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An Enzyme-Bound Intermediate in the Biosynthesis of 3-Hydroxy-3-methylglutaryl-Coenzyme A

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1. Purified 3-hydroxy-3-methylglutaryl-CoA synthase from baker's yeast (free from acetoacetyl-CoA thiolase activity) catalysed an exchange of acetyl moiety between 3′-dephospho-CoA and CoA. The exchange rate was comparable with the overall velocity of synthesis of 3-hydroxy-3-methylglutaryl-CoA. 2. Acetyl-CoA reacted with the synthase, giving a rapid 'burst' release of CoA proportional in amount to the quantity of enzyme present. The 'burst' of CoA was released from acetyl-CoA, propionyl-CoA and succinyl-CoA (3-carboxypropionyl-CoA) but not from acetoacetyl-CoA, hexanoyl-CoA, DL-3-hydroxy-3-methylglutaryl-CoA, or other derivatives of glutaryl-CoA. 3. Incubation of 3-hydroxy-3-methylglutaryl-CoA synthase with [1-^{14}C]acetyl-CoA yielded protein-bound acetyl groups. The $K_{eq.}$ for the acetylation was 1.2 at pH7.0 and 4°C. Acetyl-labelled synthase was isolated free from [1-^{14}C]acetyl-CoA by rapid gel filtration at pH6.1. The [1-^{14}C]acetyl group was removed from the protein by treatment with hydroxylamine, CoA or acetoacetyl-CoA but not by acid. When CoA or acetoacetyl-CoA was present the radioactive product was [1-^{14}C]acetyl-CoA or 3-hydroxy-3-methyl-[^{14}C]glutaryl-CoA respectively. 4. The isolated [1-^{14}C]acetyl-enzyme was slowly hydrolysed at pH6.1 and 4°C with a first-order rate constant of 0.005min^{-1}. This rate could be stimulated either by raising the pH to 7.0 or by the addition of desulpho-CoA. 5. These properties are interpreted in terms of a mechanism in which 3-hydroxy-3-methylglutaryl-CoA synthase is acetylated by acetyl-CoA to give a stable acetyl-enzyme, which then condenses with acetoacetyl-CoA yielding a covalent derivative between 3-hydroxy-3-methylglutaryl-CoA and the enzyme which is then rapidly hydrolysed to free enzyme and product.

Previous studies into the mechanism of action of 3-hydroxy-3-methylglutaryl-CoA from baker's yeast (Stewart & Rudney, 1966*a*,*b*; Middleton, 1972) suggested that an acetyl derivative of the enzyme was a normal intermediate in the biosynthesis of 3-hydroxy-3-methylglutaryl-CoA. However, direct attempts to isolate this intermediate (Stewart & Rudney, 1966*b*) were complicated by contamination of the synthase with acetoacetyl-CoA thiolase, an enzyme which Gehring *et al.* (1968) have shown will also form an acetyl derivative. The aim of the present investigation was to obtain direct evidence for the participation of an acetyl derivative of the synthase, by using a purified preparation free from acetoacetyl-CoA thiolase activity.

Experimental

Materials

Phosphate acetyltransferase (EC 2.3.1.8), CoA, NAD^+ and acetyl phosphate were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. Acid phosphatase (EC 3.1.3.2) from wheat germ was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. [1-^{14}C]Acetic anhydride (45mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 2-Oxoglutaric acid was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., and was recrystallized twice from ethyl propionate before use. DEAE-cellulose (Whatman DE 11) and glass-fibre discs (Whatman GF/C) were from W. and R. Balston Ltd., Springfield Mill, Maidstone, Kent, U.K. Cellulose ester membrane filters were obtained from the Oxoid Division of Oxo Ltd., London S.E.1, U.K. Sephadex G-25 (medium grade) was purchased from Pharmacia, Uppsala, Sweden.

Methods

Preparation and assay of substrates. Acetyl-, bromoacetyl-, propionyl- and acetoacetyl-CoA were prepared and assayed as described by Middleton & Tubbs (1972). DL-3-Hydroxy-3-methylglutaryl-, DL-3-methylglutaryl-, 3,3′-dimethylglutaryl- and glu-

