactivity

The column effluent was passed through a calcium

chloride tube packed with toluene soaked glass wool. This was connected to the exit nozzle by a short length of silicone rubber tubing. The tubes were changed at suitable intervals during the course of an analysis.

The collected fractions were washed into scintillation vials with toluene-phosphor (5ml.) and light petroleum (5ml.).

Recovery of fatty acid methyl esters averaged 80%. The recovery of [1-^{14}C] methyl palmitate was 78 ± 4.4% (S.E.M. of 5 determinations).

b) Thin Layer Chromatography

Thin layer chromatography has several distinct advantages over column chromatographic methods. Development is very rapid, and resolution can be high. Its principal disadvantage is that loading is necessarily limited.

Preparation of plates

Thin layer plates were prepared using a Shandon Unoplan spreading apparatus, to a wet thickness of 0.3mm. 35g. of silica Gel-G (Kieselgel-G, nach Stahl,)

was shaken vigorously with 70 ml. distilled water for 90 seconds. The slurry was poured into the spreader and the plates were prepared immediately. When air dry (about two hours) the plates were removed from the leveller. Before use they were activated at approximately 110°C for 30 minutes.

Development

Samples were applied to the plate by means of capillary glass tubing and were developed by ascending solvent in tanks which contained a lining of filter paper to help maintain a saturated atmosphere.

When the developing solvent had travelled about 15 cm., the plates were removed and the solvent evaporated in air.

Visualisation

i. Iodine - After drying in air, the developed chromatograms were placed in another tank containing a few crystals of iodine. Unsaturated compounds rapidly stained brown. Saturated compounds usually developed

more slowly, presumably by the solution of iodine in the lipid.

ii. Charring with sulphuric acid - the developed chromatograms were sprayed with a solution of concentrated sulphuric acid (5%) in ethanol. The plate was then transferred to an oven at 110°C for 30 minutes. Very small quantities of lipid could be made visible by this means.

iii. Rhodamine-6G - thin layer plates were prepared containing 0.25% Rhodamine-6G. The plates were developed in the normal way, and were studied under ultra-violet light. Lipid zones quenched the fluorescence of the dye and were observed as darker areas, or changed the colour of fluorescence. Positions of zones were marked and the silica gel could be scraped from the plate and eluted with chloroform or dichloromethane. In these conditions, the dye stayed bound to the silica gel. Elution procedure is described in "The measurement of radioactivity." (1a(iii)).

c) Silicic Acid Column Chromatography

A reliable procedure for the separation of relatively large quantities of lipid mixtures into constituent

classes was described in detail by Hirsch and Ahrens (1958). This type of analysis has been superseded for many applications by thin layer chromatographic techniques. Often the latter are more reproducible, are nearly always more rapid and have a high resolving power. However, a much greater quantity of material may be separated on a column.

Preparation of Silicic Acid

Mallinkrodt silicic acid, (100 mesh, labelled 'suitable for chromatographic analysis by the method of Ramsey and Patterson') was employed. The grinding described by Hirsch and Ahrens was ommitted, and 200g. of the adsorbent was suspended in methanol in a 2.0l. glass measuring cylinder. After allowing the particles to settle for 30 minutes, the methanol and the suspended silicic acid were discarded. This was repeated twice; the third time diethyl ether replacing methanol. The adsorbent was dried in air.

Preparation of the column

A column was manufactured by fitting a sintered

glass disc in a Leibig condenser so that the adsorbent was totally enclosed in the water jacket. The sinter was covered by a disc of filter paper. 1 8g. of adsorbent, which had been dried overnight at 115°C, was introduced into the column on to the filter paper disc, a little at a time and with continuous tapping. The column was developed and the lipids eluted by a stepwise gradient of diethyl ether in light petroleum.

Monitoring the column eluate

Fractions of about 25ml. were made up to a standard volume (usually 100ml.) 10ml. aliquots were reduced to 1 ml. and tested for sterols or sterol esters by the Liebermann-Burchard reaction; 50 ml. aliquots were evaporated and dried on weighed aluminium milk bottle caps, which were re-weighed; 20ml. were reduced to dryness and taken up in a minimum of chloroform: An aliquot of this was examined by thin layer chromatography. When radioactive lipids were being examined, suitable aliquots were dried in scintillation vials with nitrogen and the lipids redissolved in 5.0 ml. light petroleum and 5.0 ml. phosphor-toluene.

c) Alumina Column Chromatography

Chromatography of the unsaponifiable fraction of lipid extracts on columns of deactivated alumina was used as a rapid method for the separation of squalene from more polar compounds.

Aluminium oxide ("Camag", 100-200 mesh. obtained from Hopkin and Williams Ltd) was deactivated by stirring with methanol. The alumina was filtered on a Buchner funnel and dried overnight at 37°C. Columns (1.3 x 10cm.) were prepared by mixing the adsorbent with light petroleum and pouring the slurry onto the retaining pad of glass wool. The lipid extracts which usually contained carrier squalene, dissolved in light petroleum, were applied to the columns. Squalene was completely eluted by 20 ml. of light petroleum in quantitative yield.

8 Preparation of Derivatives

a) Squalene hexahydrochlorides

Squalene was chosen as a representative isoprenoid in much of this work because of the relative ease with which a crystalline derivative could be prepared. The

procedure adopted was a modification of the method of Heilbron, Kamm and Owens (1926), described by Langdon and Bloch (1953b).

Hydrogen chloride was generated by the action of concentrated sulphuric acid on sodium chloride (A.R.), the reaction was stimulated by warming when necessary, Acetone at -5°C (cooled by a bath of acetone in which was immersed a beaker containing an ice/salt freezing mixture) was saturated with dry hydrogen chloride. 2.0 ml. of this acetone was added to the solvent free hydrocarbon sample in a small vial. Dry hydrogen chloride was bubbled through the mixture until a precipitate appeared and then for a further sixty minutes.

The precipitates were isolated on a Hirsch funnel and were washed with anhydrous ether at 0°C. The crude hydrochlorides, as a white powder, were suspended in 2.0 ml. of acetone in a tapered centrifuge tube, warmed in a water bath for ten minutes at 50°C and in a water bath at 60°C for three minutes. The insoluble material was immediately removed by centrifuging in a bench centrifuge.

The acetone insoluble residue was washed once more with acetone (50°C) and then warmed with ethyl acetate. The material dissolved with difficulty and rapidly crystallised on standing. These crystals melted at 142° - 143°C and comprised most (70-80%) of the product. The acetone soluble fraction crystallised on standing at 4°C and melted at 105-108°C.

b) Sterol digitonides

Sterol digitonides were prepared by the method of Frantz and Bucher (1954) and Frantz, Schneider and Hinkelmann (1954). Solvent was removed from the extract containing carrier cholesterol and the solid material dissolved in 3ml. acetone-ethanol (1:1 v/v). The solution was clarified by centrifugation and the sterols precipitated by an excess of 0.5% digitonin in ethanol-water (1:1 v/v).

The mixture was left at 4°C overnight and the precipitate collected by centrifuging. The precipitate was resuspended in ethanol-water and collected again. Frantz et al. (1954) showed that the digitonide of rat liver extracts consisted largely of the cholesterol derivative. This procedure was also used to isolate sterol components of yeast incubations. In this circumstance, ergosterol was added as the carrier.

c) HMG-di-p-bromophenacyl ester

The di-p-bromophenacyl ester of HMG- was prepared in the manner described by Brodie et al. (1963).

Incubations, in which HMG was to be determined, were deproteinised by boiling with 1.0 ml. of ethanol. The precipitated protein was removed by centrifugation and the supernatant freeze dried. To hydrolyse all the CoA thioesters 0.2 ml. of 1.0N-KOH was added to each vial which was then incubated at 37°C for one hour. Aqueous solutions of HMG-CoA eluted from chromatograms were treated in the same way.

Approximately 10mg. of HMG was added to the mixture which was brought to pH 6.5 (narrow range indicator paper). 50mg. of α,p-dibromoacetophenone was added and the mixture was refluxed for three hours with sufficient ethanol to dissolve all the components. The solution was allowed to cool until oils separated out. The still hot solution was transferred to a separate flask. Boiling water was added until the mixture became slightly opalescent, when it was allowed to cool. A white precipitate formed on occasions, but more often, vigorous scratching of the vessel walls was required before solid material separated. This precipitate was isolated by centrifuging, washed once by cold 50%-ethanol and

then taken up in warm benzene (38°C). If there was any insoluble residue it was removed. The benzene solution was evaporated to dryness and the product was recrystallised from aqueous ethanol. The colourless crystals melted at 127-128°C.

d) Enzymically produced derivatives of mevalonic acid

Mevalonic acid is often regarded as the first incontrovertible isoprenoid precursor. In an attempt to demonstrate the labelling of this compound, experiments are described in which unlabelled trapping pools of mevalonic acid were added to Hevea latex and yeast systems. A similar experiment was described by Weeks (1966).

After saponification, lipid extractions were performed in the manner previously described. All aqueous washings, however, were retained. The pooled solutions were reduced in volume to approximately 100ml. by rotary evaporation at 50°C. The solution was saturated with sodium chloride and extracted three times with 25ml. aliquots of diethyl ether, or for 72 hours using continuous liquid - liquid extraction apparatus. The ether extract, which contained substrates such as acetate and malonate,

as well as mevalonate, was reduced to a small volume using a rotary evaporator and then nitrogen.

Chromatography on paper resolved mevalonate and malonate if the ethanol; ammonia; water solvent system was used: (7:2:1, Chesterton, 1966). When conducted in an acidic solvent (Butanol:Formic acid: Water, 77:11:12, Tchen, 1958) acetic acid evaporated from the solvent front.

The mevalonate eluted from a chromatogram with water, or isolated by the celite column method, was freeze dried in a small vial. Phosphorylated derivatives of such material were prepared by an enzyme preparation from the freeze dried serum of Hevea brasiliensis.

An 8% (w/v) solution of freeze dried serum in water was acidified to pH 4.5 with 1%-acetic acid (v/v). The precipitated protein was removed by centrifuging, and the supernatant was dialysed for 120 minutes against 0.05M·potassium phosphate, pH 7.3. In a total volume of 1.0 ml., incubations contained: 5mM-ATP, 5mM-$MgCl_2$, 0.2ml.

Hevea enzyme preparation, 0.1 M-potassium phosphate, pH7.3 with the freeze dried substrate taken up in 0.2ml. water.

After incubating for sixty minutes at 30°C, the protein was denatured by an equal volume of ethanol, most of which was removed by boiling. The precipitated protein was removed from suspension by centrifuging and the supernatant together with two water washings of the precipitate was transferred to vials for freeze drying. An aliquot of the residue was analysed for phosphorylated derivatives of mevalonate.

e) Preparation of Hydroxamic Acid Derivatives

Authentic acetyl hydroxamate and malonyl-mono-hydroxamate were required to standardise the assays of acetyl-CoA, malonyl-CoA and acetyl-CoA synthetase.

Acetyl-Hydroxamate

A modification of the method described by Hauser and Renfrow (1943) was used to prepare the potassium salt of acetohydroxamic acid. 0.67 moles of hydroxylamine hydrochloride in 240ml. of methanol, and 1 mole of potassium hydroxide in 140ml. of methanol were dissolved in the boiling solvents. Both solutions were slowly cooled to 30-40°C, and the alkali was slowly added to the hydroxylamine solution. The mixture was placed on ice for 60 minutes, after which the precipitated KCl was removed by filtration on a Buchner funnel. 0.33-mole of ethyl acetate was added to the hydroxylamine solution with thorough mixing. After allowing to stand overnight at room temperature, the mixture was evaporated almost to dryness using a rotary evaporator at 25°C. The yellow paste obtained was mixed with ethyl acetate and sufficient methanol to bring the solids into solution at 35°C. The cloudy solution was filtered and

allowed to stand at 4°C for 48 hours. The large needle shaped pale yellow crystals were collected by filtration and were washed with cold ethyl acetate. They were redissolved in methanol-ethyl acetate and allowed to recrystallise a second time.

Malonyl mono hydroxamate

i Potassium mono-ethyl malonate - mono-ethyl-malonate was prepared by the method of Rodionov and Bezinger (1953). 75g. of diethyl malonate was mixed with 300ml. of absolute methanol. 26.1g. of potassium hydroxide in 300ml. methanol was added and the mixture was stirred at room temperature for two hours. The solution was heated at reflux temperature for 15 minutes, and then filtered. The white precipitate that formed on cooling was collected.

ii. - Potassium-malonyl-mono-hydroxamate was prepared in a similar manner to that described for acetohydroxamate. The almost cubic crystals were light yellow in colour.

Both acetyl and malonyl derivatives gave single

ferric chloride staining spots on paper chromatography using the upper phase of Butanol: Acetic acid: Water (4:1:5,) as solvent.

9 Visualisation of Paper chromatograms

a) Hydroxamates

Hydroxamate derivatives were detected by the colour reaction with ferric chloride. The paper was dried in air and dipped in a solution of 2% ferric chloride in 95% methanol (acidified with 5ml. 1.0N-hydrochloric acid/ 100ml.). Hydroxamates appeared as red-violet areas. Hydroxylamine stained more slowly and appeared after a few hours.

b) Organic Acids

Unlabelled HMG, malonic acid or MVA was detected with an aniline-xylose reagent.

1.0g. of xylose was dissolved in 3ml. of water. 1.0ml. aniline was added and the volume was made up to 100ml. with methanol. The paper was dipped in this solution. When most of the methanol had evaporated the

damp paper was heated at 100° - 110°C. for 15 minutes. the acids appeared as dark brown areas on a lighter background.

c) Radioactive compounds

The chromatogram was stapled to a sheet of Kodirex X-ray film (Kodak Ltd.), and left in the dark for a period depending on the radioactivity. The film was developed in commercial X-ray developers supplied by May and Baker Ltd. or Kodak Ltd.

EXPERIMENTAL

1. Methods

B. Preparation and Assay of Enzymes and Enzyme Systems

I Acetyl - CoA Synthetase (E.C. 6.2.1.1.)

Partially purified preparations of acetyl-CoA synthetase were used to prepare substrate quantities of ^{14}C-acetyl-CoA, thereby avoiding the use of costly ^{14}C-acetic anhydride. The enzyme isolated from bovine heart mitochondria has been extensively examined by Webster (1965a).

Assay

The assay used by Webster depended on the measurement of the rate of disappearance of CoASH from reaction mixtures measured by the Nitroprusside method of Grunert and Philips (1951). This assay was first described by Mahler, Wakil and Bock (1953). It was not possible to use this procedure with other than highly purified preparations because of the sulphydryl compounds necessary to maintain enzyme activity. Furthermore, it was found difficult to obtain reproducible estimations of sulfhydryl content at the low levels employed in such assay conditions. To facilitate proper comparisons the assay employed throughout measured hydroxamate formation. The

details are discussed by Jones and Lipmann (1955), and the reaction mixture consisted of: CoASH (0.08 μmole); dipotassium ATP (10 μ mole); potassium acetate (20 μmole); Tris HCl buffer (100 μmole); potassium fluoride (50μmole); $MgCl_2$ (10μmole); glutathione (10μmole); neutralised hydroxylamine hydrochloride (200 μmole): Sufficient enzyme was added to form 0.1 to 0.6 μmole of acetohydroxamate in 20 minutes. The final volume was 1.0ml.

Control assays, in which Coenzyme A was omitted, were always performed simultaneously. In the more crude fractions, significant CoA-independant hydroxylamine acetylating activity occurred.

Sodium ions have been shown to inhibit this enzyme (Von Korff, 1953; Webster 1965b;): buffers were therefore the potassium salts. Disodium-ATP was converted to the potassium form with the aid of the P.T.F.E. hollow plugs described by Briggs, (1967). Washed Dowex - 50 resin in the acid form was introduced into the plug inserted in a tapered centrifuge tube. Water was removed from the resin by centrifuging. A solution of disodium-ATP was added to the resin. When spun again, cation free ATP was eluted. The resin was washed twice with water:

the combined washings were neutralised with potassium hydroxide and used immediately.

Assays were started by the addition of enzyme, and terminated by 1.5ml. of ferric chloride reagent. (10%-$FeCl_3.6H_2O$, 3.3%-TCA, in 0.66N-HCl). The precipitated protein was removed by centrifugation and the developed colour of the supernatant was determined in a Unicam SP 500 spectrophotometer.

Enzyme Purification

Method 1

The procedure described by Webster (1965a), which consisted of the isolation of beef heart mitochondria, their disruption by repeated freezing and thawing, then purification of the enzyme extracted from this source. It was not found possible to obtain an active enzyme after the freezing and thawing stage.

Method 2

Active preparations were obtained using the procedure described by Beinert, Green, Hele, Hift, Von

Korff, and Ramakrishnan (1953) from pig hearts.

The pig hearts (2), obtained freshly from the abbattoir, were washed and most of the connective tissue and fat removed. The muscle was blended with 10 volumes of cold acetone (-10°C) for 1 minute. The homogenate was filtered on a buchner funnel at a filter pump. The semi-dry filter cake was blended with a further 10 volumes of cold acetone and again filtered. When most of the acetone had been removed, the almost dry residue was pulverised by hand and then allowed to dry at room temperature in a desiccator over phosphorus pentoxide. The dry powder was stored at -15°C and retained its activity for at least six months.

The dry acetone powder was extracted with 0.02M-$KHCO_3$ (10 volumes to the original tissue weight), by stirring at 4°C for thirty minutes. The suspension was filtered through two layers of muslin, and centrifuged for 30 minutes at 5,000 g.

21g. of solid ammonium sulphate was added slowly to each 100ml. of supernatant solution, and the mixture

was stirred for thirty minutes after the salt had completely dissolved.

The precipitate was sedimented by centrifuging and discarded. A further 21g./100ml. of ammonium sulphate was added. This time the precipitate was retained and dissolved in the minimum volume of 0.02M-$KHCO_3$. The solution was stable when stored at -15°C.

Before use, the enzyme solution was further fractionated with ammoniacal saturated ammonium sulphate solution, pH 9.0.5.75ml. of ammonium sulphate was added for each 10ml. of protein solution (approximately 37% saturation). After stirring for 30 minutes, the precipitate was discarded. Further ammonium sulphate solution was then added to the supernatant (6.5ml./10ml.) to give a salt concentration of approximately 55% of saturation. The precipitate was retained and dissolved in 0.02M-$KHCO_3$. The solution was dialysed for 60 minutes against 0.02M-$KHCO_3$. Table 3 shows the activities obtained from such a preparation.

Table 3

The Purification of Acetyl-CoA Synthetase by Method 2

Fraction	sp.act.(μmole/min/mg.protein)
Potassium bicarbonate extract	0.15
21 -42g./100ml. ammonium sulphate fraction	0.28
pH 9.0 Ammonium sulphate fraction. 37% - 55% of saturation	0.43

Assay conditions are described in the text.

Method 3

The procedure described above gave reliable enzyme preparations but of relatively low specific activity. A method of fractionation was devised based on the bicarbonate extraction, but followed by stages in the purification of the crystalline enzyme (Webster, 1965a).

Starting material was an acetone powder prepared from crude beef heart mitochondria. Occasionally, an extract of an acetone powder of the whole heart was prepared as described in method 2. The specific activity of this material was lower, but the yield of protein was much higher.

Beef heart mitochondria

A fresh beef heart was minced, and each 1,000g. of this material was homogenised with 2,800ml. of 0.13M-KCl in a Waring type blendor for 30 seconds at top speed. The homogenate was centrifuged at 1,000g for 15 minutes and the crude supernatant filtered through several layers of muslin. To obtain the crude mitochondrial fraction,

the solution was centrifuged at 10,000g for 30 minutes. This procedure was basically that of Webster, Gerowin and Rakita (1965).

Purification

An acetone powder of this fraction was prepared by the previously described method, and the subsequent potassium bicarbonate extraction and first ammonium sulphate fractionation performed as for the pig heart enzyme. From this stage the procedure followed that of Webster (1965a).

The ammonium sulphate fraction was dissolved in 0.02M-potassium bicarbonate and 2-mercaptoethanol was added to a concentration of 2mM. Saturated ammonium sulphate solution (pH 8.5, 0°C) was added to the solution (5% of the solution volume) followed by alumina gel (14.5mg./ml., 15% of the solution volume). After stirring for 5 minutes, the gel was collected by centrifuging at 5,000g for 10 minutes, then discarded. This procedure removed about 40% of the protein. Protein was precipitated by 45.5g. of ammonium sulphate/100ml. of

solution , and was collected by centrifuging. The sediment was redissolved in 0.02M-$KHCO_3$, 5mM-EDTA, 2.0mM-2-mercaptoethanol and was refractionated by the addition of 67ml. of a saturated solution of ammonium sulphate at 0°C, pH 8.5 for each 100ml. of solution. The precipitate was collected after 30 minutes and discarded. The protein that was precipitated by 18g. of solid ammonium sulphate/100ml. was retained and dissolved in the minimal volume of a buffer containing 0.2M-$KHCO_3$, 0.18M-KCl, 0.05mM-EDTA and 3.0mM-2-mercaptoethanol. The solution was dialysed against this buffer for 60 minutes.

The protein solution (about 4ml.) was applied to a column of G-100 Sephadex that had been equilibrated with the same buffer. The column was developed and eluted at a rate of approximately 0.3ml./minute. 5ml. fractions were collected. Figure 1 shows the result of such a column and table 4 summarises this purification procedure.

Products of the enzymic incubation

Incubation mixtures were prepared to contain five

Table 4

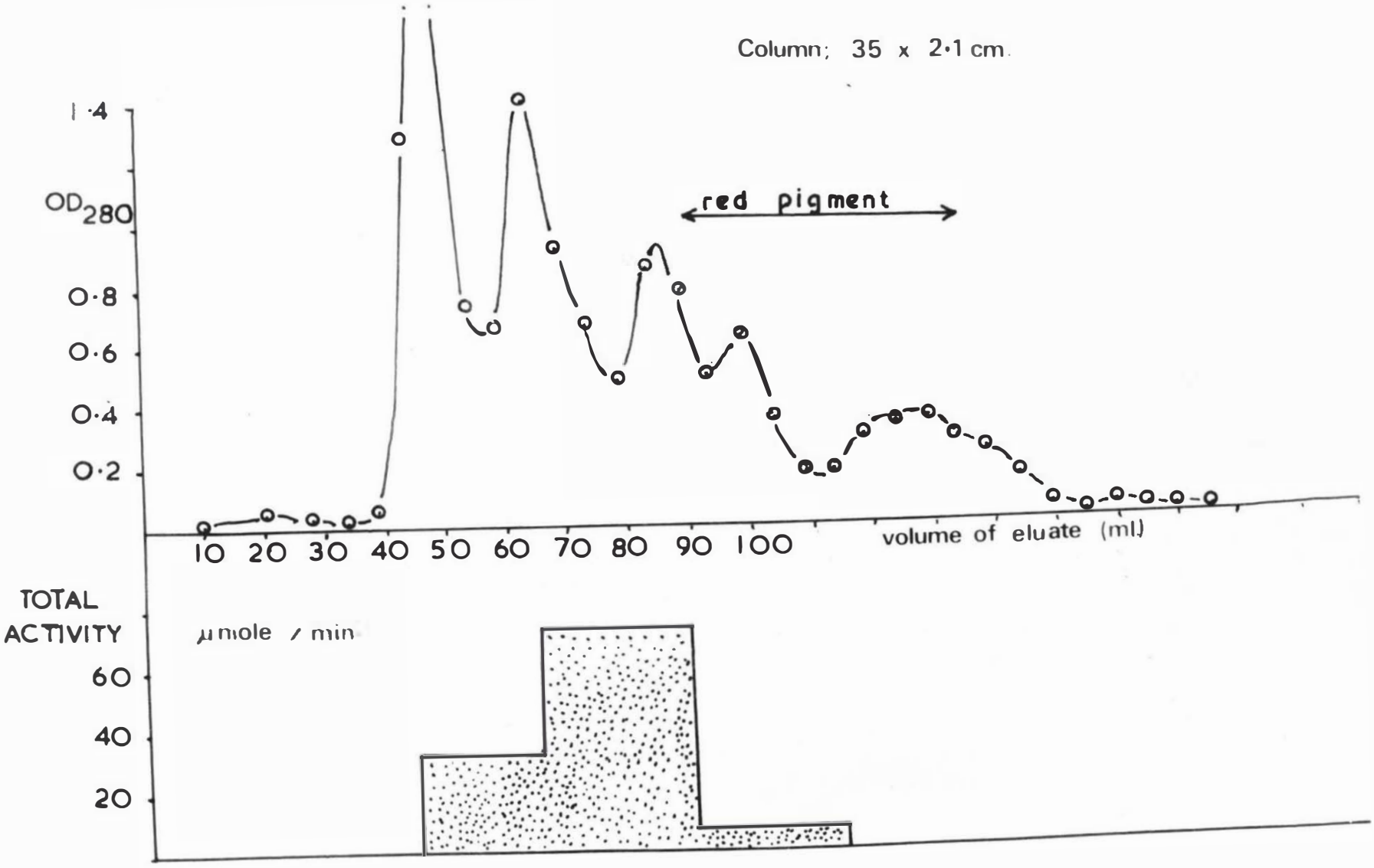
The Purification of Acetyl-CoA Synthetase by Method 3

Fraction		Protein (mg)	sp.act. (μmole/min/mg.protein)	Total Activity (sp.act x protein)	%Recovery
1st. Ammonium sulphate fractionation		1,100	0.16	176	"100"
Alumina gel/ammonium sulphate		650	0.24	156	89
2nd. Ammonium sulphate fractionation		225	0.51	114.8	65
G-100 Sephadex	I	51	0	-	
	II	72	0.38	27.36	
see fig. 1	III	60	1.2	72.0	59
	IV	50	0.12	6.0	

The assay conditions are described in the text.

FIG I

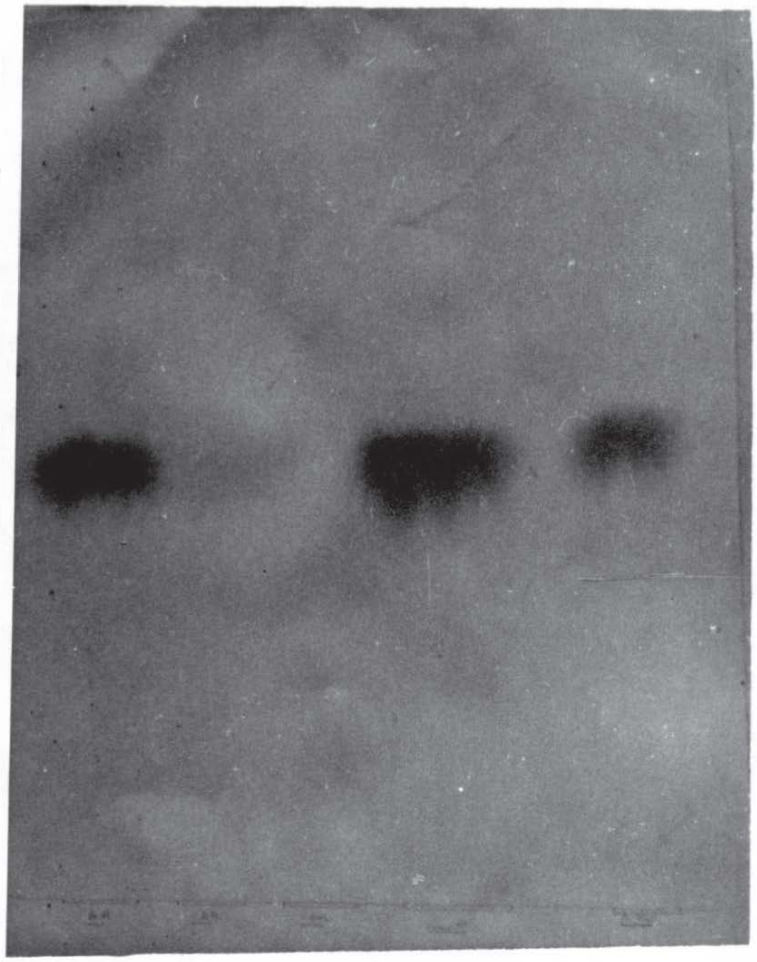
PURIFICATION OF ACETYL-CoA SYNTHETASE ON G-100 SEPHADEX



times the quantities of all reagents of the standard assay mixture, in a total volume of 5ml. The incubations were terminated by 25ml. of ethanol and the precipitated protein was sedimented by centrifuging. The pellet was rinsed with ethanol and then discarded. The supernatant solutions were reduced to dryness using a rotary evaporator at 25°C and the residues were extracted with three 2ml. aliquots of ethanol, (Stadtman and Barker, 1950)

This volume was reduced to approximately 0.2ml. by a stream of nitrogen, and the precipitated salts packed by centrifuging. 50µl. aliquots of the supernatant were chromatographed. Fig. 2 is a photographed of a chromatogram of a set of incubations on Whatman No.1 paper, stained with $FeCl_3$.

Fig. 2 ACETYL-CoA SYNTHETASE : PRODUCT OF INCUBATION WITH HYDROXYLAMINE



1 2 3 4 5

Details of incubations : see text.

1. & 5. Acetohydroxamate
2. − Co A
3. − Enzyme
4. Complete

Upper phase of n-butanol, acetic acid, water

4 : 1 : 5

Ascending. Stained by $FeCl_3$

2 Fatty acid synthetase from yeast

A soluble fatty acid synthesising enzyme was partially purified from yeast using the method described by Lynen (1962).

Assay

The disruption of the yeast and the first ammonium sulphate fractionation were performed without assay. After this stage a spectrophotometric assay was used.

A silica cell (1 cm path length) contained potassium phosphate buffer, pH 6.5 (100 μmole); cysteine, freshly prepared by the mixing of cysteine hydrochloride and potassium hydroxide (20 μmoles), NADPH (0.20 μ mole) and acetyl CoA (0.01 μ mole). Sufficient enzyme solution was added to give a linear rate of oxidation of NADPH for 5 minutes. The volume was made up to 1.49ml. with water and the reaction started with 0.01ml. of malonyl-CoA solution (0.01 μmole)

Protein was assayed by the biuret procedure, or for very dilute solutions by the absorption at 280mμ and 260mμ.

Disruption of Yeast

Several methods were used.

Hughes' Press

10g. of commercial bakers yeast was suspended in 20ml. of potassium phosphate buffer (pH 7.5 0.1M). The suspension was repidly frozen in a Hughes' press (Hughes, 1951). and forced through the orifice. Using three sets of apparatus, 100g. of yeast was eventually processed. This method was tedious, time consuming and required the broken yeast to be stored in the frozen state for a considerable length of time. When 100g. of yeast had been processed it was allowed to thaw at room temperature and the mixture was diluted to 100ml. with the phosphate buffer. Unbroken cells and large particles were removed by centrifuging at 10,000g for 60 minutes. The supernatant was used for subsequent purification of the enzyme.

Solid carbon dioxide and sand

Portions of the yeast were put into a mortar which contained an equal weight of silver sand and that had

been cooled with solid carbon dioxide. The yeast sand mixture was ground with powdered carbon dioxide, When 100g. of yeast had been treated in this way, it was allowed to thaw at room temperature. The mixture of yeast with sand was diluted by 200ml. potassium phosphate buffer (pH7.5, 0.1M), and centrifuged at 10,000g for 60 minutes. The turbid brown supernatant was used for purification of the enzyme.

This method was considerably less tedious than the use of the Hughes' press and was used for routine preparations until the homogeniser became available.

Braun Homogeniser (Typ. 2876)

The instrument was as described by Merkenschlager, Schlossman and Kurz (1957) and was manufactured by B.Braun, Melsungen. Such an apparatus was used by Lynen. 10g. of yeast with 10ml. of buffer and 50g. of ballotini beads (0.45-0.50mm.) in the homogeniser bottle were cooled to approximately -3°C by careful immersion in a bath of acetone-solid carbon dioxide. The mixture was shaken in the homogeniser for 3 minutes. The fluid and beads were transferred to centrifuge pots and centrifuged for

ten minutes at 5,000g. The fluid was decanted from the beads and centrifuged at 20,000g for 30 minutes. 100g. of yeast could be processed in a relatively short time.

Partial purification of the enzyme

The supernatant solution was diluted with twice its volume of distilled water, 19.4g. of solid ammonium sulphate was added slowly and the mixture was stirred for 30 minutes after the last addition had dissolved. The precipitated protein was removed by centrifuging at 10,000g for 15 minutes and discarded. A further slow addition of 9g. ammonium sulphate/100ml. of solution was made. This precipitate was retained and dissolved in 25ml. of distilled water.

The enzyme solution was dialysed against 5.1.0.05M-K_2HPO_4 for 3 hours, and was then diluted to give a protein concentration (biuret) of 30mg./ml. Using a glass electrode the solution was acidified to pH 5.5 with 0.2N-acetic acid. Calcium phosphate gel (15mg./ml.) was added, with stirring, to give a gel-protein ratio of 1:1. After stirring for 10 minutes, the gel was sedi-

mented by centrifuging at 5,000g for 15 minutes. After a single wash with 50ml. of water, the active enzyme was eluted from the gel by three separate 50ml. aliquots of 0.05M-potassium phosphate, pH 6.5.

The eluate thus obtained was fractionated using solid ammonium sulphate as described for the first stage. If further purification through alumina gel was to be performed immediately, the precipitate was dissolved in a minimal volume of water and dialysed against 5 l. of 1.0mM-K_2HPO_4 for 3 hours. If the enzyme preparation was to be stored, the ammonium sulphate precipitate was kept at $4^{o}C$. In this form it lost approximately 40% of its activity in one week.

Immediately before use, the dialysed solution was diluted with water to a protein concentration of 15mg./ml. and was carefully acidified to pH 6.0 with 0.1M-acetic acid. Alumina gel was added to give a gel/protein ratio of 0.24. The mixture was stirred for a further ten minutes, then the gel sedimented by centrifuging at 20,000g for 30 minutes.

Table 5

Summary of a Purification of the Fatty Acid Synthetase from 100g. of Bakers' Yeast

	Protein (mg)	Total activity (sp.act. x protein)	Sp.act. (mμmoles palmitate formed/ mg.protein/minute)
First Ammonium sulphate fraction	2,700	10,125	3.75
Calcium Phosphate gel eluate	215	2,623	12.2
Second Ammonium sulphate fraction	112	2.049	18.1
Supernatant from Alumina gel	64	1,888	29.5

The Yeast was disrupted by grinding with sand and solid

Carbon dioxide

These procedures are summarised in table 5.

3 Fatty acid synthetase from pigeon liver

Assays

a) Fatty acid synthesising activity

(i) The assay mixture contained 100 μmoles-potassium phosphate buffer, pH 7.0; 5.0 μmole-2-mercaptoethanol; 3 μmole-EDTA; 39 mμmole of [2-^{14}C] malonyl-CoA; 12.5 mμmole of acetyl-CoA, 0.3 μmole of NADPH and water and enzyme to give a volume of 1 ml.

The incubation mixture was equilibrated at 38°C in a 50ml. pyrex glass centrifuge tube, and the reaction was started by adding the enzyme (about 3 μg. of the purest preparations). After six minutes, 0.05ml. of 60% perchloric acid was added to stop the reaction. When the mixture had been diluted with one volume of ethanol, the long chain fatty acids were extracted with four 5ml. aliquots of light petroleum (b.p. 40 - 60°C); as described by Porter and Tietz (1957). This assay was used by Hsu, Wasson and Porter (1965).

ii) Later enzyme preparations (enzyme B) were assayed

by a similar method, but incorporated the modifications described by Butterworth, Yang, Bock and Porter (1967) and Butterworth, Guchhait, Baum, Olson, Margolis and Porter, (1966), and mercaptoethanol was replaced by 2mM-dithiothreitol.

A convenient and rapid assay depended on the oxidation of NADPH. The incubation, in a 1cm. cell contained the above assay mixture except that the concentration of NADPH was reduced to 0.2μmole/ml,

B HMG synthesising activity

The incubation mixtures for HMG synthesising activity consisted of : 1.0mg. enzyme; 2-mercaptoethanol (5mM), or later 2mM-dithiothreitol; [2-^{14}C] malonyl-CoA (50μM) and 50 μM-acetyl-CoA in a volume of 2ml. The incubations, at 30°C for 60 minutes were terminated by 1 ml. of 2N-KOH in 95%-methanol. The tubes were incubated at 60°C for 60 minutes to hydrolyse thioesters then the mixture was neutralised with hydrochloric acid and freeze dried. The dry salt was extracted with acetone and chromatographed on Whatman 3MM paper using the solvent described by Brodie and Porter (1960) and used by Brodie, Wasson and

Porter (1963): Isobutyric acid-0,1M-EDTA-3N-NH_4OH - Water, 62:1:12:28. Material chromatographing at the same R_f as authentic HMG was eluted with water and crystallised as the di-p-bromophenacyl ester.

When there were many samples to analyse, HMG-CoA was isolated from incubation mixtures by the method of Rudney and Ferguson (1959), (Methods D (3)). The latter procedure was eventually adopted as the standard one since the removal of salt by adsorbing the CoA esters onto charcoal greatly facilitated the subsequent chromatography.

c Assay for mevalonic acid formation

Incubation mixtures contained 1.0mg. enzyme, potassium phosphate buffer (100mM), 5mM-2-mercaptoethanol, 50μM-[2-^{14}C] malonyl-CoA, 50μM acetyl-CoA, unlabelled mevalonate (20μmole) and 0.3μmole NADPH in a volume of 2ml. The extraction procedure followed that of Lynen and Grassl. (1959). The incubation was terminated by 0.5ml. 5N-sulphuric acid and the solution was saturated with sodium sulphate. The mixture was extracted with 10 2ml. aliquots of diethyl ether. Mevalonate was isolated by celite column chromatography (Methods, D (3)) or by

paper chromatography using ethanol - ammonia - water, 7:2:1. (Chesterton, 1966).

