An Investigation into the Role of Malonyl-Coenzyme A in Isoprenoid Biosynthesis

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(Received 29 November 1972)

1. [^{14}C]Malonyl-CoA was incorporated into isoprenoids by cell-free yeast preparations, by preparations from pigeon and rat liver, and by *Hevea brasiliensis* latex. 2. In agreement with previous reports the incorporation of acetyl-CoA into isoprenoids was not inhibited by avidin and was not stimulated by HCO_3^- . In a cell-free yeast preparation addition of HCO_3^- stimulated the formation of fatty acids from acetyl-CoA and decreased the incorporation into unsaponifiable lipids. 3. The labelling patterns of β -hydroxy- β -methylglutaryl-CoA formed from [$2\text{-}^{14}\text{C}$]- and [$1,3\text{-}^{14}\text{C}$]-malonyl-CoA in rat and pigeon liver preparations were those that would be expected if malonyl-CoA underwent decarboxylation to acetyl-CoA before incorporation. 4. The labelling pattern of ergosterol formed by cell-free yeast preparations from [$2\text{-}^{14}\text{C}$]malonyl-CoA was also consistent with decarboxylation of malonyl-CoA before incorporation. 5. The incorporation of [$2\text{-}^{14}\text{C}$]malonyl-CoA into mevalonate by rat liver preparations was related to the malonyl-CoA decarboxylase activity present in the preparation.

There is experimental evidence for two possible pathways for the formation of the isoprenoid precursor β -hydroxy- β -methylglutaryl-CoA; these are shown in Scheme 1.

The steps of pathway 1 (Scheme 1) occur in yeast (Rudney, 1957; Lynen *et al.*, 1958; Rudney & Ferguson, 1957, 1959). Pathway 2 of scheme 1 has been proposed by Brodie *et al.* (1963) on the basis of experiments carried out on pigeon liver preparations. Both pathways involve the condensation of an acetyl thioester with an acetoacetyl thioester to give a β -hydroxy- β -methylglutaryl thioester. In pathway 1 this ester is β -hydroxy- β -methylglutaryl-CoA (III); in pathway 2 it is enzyme-bound β -hydroxy- β -methylglutarate. The key difference between these two possible pathways is the way in which the acetoacetyl thioester is formed.

In pathway 1 acetoacetyl-CoA (II) is formed in step A by the condensation of two molecules of acetyl-CoA. This reaction, which has an equilibrium constant of 1.6×10^{-5} at pH 7.0 (Lynen & Decker, 1957) that is clearly in favour of thiolysis, is used to produce acetyl-CoA in the oxidation of fatty acids. In pathway 2 the acetoacetyl moiety is formed bound to protein (VII), from a molecule of protein-bound malonate (V) and a molecule of protein-bound acetate with the concomitant evolution of CO_2 (step E). The overall reaction in which enzyme-bound acetoacetate is formed from malonyl-CoA and acetyl-CoA was found by Lynen (1965) to have an equilibrium constant of $6.15 \times 10^{-1} \text{ M}$ at pH 6.5. This reaction is, however, only known as a stage in fatty acid biosynthesis.

At first it appears that pathway 2 involving the formation of malonyl-CoA by the carboxylation of acetyl-CoA and its subsequent conversion into protein-bound malonate would be the most likely pathway for the biosynthesis of acetoacetate. The possibility, however, that pathway 1 occurs in Nature is made plausible by the observation by Rudney & Ferguson (1959) that the subsequent reaction (B of pathway 1) for the formation of β -hydroxy- β -methylglutaryl-CoA is essentially irreversible. Evidence for pathway 2 has been produced by Brodie *et al.* (1963), who demonstrated the formation of β -hydroxy- β -methylglutaryl-CoA from malonyl-CoA by a pigeon liver fatty acid synthetase preparation. Examination of the distribution of radioactivity among the carbon atoms of this β -hydroxy- β -methylglutaryl-CoA led to the conclusion that this substance had been synthesized from acetoacetyl-CoA previously formed by the condensation of acetyl-CoA and malonyl-CoA according to reaction E of Scheme 1. Brodie *et al.* (1964) subsequently showed that the pigeon liver fatty acid synthetase could form β -hydroxy- β -methylglutaryl-CoA from enzyme-bound malonate. Some evidence that malonyl-CoA was indeed a precursor of isoprenoids was obtained by Fimognari & Rodwell (1964, 1970), who found that [$2\text{-}^{14}\text{C}$]malonyl-CoA was incorporated into both β -hydroxy- β -methylglutaryl-CoA and mevalonate by the 5000g supernatant from a rat liver homogenate. The presence of an excess of unlabelled acetyl-CoA did not decrease the incorporation of [$2\text{-}^{14}\text{C}$]malonyl-CoA and it was therefore concluded that this malonyl-CoA was not

Scheme 1. Alternative pathways for the formation of β -hydroxy- β -methylglutaryl-CoA

decarboxylated to acetyl-CoA before incorporation. Similar observations were reported by Weeks & Kekwick (1965) in a preliminary communication; they found that $[2\text{-}^{14}\text{C}]$ malonate was incorporated into rubber by *Hevea brasiliensis* latex equally well in the presence and absence of unlabelled acetyl-CoA. There is, however, some evidence that pathway 1 of Scheme 1, which involves the formation of acetoacetyl-CoA from two molecules of acetyl-CoA, is important in isoprenoid formation. Bu'Lock *et al.* (1962) investigated the formation of 6-methylsalicylic acid and ergosterol from $[2\text{-}^{14}\text{C}]$ diethylmalonate by cultures of *Penicillium urticae*. Whereas the labelling pattern of the carbon atoms of 6-methylsalicylic acid was consistent with the incorporation of the malonyl moiety without prior decarboxylation, the labelling pattern of the ergosterol was that expected if acetyl-CoA had been formed from the diethylmalonate before incorporation. There is also some less-direct evidence that the reactions A and B of Scheme 1 may be important in isoprenoid biosynthesis, for a number of workers studying various microbial and mammalian tissue preparations found no evidence for the formation of acetoacetate and isoprenoids being biotin-dependent, as necessitated by step C of

pathway 2 in Scheme 1 (Abraham *et al.*, 1961; Bloomfield & Bloch, 1960; Fletcher & Myant, 1960).

For the CoA ester of β -hydroxy- β -methylglutamic acid to be formed by pathway 2 of Scheme 1 the enzyme-bound derivative must be converted into the CoA ester. Stewart & Rudney (1966) have, however, shown that when β -hydroxy- β -methylglutaryl-CoA is formed from acetoacetyl-CoA by a yeast preparation, the CoA originally bound to the acetoacetate remains bound to the β -hydroxy- β -methylglutaryl-CoA formed, thereby ruling out the possibility of the intermediate reactants being enzyme-bound. Nevertheless, Rudney *et al.* (1966) have shown that when the acyl carrier protein ester of acetoacetate is substituted for acetoacetyl-CoA as substrate in the yeast preparation the product is the acyl carrier protein ester of β -hydroxy- β -methylglutamic acid.

In view of the conflicting evidence for the two possible pathways for the formation of isoprenoids from acetyl-CoA, the role of malonyl-CoA as an intermediate was re-investigated. Evidence for the incorporation of malonyl-CoA into isoprenoids by a number of different tissues was obtained and an attempt was made to distinguish between the possible pathways for the incorporation of acetyl-CoA on the

basis of the labelling pattern of the β -hydroxy- β -methylglutaryl-CoA and ergosterol produced from [^{14}C]acetyl-CoA and [^{14}C]malonyl-CoA.

