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The Purification and some Properties of 3-Hydroxy-3-methylglutaryl-Coenzyme A Synthase from Baker's Yeast

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1. A purification of 3-hydroxy-3-methylglutaryl-CoA synthase from baker's yeast is described. This yields a preparation of average specific activity 2.1 units (μmol/min)/mg in which contamination by acetoacetyl-CoA thiolase is less than 0.2%. 2. The molecular weights of 3-hydroxy-3-methylglutaryl-CoA synthase and acetoacetyl-CoA thiolase from baker's yeast were determined by gel filtration on Sephadex G-200. The values obtained were 130000 and 190000 respectively. 3. 3-Hydroxy-3-methylglutaryl-CoA synthase is susceptible to irreversible inhibition by a wide variety of alkylating and acylating agents. The time-course of inhibition of the enzyme by some of these, including the active-site-directed inhibitor bromoacetyl-CoA, was studied in the presence and absence of substrates, products and product analogues. Acetyl-CoA, even when present at concentrations as low as 5 μM, gives almost complete protection. Other acyl-CoA derivatives give some protection, but only at concentrations 10–30-fold higher. 4. These results are discussed with reference to an ordered reaction pathway in which acetyl-CoA reacts to give a covalent acetyl-enzyme intermediate.

3-Hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) catalyses the condensation between acetyl-CoA and acetoacetyl-CoA to give 3-hydroxy-3-methylglutaryl-CoA:

$$CH_3\text{–}CO\text{–}SCoA + CH_3\text{–}CO\text{–}CH_2\text{–}CO\text{–}SCoA + H_2O \rightarrow \begin{array}{l} CH_2\text{–}CO\text{–}SCoA \\ | \\ CH_3\text{–}C\text{–}OH \\ | \\ CH_2\text{–}CO_2H \end{array} + CoA \qquad (1)$$

This enzyme was discovered in baker's yeast and partially purified by Ferguson & Rudney (1959), who demonstrated the occurrence and stoicheiometry of the above reaction. Rudney (1959) showed that a probable role of this enzyme in yeast was to provide 3-hydroxy-3-methylglutaryl-CoA for the synthesis of isoprenes and steroids. Stewart & Rudney (1966*a*) succeeded in further purifying the yeast enzyme, but were unable to free the preparation from contaminating acetoacetyl-CoA thiolase activity without resorting to limited proteolysis with trypsin. This procedure, though effective in removing the thiolase activity, also partially inactivated the 3-hydroxy-3-methylglutaryl-CoA synthase and increased its K_m for acetyl-CoA 100-fold. This indicated a modification of the 3-hydroxy-3-methylglutaryl-CoA synthase molecule and rendered dubious the relevance of their subsequent experiments to the mechanism of action of the native enzyme.

The present work describes a method of purification of 3-hydroxy-3-methylglutaryl-CoA synthase from baker's yeast (*Saccharomyces cerevisiae*) that yields a highly purified enzyme free from thiolase activity. A brief report of some of this work has appeared (Middleton, 1967).

Materials and Methods

Materials

Enzymes and proteins. The following enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.: alcohol dehydrogenase (EC 1.1.1.1) (from yeast), catalase (EC 1.11.1.6) (from ox liver), citrate synthase (EC 4.1.3.7) (from pig heart), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (from pig heart) and lactate dehydrogenase (EC 1.1.1.27) (from rabbit muscle).

Crystalline bovine serum albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.

Soya-bean trypsin inhibitor was the crystalline product of the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Crystalline ovalbumin was a gift from Dr. D. S. Bendall.

Apoferritin, prepared by the method of Granick & Michaelis (1943), was a gift from Dr. D. K. Apps.

3-Hydroxy-3-methylglutaryl-CoA-cleavage enzyme (EC 4.1.3.4) (from ox liver) was prepared by the method of Middleton & Apps (1969).