Purification of the enzyme

Method A. (Hsu, Wasson and Porter, 1965)

1. Preparation of the particle free supernatant.

200g. of pigeon liver was homogenised in a blendor at full speed for 30 seconds with 300ml. of a buffer which contained 0.1M-potassium phosphate pH 8,0; 0.07M- potassium bicarbonate; 1.0mM-EDTA and 1.0mM-2-mercaptoethanol. The homogenate was centrifuged at 1,500g for 15 minutes and the supernatant decanted through a double layer of muslin. The solution was centrifuged at 104,000g for 60 minutes. The particle free supernatant was divided into 40ml. portions and stored at -15°C under nitrogen.

1st ammonium sulphate fractionation

80ml. of pigeon liver supernatant solution was thawed and brought to 25% of saturation with saturated ammonium sulphate. Ammonium sulphate solutions were at 0°C and contained 3mM-EDTA and 2mM-2-mercaptoethanol.

The precipitate was removed by centrifuging at 15,000g for 30 minutes. The protein that was insoluble when the ammonium sulphate concentration was increased to 40% was dissolved in 5mM-potassium phosphate buffer, pH 7.0, to give a volume of 400ml.

Calcium phosphate gel

This solution was treated immediately with calcium phosphate gel to give a gel protein ratio of 1:2. The solution was gently mixed with a glass rod, and immediately centrifuged for 5 minutes at 4,000g. If mixing and centrifugation were not performed rapidly, much enzymic activity was lost. In a similarly rapid fashion, the gel was washed by 10ml. of 50mM-potassium phosphate buffer, pH 7.0.

DEAE-Cellulose chromatography

The enzyme solution was divided into two, and was applied to two DEAE-cellulose columns, each 10 x 3.8 cm. The columns were washed with the same buffer until the optical density of the eluate at 280mμ had fallen to less than 0.2. Active enzyme was eluted by 0.25M-buffer

at the same pH and appeared about 70ml. after the buffer had been changed. It was usually completely eluted by 130ml. This enzyme solution, referred to by Brodie *et al.* as "DEAE enzyme" was used in some experiments.

2nd ammonium sulphate fractionation

The active eluate from the DEAE-cellulose columns was brought to an ammonium sulphate concentration of 26% by adding saturated ammonium sulphate solution at pH 7.0 (containing 2-mercaptoethanol and EDTA). The precipitate was discarded. The protein that was insoluble at 32% of saturation was retained and dissolved in a minimal volume of 0.25M-potassium phosphate buffer, pH 7.0 containing 2-mercaptoethanol and EDTA.

G-100 Sephadex

A column of G-100 sephadex (22 xlcm.) was equilibrated with 0.25M-potassium phosphate buffer containing 3mM-EDTA and 2mM-mercaptoethanol. The enzyme from the previous stage was applied and eluted with the same buffer at a flow rate of 15ml./hr., and collected in 0.5ml. fractions.

The purified enzyme was not stable, and experiments with it were performed immediately.

Method B

The stages in the preparation of the pigeon liver fatty acid synthetase by this method were the same as in that described above. The procedure was modified in that all buffers and solutions now used 1mM-dithiothreitol instead of mercaptoethanol. Yang, Butterworth, Bock and Porter (1967) showed that dithiothreitol stabilised the enzyme and markedly increased its specific activity. DEAE-cellulose chromatography was modified in that protein was applied to a column 2 x 25cm., and eluted by a linear gradient of phosphate buffer 0.05 - 0.2M. The enzyme was eluted at 0.1 - 0.15M-phosphate.

Table 6 compares the best preparations obtained by each method. It should be noted that in this particular preparation of enzyme B, livers were obtained from pigeons deprived of food for 24 hours, and then refed for 24 hours. From other preparations the activity usually obtained by method A was 37 - 44mμ mole/ palmitate formed/mg.protein/min. and from method B, 65 -

- 72 mμmole/mg. protein/min.

Table 6

A Comparison of the Most Active Preparations of Pigeon Liver Fatty Acid Synthetase prepared by Method A and Method B

	Protein (mg.)		Total Units		Specific Activity		Yield(%)	
	A	B	A	B	A	B	A	B
PARTICLE FREE SUPERNATANT	3,450	4,110	8,280	51,375	2.4	12.5	100	100
1st $(NH_4)_2SO_4$ FRACTIONATION	1,026	1,350	5,027	24,570	4.9	18.2	61	48
CALCIUM PHOSPHATE GEL	847	982	4,066	13,453	4.8	13.7	49	26
DEAE - CELLULOSE	92	115	2742	7,498	29.8	65.2	33	15
2nd. $(NH_4)_2SO_4$ FRACTIONATION	42	78	2,436	7,137	58.0	91.5	29	14
G-100 SEPHADEX	16.5	48	1,025	5,928	62.1	123.5	12	11.5

Total units = Specific activity x Protein

Specific activity calculated as mμmoles palmitate formed/mg. protein/minute.

Fatty acid synthesis was assayed by the radiochemical procedure in both preparations.

4. HMG-CoA Cleavage enzyme (β-hydroxy-β-methyl glutaryl-CoA acetoacetate lyase E.C.4.1.3.4.)

This enzyme was used for the initial stage in the degradation of HMG-CoA. Stegink and Coon (1968) have published details of a purification procedure which gave an enzyme of high specific activity and a 2,000 fold purification from bovine liver. However, Brodie et al. (1964) used a preparation donated by M. J. Coon and apparently prepared by his earlier procedure, (Bachhawat, Robinson and Coon, 1955)

Assay (Stegink and Coon, 1968)

1. HMG-CoA $\longrightarrow$ Acetyl-CoA + Acetoacetate
2. Acetyl-CoA + Oxalacetate + H_2O $\longrightarrow$ Citrate + CoASH + H^+
3. L-malate + NAD^+ $\rightleftharpoons$ Oxalacetate + NADH + H^+

HMG-CoA + L-malate + NAD^+ + H_2O $\rightleftharpoons$ Citrate + acetoacetate + CoASH + NADH + $2H^+$

Since reactions 1. and 2. are essentially irreversible, the equilibrium of the over all reaction lies far to the right. Thus the rate of NAD^+ reduction, when there is an excess of HMG-CoA, citrate condensing enzyme, malate, and

malate dehydrogenase depends on the rate of cleavage of HMG-CoA.

NADPH was added to the incubation mixture to facilitate the complete conversion of malate to citrate (Pearson, 1965).

The assay mixture consisted of 200 μmole-Tris/HCl buffer, pH 8.1; 10 μmole-$MgCl_2$; 5 μmole-cysteine, pH 7.0; 2.5 μmole-L malate; 1.4 μmole-NAD^+; 0.05 μmole-NADH; 2μg. citrate synthetase; 2μg. malate dehydrogenase; 0.7 μmole HMG-CoA and cleavage enzyme and water to give a total volume of 1.0ml. The reaction was started by the addition of the HMG-CoA after the mixture had equilibrated at 37°C.

Preparation of the Enzyme

To enable direct comparison with the results of Rudney and Ferguson (1959), and of Brodie et al. (1964), the enzyme was prepared as described by Bacchawat et al. (1955).

Chilled, fresh pig hearts were defatted and minced. Each 500g. of the minced tissue was blended with 500ml.

0.5M-potassium chloride for three minutes at top speed. The suspension, diluted with 500ml. 0.5M-potassium chloride and 10ml. 1M, pH 7.4 potassium phosphate buffer was stirred for thirty minutes.

The mixture was transported to a room maintained at -20°C and 1.0 l. of a solution of ethanol-0.5M-potassium chloride (1:1 v/v) was added, with stirring, over a period of one hour. The mixture was centrifuged at 1,000g (-5°C) for two hours. The red supernatant, which was quite clear, was dialysed for 16 hours against 40.0l. of 0.04M-potassium chloride which contained 0.025M-potassium phosphate buffer, pH 7.4 and 1mM-cysteine.

45.4g. of solid ammonium sulphate was added slowly for each 100ml. of extract. After the salt had completely dissolved the mixture was stirred for another thirty minutes and then centrifuged at 10,000g for 15 minutes. The supernatant was discarded, while the precipitate was dissolved in the minimum of 0.1M Tris/HCl buffer, pH 7.4 50.8g. of solid ammonium sulphate

was added to this solution which had been diluted to 150ml. with the same buffer. The precipitated protein was collected and redissolved in the Tris buffer containing cysteine, against which it was dialysed for 24 hours.

After diluting the enzyme solution with an equal volume of distilled water, 1M-acetic acid was added carefully, with constant stirring, to bring the pH to 5.8 The mixture was stirred for 15 minutes, allowed to stand for 1 hour and then centrifuged at 20,000g for 30 minutes. The sediment was dissolved in 30ml. of 0.1M Tris/HCl pH 7.4 containing 1.0mM-cysteine.

4.6ml. of saturated ammonium sulphate solution ($4^{o}C$) was added and the precipitate was discarded. The precipitate obtained when 9.7ml. of the salt solution was added was retained and dissolved in 10ml. Tris/Hcl pH 7.4, 1mM-cysteine.

68mg. of protein with a specific activity of 0,17 µmole/min/mg. protein was obtained from 500g. of minced heart. The solution in Tris buffer was stored frozen

at -15°C.

5 HMG-CoA condensing enzyme (β-hydroxy-β-methyl glutaryl--CoA acetoacetyl-CoA lyase (CoA-acetylating E.C.4.1.3.5.)

Degradation of ^{14}C-HMG-CoA with HMG-CoA cleavage enzyme required the preparation of ^{14}C-HMG-CoA of known radioactivity and in which the labelled carbon atoms were in known positions. This was possible by employing HMG-CoA condensing enzyme to make HMG-CoA from ^{14}C-acetyl -CoA and unlabelled acetoacetyl-CoA.

Assay

The assay (Stewart and Rudney, 1966a) depended on the disappearance of the enolate absorption of acetoacetyl-CoA at alkaline pH (Stern, Ochoa and Lynen, 1952). Assay mixtures contained 200μmoles Tris/HCl, pH 8.2; 0.2μmole acetoacetyl-CoA enzyme and water to 1.5ml.

The rate of deacylation of acetoacetyl-CoA was followed for three minutes in a Beckman DB spectrophotometer fitted with a chart recorder. 0.2μmole of acetyl-

CoA was added and the increase in the rate of decrease in optical density was observed.

The molar extinction coefficient of acetoacetyl--CoA at pH 8.2 and 310mμ was taken as 3,600 (Stern et al, 1952)

Isolation of the enzyme from bakers' yeast

The procedure adopted was described by Ferguson and Rudney (1959).

Bakers' yeast was stirred with toluene for 4.5 hours (40ml. toluene/500g. yeast) at 37°C. After this time the yeast had lysed and was quite liquid. The mixture was diluted with an equal volume of cold water and centrifuged at 12,000g for 20 minutes.

Dowex - 1 resin, in the bicarbonate form, was added to the supernatant solution. 1 ml. of packed resin (200 - 400 mesh) was added for every 160mg. of protein (absorption at 260 mμ and 280 mμ). The mixture was agi-

tated for 20 minutes when, after the resin had settled, the supernatant was filtered through glass wool. 351g. of solid ammonium sulphate for each litre of filtrate was added, and the mixture stirred for 30 minutes after the ammonium sulphate had dissolved. The precipitate was removed by centrifuging and the ammonium sulphate concentration was increased by adding 103g. of salt to each litre of the solution. The precipitate was isolated after 60 minutes and was taken up in 50ml. 1mM-EDTA. The suspension was dialysed for 2 hours against four changes, (2 l.) of 1mM-EDTA. The protein concentration was adjusted to 10mg./ml. (biuret), and calcium phosphate gel was added to give a gel-protein ratio of 7:18. The suspension was stirred for 15 minutes and the gel, which had adsorbed HMG-Condensing enzyme, was separated by centrifuging for 10 minutes at 10,000g.

The enzyme was eluted by suspending the gel twice, for 30 minutes, in 75ml. of 0.3M-potassium phosphate buffer, pH 7.5. The nucleic acid content of the solution was determined spectrophotometrically (Layne, 1957). 2% protamine sulphate solution was added, in stages of

0.01ml/mg. nucleic acid until the OD_{280}/OD_{260} exceeded 1.3:1. Each precipitate was removed by centrifuging.

The protein was recovered by adding solid ammonium sulphate until the solution was 80% saturated. The precipitate obtained in this way and stored at -15°C under saturated ammonium sulphate solution at pH 7.0 lost approximately 10% of its activity overnight.

Removal of Thiolase activity

The ammonium sulphate precipitate was dissolved in a minimal volume of water and dialysed for 2 hours against 1mM-EDTA. The protein concentration was adjusted to 10mg./ml. (biuret). 10mM-iodoacetamide was added to give a final concentration of 1mM. The mixture was incubated at 37°C, samples being withdrawn every five minutes to assay for thiolase activity (CoASH was substituted for acetyl-CoA in the assay mixture). When this activity had disappeared (20 minutes and 25 minutes in the two preparations), neutralised cysteine was added to give a final concentration of 5mM.

Protein was precipitated by pouring the solution into saturated ammonium sulphate at pH 7.0 and 0°C. This precipitate was dialysed against 1mM-EDTA and adsorbed again onto calcium phosphate gel. Protein was eluted by two 30ml. aliquots of 0.2M-potassium phosphate buffer, pH 7.6.

The eluant was precipitated with saturated ammonium sulphate and when stored under a neutralised saturated solution of this salt at -15°C, the enzyme lost only 8% of its activity in six weeks.

The procedure is summarised in table 7.

At some stages of the purification, the recoveries of activity were more than 100% of the previous stage. This was probably due to the removal of competing enzymes, such as thiolase, and acetoacetyl-CoA deacylase.

Table 7

Summary of the Purification of HMG-CoA Condensing Enzyme from 600g. of Bakers' Yeast

Yeast	Total Protein (mg.)	Apparent sp.acy. units/mg.protein	Total activity($\times 10^3$)	Yield%
Crude yeast supernatant	21×10^3	0.7	147	"100"
Dowex-1-bicarbonate	17.2×10^3	1.6	275	187
1st. $(NH_4)_2SO_4$ fractionation	5.75×10^3	5.3	304	207
1st. calcium phosphate fractionation	1.2×10^3	16.5	198	13.4
Protamine sulphate	870	21.1	184	12.5
Iodoacetamide	742	8.2	608	4.1
2nd. calcium phosphate gel fractionation	67	33.4	224	1.6

The unit was defined by Rudney & Ferguson (1959) as corresponding the the utilisation of approximately 5mμmole of acetoacetyl-CoA/minute.

6 Cell free yeast system

The yeast system described below was used in the initial studies on the incorporation of labelled substrates into lipids, for the squalene labelling experiments as well as for measurements of decarboxylase activity and the effect of avidin.

Growth of yeast

Bakers' yeast cultures (derived from commercial samples supplied by the Distillers. Co. Ltd.) were maintained on 3% agar slopes containing malt extract (0.3%), 1%-glucose and 0.5% - peptone, yeast extract (0.3%).

Yeast was grown in 2.0 l. flasks each containing 500ml. of medium which consisted of (per litre in distilled water) :-

3.0g.-KH_2PO_4; $MgSO_4 7H_2O$ - 1.0g.; 0.25g.-$CaCl_2 5H_2O$; $MnSO_4 7H_2O$ - 2.75mg.; $FeSO_4 7H_2O$; 0.5mg; 20g. - glucose; $(NH_4)_2 SO_4$ - 2.0g.; 2.0g. - yeast extract; D-biotin - 0.01mg; Thiamine hydrochloride - 1.0mg, and $CuSO_4 . 5H_2O$-0.10mg.

The flasks were incubated at 30°C for two days on a rotary shaker when the yeast was harvested by centri-

fuging at 1,500g for 30 minutes. The cells were washed once with distilled water at 4°C.

Disruption of yeast

The pellet, from two flasks, which contained about 15ml. of packed cells was suspended in a half volume potassium phosphate buffer (0.1M, pH 7.0). frozen and disrupted in a Hughes' Press. The broken yeast was transferred to a plastic centrifuge tube where it was allowed to thaw. An equal volume of buffer was added and the suspension was centrifuged at 2,000g for 30 minutes. The brown, strongly turbid supernatant was carefully withdrawn by a teat-pipette and transferred to another tube in which it was centrifuged at 12,000g for 60 minutes.

The supernatant solution was used as the enzyme system, and contained all the enzymes necessary for the incorporation of acetate into fatty acids and unsaponifiable lipids.

Incubations.

Aerobic incubations were performed in pyrex glass centrifuge tubes fitted with ground glass stoppers.

Incubations could then be terminated with the lipid extracting solvents. If the chloroform-methanol procedure was being employed, the extractions were completed without the transfer of the mixture to other vessels.

Anaerobic systems were incubated in Warburg vessels in a Warburg shaker. The mixture was gassed beforehand, and the apparatus was continually flushed with nitrogen.

7 Rat liver enzyme systems

a) Knauss, Porter and Wasson (1959) and Witting and Porter (1959) described a rat liver system that contained the enzymes necessary to convert acetate into squalene and sterols. This preparation was adopted to study the biosynthesis of squalene from malonyl and acetyl derivatives.

Preparation of the enzyme system

Livers were removed from freshly killed rats and chilled in ice cold homogenising buffer:- 0.1M-potassium phosphate, pH 7.4; 30mM-nicotinamide and 4mM-$MgCl_2$. The livers were blotted and rapidly weighed, then homogenised with an equal volume of buffer in a Potter-Elvehjem

homogeniser with a loosely fitting teflon pestle. Six full strokes only were used. The homogenate was strained through a nylon mesh to remove large particles, blood vessels and connective tissue, and then centrifuged at 1,000g for 15 minutes to remove unbroken cells and nuclei. The supernatant was carefully transferred by a Pasteur pipette to a second tube and centrifuged for 15 minutes at 9,000g. The supernatant from this centrifugation was centrifuged for thirty minutes at 104,000g.

The pellet from the high speed spin clearly consisted of an opalescent lower layer, presumably glycogen, and an upper layer which was red and almost transparent. The latter was carefully resuspended in buffer by a glass spatula, and then homogenised in a small Potter-Elvejhem homogeniser. This fraction was centrifuged again at 104,000g for 30 minutes and resuspended in 0.1 volume of buffer with respect to the 9,000g supernatant using a Potter homogeniser powered by hand. This fraction was referred to as "microsomal fraction"

The clear red supernatant was used as the source of soluble enzymes. Saturated ammonium sulphate solution (pH 7.0, 0°C) was added to give a solution that was 40% saturated with this salt. After stirring for 15 minutes the precipitated protein was collected by centrifuging and discarded. Sufficient ammonium sulphate solution was added to give 80% saturation and after stirring for thirty minutes the protein precipitate was collected. The precipitate was dissolved in a minimum of buffer and dialysed for two to three hours against 5mM-potassium phosphate, pH 7.0.

These enzyme fractions were invariably used on the day of preparation, although Knauss et al. (1959) reported that they could be stored for up to twenty days on solid carbon dioxide.

Incubations

Details of the incubation systems are given with the experimental results. Usually, the system described by Knauss et al. (1959) was used without modification.

Anaerobic incubations were done in tightly

stoppered glass centrifuge tubes. Oxygen free nitrogen was bubbled through the incubation mixtures at 37°C for fifteen minutes before the addition of the enzyme - which over a stream of nitrogen had been passing. After the incubation had been started by adding the enzymes, the tubes were flushed with nitrogen and were tightly stoppered.

Incubations were terminated and the lipids extracted by 3.75 volumes of chloroform-methanol (1:2), containing squalene and/or cholesterol. The total lipid extracts were saponified, and squalene and cholesterol were separated from the unsaponified fraction on columns of deactivated alumina. The radioactivity of the squalene was determined, as the hexahydrochloride, and of the cholesterol as the digitonide.

b Rat liver unwashed microsomal system

Rudney (1957) described this system which was very active in the biosynthesis of HMG.

The microsomal pellet obtained as described above was resuspended in 1mM-EDTA. This preparation was used to study the synthesis of ^{14}C-HMG-CoA.

C. Preparation of a rat liver system in an isotonic medium

In the light of experiments which suggested that malonyl-CoA decarboxylase may be concentrated in the mitochondrial fraction, a modified rat liver system was used in an attempt to preserve mitochondrial integrity and thus lower the level of malonyl - CoA decarboxylase in the soluble protein and microsomal fractions. Scholte (1969) has published a detailed study of the subcellular distribution of malonyl-CoA decarboxylase, and his conclusion is that it is primarily a mitochondrial enzyme.

Tissue fractionation

A rat liver was fractionated essentially as described by DeDuve, Pressman, Gianetto, Wattiaux and Applemans (1955), as modified by Sedgwick and Hubscher (1965) and Michell and Hawthorne (1965).

The liver of a rat, which had fasted for twelve hours prior to death, was blotted weighed and cut into small pieces. The tissue was homogenised in three volumes of 0.3M sucrose-2mM-EDTA and then diluted with an equal volume of this solution. The homogenate was centrifuged at 1,000g for ten minutes. The sediment discarded the

supernatant was filtered through two layers of fine nylon mesh and then recentrifuged at 4,300g for ten minutes. The sediment was designated the mitochondrial fraction which was washed twice with the sucrose-EDTA solution. The 4,300g supernatant, together with mitochondrial washings, was centrifuged at 11,700g for twenty minutes to give a lysozomal fraction which was washed once and discarded. The 11,700g supernatant combined with the washing was centrifuged at 104,000g to give a microsomal fraction and a particle free supernatant. The former was washed once.

In some experiments, the mitochondrial suspension was sonicated for thirty seconds, and then recentrifuged at 4,300g. The supernatant of this preparation was recentrifuged at 104,000g for 60 minutes to give a broken mitochondrial fraction.

8 Assay and Purification of Avidin.

Assay

A rapid and sensitive assay for avidin was described by Green (1963), which depends on the difference between the ultra violet absorption spectra of avidin and the avidin-biotin complex. This assay was made possible by the preparation of pure avidin by Melamed and Green (1963) which was shown to bind three moles of biotin per mole, and to have a molecular weight of 53,000.

Crystalline biotin was obtained from Sigma Ltd. This material melted sharply at 230-231°C (uncorrected). The published melting point is 232-233°C (The Merck Index, 7th edition, p.171).

Measurements were made by a Unicam S.P.700 recording spectrophotometer. Avidin solution, in 50mM-phosphate buffer pH 6.8, was placed in both reference and sample cuvettes. Small aliquots of biotin were added to the sample and an equal volume of buffer to the reference cell. The difference spectrum was measured between each addition and the amount of biotin required

to saturate the avidin was determined.

Assuming the molecular weight of avidin to be 53,000 and one mole of avidin to combine with three moles of biotin, the purity of the avidin could be determined easily. An assay is shown in Figure 3.

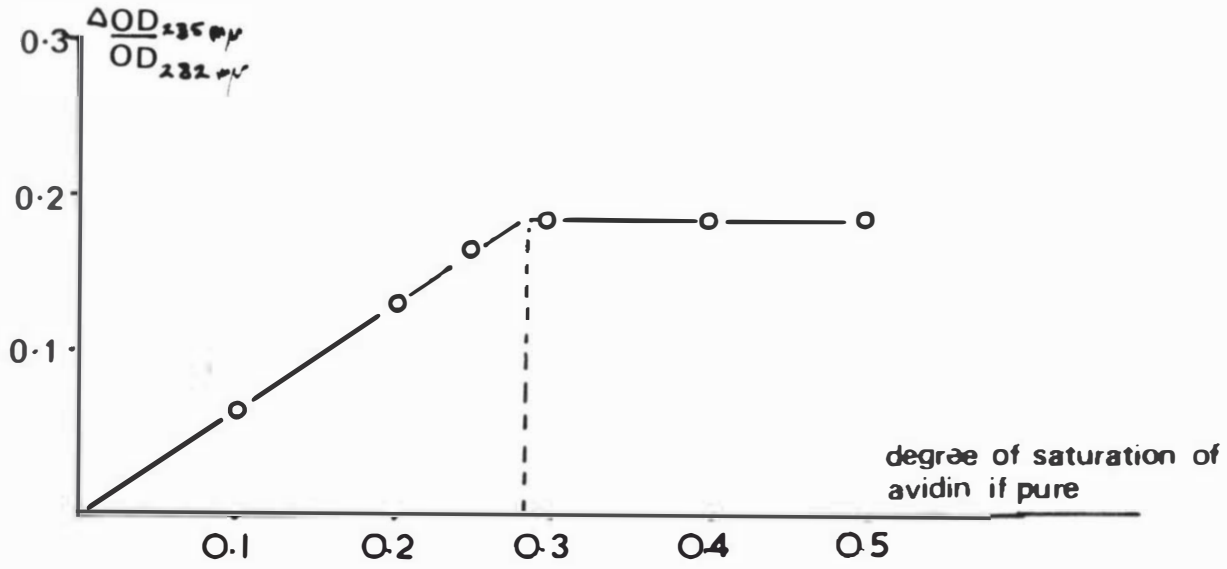
Purification

Avidin was obtained from the Nutritional Biochemicals Corporation and was purified by chromatography on columns of CM-cellulose.

The crude avidin was dissolved, with difficulty, in distilled water (10mg./100ml.). A filter cake of moist CM-cellulose, which had been washed with 2mM-EDTA and then water after the standard precycling procedure, was stirred into the solution (5.0g. of dry cellulose). The suspension was brought to pH 6.3 by 2N-sulphuric acid, and then stirred for 30 minutes. The cellulose was allowed to settle, the supernatant was carefully decanted. The latter was treated with another batch of 2.5g. of CM-cellulose. The combined batches of CM-cellulose with its

Fig 3

Spectrophotometric Titration of Avidin with Biotin



Avidin mμ moles	Biotin	$\Delta OD_{235m\mu}$	$OD_{282m\mu}$	$\frac{\Delta OD_{235m\mu}}{OD_{282m\mu}}$
8 0	2·4	0·030	0·450	0·066
"	4·8	0·060	0·450	0·132
"	6·0	0·073	0·440	0·165
"	7·2	0·080	0·435	0·184
"	9·6	0·077	0·425	0·181
"	12·0	0·076	0·415	0·183

1 mole impure avidin ≡ 0·855 mole biotin.

1 mole pure avidin ≡ 3·0 mole biotin

∴ avidin is 28·5 % pure

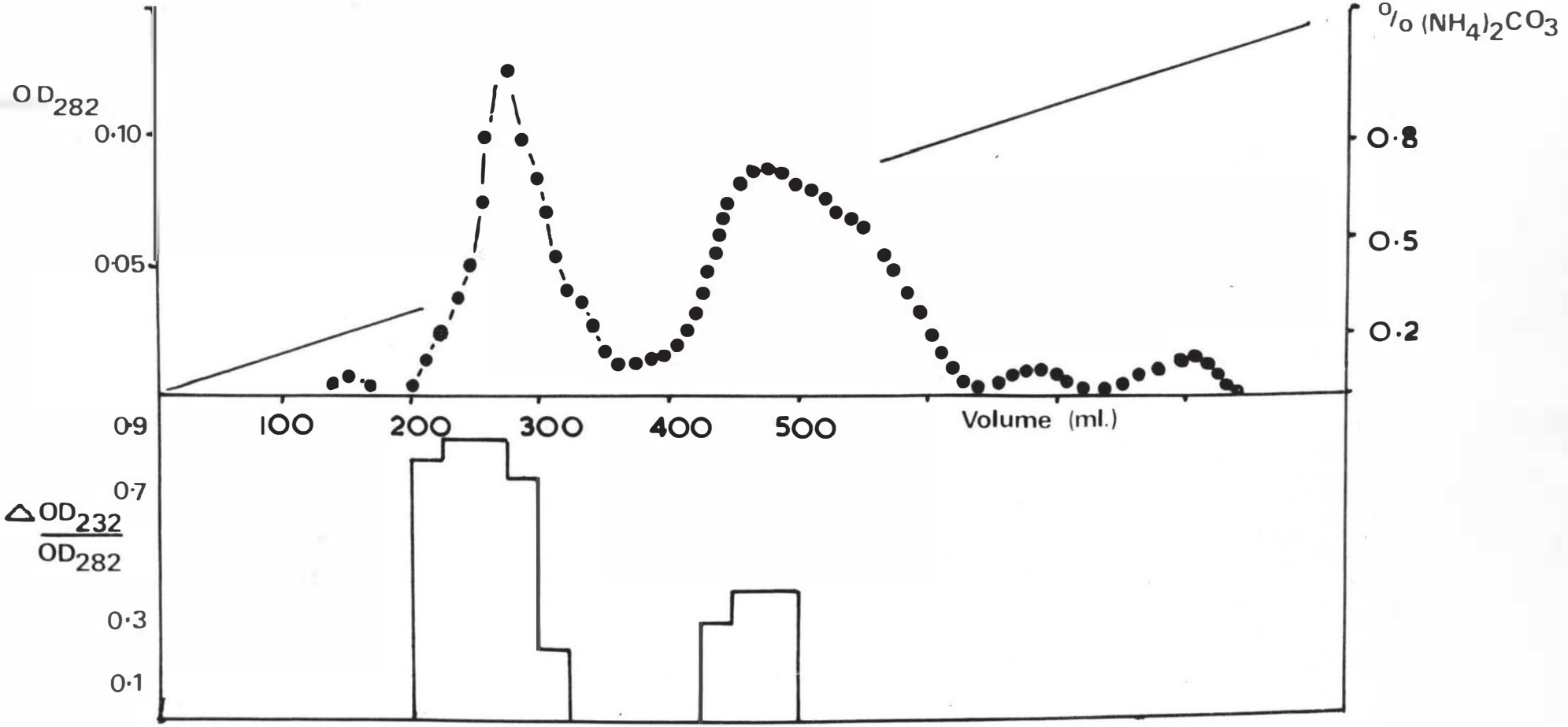
adsorbed avidin, was suspended in 25mM-ammonium acetate buffer, pH 9.0 and poured into a column which contained fresh CM-cellulose (2.5g. dry weight) The column was allowed to pack under gravity and was washed with 500ml. acetate of ammonium solution.

Protein was eluted by a linear gradient of ammonium carbonate (0.1% - 1%) in a total volume of one litre (figure 4).

The eluted peaks were neutralised, degassed and freeze dried.

Two separate batches of avidin were treated in this way; the initial purity of which were 28% and 18% avidin. This was raised to 90-95% in both cases as determined by the spectrophotometric assay.

Chromatography of Commercial Avidin on CM-Cellulose Fig. 4



See text for details of method. Approximately 10 mg. crude avidin was chromatographed on a 2 x 20cm. column.

9 Assays of malonyl-CoA decarboxylase

a. Spectrophotometric procedure

The spectrophotometric procedure was useful in that it gave a measure of the initial rate of a reaction, and avoided the use of radioactively labelled malonyl-CoA. However, in general the radiochemical assay was preferred. This gave very reproducible results and was a much simpler system to prepare.

The continuous spectrophotometric assay depends on the reaction of acetyl-CoA, formed by the decarboxylation of malonyl-CoA with oxalacetate to form citrate. In effect, the assay is identical to that used for the assay of HMG-CoA cleavage enzyme (see Experimental Section I B(4).)

malonyl-CoA $\longrightarrow$ Acetyl-CoA + CO_2

acetyl-CoA + oxalacetate + $H_2O \longrightarrow$ citrate + CoA + H^+

L-malate + $NAD^+ \rightleftharpoons$ oxalacetate + NADH + H^+

Malonyl-CoA + L-malate + NAD^+ + $H_2O \rightleftharpoons$ citrate + CO_2 + NADH + H^+

In a 1cm. silica cell, the reaction mixture was 50 µmole potassium phosphate buffer, pH 7.5, 2mM-dithiothreitol, 2mM-EDTA, 2.5 µmole-L-malate, 1.4 µmole-NAD^+, 0.05 µmole NADH, 2µg.-malate dehydrogenase, 2µg. citrate synthase, and 5mg. of bovine albumen in a total volume of 1 ml.

The mixture was equilibrated at 37°C. The change in optical density at 340mµ when the decarboxylating enzyme was added was noted. The protein concentration was adjusted with buffer to give a linear rate for 5 minutes.

NADH was added so that the equilibria gave rise to complete convertion to citrate, (Pearson, 1965).

b. Radiochemical Procedure

The radiochemical assay also used the citrate cleavage enzyme to convert acetyl-CoA into citrate. This was necessary to avoid a possible recarboxylation of acetyl-CoA in crude systems which may have contained acetyl-CoA carboxylase.

The incubation mixture contained 300μmole of potassium phosphate buffer, pH 6.8, 2.0mM-dithiothreitol, 2.0mM-EDTA, 30μmole of oxalacetate, 5μg. citrate synthase of specific activity 170μmole/min./mg. protein., 200mμmole of (1-^{14}C) malonyl-CoA and 6mg. of bovine albumin.

The reaction, in a volume of 1 ml. was started by the addition of the decarboxylating enzyme. The mixture was incubated for 30 minutes at 37°C in a Warburg flask, the centre well of which contained 0.2ml. -hyamine hydroxide (1.0M, in methanol). After thirty minutes the reaction was stopped by 0.2ml. of 2N-hydrochloric acid tipped from a side arm. Control flasks which did not have decarboxylating enzyme were always incubated simultaneously because a significant amount of malonyl-CoA was decarboxylated non-enzymically by the acid used to terminate the incubation and to release the carbon dioxide.

The decarboxylase was diluted so that not more than 100mμmole of malonyl-CoA were decarboxylated during the course of the incubation.

10 Incubations of the Latex of Hevea brasiliensis

Fresh latex was obtained from Hevea brasiliensis plants cultivated under glass at a temperature of 28°C.

Natural light was supplemented by fluorescent lighting so that a constant length of daylight could be maintained.

Latex was obtained by making a diagonal cut through the bark to penetrate the latex vessels. The latex was collected in a teat pipette and transferred to a collecting tube. Often, more than one tree had to be tapped to obtain sufficient fluid for an experiment. The latex samples were combined and thoroughly mixed before being added to the previously prepared incubation tubes.

The substrates for each incubation was pipetted into 3ml. incubation tubes and frozen. The tubes were dried in vacuo, weighed and transported to the greenhouse where a measured volume of latex was added to each from a fast flowing graduated pipette. The tubes were gently shaken to dissolve the substrate.

EXPERIMENTAL

1 Methods

C Preparation and Purification of Coenzyme A and Coenzyme A thioesters.

a) Isolation and Purification of Coenzyme A

Coenzyme A was first detected as such in liver preparations (Lipmann, 1945) where it was shown to be necessary for the acetylation of aromatic amines. Lipmann and Kaplan (1946) subsequently showed that the cofactor responsible for the acetylation of choline in the brain was identical with the liver coenzyme. Impure preparations were obtained from Hog liver by precipitating the mercury and barium salts, (Lipmann, Kaplan, Novelli, Tuttle and Guirard, 1950). DeVries, Govier, Evans, Gregory, Novelli, Soodak and Lipmann (1950) used charcoal columns in obtaining CoASH from Streptomyces fradiae. This technique was extended by Beinert, Von Korff and Green (1953) and by Kornberg and Stadtman (1953) in isolation procedures from yeast and by Gregory, Novelli and Lipmann (1952) from S.fradiae. The latter method involved a reductive step in the procedure hence eliminating the mixed disulphides of CoA.

The best readily available source is yeast (Beinert et al. 1953) and an isolation procedure which involves chromatography on charcoal and on Dowex-1(formate) but

no reduction was described in detail by Kornberg and Stadtman (1953, 1957). The essentials of the procedure described by these authors were adopted, but modified extensively in detail.

Assay

Thiol-group assay

Ellman (1959) observed that di-(5-carboxy-4-nitro phenyl) disulphide was reduced by sulphydryl groups to an aromatic thiol with an intense yellow colour. Jocelyn (1962) described a procedure for the estimation of non-protein thiols, utilising this reaction.

The thiol sample in aqueous solution (0.5ml.) was mixed with 1.5ml. of the thiol reagent (1mM, in pH 6.8 phosphate buffer, I = 0.2.) The yellow colour was read at 412 mμ in a Unicam SP. 500 spectrophotometer. Glutathione (Sigma Ltd) or N-acetyl cysteine (B.D.H. Ltd) were used as standards.

b. Enzymic Assay

An assay used by the earlier workers relied on the CoA dependent acetylation of sulphanilamide by a

pigeon liver enzyme, in the presence of ATP. This is a fairly sensitive and rapid assay using readily available starting materials, but suffers from the disadvantage that dephospho-CoA can be rephosphorylated by the pigeon liver enzymes used in the assay. Dephospho-CoA is therefore assayed by this system (Wang, Shuster and Kaplan, 1952). An assay which utilises the CoA dependent arsenolysis of acetyl phosphate by the transacetylase from Clostridium kluyveri does not register dephospho-CoA (Stadtman, Novelli and Lipman, 1951).

Despite this objection, the acetylating enzyme system was used. The enzyme system and all materials were readily available. The biological acitivity of the CoA was tested by the acetyl-CoA synthetase enzyme.

The assay procedure is fully described by Novelli (1957).

Isolation of CoA

a. Extraction of yeast

Pressed bakers yeast was added to boiling water

(5 volumes). The suspension was continually stirred, brought back to boiling point and boiled vigorously for 5 minutes. The boiling suspension was poured on to an equal volume of crushed ice. Cellular debris was removed by centrifuging the mixture at 2,000g for thirty minutes.

b. Adsorption on Charcoal

Columns of charcoal were prepared from B.D.H. animal charcoal, labelled 'Granular, for Filters'. The granules were added to excess of 4N - hydrochloric acid (1:10 w/v). When effervescence had ceased a vacuum was applied (filter pump) until the charcoal had settled. This usually took 24 - 36 hours. The supernatant was removed and the granular charcoal poured into two-litre measuring cylinders containing distilled water. When the granules had settled the supernatant was decanted. Small particles were removed in this way. The granular charcoal was packed in two columns (each 5cm. x 20cm), which were then washed with distilled water until the pH of the effluent had reached 5.