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taryl-CoA were prepared and assayed as described by Middleton (1972). Desulpho-CoA was prepared by the method of Chase *et al.* (1966) and assayed by its E_{260}, by using the extinction coefficient for adenine nucleotides of 16.4×10^3 litre·mol^{-1}·cm^{-1} (Stadtman, 1957). Acetyl-3′-dephospho-CoA was prepared from 3′-dephospho-CoA by reaction with acetic anhydride as for acetyl-CoA (Simon & Shemin, 1953). The 3′-dephospho-CoA was made from CoA by the removal of the 3′-phosphate group by the method of Brown (1963). After acetylation of the crude 3′-dephospho-CoA, it was purified and separated from acetyl-CoA and other derivatives on a column of DEAE-cellulose by the method of Moffatt & Khorana (1961). Acetyl-CoA and acetyl-3′-dephospho-CoA were determined in solutions containing both by a method based on an assay for acetyl-CoA (Stadtman, 1952). In this method measurement is made of the decrease in E_{232} associated with the hydrolysis of acetyl-CoA and acetyl-3′-dephospho-CoA brought about by phosphate acetyltransferase in the presence of arsenate. It was found that small amounts (0.25mg/ml) of phosphate acetyltransferase will only utilize acetyl-CoA as substrate but that larger amounts of the enzyme (6.5mg/ml) will use acetyl-3′-dephospho-CoA as well. This is in accord with the findings of Michal & Bergmeyer (1963) for CoA and 3′-dephospho-CoA. A 1cm light-path silica cuvette contained 600μmol of potassium arsenate buffer, pH7.6, up to 0.3μmol of potassium arsenate buffer, pH7.6, up to 0.3μmol of acetyl- and acetyl-3′-dephospho-CoA and water to 2.0ml. The decrease in E_{232} on addition of 0.5μg of phosphate acetyltransferase was a measure of the acetyl-CoA content. When there was no further change in absorbance a further 13μg of the enzyme was added. This caused a further decrease in absorbance, which was a measure of the acetyl-3′-dephospho-CoA. The concentrations of acetyl-CoA and acetyl-3′-dephospho-CoA were determined from the absorbance changes by using the fact that the difference between the molar extinction coefficients of acetyl-CoA (and acetyl-3′-dephospho-CoA) and its hydrolysis products at 232nm is 4.5×10^3 litre·mol^{-1}·cm^{-1} (Stadtman, 1957). [1-^{14}C]Acetyl-CoA was prepared from CoA and [1-^{14}C]acetic anhydride by the method of Simon & Shemin (1953) except that the anhydride was first dissolved in dry ether. The product was purified by chromatography on DEAE-cellulose by the method of Moffatt & Khorana (1961).

Preparation and chromatography of hydroxamates. Acetyl- and DL-3-hydroxy-3-methylglutaryl-hydroxamate were prepared by the reaction of the respective anhydride with neutral hydroxylamine. Chromatography of radioactive hydroxamates was carried out on Whatman no. 1 paper in the ascending mode. Controls of acetyl- and DL-3-hydroxy-3-methylglutarylhydroxamate were also run. The solvent system was a mixture of propan-1-ol and $(NH_4)_2CO_3$ in the ratio 2:1 (v/v), the latter consisting of 2 parts of aq. 10% (w/v) $(NH_4)_2CO_3$ and one part of 5M-NH_3. The paper was run for 18h at room temperature and then dried and the control hydroxamates were detected by spraying with $FeCl_3$ [10% (w/v) in ethanol]. The paper containing the radioactive material was cut into 1cm × 3cm strips at right angles to the direction of flow from the point of application up to the solvent front. These strips were then placed in 4ml of toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene and counted for radioactivity in a Nuclear–Chicago Unilux liquid-scintillation counter.

Determination of [1-^{14}C]acetyl moiety bound to protein. Acetylated protein was prepared by incubating an excess of [1-^{14}C]acetyl-CoA with purified 3-hydroxy-3-methylglutaryl-CoA synthase (0.6–3mg) in 1.0ml of 0.1M-potassium phosphate buffer, pH7.0, at 4°C for 1min. The incubation mixture was then either immediately deproteinized with trichloroacetic acid (final concn. 5%, w/v) or was first passed through a column of Sephadex G-25 (20cm × 2cm diam., equilibrated with 0.1M-potassium phosphate, pH6.1 at 4°C) and subsequently collected in 2ml fractions and 0.1–0.5ml samples were deproteinized. The precipitated protein was collected on cellulose ester membranes or glass-fibre discs, washed with cold 1% (w/v) trichloroacetic acid, dried and counted for radioactivity in a Nuclear–Chicago gas-flow counter. In the case of the gel-filtered material the radioactivity released during hydrolysis of the acetyl-protein was determined by extracting the acid washings three times with ether, then back-extracting into 0.1M-NaOH and counting the radioactivity of 0.2ml of the neutralized aqueous fraction in 3ml of a solution prepared from 1 part of Triton X-100 and 3 parts of toluene and containing 0.5% (w/v) 2,5-diphenyloxazole.

Purification of enzymes. 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was purified by the method of Hayakawa *et al.* (1964) up to a specific activity of 4units/mg. 3-Hydroxy-3-methylglutaryl-CoA synthase was purified and assayed by the method of Middleton & Tubbs (1972). The final specific activity was 2units/mg. The preparation had a maximum acetoacetyl-CoA thiolase activity of 0.005unit/mg.