Chemicals. CoA, NAD^+ and NADH were obtained from Boehringer Corp. (London) Ltd. Oxaloacetic acid was from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. 2-Mercaptoethanol was obtained from Eastman–Kodak Co., Rochester, N.Y., U.S.A. Dithiothreitol was obtained from Calbiochem (London) Ltd., London W.1, U.K. *N*-Ethylmaleimide and 3-hydroxy-3-methylglutaric acid were the products of Sigma Chemical Co., St. Louis, Mo., U.S.A. Hydroxymethylglutaric anhydride was prepared by the method of Hilz *et al.* (1958). 5,5′-Dithiobis-(2-nitrobenzoic acid) and diketen were obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

Phenylarsenious oxide was a gift from Sir Rudolph A. Peters, F. R. S. Other reagents were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

DEAE-cellulose (Whatman grade DE32) was obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Sephadex G-25 (medium grade), G-150 and G-200 were obtained from Pharmacia, Uppsala, Sweden; Sephadex G-150 and G-200 were allowed to swell for 1 week before packing into columns. To ensure even packing a well-stored slurry of gel was added very slowly to the top of a slow-running column, initially filled with buffer and containing a layer of ballotini beads over the sinter.

Calcium phosphate gel was prepared as described by Keilin & Hartree (1938).

Buffers. Tris (Trizma Base; Sigma Chemical Co.) was neutralized with HCl. KH_2PO_4 buffer was adjusted to the desired pH with KOH.

Methods

Preparation and assay of substrates. Acetyl-CoA, propionyl-CoA and DL-3-hydroxy-3-methylglutaryl-CoA were prepared by treating CoA with the appropriate acid anhydride (Simon & Shemin, 1953). Acetoacetyl-CoA was prepared by the method of Wieland & Rueff (1953). The reaction of CoA with diketen (freshly distilled under reduced pressure) was measured by the disappearance of thiol, assayed by the method of Ellman (1959). Bromoacetyl-CoA was prepared by the method of Chase & Tubbs (1969). Desulpho-CoA was prepared by the method of Chase *et al.* (1966).

Acetyl-CoA was assayed by the method of Chase (1967*a*). Bromoacetyl-CoA was determined by the method of Chase & Tubbs (1969). Propionyl-CoA was assayed by the method of Chase (1967*b*). Acetoacetyl-CoA was determined by the method of Decker (1963). DL-3-Hydroxy-3-methylglutaryl-CoA was determined by its E_{260} by using the extinction coefficient for adenine nucleotides of 16.4 litre·mol^{-1}·cm^{-1} (Stadtman, 1957). The naturally occurring isomer was determined by the method of Knappe (1957), but modified by the inclusion of 0.1 μmol of NADH to correct for non-stoicheiometry of the assay (Pearson, 1965). The 3-hydroxy-3-methylglutaryl-CoA cleavage enzyme used in this assay had a specific activity of 0.63 unit/mg.

Assay of 3-hydroxy-3-methylglutaryl-CoA synthase and acetoacetyl-CoA thiolase (EC 2.3.1.9). Both these enzymes use acetoacetyl-CoA as one substrate and their assay can be conveniently based on the extinction of the enol form of this thiol ester at 303 nm (Lynen, 1953). The apparent extinction coefficient of acetoacetyl-CoA was shown by Stern (1956) to be a function of the pH and the Mg^{2+} concentration of the medium. To achieve a reasonably high sensitivity in the assay of these enzymes, activity was measured in the presence of Mg^{2+}, although this has no direct effect on the enzymes concerned. In the standard assay a 1 cm light-path silica cuvette contained: 50 mM-tris chloride, pH 8.2, 20 mM-$MgCl_2$ and 0.016 mM-acetoacetyl-CoA in a 2.0 ml volume. To measure 3-hydroxy-3-methylglutaryl-CoA synthase activity the acetoacetyl-CoA hydrolysis rate was observed at 303 nm after addition of the enzyme; acetyl-CoA (0.043 mM) was then added, and the increase in acetoacetyl-CoA disappearance was taken to represent the 3-hydroxy-3-methylglutaryl-CoA synthase activity. To assay acetoacetyl-CoA thiolase an identical procedure was used but the system was completed by the addition of CoA (0.1 mM) instead of acetyl-CoA.