The dark yellow turbid yeast extract was acidified to pH 3.0 with hydrochloric acid and passed through a

column, the effluent of which was passed through the second. The white, milky effluent was discarded, and the column was washed with 10.1 of distilled water and 1 l. of an aqueous solution of acetone (40% v/v).

Coenzyme -A was eluted by 40% aqueous acetone containing 1 ml. 0.880 ammonia/l. (Table 8).

c. Precipitation by acetone

The fractions containing CoA were pooled and acidified (pH 1.7) with 5N-hydrochloric acid. Six volumes of cold acetone (4°C, technical grade) were added and the precipitate was allowed to form for three days at 4°C. The acetone powder was recovered by filtration through a double layer of Whatman 544 filter paper on a Buchner funnel, washed with cold acetone (A.R.) followed by cold diethyl-ether and then dried in a desiccator over phosphorus pentoxide. Recovery of CoA at this stage was usually about 65%.

d. Dowex-1-formate chromatography

Dowex-1 (x2) 200-400 mesh resin was washed three times by suspending in 3N-hydrochloric acid (1:6 w/v),

allowing the resin to settle and decanting the supernatant. The resin was transferred to a column (4 x 15 cm) and washed with 3 M-sodium formate (1 l.) and distilled water (5.l.). The acetone powder was dissolved in 100ml. water and brought to pH 8.1 with 2N-KOH. The solution was clarified by centrifuging and applied to the column which was then washed with 250ml. of water. The column was developed and eluted with 0.6M-formic acid - 0.3M-ammonium formate. Fractions of 200ml. were collected.

e. Concentration

The CoA containing fractions were pooled and passed through a second charcoal column (2cm x 20cm). CoA was eluted with ammoniacal acetone as before.

Ammonia and acetone were removed from the solution by rotary evaporation, and the aqueous solution of CoA was freeze dried and stored at -15°C.

f. Chromatography on DEAE-cellulose

10mg. of the dry powder was dissolved in water (10ml.) and reduced by adding 0.5ml. of an 0.5% solution of potassium borohydride in distilled water. After heating in a boiling water bath for one minute, the mixture

was cooled in an ice/salt bath. Excess potassium borohydride destroyed by 0.5ml. of 1% acetic acid, the solution was diluted to 25ml. and applied to a column of DEAE-cellulose which had been equilibrated with 0.02M-KCl, and 0.003N-HCl. The column was developed and eluted by a linear gradient of 0.02M-KCl to 0.15M-KCl in a total volume of 2l.

The fractions containing CoA were pooled and were passed through a column of Dowex-50 (H^+), and then reduced to a small volume by a rotary evaporator. The solution was freeze dried.

Analysis of sulphydryl content by the Jocelyn procedure and of adenine by absorption at 260mμ gave an adenine to sulphydryl ratio of unity.

Paper chromatography using absolute ethanol-0.1M-sodium acetate (1/1) and in isobutyric acid-ammonia (.880)-water-0.1M-EDTA (62:2.3:37.7:1) gave only one component that could be detected by observation in ultra violet light.

When compared with a commercial sample of CoA the samples purified here were 115% as active in the sulphanilamide assay (average of 5 preparations).

When used for the enzymic preparation of ^{14}C-acetyl-CoA using the acetyl-CoA synthetase enzyme, or for the preparation of unlabelled acetyl-CoA using acetic anhydride, the yield of product did not differ significantly from that obtained when commercially obtained CoA was used.

Table 8 Elution of Coenzyme-A from a Charcoal Column

Fraction (1l.)	pH	CoA (% of applied)
1	3.3	0
2	3.3	0
3	3.4	0
4	3.8	0
5	3.8	0
6	3.9	3
7	4.0	12
8	4.1	24
9	4.3	23
10	5.0	12
11	7.4	7
12	8.7	4
13	9.4	2
14	9.5	0
15	9.6	0

Table 9 Elution of Coenzyme-A from a Dowex-1-Formate column

Fraction (200 ml)	adenine µmoles	CoA µmoles
1	2,300	0
2	2,400	0
3	2,350	0
4	1,200	0
5	770	0
6	425	0
7	545	0
8	432	0
9	354	0
10	343	0
11	152	17
12	142	38
13	112	57
14	110	62
15	75	47
16	74	33
17	63	23
18	45	18
19	40	11
20	37	9
21	35	6
22	33	5

These data are presented in the same manner as the data of Kornberg and Stadtman (1957) to facilitate comparison.

Preparation and Purification of Coenzyme A Thioesters

1.(i) General Assays

a. Thiol Group

A sample of CoA thioester was incubated with 0.1 ml. of 0.1N-KOH at 50°C for 30 minutes. At this time the mixture was neutralised with 0.1 ml. of 0.1N-hydrochloric acid, and made up to a suitable volume with phosphate buffer, pH 6.8 I=0.2. Aliquots containing up to 0.3 μmole in 0.5ml. were assayed for sulphydryl groups using di-(5-carboxy-4-nitrophenyl) disulphide. Blank values determined from unhydrolysed samples, were subtracted.

b. Hydroxamate formation

Lipmann and Tuttle (1945) showed that esters readily form hydroxamate derivatives which give an intense red colour with ferric ions. The procedure is not applicable to acetoacetyl-CoA which forms methyl isoxazolone on reaction with hydroxylamine (Avena and Kuman, 1962) but was applied as described by Stadtman (1957) to assays of acetyl-CoA, malonyl-CoA and HMG-CoA.

(ii). CoA-Thioesters

0.5ml. of neutralised hydroxylamine reagent (freshly prepared by mixing equal volumes of 3.3M-hydroxylamine hydrochloride and 3.5M-sodium hydroxide) was added to 1 ml. of the thioester sample (0.5 - 3.0 μ moles). The solution was incubated for ten minutes at room temperature. 1.5ml. of ferric chloride reagent (10% $FeCl_3.6H_2O$, 3.3% trichloracetic acid in 0.66N-hydrochloric acid) was added. The colour that had developed after ten minutes was read against a reagent blank at 510 mμ, and the amount of hydroxamate formed was determined by comparison with parallel determinations of standard solutions.

(iii) Monothiophenol-malonate

Hydroxamate assay of monothiophenol-malonate was performed by a modification by Tame and Dils (1967) of the method described by Pilz. (1958).

1.0ml. of 1M-hydroxylamine hydrochloride, adjusted to pH 7.6 with potassium hydroxide, was added to 3.0ml. of ethanol-diethyl ether (3:1 v/v) containing 0.5 to 3.0 μ moles of sample. This was incubated at room temperature for thirty minutes when 0.5ml. of 1.35N-hydro-

chloric acid and 0.5ml. of 1.4M-$FeCl_3$ in 0.2N-hydrochloric acid, were added. The optical density at 540 mμ was measured after shaking.

Ultra Violet Absorption Assays

a. Difference in absorption at 232 mμ

Measurement was made of the decrease in optical density at 232 mμ when the thioester bond of CoA esters were hydrolysed, (Stadtman, 1953; Lynen 1953 and Lynen and Ochoa, 1953) The test solution containing 0.02 to 0.1 μmoles of thioester was diluted to 1 ml. with water in a silica spectrophotometer cell. The optical density at 232 mμ was measured. 0.1 ml. 2N-potassium hydroxide was added to the sample and reference cuvettes and allowed to hydrolyse the thioester for 60 minutes at room temperature. 0.1ml-2N hydrochloric acid was added to both cuvettes and the volumes made up to 2ml with 0.1M-phosphate buffer, pH 7.0. The optical density at 232 mμ was again measured. The concentration of thioester was calculated from the difference in Molar extinction coefficient between ester and CoA. ΔE_{232} was taken as 4.5×10^3 cm^2/Mole (Stadtman, 1957).

b. Absorption of acetoacetyl-CoA at 303 mμ

Lynen, Wessely, Wieland and Rueff (1952) discovered that acetoacetic thioesters absorb light strongly between 280 mμ and 320 mμ with maxima at about 303 mμ. Stern, Coon and Del Campillo (1956) showed that the intensity of absorption was greatly increased by the presence of M_g^{++}. The optical properties of acetoacetyl-CoA were investigated in detail by Stern (1956).

The ultra-violet absorption spectrum of the sample was determined at pH 8.2 in the presence of 5mM-$MgCl_2$ and 0.1M-Tris/HCl buffer, Hydrochloric acid was added to give a final concentration of 0.1N and the spectrum was again determined. The molar-extinction coefficient of the enolchelate absorption at 310 mμ was given by Stern (1956) as 11.4 x 10^3 cm^2/mole.

Assay of acetyl-COA using citrate synthetase and malate dehydrogenase enzymes

Acetyl-CoA was assayed by following the reduction of NAD^+ concomitant with the condensation of acetyl-CoA with oxaloacetate. The reaction was catalysed by citrate condensing enzyme, coupled with the oxidation of malate to oxalacetate using malate dehydrogenase. The condi-

tions for the assay are described as for the assay of HMG-CoA cleavage enzyme, with the exception that the Tris buffer was replaced by 0.05M-phosphate buffer, pH 7.2.

Assay of malonyl-CoA by fatty acid synthetase

The spectrophotometric assay for the yeast fatty acid synthetase was used, except that the enzyme was in excess and the reaction was allowed to go to completion. Since each molecule of malonyl-CoA requires two of NADPH for fatty acid synthesis, the amount of malonyl-CoA was easily determined.

The enzymic assays were useful for determinations of small quantities of thioester (0.005 - 0.02 μmoles)

2 Preparation of malonyl-CoA

Malonyl-CoA and ^{14}C-malonyl-CoA were prepared via the monothiophenol ester according to the method of Trams and Brady (1960).

a. Preparation of ^{14}C-malonic acid from ^{14}C-sodium malonate

Radioactive malonic acid was supplied as the sodium salt, and it was necessary to prepare the free acid for

The esterification with thiophenol. It was possible simply to treat the sodium malonate with concentrated hydrochloric acid, but much better yields of the monothiophenol ester were obtained using the following procedure.

Dowex-50 (H^+) resin was washed in the hollow P.T.F.E. plugs (Briggs, 1967), as used for the preparation of dipotassium-ATP in the assay of acetyl-CoA synthetase. The sodium malonate, in a minimum of water, was quantitatively applied to the resin. The free malonic acid, in solution, was removed by centrifugation. 0.5ml. aliquots of water were used to wash the resin until no further radioactivity was eluted.

The aqueous solution of malonic acid was freeze dried in the vessel to be used for the esterification reaction.

b. Preparation of monothiophenol malonate

Glassware was dried overnight at 110°C. Thiophenol and NN-dimethylformamide (DMF) were freshly redistilled. Malonic acid and dicyclohexylcarbodiimide

(DCC) were stored in a desiccator.

Unlabelled thiophenol malonate was prepared from 1mM (104mg.) of malonic acid. Preparations using ^{14}C labelled material were invariably on a much smaller scale; values in parentheses refer to such preparations.

100mg. of malonic acid (2.3mg) were dissolved in 5ml. (0.1 ml) of DMF. 0.11ml (0.025ml.) of redistilled thiophenol was added. The reaction flask was flushed with nitrogen and a bypass dropping funnel, which contained 500mg. of DCC in 5ml. of DMF, was fitted. The DCC solution was added slowly over the course of one hour to the stirred reaction mixture. The flask was continually flushed with nitrogen and cooled in an ice bath.

Small scale radioactive preparations were done in a tightly stoppered flask to which the DCC (10mg. in 0.1 ml. DMF) was added from a syringe over the same period of time. The flask was flushed with nitrogen each time an addition was made.

The reactions mixture was stirred for three hours

after the additions of DCC had been completed. 10ml. (0.2ml.) of water was added and the mixture stirred for a further 15 minutes. The heavy precipitate obtained in the larger scale preparations was removed by filtration through a double layer of Whatman 544 filter paper in a small Hirsch funnel. The precipitate was washed with 5ml. of water and the washings and the filtrate were combined. Small scale preparations did not give sufficient precipitate to enable it to be separated in this way, the solution was diluted to 5ml. with water.

The aqueous solutions were carefully acidified to pH 3.5 - 4.0 with dilute hydrochloric acid and extracted 6 times with 10ml. aliquots of diethyl ether. The ethereal solution was washed with 0.01N-hydrochloric acid and then with water until there was no longer an acid reaction to indicator paper. Usually 5 x 5ml. was adequate.

Large scale unlabelled preparations were stirred with activated charcoal, then filtered, and subsequently dried for one hour at -15°C over anhydrous sodium sulphate. The activated charcoal step was ommitted with

small scale preparations. The yields obtained at this stage were usually 45 - 50% for the large scale preparation, and 30-40% for the small scale preparation with respect to malonic acid.

Transesterification to Coenzyme A

CoA was dissolved in 0.2ml. of water in a tapered glass centrifuge tube. 0.1ml. of a 0.5% (w/v) solution of potassium borohydride was added and the mixture heated on a boiling water bath for 30 seconds, then cooled rapidly by an ice-salt bath. 0.1 ml. of 1% acetic acid was added to destroy excess potassium borohydride. 0.2ml. of a saturated solution of $KHCO_3$ at 0^oC was added and then pH was checked using narrow range indicator paper. If the solution was not at pH 8.0, more $KHCO_3$ solution was added.

i. Unlabelled preparations

An aliquot of the diethyl ether solution containing a ten fold molar excess of monothiophenol-malonate was added to the CoA solution. The tube was flushed with nitrogen and agitated at 0^oC for 30 minutes.

ii. Labelled preparations

A 1.5 fold molar excess of the thiophenol ester was used.

The reaction mixtures were acidified to pH 5.0 with 0.05N-hydrochloric acid, with care to avoid frothing. The aqueous phase was extracted 5 times with 3ml. aliquots of diethyl ether. Ether was removed by passing a stream of nitrogen through the solution and the malonyl-CoA solution was freeze dried.

An alternative procedure from Lynen's laboratory (1962) has several advantages over the preparation described here.

Coenzyme A is acylated by S-malonyl-N-capryl-cysteamine, and the capryl cysteamine formed during the reaction is removed by continuous ether extraction. The equilibrium is therefore displaced in favour of the acylation reaction. It has been reported that this preparation does not give rise to acetyl-CoA (Weeks, 1969; Williamson, 1969, personnal communications).

This method, therefore, is probably now the method of choice.

iii) Preparation of Acetyl-CoA

Acetylation of CoASH by acetic anhydride

Unlabelled acetyl-CoA was prepared by a modification of the general method described by Simon and Shemin (1953), usually in batches of 5 μmole.

The solution of CoA was reduced by potassium borohydride as described for malonyl-CoA. 0.05ml. of freshly distilled acetic anhydride was added and the tube was flushed with nitrogen, stoppered, and thoroughly mixed.

After 15 minutes, 0.01 ml. was withdrawn and tested for sulphydryl groups. Nearly always, this was negative. If not, a further 0.05ml. of acetic anhydride was added, the pH being adjusted with $KHCO_3$ if necessary. When there was no longer any sulphydryl reaction 0.05N-hydrochloric acid was added, with care, to lower the pH to 4.0. The solution was left at room temperature for 15 minutes to ensure that excess acetic anhydride had been hydrolysed, and then was diluted to 25ml. After removal of an aliquot for assay, the thioester was purified by chromatography on DEAE-cellulose.

Enzymic preparation of ^{14}C - acetyl CoA

^{14}C - acetyl-CoA was prepared enzymically using

acetyl-CoA synthetase. Incubation mixtures for the preparation were:- 3.0 μmole CoA, 7.0 μmole -^{14}C-acetate, 7.0 μmoles dipotassium ATP, 10 μmole 2-mercaptoethanol, 10 μmole $MgCl_2$ 1-10mg. protein of enzyme solution depending on its activity, 100 μmole of Tris/HCl buffer (pH 8.2) and 50 μmole of potassium fluoride in a total volume of 1 ml.

The incubation was started by adding the enzyme. The procedure eventually adopted for the isolation of acetyl-CoA was to terminate the reaction after sixty minutes with an equal volume of ethanol. After ten minutes at room temperature the precipitate was removed by centrifuging and was rinsed twice with ethanol-water (1:1 v/v). The supernatant and washings were combined and freeze dried.

Purification of acetyl-CoA and malonyl-CoA

Paper chromatography

The freeze dried thioester was dissolved in the minimum of water and passed through a column of G-10 sephadex (25 x 2.5cm) that was in equilibrium with water. CoA and its esters appear in the void volume of such columns while salts are retained.

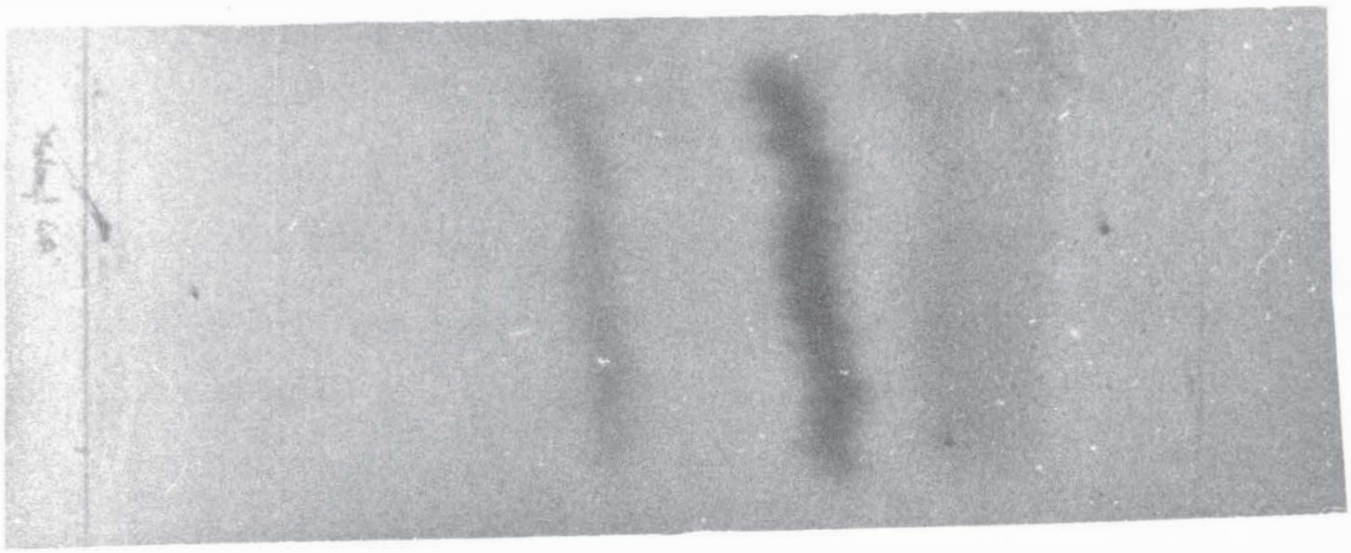
The product could be chromatographed on Whatman 3MM paper. Malonyl-CoA separated from acetyl-CoA and free malonic acid but not from CoA using 2.28ml. 28%-NH_4OH, 37.7ml. water; 62ml. isobutyric acid; 1 ml. 0.1M-EDTA pH 4.5. Figure 5 shows a photograph of such a separation. Rechromatography in a second solvent system 50ml. 0.1M-sodium acetate, pH4.5; 50ml. absolute ethanol enabled uncontaminated malonyl-CoA to be isolated, (Brodie and Porter, 1960). Acetyl-CoA was purified using the same solvent systems.

Chromatography on DEAE-cellulose

The routine method of purification employed a column chromatographic procedure. The conditions were essentially those of Moffatt and Khorana (1961) but

Fig 5 CHROMATOGRAPHY OF AN AGED SAMPLE OF

[2-^{14}C]-MALONYL-CoA



1	malonic acid	iso-butyric acid	62
2	CoA	3N-NH_4OH	12
3	malonyl-CoA	0·1M EDTA pH 4·5	1
4	acetyl-CoA	water	28

Whatman no.1 Ascending

were extensively modified in detail.

The desalted solution was applied to a column of DEAE-cellulose (Cl^-) in equilibrium with 0.003N-hydrochloric acid, 0.02M-KCl. After washing the column with 100ml. of this solution, the column was developed by a linear gradient of KCl. (0.02M-0.15M in a total volume of 2l.) CoA was eluted from a 20 x 2cm. column by 0.05 - 0.06M-salt, acetyl-CoA by 0.065 - 0.075M-KCl and malonyl - CoA by 0.09 - 0.10M-KCl. Figure 6 shows a column chromatogram of an enzymic preparation of acetyl-CoA, and a preparation of malonyl-CoA.

The appropriate fractions were pooled and immediately brought to pH 6.5 with dilute KOH. The volume was reduced by freeze drying until the solutions could be desalted by passage through a column of G-10 Sephadex.

The freeze dried eluates were viscous and could not be applied to G-10 Sephadex columns in less than 2 - 3ml. Satisfactory separation of thioesters from salt was obtained using a column 30 x 3cm. in equili-

brium with distilled water.

Occasionally, acetyl-CoA did not separate completely from Coenzyme-A, and rechromatography was necessary.

Thioester peaks were identified by the preparation and chromatography to the hydroxamate derivatives.

Disadvantages of DEAE cellulose column chromatography

1. The pH is approximately 2.7. Malonyl-CoA is labile to acid catalysed decarboxylation. It was found, however, that if the column chromatography was carried out immediately, in the cold, the loss of malonyl-CoA was not more than 8%.

2. Acetyl-CoA did not always separate completely from coenzyme A.

Because of the acid conditions, this method could not be used to purify acetoacetyl-CoA.

Advantages of DEAE cellulose column chromatography over paper chromatography

1. Recovery of material was usually greater than 90%

while yields from paper chromatography rarely exceeded 60%.

2 The technique, with a given column, gave extremely reproducible results.

3 The need to apply large volumes of liquid to paper, and the subsequent elutions were avoided. The column procedure was therefore much more rapid.

4. The column effluent was easily monitored, either for radioactivity, or for absorption at 260 mμ.

Stability and Storage of Acetyl and Malonyl-CoA

Both thioesters were very labile to alkaline hydrolysis which was complete within 15 minutes at pH 10 at 30°C,

In general, the substrates were used as soon as possible after preparation. When storage was necessary, the freeze dried material was kept at -20°C.

Freshly prepared monothiophenol malonate gave rise to a trace of acetyl-CoA, which was detected by chromatography of the hydroxamate derivatives. When stored in dry ether at -15°C, the level of acetyl-CoA

had risen to 8% after twelve months. A sample stored for two years contained 17% of the acetyl species. The importance of careful purification of malonyl-CoA was therefore obvious.

Malonyl-CoA was routinely examined for its activity in fatty acid synthesis using the fatty acid synthetase isolated from yeast.

[1,3-^{14}C] malonyl CoA and [2-^{14}C] malonyl CoA used for the determination of the ratios of their incorporation into squalene were further checked by separate incubations (using the complete assay mixture) with the enzyme in Warburg vessels. The centre wells contained 0.2ml. 1M-hyamine hydroxide in methanol. Incubations were terminated by 0.2ml. 2N-sulphuric acid. The ^{14}C-carbon dioxide collected and the fatty acid synthesised from the substrates were assayed, (table 29).

(v) Recovery of ^{14}C-malonic acid

The esterification of malonic acid with thiophenol never gave a yield higher than 60% and only rarely

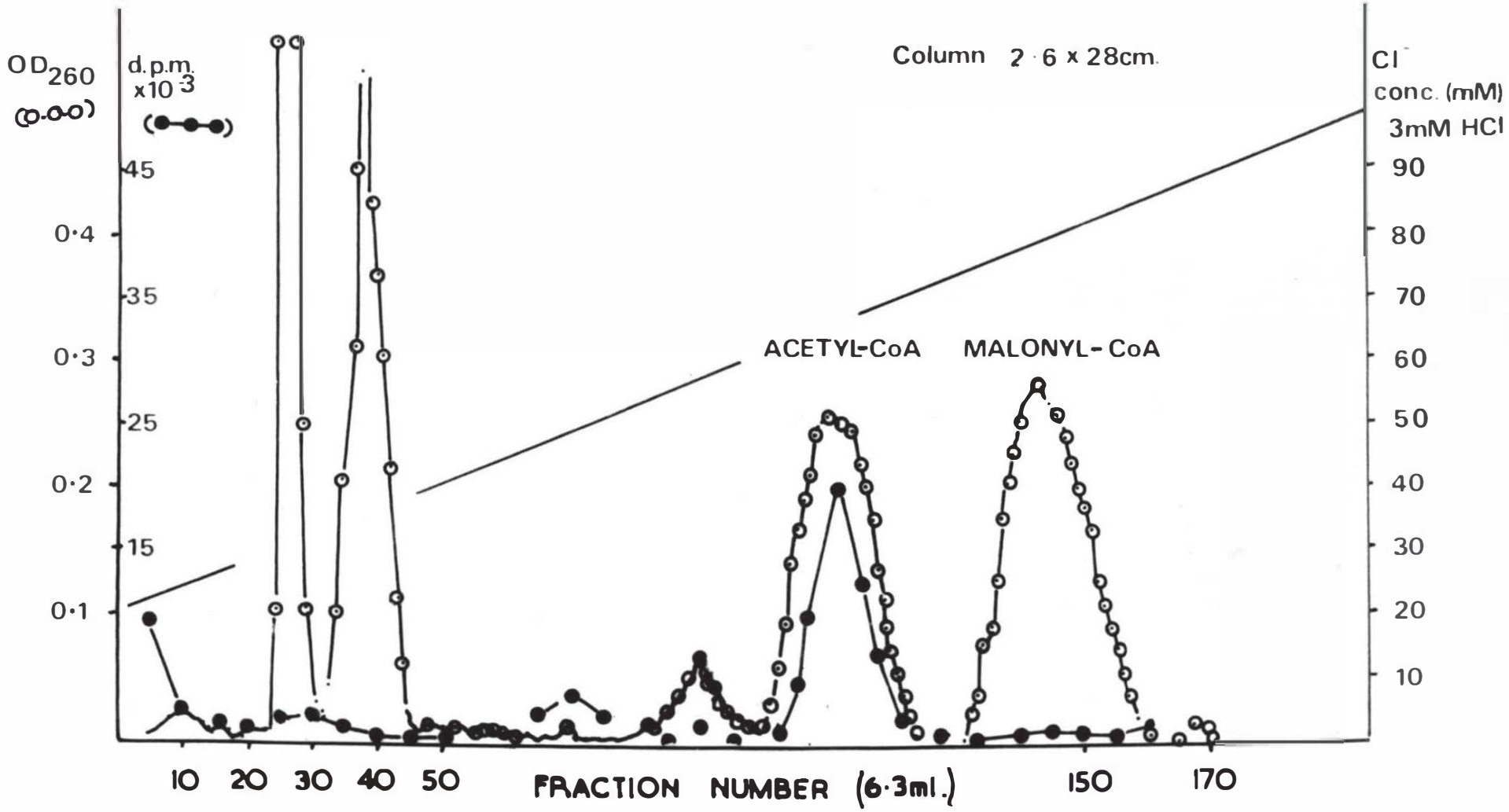
higher than 50%. ^{14}C -malonic acid was therefore recovered from the aqueous solutions after extraction of monothiophenol malonate.

Dowex - 1 resin was washed with 3M-sodium formate, 0.1M-formic acid and with water until the washings were neutral. A small column (0.6 x 10 cm) was prepared of this material.

The aqueous solution containing malonic acid was applied to the column, which was washed with 25ml. of distilled water.

Malonic acid was completely eluted by 50ml.4N-formic acid, and the solution was freeze dried.

Fig 6 Chromatography of the Products of the Acetyl-CoA Synthetase Reaction with [2-^{14}C]Acetate; and unlabelled Malonyl-CoA



Ester peaks identified by the preparation and chromatography of hydroxamate derivatives (Stadtman and Barker 1950)

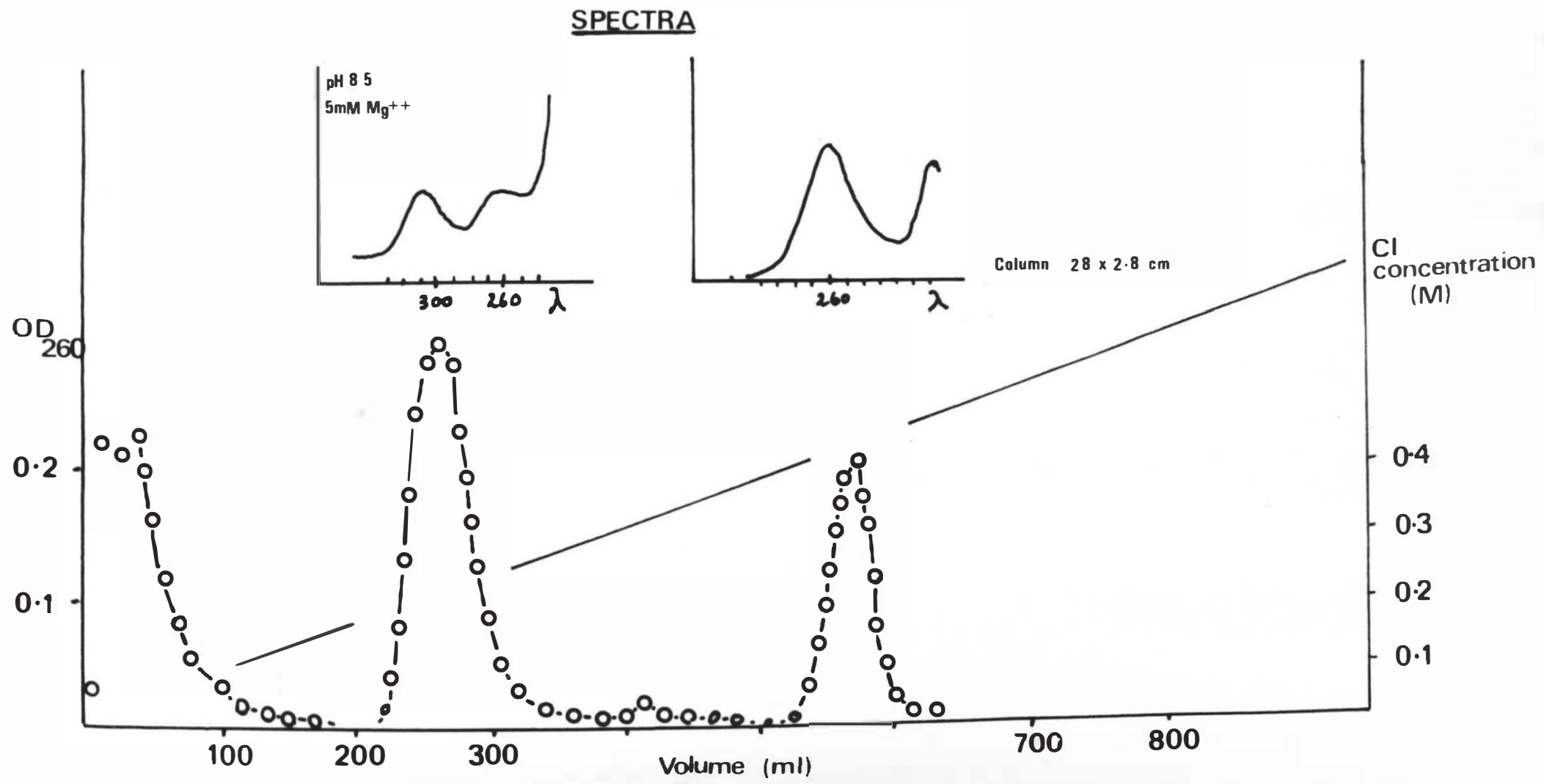
(VI) Preparation of acetoacetyl-CoA

Acetoacetyl-CoA was prepared by the action of diketene on reduced CoA. (Lynen, 1953; Ferguson and Rudney, 1959). The procedure was identical to that used for the preparation of acetyl-CoA with acetic anhydride.

Purification

The DEAE-cellulose column method described for the purification of acetyl-CoA and malonyl-CoA was not employed because of the risk of acid catalysed decarboxylation of acetoacetyl-CoA. A modified procedure was derived from that of Stewart and Rudney (1966). DEAE cellulose (Cl^-) was equilibrated with 0.02M-NaCl and pH 6.0, 50mM-phosphate buffer. The desalted acetoacetyl- CoA solution was applied and the column was developed by a linear gradient to 0.75 M-NaCl in a total volume of 1 l. The peak eluted at 0.2M NaCl was identified as acetoacetyl--CoA by its absorption spectrum (Stern, 1956). The pooled fractions were concentrated; desalted by passage through a column of Sephadex G-10, and then freeze dried. Sulphydryl reacting material was absent from this fraction.

Fig 7 CHROMATOGRAPHY OF ACETOACETYL-CoA ON DEAE-CELLULOSE



(VII) Preparation of HMG-CoA

Unlabelled HMG-CoA was prepared by the reaction of HMG-anhydride on reduced CoA.

Preparation of HMG-anhydride

The procedure was essentially that described by Hilz, Knappe, Ringelmann, and Lynen (1958), but was done on a much smaller scale.

45mg. of HMG (dried over P_2O_5) was dissolved in 1 ml. of benzene which had been dried by sodium-metal wire. 0.5ml. of freshly redistilled acetic anhydride was added and the mixture was heated under a reflux condenser on a water bath for one hour. At the end of this time excess acetic anhydride and benzene solvent were removed by rotary evaporation at approximately 30°C. The residue was redissolved in hot anhydrous benzene and the solution filtered through a previously warmed glass wool plug. Crystals, in the form fine colourless needles, appeared as the solution cooled. After two recrystallisations, the yield was 21mg. or 51% with respect to HMG. The uncorrected melting point was 102°-104°C.

Preparation of HMG-CoA

CoA was reduced by KBH_4 and brought to pH 8.0 with saturated potassium bicarbonate solution at 0°C. A ten-fold molar excess of HMG-anhydride crystals was added directly to this solution which was agitated under nitrogen for 60 minutes in an ice bath. The pH was adjusted to 6.5 with dilute hydrochloric acid and the solution was freeze dried.

Preparation of specifically labelled HMG-CoA

HMG-CoA labelled in carbons 5 or 6 was prepared by the condensation of [2-^{14}C] or [1-^{14}C] acetyl-CoA with unlabelled acetoacetyl-CoA, catalysed by the yeast HMG-CoA condensing enzyme, (Rudney and Ferguson, 1959).

Incubations contained 4 μmole of acetoacetyl-CoA, 4 μmole ^{14}C-acetyl-CoA, 400-μmole Tris/HCl buffer, pH8.5; 10mg. of bovine albumin, and 4mg. protein of enzyme solution in a total volume of 2ml. After 60 minutes the reaction was stopped by 0.2ml. 1N-acetic acid and by heating in a boiling water bath, for 30 seconds. Denatured protein was removed by centrifuging and was rinsed once with water. The combined superna-

tant were brought to pH 6.5 with dilute KOH and were desalted by passage through a column of G-10 Sephadex.

Purification of HMG-CoA

HMG-CoA was purified using the same column conditions as were used to purify acetoacetyl-CoA. HMG-CoA was eluted by 0.25M-NaCl.

EXPERIMENTAL

1 Methods

D Degradative Procedures

1 Kuhn - Roth oxidation, for C-methyl groups

A procedure for the determination of C-methyl groups was introduced by Kuhn and Roth (1933)., and consists of oxidative degradation of the molecule by a mixture of chromic and sulphuric acids at high temperatures. Methyl groups attached to carbon form acetic acid, which in the conditions of the reaction is not further oxidised. In the standard procedure, oxidation takes place in a Kjeldahl digestion flask fitted with a condenser, the mixture is heated for 90 minutes when excess chromic acid is reduced by hydrazine. The acetic acid is then distilled off.

Lemieux and Purves (1947) described a modification in which the acetate was removed by continuous steam distillation. Using this method nearly theoretical yields of acetic acid were obtained from 6-deoxy-l-glucose tetraacetate. Madsen, Abraham and Chaikoff (1964) used a similar method to degrade fatty acids and reported that the acetate was derived from only the methyl terminal end of the molecule. Rognstad, Woronsberg and Katz (1968) found that, although acetate could be recovered in 80% yield, a significant fraction

of the acetate produced arose from the centre of the molecule, and that tritium was labilised from the terminal methyl end.

The procedure adopted was that described by Lemieux and Purves (1947), and was used to isolate the methyl groups of Ergosterol and some of its oxidation products (Bu'lock, Smalley and Smith, 1962)

Method

The sample was introduced into a round bottomed flask. As a carrier 0.05 ml. of acetic acid was added. 10ml. of aqueous 30% CrO_3 was added, followed by 1 ml. of concentrated sulphuric acid. The flask was immersed in an oil bath, and was fitted with a dropping funnel containing water, a water cooled condenser and a nitrogen inlet. Nitrogen was bubbled through the solution at the rate of approximately 3 bubbles a second.

The temperature of the bath was raised to 155°C over a period of 30 minutes. The distillate (distillation commenced at about 140°C) was collected in a 50ml. graduated cylinder containing 5ml. 0.1N-NaOH. After 5ml.

had been collected 5ml. of water was run into the reaction flask from the dropping funnel. This was repeated as each five ml. of distillate was collected, to a total of 55ml. The condenser was rinsed with a further 5ml. of water which was added to the distillate.