Materials and Methods

Materials

Radioactive materials. These were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Special chemicals. CoA was obtained from Sigma Ltd., London S.W.6, or was prepared from baker's yeast by the procedure of Kornberg & Stadtman (1957). The material from either source was reduced with KBH_4 ; CoA (10mg) was dissolved in water (10ml) and reduced by the addition of KBH_4 solution (0.5%) in water, the mixture was heated at 100°C for 1 min, cooled in an ice bath and the excess of KBH_4 was destroyed by adding acetic acid (1%, 0.5ml). The solution was then diluted to 25ml, and subjected to chromatography on a DEAE-cellulose column essentially by the procedure of Moffatt & Khorana (1961). The column (2.6cm $\times$ 28cm) was equilibrated with 0.02M-KCl containing 3mm-HCl and was developed with a linear gradient of KCl concentration increasing to 0.15M. The CoA in the eluate was assayed by the procedure of Novelli (1957). Acetyl-CoA and [^{14}C]acetyl-CoA were prepared from CoA (which had previously been purified) and acetic anhydride by the procedure of Simon & Shemin (1953) for the preparation of succinyl-CoA. [^{14}C]Acetyl-CoA was also prepared biosynthetically from [$1\text{-}^{14}\text{C}$] and [$2\text{-}^{14}\text{C}$]acetate by using a preparation of pig heart acetyl-CoA synthetase (Beinert *et al.*, 1953). CoA (3 μmol) was incubated with [^{14}C]acetate (7.0 μmol), dipotassium ATP (7.0 μmol), 2-mercaptoethanol (10 μmol), MgCl_2 (10 μmol), Tris-HCl buffer, pH 8.2 (100 μmol), KCl (50 μmol) and acetyl-CoA synthetase (1–10mg of protein), in a total volume of 1 ml. The incubation was terminated after 1 h by adding an equal volume of ethanol, the precipitate was washed twice with 50% (v/v) aq. ethanol, and the supernatant and washings were combined and freeze-dried.

Acetyl-CoA prepared by either procedure was further purified; the crude material was de-salted on a Sephadex G-10 column (25cm $\times$ 2.5cm) equilibrated with water, the salt-free CoA esters being eluted from the column with water in the void volume. The material was then subjected to chromatography by the same procedure used for CoA. The appropriate fractions were again de-salted on the Sephadex G-10 column, freeze-dried, and stored at -20°C .

Malonyl-CoA and [^{14}C]malonyl-CoA were prepared via monothiophenol malonate by the procedure of Trams & Brady (1960), the crude product being subjected to chromatography on DEAE-cellulose as described for CoA. In this system CoA was eluted

with 0.05–0.06M-KCl, acetyl-CoA with 0.065–0.075M-KCl and malonyl-CoA with 0.09–0.1M-KCl. The purified substrate was de-salted and freeze-dried in the same way as acetyl-CoA.

Specifically labelled β -hydroxy- β -methyl-[5- ^{14}C] and -[6- ^{14}C]glutaryl-CoA samples were prepared from [2- ^{14}C] and [1- ^{14}C]acetyl CoA respectively, by means of yeast β -hydroxy- β -methylglutaryl-CoA synthetase prepared by the procedure of Rudney & Ferguson (1959). Acetoacetyl-CoA (4 μmol), [^{14}C]acetyl-CoA (4 μmol), bovine serum albumin (10mg) and Tris-HCl buffer, pH 8.5 (400 μmol) were incubated with the purified condensing enzyme (4mg of protein) in a total volume of 2.0ml at 30°C for 1 h. Acetic acid (0.2ml, 1.0M) was added to stop the reaction, then the mixture was heated to 100°C on a water bath for 1 min; the precipitated protein was centrifuged down, washed with water, and the combined supernatant and washings were adjusted to pH 6.5 and de-salted on Sephadex G-10. This crude material was purified by chromatography on a DEAE-cellulose column by a modification of the procedure of Stewart & Rudney (1966). The column (28cm $\times$ 2.8cm) was equilibrated with 0.02M-NaCl in 50mm-sodium phosphate buffer, pH 6.0. The de-salted CoA ester was applied to the column and eluted with a linear gradient of NaCl increasing to 0.75M by using two 500ml reservoirs. The purified material was again de-salted on Sephadex G-10, freeze-dried and stored at -20°C .

Acetoacetyl-CoA. Acetoacetyl-CoA was prepared by the procedure of Decker (1955) as described by Lynen *et al.* (1958), in which reduced CoA is treated with diketene. The crude material was de-salted and purified on a DEAE-cellulose column as described above for β -hydroxy- β -methylglutaryl-CoA. The peak that was eluted at 0.2M-NaCl was identified as acetoacetyl CoA by its absorption spectrum (Stern, 1956).

Avidin. Commercial avidin obtained from the Nutritional Biochemical Corporation, Ohio, U.S.A., was purified by chromatography on CM-cellulose at pH 9.0 by the procedure of Melamed & Green (1963). The purified material was shown to contain 90–95% avidin by using the spectrophotometric procedure of Green (1963).

Biotin. Crystallized biotin (m.p. $230\text{--}231^\circ\text{C}$ uncorr.) was obtained from Sigma Ltd., London S.W.6, U.K.

Methods

Isolation and degradation of ergosterol. [^{14}C]Ergosterol was isolated by the procedure of Bu'Lock *et al.* (1962), and was recrystallized to constant specific radioactivity with carrier ergosterol (m.p. $160\text{--}163^\circ\text{C}$ uncorr.). The crystals were oxidized with permanganate and periodate [Lemieux & von Rudloff

(1955), as modified by von Rudloff (1956)] as carried out by Bu'Lock *et al.* (1962). The 2,3-dimethylbutyric acid derived from the side chain of the ergosterol was isolated by the procedure of Hanahan & Wakil (1953). The remaining C₂₂ diketo acid derived from the sterol nucleus was isolated by ether extraction of the residue remaining after steam distillation. Carrier ergosterol (10mg) was added to the extract, and was recrystallized from ethanol-benzene (3:1, by v/v), to remove undegraded radioactive ergosterol. The mother liquor from the recrystallization was evaporated to dryness, then dissolved in the minimum volume of diethyl ether, and applied to a Silica Gel G (Merck, supplied by Anderman and Co. Ltd., London S.E.1, U.K.) t.l.c. plate 0.3mm thick. The plate was developed in light petroleum (b.p. 40–60°C)-diethyl ether (3:2, v/v); ergosterol had *R_F* value of 0.2. The radioactivity remained at the origin, and was eluted with diethyl ether. Control experiments showed that about 92% of the radioactivity present in ergosterol derived from incubations with [2-¹⁴C]malonyl-CoA or [2-¹⁴C]acetate could be accounted for in the two isolated products.

Ergosterol and its degradation products were oxidized to acetic acid by the Kuhn & Roth (1933) procedure as modified by Lemieux & Purves (1947).

The carboxyl carbon of acetate produced by the Kuhn & Roth (1933) procedure was oxidized to CO₂ and the methyl carbon converted into methylamine by the Schmidt (1923) reaction, the procedure being essentially that of Madsen *et al.* (1964). Reactions were carried out in a Warburg flask, and Hyamine hydroxide was used to trap the evolved CO₂. The radioactivity present in the methylamine was also measured. For this purpose, after quantitative removal of the contents of the centre well the material in the main compartment of the Warburg flask was transferred quantitatively to a 25ml round-bottomed flask with water, the solution was then made alkaline with 2M-NaOH and was distilled into 0.2ml of 5M-H₂SO₄ in the main compartment of a Warburg flask, 5ml of the distillate being collected. Potassium permanganate (1–5ml, 10%, v/w) and H₂SO₄ (0.2ml, 5M) was added, the flask was sealed with a rubber cap and was heated at 90°C for 30min and then cool Hyamine hydroxide (3ml) was added to the centre well through the rubber cap with a syringe. After 2h the Hyamine hydroxide was quantitatively removed and assayed for radioactivity.