Under these conditons the apparent extinction coefficient for acetoacetyl-CoA was 20 litre·mol^{-1}·cm^{-1} at 303 nm.

All spectrophotometric determinations and initial-rate measurements were performed with a Beckmann DK-2 recording spectrophotometer at 30°C.

Protein determination. The biuret method was used (Gornall *et al.*, 1949). During the purification procedure the direct spectrophotometric method of Warburg & Christian (1941) was used to monitor protein content after the DEAE-cellulose step.

Units of enzyme activity. A unit of enzyme activity is defined as the amount of enzyme necessary to transform 1 μmol of substrate into product/min under the conditions quoted.

Results

Purification of 3-hydroxy-3-methylglutaryl-CoA synthase from baker's yeast

Autolysis. Fresh baker's yeast was crumbled into a stainless-steel beaker and toluene was added [110 ml/kg (50 ml/lb) of yeast]. The mixture was placed in a water bath at 55°C and stirred until the yeast was at 38°C (liquefaction occurred when the temperature was about 30°C). The yeast was then incubated at 38°C for 8–12 h. Within these limits the

yield and specific activity of the 3-hydroxy-3-methylglutaryl-CoA synthase remain approximately constant; further incubation caused losses.

An equal volume of cold glass-distilled water [on average 900ml/kg (410ml/lb) of yeast] was then added with stirring, and the cell debris was removed by centrifugation at 15000*g* for 20min to yield a light-yellow extract.

Ethanol fractionation. To the extract at room temperature (18–20°C), cold (−5°C) 96% ethanol was slowly added, with stirring, to a final concentration of 25% (v/v). The preparation was then stirred at room temperature for 2h. The copious white precipitate of denatured protein was then removed by centrifugation at 10000*g* for 20min at room temperature. The clear supernatant was cooled to −3°C in a cold bath and left at this temperature for 40min, with occasional stirring. The reddish-brown sticky precipitate was collected by centrifugation (2000*g* for 20min) at −3°C. It was dissolved in 0.01M-potassium phosphate buffer, pH7.8, to give a final volume of 100ml/kg (45ml/lb) of starting material. All subsequent steps were performed at 4°C.

Precipitation at pH5.6. Sodium acetate buffer, pH4.3 (2M), was added slowly, with stirring, to the cold ethanol fraction to adjust the pH to 5.6. The precipitate was collected and dissolved in 0.05M-potassium phosphate buffer, pH7.8.

DEAE-cellulose chromatography. The pH5.6 fraction was diluted with 0.01M-potassium phosphate buffer, pH7.8, to give a protein concentration of 30mg/ml. It was then applied to a column of DEAE-cellulose previously equilibrated with 0.01M-potassium phosphate buffer, pH7.8, containing 0.5mM-dithiothreitol. The packed volume of the column was 0.08ml/mg of protein applied. After a washing with 1 column volume of the above buffer the enzyme was eluted with 0.075M-potassium chloride in the buffer. Although the yield for this step was low, only a single peak of activity was found and higher salt concentrations failed to elute more enzyme.

Calcium phosphate-gel adsorption. The DEAE-cellulose eluate was immediately adjusted to pH6.8 with 1M-acetic acid, and calcium phosphate gel was added in the proportion 1.3mg/mg of protein present. After stirring for 5min the gel was collected at 2000*g* and the supernatant discarded. The enzyme was immediately eluted from the gel with 0.2M-potassium phosphate buffer, pH8.3, in two batches. It was important that this procedure was done swiftly, as the enzyme activity recoverable from the gel decreased rapidly with time. However, it was found subsequently that the presence of 20–30% (v/v) glycerol stabilizes the enzyme on the gel at this stage. After this step the enzyme could be stored for some weeks without loss of activity at −15°C in the presence of 25% (v/v) glycerol.