Recovery of label

a. Distillation

The above procedure was applied to a sample of [2-^{14}C] sodium acetate, the result is shown in table 10:

Table 10

Distillation of 5mg. [2-^{14}C] sodium acetate

	Radioactivity applied	Radioactivity recovered	%age recovered in distillate
1.	122,400	122,200	99.9
2.	252,300	249,700	98.9
3.	372.100	371.800	99.9

Table 11

Recovery of Labelled Carbon from the Kuhn-Roth oxidation of 5mg. [1-^{14}C] palmitic acid

Radioactivity applied d.p.m.	Radioactivity recovered d.p.m.	Expected recovery	% recovered
22,350	110	o	0.49
21,490	74	0	0.34
22,470	92	0	0.41

Table 12

Recovery of labelled Carbon from the Kuhn-Roth Oxidation of 5mg. [U-^{14}C] Palmitic acid

Radioactivity applied d.p.m.	Radioactivity recovered d.p.m.	% recovered	theoretical % recovery	% of theoretical recovered
11,220	609	5.42	6.24	86.8
11,670	605	5.18	6.24	83.1
11,400	598	5.25	6.24	84.1

b. Oxidation

Carboxyl labelled palmitic acid and uniformly labelled palmitic acid were subjected to the oxidation and distillation procedure.

The recoveries are reported in tables 11 and 12.

2. Degradation of Acetate

a. Determination of the carboxyl carbon by the Schmidt reaction

Carboxyl carbon of acetate was determined by a modification of the Schmidt reaction (1923). Acetate was derived from Kuhn-Roth oxidation or by the hydrolysis of acetyl-CoA that had been produced by the HMG-CoA cleavage enzyme.

The method was essentially as described by Katz, Abraham and Chaikoff (1955) and Madsen, Abraham and Chaikoff (1964) where the reactions were done in Warburg vessels and hyamine hydroxide was used to trap carbon dioxide.

The solution of sodium acetate was evaporated to

dryness and the salt was taken up in 1 ml. of water, a known aliquot of which was transferred to a Warburg flask. The solution was evaporated to dryness in an oven, the flask being tilted so that the deposit was localised in one position.

50mg. of sodium azide was added at this locus, and the flask was capped with a rubber seal. 0.5 ml. of a mixture of A.R. concentrated sulphuric acid and fuming sulphuric acid (1:3 v/v) was added via a syringe and the salts were allowed to dissolve at room temperature. When this had happened, the flasks were placed in a water bath at 80°C for two hours. When cool, 0.3ml. of 1.0M-hyamine hydroxide in methanol was injected into the centre well. Carbon dioxide was assayed after two hours at room temperature.

b. Determination of the methyl carbon of acetate by oxidation of methylamine

The contents of the Schmidt reaction flasks were quantitatively transferred to 25ml. round bottomed flasks with the aid of water. The solution was made alkaline with 2N-sodium hydroxide and concentrated by

Table 13

Recovery of Radioactive Carbon from the Schmidt degradation of ^{14}C-Acetate

	Radioactivity applied (d.p.m.)	Radioactivity collected (d.p.m.)	%age recovered
[1-^{14}C] Acetate	22,350	20,122	90
	21,870	19,350	88.4
[2-^{14}C] Acetate	32,450	82	0.25
	32,370	125	0.38

Table 14

Recovery of labelled carbon from the oxidation of Methylamine derived from the Schmidt degradation of Acetate

Radioactivity applied (d.p.m.)	Radioactivity collected (d.p.m.)	% recovered
FROM [2-^{14}C] ACETATE		
28,740	25,430	88.5
27,860	23,980	86.1

distillation. 5ml. of the distillate was collected in 0.2ml of 10.0N-sulphuric acid contained in the main compartment of a Warburg flask. 12ml. of 10N-sulphuric acid and 1.5ml. of 10%-potassium permanganate was added. The flask, sealed with a rubber cap, was heated for 30 minutes at 90°C. When cool, 0.3ml. - hyamine hydroxide was added to the centre well from a syringe.

3 Isolation and Degradation of HMG-CoA

The radioactivity on carbons 1,2,3-5, and 6 of HMG-CoA synthesised by rat and pigeon liver enzyme systems, and by yeast HMG-CoA condensing enzyme was determined by an extension of the procedures used by Rudney and Ferguson (1959) and by Brodie et al. (1964)

Isolation of HMG-CoA

HMG-CoA was isolated from incubations by the procedure described by Rudney and Ferguson (1959). Reactions were stopped by adding a one-tenth volume of 1N-acetic acid and then heating in a boiling water bath for 2 minutes. Coagulated protein was removed by centrifuging and was rinsed once with water. The combined supernatants were diluted to 25ml. and acidified to pH 1

with 1N hydrochloric acid and then passed through a small column of granular charcoal (2 x 10 cm) as used for the isolation of CoA from boiled yeast extract (see methods section 3). The column was washed with 50ml. cold water. CoA and CoA esters were eluted with ethanol-water-0.880 ammonia (1:1+2ml/l.) The solution was freeze dried after the pH of the eluate had been lowered to 6.0.

The residue was dissolved in a minimum of water and chromatographed on Whatman No.3 paper with 1M-ammonium acetate pH 4.7:Ethanol (1:7 v/v). HMG-CoA chromatographed at R_f 0.1-0.2, acetyl-CoA at 0.25-0.30, and malonyl-CoA at R_f 0.2. If malonyl-CoA was present the HMG-CoA area was eluted and rechromatographed in the same system. The HMG-CoA that was eluted from the paper was freeze dried.

Degradation of HMG-CoA

a. Carbons 3-5 and 6

The radioactive HMG-CoA was diluted with unlabelled HMG-CoA and transferred to the main compartment of a Warburg flask. The incubation mixture was fortified

with $MgCl_2$ (10mM) and Cysteine pH 7.5 (10mM) and Tris/ /HCl buffer (100mM, pH 8.0). The flasks were sealed with rubber serum caps and HMG-CoA cleavage enzyme was added to the main compartment from a side arm. After incubating the mixture for three hours at 37°C the reaction was terminated by 0.5ml. of 4N-hydrochloric acid injected through the rubber cap.

2ml. 0.5% 2,4-dinitro-phenyl-hydrazine in 2N-HCl was injected into the main compartment and the mixture was heated at 60°C for 60 minutes to ensure that the acetoacetate had been completely decarboxylated. When cool, 0.2ml. of 1.0M-hyamine hydroxide in methanol was injected into the centre well.

After two hours at room temperature, the radioactivity in the carbon dioxide absorbed in the hyamine hydroxide was assayed. A second aliquot of 2,4-dinitro-phenyl-hydrazine and authentic acetone -2,4-dinitro-phenyl-hydrazone were added to the main compartment and the solution was extracted with carbon tetrachloride as described by Lehninger (1957). The solution was concentrated and chromatographed in an ascending system on

Whatman No 1 paper using the solvent system described by El Hawary and Thomson (1953), of n-butanol - ethanol - ammonia (.880) 7:1:2.

The acetone 2,4-dinitrophenylhydrazone, which chromatographed near the solvent front was eluted with carbon tetrachloride and dried on discs of filter paper. The radioactivity of these was assayed by geiger counting; the activity was determined by comparison of the count rate with that obtained from known aliquots of [2-^{14}C] acetate similarly dried on paper.

b) Carbons 1 and 2

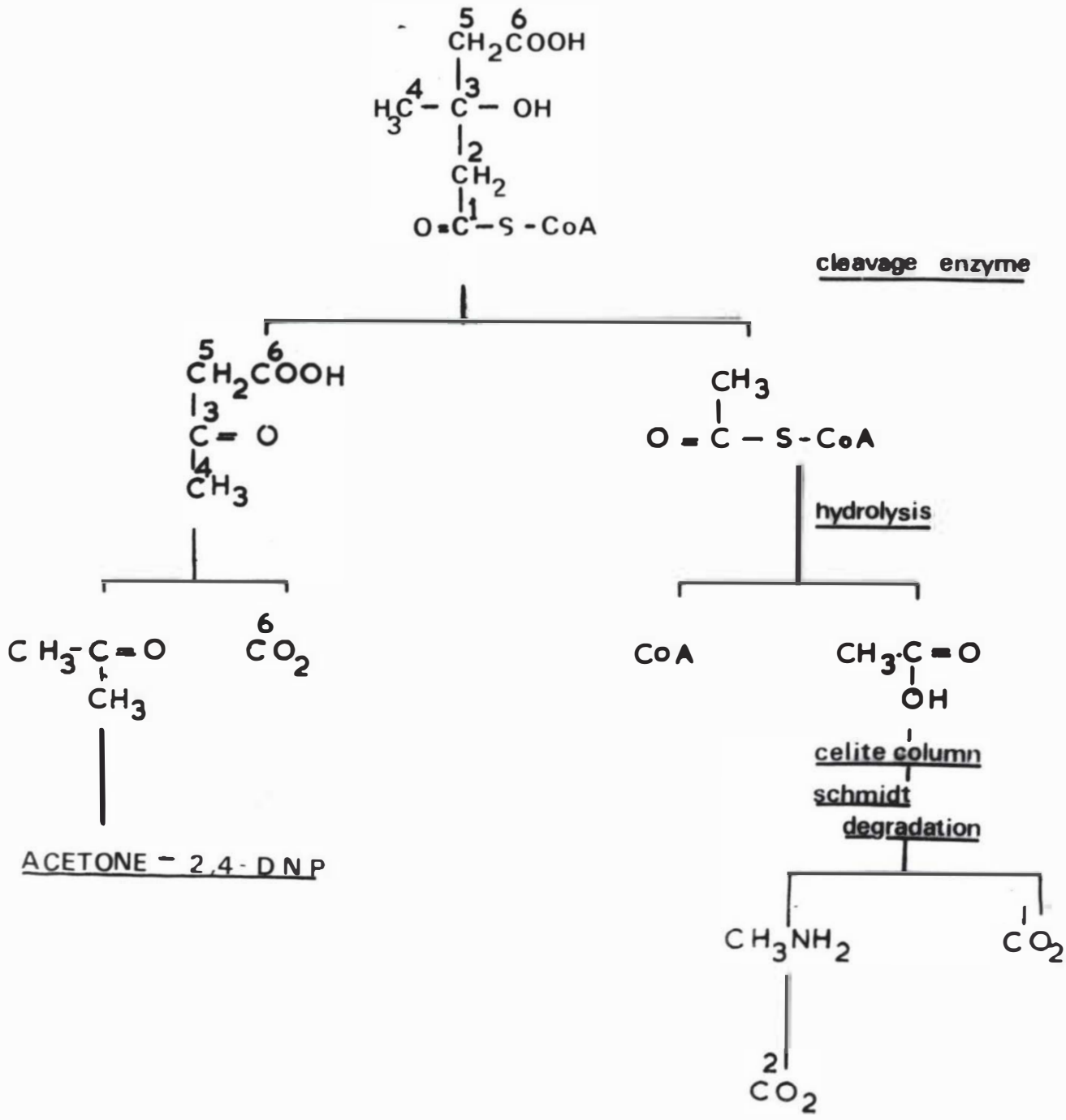
Duplicate incubations of cleavage enzyme to the above were terminated with 0.5ml. 2N-hydrochloric acid. After one hour at 60^{o}C, the pH was raised to 10 and a stream of nitrogen was passed through the solution to remove acetone and carbon dioxide. The solution was incubated for another hour to ensure the hydrolysis of the thioesters. The solution was then evaporated to dryness and acetate was isolated by celite column chromatography.

The sodium acetate that had been isolated in this manner was degraded by the Schmidt azide reaction, and by permanganate oxidation of the resulting methylamine.

The scheme (5) displays the steps of this degradation.

Scheme 5

STAGES OF THE DEGRADATION OF HMG-CoA



4 Degradation of Ergosterol

Bu'lock, Smalley and Smith (1962) showed that, in Penicillium urticae, diethyl malonate was incorporated into fatty acids and 6-methyl salicyclic acid, but that acetate was the sole precursor of ergosterol. The procedure described below used similar techniques to those described by Bu'lock et al.

a Isolation of ergosterol

Ergosterol was isolated from incubations of the yeast system with ^{14}C-malonyl-CoA, ^{14}C-acetyl-CoA or ^{14}C-acetate. The unsaponifiable fraction was diluted with 10mg. of unlabelled ergosterol and dissolved in 100ml. of warm (45°C) ethanol-benzene (3:1 v/v). Insoluble material was removed by filtering through a previously warmed glass wool plug. The solution was cooled to room temperature and then left at 4°C overnight. The crystals were collected on a Hirsch funnel and were crystallised for a second time from the same solvent system. The uncorrected melting point was 160° - 163°C. The published corrected melting point is 163°C - 164°C (Hanahan and Wakil, 1953).

b. Permanganate - periodate oxidation

Lemieux and Von Rudloff (1955) introduced a procedure for the quantitative cleavage of olefinic bonds.

As soon as permanganate has completed the initial oxidation

$$R^1 - CH = CH - R^{11} \longrightarrow R^1 - \underset{OH}{CH} - \underset{OH}{CH} - R^{11}$$

periodate completes the glycol fission, and at the same time reoxidises Mn (V) back to permanganate.

Von Rudloff (1956) applied a modification of the procedure to a variety of compounds that were insoluble in water.

Stock oxidising solution consisted of 20.86g. of sodium metaperiodate and 250ml. of 0.01M-potassium permanganate, in 1 l. distilled water.

2ml. of this solution was pipetted into a 100 ml. conical flask, followed by 1 ml. of K_2CO_3 solution (2.5 g/l.) t-butanol and water to give a volume of 9ml. 3 - 5mg. of crystalline ergosterol dissolved, with warming, in t-butanol was added slowly and with shaking.

In the total volume of 10ml. there was 5ml. of t-butanol.

The mixture was shaken for 24 hours at room temperature after which 1g. $NaHCO_3$ and 5ml. 0.1N-sodium arsenite were added. When the permanganate colour had disappeared the solution was diluted to 100ml. and acidified to pH 2.0 with H_2SO_4.

Separation of oxidation products

a. 2,3-dimethyl butyric acid (Hanahan and Wakil, 1953)

The acidified solution was poured into a 250ml. round bottomed flask and steam distilled. 50ml. of distillate was collected. The distillate was acidified and extracted with diethyl ether. The extract was dried over anhydrous sodium sulphate and the ether evaporated with a stream of nitrogen. The colourless liquid that remained was neutralised with 1N-NaOH and allowed to crystallise on a watch glass. The product was recrystallised once from water.

b. Derivatives of the steroid nucleus

Bu'lock et al. (1962) described the oxidation product of the steroid nucleus as a C-22 keto acid.

The residue after steam distillation was extracted with diethyl ether in a continuous liquid/liquid extraction apparatus for 72 hours. The ether extract was dried by anhydrous sodium sulphate and evaporated to dryness with a stream of nitrogen. The dry residue was diluted with 10mg. of unlabelled ergosterol, and the solid material was dissolved in warm ethanol-benzene (3:1, v/v). Crystals of ergosterol were collected after 24 hours and were rinsed with cold benzene. This procedure reduced the specific activity of the undegraded ergosterol. The mother liquor was taken to dryness and dissolved in a minimum of diethyl-ether. The solution was applied to a thin layer chromatogram plate (silica gel G, 0,3 mm) which was developed by light petroleum - diethyl ether 60:40 (v/v). In this system, ergosterol chromatographed with an R_f of about 0.2. The material remaining at the origin was eluted with diethyl ether.

Recovery of radioactivity

Table 15 shows the total radioactivity recovered in six separate determinations. There was little difference in the recovery of radioactivity from ergosterol derived from acetyl-CoA or malonyl-CoA.

The dimethyl-butyric acid fragment, the undegraded ergosterol and the nucleus oxidation products were degraded further by Kuhn - Roth oxidation as described previously. The distribution of label between the carboxyl and methyl carbons of the acetate was also determined.

Table 15

The Total Recovery of Radioactivity from the Permanganate-Periodate Oxidation of Ergosterol

^{14}C-Ergosterol oxidised (d.p.m.)	Recovered as 2,3-dimethyl butyric acid (d.p.m.)	Recovered as Ergosterol (d.p.m.)	Recovered from T.L.C. (d.p.m.)	% recovered
1) Ergosterol derived from [2-^{14}C] Acetyl-CoA				
82,400	16,750	8,470	51,180	93
50,730	9,200	9,280	28,780	94
69,200	12,920	11,650	40,300	94
2) Ergosterol derived from [2-^{14}C] Malonyl-CoA				
87,200	17,140	10,960	51,000	93
83,300	16,180	7,490	53,900	93
52,100	9,700	8,990	27,730	89

EXPERIMENTAL

2. RESULTS

A. The Incorporation of Malonyl-CoA into Lipids by Cell Free preparations of *Saccharomyces cerevisiae* and *Hevea brasiliensis*.

The Incorporation of [2-^{14}C] Malonyl-CoA and [2-^{14}C] Acetyl-CoA into Lipids by the Cell Free Extract of Yeast

The experiments described by Klein and Booher (1955) showed that lipids could be synthesised from acetate by cell free extracts of yeast. The initial experimental approach to this work was to use a similar enzyme system to obtain information on the utilisation of malonyl-CoA with reference to isoprenoid synthesis. To this end a comparison with the incorporation of acetyl-CoA was attempted.

The incorporation of acetyl-CoA and malonyl-CoA into the saponifiable and unsaponifiable fraction was determined with varying incubation times. The details of the incubation conditions are given in figures 8, and 9. The systems contained no added cofactors.

The rate of incorporation of malonyl-CoA into the fatty acid fraction was much greater than that of acetyl-CoA while the rates of incorporation into

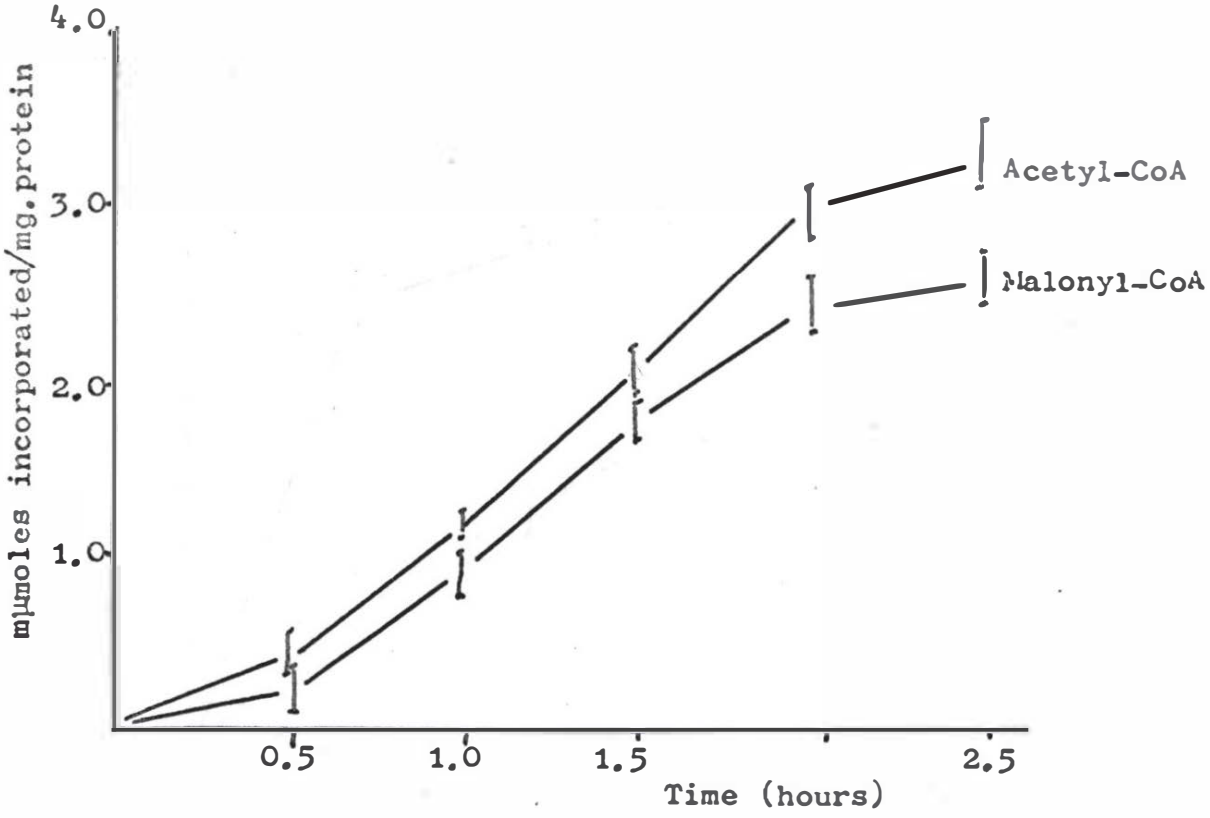
the unsaponifiable fraction were similar.

Since the pathway envisaged by Brodie et al, (1963, 1964) involves the condensation of one molecule of malonyl thioester with two of the acetyl derivative a direct comparison of the level of incorporation into the products does not accurately reflect the relative importance of the possible pathways. After 2.5 hours 2.6 mμmoles/mg. protein of malonyl-CoA had been incorporated into the unsaponifiable lipids compared to 3.3 mμmoles/mg. protein of acetyl-CoA. However, if the Brodie pathway was operating, this represents 2.6 x 3 mμmoles of 2-C units. The situation is complicated, however, by the possibility of the decarboxylation of the malonyl-CoA. Furthermore, the size of the acetyl -CoA pool must affect the result and in this experiment the acetyl-CoA concentration may be limiting.

The incorporation of malonyl-CoA into the yeast unsaponifiable lipids suggested that it might indeed be an isoprenoid precursor. Weeks (1966) observed that malonate was incorporated into the rubber and unsaponifiable lipid fractions of Hevea latex, although he reported that it was a better precursor of fatty

Figure 8

TIME COURSE OF THE INCORPORATION OF [2-^{14}C] MALONYL-CoA AND [2-^{14}C] ACETYL-CoA INTO YEAST UNSAPONIFIABLE LIPIDS



Incorporation of substrate expressed as mμmole/mg. protein.

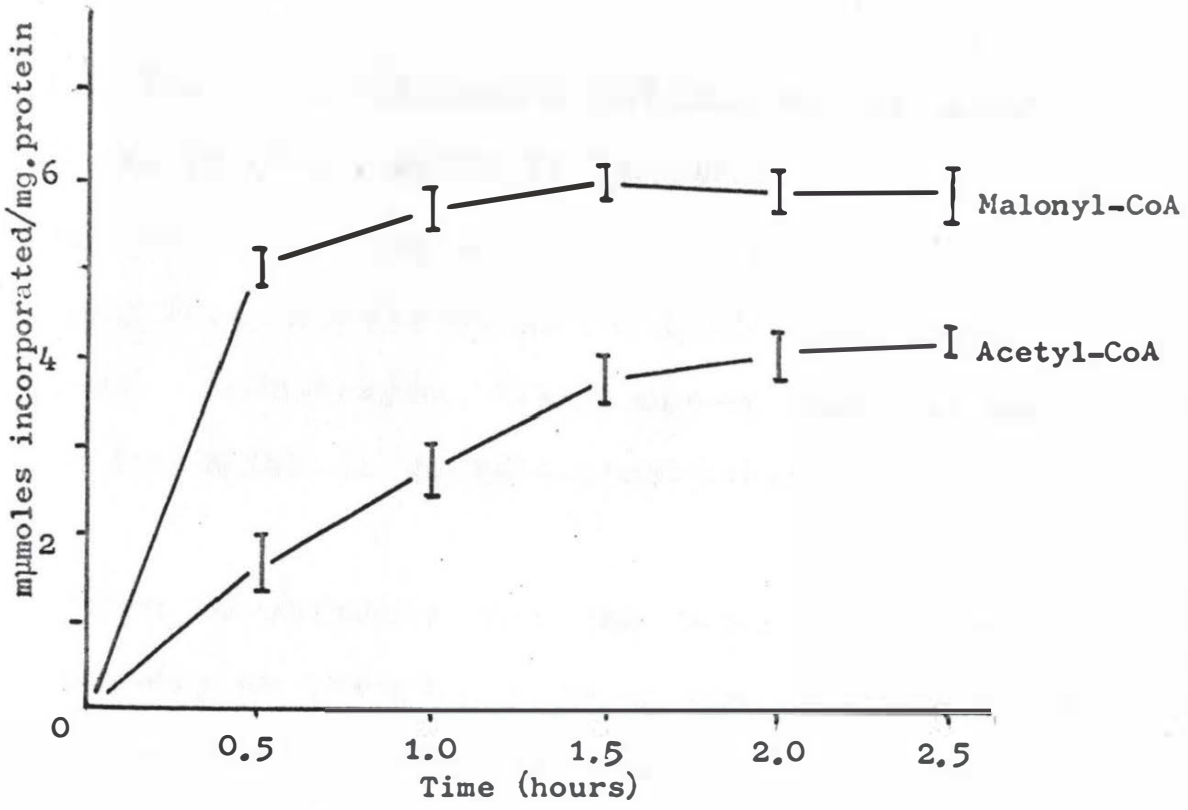
Time (hours)	0.5	1.0	1.5	2.0	2.5
Malonyl-CoA	0.23	0.90	1.80	2.40	2.60
Acetyl-CoA	0.45	1.22	2.10	3.00	3.30

Results are the mean of three experiments. Range of data is shown in the graph.

Incubations contained 0.25ml. yeast homogenate, 1.0mM-[2-^{14}C] Malonyl-CoA or acetyl-CoA (0.75μC); Potassium phosphate buffer, pH 7.0 200mM in a final volume of 1.0ml. Temperature 30°C.

Figure 9

TIME COURSE OF THE INCORPORATION OF [2-^{14}C] MALONYL-CoA AND [2-^{14}C] ACETYL-CoA INTO YEAST SAPONIFIABLE LIPIDS



Incorporation of substrate expressed as mμmole/mg. protein

Time (hours)	0.5	1.0	1.5	2.0	2.5
Malonyl-CoA	5.1	5.8	6.0	6.0	5.9
Acetyl-CoA	1.7	2.65	3.8	4.1	4.2

Results are the mean of three experiments. Range of data is shown in the graph.

Incubations contained 0.25ml. yeast homogenate, 0.5mM-[2-^{14}C] malonyl-CoA or acetyl-CoA (0.75 μC.), Potassium phosphate buffer, 200mM, pH 7.0. Temperature 30°C

acids. The incorporation of malonyl-CoA into latex lipids was therefore investigated.

The incorporation of acetyl-CoA and malonyl-CoA into lipids by the latex of Hevea brasiliensis

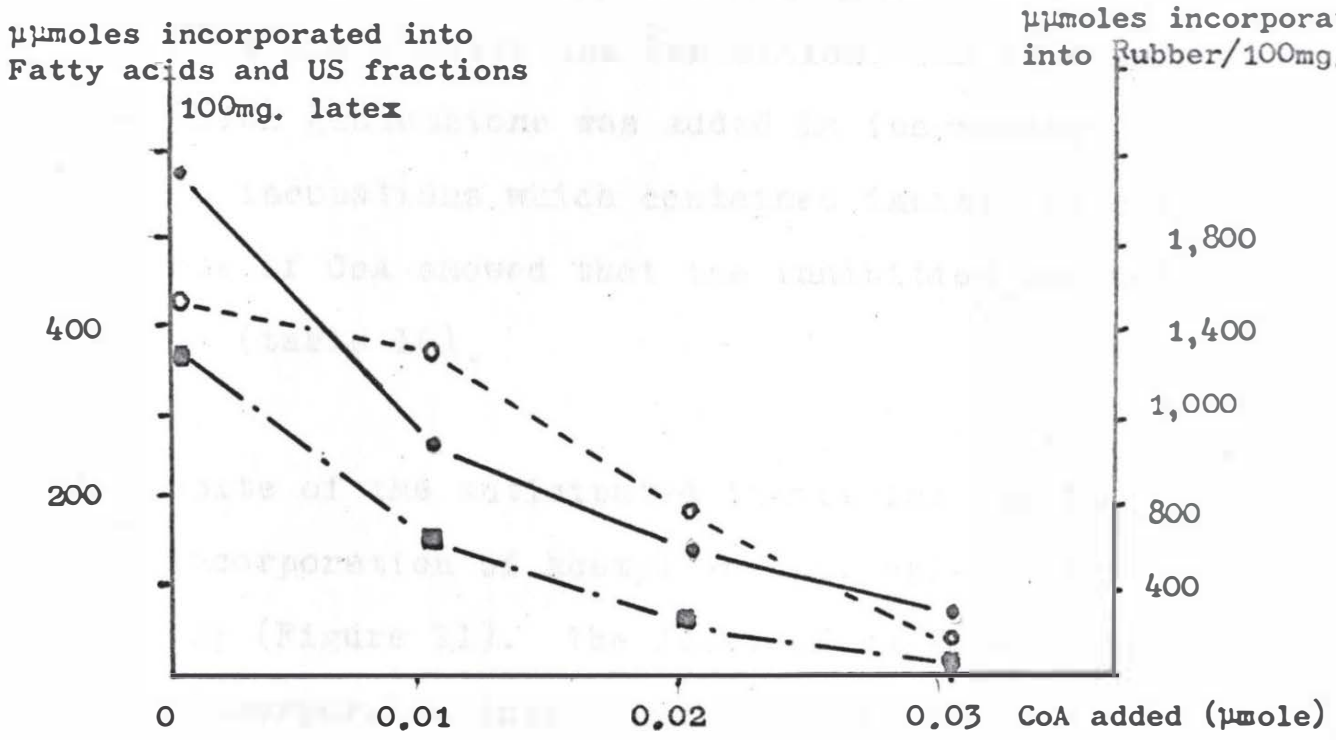
Weeks (1966) and Fournier (quoted by Weeks) reported negligible incorporation of acetyl-CoA into latex lipids. Furthermore, Weeks showed that CoA was a powerful inhibitor of acetate incorporation.

The first experiments with the latex system were directed towards an investigation of the CoA inhibition, since any incubation with a substrate concentration of a CoA ester would introduce an inhibitory concentration of CoA. i.e. utilisation of 10mμmoles of substrate for synthetic purposes would leave that amount of CoA in the incubation mixture.

The effect of increasing the concentration of CoA in incubations of Hevea latex on the incorporation of acetate into the fatty acid, unsaponifiable and rubber fractions is shown in figure 10. Incorporation into all three fractions was strongly inhibited. If the

Figure 10

THE EFFECT OF ADDED COENZYME-A ON THE INCORPORATION OF [2-^{14}C] ACETATE INTO THE RUBBER , FATTY ACID (FA), AND UNSAPONIFIABLE LIPID (US) FRACTIONS OF HEVEA LATEX



Incorporation expressed as μμmole substrate incorporated/100mg. latex.

CoA added (μmole)	0	0.01	0.02	0.03
Rubber	2,150	940	580	290
FA fraction	432	360	183	55
US fraction	379	157	69	21.5

Incubations of 0.1ml. latex for 60 minutes at 30°C; with the freeze dried substrate - 1.5mM [2-^{14}C] acetate, 1μc.

Results are the mean of two determinations.

------ = Fatty acid fraction
_____ = Rubber
— . — = Unsaponifiable lipid fraction

inhibition was due to a non-specific effect of a thiol compound, the addition of glutathione might compete with the CoA and relieve the inhibition. An experiment in which glutathione was added in increasing amounts to incubations which contained inhibitory concentrations of CoA showed that the inhibition was not relieved, (table 16).

In spite of the anticipated inhibition due to CoA the incorporation of acetyl and malonyl-CoA was determined; (Figure 11). The level of radioactivity that was incorporated into rubber, unsaponifiable lipid and fatty acids from acetyl-CoA was always much lower than that obtained from control incubations using acetate. In this system malonyl-CoA was neither incorporated into rubber nor into the unsaponifiable fraction but was extensively incorporated into the fatty acid components. This indicated that the malonyl-CoA was viable for synthesis, despite the inhibitions to be expected from CoA. The lack of any incorporation into isoprenoid compounds was in complete contrast to the results obtained from the yeast system.

Table 16

The Effect of Adding Glutathione on the incorporation of Acetate by CoA containing Hevea latex Incubations

Concentration of Glutathione		0	0.01	0.03	0.05	0.1
Incorporation of Acetate µµmoles/100mg. latex	Rubber	263	269	274	267	279
	FA	47	59	62	65	65
	US	24	24	27.5	28	26.5

Incubations contained 0.03µmoles of CoASH; 1.5mM- [2-^{14}C] acetate (1µC.) and 0.1ml. latex

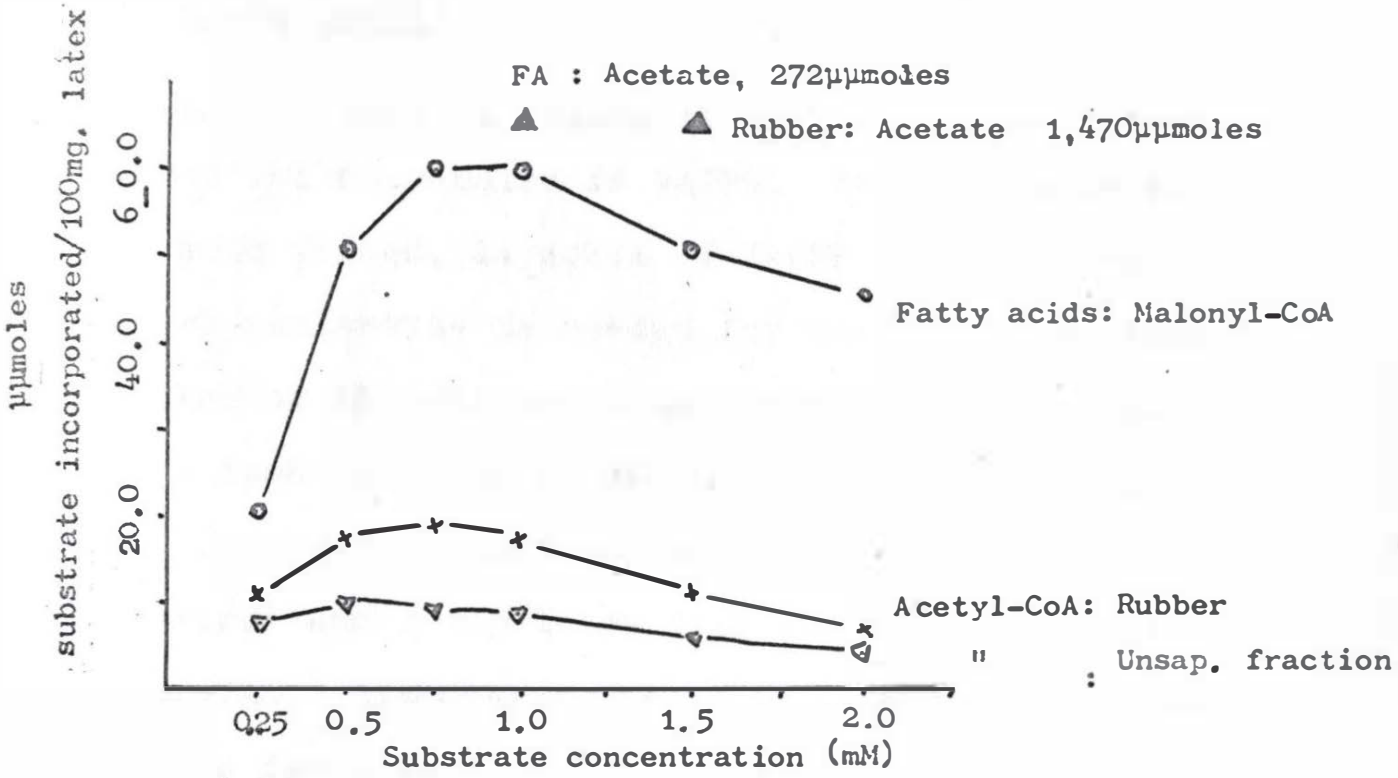
Results are the mean of 4 determinations

FA = Fatty acid fraction

US = Unsaponifiable lipid fraction

Figure 11

INCORPORATION OF [2-^{14}C] MALONYL-CoA and [2-^{14}C] ACETYL-CoA into the RUBBER, FATTY ACID (FA) and UNSAPONIFIABLE LIPID (US) FRACTIONS of HEVEA LATEX, COMPARED WITH THE INCORPORATION OF [2-^{14}C] ACETATE



Incorporations expressed as µµmole of substrate incorporated/100mg. latex.

Concentration of Substrate		0.25	0.50	0.75	1.0	1.5	2.0
Malonyl-CoA	Rubber	-	not significant				-
	FA	210	503	600	598	508	455
	US	-	not significant				-
Acetyl-CoA	Rubber	105	178	190	171	103	68
	FA	27	27	24	16	-	-
	US	86	102	97	90	63	48
Acetate	Rubber	-	815	-	1,135	1,470	1,380
	FA	-	196	-	272	223	197
	US	-	181	-	237	194	172

Incubations contained 0.1ml. latex and the freeze dried substrate.

1 hour at 30°C.

The results are the average of 3 determinations.