Determination of CoA. CoA and the stoicheiometric 'burst' release of CoA (from acetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA synthase) were determined by the following modification of the fluorimetric assay of Garland *et al.* (1965). In a 1.0ml total volume a 1cm path-length fluorescence cell contained at 25°C: potassium phosphate, pH6.4, 100μmol; $MgCl_2$, 1μmol; EDTA, 0.5μmol; NAD^+, 0.37μmol; 2-oxoglutarate, 1μmol; 2-oxoglutarate

dehydrogenase, 35μg. The assay was carried out in an Aminco–Bowman spectrofluorimeter, the NADH fluorescence being excited by light at 340nm and the fluorescence measured at 460nm and recorded on a Bryans high-speed recorder. Under the conditions of the assay 1nmol of CoA gave a fluorescence increase of 5.2cm (2.05in) with a 'noise' level equivalent to 0.02nmol of acetyl-CoA. No acyl-CoA deacylase activity was detectable in the oxoglutarate dehydrogenase preparation.

Protein determination. Both the biuret (Gornall *et al.*, 1949) (with crystalline bovine serum albumin as a standard) and the direct spectrophotometric method (Warburg & Christian, 1941) were used.

Units of enzyme activity. A unit of enzyme activity transforms 1μmol of substrate into product/min under the conditions quoted.

Results

Acetyl-3'-dephospho-CoA/CoA exchange

This is shown in Fig. 1. In the presence of purified 3-hydroxy-3-methylglutaryl-CoA, acetyl-CoA was synthesized from acetyl-3'-dephospho-CoA and CoA at a rate proportional to the amount of enzyme present (Fig. 1*b*). The exchange did not proceed to the theoretical limit, perhaps owing to the presence of an acetyl-CoA hydrolase activity in the synthase preparation. The initial exchange rate under the conditions of Fig. 1 was 22nmol of acetyl moiety transferred/min per unit of synthase. The rate of the enzyme-catalysed condensation of the same concentration of acetyl-3'-dephospho-CoA under the standard assay conditions was 23nmol/min per unit of synthase.

Stoicheiometric release of CoA

The extremely sensitive fluorimetric assay for CoA, using 2-oxoglutarate dehydrogenase (Garland *et al.*, 1965), was used to detect CoA release when substrate quantities of purified 3-hydroxy-3-methylglutaryl-CoA synthase were mixed with acetyl-CoA. To minimize acetyl-CoA hydrolase activity and yet maintain high oxoglutarate dehydrogenase activity the experiments were carried out at pH6.4. In the absence of the synthase and also in its presence (but see below) the system gave accurate increments of NADH fluorescence for quantities of CoA down to 0.1nmol. When CoA-free acetyl-CoA was added in the absence of synthase no significant response was detected, but in the presence of the synthase a rapid 'burst' of CoA (measured as NADH increment) was observed (Fig. 2), followed by a slower, steady-state release of CoA. After the occurrence of the 'burst', further addition of acetyl-CoA caused no more rapid increments in fluorescence. The 'burst' was proportional to the amount of synthase present (Fig. 3) and did not occur if heat-inactivated synthase was used. This rapid stoicheiometric release of CoA was interpreted as indicating the acetylation of the synthase by acetyl-CoA. In accord with the proposed mechanism of action the synthase gave no rapid 'burst' of CoA from acetoacetyl-CoA (the second substrate) or 3-hydroxy-3-methylglutaryl-CoA (the product). However, it was of interest to find a 'burst' when the synthase reacted with succinyl-CoA (3-carboxypropionyl-CoA) or propionyl-CoA (Fig. 4), because neither of these are substrates. Longer-chain analogues of propionyl-CoA (such as hexanoyl-CoA) or of succinyl-CoA (such as glutaryl-CoA and its 3-

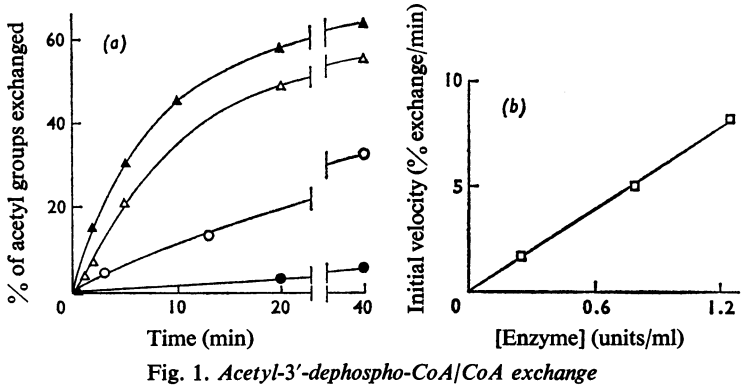


Fig. 1. *Acetyl-3'-dephospho-CoA/CoA exchange*

(*a*) Time-course of the exchange. Acetyl-3'-dephospho-CoA (1μmol) and CoA (0.5μmol) were incubated in 0.5ml of 0.1-M Tris–HCl, pH8.2 at 30°C in the presence of the following amounts of purified enzyme (2units/mg): ●, none; ○, 0.25 unit; △, 0.8unit; ▲, 1.25 units. The exchange was started by the addition of the acetyl-3'-dephospho-CoA. (*b*) Dependence of the initial rate of exchange on enzyme concentration. Conditions were as above. For further details see the Experimental section.