Gel filtration on Sephadex G-150. The preparation was first freed from glycerol by passage through a Sephadex G-25 column and then concentrated by rotary evaporation at 28°C. The enzyme was then loaded on a Sephadex G-150 column (3.7cm × 64cm) equilibrated with 0.02M-tris chloride buffer, pH7.5, containing 0.5mM-dithiothreitol and 10% (v/v) glycerol. Not more than 10ml of gel eluate was loaded in any one run. Fractions (10ml) were collected and those purified more than threefold by the procedure were pooled. The pooled eluate was then concentrated by rotary evaporation until the glycerol concentration was about 40–50% (v/v) and stored at −15°C. A typical purification starting from 9.1kg (20lb) of bakers yeast is summarized in Table 1. The enzyme at all stages lost activity on freezing and thawing and the purified enzyme also lost activity rapidly if stored at 4°C in the absence of glycerol. The presence of 10% (v/v) glycerol is necessary if the best yields are to be obtained from the gel-filtration step. The purified concentrated enzyme, if stored in 30% glycerol at −15°C in the presence of dithiothreitol, retained complete activity for periods of up to 3 months.

The 300-fold purified enzyme prepared by the method given above is a colourless protein with a specific activity of about 2.1 units/mg. The preparation had an acetoacetyl-CoA thiolase activity of

Table 1. *Purification of 3-hydroxy-3-methylglutaryl-CoA synthase from yeast*

This is a typical result starting from 9.1kg (20lb) of fresh baker's yeast. Experimental details are given in the text.

Procedure	Volume (ml)	Total activity (units)	Protein concn. (mg/ml)	Specific activity (units/mg)	Yield (%)
Centrifuged autolysate	12110	2900	37	0.007	(100)
Ethanol fractionation	950	2460	65	0.04	85
pH5.6 precipitation	210	2000	60	0.16	70
DEAE-cellulose column	2300	810	0.52	0.47	28
Calcium phosphate gel	40	470	16.5	0.7	16
Sephadex G-150 (after concentration)	18	350	9.2	2.1	12

0.004 unit/mg and an acetoacetyl-CoA hydrolase activity of 0.015 unit/mg. The preparation contained no detectable 3-hydroxy-3-methylglutaryl-CoA-cleavage enzyme activity, but had an acetyl-CoA hydrolase activity of 0.004 unit/mg at pH 8.2 and 30°C, in the presence of 1.0mM-acetyl-CoA. Thus, unlike the preparation used by Stewart & Rudney (1966*a*), this 3-hydroxy-3-methylglutaryl-CoA synthase, although not homogeneous, was virtually free from contaminating enzyme activities.

Properties of 3-hydroxy-3-methylglutaryl-CoA synthase purified from baker's yeast

Molecular-weight determination by gel filtration. The method of Andrews (1965) was used to obtain a value for the molecular weights of 3-hydroxy-3-methylglutaryl-CoA synthase and acetoacetyl-CoA synthase from baker's yeast. A sample from the fraction precipitated at pH 5.6 in the purification procedure for 3-hydroxy-3-methylglutaryl-CoA synthase containing these enzymes with specific activities of 0.16 and 0.02 unit/mg respectively was run through a calibrated Sephadex G-200 column; the location of the enzymes in the eluate was determined by the standard assays. The column (2cm×30cm) was equilibrated with 0.02M-tris chloride buffer, pH 7.2, containing 0.1M-KCl, and was calibrated with soya-bean trypsin inhibitor, ovalbumin, bovine serum albumin, lactate dehydrogenase (EC 1.1.1.27), alcohol dehydrogenase (EC 1.1.1.1), catalase (EC 1.11.1.6) and apoferritin. The molecular weights of 3-hydroxy-3-methylglutaryl-CoA synthase and acetoacetyl-CoA thiolase determined from their elution volumes were 130000 and 190000 respectively. There was no evidence for the existence of a high-molecular-weight complex between these two enzymes as suggested by Stewart & Rudney (1966*a*).

Effects of irreversible inhibitors. In all these investigations the 3-hydroxy-3-methylglutaryl-CoA synthase was freed from dithiothreitol by passage through Sephadex G-25 into 10mM-tris chloride buffer, pH 7.5. If necessary the enzyme was concentrated by rotary evaporation.