Isolation and degradation of β-hydroxy-β-methylglutaryl-CoA. The procedure of Rudney & Ferguson (1959) was used to isolate β-hydroxy-β-methylglutaryl-CoA; the material chromatographed with *R_F* 0.1–0.2 on Whatman no. 3 paper in 1.0M-ammonium acetate (pH 4.7)-ethanol (1:7, v/v). The material having this *R_F* value obtained from incubations containing malonyl-CoA was eluted and re-chromatographed in the same system.

To measure the radioactivity in C-3, C-5 and C-6 the β-hydroxy-β-methyl[¹⁴C]glutaryl-CoA was degraded with the cleavage enzyme (β-hydroxy-β-methylglutaryl-CoA acetoacetate lyase, EC 4.1.3.4) prepared by the procedure of Bachhawat *et al.* (1955). The β-hydroxy-β-methyl[¹⁴C]glutaryl-CoA was diluted with the unlabelled compound and transferred to the main compartment of a Warburg flask containing MgCl₂ (10mm), cysteine, pH 7.5 (10mm), and Tris-HCl buffer, pH 8.0 (100mm), together with the cleavage enzyme in the side arm. The flasks were sealed with a rubber cap, and the cleavage enzyme, (1.5mg of protein; sp. enzyme activity 0.17 μmol/min per mg) was added from the side arm; the mixture was incubated for 3h at 37°C, then the reaction was stopped by adding 0.5ml of HCl (4M) through the rubber cap with a syringe. The acetoacetate derived from the β-hydroxy-β-methylglutaryl-CoA was converted into acetone dinitrophenylhydrazone by injecting 2ml of 2,4-dinitrophenylhydrazine (0.5% in 2M-HCl) into the main compartment, and heating the flask at 50°C for 2h. To recover the CO₂, the flask was cooled and 2.0ml of Hyamine hydroxide solution (1.0M in ethanol) was injected through the rubber cap into the centre wall. After 2h, the contents of the centre wall were quantitatively removed, and the absorbed CO₂ was assayed in the scintillation counter. The acetone 2,4-dinitrophenylhydrazone in the centre main compartment was then purified. A further 2.0ml of 2,4-dinitrophenylhydrazone was added to the main compartment, together with authentic acetone 2,4-dinitrophenylhydrazone. The acetone 2,4-dinitrophenylhydrazone was then extracted with CCl₄ by the procedure of Lehninger (1957). The concentrated extract was subjected to chromatography in butan-1-ol-ethanol-aq. NH₃ (sp.gr. 0.880) (7:1:2, by vol.) by the procedure of El Hawary & Thompson (1953). The radioactivity of the acetone dinitrophenylhydrazone was assayed on the paper with an end-window Geiger counter.

The radioactivity in C-1 and C-2 of β-hydroxy-β-methylglutaryl-CoA was assayed by isolating acetate derived from the acetyl-CoA produced by the cleavage enzyme. Incubations of β-hydroxy-β-methylglutaryl-CoA with the cleavage enzyme were carried out as described above and stopped by the addition of 0.5ml of HCl (2M), and were heated to 60°C for 1h. The mixture was adjusted to pH 10.0 and acetone was removed by bubbling a stream of N₂ through. To ensure complete hydrolysis of the thioesters the solution was incubated for a further 1h at 60°C. The solution was then evaporated to dryness, redissolved in a small volume of water and the acetate extracted by chromatography on a Celite column. The distribution of the radioactivity in the sodium acetate thus isolated was determined by the Schmidt (1923) degradation followed by oxidation of the resulting methylamine as described above.

Yeast cell-free enzyme system. Baker's yeast (*Saccharomyces cerevisiae*) for the preparation of cell-free enzyme systems was grown on a medium containing 3.0g of KH_2PO_4 , 1.0g of MgSO_4 , 0.25g of $\text{CaCl}_2 \cdot 5\text{H}_2\text{O}$, 2.75mg of MnSO_4 , 0.5mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10mg of $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$, 20g of glucose, 2.0g of $(\text{NH}_4)_2\text{SO}_4$, 2.0g of yeast extract, 1.0mg of thiamin hydrochloride and 0.01mg of D biotin in 1 litre of water. After 2 days of growth at 30°C the cells were harvested and washed with distilled water, and 10g of the wet yeast paste was mixed with 10ml of potassium phosphate buffer (pH 7.5, 0.117M) and 50g of Ballotini beads (0.45–0.5mm) before being disrupted at -30°C in a Braun homogenizer [B. Braun, Milsungen, W. Germany, as described by Merckenschlager *et al.* (1957)] for 3min. The fluid and beads were centrifuged at 5000g at 0°C , then the supernatant was centrifuged at 20000g for 30min. This supernatant solution contained all the enzymes necessary for the incorporation of acetate into fatty acids and unsaponifiable lipids.

Rat liver enzyme system. A system containing all the enzymes necessary to convert acetate into squalene and sterols was prepared from rat liver by the procedure of Knauss *et al.* (1959).

To study the biosynthesis of β -hydroxy- β -methylglutaryl-CoA an unwashed rat liver microsomal system was prepared by the procedure of Rudney (1957), the microsomal pellet being suspended in 1.0mM-EDTA.

Fractionation of rat liver homogenate. To obtain a system that would form mevalonate from acetate in which the malonyl-CoA decarboxylase activity was minimal rat liver homogenate was prepared and fractionated by the procedure of de Duve *et al.* (1955). The particle-free supernatant obtained by centrifugation at 104000g was fractionated with $(\text{NH}_4)_2\text{SO}_4$ and the fraction precipitated at 40–80% saturation was taken together with a minimal amount of microsomal protein.

Assay of formation of β -hydroxy- β -methylglutaryl-CoA. Incubation mixtures for the assay of β -hydroxy- β -methylglutaryl-CoA formation contained 1.0mg of enzyme protein, 5mM-mercaptoethanol or 2mM-dithiothreitol and 25mM-[2- ^{14}C]malonyl-CoA or 25mM-[2- ^{14}C]acetyl-CoA, in a total volume of 2.0ml. The mixture was incubated at 30°C for 1h and the β -hydroxy- β -methylglutaryl-CoA was assayed by the procedure of Rudney & Ferguson (1959).

Triacetic acid lactone. Formation of this was assayed in the incubation mixtures used for the assay of β -hydroxy- β -methylglutaryl-CoA (see above). The incubation was stopped by the addition of 0.25ml of 0.1M-HCl, and the mixture was saturated with anhydrous Na_2SO_4 . The triacetic acid lactone was extracted with ten 3ml portions of diethyl ether, and after evaporation of this ether, the residue was dissolved in 0.1ml of methanol. The material was identi-

fied by its chromatographic characteristics by the following procedures.

(a) T.l.c. on silica gel G in chloroform–acetic acid (21:4, v/w) in which the suspected triacetic acid lactone had an R_F value of 0.35 in agreement with the value obtained in this solvent by Yalpani *et al.* (1969) for the chemically synthesized compound.

(b) Paper chromatography on Whatman no. 1 paper in butan-1-ol saturated with 0.1M-sodium acetate buffer pH 5.5; the material had R_F 0.3–0.4 which is similar to that obtained by Yalpani *et al.* (1969) for chemically synthesized triacetic acid lactone.

(c) Paper chromatography on Whatman no. 1 paper in 2-methylpropionic acid–3M- NH_3 –0.1M-EDTA–water (62:12:1:28, by vol.); the major component had R_F 0.85–0.95 in agreement with the value reported by Brodie *et al.* (1964) for authentic triacetic acid. For routine assay the material separating with R_F 0.3 in system (a) was scraped from the plate and assayed by scintillation counting.

Malonyl-CoA decarboxylase. Crude preparations of malonyl-CoA decarboxylase were obtained from rat liver mitochondria. These were prepared by the procedure of de Duve *et al.* (1955) and were then sonicated for 30s in an MSE ultrasonic disintegrator at 360W, and centrifuged at 4300g for 10min. The supernatant was then centrifuged at 104000g for 50min, and the supernatant from this centrifugation contained the malonyl-CoA decarboxylase.