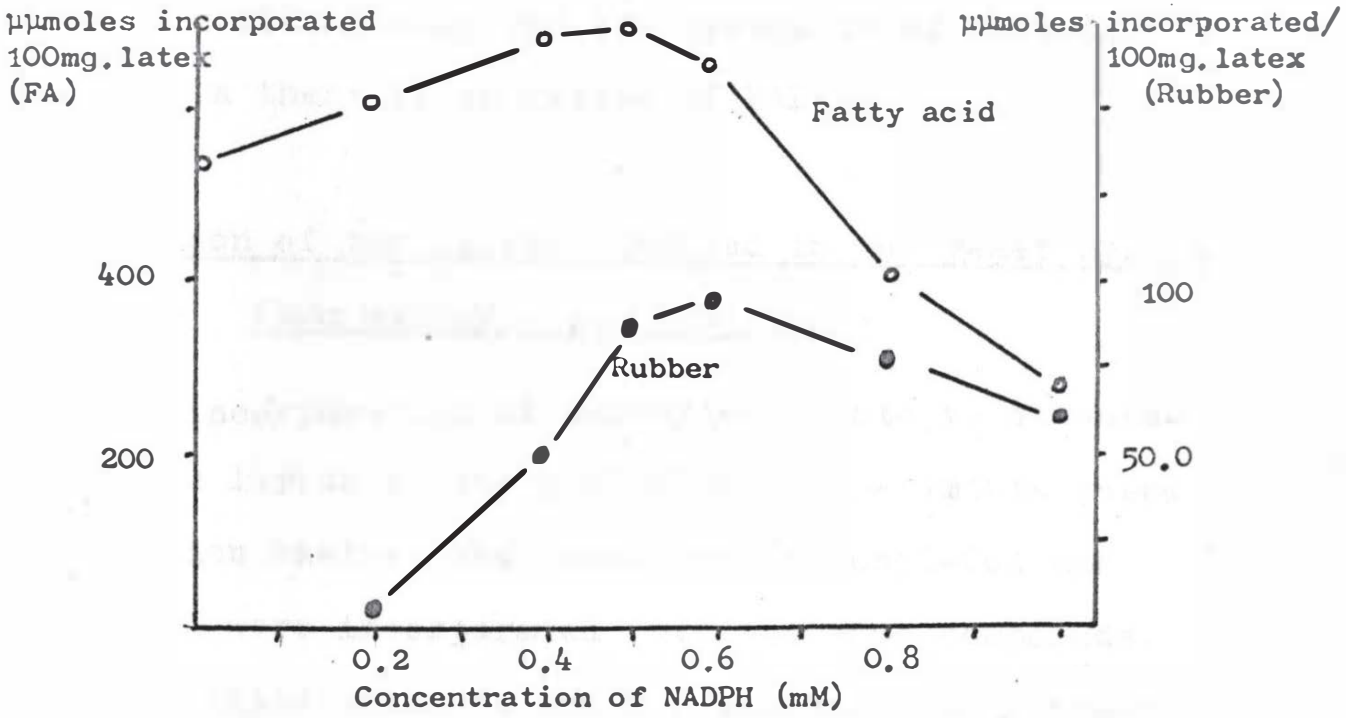
The effect of NADPH on the incorporation of [2-^{14}C] malonyl-CoA into fatty acids and rubber by the latex of Hevea brasiliensis

A cofactor that is common to the formation of both fatty acids and isoprenoids is NADPH. For each mole of palmitic acid formed, 14 moles of NADPH are required. The reduced nucleotide is needed for isoprenoid synthesis at the stage of HMG-CoA reductase and 2 moles are required for each molecule of MVA produced. In a cell free rat liver system that was active in the synthesis of cholesterol and fatty acids from acetate, Fletcher and Myant (1961) discovered that a reciprocal relationship between fatty acid and cholesterol synthesis could be almost abolished by adding ATP.

Figure 12 is the result of incubating Hevea latex with [2-^{14}C] malonyl-CoA and increasing concentrations of NADPH. Label from malonyl-CoA was incorporated into rubber in these conditions, but the incorporation into unsaponifiable lipids was too low to be determined accurately with the specific activities of malonyl-CoA that were available. However, the maximum level of incorporation into rubber from malonyl-CoA was still less than 10% of that achieved from acetate in un-

Figure 12

THE EFFECT OF NADPH ON THE INCORPORATION OF [2-^{14}C] MALONYL-CoA INTO RUBBER AND FATTY ACIDS BY HEVEA LATEX



Incorporations expressed as µµmole/100mg. latex

Concentration of NADPH	0.0	0.2	0.4	0.5	0.6	0.8	1.0
FATTY ACIDS	546	610	680	692	652	403	279
RUBBER		6.5	51	88	93	76	63

Incubations contained 0.1ml. latex and freeze dried substrate

([2-^{14}C] Malonyl-CoA 0.5mM, 0.5µc.)

The results are the mean of 3 determinations

60 minutes at 30°C

fortified incubations.

The results suggest that malonyl-CoA is used for the formation of rubber but that in latex this substrate is used preferentially for the synthesis of fatty acids except when there is an excess of NADPH.

A comparison of the lipids labelled in the yeast system from malonyl- and acetyl-CoA

The incorporation of malonyl-CoA into yeast unsaponifiable lipids in the preliminary experiments posed the question whether the label from malonyl-CoA and acetyl-CoA were incorporated into the same compounds. The total lipid extracts and the unsaponifiable fractions from yeast incubations with ^{14}C-acetyl-CoA and ^{14}C-malonyl-CoA were therefore examined by silicic acid column chromatography, by thin layer chromatography, by gas liquid chromatography and by the preparation of derivatives.

Chromatography on columns of silicic acid

Total lipid extracts were mixed with samples of squalene, ergosterol, cholesterol stearate, palmitic

acid and glycerol trioleate. The mixture was fractionated by elution from a column of 18g. of silicic acid as described in the methods section (A7) Figure 13 compares the distribution of radioactivity in the lipids from incubations with [2-^{14}C] acetyl-CoA and [2-^{14}C] malonyl-CoA.

The pattern is very similar, the most striking difference being the much higher level of radioactivity in the free fatty acid fraction derived from the malonyl-CoA incubations.

Gas liquid chromatography

Aliquots of the radioactive hydrocarbon fraction containing authentic squalene were subjected to gas-liquid chromatography on a column of 5% PEGA/95% celite at 195°C. Fractions emerging from the column were collected in calcium chloride tubes containing toluene wetted glass wool and were assayed for radioactivity.

Figure 14 shows the mass trace and histograms of the radioactivity eluted from the column. Both acetyl-CoA and malonyl-CoA incubations gave rise to material that chromatographed with squalene. More proof that

Fig. 13

SILICIC ACID COLUMN CHROMATOGRAPHY of the Lipid Extracts from Incubations of a Cell Free Yeast System with [2-^{14}C]Malonyl-CoA or [2-^{14}C]Acetyl-CoA

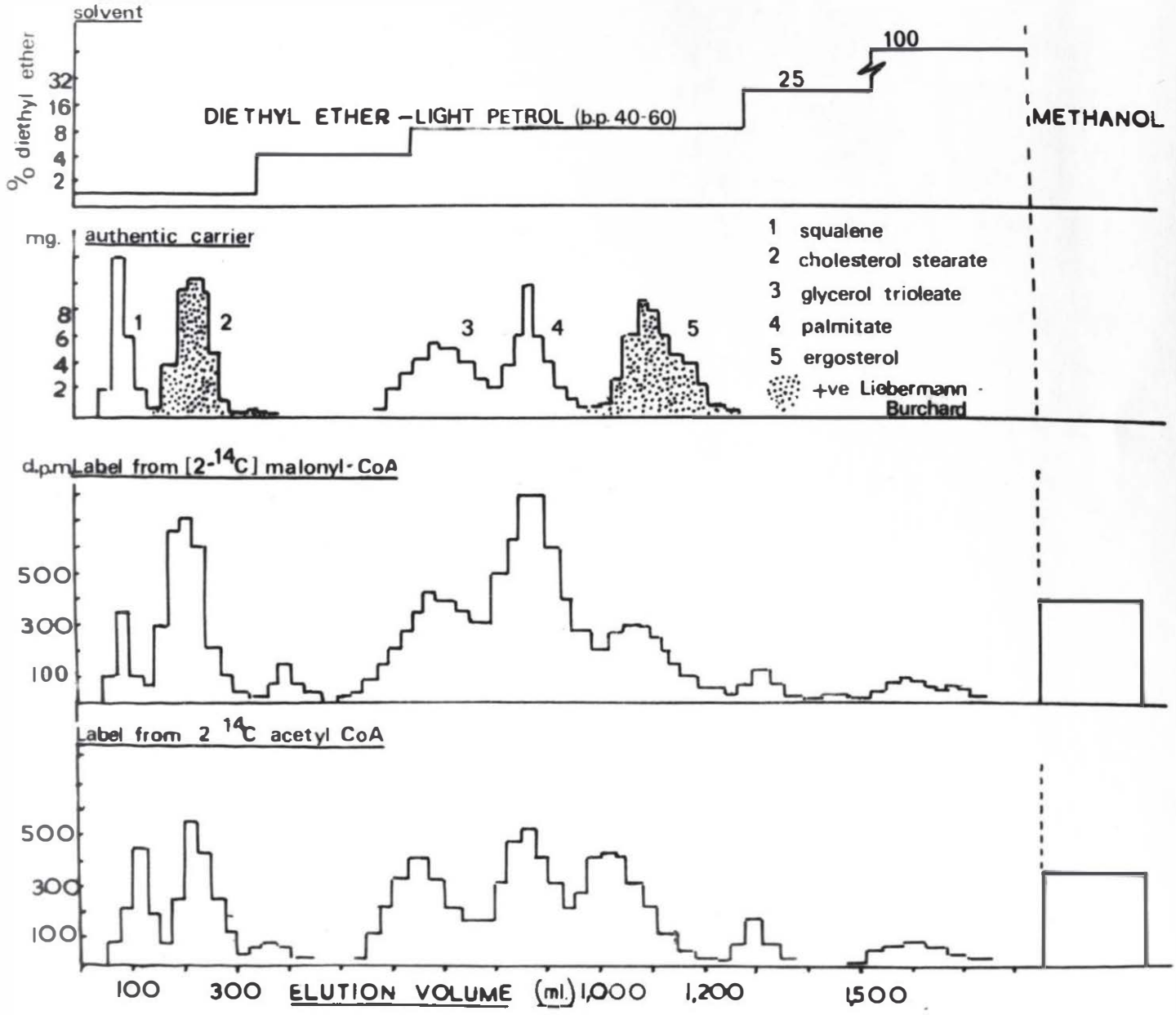
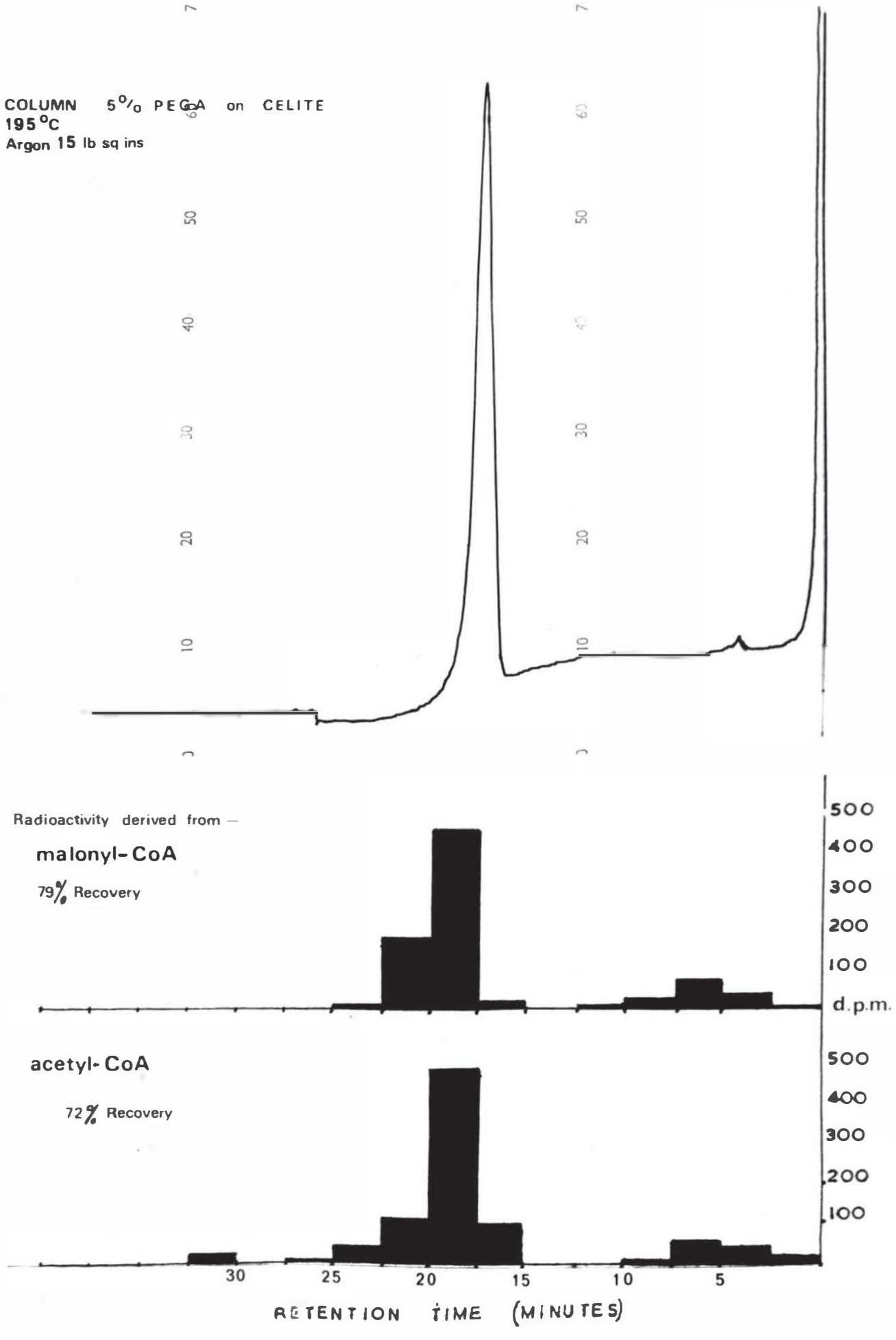


FIG 14

G L C of Hydrocarbon Fractions from a Silicic acid Column with Squalene



this radioactive non-polar compound was indeed squalene was obtained by preparing the crystalline hexahydrochloride derivatives.

Preparation of Derivatives

The hydrocarbon fractions were pooled and 75 mg. of authentic squalene was added. The hexahydrochloride derivatives were prepared by the method described in part A of the methods section and the isomer that crystallised from ethyl acetate was recrystallised several times. Table 17 shows that both acetyl-CoA and malonyl-CoA derived material crystallised to a constant specific radioactivity with the authentic compound.

The digitonide derivative of ergosterol was prepared from the sterol and sterol ester fractions from the silicic acid column. This material was pooled and diluted with 50mg. of chromatographically homogenous ergosterol. The mixture was saponified to hydrolyse the sterol ester and the digitonide derivative was prepared. Table 18 describes the result from two preparations of the digitonide of ergosterol labelled

Table 17

Cocrystallisation of Squalene Hexahydrochloride with the derivative of the Hydrocarbon Fraction from Silicic Acid Column Chromatography

Crystallisation number			1	2	3	4	5	6
Source of ^{14}C label	[2-^{14}C]malonyl-CoA	Weight (mg.)	62.5	52.3	50.1	46.6	43.4	-
		sp.act.(d.p.m./mg.)	192	167	165	166	164	-
	[2-^{14}C]acetyl-CoA	Weight (mg.)	69.7	56.4	49.1	47.2	44.3	42.8
		sp.act.(d.p.m./mg.)	214	198	185	183	182	182

Table 18

The Preparation of the Digitonide Derivatives of Labelled Yeast Sterols diluted with Ergosterol

Source of ^{14}C label	[2-^{14}C] Acetyl-CoA		[2-^{14}C] Malonyl-CoA	
	1	2	1	2
Weight of Sterols (mg.)	42.1	46.3	43.2	44.1
Specific activity (d.p.m./mg.)	530	592	583	578
Weight of Digitonide (mg.)	117.3	118.3	117.8	118.2
Specific activity (d.p.m./mg.)	139	177	168	163
Recovery of Radioactivity in Digitonides (%)	75.0	76.3	79.2	76.1

from malonyl-CoA and two preparations labelled from acetyl-CoA. The recoveries of radioactivity in the digitonides were very similar.

Thin layer chromatography

The final technique used to survey the nature of the lipids that had become labelled from the two substrates was thin layer chromatography. A comparison of the distribution of the radioactivity in the two samples of this unsaponifiable fraction is displayed in figure 15. The labelling pattern in the two solvent systems used was almost identical.

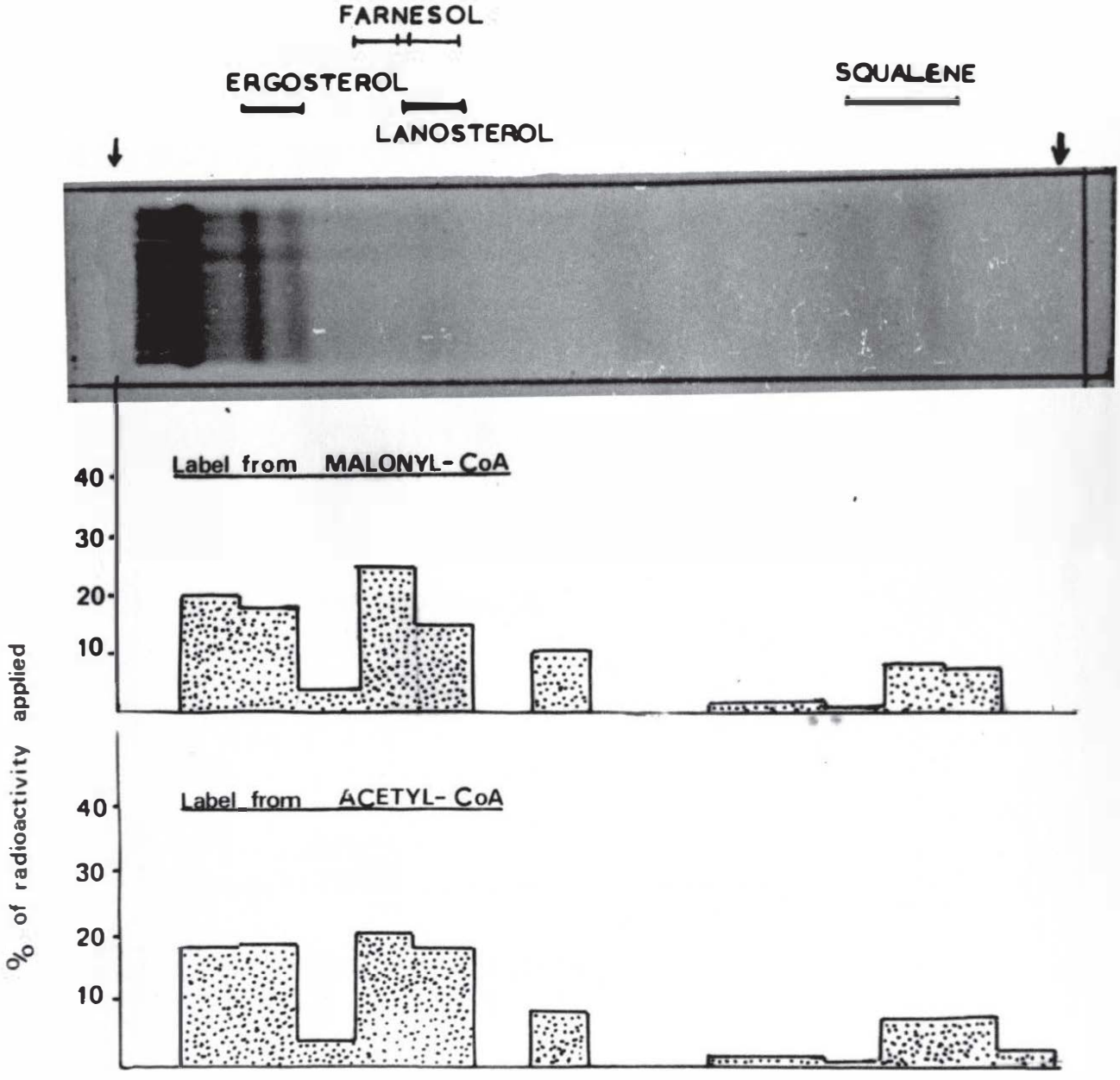
The results of these analyses have demonstrated that the labelling of the lipids did not depend on the labelled precursor used. Label from malonyl-CoA was incorporated into isoprenoid compounds, specifically squalene and ergosterol in the same way as label from acetyl-CoA.

The incorporation of label from [2-^{14}C]malonyl-CoA into mevalonic acid by Yeast and Hevea latex

Mevalonic acid is often regarded as the first in-

Fig 15 a

T.L.C. of UNSAPONIFIABLE YEAST LIPIDS FROM INCUBATIONS WITH [2 -^{14}C] MALONYL- CoA or [2 -^{14}C] ACETYL - CoA



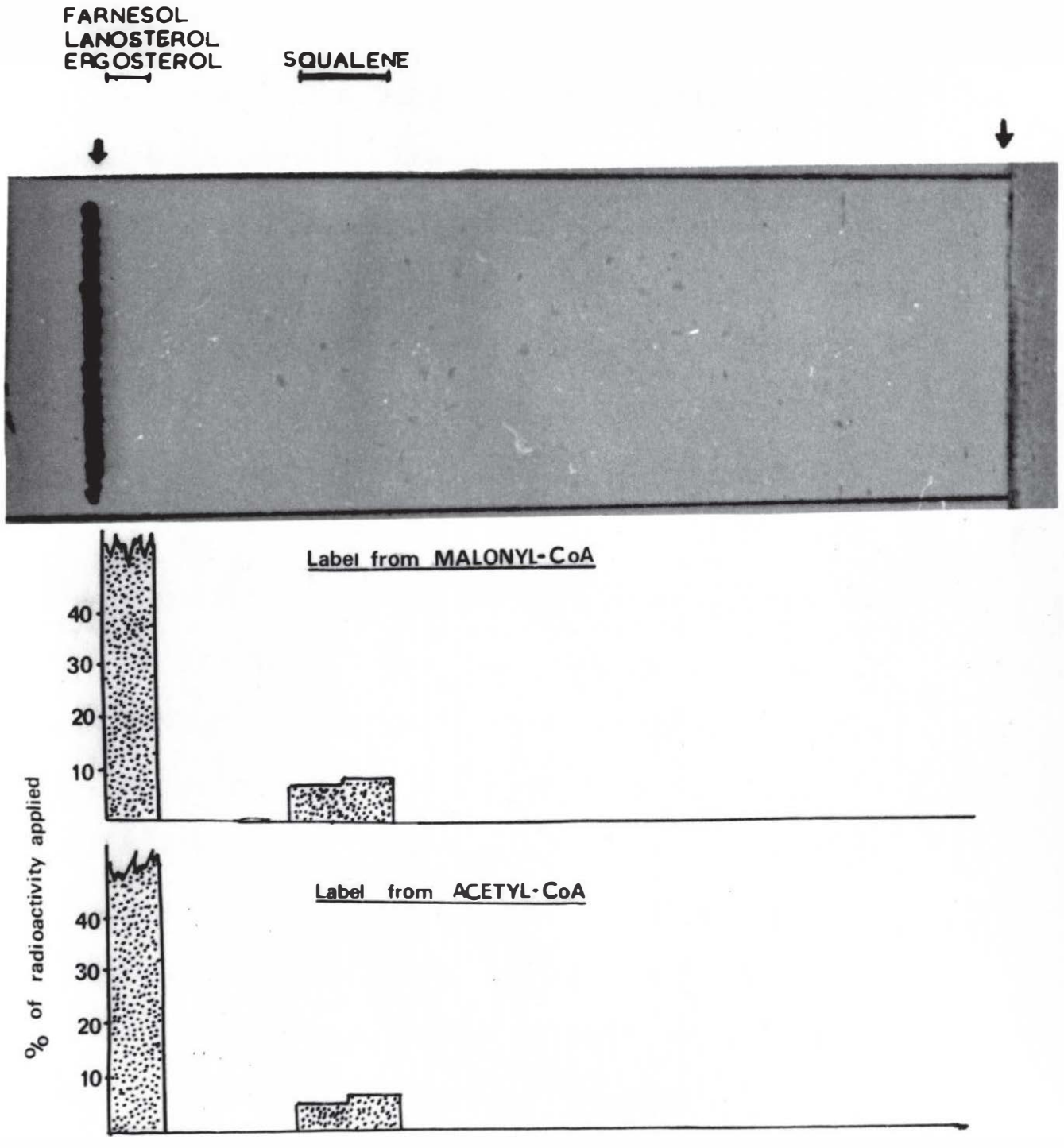
SOLVENT : LIGHT PETROLEUM - DIETHYL ETHER

60 40

SILICA - GEL- G .... 0·3·mm

Fig 15b

T L C of UNSAPONIFIABLE YEAST LIPIDS from INCUBATIONS with [2 -^{14}C] MALONYL-CoA or [2 -^{14}C] ACETYL-CoA



SOLVENT : LIGHT PETROLEUM

SILICA GEL-G 0.3 mm.

controvertible isoprenoid precursor. Further evidence for the utilisation of malonyl-CoA in isoprenoid synthesis was sought from experiments designed to trap any mevalonic acid that may be formed in incubation mixtures.

Table 19 shows the effect of the addition of unlabelled mevalonic acid on the incorporation of [2-^{14}C] malonyl-CoA and [2-^{14}C]acetyl-CoA into the fatty acids and unsaponifiable lipid fractions from the yeast system. Although fatty acid synthesis was not significantly affected, the incorporation from both substrates into the unsaponifiable fraction was markedly diminished.

Mevalonic acid was extracted from incubations and chromatographed on paper. (see methods section A, "enzymically produced derivatives of mevalonic acid"). The radioactive material that chromatographed with mevalonic acid was eluted and incubated with the MVA-kinase preparation from Hevea freeze dried serum. Figure 16 is a photograph of the autoradiograms of a chromatogram of the diethyl ether extract of an incubation of the yeast enzyme with [2-^{14}C] malonyl-CoA and unlabelled MVA, and a second chromatogram showing

Table 19

The Effect of unlabelled Mevalonate on the Incorporation of [2-^{14}C] Malonyl-CoA and [2-^{14}C] Acetyl-CoA Into Lipids by a Cell Free Yeast Preparation

Mevalonate added (µmoles)		0	5	10	20
Incorporation into fatty acids (mµmoles)	Acetyl-CoA	3.70	3.62	3.58	3.65
	Malonyl-CoA	5.90	5.90	5.72	5.81
Incorporation into the unsaponifiable lipids (mµmoles)	Acetyl-CoA	2.32	1.34	0.47	0.26
	Malonyl-CoA	2.17	1.19	0.32	0.22

Results are the mean of 3 determinations. Incubations were for 2 hours at 30°C. and contained 0.25ml. yeast preparation, 1.0mM- [2-^{14}C] Malonyl-CoA or 1.0mM- 2-^{14}C acetyl-CoA; 200mM-potassium phosphate buffer, pH 7.0. in a volume of 0.8ml.

Specific activity of the substrates was 1.75µC/µmole.

the result of the incubation with MVA kinase. The production of material chromatographing as MVAP is good evidence that the original labelled material was MVA.

A similar experiment was performed with Hevea latex. The diethyl ether extracts of incubations of latex with malonyl-CoA in the presence of unlabelled mevalonic acid were chromatographed in the butanol-formic acid-water, system (77:11:12). The radioactive material that chromatographed in the region of mevalonic acid was eluted and incubated with the mevalonic kinase preparation. On rechromatography in the ethanol-ammonia-water system (7:2:1) the result shown in figure 17 was obtained. Mevalonate 5 Phosphate was not formed. Moreover, in this solvent system, the radioactive material did not chromatograph at the same R_f as mevalonic acid.

It was thought that this observation might be analogous to those of Brodie and Porter (1960) who, using a pigeon liver system in the absence of NADPH observed the incorporation of ^{14}C-malonyl-CoA into several unidentified components. The most important

Fig 16

RADIOAUTOGRAM of the DIETHYL ETHER EXTRACT of an incubation of a YEAST SYSTEM with [2-^{14}C]MALONYL-CoA and an MVA TRAPPING POOL (1); and (2) the RESULT of treating the MATERIAL CHROMATOGRAPHING as MVA with HEVEA MVA-KINASE

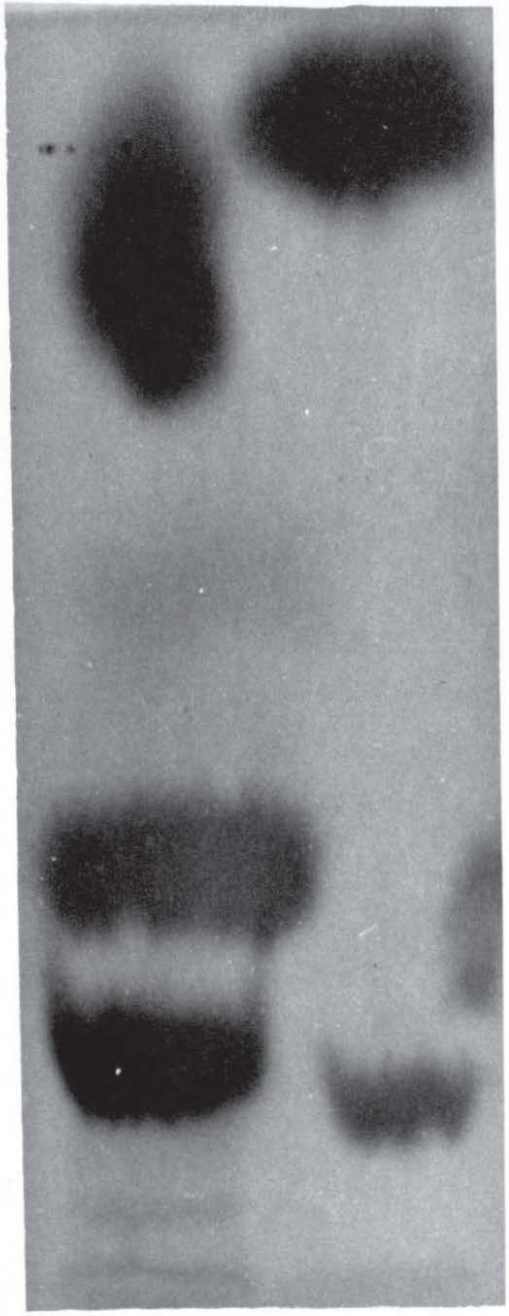
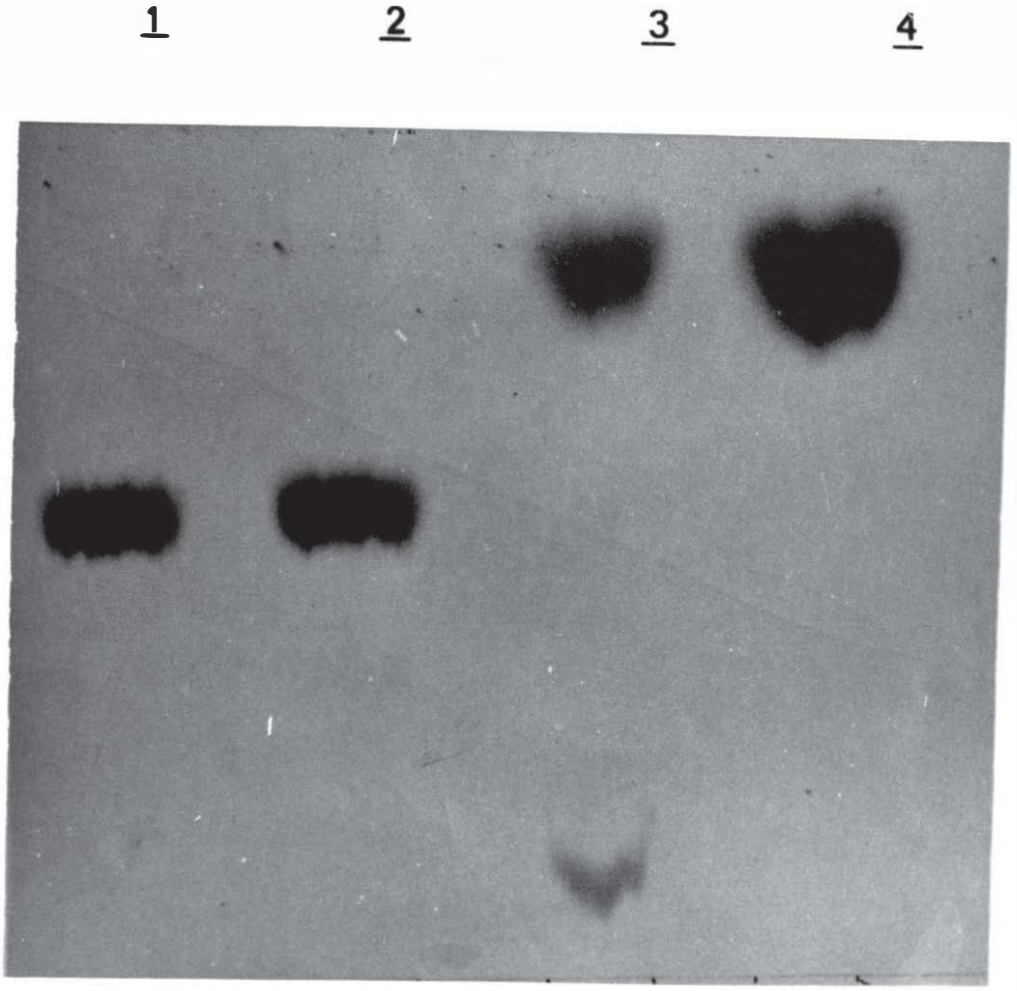


Fig 17

RADIOAUTOGRAM of EXTRACTS of LATEX INCUBATIONS with [2-^{14}C] MALONYL CoA and an UNLABELLED POOL of MVA



1 Material chromatographing with MVA in Butanol : Formic acid : Water

2 1 Treated with MVA kinase

3 MVA treated with MVA kinase

4 MVA

SOLVENT :– ETHANOL : AMMONIA : WATER 7 : 2 : 1

of these was later identified as HMG-CoA, which disappeared when NADPH was added to the incubation mixture.

To check this possibility, authentic unlabelled HMG was chromatographed in the same solvent systems and was found to have the same R_f as the material thought to be HMG (0.7 in butanol: formic acid: water, 0.55 - 60 in ethanol: ammonia: water).

The radioactive components were therefore pooled and diluted with 10 mg. of authentic HMG. The di-p-bromophenacyl ester was prepared and recrystallised:

No. of recrystallisation	1	2	3	4
weight of derivative (mg.)	8.5	6.3	5.4	4.2
specific activity (d.p.m./mg)	162	143	147	142

Incubations of [2-^{14}C] malonyl-CoA with the latex system were performed in the presence of 0.5mM NADPH and an unlabelled trapping pool of MVA. The incorpo-

ration of malonyl-CoA into rubber and fatty acids in this experiment is shown in table 20. The diethyl ether extract of this material contained labelled components that chromatographed as mevalonate and which gave rise to mevalonate-5-phosphate when incubated with the MVA-kinase preparation.

In both yeast and latex, therefore, malonyl-CoA gave rise to mevalonic acid. The addition of the unlabelled mevalonic acid causing a diminution in the incorporation into the unsaponifiable fraction in yeast or into the rubber fraction in Hevea latex. NADPH was necessary for the incorporation of malonyl-CoA into isoprenoid compounds in Hevea. In the absence of NADPH mevalonate was not formed, but malonyl-CoA gave rise to material which was probably HMG-CoA. (the thio-ester bond would have been hydrolysed at the saponification stage in the extraction) These results are analogous to those of Brodie and Porter (1960) obtained from a pigeon liver system, and may be interpreted as supporting their postulate of the direct involvement of malonyl-CoA in isoprenoid synthesis. The other interpretation i.e. i.e. decarboxylation of malonyl-CoA prior to the use of

Table 2o

The Effect of unlabelled Mevalonate on the incorporation of [2-^{14}C] Malonyl-CoA and [2-^{14}C] Acetyl-CoA into rubber and the Fatty Acid fraction by the Latex of Hevea brasiliensis

Mevalonate added (µmoles)		0	0.5	1.0	2.0
Incorporation into fatty acids (µµmoles/100mg.latex)	Acetyl-CoA	13.2	14.7	14.7	14.8
	+NADPH	18.5	18.8	18.3	17.9
	Malonyl-CoA	573	562	569	557
	+NADPH	624	643	627	630
Incorporation into rubber (µµmoles/100mg.latex)	Acetyl-CoA	263	131	32	24
	+NADPH	308	183	52	39
	Malonyl-CoA	-	not	significant	-
	+NADPH	73	32	9.2	7.8

Incubations contained 0.1ml. latex and the freeze dried substrate:

0.5mM-[2-^{14}C]malonyl-CoA or 0.5mM-[2-^{14}C]acetyl-CoA (0.80µC).

60 minutes at 30°C.

acetyl-CoA for the initial condensation, is not excluded.

The effect of carbon dioxide on the incorporation of [2-^{14}C]- acetate, [2-^{14}C]acetyl-CoA and [2-^{14}C] malonyl -CoA into lipids by the yeast system.

Weeks observed that some cofactors influenced the incorporation of acetate into fatty acid and rubber fraction of latex in opposing directions. Carbon dioxide and ATP had a pronounced effect. Reciprocal relationships of this kind have been observed in other systems, e.g. Klein (1957) in yeast, and Fletcher and Myant (1961) in cell free rat liver preparations. The effect of carbon dioxide was particularly striking in both cases.

Experiments were designed to test the effect of this factor on the incorporation of malonyl-CoA acetyl-CoA and acetate by the yeast system.

Yeast homogenate was incubated with the substrate in the main compartment of Warburg flasks. When incubations were to be carbon dioxide free, the phosphate buffer (pH 7.0) was freshly prepared from distilled water that had been boiled for thirty minutes. The centre wells of the vessels contained 0.2ml. of a saturated solution of potassium hydroxide and a wick of Whatman 544 filter

paper. The pH of each incubation mixture was checked with narrow range indicator paper.

The results of incubations which were conducted in carbon dioxide free solutions or in which bicarbonate was added are given in table 21.