Susceptibility to reagents for thiol groups. Purified 3-hydroxy-3-methylglutaryl-CoA synthase was incubated at pH 8.2 and 30°C for 1 min in the cuvette with a number of such reagents before addition of both substrates for activity determination. The results are shown in Table 2. Particularly potent inhibitors are *p*-chloromercuribenzoate, *N*-ethylmaleimide and bromoacetyl-CoA. Further experiments involving the kinetics of inhibition by these agents are reported below.

The presence of 2μM-CoA greatly increased the inhibition by arsenite and phenylarsenious oxide. This effect appeared to be specific for CoA; thus inclusion of 50μM-2-mercaptoethanol did not have any effect. However, it is the thiol moiety of CoA that is required for the potentiation of inhibition by arsenical compounds, as 50μM-desulpho-CoA was found to be ineffective.

Table 2. *Effect of treatment with thiol-group reagents on the activity of 3-hydroxy-3-methylglutaryl-CoA synthase*

3-Hydroxy-3-methylglutaryl-CoA synthase (0.02 unit; specific activity 2.5 units/mg) and the inhibitor were incubated together for 1 min in the cuvette under the conditions of the standard assay. The activity remaining was then determined by the addition of both substrates. Exposure to 5,5′-dithiobis-(2-nitrobenzoate) was done by incubating 3-hydroxy-3-methylglutaryl-CoA synthase (0.5 unit/ml) together with the reagent in the assay buffer at 30°C in a separate vessel. After 1 min of exposure a 10μl sample was withdrawn from the reaction mixture and immediately tested for enzyme activity.

Irreversible inhibitor	Concn. of inhibitor (μM)	Residual activity (% of control)
Iodoacetamide	10000	84
Bromoacetyl-CoA	2	0
	0.2	46
N-Ethylmaleimide	2	16
5,5′-Dithiobis-(2-nitrobenzoate)	200	51
p-Chloromercuribenzoate	2	16
Cd^{2+} (as $CdSO_4$)	5	9
	1	30
Arsenite (as $NaAsO_2$)	500	90
Arsenite+CoA (2μM)	500	25
Phenylarsenious oxide	50	75
Phenylarsenious oxide+CoA (2μM)	50	41

Inhibition by p-chloromercuribenzoate and N-ethylmaleimide. In these experiments the time-course of inhibition was investigated in the presence and absence of substrates, products and various substrate and product analogues. The enzyme was incubated in the presence or absence of the CoA derivative and the reaction started by addition of the relevant inhibitor. At various times a sample was removed for immediate assay. The dilution in the assay system in the presence of substrates prevented further inhibition. The results are expressed as percentage of the control activity in a system identical but for the omission of inhibitor.

The time-course of inhibition by *p*-chloromercuribenzoate is shown in Fig. 1. Acetyl-CoA protects very effectively against loss of activity, even at extremely low concentrations. DL-3-Hydroxy-3-methylglutaryl-CoA and propionyl-CoA give slight protection at higher concentrations, but none at 5μM. Desulpho-CoA, which, like CoA, competes with acetyl-CoA for 3-hydroxy-3-methylglutaryl-CoA synthase (Chase *et al.*, 1966), did not protect at all. Owing to the effect of Hg^{2+} ions on the hydrolysis of acetoacetyl-CoA (Stern, 1956), the inclusion of this substrate in the preincubation could not be attempted. In accord with the findings of Sanner & Pihl (1962) *p*-chloromercuribenzoate did not catalyse the hydrolysis of acetyl-, propionyl- and 3-hydroxy-3-methylglutaryl-CoA, indicating that any protection against inhibition was due to these compounds acting as intact thiol-esters and not as potential sources of CoA.