Pigeon liver fatty acid synthetase. Pigeon liver fatty acid synthetase was prepared either by the procedure of Hsu *et al.* (1965) or by the method of Yang *et al.* (1967). The latter preparation was consistently more active than the former in the synthesis of palmitate from malonyl-CoA and acetyl-CoA. Neither method of preparation gave a product that synthesized β -hydroxy- β -methylglutaryl-CoA from malonyl-CoA and acetyl-CoA. The first $(\text{NH}_4)_2\text{SO}_4$ fraction in the preparation of Yang *et al.* (1967) was therefore used to study β -hydroxy- β -methylglutaryl-CoA synthesis by pigeon liver.

Lipid extraction. Lipids were extracted from incubations of yeast, rat and pigeon liver systems by the procedure of Bligh & Dyer (1959) as modified by Galliard *et al.* (1965). The chloroform used in the extraction procedure was evaporated and the lipids saponified by refluxing them with 50ml of 2M-KOH in 95% (w/v) methanol for 18h. The methanol was removed by evaporation and the non-saponifiable material was extracted ($\times 4$) with light petroleum (b.p. 30 – 40°C , low in aromatic hydrocarbons). Contaminating labelled substrate was removed by washing the extract three times with water. These washings were combined with the remaining aqueous extract, concentrated under reduced pressure and acidified to pH 2. This acid solution was extracted three times with 25ml portions of light petroleum

(b.p. 30–40°C) and the extract washed with water, dried and concentrated to 3 ml under a stream of N₂. Fatty acids were esterified with an excess of diazomethane in ether (James, 1960). When [¹⁴C]acetate was the substrate the product was dissolved in 20 ml of methyl acetate and evaporated to dryness in vacuum; this was repeated a further four times to obtain fatty acid methyl esters of constant specific radioactivity (Rowe, 1964).

Latex incubations. Latex was obtained from *Hevea brasiliensis* trees grown under glass in the University of Birmingham Botanical Garden. Each incubation contained 0.1 ml of fresh latex which was added to the freeze-dried substrate and cofactors.

Rubber extraction. The procedure used was that of Weeks (1966). Latex incubations were terminated by the addition of 2 ml of chloroform-methanol (4:1, v/v) in which the lipids and rubber of the latex were dissolved. The solution was transferred to a round-bottomed flask and the solvent was evaporated leaving a thin film of rubber and lipid over the side of the flask. The lipids in the flask were saponified by the above procedure, and the alcoholic extracts containing saponified and unsaponifiable lipid were carefully decanted, leaving the rubber adhering to the walls of the saponification vessels. The rubber was washed in the flask three times with methanol and the washings were combined with the extracted lipid material. The rubber was then dissolved in 50 ml of chloroform, and washed seven times with water to remove contaminating substrate; this treatment yielded rubber of constant specific radioactivity almost free of contamination with the radioactive precursor.

Measurement of radioactivity. β -Scintillation counting was carried out in a Nuclear-Chicago model 721 β -scintillation spectrometer. The radioactive lipid samples were dissolved in 5 ml of light petroleum (b.p. 40–60°C) and added to 5 ml of phosphor solution containing 0.3% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene. For aqueous samples up to 1 ml of solution was mixed

with 10 ml of a solution consisting of a mixture of the above phosphor solution (1 vol.) and Triton X-100 (2 vol.). The counting efficiency was measured by the channels-ratio method.

Protein determination. The protein content of the enzyme preparation was determined by the biuret procedure (Weichselbaum, 1947).

Isolation of mevalonate. The mevalonate formed from radioactive precursors in the presence of a trapping pool of non-radioactive mevalonate was isolated by the procedure of Brodie *et al.* (1963).

Isolation of squalene. The squalene formed was isolated and characterized as the hexachloride by the procedure of Langdon & Bloch (1953).

Results

Formation of lipids from malonyl-CoA

An initial survey of the incorporation of [2-¹⁴C]-malonyl-CoA into isoprenoids by a number of tissue preparations was made. The results in Fig. 1 show that a cell-free preparation of yeast incorporated [2-¹⁴C]malonyl-CoA into both the saponifiable and non-saponifiable lipid fractions. The incorporation of [2-¹⁴C]malonyl-CoA into the unsaponifiable lipid fraction was about 0.6 times that of [2-¹⁴C]acetyl-CoA.

Somewhat similar results were obtained when rubber latex was incubated with [2-¹⁴C]malonyl-CoA (Table 1) although in initial experiments no incorporation of radioactive label into rubber could be detected despite incorporation into saponifiable lipid. When, however, latex was supplemented with NADPH, a significant incorporation of [2-¹⁴C]-malonyl-CoA into rubber became evident.

Formation of mevalonate from malonyl-CoA

When a trapping pool of unlabelled mevalonate was added to a cell-free yeast extract and the incorporation of [2-¹⁴C]malonyl-CoA and [2-¹⁴C]acetyl-CoA into saponifiable and unsaponifiable lipids

Table 1. Incorporation of [2-¹⁴C]malonyl-CoA, [2-¹⁴C]acetyl-CoA and [2-¹⁴C]acetate into lipid fractions by *H. brasiliensis* latex

The substrates (0.5 mM, specific radioactivity 1.0 Ci/mol) were incubated with 0.1 ml of latex at 30°C for 1 h. The results show the range of three separate incubations; latex from the same tapping was used.

Substrate	Incorporation into lipid fraction (nmol/100 mg of latex)		
	Fatty acid	Unsaponifiable lipid	Rubber
[2- ¹⁴ C]Malonyl-CoA	480–520	Not detectable	Not detectable
[2- ¹⁴ C]Malonyl-CoA + NADPH (0.5 mM)	630–680	Not detectable	84–92
[2- ¹⁴ C]Acetyl-CoA	24–29	96–100	170–196
[2- ¹⁴ C]Acetate	186–206	172–190	790–830

Fig. 1. Time-course of the incorporation of [2-¹⁴C]-malonyl-CoA and [2-¹⁴C]acetyl-CoA into yeast lipids

Cell-free yeast preparations (0.2ml) were incubated with [2-¹⁴C]malonyl-CoA and [2-¹⁴C]acetyl-CoA each at a concentration of 1.0mM and sp. radioactivity of 0.75Ci/mol, with potassium phosphate buffer, pH 7.0, 200mM, in a total volume of 1.0ml at 30°C. Points are the means of three replicates and bars give the range of values obtained. Formation of saponifiable lipid from: ○, [2-¹⁴C]malonyl-CoA; ●, [2-¹⁴C]acetyl-CoA. Formation of unsaponifiable lipid from: △, [2-¹⁴C]malonyl-CoA; ▲, [2-¹⁴C]acetyl-CoA.

was investigated, it was found that, although the incorporation of either radioactive substrate into the saponifiable fraction was unaffected by increasing concentrations of mevalonate, the incorporation of radioactivity from both substrates into the non-saponifiable fraction was decreased about tenfold in the presence of 25mM-mevalonate. When an ether extract of the incubations with [2-¹⁴C]malonyl-CoA was prepared, a radioactive material was obtained that had the same chromatographic characteristics as authentic mevalonate. This radioactive material was eluted from the paper chromatograms and incubated with a preparation of *Hevea* mevalonate kinase and ATP (Williamson & Kekwick, 1965). The products of this second incubation had the same chromatographic characteristics as 5-phosphomevalonate on paper chromatography in ethanol-ammonia-water (7:2:1, by vol.) (Chesterton & Kekwick, 1968).