The removal of carbon dioxide from the incubations almost abolished fatty acid synthesis from acetate and acetyl-CoA. When this occurred the incorporation into unsaponifiable lipid increased considerably. The incorporation of malonyl CoA was not affected by the absence of carbon dioxide.

(Klein (1957); Abraham, Matthes and Chaikoff (1961); Fletcher and Myant (1961) and Fimognari and Rodwell (1964) interpreted their observations of similar inverse relationships as indicating that malonyl-CoA does not play a significant role in isoprenoid synthesis. Weeks (1966), however, pointed out that if the control between mevalonate synthesis and fatty acids synthesis is at the acetoacetate level as postulated by Brodie, Wasson and Porter (1963)

Table 21

The Effect of Carbon Dioxide on the Incorporation of [2-^{14}C] Acetate, [2-^{14}C] Acetyl-CoA and [2-^{14}C] -Malonyl-CoA into Lipids by a Cell Free Yeast System

System		μμmoles incorporated/mg. protein					
		Acetate		Acetyl-CoA		Malonyl-CoA	
		S	US	S	US	S	US
Phosphate buffer	gas phase air	172	103	483	240	683	187
"	air -CO_2	18	437	28	973	685	191
"	air -CO_2, +0.1mM-$KHCO_3$	185	97	546	203	684	187
"	air -CO_2, +0.5mM-$KHCO_3$	192	96	543	208	685	193
"	air -CO_2, +1.0mM-$KHCO_3$	191	98	547	209	691	189

0.25ml. of yeast preparation was incubated with 1mM-substrate (0.75μC); 200mM-potassium phosphate buffer, pH 7.2 and potassium bicarbonate (where used) in a volume of 0.8ml. for 2 hours at 30°C.

S = Saponifiable lipid fraction

US = Unsaponifiable lipid fraction.

then a stimulation of the formation of malonyl-CoA by ATP or bicarbonate would increase the level of malonyl-CoA relative to acetyl-CoA and stimulate fatty acid synthesis at the expense of mevalonate synthesis.

The presence of such an inverse relationship, therefore, cannot be interpreted as confirmation or refutation of the role of malonyl-CoA in the biosynthesis of isoprenoids.

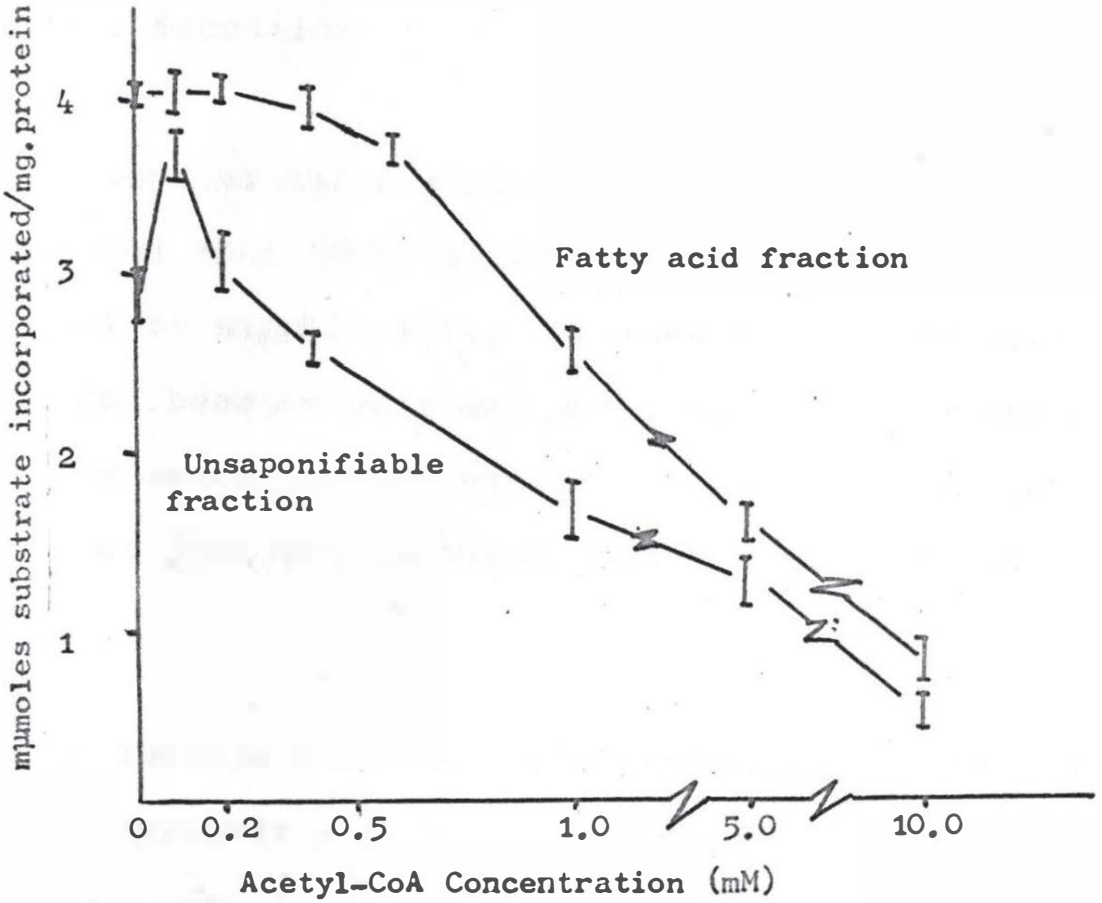
The effect of an unlabelled acetyl-CoA pool on the incorporation of malonyl-CoA into lipids by yeast

If malonyl-CoA is used as such for the initial condensation with acetyl-CoA the incorporation of malonyl-CoA should not be significantly diminished in the presence of unlabelled acetyl-CoA. Conversely, if malonyl-CoA is converted to acetyl-CoA before incorporation, the label would be diluted and incorporation would decrease.

The effect of increasing the concentration of unlabelled acetyl-CoA on the incorporation of malonyl-CoA into the lipid fractions of a yeast incubation is shown in figure 18.

Figure 18

THE EFFECT OF AN UNLABELLED POOL OF ACETYL-CoA ON THE INCORPORATION OF [2-^{14}C] MALONYL CoA INTO THE FATTY ACID AND UNSAPONIFIABLE FRACTIONS OF YEAST LIPIDS



Incorporations expressed as mμmole substrate/mg. protein

Acetyl-CoA concentration (mM)	0	0.1	0.2	0.5	1.0	5.0	10.0
Unsaponifiable lipid fraction	2.8	3.65	3.10	2.55	1.67	1.20	0.42
Fatty acid fraction	4.02	4.0	3.90	3.78	2.54	1.52	0.83

Incubations contained 1.0mM-[2-^{14}C]malonyl-CoA, 200mM-potassium phosphate buffer pH 7.2 and 0.25ml. yeast homogenate in a volume of 0.75ml.

Results are the average of 4 determinations, the range of data is shown in the graph

A low concentration of acetyl-CoA with respect to malonyl-CoA (1:10) stimulated incorporation into the unsaponifiable fraction. As the acetyl-CoA concentration increased the incorporation of malonyl-CoA into both fractions decreased.

If the initial acetyl-CoA pool were large, it would be expected that fatty acid synthesis from malonyl-CoA would not be significantly affected by the addition of acetyl-CoA because only one acetyl-moiety is required for every seven malonyl esters. Alternatively, if the acetyl-CoA pool was initially small some stimulation could occur.

The incorporation of malonyl-CoA *per se* into isoprenoid compounds would be stimulated in conditions of a limiting acetyl-CoA pool, by the addition of acetyl-CoA.

The results obtained are difficult to interpret in these terms. There was indeed a stimulation of the incorporation of malonyl-CoA at a low concentration of acetyl-CoA but at higher concentrations acetyl-CoA

became inhibitory.

Furthermore, incorporation into the fatty acid fraction was also inhibited. A possible explanation is that the [2-^{14}C] malonyl-CoA was diluted by the carboxylation of unlabelled acetyl-CoA. The situation is further complicated by the inhibitory effects of free CoA as is shown in table 22.

Table 22

The Effect of CoASH on the incorporation of [2-^{14}C] Malonyl-CoA into the Fatty Acid fraction (FA) and the Unsaponifiable lipid fraction(US) by the Cell Free Yeast System

Concentration of CoASH (mM)		0	0.1	0.5	1.0	5.0	10.0
Malonyl-CoA incorporated mμmoles/mg. protein	FA	4.62	4.63	4.32	3.38	1.85	0.76
	US	2.40	2.10	1.95	1.83	1.36	0.52

Incubations consisted of 0.25ml. of yeast preparation, 1.0mM-[2-^{14}C] malonyl-CoA (0.5μC) 500mM-potassium phosphate buffer, pH 7.3, and 1.0mM-ATP.
The pH of each incubation mixture was checked with narrow range indicator paper.

The result is the average of 3 determinations.

EXPERIMENTAL

2 RESULTS

B The Role of a Biotin dependent Carboxylation Reaction in the Synthesis of Isoprenoids from Acetate

The role of carboxylation reactions in the synthesis of isoprenoids and fatty acids from acetate or acetyl-CoA.

The inverse relationship between unsaponifiable-lipid and fatty acid synthesis induced by carbon dioxide (section A) suggested that a further study of the role of the carboxylation of acetyl-CoA in the synthesis of the two classes of lipids would provide information on the role of malonyl-CoA in isoprenoid synthesis. This reciprocal effect was first observed by Klein (1957)

Wakil, Titchener and Gibson (1958) showed that a biotin rich fraction of avian liver was essential for fatty acid synthesis and was selectively inhibited by avidin. Avidin has been used on several occasions in investigations on pathways of synthesis from acetate. Fletcher and Myant (1961) observed that fatty acid synthesis in cell free rat liver preparations was inhibited by avidin, but incorporation of label into cholesterol was not greatly reduced. Abraham, Matthes and Chaikoff (1961) concluded from a study on acetoacetate synthesis in rat liver and mammary gland that the condensation of two acetyl-CoA molecules for acetoacetate synthesis does not involve CO_2 fixation and that

the major pathway for fatty acid synthesis in rat liver and mammary gland depends entirely upon a carboxylation reaction in which, presumably, malonyl-CoA is formed.

Weeks (1966) observed that the incorporation of acetate into fatty acids by the latex of Hevea brasiliensis was markedly depressed in the presence of avidin. The incorporation of acetate into rubber was also inhibited quite significantly. These inhibitions were relieved by prior incubation with biotin.

Any inhibition by avidin must be reversible by prior treatment of the avidin by an excess of biotin. If this does not occur, the inhibition recorded cannot be claimed to be a specific inhibition of a biotin dependent carboxylation. Furthermore, when this reaction is bypassed by malonyl-CoA, it must be shown that avidin has no effect on fatty acid synthesis.

Incorporation of malonyl-CoA when synthesis from acetate is inhibited indicates that the route is:

a via malonyl-CoA without prior decarboxylation

b via acetyl-CoA as such without subsequent

carboxylation

In the circumstance of the incorporation from acetate or acetyl-CoA in the presence of avidin, pathway b must be operating.

The avidin used in the experiments described was purified by chromatography on CM-cellulose as described in the Methods section (B). The preparations were 92-96% pure avidin as assayed by spectrophotometric titration with biotin.

The effect of avidin on the incorporation of [2-^{14}C] acetate into rubber, unsaponifiable lipids and fatty acids by Hevea latex

Table 23 shows the result of increasing the concentration of avidin on the incorporation of acetate into rubber, unsaponifiable lipids and fatty acids. In a separate experiment, the concentration of avidin was constant at a level which almost completely inhibited the incorporation into fatty acids while the concentration of biotin was increased.

Table 23

The Effect of Avidin on the Incorporation of [2-^{14}C] Acetate into Rubber, Unsaponifiable lipid (US) and Fatty acid (FA) by the latex of Hevea brasiliensis

a) The Effect of Avidin

Avidin Concentration (mμM)		0	0.5	1.0	1.5	3.0	5.0	10
Incorporation of acetate μμmoles/100mg. latex	Rubber	751	796	833	824	817	810	808
	US	38.1	39.6	44.0	43.1	37.9	39.8	39.0
	FA	22.5	10.1	4.30	2.50	1.95	1.76	1.69

b) The Effect of Biotin + Avidin

Avidin concentration (mμM)		3	3	3	3	3	3	3
Biotin concentration (mμM)		0	1	3	6	9	12	15
Incorporation of acetate μμmoles/100mg. latex	Rubber	937	932	913	918	903	910	907
	US	42.6	41.7	43.1	41.8	38.0	38.2	38.7
	FA	1.43	1.51	1.50	1.72	8.73	24.3	31.2

Incubations were for 60 minutes at 30°C and contained 0.1ml. latex, 1.5mM-[2-^{14}C] acetate and the avidin and biotin. Substrates were freeze dried in the incubation vial.

Fatty acid synthesis was reduced to less than 10% of its former level in the absence of avidin, but there was no marked effect on the incorporation into rubber or unsaponifiable lipids.

The results are clearly difficult to reconcile with those obtained by Weeks. The only difference in experimental conditions was that in this case, a highly purified preparation of avidin was used. The inhibition of incorporation into fatty acids synthesis was greater than that reported by Weeks.

This result stimulated an examination of the effect of avidin in the yeast homogenate system and in a rat liver system; the results are shown in figures 19 and 20.

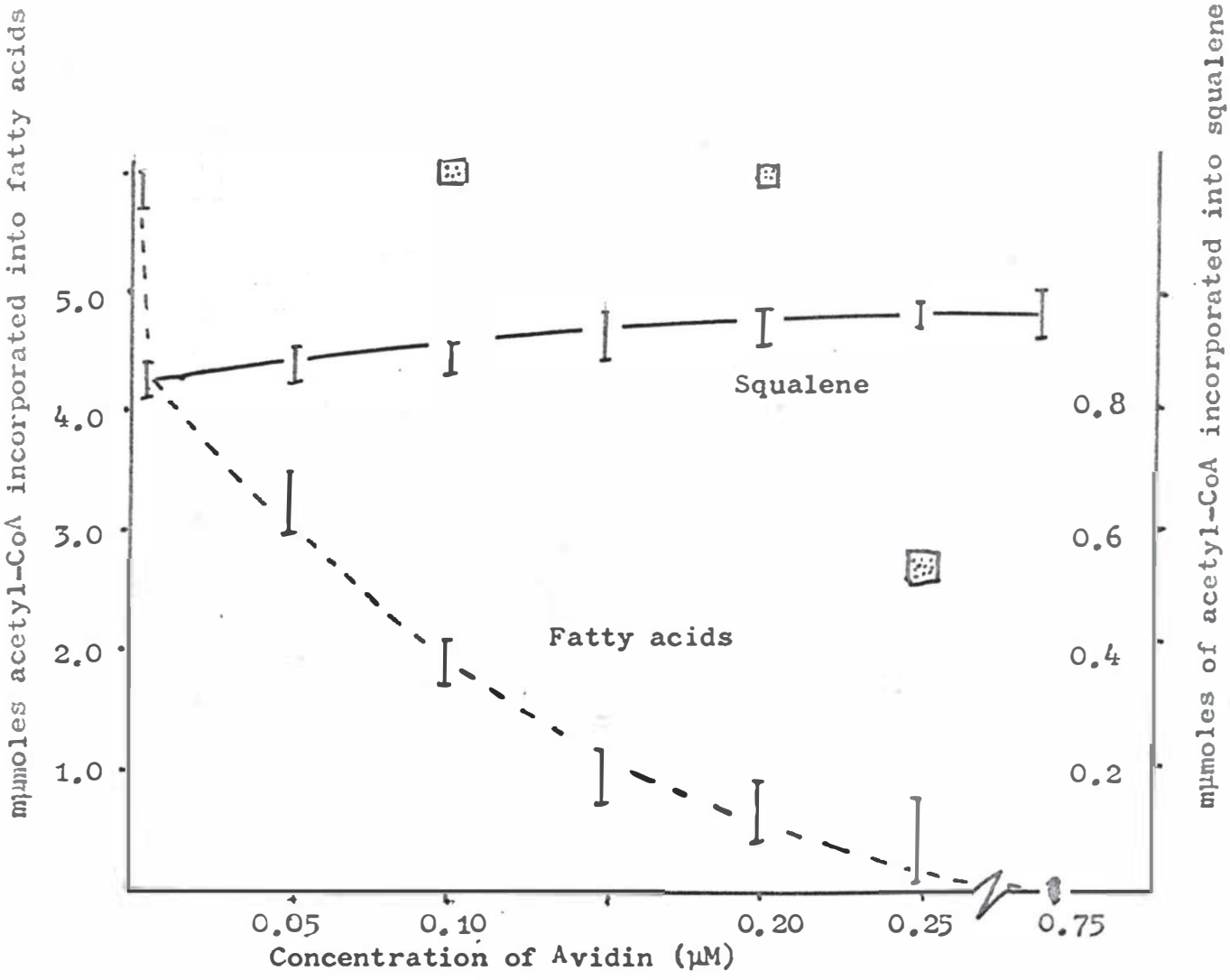
Further experiments were done to test the effect of avidin on the incorporation of malonyl-CoA into the fatty acid and unsaponifiable lipid fractions. There was no significant effect on the incorporation into unsaponifiable lipid, but a relatively small inhibition of the incorporation into fatty acids, (Table 24).

The sensitivity of the carboxylation reaction to avidin was clearly shown by the inhibition of the incorporation of acetate and acetyl-CoA into the fatty acid fraction by the rat liver and yeast systems. In neither case was there any effect on incorporation into squalene.

Since it is apparent that malonyl-CoA is not an obligatory precursor of isoprenoid compounds in these systems it is reasonable to postulate that incorporation into these components occurs after malonyl-CoA has been decarboxylated. If this is the case, the inhibition of the incorporation of malonyl-CoA into fatty acids can be explained by removal of malonyl-CoA by malonyl-CoA decarboxylase, recarboxylation being inhibited in the presence of avidin.

Figure 19

THE EFFECT OF AVIDIN ON THE INCORPORATION OF [2-^{14}C] ACETYL CoA INTO SQUALENE AND FATTY ACIDS BY A CELL FREE YEAST SYSTEM

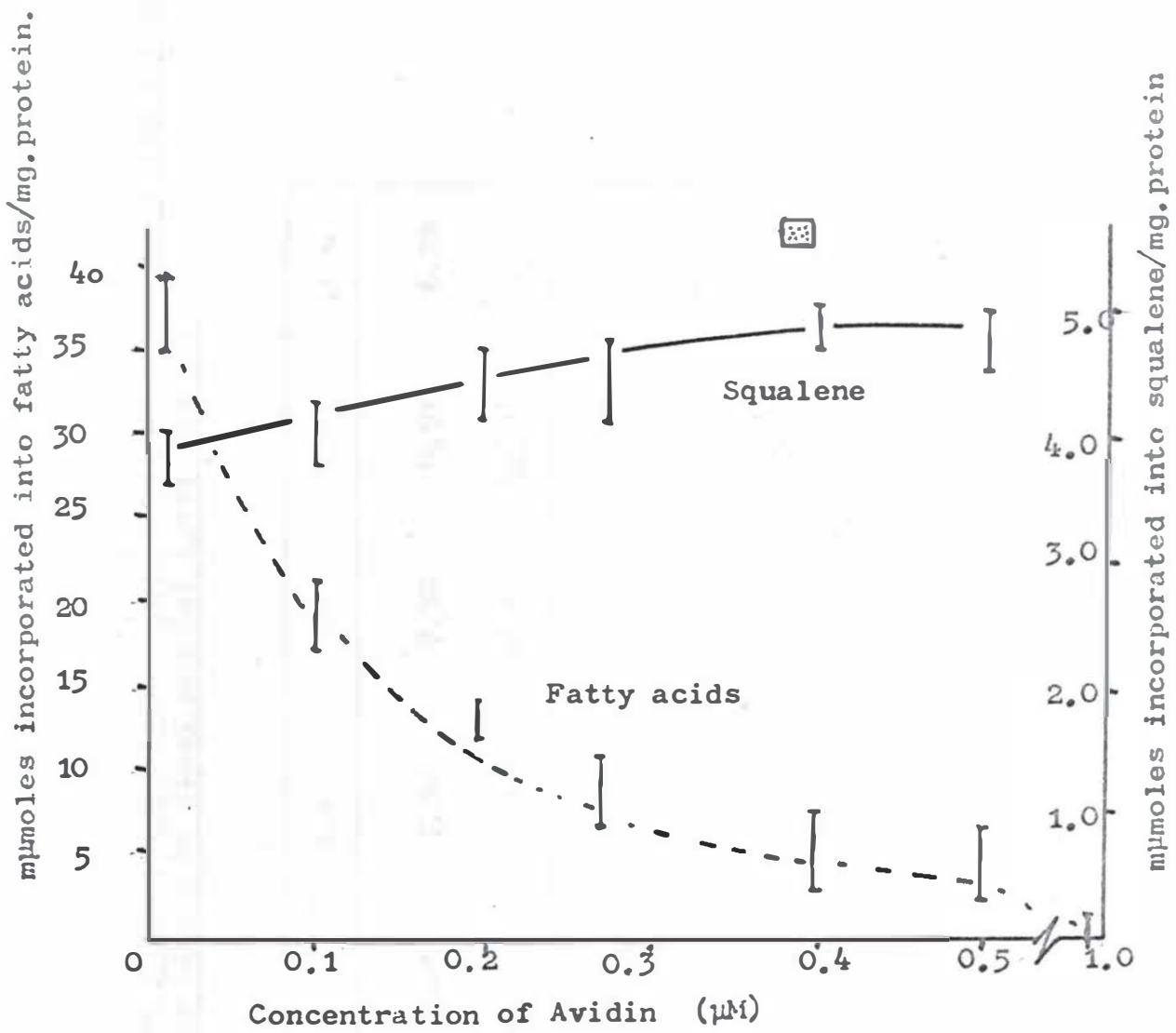


Incubations consisted of 0.50ml. of yeast homogenate; 1.0mM-$KHCO_3$; 0.5mM-NADPH; 1.5mM-ATP; Avidin and Biotin in a volume of 1.25ml. Incubations were anaerobic for 2 hours at 30°C. Squalene was isolated as the hexahydrochloride derivative.

represent the incorporation of substrate into fatty acids in the presence of 0.75μM-biotin.

Figure 20

THE EFFECT OF AVIDIN ON THE INCORPORATION OF [2-^{14}C] ACETYL-CoA into FATTY ACIDS AND SQUALENE BY A RAT LIVER SYSTEM



The range of values from three experiments is shown in the graph.

Incubations contained 1.5ml. 104,000g supernatant from liver homogenate (see text), and 2.0mg. microsomal protein. 500mM-potassium phosphate buffer, pH 7.3; 20mM-$MgCl_2$; 0.5mM-$MnCl_2$; 10mM-glutathione; 3.5mM-$KHCO_3$; 1.0mM-NADPH; 2.0mM-ATP and 2.0mM-potassium acetate. (After Abraham et al. 1961). Incubations were anaerobic.

——— = Incorporation into squalene

- - - - = Incorporation into fatty acids

Represents incorporation into fatty acids in the presence of 1.5mM-biotin.

Table 24

The Effect of Avidin on the Incorporation of [2-^{14}C] malonyl-CoA into Fatty acids and Unsaponifiable Lipids by Yeast and Rat Liver Systems

Avidin Concentration (μM)		0·0	0·1	0·2	0·5
Malonyl-CoA incorporated into fatty acids (mμmole/mg.protein)	Yeast	7.50	7.32	6.91	6.72
	Rat	98.7	93.8	92.7	87.2
Malonyl-CoA incorporated into unsaponifiable lipids (mμmol/mg. protein)	Yeast	2.41	2.39	2.39	2.68
	Rat	12.1	11.8	11.9	12.4

Yeast incubations as for Figure 19, but were aerobic. Rat liver incubations as for figure 20, but were aerobic.

Each incubation contained 0.5mM- 2-^{14}C Malonyl-CoA and 0.05mM-acetyl-CoA, and were terminated after two hours at 37°C (rat) or 30°C (yeast).

Results are the average of three experiments.

EXPERIMENTAL

2 RESULTS

C The Route of Incorporation of Malonyl-CoA into Isoprenoids Determined by Examination of Labelling Patterns.

1 The labelling of Squalene from acetate and from malonyl-CoA

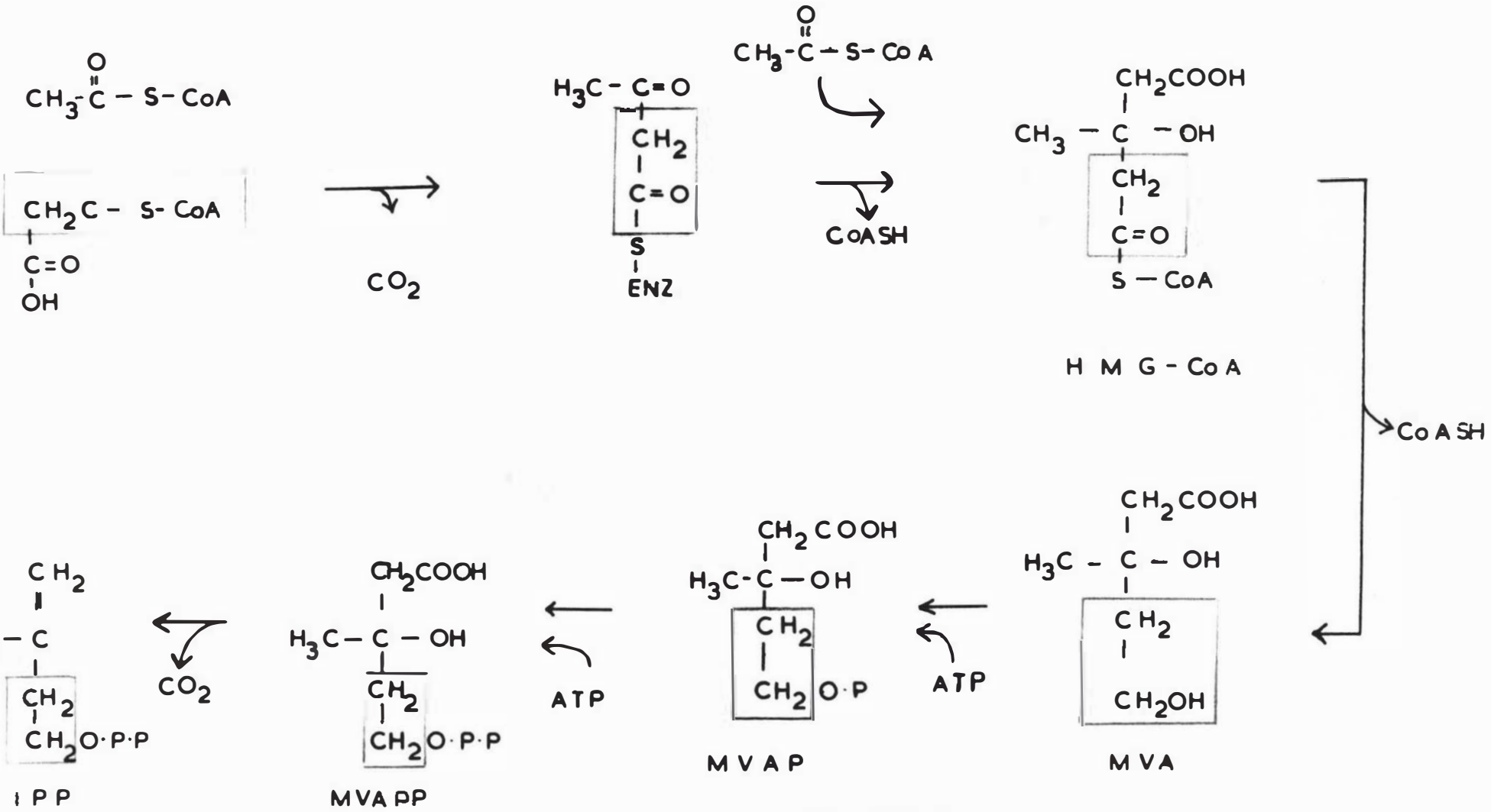
The results described in section A and B have indicated that label from [2-^{14}C] malonyl-CoA is indeed incorporated into isoprenoid compounds. The effect of avidin indicated that malonyl-CoA as such was not being incorporated, rather, that this substrate was being converted first of all into acetyl-CoA.

Scheme 6 traces the path of carbon from malonyl-CoA in the initial stages of isoprenoid synthesis if it were to follow the route proposed by Brodie et al. (1963, 1964). The incorporation of methylene labelled carbon and carbon labelled in the carboxyl position will be different, since the label in the latter is divided between the free carboxyl - which is lost - and the esterified atom. Consequently, the ratio of incorporation from [2-^{14}C] malonyl-CoA to the incorporation from [1,3-^{14}C] malonyl-CoA will be 2:1 if the substrates are of the same specific activity and if malonyl-CoA condenses with unlabelled acetyl-CoA. Alternatively, if the malonyl-CoA is completely decarboxylated prior

Scheme 6

BIOSYNTHESIS OF ISOPRENOIDS FROM MALONYL-CoA

ACCORDING TO BRODIE, WASSON & PORTER (1963)



to condensation and labelled acetyl-CoA derived from malonyl-CoA condenses with labelled acetyl-CoA also derived from labelled malonyl-CoA the ratio will be 3:1. [2-^{14}C] acetate and [1-^{14}C] acetate will be incorporated in the ratio of 3:2. Unambiguous information about the pathway being followed should be obtained by determining the ratios of the incorporation of the [1,3-^{14}C] and the [2-^{14}C] labelled substrates into a pure isoprenoid compound.

Squalene was chosen as the test material because the authentic compound of high purity was readily available. Furthermore, squalene is easily isolated by adsorption chromatography and its crystalline hexahydrochloride derivatives prepared. The yeast system described previously has been shown to incorporate acetate, acetyl-CoA and malonyl-CoA into squalene. Knauss et al. (1959) described a cell free rat liver enzyme system that incorporated acetate into squalene. The preparation of this system is described in Methods B (7).

The incorporation of acetate into isoprenoids by the rat liver system of Knauss et al.

The incubations, in a final volume of 2.5ml., contained: 3mg. microsomal protein, 10mg. soluble protein (40% - 80% ammonium sulphate fraction), 1.0 μmole -ATP, 2.5 μmole $MgCl_2$ 7.0 μmole-glucose-1-phosphate, 0.3 μmole-NAD^+, 0.3μmole-$NADP^+$, 0.025μmole-CoA 10.0 μmole-glutathione and 500 μmole potassium phosphate buffer pH 7.0. Radioactive acetate was added to a final concentration of 2.0mM.

After two hours incubation the total lipids were extracted. 100mg. squalene and 100mg. cholesterol were added as carriers and were separated on columns of deactivated alumina. Table 25 and 26 give the specific activity of the squalene hexahydrochloride and cholesterol digitonide derivatives.

The system was therefore active in isoprenoid synthesis from acetate.

Experiments were done in which the ratio of the

incorporation of [2-^{14}C] acetate and [1-^{14}C] acetate was determined. The results of these experiments are given in table 27. A similar experiment was performed using the yeast system.

In both the rat and the yeast systems the ratio of incorporation from [2-^{14}C] and [1-^{14}C] acetate approached 1.50, which was the theoretical result.

Table 25

Incorporation of [2-^{14}C] Acetate into Squalene and Cholesterol by the Rat Liver Enzyme System (after Knauss et al. ,1959)

AEROBIC INCUBATIONS

Time of Incubation (minutes)	30	90	150	180
Specific activity of squalene hexahydrochloride (d.p.m./mg.)	42 (2)	105 (2)	100±6.3 (5)	96.7±7.2 (5)
Specific activity of cholesterol digitonide (d.p.m./mg.)	67 (2)	232 (2)	375±13.7(5)	382±15.6 (5)

Incubations contained 10mg. soluble protein (40 -80% ammonium sulphate fraction) and 3mg. microsomal protein. 1.0µmole ATP; 2.5µmole $MgCl_2$; 0.3µmole NAD^+; 0.3µmole $NADP^+$; 0.025µmole CoASH; 10.0µmole glutathione and 500µmole potassium phosphate buffer, pH 7.0 in a total volume of 2.5ml. Acetate was added to a concentration of 2.0mM (0.5µC./µmole).

Table 26

Incorporation of [2-^{14}C] Acetate into Squalene and Cholesterol by the Rat Liver Enzyme System

ANAEROBIC CONDITIONS

Time of Incubation (minutes)	30	60	90	120	150	180
Specific activity of squalene hexahydrochloride d.p.m./mg.	78 (2)	162 (2)	265 (2)	347 (2)	422±15.5 (5)	426±14.6 (5)
Specific activity of cholesterol digitonide d.p.m./mg.	4.6 (2)	10 (2)	21 (2)	28 (2)	35±4.3 (5)	36±4.9 (5)

(n) .. average of n determinations

± n .. Standard error of the mean of n determinations

Legend to Table 27

The rat liver system incubations consisted of:-
3mg. microsomal protein; 10mg. soluble protein (40%-80% ammonium sulphate fraction); 1.0µmole ATP; 2.5µmole $MgCl_2$; 7.0µmole glucose-1-phosphate; 0.3µmole NAD^+; 0.3µmole $NADP^+$; 0.025µmole CoASH; 10.0µmole glutathione and 500µmole potassium phosphate buffer, pH 7.0. Radioactive acetate was added to a final concentration of 2.0mM (0.1µC./µmole), in a volume of 2.5ml. The results are from 3 determinations. of incubations of 2.5 hours at 37°C.

The Yeast system consisted of 10mg. protein of the 12,000g supernatant, and was fortified with 0.0 25µmole-CoASH; 0.3µmole NADPH; and 1µmole ATP in a volume of 2.5ml. Acetate was at a final concentration of 2.0mM (2.0µc/µmole).

Incubations were anaerobic.

Table 27

The Ratio of the Incorporation of [2-^{14}C]: [1-^{14}C] Acetate into Squalene (isolated as the hexahydrochloride derivative) by Cell Free Systems from Rat Liver and Yeast

		RAT LIVER				YEAST			
No. of times crystallised		1	2	3	4	1	2	3	4
A Incorporation of [2-^{14}C] Acetate	Total d.p.m.	15,900	14,210	14,027	13,188	42,350	41,250	39,000	36,500
	sp.act. d.p.m./mg.	162.1	156.3	164.3	160.7	385	362	366	357
B Incorporation of [1-^{14}C] Acetate.	Total d.p.m.	9,085	8,902	8,800	8,130	28,957	27,270	25,870	24,027
	sp.act. d.p.m./mg.	102.9	107.7	109.6	107.3	262	247	243	244
Ratio of specific activities A:B		1.57	1.46	1.49	1.50	1.47	1.47	1.51	1.47

The incorporation of malonyl-CoA into squalene by the cell free yeast system

The results of experiments in which malonyl-CoA, labelled in either the [1,3] carbon or [2] carbon atom was substituted for acetate in the yeast and the rat liver system are shown in table 28. Acetyl-CoA (0.05mM) was added to both yeast and rat liver systems. CoA was omitted from incubations containing CoA esters.

In the yeast system there was a much lower incorporation of label from the carboxyl carbon of malonyl-CoA than anticipated to give a $[2\text{-}^{14}C] / [1,3\,^{14}C]$ ratio of 9.2:1. In the rat liver experiments the opposite was the case for here a ratio of 1.3:1 was found.

This result is clearly different from that obtained from acetate and the interpretation in terms of utilisation of malonyl-CoA itself or prior decarboxylation is difficult.

All the incubations initially contained acetyl-CoA, for if malonyl-CoA were not being decarboxylated this

Legend to Table 28

Incubation conditions were as for table 27, but malonyl-CoA replaced acetate, CoASH was omitted and the yeast incubations contained 25mg. protein. Specific activity of malonyl-CoA was adjusted to 1μC./μmole.

The ratio of the specific activity of the squalene hexahydrochloride derived from -

$[2\text{-}^{14}C]$Malonyl-CoA : $[1,3\text{-}^{14}C]$Malonyl-CoA

is:- Rat - 1.31 : 1 ; Yeast - 9.20 : 1.

Table 28

The Ratio of the Incorporation of [2-^{14}C] Malonyl-CoA to the Incorporation of [1-3-^{14}C] Malonyl-CoA into Squalene by the RAT and YEAST Enzyme systems.

		RAT	YEAST
Incorporation of [2-^{14}C]-Malonyl-CoA	Total d.p.m.	13,622±1350 (9)	98,770±5,200 (6)
	sp. act. d.p.m./mg.	174±12.7 (9)	1,224±92 (9)
Incorporation of [1,3-^{14}C] Malonyl-CoA	Total d.p.m.	10,824±852 (9)	11,440±870 (9)
	sp. act. d.p.m./mg.	131±8.5 (9)	133.0±7.9(7)

would be necessary for isoprenoid synthesis. If decarboxylation were occurring the concentration of acetyl-CoA as well as the specific activity of the acetyl-CoA pool would increase during the incubation. The experiment which is described below was designed to see if there was any change in the ratios of incorporation as the incubations proceeded.