The inhibition by *p*-chloromercuribenzoate was reversed by incubation with thiols, CoA being the most effective. Thus, as shown in Fig. 2, reactivation occurs spontaneously under the conditions of the

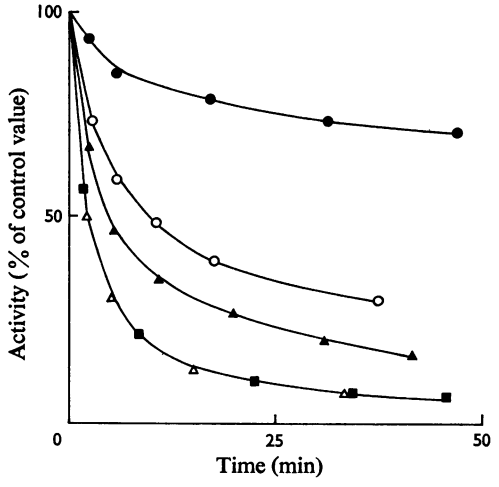

Fig. 1. *Time-course of inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by p-chloromercuribenzoate*

3-Hydroxy-3-methylglutaryl-CoA synthase (specific activity 2 units/mg) was incubated at 0°C in 0.1M-potassium phosphate buffer, pH7.0, at a concentration of 0.5 unit/ml. CoA compounds, if present, were added 1min before addition of *p*-chloromercuribenzoate. Samples (20μl) were removed at the times indicated after the addition of inhibitor and immediately assayed for enzyme activity. Additions were: *p*-chloromercuribenzoate, 60μM (△); *p*-chloromercuribenzoate+desulpho-CoA, 122μM (■); *p*-chloromercuribenzoate+DL-3-hydroxy-3-methylglutaryl-CoA, 60μM (▲); *p*-chloromercuribenzoate+propionyl-CoA, 60μM (○); *p*-chloromercuribenzoate+acetyl-CoA, 5μM (●). All concentrations are final values in the system. Results are expressed as percentages of the appropriate controls in which the inhibitor was omitted.

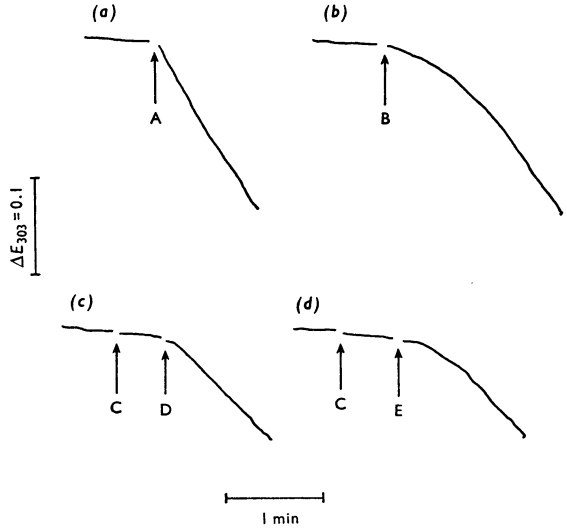

Fig. 2. *Reactivation of p-chloromercuribenzoate-treated 3-hydroxy-3-methylglutaryl-CoA synthase*

Preincubation conditions were as described in Fig. 1 but with 3-hydroxy-3-methylglutaryl-CoA synthase at a final concentration of 2 units/ml. The figure shows spectrophotometer traces representing the progress curves resulting from the following 10μl additions to the standard assay system containing both substrates: (*a*) enzyme, from a control preincubation that did not contain *p*-chloromercuribenzoate, added at A; (*b*) partially inhibited enzyme, after 15min exposure to *p*-chloromercuribenzoate, added at B; (*c*) totally inhibited enzyme, after 60min exposure to *p*-chloromercuribenzoate, added at C; then CoA added at D to give a final concentration of 5μM in the cuvette; (*d*) inhibited enzyme, as in (*c*), added to the cuvette at C; then 2-mercaptoethanol, to a final concentration of 100μM, added at E.

assay, as CoA is released during the reaction. Complete reactivation by thiol compounds was only obtained with freshly inhibited enzyme.