When a similar experiment was carried out to investigate the formation of mevalonate from [2-¹⁴C]-malonyl-CoA in *Hevea* latex serum, radioactive

material chromatographing as mevalonate could not be demonstrated by similar criteria; there was, however, radioactivity in the component chromatographing as β -hydroxy- β -methylglutarate. The radioactive components were pooled and diluted with authentic β -hydroxy- β -methylglutarate and the dibromophenacyl ester was synthesized and crystallized to constant specific radioactivity. When [2-¹⁴C]-malonyl-CoA was incubated with latex in the presence of 0.5mM-NADPH, however, a diethyl ether extract of the incubation mixture again contained radioactive material which chromatographed as mevalonate, but this fraction gave rise to material which chromatographed as 5-phosphomevalonate after treatment with *Hevea* mevalonate kinase and ATP.

Evidence for the carboxylation of acetyl-CoA

From the above results it was clear that in both yeast and *Hevea* latex malonyl-CoA could be incorporated into isoprenoid substances via mevalonate. Evidence was therefore sought to establish whether a biotin-dependent carboxylation of acetyl-CoA was an obligatory step in mevalonate formation. The effect of CO₂ and HCO₃⁻ on the incorporation of acetate, acetyl-CoA and malonyl-CoA into the saponifiable and non-saponifiable lipids by the cell-free yeast preparation was investigated. The results of a typical experiment (Table 2) show that whereas the incorporation of acetyl-CoA into fatty acids was stimulated by increasing the HCO₃⁻ concentration, that into the unsaponifiable lipids decreased, and the incorporation of malonyl-CoA was unaffected. These results suggest that there is not an obligatory carboxylation stage in the formation of isoprenoids from acetyl-CoA.

More direct evidence about the role of an acetyl-CoA carboxylation in isoprenoid biosynthesis was obtained from an investigation of the effect of avidin on the biosynthesis of isoprenoids from acetyl-CoA. Fig. 2 shows the effect of avidin on the incorporation of acetyl-CoA in fatty acids and squalene by a cell-free yeast preparation. In both this and rat liver preparations the biosynthesis of fatty acids was markedly sensitive to inhibition by avidin but the synthesis of squalene was quite unaffected.

Effect of acetyl-CoA on the incorporation of [2-¹⁴C]-malonyl-CoA into lipids

Earlier workers (Fimognari & Rodwell, 1970) found that the incorporation of [2-¹⁴C]malonyl-CoA into mevalonate by the 10000g supernatant of a rat liver homogenate was unaffected by the addition of unlabelled acetyl-CoA and concluded that this was evidence that malonyl-CoA did not undergo decarboxylation to acetyl-CoA before incorporation

Table 2. *Effect of HCO_3^- on the formation of lipids in the cell-free yeast preparation*

The cell-free yeast preparation (0.25 ml) was incubated with the substrate (1 mM, $0.75 \mu\text{Ci}$) and potassium phosphate buffer (0.2M), pH 7.2, in a Warburg flask (total volume 0.8 ml) for 2 h at 30°C . When the incubations were to be CO_2 -free the phosphate buffer was freshly prepared from water which had been boiled for 30 min and the centre wells of the Warburg flasks contained 0.2 ml of saturated KOH and a filter-paper wick.

Substrate	KHCO_3 concentration (mM)	Gas phase ...	^{14}C incorporated (pmol/mg of protein)				
			Air with CO_2	CO_2 -free air			
				0	0.1	0.5	1.0
[^{14}C]Acetate	Lipid fraction	Unsaponifiable	103	437	97	96	98
		Saponifiable	172	18	185	192	191
[^{14}C]Acetyl-CoA	Lipid fraction	Unsaponifiable	240	973	203	208	209
		Saponifiable	483	28	546	543	547
[^{14}C]Malonyl-CoA	Lipid fraction	Unsaponifiable	187	191	187	193	189
		Saponifiable	683	685	684	685	691

Fig. 2. *Effect of avidin on the incorporation of [^{14}C]-acetyl-CoA into squalene and fatty acids by the cell-free yeast preparation*

Cell-free yeast preparation (0.5 ml) was incubated with [^{14}C]acetyl-CoA (1.0 mM, sp. radioactivity 0.75 Ci/mol), KHCO_3 (1.0 mM), NADPH (0.5 mM) and ATP (1.5 mM) together with avidin in the concentration given, in a total volume of 1.5 ml. The addition of biotin (0.75 μM) completely reversed the inhibition obtained with avidin at concentrations up to 2.0 μM . The results are the mean of three replicates, the range of which is shown. Incorporation of: ○, [^{14}C]acetyl-CoA into squalene; ●, [^{14}C]acetyl-CoA into fatty acids; Δ, [^{14}C]acetyl-CoA into fatty acids in the presence of 0.75 μM -biotin.

into mevalonate. The effect of the presence of unlabelled acetyl-CoA on the incorporation of [^{14}C]malonyl-CoA into the saponifiable and unsaponifi-

Fig. 3. *Effect of unlabelled acetyl-CoA on the incorporation of [^{14}C]malonyl-CoA into the fatty acid and unsaponifiable fraction of yeast lipids*

Incubations contained 1.0 mM-[^{14}C]malonyl-CoA, 200 mM-potassium phosphate buffer, pH 7.2, and 0.25 ml of yeast homogenate in a total volume of 0.75 ml. Results are the average of four determinations, the range of which is shown. ●, Fatty acids; Δ, unsaponifiable lipid.

able lipid fractions by the cell-free yeast preparation was therefore investigated. The results in Fig. 3 show that low acetyl-CoA concentrations stimulated the incorporation of [^{14}C]malonyl-CoA into the unsaponifiable fraction and had no effect on the

Scheme 2. *Expected labelling pattern of β -hydroxy- β -methylglutaryl-CoA formed from [2- 14 C]- and [1,3- 14 C]-malonyl-CoA by two pathways*

The subsequent degradation of the β -hydroxy- β -methylglutaryl-CoA is also shown. The labelling of C atoms is indicated by ■ and ●. To simplify presentation of the Scheme, details of reactions have been omitted.

incorporation into the saponifiable fraction; higher concentrations of unlabelled acetyl-CoA, however, inhibited the incorporation of [2-¹⁴C]malonyl-CoA into both the saponifiable and the unsaponifiable lipid fractions.

From these results it appeared that no clear conclusions about the role of malonyl-CoA in isoprenoid biosynthesis could be drawn from the effect of acetyl-CoA on the incorporation of malonyl-CoA.

Labelling pattern of β -hydroxy- β -methylglutaryl-CoA

Further evidence about the route of incorporation of malonyl-CoA into isoprenoids was sought by ascertaining the labelling pattern of the β -hydroxy- β -methylglutaryl-CoA produced from [2-¹⁴C]malonyl-CoA, [1,3-¹⁴C]malonyl-CoA and [2-¹⁴C]acetyl-CoA. Scheme 2 indicates that if pathway 1 is operative, that is, if malonyl-CoA is decarboxylated before incorporation, the labelling pattern of β -hydroxy- β -methylglutaryl-CoA formed from [2-¹⁴C]malonyl-CoA will be identical with that formed from [2-¹⁴C]acetyl-CoA, radioactivity being distributed

between C-2, C-4 and C-5. If [1,3-¹⁴C]malonyl-CoA is the substrate and it is incorporated by pathway 1, then radioactivity will be present in C-1, C-3 and C-6. If, however, malonyl-CoA is a direct precursor of acetoacetyl-CoA according to pathway 2, with [2-¹⁴C]malonyl-CoA as the substrate, radioactivity would be found exclusively in C-2 and the radioactivity of [1,3-¹⁴C]malonyl-CoA as substrate would be in C-1.

The results of a series of experiments in which the labelling pattern of β -hydroxy- β -methylglutaryl-CoA formed by rat liver microsomal preparations was measured are shown in Table 3. The labelling pattern of β -hydroxy- β -methyl-[5-¹⁴C]- and [6-¹⁴C]-glutaryl-CoA formed from [2-¹⁴C]acetyl-CoA or [1-¹⁴C]-acetyl-CoA respectively and acetoacetyl-CoA by a preparation of the yeast β -hydroxy- β -methylglutaryl-CoA condensing enzyme was investigated as a control on the degradation procedure (Table 3).