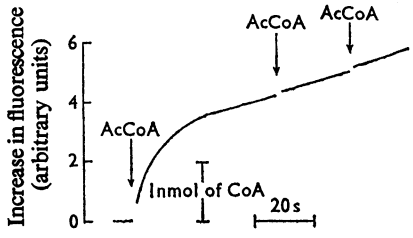


Fig. 2. *Synthase-catalysed 'burst' release of CoA from acetyl-CoA*

Recorder trace of the fluorescence of NADH at 460nm emitted by a 1ml assay system (see the Experimental section) containing 0.42mg of purified 3-hydroxy-3-methylglutaryl-CoA synthase (2units/mg). Acetyl-CoA (3.9nmol batches) was added as indicated. The following abbreviations are used here and in Fig. 4: AcCoA, acetyl-CoA; SuccCoA, succinyl-CoA; AcacCoA, acetoacetyl-CoA; HMGCoA, DL-3-hydroxy-3-methylglutaryl-CoA.

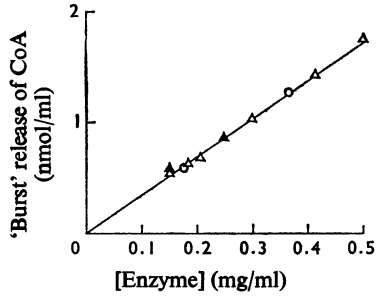


Fig. 3. *Relation between CoA 'burst' and synthase concentration*

'Bursts' of CoA were determined at different concentrations of 3-hydroxy-3-methylglutaryl-CoA synthase by using various acyl-CoA compounds: △, acetyl-CoA; ○, propionyl-CoA; ▲, succinyl-CoA. For details of the assay see the Experimental section.

methyl-substituted derivatives) gave no reaction. The magnitudes of the 'bursts' with propionyl-CoA and succinyl-CoA were equal to that obtained with acetyl-CoA (1mol of CoA released from acyl-CoA by 295000g of protein; Fig. 3) and preparations that had previously reacted with succinyl-CoA did not give another 'burst' when mixed with acetyl-CoA. However, the 'burst' of CoA from propionyl-CoA was demonstrably slower than the reactions with succinyl- and acetyl-CoA (Fig. 4).

The discovery that succinyl-CoA can acylate the synthase (thus releasing the 'burst' of CoA) explained a peculiar result shown in Fig. 5: the increment in fluorescence resulting from the first addition of CoA

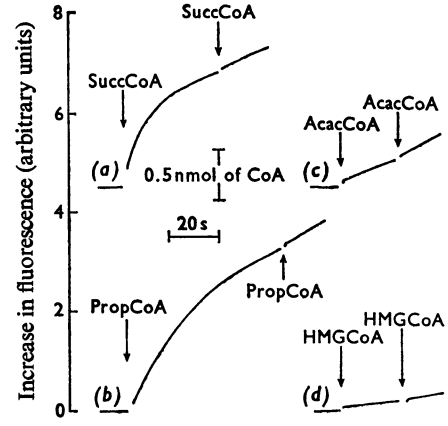


Fig. 4. *Stoicheiometric CoA release from various acyl-CoA compounds*

Recorder traces of the fluorescence of NADH at 460nm emitted by 1ml assay systems, as described in Fig. 2, containing: (*a*) 0.15mg of synthase with additions, as indicated, of succinyl-CoA (4nmol batches), (*b*) 0.18mg of synthase with additions, as indicated, of propionyl-CoA (PropCoA) (4.2nmol batches), (*c*) 0.18mg of synthase with additions, as indicated, of acetoacetyl-CoA (6nmol batches) and of DL-3-hydroxy-3-methylglutaryl-CoA (5.8nmol batches).

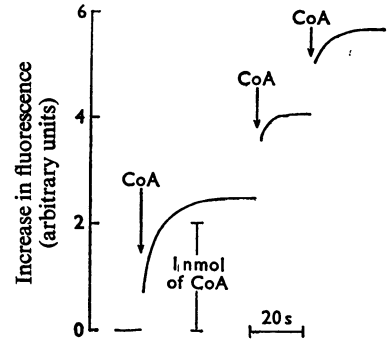


Fig. 5. *Assay response to CoA in the presence of synthase*

Recorder trace of the fluorescence of NADH at 460nm emitted by a 1ml assay system, as described in Fig. 2, containing 0.19mg of purified 3-hydroxy-3-methylglutaryl-CoA synthase. The additions of CoA (0.8nmol batches) were as indicated.