The time-course of inhibition by *N*-ethylmaleimide is shown in Fig. 3. This inactivation was not reversed by subsequent treatment with excess of thiol. As with *p*-chloromercuribenzoate there was a rapid inactivation of the enzyme, against which 5 μM-acetyl-CoA gave almost complete protection. Other acyl-CoA compounds at such concentrations had no effect, but at 100 μM or above some protection was observed with acetoacetyl-, propionyl- and 3-hydroxy-3-methylglutaryl-CoA. Even at these high concentrations desulpho-CoA had no effect.

To check that *p*-chloromercuribenzoate and *N*-ethylmaleimide were inhibiting by acting at the same site, enzyme inactivated by *p*-chloromercuribenzoate was tested for resistance to inhibition by *N*-ethylmaleimide. The result is shown in Fig. 4. The *p*-chloromercuribenzoate-treated enzyme was reactivated in the cuvette by incubation with 100 μM-2-mercaptoethanol before the addition of substrates. *p*-Chloromercuribenzoate was found to give marked protection against inactivation by *N*-ethylmaleimide.

Inhibition by bromoacetyl-CoA. The results of inhibition by a low concentration of bromoacetyl-CoA and the effects of various CoA derivatives on the process are shown in Fig. 5. Inhibition by bromoacetyl-CoA was not reversed by thiols. Once again 1 μM-acetyl-CoA gave good protection, whereas much higher concentrations of other acyl-CoA compounds were required. Unlike its behaviour with *p*-chloromercuribenzoate and *N*-ethylmaleimide, inclusion of desulpho-CoA in the preincubation system was found to give some protection from bromoacetyl-CoA.

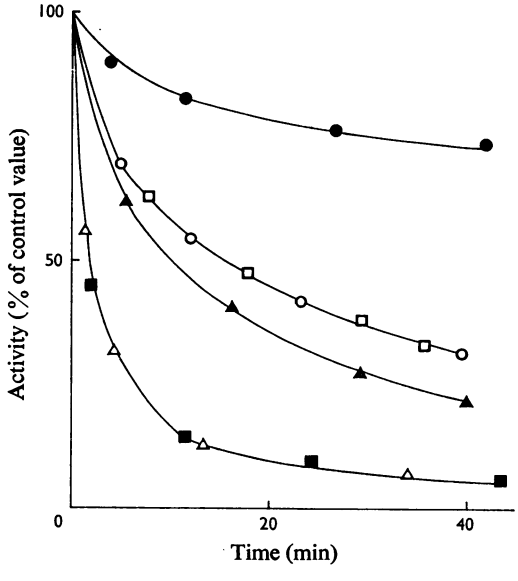

Fig. 3. *Time-course of inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by N-ethylmaleimide*

Preincubation conditions were as described in Fig. 1. Samples (20 μl) were removed as indicated after the addition of *N*-ethylmaleimide and immediately assayed for enzyme activity. Additions to the system were: *N*-ethylmaleimide, 50 μM (△); *N*-ethylmaleimide+desulpho-CoA, 122 μM (■); *N*-ethylmaleimide +DL-3-hydroxy-3-methylglutaryl-CoA, 110 μM (▲); *N*-ethylmaleimide+propionyl-CoA, 143 μM (○); *N*-ethylmaleimide+acetoacetyl-CoA, 128 μM (□); *N*-ethylmaleimide+acetyl-CoA, 5 μM (●). All concentrations were final values. Results are expressed as percentages of the appropriate controls from which *N*-ethylmaleimide was omitted.

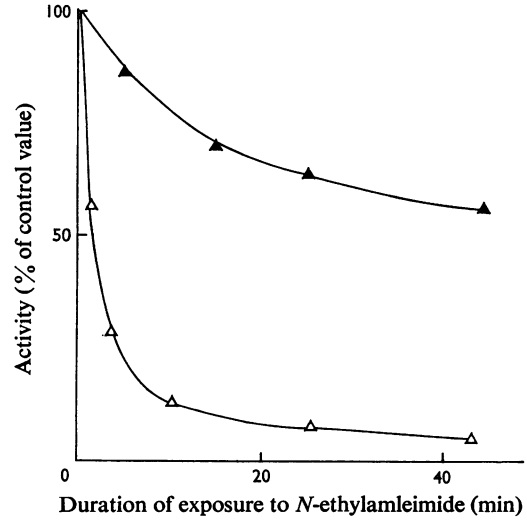

Fig. 4. *Effect of prior preincubation with p-chloromercuribenzoate on inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by N-ethylmaleimide*