The labelling pattern of β -hydroxy- β -methylglutaryl-CoA formed by the rat liver systems from [2-¹⁴C]malonyl-CoA is seen to be the same as that of the same substance formed from [2-¹⁴C]acetyl-CoA,

Table 3. *Labelling pattern of β -hydroxy- β -methylglutaryl-CoA*

β -Hydroxy- β -methylglutaryl-CoA labelled at C-5 or C-6 was prepared for reference by condensation of [2-¹⁴C]acetyl-CoA or [1-¹⁴C]acetyl-CoA with unlabelled acetoacetyl-CoA catalysed by the yeast β -hydroxy- β -methylglutaryl-CoA condensing enzyme (preparation procedure is given in 'Methods'). Incubations contained 2.0mM-acetoacetyl-CoA, 2.0mM-[¹⁴C]acetyl-CoA (sp. radioactivity 2 μ Ci/ μ mol), 200mM-Tris buffer, pH8.5, 10mg of bovine serum albumin and 4mg of enzyme preparation in a total volume of 2.0ml. For the rat liver microsomal preparation, incubations contained 0.25mM radioactive substrate (sp. radioactivity 2 μ Ci/ μ mol), 5mM-MgCl₂, 5mM-cysteine, pH7.0, 150mM-potassium phosphate buffer, pH7.2, and 10mg of microsomal protein. For the pigeon liver system incubations contained 0.5mM radioactive substrate (sp. radioactivity 3.0 μ Ci/ μ mol), 1mM-EDTA, 2mM-dithiothreitol, 2mM-KHCO₃, 50mM-potassium phosphate buffer, pH7.0, 2mM-MgCl₂ and 15mg of partially purified fatty acid synthetase protein in a total volume of 2.0ml. The results for the distribution of radioactivity in β -hydroxy- β -methylglutaryl-CoA formed by the yeast condensing enzyme and the rat liver microsomal preparation are given $\pm$ s.e.m. of six determinations; those for pigeon liver are the mean of three determinations.

Substrate	Radioactivity (%)				Total radioactivity recovered (%)
	C-1	C-2	C-3, C-4, C-5	C-6	
Yeast condensing enzyme					
[2- ¹⁴ C]Acetyl-CoA + acetoacetyl-CoA (2.0mM)	<1	<1	98.0 $\pm$ 2.8	<1	82.0 $\pm$ 3.5
[1- ¹⁴ C]Acetyl-CoA + acetoacetyl-CoA (2.0mM)	<1	<1	3.4 $\pm$ 0.8	96.0 $\pm$ 5.3	84.9 $\pm$ 4.8
Rat liver microsomal preparation					
[2- ¹⁴ C]Acetyl-CoA	4.7 $\pm$ 1.9	29.8 $\pm$ 2.1	62.0 $\pm$ 1.7	3.5 $\pm$ 0.2	78.0 $\pm$ 4.3
[2- ¹⁴ C]Acetyl-CoA + acetoacetyl-CoA (2.0mM)	1.3 $\pm$ 0.09	7.6 $\pm$ 0.81	90.0 $\pm$ 3.2	1.8 $\pm$ 0.11	81.0 $\pm$ 3.6
[2- ¹⁴ C]Malonyl-CoA	1.0 $\pm$ 0.08	31.5 $\pm$ 1.8	67.0 $\pm$ 9.3	1.5 $\pm$ 0.06	83.2 $\pm$ 4.9
[1,3- ¹⁴ C]Malonyl-CoA	31.4 $\pm$ 2.0	1.0 $\pm$ 0.07	29.5 $\pm$ 1.87	39.0 $\pm$ 2.10	77.5 $\pm$ 5.2
Soluble pigeon liver system					
[2- ¹⁴ C]Acetyl-CoA	2.4	29.7	67.2	3.7	81
[2- ¹⁴ C]Malonyl-CoA	1.2	32.2	66.9	1.3	76

in accord with pathway 1; the labelling pattern of the β -hydroxy- β -methylglutaryl-CoA produced from [2- 14 C]malonyl-CoA by this system was also the one predicted if pathway 1 were operative.

When a large pool of unlabelled acetoacetyl-CoA was added to rat liver systems incorporating [2- 14 C]acetyl-CoA into β -hydroxy- β -methylglutaryl-CoA, 90% of the radioactivity was recovered in the fragment containing C-3, C-4 and C-5 and 7.0% in C-2 of the product (Table 3). This indicated that C-1 and C-2 have originated from the acetoacetyl-CoA added as would be expected from the specificity of the condensing enzyme (Rudney & Ferguson, 1959), and that there has been little randomization of the labelling of β -hydroxy- β -methylglutaryl-CoA by the formation of acetyl-CoA by action of the β -hydroxy- β -methylglutaryl-CoA cleavage enzyme which might be present in the preparation.

Further evidence for the decarboxylation of [2- 14 C]malonyl-CoA to [2- 14 C]acetyl-CoA before conversion into β -hydroxy- β -methylglutaryl-CoA was obtained by measuring the effect of unlabelled acetyl-CoA on the incorporation of [2- 14 C]malonyl-CoA into β -hydroxy- β -methylglutaryl-CoA. As is shown in Table 4, addition of unlabelled acetyl-CoA decreased the incorporation of [2- 14 C]malonyl-CoA, but low concentrations of acetoacetyl-CoA increased the incorporation of this radioactive substrate into β -hydroxy- β -methylglutaryl-CoA.

Because of the discrepancy between the above results and those reported by Brodie *et al.* (1963) for the labelling pattern of the β -hydroxy- β -methylglutaryl-CoA formed from [2- 14 C]malonyl-CoA by a pigeon liver fatty acid synthetase preparation, the behaviour of this system was reinvestigated. The purified preparation of fatty acid synthetase prepared according to the procedure described by Brodie *et al.* (1963), in which the final stage is elution of the enzyme from a DEAE-cellulose column by 0.25M-potassium phosphate buffer, pH7.0, did not, however, form

appreciable amounts of β -hydroxy- β -methylglutaryl-CoA. The synthesis of β -hydroxy- β -methylglutaryl-CoA by preparations obtained at various stages in the purification of pigeon liver fatty acid synthetase by the procedures of Yang *et al.* (1967) and Hsu *et al.* (1965) were therefore compared with the synthesis of fatty acids and of triacetic acid lactone by the same preparations.

The results in Table 5 show that the synthesis of β -hydroxy- β -methylglutaryl-CoA by the more highly purified preparations is very small. The labelling pattern of β -hydroxy- β -methylglutaryl-CoA formed by the fraction precipitated by 20–40%-saturated $(\text{NH}_4)_2\text{SO}_4$ from [2- 14 C]malonyl-CoA was therefore investigated. Table 3 shows the labelling pattern of the β -hydroxy- β -methylglutaryl-CoA thus formed was the same as that obtained with the same substrate in the rat liver system and agreed with the labelling pattern given when [2- 14 C]acetyl-CoA was the substrate. From these experiments it appeared therefore that malonyl-CoA underwent decarboxylation before incorporation into β -hydroxy- β -methylglutaryl-CoA.