to a system containing synthase was significantly greater than subsequent increments, all of which were now identical with those obtained in a system lacking the synthase. The difference between the first and subsequent fluorescence changes is, under the conditions of Fig. 5, equivalent to 79% of the 'burst'

that would have occurred had acetyl- or succinyl-CoA been added instead of CoA. It appears therefore that the succinyl-CoA that is produced from the reaction of CoA with the oxoglutarate dehydrogenase can go on to react with the synthase yielding some succinyl-enzyme plus further CoA:

$$CoA + NAD^+ + 2\text{-oxoglutarate} \rightarrow NADH + CO_2 + \text{succinyl-CoA}$$

$$\text{Succinyl-CoA} + \text{synthase} \rightarrow \text{succinyl-synthase} + CoA$$

The process would presumably continue until all the synthase was succinylated. Thus it was extremely important that the concentration of contaminating CoA in the thiol esters used in this experiment should be low compared with the amount of synthase present.

Titration of 3-hydroxy-3-methylglutaryl-CoA synthase with [1-^{14}C]acetyl-CoA

Portions of purified synthase were incubated with a range of concentrations of [1-^{14}C]acetyl-CoA at pH7.0, 4°C. The reaction was terminated after 1min by the addition of trichloroacetic acid, and the precipitated protein collected on membrane filters, washed and counted for bound radioactivity. The results in Fig. 6 show that the bound radioactivity increased with increasing amounts of [1-^{14}C]acetyl-CoA until a plateau value was reached, which was proportional to the amount of protein present in the incubation. One mol of [1-^{14}C]acetyl group was bound for every 285000g of protein present, a value very close to the amount of CoA released during the acetylation (see the previous section). Pretreating the enzyme with sufficient bromoacetyl-CoA to inactivate the synthase totally abolished the ability to bind [1-^{14}C]acetyl groups. Since bromoacetyl-CoA is a potent active-site-directed inhibitor of the synthase (Middleton & Tubbs, 1972) this result confirms that the acetylation being observed involves the synthase and is a specific reaction. The approximate equilibrium constant for the acetylation was determined from the data of Fig. 6 to be 1.2.

Isolation and characterization of acetyl-synthase

As shown in Fig. 7, preincubation of synthase with [1-^{14}C]acetyl-CoA followed by gel filtration at 4°C into 0.1M-potassium phosphate, pH6.1, gave a peak of radioactivity which emerged coincidentally with the enzyme activity and in front of the main peak of low-molecular-weight radioactivity. Throughout this first peak the ratio of radioactivity to enzyme activity was constant. The radioactivity in this protein peak was virtually completely precipitated by trichloroacetic acid, but pretreatment with neutral 1M-hydroxylamine at 4°C caused the release of most of the radioactivity from the protein. Similarly, pretreatment of the radioactive protein peak with

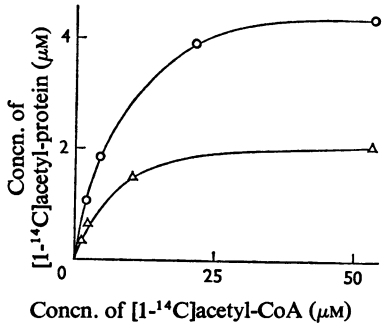


Fig. 6. *Titration of synthase with [1-^{14}C]acetyl-CoA*

Purified synthase (△, 0.6mg/ml; ○, 1.2mg/ml) was incubated at 4°C, pH7.0, for 1min with increasing concentrations of [1-^{14}C]acetyl-CoA. The reaction was terminated with cold trichloroacetic acid and the protein-bound radioactivity determined. For full details see the Experimental section.

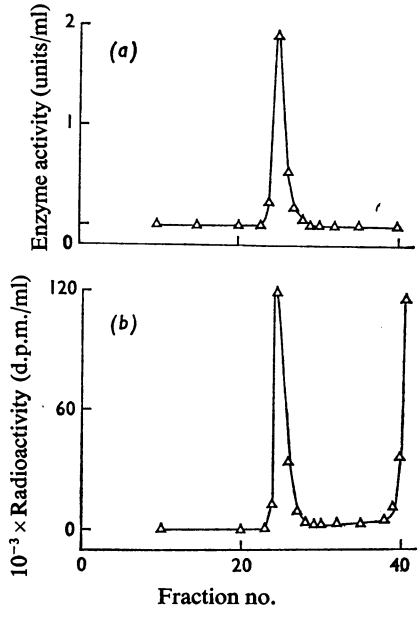


Fig. 7. *Isolation of [^{14}C]acetyl-labelled synthase*

Purified synthase (2.5mg) was incubated with [1-^{14}C]-acetyl-CoA (85nmol), and then rapidly gel-filtered on Sephadex G-25 into 0.1M-potassium phosphate, pH6.1, at 4°C. Fractions (2ml) were collected and assayed for (*a*) synthase activity and (*b*) total radioactivity.