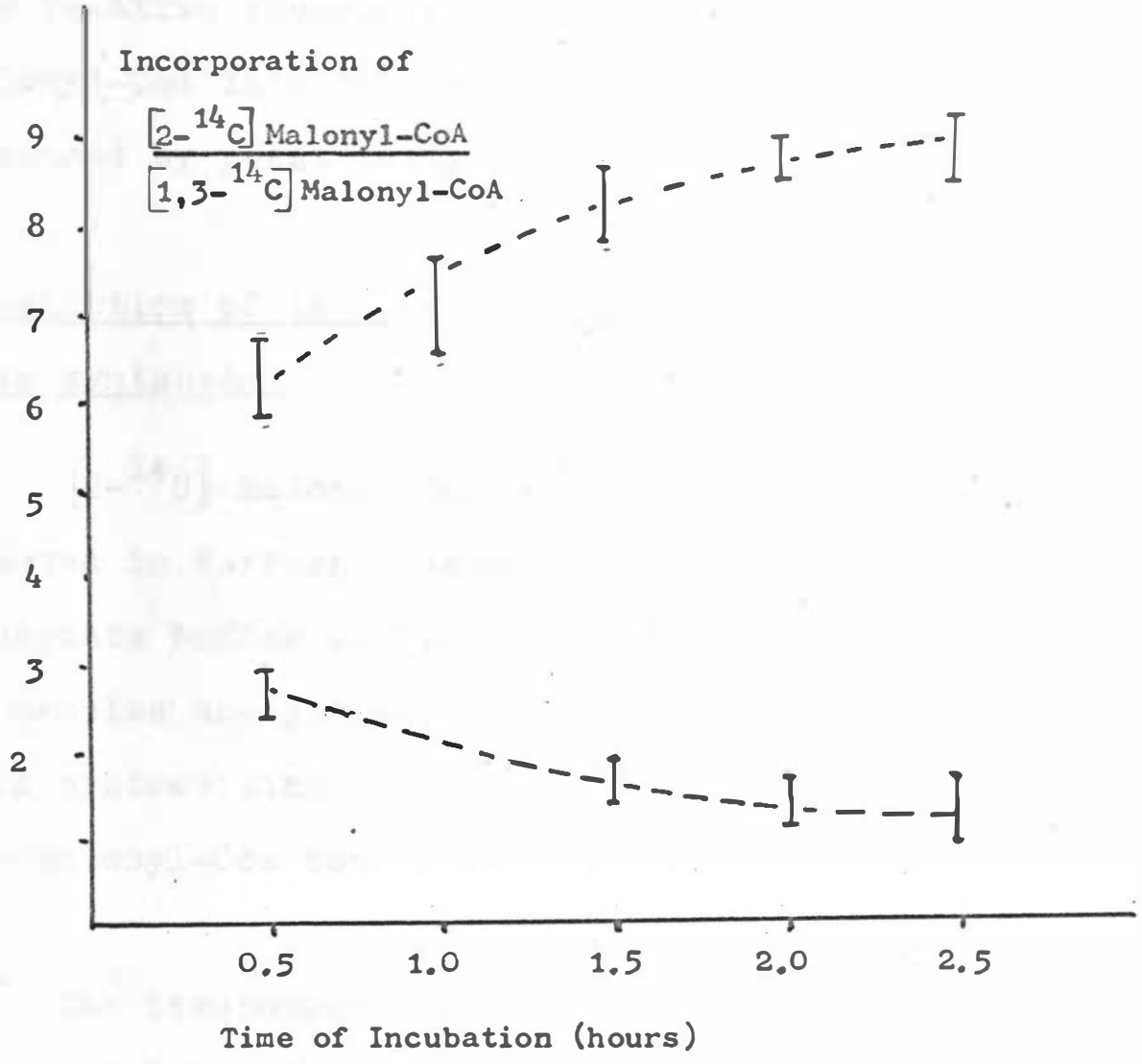
Variation of the ratio of [2-^{14}C] malonyl-CoA to [1,3-^{14}C] malonyl-CoA incorporated with time of incubation.

Incubations were set up in the same manner as above, but were increased in scale five fold. Aliquots were withdrawn at half hourly intervals and after the addition of carrier squalene and its isolation by chromatography on columns of deactivated alumina, the hexahydrochloride derivatives were prepared. The result is shown in figure 21.

That there was indeed a change in the relative incorporations during the incubations does not help the interpretation of the results. The marked decrease

Figure 21

THE VARIATION OF THE INCORPORATION OF [2-^{14}C] MALONYL-CoA RELATIVE TO THE INCORPORATION OF 1,3-^{14}C MALONYL-CoA INTO SQUALENE WITH TIME OF INCUBATION IN THE RAT AND YEAST SYSTEMS



Incubation conditions were as described in table 27, but on a larger scale (see text).

Aliquots were withdrawn at the stated times and the incorporation of substrate into squalene (as the hexahydrochloride derivative) was determined.

in the incorporation of carboxyl labelled carbon by the yeast preparation as the incubation proceeded suggested that in inhibiting impurity was present in the malonyl-CoA preparation. In order to check this, the relative incorporation of the two carbons from malonyl-CoA into the fatty acids and carbon dioxide produced by yeast fatty acid synthetase was measured.

Examination of labelled malonyl-CoA by the yeast fatty acid synthetase

[2-^{14}C] malonyl-CoA or [1,3-^{14}C] malonyl was incubated in Warburg flasks with 200 μmoles potassium phosphate buffer pH 7.0 5 μmoles 2-mercaptoethanol, 20 mμmoles acetyl-CoA 0.3 μmoles NADPH and excess fatty acid synthesising enzyme in a total volume of 1ml. The malonyl-CoA concentration was 50μM.

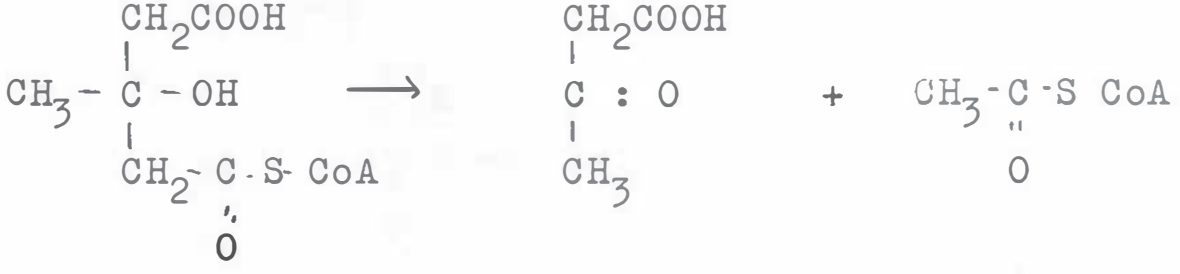
The incubations were stopped after 15 minutes by adding 0.1ml. of 60% perchloric acid from a side arm of the flask. The carbon dioxide released was collected in 0.2ml. 1.0M-hyamine hydroxide in methanol contained in the centre well of the flask. Fatty acids were extracted as described in the assay of the pigeon

liver fatty acid synthetase (Methods B 2). Table 29 shows the incorporation of radioactivity into fatty acids and carbon dioxide.

The distribution of ^{14}C label in the malonyl-CoA of both species was that predicted by theory. The anomolous relative incorporations into squalene therefore remained unexplained, but were unlikely to be due to an impurity in the malonyl-CoA.

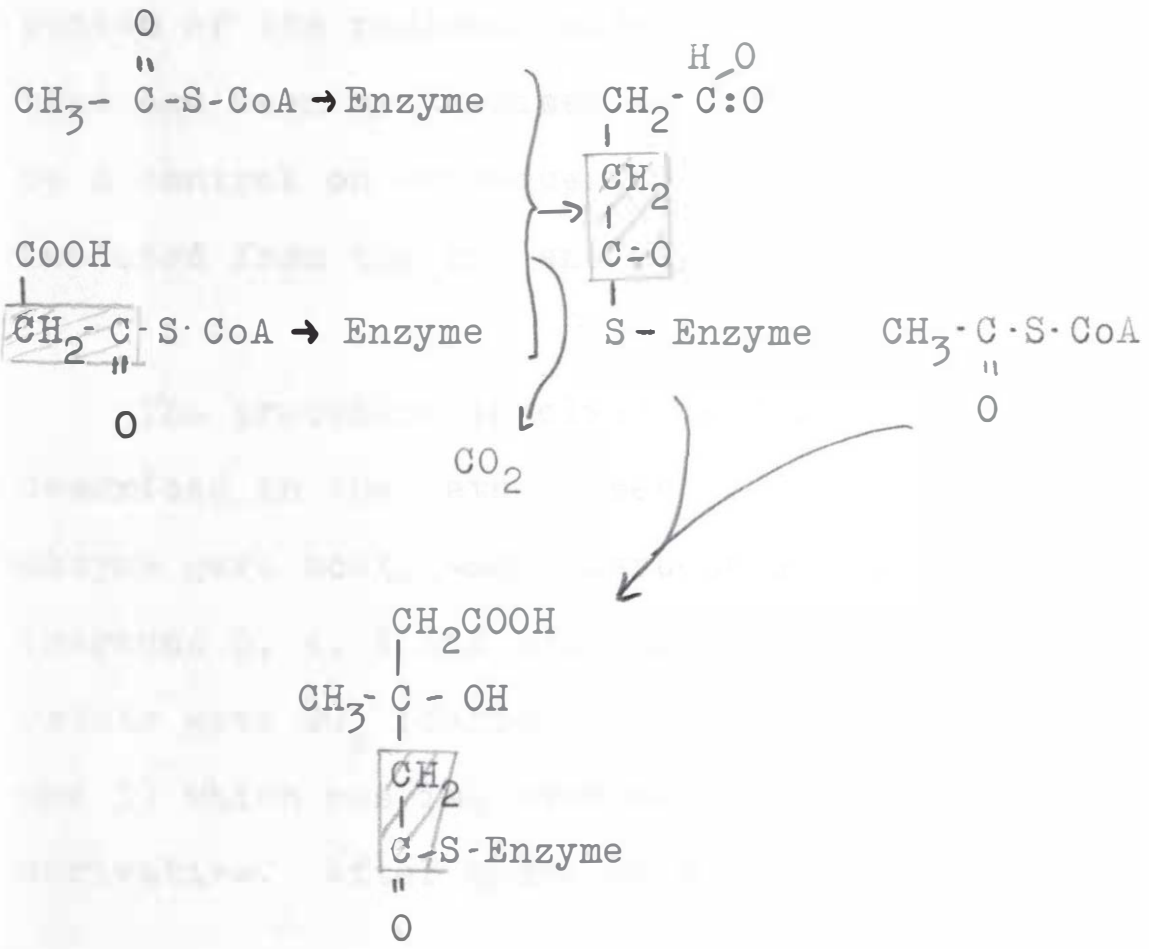
2 Degradation of HMG-CoA

Brodie *et al*. (1964) showed that HMG-CoA synthesis by a pigeon liver preparation in the absence of NADPH was dependent on malonyl-CoA. They confirmed this by degrading HMG-CoA with HMG-CoA cleavage enzyme. Bacchawat *et al*. (1955) demonstrated that this enzyme degraded HMG-CoA specifically, as shown:



This was confirmed by Rudney and Ferguson (1959) who used cleavage enzyme prepared by the method of Bachhawat *et al*. (1955) to degrade HMG-CoA that had been synthesised by an HMG-CoA condensing enzyme from yeast, (see Methods, B (4,5).

In the scheme below, the part of the molecule derived from malonyl-CoA in the Brodie *et al*. pathway is shaded.



Partial degradation of HMG-CoA synthesised by HMG-CoA condensing Enzyme

HMG-CoA synthesised by a partially purified condensing enzyme (Ferguson and Rudney, 1959) was isolated as described by Rudney and Ferguson (1959) (Methods, D 3). Since the substrates for this reaction were acetoacetyl-CoA and either [1-^{14}C]acetyl-CoA or [2-^{14}C] acetyl-CoA the label in the product was in position 6 or 5. The subsequent determination of the distribution of the radioactivity in the HMG-CoA molecule that had been synthesised in this way was intended to be a control on subsequent degradations of HMG-CoA isolated from the rat and pigeon liver systems.

The procedure involved in the degradation is described in the methods section (D 3). Cleavage enzyme gave acetyl-CoA (carbons 1 and 2) and acetoacetate (carbons 3, 4, 5 and 6). Decarboxylation of the acetoacetate gave CO_2 (carbon 6) and acetone (carbons 3, 4 and 5) which was isolated as the dinitrophenylhydrazone derivative. After hydrolysis of the thioester bond

the acetate was degraded by the Schmidt azide reaction to give C-1 of the HMG-CoA as CO_2. The resulting methylamine was further oxidised by potassium permanganate to give C-2 as CO_2.

The result of such a degradation of HMG-CoA synthesised by condensing enzyme is shown in table 30. The distribution of radioactivity in the molecule was found to be in accord with that predicted from theory, and demonstrated that label was not randomised by the cleavage enzyme preparation.

Degradation of HMG-CoA isolated from a rat liver system.

HMG-CoA was isolated from incubations of [2-^{14}C] acetyl-CoA with the unwashed microsomal system (Rudney, 1957; see methods B(7)) The degradations were performed as before and the result is shown in table 31. As expected, the label was distributed between carbon 2 and carbons 3+4+5 in the ratio of approximately 1:2. When a large pool of acetoacetyl-CoA was added, 90% of the radioactivity was located in C3+4+5 and 7.6% in C-2. This indicated that the randomisation of label by

Table 30

Degradation of the HMG-CoA Synthesised by Yeast Condensing Enzyme from [1-^{14}C] or [2-^{14}C] Acetyl-CoA and Acetoacetyl-CoA

Position of Label in HMG-CoA	% of recovered radioactivity				
	C -1	C - 2	C - 3+4+5	C - 6	Total recovery(%)
C - 5	< 1	< 1	98±2.8	< 1	82.0±3.5
C - 6	<1	< 1	3.4±0.8	96±5.3	84.9±4.8

Results are ± Standard error of the mean of 6 determinations

Table 31

Degradation of HMG-CoA Isolated from Incubations of [2-^{14}C] Acetyl-CoA with Unwashed Rat Liver Microsomes (Rudney, 1957)

a)

	C -1	C - 2	C - 3+4+5	C - 6	Recovery (%)
% of total recovered radioactivity	4.7±1.9	29.8±2.1	62±1.7	3.5±0.2	78±4.3

b) Degradation of HMG-CoA synthesised from 2-^{14}C acetyl-CoA + unlabelled 2.0mM-acetoacetyl-CoA

1.3±0.09	7.6±0.81	90±3.2	1.8±0.11	81±3.6

Incubations (2 hours at 37°C) contained 0.25mM-[2-^{14}C] acetyl-CoA, 0.5μC.; 5mM-$MgCl_2$; 2.0mM-cysteine pH 7.0; 200mM-potassium phosphate buffer, pH 7.2 and 10mg. of microsomal protein, in a volume of 1.5ml.

The results are ± Standard Error of the Mean of 6 Determinations.

cleavage enzyme and resynthesis of HMG-CoA from the acetyl-CoA produced was not sufficiently rapid to invalidate results obtained with malonyl-CoA.

[2-^{14}C] malonyl-CoA was substituted for acetyl-CoA and incorporation of radioactivity into material chromatographing with HMG-CoA in the ammonium acetate pH 4.7 - ethanol system (Rudney and Ferguson, 1959) is shown in table 32. The effect of the addition of unlabelled acetyl-CoA to incubations containing malonyl-CoA is shown, also in table 32. Incorporation of radioactivity from malonyl-CoA into HMG-CoA was decreased.

Degradation of HMG-CoA from incubations with [2-^{14}C] malonyl-CoA or [1,3-^{14}C] malonyl-CoA gave the result shown in table 33. The pattern did not differ significantly from that obtained by the degradation of HMG-CoA synthesised from acetyl-CoA in this system.

This result from a crude rat liver system is in direct conflict with those obtained by Brodie et al. with a pigeon liver system that had been partially purified for fatty acid synthesis. Those authors

Table 32

Incorporation of Radioactivity from Malonyl-CoA into HMG-CoA by Unwashed Microsomes of Rat Liver

Incubation conditions as for Table 31

System		Incorporation mμmoles/mg. protein
2-^{14}C Malonyl-CoA	0.75mM	24.5
+ 0.75mM-acetyl-CoA		17.5
+ 3.00mM-acetyl-CoA		12.5
+ Acetoacetyl-CoA	0.1mM	32.5
"	0.2mM	42.3
"	0.5mM	27.8
"	1.0mM	21.2

Table 33

Degradation of HMG-CoA synthesised by Unwashed Rat Liver Microsomal Preparations from [2-^{14}C] Malonyl-CoA or [1,3-^{14}C] Malonyl-CoA

Origin of Label	% of recovered radioactivity C - 1	C - 2	C - 3+4+5	C - 6	Total Recovery(%)
[1,3-^{14}C] Malonyl-CoA	31.4±2.0	<1	29.5±1.87	39.0±2.10	77.5±5.2
[2-^{14}C] Malonyl-CoA	<1	31.5±1.8	67.0±9.3	1.5±0.06	83.2±4.9

Incubation conditions were as for Table 31, except that malonyl-CoA was substituted for acetyl-CoA.

The Results are ± the Standard error of the mean of 6 determinations.

found that label from malonyl-CoA was localised exclusively in carbons 1 and 2 of the HMG molecule. This accorded with their proposed mechanism of the biosynthesis of the acetoacetyl moiety for isoprenoid synthesis. Also in conflict with Brodie et al. is the effect of acetyl-CoA on the incorporation of malonyl-CoA into HMG-CoA.

These results may, in fact, be explained by the randomisation of label in the CoA molecule by cleavage of HMG-CoA to acetyl-CoA and acetoacetate. Condensation of acetyl-CoA to form acetoacetyl-CoA and another condensation to give HMG-CoA would randomise the label. When relatively high concentrations of acetoacetyl-CoA were added to such incubations (tables 31b and 32), 90% of the recovered radioactivity was in carbons 3+4+5. Although 7.6% was in carbon 2 of the acetate derivative, the randomisation was not sufficient to bring about an even distribution throughout the molecule.

It was clearly necessary to obtain a more direct comparison with the experiments of Brodie et al.

The degradation of HMG-CoA isolated from a pigeon liver system

HMG-CoA was isolated from incubations of [2-^{14}C] malonyl-CoA with the first ammonium sulphate fraction from the purification of the pigeon liver fatty acid synthetase (preparation B, see methods B (3)). The degradation is shown in table 34. This experiment was not directly comparable with those of Brodie *et al.* since they used the eluate from DEAE-cellulose chromatography. This fraction was shown to be an unreliable source of HMG-CoA synthesising activity; this will be discussed in a later section.

The result was very similar to that obtained from the rat system and therefore dissimilar to those obtained by Brodie *et al.*

Table 34

Degradation of HMG-CoA Isolated from Incubations of [2-^{14}C] Malonyl-CoA or [2-^{14}C] Acetyl-CoA with the 20 - 40% Ammonium sulphate fraction of Pigeon Liver

Source of Labelled Carbon	% of recovered radioactivity				
	C-1	C - 2	C - 3+4+5	C - 6	Total Recovery(%)
2-^{14}C Malonyl-CoA	1.2	32.2	66.9	1.3	76
2-^{14}C Acetyl-CoA	2.4	29.7	67.2	3.7	81

Results are the average of 3 determinations.

Incubations contained 15mg.-protein; 1mM-EDTA; 1mM-dithiothreitol; 2mM-$KHCO_3$; 50mM-potassium phosphate buffer, pH7.0. 0.5mM- [2-^{14}C] Malonyl-CoA (sp.ast. 3.0µC./µmole) + 0.1mM-Acetyl-CoA; 2mM-$MgCl_2$ in a final volume of 2ml.

3 The partial degradation of Ergosterol from Incubations of [2-^{14}C] acetate and [2-^{14}C]malonyl-CoA with cell free extracts of yeast.

The isolation of ^{14}C-ergosterol from incubations of the yeast system with labelled precursors (described in section A) afforded an opportunity for the degradation of another isoprenoid compound.

Bu'Lock, Smalley and Smith (1962) demonstrated that although the malonyl moiety was a precursor of fatty acids and 6-methyl-salicylie acid in Penicillium urticae, the synthetic pathway of ergosterol was different. They showed that the incorporation of label into ergosterol was similar to the incorporation into the acetate "primer" terminals of fatty acids and 6-methyl-salicylic acid. This suggested that the malonyl-moiety was being converted to an acetyl intermediate before use. The procedures outlined by Bu'Lock et al. were applied to ergosterol isolated from yeast, and are described in full in methods section D(4).

The first step was to cleave the side chain at

the olefinic bond using an oxidising mixture of permanganate and periodate (Lemieux and Von Rudloff, 1955). The dimethyl butyric acid fragment was removed by steam distillation and further degraded by Kuhn-Roth oxidation. The radioactivity in the carboxyl and methyl carbon atoms of the Kuhn-Roth acetic acid was determined by the Schmidt azide reaction followed by permanganate oxidation of the resulting methylamine.

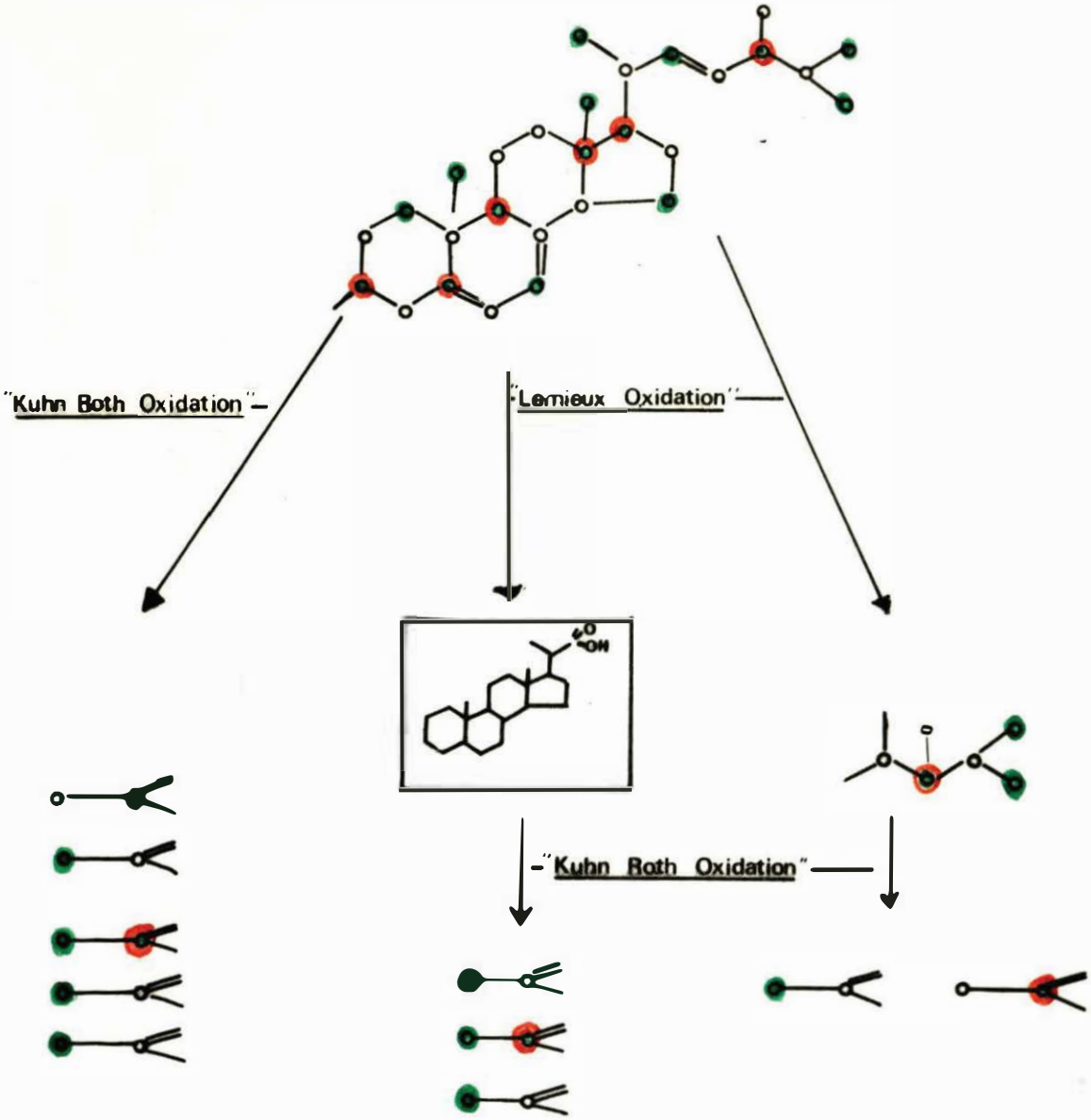
The undegraded ergosterol as well as the larger fragment from the Lemieux oxidation were themselves subjected to Kuhn-Roth degradation and the distribution of radioactivity in the resulting acetic acid was determined.

The position of radioactive carbon in the ergosterol molecule can be predicted if the substrate is known to have its radioactivity in either the methyl or the carboxyl carbon. Consequently, the expected ratio of the radioactivity in the carboxyl carbon to the radioactivity in methyl carbon of the Kuhn-Roth acetic acid can be determined. According to the

Brodie *et al*. pigeon liver pathway, only two carbon atoms from every five are derived from malonyl-CoA. The ratio of the radioactivity from this source in the carboxyl:methyl carbon atoms can be calculated. Comparison with the ratios actually observed should, therefore, indicate the route followed from malonyl-CoA. This is illustrated in scheme 7.

The ratios expected and those found are shown in table 35. The ratio of the radioactivity found in the carboxyl groups to that found in the methyl carbons when malonyl-CoA was used as a substrate was very similar to that found when acetate was the precursor. The actual ratios approach the theoretically deduced values for acetate as a precursor and are very similar to the results of Bu'Lock *et al*. obtained with a different system. This finding strengthens the conclusion reached from the previous results that malonyl-CoA is decarboxylated before being used for isoprenoid synthesis.

Scheme 7 Partial Degradation of Ergosterol. (after Bu'Lock et.al., 1962)



Carbon atoms that would be labelled from [2-^{14}C]acetate according to: 1. The classical pathway (Rudney, 1957) are designated ●; and those that would be labelled from [2-^{14}C]malonyl-CoA (2) according to the Brodie et. al.(1963) pathway (see scheme 6.) ○—

∴ Ratio of radioactivity of METHYL carbon to CARBOXYL carbon of Kuhn-Roth acetic acid :-

	1	2
Ergosterol	2 : 1	0 : 1
Dimethyl butyric acid	1 : 1	0 : 1
Nuclear fragment	3 : 1	0 : 1

Table 35

The Ratios of the Radioactivity Found in the Carboxyl Carbon to the Radioactivity Found in the Methyl Carbon of the Kuhn-Roth Acetic acid Derived from Ergosterol and its Oxidation Products

Precursor		Source of Acetic Acid		
		ERGOSTEROL	2,3-dimethyl Butyric acid	Nuclear Fragment
[2-^{14}C] ACETATE	COOH/CH_3 - Found	0.48±0.08	1.21±0.17	0.41±0.11
	COOH/CH_3 - Expected if substrate used as such	0.50	1.0	0.33
[2-^{14}C] MALONYL-CoA	COOH/CH_3 - Found	0.49±0.11	1.19±0.15	0.39±0.12
	COOH/CH_3 - Expected if substrate used as such	1/0	1/0	1/0

Results are ± Standard Error of the Mean for 6 determinations

EXPERIMENTAL

2. RESULTS

D. Studies of the Decarboxylation of Malonyl-CoA by Rat Liver and Yeast Systems.

The Enzymic Decarboxylation of Malonyl-CoA

The results this far have shown that in the preparations used, malonyl-CoA is decarboxylated to acetyl-CoA before incorporation into isoprenoids. It therefore became of interest to investigate the distribution of this enzymic activity in the subcellular components of the preparations used.

The enzymic decarboxylation of malonate in rat kidney was demonstrated by Hayaishi (1955), in rat liver, rat heart and disphragm by Nakada, Wolfe and Wicke (1957) and in yeast, moulds and bacteria by Hayaishi (1954), Wolfe and Rittenberg (1954). Highly purified fatty acid synthetase enzymes are often contaminated by this activity (e.g. Vagelos and Alberts, 1960; Burton, Haavik and Porter, 1968). Lynen, Oosterhelt, Schweizer and Willecke (1967) reported that malonyl-CoA decarboxylation was a latent property of the fatty acid synthetase from yeast which appeared after treatment of the enzyme with iodoacetamide.

Lorch, Abraham and Chaikoff (1963) observed that in rat liver, the activity was concentrated in the mi-

crosomal fraction. The subcellular distribution of the enzyme has been determined by Scholte (1969). Using marker enzymes to characterise the fractions he found that malonyl-CoA decarboxylase was located principally in the mitochondria.

These reports suggested that malonyl-CoA decarboxylase was essentially a particle bound enzyme and that it might be possible to remove it from the preparations.

The decarboxylation of malonyl-CoA by the rat liver system of Knauss et al. (1959) and by the cell free yeast system.

The decarboxylation of malonyl-CoA by the rat liver system (40-80% ammonium sulphate fraction) and the cell free yeast system is shown in table 36. These were the systems used to determine the ratios of the labelling of squalene (results section C(1)). The initial rates of the decarboxylation reaction were measured by the spectrophotometric assay and the total malonyl-CoA decarboxylated in the stated time intervals

Table 36

Decarboxylation of Malonyl-CoA by cell free Rat Liver and Yeast Systems

a) Spectrophotometric Assay

	RAT Liver System	Yeast
Initial Rate	2.35mμmole/min.mg. (3)	1.30mμmole/min./mg. (3)

b) Radiochemical Assay

Time (minutes)	5	10	15	30	60	5	10	15	30	60
mμmoles decarboxylated/ mg. protein	12.0	24.2	34.9 (2)	65.2	112.6	6.6	14.5	18.9 (2)	37.5	74.2

Yeast assays at 30°C, Rat liver assays at 37°C

Results are the mean of (n) determinations

was determined by the radiochemical procedure.

The finding that 1.46 μmoles of malonyl-CoA would be decarboxylated by a rat liver incubation containing 13mg. of protein (as in the squalene experiments) and 0.74 μmoles by a 10 mg. yeast incubation in 60 minutes demonstrated that the activity of the enzyme was more than sufficient to account for all of the incorporation of malonyl-CoA into isoprenoids.

The distribution of decarboxylase activity in the same systems was investigated. The standard rat liver ammonium sulphate fractionation and microsomal washing procedure was followed by the radiochemical assay for malonyl-CoA decarboxylase in each component of the system. The cell free yeast homogenate (12,000g supernatant) was centrifuged at 104,000g for 90 minutes. The supernatant and particulate fractions were assayed for decarboxylase. The results of these determinations are presented in table 37, and show that malonyl-CoA decarboxylase activity is concentrated in the paticulate fractions of both preparations. The activity in the supernatant was still too high, however,

Table 37

The Distribution of Malonyl-CoA Decarboxylase in the Rat Liver System (Knauss et al, 1959) and The Yeast System

RAT LIVER		YEAST	
mμmole malonyl-CoA decarboxylated/ mg. protein/30 minutes.		mμmole malonyl-CoA decarboxylated/ mg. protein/30 minutes	
0 - 40% ammonium sulphate fraction	3.2	Supernatant	16.7
40 - 80% ammonium sulphate fraction	2.2	Microsomal fraction	21.8
Microsomal fraction	17.5		

Assayed by the radiochemical method. For details see text

to be effective in limiting the incorporation of malonyl-CoA into isoprenoid materials by preventing decarboxylation.

Since no precautions were taken to preserve the integrity of the mitochondria in the liver preparation, and because Scholte (1969) showed that malonyl-CoA decarboxylase was located in the mitochondria, it seemed possible that much of the microsomal decarboxylase activity was due to contamination by mitochondrial fragments.

To investigate this possibility, rat livers were fractionated essentially according to DeDuve et al. (1955) as modified by Michell and Hawthorne (1965). The procedure is described in methods section B (7c). It was hoped that this preparation would conserve mitochondrial integrity and prevent contamination of the microsomal pellet with mitochondrial fragments. The results of the assay of each fraction for malonyl-CoA decarboxylase activity (table 38) were in essential agreement with the published data of Scholte (1969)

Table 38

Distribution of Malonyl-CoA Decarboxylating Activity in Rat Liver

Assayed by the Radiochemical procedure.

Total Activity	4.42 mμmole/min./mg.protein
Nuclear fraction	1.90 "
Mitochondrial fraction	2.10 "
Whole supernatant	0.05 "
0-40% $(NH_4)_2SO_4$ fraction	0.038 "
0-80% $(NH_4)_2SO_4$ fraction	0.011 "
Microsomal fraction	0.030 "
% Recovered	91.5

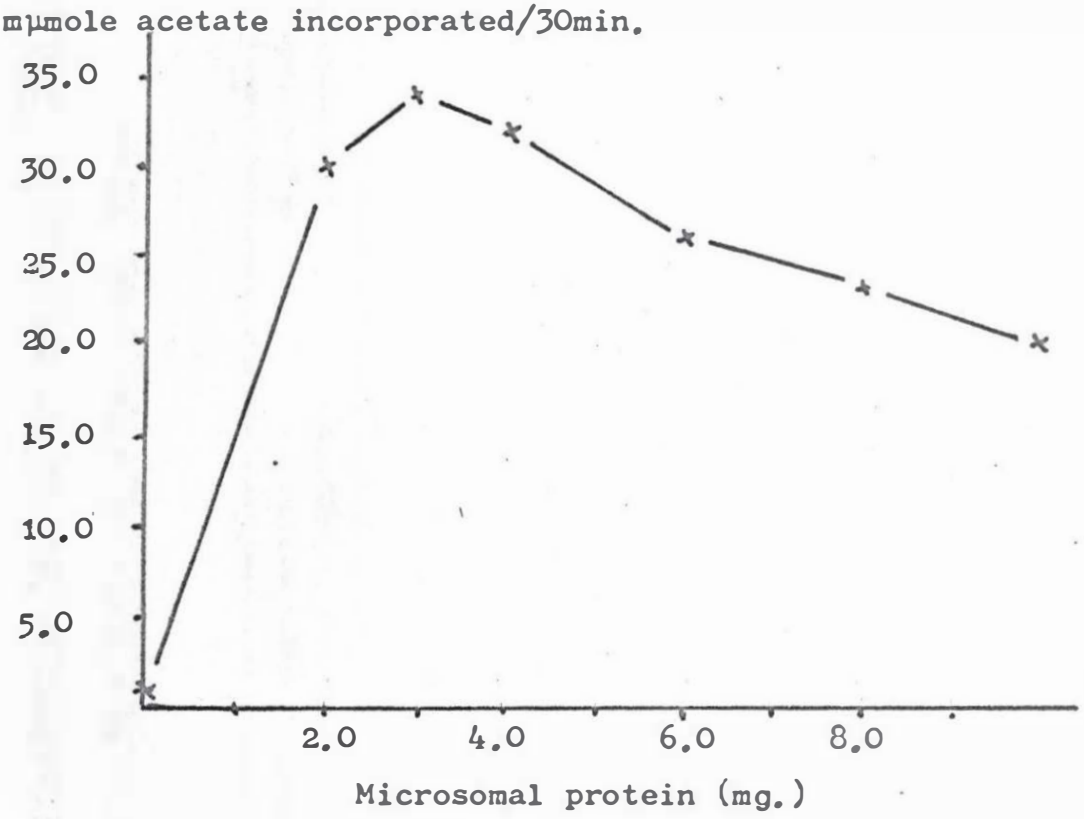
In these conditions of isolation most of the decarboxylase activity was present in the mitochondrial fraction. There was still significant activity in the microsomes, however, and since microsomal protein is required for the convertion of HMG-CoA to MVA, the minimal level of microsomal protein required to give a reproducible incorporation of acetate into mevalonate was established. Carrier mevalonate was added to the incubations which contained 10mg. soluble protein and varying amounts of microsomal material. Mevalonate was isolated by the celite column method; the incorporations are shown in figure 22.

<u>The incorporation of [2-^{14}C] malonyl-CoA into mevalonate by rat liver system with reduced malonyl-CoA decarboxylase activity</u>

[2-^{14}C] malonyl-CoA or [2-^{14}C] acetate was incubated with the rat liver system prepared as described above. Each incubation contained the optimal amount of microsomal protein (3mg./2.5ml.), and a trapping pool of 50 μmoles of unlabelled MVA. A duplicate series of incubations contained in addition a sus-

Figure 22

THE MICROSOMAL REQUIREMENT FOR THE INCORPORATION OF [2-^{14}C] ACETATE INTO A TRAPPING POOL OF MEVALONATE BY A RAT LIVER SYSTEM (Knauss et al.,



Incorporation of substrate expressed as mμmole/30 minutes.

Microsomal protein added	0	1	2	3	4	6	8	10
Incorporation	2.1	14.0	30.3	34.6	32.2	26.6	23.1	2o.0

In 2.5ml. incubations contained 1omg. soluble protein (4o-80% ammonium sulphate); 2.0mM- [2-^{14}C] sodium acetate; 0.025μmole CoASH; ATP-1.0μmole; 2.5μmole-$MgCl_2$; 10μmole-glutathione; 250umole pH7.0 potassium phosphate buffer; 0.4μmole-NADP$^+$; 0.3-μmole NAD^+; 7.0μmole-glucose-1-phosphate and 50μmole of unlabelled potassium mevalonate.

Each result is the mean of 3 determinations.

Table 39

The Effect Of Malonyl-CoA Decarboxylase on the Incorporation of [2-^{14}C]Malonyl-CoA or [2-^{14}C] Acetate into Mevalonate by a Rat Liver System

Decarboxylase activity mμmole/minute	Acetate incorporated into mevalonate(mμmole)	Malonyl-CoA incorporated into mevalonate (mμmole)
0.2	35.2 (3)	14.3 (3)
18.3 (+sonicated mitochondria)	36.1 (3)	36.4 (3)

Incubation for 30 minutes at 30°C, incubation conditions as figure 22. Mevalonate was isolated by celite column chromatography.

The mean of (n) determinations.

pension of a mitochondrial fraction from the same livers (2mg. protein) that had been disrupted by sonicatation.