Labelling pattern of ergosterol

Information about the consistency of the above observations in different systems and with terminal isoprenoid products was then sought by examining the labelling pattern of ergosterol formed from [2- 14 C]malonyl-CoA and [2- 14 C]acetate by the cell-free yeast system. The experimental techniques were similar to those employed by Bu'Lock *et al.* (1962). A diagram of the procedure used to degrade the isolated ergosterol is shown in Scheme 3. The olefinic bond of the side chain was cleaved to give dimethylbutyric acid by the procedure of Lemieux & von Rudloff (1955). This dimethylbutyric acid and the remaining nuclear fragment were each subjected to a Kuhn-Roth (1933) oxidation and the radioactivity of the resultant acetic acid was determined; the distribution

Table 4. Effect of unlabelled acetyl-CoA and acetoacetyl-CoA on the incorporation of [2- 14 C]malonyl-CoA into β -hydroxy- β -methylglutaryl-CoA by rat liver microsomal preparations

The incubation mixture and conditions except for the substrates were as given in Table 3. The concentration of malonyl-CoA was 0.75 mM in each experiment; the results are the mean of three determinations.

Substrates	Incorporation (nmol/mg of protein)
[2- 14 C]Malonyl-CoA (0.75 mM)	24.5
[2- 14 C]Malonyl-CoA + acetyl-CoA (0.75 mM)	17.5
[2- 14 C]Malonyl-CoA + acetyl-CoA (3.0 mM)	12.5
[2- 14 C]Malonyl-CoA + acetoacetyl-CoA (0.1 mM)	32.5
[2- 14 C]Malonyl-CoA + acetoacetyl-CoA (0.2 mM)	42.3
[2- 14 C]Malonyl-CoA + acetoacetyl-CoA (0.5 mM)	27.8
[2- 14 C]Malonyl-CoA + acetoacetyl-CoA (1.0 mM)	21.2

Table 5. *Synthesis of β -hydroxy- β -methylglutaryl-CoA by purified preparations of pigeon liver fatty acid synthetase*

Assays of fatty acid, triacetic acid lactone and β -hydroxy- β -methylglutaryl-CoA synthetase activities were carried out according to the procedures given in 'methods'. Results are given for four separate preparations of pigeon liver fatty acid synthetase. Preparations 1A and 2A were obtained from duplicate purifications according to the procedure of Yang *et al.* (1967) in which all the solutions used contain 1.0mM-dithiothreitol. Preparations 1B and 2B were obtained from duplicate purifications carried out by the essentially similar procedure of Hsu *et al.* (1965) in which the solutions contained 1.0mM-2-mercaptoethanol.

Purification stage	Malonyl-CoA incorporated (nmol/min per mg of protein)											
	Fatty acid				Triacetic acid lactone				β -Hydroxy- β -methylglutaryl-CoA			
	1A	2A	1B	2B	1A	2A	1B	2B	1A	2A	1B	2B
Particle-free supernatant	12.5	8.3	2.4	4.6	2.0	—	—	—	—	—	—	—
(NH ₄) ₂ SO ₄ (20–40% saturated)	18.2	12.3	4.9	6.5	3.4	2.1	1.3	1.4	1.2	1.0	—	—
Calcium phosphate gel	13.7	10.1	4.8	6.5	1.4	1.1	1.3	1.6	1.1	1.0	—	4.1
DEAE-cellulose eluate	65.2	36.1	29.8	23.0	16.4	10.8	7.4	8.2	0.1	0.3	8.4	3.7
(NH ₄) ₂ SO ₄ (26–30% saturated)	91.5	42.2	58.0	54.1	—	9.7	11.4	9.1	—	0.2	5.1	3.6
Sephadex G-100	123.5	67.2	62.1	58.2	29.4	13.8	15.2	14.9	—	0.1	1.8	0.4

of this radioactivity between the carboxyl and methyl carbon atoms was determined by assaying the radioactivity present in the methylamine and CO₂ produced by the Schmidt (1923) degradation. Scheme 3 shows the distribution of the label to be expected in the fragment formed by degradation of ergosterol synthesized from [2-¹⁴C]malonyl-CoA or [2-¹⁴C]acetate by pathways 1 or 2. The results given in Table 6 show that the labelling tallies closely with that which would be predicted if acetoacetyl-CoA is formed from two acetyl-CoA molecules and not from a malonyl-CoA molecule and an acetyl-CoA molecule. The labelling patterns given by [2-¹⁴C]malonyl-CoA and [2-¹⁴C]acetate were closely similar.

Location of malonyl-CoA decarboxylase

The remaining point to be investigated was whether the activity and location of malonyl-CoA decarboxylase in the tissues examined were such as would explain the observed incorporation of malonyl-CoA via acetyl-CoA. When the decarboxylation of malonyl-CoA in the rat liver system used for studying squalene formation (Knauss *et al.*, 1959) was investigated it was found that under the experimental conditions used for the incubation 1.46 μ mol of malonyl-CoA was decarboxylated/h. Analogous experiments with the yeast system gave a decarboxylation rate of 0.74 μ mol/h. Both these activities are sufficient to account for the observed incorporation of malonyl-CoA.

Experiments were then carried out to attempt to control the activity of malonyl-CoA decarboxylase present in the rat liver system. Scholte (1969) has shown that the malonyl-CoA decarboxylase is a mitochondrial enzyme. When the fractionation procedure of Scholte (1969) was repeated it was found that 90% of the malonyl-CoA decarboxylase activity was in the mitochondrial fraction, but the microsomal fraction still possessed some decarboxylase activity. No precautions are taken to preserve the integrity of the mitochondria in the preparation of the rat liver system of Knauss *et al.* (1959), used for the study of squalene formation. The malonyl-CoA decarboxylase activity found in the preparation could therefore have arisen from degradation of the mitochondria.

To obtain a system in which the malonyl-CoA decarboxylase activity was minimal but which would form mevalonate from acetate, rat livers were fractionated by the procedure of de Duve *et al.* (1955). The 104000g supernatant was then fractionated with (NH₄)₂SO₄ and the 40–80%-saturated (NH₄)₂SO₄ fraction was taken. This required the addition of microsomal protein to obtain synthesis of mevalonate from acetate, and the optimum incorporation was obtained when 1.2mg of microsomal protein/ml was added to an incubation containing 10mg of protein from the soluble (NH₄)₂SO₄ fraction. The effect of the

Scheme 3. Degradation of ergosterol formed by two pathways

Carbon atoms marked □ and Δ would be labelled when [2-¹⁴C]acetate was the substrate and pathway 1 was operative (Scheme 2). Carbon atoms marked Δ would be labelled when [2-¹⁴C]malonyl-CoA was the substrate and pathway 2 was operative (Scheme 2).

Table 6. Labelling patterns of acetic acid derived from ergosterol formed by the cell-free yeast preparation from [2-¹⁴C]acetate and [2-¹⁴C]malonyl-CoA

Cell-free yeast preparation (1.5ml, 18.0mg of protein) was incubated with either 1.0mM-[2-¹⁴C]acetate or 1.0mM-[2-¹⁴C]malonyl-CoA (sp. radioactivity of both substrates 1.75 Ci/mol) in 0.2M-potassium phosphate buffer in a total volume of 5ml at 30°C for 1.5h. After saponification of the extracted lipid the unsaponifiable lipid fraction was dissolved in 100ml of ethanol-benzene (3:1, v/v) together with 10mg of carrier ergosterol at 45°C. Crystals of ergosterol were harvested after the solution had remained at 4°C for 18h, and were recrystallized to constant specific radioactivity from the same solvent system. Details of the degradation procedure are given under 'Methods'. Results are means ±S.E.M. for six determinations.