CoA or acetoacetyl-CoA released most of the protein-bound radioactivity. These results are presented in Table 1. The supernatants resulting from [1-^{14}C]-acetyl-protein pretreatment with CoA and with acetoacetyl-CoA were kept for characterization of the released radioactive compound. The trichloroacetic acid was first removed by ether extraction, then 0.5μmol of acetyl- and DL-3-hydroxy-3-methylglutaryl-CoA were added to each supernatant. The fractions were then converted into hydroxamates by treatment with neutral 1M-hydroxylamine for 30min. After freeze-drying, the hydroxamates were extracted with ethanol, concentrated in a stream of air and chromatographed on Whatman no. 1 paper together with non-radioactive samples of acetyl- and 3-hydroxy-3-methylglutarylhydroxamates. After chromatography the marker hydroxamates were detected with a $FeCl_3$ spray, and the lengths of paper in which the radioactive material had run were cut into 1cm × 3cm strips and counted for radioactivity. The results, shown in Fig. 8, indicate that the supernatant from

Table 1. *Properties of [^{14}C]acetyl-labelled synthase*

Purified synthase (1.0mg) was incubated with [1-^{14}C]-acetyl-CoA (10nml) and then rapidly gel-filtered on Sephadex G-25 into 0.1M-potassium phosphate, pH6.1, at 4°C. Fractions containing enzyme activity were pooled and their radioactivity was determined. Protein-bound radioactivity was determined after collection of acid-precipitated protein on membrane filters. A sample of the column eluate (1ml) was pretreated with neutral hydroxylamine for 30min at 4°C before determination of protein-bound radioactivity. Two more samples each containing 35000d.p.m. were pretreated with CoA or acetoacetyl-CoA for 10min at 4°C before acid precipitation and counting of the protein-bound radioactivity. In these latter two cases the deproteinized extracts were retained for further experiments (see Fig. 8).

Sample	Radioactivity found (d.p.m./ml of column eluate)
Column eluate	5760
Acid-precipitated protein from column eluate	5200
Acid-precipitated protein from column eluate after treatment with:	
Neutral hydroxylamine (1M)	296
CoA (1.35mM)	357
Acetoacetyl-CoA (75μM)	290

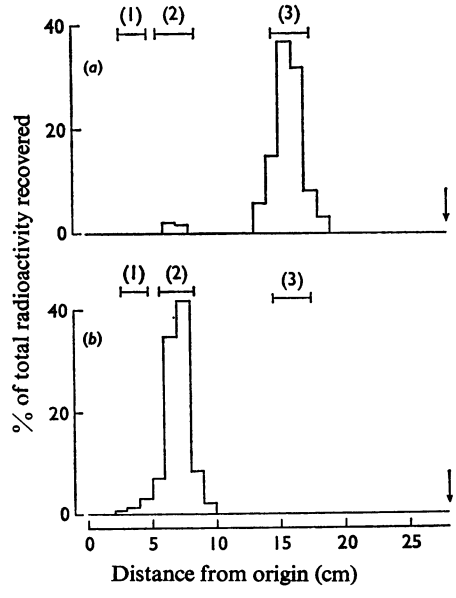


Fig. 8. *Identification of the products derived from treatment of [^{14}C]acetyl-enzyme with CoA or acetoacetyl-CoA*

[^{14}C]Acetyl-enzyme, isolated as described in Fig. 7, was treated with CoA or acetoacetyl-CoA (see Table 1 for details), deproteinized and mixed with neutral hydroxylamine. After freeze-drying the hydroxamates were extracted into ethanol and chromatographed on paper as described in the Experimental section. The distribution of radioactivity on the paper is plotted as a function of the distance from the origin. The solvent front is indicated by an arrow. The positions of marker hydroxamates are shown by the numbered horizontal bars: 1, the compound resulting from reaction between acetoacetyl-CoA and neutral hydroxylamine. 2, DL-3-hydroxy-3-methylglutarylhydroxamate; 3, acetylhydroxamate; (*a*) After pretreatment with CoA; (*b*) after pretreatment with acetoacetyl-CoA.

the CoA pretreatment now corresponds chromatographically to [^{14}C]acetylhydroxamate, whereas that from the acetoacetyl-CoA pretreatment chromatographs as 3-hydroxy-3-methyl[^{14}C]glutarylhydroxamate. The recoveries of total radioactivity were 67 and 69% respectively. Thus the [^{14}C]acetyl-protein isolated on Sephadex G-25 behaves as the acetyl derivative of 3-hydroxy-3-methylglutaryl-CoA synthase, carrying out the following partial reactions:

$$CH_3\text{–}CO\text{–}X\text{–}E + CoA \rightarrow CH_3\text{–}CO\text{–}SCoA + E \quad (1)$$

$$CH_3\text{–}CO\text{–}X\text{–}E + CH_3\text{–}CO\text{–}CH_2\text{–}CO\text{–}SCoA + H_2O \rightarrow CH_3\text{–}C(CH_2\text{–}COSCoA)(CH_2\text{–}CO_2H)\text{–}OH + E \quad (2)$$

where X– is the group at the enzyme-active centre that is acetylated.