The result (table 39) was that when the malonyl-CoA decarboxylase activity was lowered to approximately 2% of its former value, the rate of decarboxylation of malonyl-CoA became the limiting factor in the incorporation of malonyl-CoA into mevalonic acid. The addition of a sonicated mitochondrial suspension restored the incorporation to levels observed when acetate was the precursor.

Conclusions

It has been shown that the activity of malonyl-CoA decarboxylase is a profound influence on the incorporation of malonyl-CoA into mevalonate by a rat liver system. When this result is considered with the data on the effect of avidin and with the results of the degradations of HMG-CoA and ergosterol, it is convincing evidence that synthesis of isoprenoid compounds from malonyl-CoA occurs only after decarboxylation of

of acetyl-CoA.

Fimognari and Rodwell (1964) and Fimognari (1965) reported that acetyl-CoA and malonyl-CoA were equally good precursors of isoprenoids in a liver homogenate. In a 14,000g supernatant, however, acetyl-CoA was a better precursor than malonyl-CoA. This observation is in accord with the results reported in this work, the decrease in incorporation in the 14,000g supernatant probably being due to the removal of decarboxylase activity.

The reported synthesis of HMG-CoA by the pigeon liver enzyme remains to be explained. In an attempt to do this some properties of the pigeon liver fatty acid synthetase were examined.

EXPERIMENTAL

2 RESULTS

E Studies on the Purification of the Pigeon Liver Fatty Acid Synthetase

Studies with the purified fatty acid synthetase from pigeon liver

The methods used for the purification of this enzyme are described in Methods B (3). Since the two conditions used in the isolation gave enzymes of differing activities and stabilities, they are referred to as preparations A and B. A comparison of the purification of the two types is given in table 6.

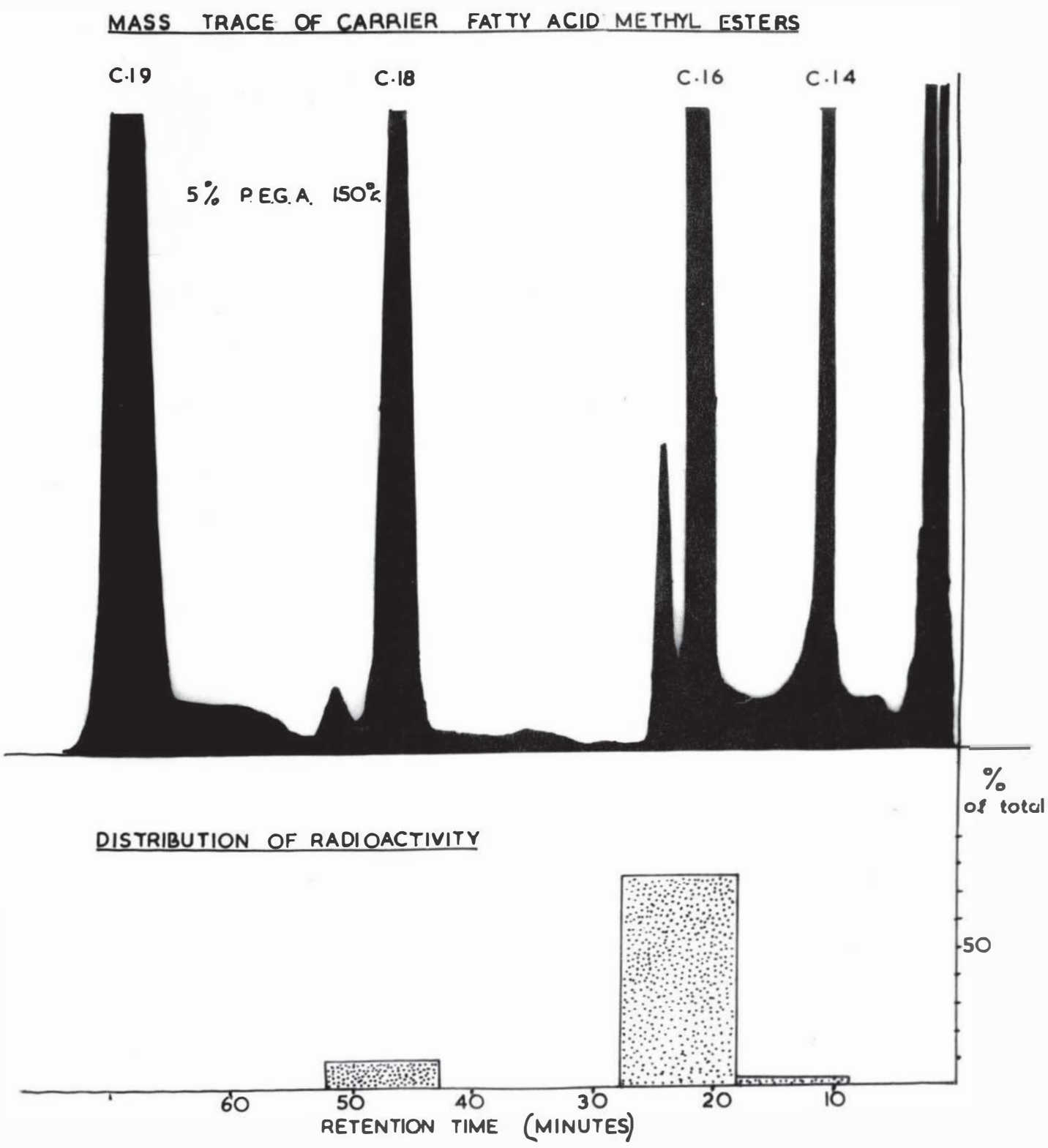
To ensure a valid comparison with the enzyme used by Brodie *et al.*, some of its properties were determined.

Nature of fatty acids synthesised

The radiochemical assay was terminated by the addition of 5ml. 2N-KOH in 95% methanol. An aliquot of carrier fatty acids was added and the mixture saponified. After acidification and extraction of fatty acids with light petroleum, the mixture was methylated and analysed by gas liquid chromatography. The radioactive methyl esters were collected as described in

FIG 23

G L C Analysis of products of the Pigeon liver Fatty Acid Synthetase



the methods section A (1). The distribution of radioactivity in the fractions collected is shown in figure 23. 90% of the radioactivity was in palmitic acid, most of the remaining 10% chromatographed with stearic acid.

Synthesis of Triacetic acid lactone

Triacetic acid lactone (TAL) has been shown to be a product of several fatty acid synthesising enzymes. Bressler and Wakil (1962) showed that this product was formed by a pigeon liver enzyme and Brock and Bloch (1966) demonstrated the formation of the same compound by Escherichia coli. Yalpani, Willecke and Lynen (1969) described the formation of the same product by the yeast fatty acid synthetase. The assay system used was that described in methods B (3) for HMG-CoA formation. The reaction was stopped by adding 0.25ml. 1N-HCl and the incubation mixture was saturated with anhydrous sodium sulphate. TAL was extracted with 10 2ml. aliquots of diethyl ether.

The ether was evaporated and the residue was taken up in 0.1ml. of methanol. The solution was

chromatographed in three systems and the R_f of the radioactive material was compared with the published values.

a Thin layer chromatographic analysis on Silica Gel G

A major radioactive component at an R_f of 0.3 to 0.35 in the solvent system chloroform-acetic acid 21;4. This corresponds to the data of Yalpani et al. (1969) for TAL.

b Paper chromatography (Whatman No 1 paper)

A major component was observed at R_f 0.3-0.4 in the solvent system butanol saturated with 0.1M-sodium acetate. This corresponds with TAL from the data of Yalpani et al. (1969)

c In the solvent system Isobutyric acid, 3N-NH_4OH 0.1M-EDTA, Water, 62:12:1:28) the major radioactive product was at R_f 0.85 - 0.95. This corresponded to the data for TAL obtained by Brodie et al. (1964)

Examination for the formation of mevalonic acid

The assay mixture is described in methods B (3) and contained 20 μmole of unlabelled mevalonic acid. Extraction was as described and chromatography on paper

or passage through a celite column (methods A (3c)) failed to demonstrate the formation of mevalonic acid.

Examination for the formation of HMG-CoA

The assay system and extraction procedure are described in Methods B (3) and D (3). Purified preparations (A or B) were examined for the synthesis of HMG-CoA. The HMG-CoA synthesising activities were extremely variable from enzyme preparation, and in the preparations most highly purified for fatty acid synthesis, the HMG-CoA activity disappeared completely. The activities for fatty acid synthesis, Triacetic acid lactone synthesis and HMG-CoA synthesis were therefore determined at each stage of the purification. Table 40 shows the results of two such experiments with preparation B and table 41 the results of similar experiments with preparation A. In neither case does the activity for HMG-CoA synthesis copurify with the activity for fatty acid synthesis or TAL synthesis.

Conclusions

The conclusion from these experiments is that

the reported synthesis of HMG-CoA by the pigeon liver fatty acid synthetase was due to contaminating enzymes, for in the best preparations this activity can be abolished completely. Furthermore, the HMG-CoA synthesised in a crude pigeon liver system did not conform to a malonyl-CoA dependent labelling pattern (section C)

In other respects, the enzyme preparations behaved in a very similar manner to those reported from Porter's laboratory.

A personal communication from J. W. Porter has confirmed that HMG-CoA synthesising activity does not copurify with the fatty acid synthesising activity. Since the purified enzyme has been shown to possess a malonyl-CoA decarboxylase activity, (e.g. Burton et al. 1968) it is difficult to see how the synthesis of HMG-CoA could be shown to be dependent on malonyl-CoA.

Table 40

Purification of Pigeon Liver Fatty Acid Synthetase (B) with Assays for Triacetic Acid Lactone (TAL) and β-Hydroxy-β-methyl-glutaryl-CoA (HMG-CoA)

	Specific Activity (mμmole/min./mg.protein)					
	Fatty Acid		TAL		HMG-CoA	
	1	2	1	2	1	2
Particle free supernatant	12.5	8.3	1.95	-	-	-
1st. Ammonium sulphate fraction	18.2	12.3	3.4	2.1	1.2	0.97
Calcium Phosphate gel	13.7	10.1	1.4	1.1	1.1	0.98
DEAE-cellulose	65.2	36.0	16.4	10.8	0.1	0.34
2nd. Ammonium sulphate fractionation.	91.5	42.2	-	9.7	-	0.15
G-100 Sephadex	123.5	67.2	29.4	13.8	-	0.1

The results of two separate purifications are shown.

Fatty acids were assayed by the radiochemical procedure. See text for other assays.

Table 41

Purification of Pigeon Liver Fatty Acid Synthetase (A) with Assays for Triacetic Acid Lactone (TAL) and β-Hydroxy-β-methyl-glutaryl-CoA (HMG-CoA)

	Specific Activity (mμmole/min./mg.protein)					
	Fatty Acid		TAL		HMG-CoA	
	1	2	1	2	1	2
Particle Free Supernatant	2.4	4.6	-	-	-	-
1st. Ammonium Sulphate fractionation	4.9	6.5	1.3	1.4	-	-
Calcium Phosphate Gel	4.8	6.5	1.3	1.6	-	4.1
DEAE-Cellulose	29.8	23.0	7.4	8.2	8.4	3.7
2nd. Ammonium Sulphate fractionation	58.0	54.1	11.4	9.05	5.1	3.6
G-100 Sephadex	62.1	58.2	15.2	14.9	1.8	0.4

The results of two separate purifications are shown.

Fatty acid synthesis was assayed by the radiochemical procedure. See text for other assays

Summary.

The work of J. W. Porter's group with the pigeon liver fatty acid synthetase implicated malonyl-CoA as one of the initially condensing molecules of isoprenoid synthesis. Evidence for this mechanism in other systems was conflicting - and despite the thermodynamic advantages the significance of the pathway was in doubt. The object of this investigation, therefore, was to determine the significance of the new (malonyl-CoA) pathway for the synthesis of mevalonic acid.

The initial experiments, principally with a cell free yeast system, indicated that malonyl-CoA was indeed incorporated into isoprenoid compounds: and more evidence that malonyl-CoA was an isoprenoid precursor was obtained from the observation that ^{14}C-malonyl-CoA was incorporated into trapping pools of mevalonate by the yeast system (and by Hevea latex in the presence of added NADPH). On the other hand, there was some evidence in favour of the alternative explanation: that malonyl-CoA was decarboxylated and acetyl-CoA was incorporated as such. Thus bicarbonate stimulated the

incorporation of the acetyl-moiety into fatty acids but not into the unsaponifiable lipid fraction; and when carbon dioxide was removed from the system, incorporation of these substrates into the fatty acids was markedly inhibited.

Later experiments therefore, were designed to find out if malonyl-CoA was decarboxylated before incorporation as acetyl-CoA. The addition of an unlabelled pool of acetyl-CoA to yeast incubations with [2-^{14}C] malonyl-CoA gave indecisive results - for although a low concentration of acetyl-CoA did stimulate the labelling of unsaponifiable lipids from malonyl-CoA, raising the concentration of acetyl-CoA was inhibitory to the incorporation of this substrate, into this fraction and into fatty acids. Other results that were difficult to interpret came from the determinations of the relative incorporation of [1,3-^{14}C] and [2-^{14}C] malonyl-CoA into squalene by the rat liver and yeast systems; however, experiments in which the distribution of radioactivity within the HMG-CoA and ergosterol molecules were determined clearly showed that the distribution was that to be expected from the incorporation

of the acetyl-CoA moiety as such. Incorporation of malonyl-CoA *per se* would have given quite different labelling patterns (see schemes 6 and 7). These data are not necessarily unequivocal since it is probable that the relatively crude systems contained HMG-CoA cleavage enzyme which would have led to a randomisation of label throughout the molecule; but it was shown in the rat liver system that HMG-CoA synthesised from ^{14}C - acetyl-CoA and acetoacetyl-CoA was not sufficiently randomised to negate the conclusion that malonyl-CoA was probably decarboxylated before incorporation. Further experiments which supported this contention were those in which avidin was added to incubations of acetate or acetyl-CoA. That in the rat, yeast and *Hevea* systems fatty acid synthesis could be abolished or very much reduced while incorporation into squalene or rubber was not diminished at all indicated the absence of a biotin dependent carboxylation reaction.

If it were true that decarboxylation of malonyl-CoA accounted for isoprenoid synthesis from this substrate two conditions are required to be true:-

a. Decarboxylating activity must be at a sufficiently high level

b. Removal of this activity must inhibit the incorporation of malonyl-CoA.

Both requirements were met, for malonyl-CoA was decarboxylated at a rate great enough to account for the incorporation into the isoprenoid fraction of both the rat and yeast systems. The decarboxylating activity could be considerably reduced in the soluble protein and microsomal fractions by employing conditions in which the mitochondrial integrity was conserved. In these circumstances the incorporation of malonyl-CoA into mevalonate was decreased, although the incorporation of acetate was not diminished.

The observations that led to the proposal of the malonyl-CoA pathway were made on a pigeon liver system purified for fatty acid synthesis. The accumulated data in this work had shown that the incorporation of malonyl-CoA into isoprenoids could be ascribed to a prior decarboxylation of malonyl-CoA. Therefore, some properties of the purification of the pigeon liver

fatty acid synthetase were reexamined. It was shown that the activity for fatty acid synthesis and Triacetic acid lactone synthesis did not copurify with that for the formation of HMG-CoA.

General Discussion

Some of the results that have been described can explain data reported by other groups. Porter et al. (1964) described experiments in which ^{14}C-malonyl-CoA + acetyl-CoA or ^{14}C-acetyl-CoA + malonyl-CoA were incubated with a rat liver homogenate. The radioactivity that was incorporated into unsaponifiable compounds was resolved on columns of deactivated alumina and the distribution was shown to be similar whether the radioactivity originated in malonyl-CoA, acetyl-CoA or mevalonic acid. If malonyl-CoA were decarboxylated before incorporation - as suggested in this thesis - such a result was that to be expected. Similarly Fimognari and Rodwell (1964) and Fimognari (1965) showed that acetyl and malonyl-CoA were both good precursors of mevalonate in a rat liver homogenate, but acetyl-CoA was a much better precursor in a 14,000g supernatant. The results of Scholte (1969) and in this work, have shown that malonyl-CoA decarboxylase activity is concentrated in the mitochondrion; a 14,000g supernatant, therefore, would contain much less decarboxylase than the whole homogenate (if the mitochondria had remained intact).

The most important results which conflict with published data are those obtained from some studies of the purification of the pigeon liver fatty acid synthetase. In the absence of NADPH, material with the chromatographic characteristics of TAL, and small quantities of HMG-CoA were synthesised by the pigeon liver enzyme. In the best preparations, the eliminations of HMG-CoA synthesising activity showed that this activity was due to other contaminating enzymes. In a personal communication, Porter has agreed that this is the case and reported that another protein fraction as well as the fatty acid synthetase was required for HMG-CoA synthesis from acetyl-CoA and malonyl-CoA. Presumably, this is a condensing enzyme that will react with acetoacetyl-enzyme. Brodie *et al.* showed that HMG-CoA synthesis was dependent on malonyl-CoA, however, their preparations were probably contaminated by malonyl-CoA decarboxylase. Burton, Haavik and Porter (1968) reported that a more highly purified preparation than that used by Brodie *et al.* synthesised 4.7 mμmole palmite/mg.protein/minute in the absence of acetyl-CoA. The decarboxylase activity of the less highly purified preparation was not reported, but this activity would

have been sufficient to account for the low level of incorporation into HMG-CoA described in this thesis. Yalpani, Willecke and Lynen (1969) doubted the physiological significance of TAL formation by fatty acid synthesising enzymes: sufficient NADPH would normally be available to reduce the acetoacetyl derivative to the β-hydroxybutyryl derivative. The same argument can be applied to the synthesis of HMG-CoA by such a system.

The complete difference of the labelling patterns of HMG-CoA synthesised by the DEAE-enzyme of Brodie et al. and those reported in this work can only be explained by the differences in the enzyme used. Although complete randomisation of the molecule is a possibility, this did not occur with the rat liver system.

The relative incorporation of malonyl-CoA labelled in the two possible ways into squalene by rat and yeast systems gave results that were difficult to interpret and did not follow any anticipated pattern. The experiments with yeast indicated that much less radioactivity was retained from the carboxyl-carbon than could be deduced from any biosynthetic pathway. A com-

plete decarboxylation of malonyl-CoA would have eliminated 50% of the radioactivity and lowered the ratio of [1,3-^{14}C] malonyl-CoA to [2-^{14}C] malonyl-CoA incorporated to 1:3. The ratio observed was 1:9.2. The situation in the rat liver system was completely different, for here, more carboxyl carbon was retained than anticipated. The latter may possibly be explained by postulating that the radioactive carbon dioxide formed from the decarboxylation of malonyl-CoA, was retained in the system by a recarboxylation of acetyl-CoA. If an acetyl-CoA deacylase as well as an acetyl-CoA synthetase were present, the radioactivity in the acetyl-CoA pool might well be increased. No such explanation for the yeast results can be proposed. It is unlikely that an inhibiting impurity was present in the carboxyl labelled malonyl-CoA preparations, since:

a) It was not revealed by the yeast fatty acid synthetase

b) The substrates were purified by chromatography on DEAE cellulose, and

c) The same batch of [1,3-^{14}C] monomalonyl thiophenol ester was used in experiments with both rat and yeast systems. The observed ratios depended on the duration

of the incubations; although this did not give any direct information about the anomolous results, it indicated that an accumulative process may have been responsible.

The experiments in which avidin was used as an inhibitor of biotin dependent carboxylation reactions showed that such a carboxylation was not involved in the synthesis of isoprenoid materials from acetate. This observation is in accord with some reports from other laboratories. (e.g. Jacobsohn and Corley, 1957) but disagrees with the results of Foster and Bloom (1963) and Weeks (1966). However, Foster and Bloom doubted that the inhibition observed by them was a genuine effect and suggested that it may have been due to a comtaminant in the avidin. This is probably not the explanation of Weeks' results since his incubations were reversed by prior incubation of the avidin with biotin: that is, unless biotin exerted some other protective effect than that usually accepted.

The conclusion that had been reached - that carboxylation of acetate is not a significant reaction in

isoprenoid synthesis - is implied by the composition of the rat liver system of Knauss et al. (1959), which was used for much of the work involving the isolation of squalene. The system consisted of a microsomal component and a 40% - 80% ammonium sulphate fraction of the soluble proteins. Since acetyl-CoA carboxylase precipitates below 25% of saturation with this salt (e.g. Gregolin, Ryder and Lane, 1968; Miller and Levy, 1969), it is unlikely that the 40% - 80% fraction would be active in the carboxylation of acetyl-CoA, and so the acetyl-CoA used in this system was probably incorporated as such.

The reciprocal relationship between the incorporation of acetate into fatty acids and unsaponifiable lipid induced by the addition of bicarbonate or the removal of carbon dioxide in the yeast experiments was similar to the effect observed by Klein (1957) with yeast and by Weeks (1966) with Hevea latex. It was pointed out in the results section that such relationships have been interpreted as evidence that malonyl-CoA is not a precursor of isoprenoids, Weeks (1966) did not agree with this interpretation; it was argued

that an increase in the level of malonyl-CoA with respect to acetyl-CoA would stimulate fatty acid synthesis at the expense of isoprenoid synthesis if the control were at the acetoacetyl-enzyme level, (see scheme 4). However, if this were true, it is difficult to understand the mechanism by which isoprenoid synthesis could be stimulated in conditions where malonyl-CoA formation is inhibited. The postulated malonyl-CoA pathway requires that two carbon atoms from every five of the isoprene unit are derived from this substrate.

The conclusion from this work was that acetyl-CoA is incorporated into isoprenoid compounds as such without carboxylation to malonyl-CoA; the observed incorporation of malonyl-CoA was ascribed to the activity of malonyl-CoA decarboxylase in the system. As a consequence, there are problems which have still to be answered, some of these are discussed in the following sections.

The Locus of Mevalonic Acid Synthesis and its Relationship to Ketogenesis

One of the difficulties of the classical pathway of mevalonate synthesis is the distribution of the enzymes responsible for the degradation and the formation of HMG-CoA. The situation is complicated by the role of HMG-CoA in the formation of ketone compounds. Lynen et al. (1958) discovered that two enzyme fractions are required for the formation of acetoacetate from acetoacetyl-CoA and that acetyl-CoA was required in catalytic quantities. These fractions were later identified as HMG-CoA condensing enzyme and HMG-CoA cleavage enzyme; the two reactions working together in liver were envisaged as forming an "HMG-CoA" cycle. The activity of the condensing enzyme was found by Bucher et al. (1960) to lie mainly in the mitochondria, while only a relatively small proportion was observed in the microsomes. In a similar way, the cleavage enzyme was exclusively located in the mitochondria. Rudney (1957) was able to demonstrate the formation of HMG- in a microsomal fraction of beef liver using isotopic techniques which were of a far higher sensitivity than the

optical methods of Bücher et al. It is important to note that the total activity in the microsomal fraction of the liver was nearly 15 times greater than the activity of the whole homogenate. At that time, he suggested that this may be due to the metabolism of HMG- to other compounds in the unfractionated system. Furthermore, Rudney was able to show synthesis of HMG from acetyl-CoA by washed rat liver microsomes. This implies that the enzyme catalysing the condenation of two molecules of acetyl-CoA to produce the substrate for the condensing enzyme is located in the microsomal fraction.

Siperstein and Fagan (1966) also concluded that mevalonate synthesis was a microsomal function, but also observed some synthesis in the particle free fraction. The rat liver system described in this thesis to which an unlabelled trapping pool of mevalonate was added exhibited a distinct microsomal requirement. Synthesis of mevalonate in the absence of microsomes was very low indeed, (see figure 22). In support of this, Linn (1967a,b) demonstrated that the reduction of HMG-CoA occurred in the microsomal fraction of rat liver and Chesterton (1968) demonstrated that the formation of

cholesterol and its immediate precursors from mevalonate *in vivo* occurred in the microsomal fraction.

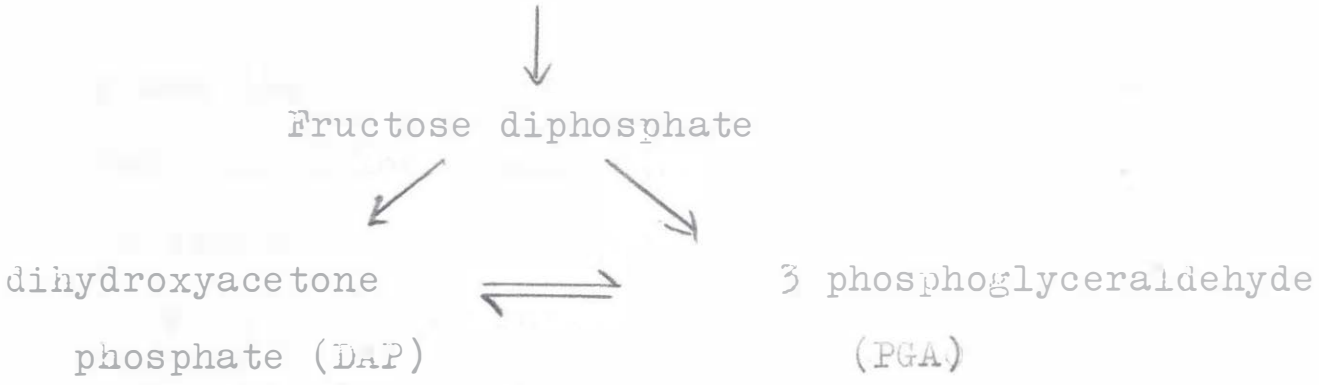
While there was considerable doubt about the role of the HMG-CoA condensing and cleavage enzymes in ketogenesis, it now seems that in liver they play a major part in the formation of acetoacetate. Caldwell and Drummond (1963) fractionated the acetoacetate producing system from beef liver. Heating at 90°C destroyed 90% of the acetoacetate forming activity which was restored by the addition of HMG-CoA condensing enzyme. Column fractionation techniques removed HMG-CoA cleavage enzyme and acetoacetate forming activity which was restored on the addition of HMG-CoA cleavage enzyme. Sauer and Erfle (1966) showed that acetoacetyl-CoA deacylase activity in mitochondria depended on the presence of acetoacetyl-glutathione (the glutathione derived from commercial preparation of CoASH.) Purified acetoacetyl-CoA was not deacylated; all the acetoacetate formed was derived from HMG-CoA.

Therefore, in the absence of a complete study of

the subcellular distribution of these enzymes it seems that acetoacetate production is a function of the mitochondria carried out by the abundant condensing and cleavage enzymes. Isoprenoid synthesis occurs in the microsomal fraction where there is a lower level of condensing enzyme and where cleavage enzyme is es ten-tially absent.

The yeast HMG-CoA condensing enzyme is difficult to prepare uncontaminated by thiolase activity, and in 1966 Stewart and Rudney discussed the possibility that both activities were located on the same protein. It is easy to see that a juxtaposition of the thiolase and condensing enzyme would be a favourable situation for HMG synthesis, and would be an approach to the "multi-enzyme complex" concept.

Garfinkel (1963) discussed an analogous situation that occurs with the enzymes aldolase and triosephos-phate isomerase, These are difficult to isolate inde-pendently and were once thought to be only one protein (Meyerhof, 1951).



In the presence of excess glucose, the total flux through the system in mouse ascites tumour cells was measured as 27 μmoles of hexose per second. It was calculated that for this flux to occur, the concentration of DAP should be greater than the equilibrium value deduced from kinetic studies of the enzyme (22 DAP : 1PGA). Experimental determinations showed, however, that PGA is in fact at a concentration 50 times that of DAP. This suggested a compartmentation effect. Garfinkel proposed a model in which the active sites of the two enzymes were adjacent so that only one molecule could be present at the active site at any time. A fructose diphosphate split by the aldolase results in the two triose phosphates at the joint active site. The enzyme kinetics showed that DAP is more tightly bound than PGA which could diffuse away. Since the bound DAP is near the site of the isomerase, it is likely that it would be converted to PGA: only when the site is completely

empty can the reverse reaction occur. The net result is that a high local concentration of DAP is maintained at the active site. The system was described by Garfinkel in the form of differential equations, and such a model was shown by computer analysis to fit the observations.

In a thiolase - condensing enzyme system the acetoacetyl-CoA formed would immediately be converted into HMG-CoA. The effect of a restricted access to the active site coupled with the rapid removal of acetoacetyl-CoA would be to maintain a high local concentration of acetyl-CoA.

The particular yeast thiolase isolated by Stewart and Rudney has some different properties to other thiolase enzymes that have been studied; it is possible that the thiolase of the postulated condensing enzyme-thiolase system from animal tissues is not identical with the thiolase associated with the degradative pathway.

The Decarboxylation of Malonyl-CoA

The decarboxylation of malonyl-CoA can account for the observed incorporation of this substrate into iso-

independent of the addition of acetyl-CoA as a primer for fatty acid synthesis.

The malonyl-CoA concentration in fatty acid synthesising systems has been implicated as an important factor in the regulation of the chain lengths of the fatty acids produced. Smith, Easter and Dils (1966) and Bartley, Abraham and Chaikoff (1967) showed that the concentration of malonyl-CoA directly influences the chain lengths of fatty acids synthesised by rabbit mammary gland systems. Easter and Dils (1968) suggested that in this tissue, although not in rat liver, the stimulation of synthesis and increase in chain length induced by the addition of microsomes can partly be explained by the association of this fraction with acetyl-CoA carboxylase. Margolis and Baum, (1968) suggested that in vivo acetyl-CoA carboxylase was associated with the microsomal structures in pigeon liver and Miller and Levy (1969) presented some circumstantial evidence that rat mammary gland acetyl-CoA carboxylase was located in the microsomal fraction, for freezing and thawing and the addition of Mg^{++} increased the activity of the enzyme.

Malonyl-CoA decarboxylase, by influencing the concentration of malonyl-CoA, may regulate fatty acid chain length. There is some other evidence in support of this hypothesis. Mehlman (1968) reported that the rate of malonyl-CoA decarboxylation depends on the ratio of the concentrations of malonyl-CoA and acetyl-CoA. Popjak and Tietz (1955) observed the stimulation of long fatty acid synthesis by malonate and since malonate itself was not directly incorporated into the fatty acids, Dils and Popjak (1962) suggested that the stimulation might be due to the suppression of the decarboxylation or deacylation of malonyl-CoA.

Despite all these observations, it seems doubtful that malonyl-CoA decarboxylase itself is the agent for the control of malonyl-CoA concentration. From the investigations of Scholte (1969) most of the decarboxylating activity of rat liver is located intramitochondrially while acetyl-CoA carboxylase and the enzymes for long chain fatty acid synthesis are isolated from the soluble fraction of the cell. Hülsman (1966) showed that acetyl-CoA was carboxylated by propionyl-CoA carboxylase in rabbit heart sarcosomes, and that acetyl-CoA

carboxylase was absent from these organelles. It is possible that mitochondrial malonyl-CoA decarboxylase functions simply to remove the malonyl-CoA formed in this way.

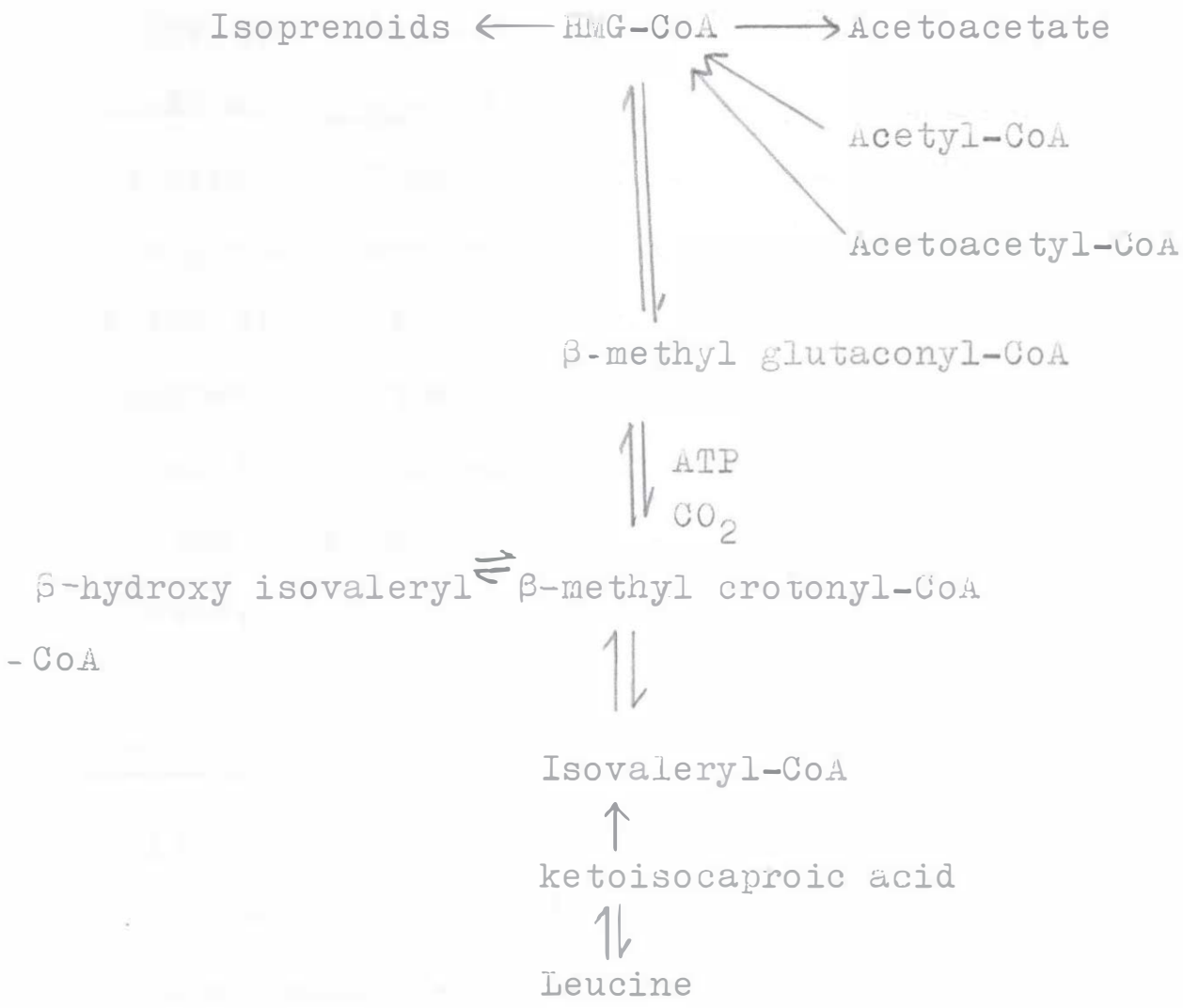
The role of Biotin in Isoprenoid Synthesis

The biotin requirement for long chain fatty acid synthesis in soluble systems has been well demonstrated, but it is significant that *in vivo* studies of biotin deficiency states in animals did not lead to a recognition of a requirement for this coenzyme in fatty acid synthesis, (e.g. see Terroine, 1960). Consequently, reports of an in-vivo requirement or lack of it for cholesterol synthesis are not very meaningful. Thus: Guggenheim and Olson (1952) found that the cholesterol content of the liver and adrenal glands was the same in biotin deficient rats as in the controls but there was a lower incorporation of ^{14}C acetate into the cholesterol of the livers of the deficient animals. Gram and Okey (1958) found that in the deficient animals a greater proportion of the ^{14}C label was respired and recovered as carbon dioxide. An *in vivo* study of the incorporation of ^{14}C-acetate into lipid fractions by biotin deficient and normal chicks by Belnave and Brown (1967) demonstra-

ted that, in the latter, label was incorporated into cholesterol at a later time than the acetate utilisation period. It was suggested that the low ^{14}C content of the liver cholesterol in this group was because this compound was being derived from a precursor, probably triglyceride, which was being broken down to C-2 units and radioactivity diluted via the acetyl-CoA pool. The higher initial activity of cholesterol found in the biotin deficient chicks suggested that more of the radioactive units were being used during the acetate utilisation period, such observations may be interpreted as an indication that biotin was not involved.

Rajalakshmi, Sarma and Sarma (1964) found a stimulation of the incorporation of acetate into cholesterol when biotin was administered to biotin deficient rats. These authors interpreted their results as an indication that acetate was being incorporated into cholesterol via the C-5 branched chain acids. It had been shown in earlier work that radioactivity from leucine is incorporated into cholesterol (Bloch, Clark and Harary, 1954) and that acetate is incorporated into β-methyl-glutaconic acid and β-hydroxy-isovaleric acid

(Rabinowitz and Gurin, 1954; Rabinowitz, 1955):



The mechanism of the carboxylation reaction was investigated by Lynen's group (e.g. Lynen, Henning, Bublitz, Sorbo and Kröplin-Rueff, 1958; Knappe, et al, 1959) and was shown to be a biotin dependent reaction. β methyl-crotonyl CoA carboxylase is an interesting enzyme for it seems to be the only biotin dependent

carboxylase that will carboxylate free biotin.

The alternative interpretation of the results of Rajalakshmi *et al*. is that the administration of biotin stimulated the formation of malonyl-CoA, which was used for isoprenoid synthesis. This view is not consistent with the observation of Jacobsohn and Corley (1957), who showed that the incorporation of acetate into cholesterol in biotin deficient rats was not affected, while the incorporation of β-methyl-crotonyl-CoA was inhibited.

To conclude

It has been demonstrated that in the *in vivo* systems used for the work described in this thesis, the postulated malonyl-CoA pathway is not a significant factor in isoprenoid synthesis.

The extrapolation of such results to *in vivo* conditions may not necessarily be justified. The techniques are becoming available to enable subcellular components to be isolated in a state of high purity (e.g. zonal centrifugation) and knowledge of 'molecular

anatomy' is rapidly increasing. Such techniques, when applied to the intracellular distribution of the various activities required for isoprenoid synthesis in systems known to correspond to the in vivo state, may finally answer the question.

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