Origin of Kuhn-Roth acetic acid	Ratio of $\frac{\text{carboxyl}}{\text{methyl}}$ radioactivity		
	Ergosterol	2,3-Dimethylbutyric acid	Nuclear fragment
Precursor			
[2- ¹⁴ C]Acetate	0.48 ± 0.08	1.21 ± 0.17	0.41 ± 0.11
Predicted for 'acetyl-CoA' pathway 1	0.5	1.0	0.33
[2- ¹⁴ C]Malonyl-CoA	0.49 ± 0.11	1.19 ± 0.15	0.39 ± 0.12
Predicted for 'malonyl-CoA' pathway 2	1/0	1/0	1/0

Table 7. *Effect of disrupted mitochondria on the formation of mevalonate by rat liver supernatant*

The saturated $(\text{NH}_4)_2\text{SO}_4$ fraction and the microsomal preparation were obtained from fractions of a rat liver homogenate prepared by the procedure of de Duve *et al.* (1955). Incubations contained 10mg of soluble protein [40–80% saturated $(\text{NH}_4)_2\text{SO}_4$ step], microsomal protein (3 mg), CoA (0.01 mM), ATP (0.4 mM), MgCl_2 (1.0 mM), GSH (4 mM), potassium phosphate buffer, pH 7.0 (100 mM), NADP^+ (0.16 mM), NAD^+ (0.12 mM), glucose 1-phosphate (2.8 mM), potassium mevalonate (20 mM) and radioactive substrate (2 mM), in a total volume of 2.5 ml. The preparation of disrupted mitochondria added contained 2 mg of protein. The mixtures were incubated for 30 min at 30°C. The amounts of substrate incorporated are the mean of three determinations.

	Malonyl-CoA decarboxylase activity (nmol/min per mg of protein)	[2- ^{14}C]Acetate incorporated into mevalonate (nmol)	[2- ^{14}C]Malonyl-CoA incorporated into mevalonate (nmol)
$(\text{NH}_4)_2\text{SO}_4$ (40–80% saturated) + microsomal preparation	0.02	35.2	14.3
As above but with disrupted mitochondria	1.25	36.2	36.4

addition of sonically disrupted mitochondria to such a system forming mevalonate is shown in Table 7. Whereas the incorporation of [2- ^{14}C]malonyl-CoA is markedly stimulated by the addition of this material, that of acetyl-CoA is unaffected.

The incorporation of [2- ^{14}C]malonyl-CoA observed in the absence of added malonyl-CoA decarboxylase was somewhat greater than expected from the endogenous activity of malonyl-CoA decarboxylase assayed. Since the total incorporation observed was only 0.3% of the substrate present it is probable that the additional incorporation arose from a small amount of [2- ^{14}C]acetyl-CoA present in the [2- ^{14}C]malonyl-CoA preparation.

Discussion

The initial experiments showed that malonyl-CoA was incorporated into isoprenoids via mevalonate in a number of tissues, but the evidence for the obligatory carboxylation of acetyl-CoA was not convincing. Although avidin had a marked inhibitory effect on the biosynthesis of fatty acids from acetate no inhibition of isoprenoid biosynthesis could be detected nor was there any evidence of an increased biosynthesis of isoprenoids arising from an acetyl-CoA-sparing effect, which might operate when fatty acid biosynthesis was blocked. Fletcher & Myant (1960) have obtained similar results by comparing the biosynthesis of fatty acids and cholesterol from [1- ^{14}C]acetate by rat liver preparations in the presence and absence of avidin. In contrast, Bloomfield & Bloch (1960) reported that addition of biotin to an incubation mixture containing biotin-deficient yeast cells caused stimulation of fatty acid biosynthesis that was accompanied by a marked decrease in the [1- ^{14}C]acetate incorporated into the total non-saponifiable lipid. Abraham *et al.* (1961) have also shown that

avidin stimulated the formation of acetoacetate from [1- ^{14}C]acetate when fatty acid synthesis was inhibited in rat liver and mammary gland preparations. On the other hand, the present results clearly show that inhibition of fatty acid synthesis by removal of CO_2 resulted in increased isoprenoid formation from acetate and acetyl-CoA, as was originally observed by Klein (1957). The inhibition of isoprenoid biosynthesis by unpurified commercial preparations of avidin observed by Foster & Bloom (1963) and by Weeks & Kekwick (1965) seems to be due to contaminants in these preparations.

If malonyl-CoA was decarboxylated to acetyl-CoA before incorporation into isoprenoids addition of unlabelled acetyl-CoA to incubation mixtures containing [2- ^{14}C]malonyl-CoA would be expected to decrease the observed incorporation. In crude systems the effect of acetyl-CoA on such incubations was shown to depend upon its concentration; at high concentrations of acetyl-CoA the incorporation of [2- ^{14}C]malonyl-CoA into both fatty acids and unsaponifiable lipid was stimulated, whereas at low concentrations the incorporation of [2- ^{14}C]malonyl-CoA into isoprenoids was inhibited and the incorporation into fatty acids was unaffected. These results may possibly explain why Fimognari & Rodwell (1970) observed that acetyl-CoA did not decrease the incorporation of [2- ^{14}C]malonyl-CoA into mevalonate by a rat liver preparation, but these workers did report that a five- to ten-fold molar excess of acetyl-CoA caused no inhibition, which is in contrast to the present results.

The labelling pattern of β -hydroxy- β -methylglutaryl-CoA, formed both by rat liver preparations from [2- ^{14}C] and [1,3- ^{14}C]malonyl-CoA and by pigeon liver preparations from [2- ^{14}C]malonyl-CoA, is consistent with pathway 1 of Scheme 1, involving the decarboxylation of malonyl-CoA to acetyl-CoA before incorporation. These results are clearly at

variance with the results of Brodie *et al.* (1963). To obtain any appreciable incorporation of [^{14}C]-malonyl-CoA into β -hydroxy- β -methylglutaryl-CoA we had to use a much less purified sample of pigeon liver fatty acid synthetase than was used by Brodie *et al.* (1963), but this in itself suggests that purified pigeon liver fatty acid synthetase does not form β -hydroxy- β -methylglutaryl-CoA. It is therefore improbable that the enzyme-bound intermediates described by Brodie *et al.* (1964) are of any importance in the biosynthesis of β -hydroxy- β -methylglutaryl-CoA. More direct evidence about this has been produced by Rudney *et al.* (1966), who showed that the CoA of substrate acetoacetyl-CoA remained bound to β -hydroxy- β -methylglutaryl-CoA when this substance was formed.

The relative incorporation of [$2\text{-}^{14}\text{C}$]malonyl-CoA into the carboxyl and methyl carbons of the acetic acid obtained from the side chain and nuclear fragments by Kuhn-Roth oxidation of ergosterol synthesized by yeast is in very close agreement with the results of similar experiments by Bu'Lock *et al.* (1962) on the ergosterol formed from diethyl[$2\text{-}^{14}\text{C}$]malonate by cultures of *Penicillium urticae*. It is particularly remarkable that these workers note that the overall amount of incorporation of diethyl[$2\text{-}^{14}\text{C}$]malonate was consistent with the amount of decarboxylation taking place in the cultures, deduced from the labelling of oleic acid and palmitic acid and of 6-methylsalicylic acid formed simultaneously in the cultures.

Although the present results appear to preclude the direct incorporation of malonyl-CoA into isoprenoids as outlined in pathway 2 of Scheme 1, it is of course possible, as noted by Bu'Lock *et al.* (1962), that the labelled C_2 units may have been derived from malonate without prior decarboxylation to acetyl-CoA. There appears to be, however, no direct experimental evidence for such a pathway.

If, as now appears, malonyl-CoA and β -hydroxy- β -methylglutaryl-CoA are concerned with quite distinct pathways, it also follows that the principal control points of isoprenoid and fatty acid biosynthesis do not overlap. The carboxylation of acetyl-CoA to malonyl-CoA is an important rate-determining step in fatty acid biosynthesis (Ganguly, 1960); cholesterol biosynthesis is subject to end-product regulation at the stage of the reduction of β -hydroxy- β -methylglutaryl-CoA to mevalonate (Siperstein & Fagan, 1966; White & Rudney, 1970) and also probably at the stage of the formation of β -hydroxy- β -methylglutaryl-CoA from acetoacetyl-CoA and acetyl-CoA (White & Rudney, 1970; Shepherd & Booth, 1971).

We wish to express our gratitude to the Science Research Council for financial support.

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