Factors affecting the stability of isolated acetyl-synthase

[^{14}C]Acetyl-synthase, prepared as described above, shows a slow first-order loss of radioactivity (Fig. 9) at a rate of 0.005min^{-1} at 4°C and pH6.1. When acid-denatured [in 1.5% (w/v) trichloroacetic acid] the hydrolysis rate is decreased to 0.0004min^{-1} at 0°C. At pH7 the hydrolysis rate of the native acetyl-enzyme was increased markedly but the loss of radioactivity was no longer first order (Fig. 9). The logarithmic curve could, however, be resolved into fast (0.05min^{-1}) and slow (0.003min^{-1}) first-order rate constants.

Surprisingly, a low concentration of the chemically unreactive CoA analogue, desulpho-CoA (Chase *et al.*, 1966), which contained no thiol group, caused a rapid release of radioactivity from the acetyl-enzyme at pH6.1 (Fig. 9). This action appeared to be a stimulation of hydrolysis, because the radioactivity released was now extractable into ether, a finding incompatible with the formation of any [^{14}C]acyl-CoA derivative. As with the increased hydrolysis rate caused by higher pH (see above), the loss of radioactivity was not now first order but could be resolved into a fast (0.3min^{-1}) and a slow (0.004min^{-1}) first-order rate constant. It is not known why procedures that stimulated hydrolysis produced biphasic logarithmic plots, but it was noted that the slower portion of the curve could always be extrapolated back to around 30% of the initial bound radioactivity. This could imply the presence of a 'crippled' species of acetyl-enzyme.

Discussion

On the basis of kinetic and other indirect experiments Stewart & Rudney (1966*a*,*b*) and Middleton & Tubbs (1972) proposed that the synthesis of 3-hydroxy-3-methylglutaryl-CoA proceeded via an acetyl-enzyme, as in eqns. (3) and (4):

$$E + CH_3\text{–}CO\text{–}SCoA \rightarrow CH_3\text{–}CO\text{–}X\text{–}E + CoA \quad (3)$$

$$CH_3\text{–}CO\text{–}X\text{–}E + CH_3\text{–}CO\text{–}CH_2\text{–}CO\text{–}SCoA + H_2O \rightarrow CH_3\text{–}\overset{\displaystyle CH_2\text{–}CO\text{–}SCoA}{\overset{|}{\underset{\displaystyle CH_2\text{–}CO_2H}{\underset{|}{C}}}}\text{–}OH + E \quad (4)$$

where X– is the group at the enzyme-active centre that is acetylated.

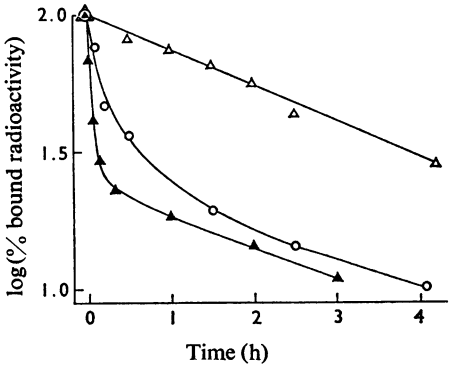


Fig. 9. *Stability of isolated* [^{14}*C*]*acetyl-enzyme*

[^{14}C]Acetyl-enzyme was isolated as described in Fig. 7. Samples containing about 1 mg of protein were used in the following experiments: △, incubated at 4°C, pH6.1; ○, rapidly adjusted to pH7.0 with strong KOH then incubated at 4°; ▲, desulpho-CoA added (at zero time) to a final concentration of 11.5μM, then the incubation carried out at 4°C, pH6.1. Zero-time samples (0.5ml, containing about 9000d.p.m.) were taken as soon as the additions were made and further samples were taken as indicated. Samples were pipetted into cold 5% (w/v) trichloroacetic acid, collected and washed on membrane filters and counted for radioactivity.

The occurrence of the first partial reaction (eq. 3) has now been confirmed by (*a*) the demonstration of the acetyl exchange between CoA moieties, (*b*) the stoicheiometric release of CoA from acetyl-CoA when substrate quantities of enzyme are mixed with acetyl-CoA, and (*c*) the isolation of [^{14}C]acetyl-enzyme and its conversion into free enzyme and [^{14}C]acetyl-CoA in the presence of CoA. Similarly, the identification of the acetyl-enzyme as acetyl-synthase has been shown by the synthesis of 3-hydroxy-3-methyl[^{14}C]glutaryl-CoA when isolated [^{14}C]acetyl-enzyme reacts with acetoacetyl-CoA as predicted by eqn. (4) above. Although the preparation of synthase used in this study was not homogeneous (Middleton & Tubbs, 1972) the above results with isolated [^{14}C]acetyl-enzyme, the close agreement between the stoicheiometric release of CoA and the amount of [^{14}C]acetyl group bound to the enzyme and the specificity found for the 'burst' release of CoA by the enzyme all substantially confirm the predicted mechanism of action of 3-hydroxy-3-methylglutaryl-CoA synthase.

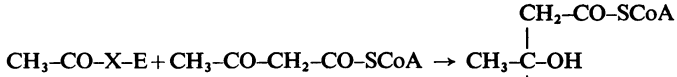
Although the nature of the ester bond involved in the linkage of acetyl moiety to the enzyme has not been investigated in detail, the extreme sensitivity of the unacetylated enzyme to denaturation by low concentrations of thiol-specific reagents (Middleton & Tubbs, 1972) suggests that it is a thiol ester. Further support for this view is given by the results

in Table 2. Here [1-^{14}C]acetyl-synthase, denatured by trichloroacetic acid and collected on a glass-fibre disc, lost all bound radioactivity after exposure to performic acid vapour. Performic acid oxidizes thiol esters to the corresponding sulphonic acids, releasing the carboxylic acid component:

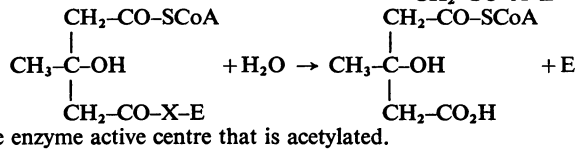
$$R\text{–}COS\text{–}R \xrightarrow{HCOOOH} R\text{–}CO_2H + R\text{–}SO_3H$$

Since acetic acid is volatile, if generated by this reaction it would be easily removed. Lynen (1967) has used this test to distinguish between thiol esters and oxygen esters involved in the linkage of acetyl units to fatty acid synthase. With the acetyl derivative of 3-hydroxy-3-methylglutaryl-CoA synthase the lability of the linkage to performic acid oxidation rules against involvement of an oxygen ester and suggests a thiol ester bond.

The surprising stimulation of hydrolysis of [^{14}C]-acetyl-synthase by desulpho-CoA suggests that the mere binding of a CoA moiety (even if incapable of chemical reaction) will give rise to a conformational change in the acetyl-enzyme leading to acceleration of hydrolysis. This behaviour appears analogous to that of acetyl-chymotrypsin, the hydrolysis of which is stimulated by the binding of the tryptophan analogue indole (Foster, 1961) and by the substrate *N*-acetyl-L-tyrosine ethyl ester (Bender *et al.*, 1964), both of which contain the aromatic grouping necessary for high chymotryptic activity. For the synthase the result implies that the enhancement of acetyl-enzyme hydrolysis may be a model of a possible last step (reaction 6, below) in the normal overall reaction: namely, the hydrolysis of an enzyme–3-hydroxy-3-methylglutaryl-CoA covalent intermediate:



(5)



(6)

where X– is the group at the enzyme active centre that is acetylated.

We therefore propose that the ester linkage present in the acetyl-enzyme is maintained until after the condensation with acetoacetyl-CoA and that the presence of the enzyme-bound CoA moiety is required before the rapid hydrolysis of the linkage can take place. Thus the complex formed between desulpho-CoA and acetyl-enzyme could resemble the proposed new intermediate (above) sufficiently to result in an increase in the rate of acetyl-enzyme hydrolysis. In an analogous experiment with the [^{32}P]phospho-enzyme derivative of ATP citrate lyase (EC 4.1.3.8) Das *et al.* (1971) observed that desulpho-CoA (in the absence of CoA) would cause the rapid release of bound radioactivity without participating chemically in the reaction.

Table 2. *Treatment of [^{14}C]acetyl-labelled synthase with performic acid vapour*

Purified synthase (0.3mg) was added to systems containing (*a*) 4nmol and (*b*) 100nmol of [1-^{14}C]acetyl-CoA/μl in 0.25ml of 0.1M-potassium phosphate, pH7.0, at 4°C. After 30s the reaction was stopped by 50μl of 20% (w/v) trichloroacetic acid. Duplicate 100μl samples were taken from systems (*a*) and (*b*), the protein was collected on glass-fibre paper discs (Whatman GF/C) and washed with cold 1% (w/v) trichloroacetic acid and dried. As controls, duplicate samples of [1-^{14}C]acetyl-CoA were dried on to two more discs of glass-fibre paper. One each of the radioactive-protein-containing duplicate discs (*a*) and (*b*) and one of the [1-^{14}C]acetyl-CoA-containing controls were exposed to performic acid vapour overnight at 20°C. All samples were then left over KOH pellets *in vacuo* for 18h to remove any volatile acids. Samples were then counted for radioactivity in a Nuclear–Chicago gas-flow counter.

Sample	[1-^{14}C]Acetyl moiety present in sample (pmol)	
	Untreated	Performic acid-treated
[1-^{14}C]Acetyl-CoA control	200	2.4
[1-^{14}C]Acetyl-enzyme (*a*)	250	1.0
[1-^{14}C]Acetyl-enzyme (*b*)	530	2.3

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