△, 3-Hydroxy-3-methylglutaryl-CoA synthase (0.5 unit/ml)+*N*-ethylmaleimide (50 μM) as described in Fig. 3, expressed as a percentage of a control containing no *N*-ethylmaleimide. ▲, 3-Hydroxy-3-methylglutaryl-CoA synthase (0.5 unit/ml)+*p*-chloromercuribenzoate (80 μM) incubated as described in Fig. 1 for 60 min before the addition of *N*-ethylmaleimide (50 μM final concentration) at zero time. Results are expressed as a percentage of a control from which *N*-ethylmaleimide was omitted. In each case 20 μl samples were taken at the appropriate times and incubated for 1 min in the standard assay system with 100 μM-mercaptoethanol to reverse the *p*-chloromercuribenzoate inhibition before assay by the addition of substrates.

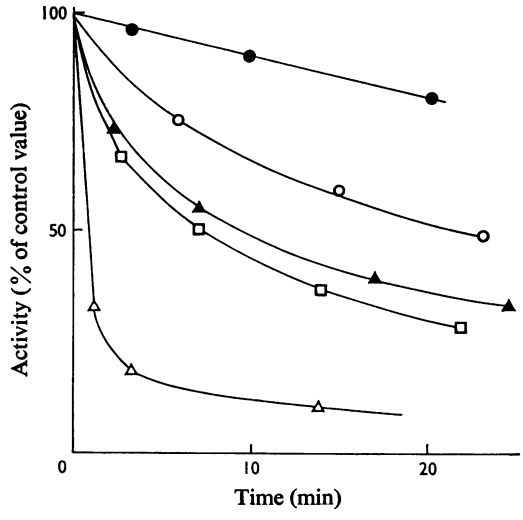

Fig. 5. *Time-course of inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase by bromoacetyl-CoA*

Preincubation conditions were as described in Fig. 1 but 3-hydroxy-3-methylglutaryl-CoA synthase was at a final concentration of 0.3 unit/ml. Samples (25μl) were removed at the times indicated and assayed immediately. Preincubation systems contained: bromoacetyl-CoA, 5μM (△); bromoacetyl-CoA+ desulpho-CoA, 122μM (□); bromoacetyl-CoA+ propionyl-CoA, 60μM (▲); bromoacetyl-CoA+ acetoacetyl-CoA, 64μM (○); bromoacetyl-CoA+ acetyl-CoA, 1μM (●). All concentrations are final values. Results are expressed as percentages of the appropriate controls in which bromoacetyl-CoA was omitted.

Discussion

The procedure described above enables the preparation of a highly purified yeast 3-hydroxy-3-methylglutaryl-CoA synthase containing not more than 0.2% contamination by acetoacetyl-CoA thiolase. Stewart & Rudney (1966*a*), commenting on their inability to separate these two enzyme activities, suggested a possible juxtaposition of thiolase and synthase activity in a protein complex analogous to the fatty acid synthase complex of yeast (Lynen, 1961). Although the present work does not disprove this possibility under the conditions of the intact cell, it does show that the two components of the putative complex are separable only by using mild procedures.

The purified 3-hydroxy-3-methylglutaryl-CoA synthase has been found to be extremely susceptible to reagents for thiol groups. Although these reagents are not so specific as to exclude reaction with other types of residue in the protein molecule, inhibition, such as found in this study, by a range of reagents each capable of reaction in different ways with thiol groups, could be taken to imply the requirement for such a group for enzyme activity.

As discussed by Webb (1966), irreversible inhibition by Cd^{2+} ions and by arsenical compounds has been used as a criterion for the detection of enzymes the activity of which depends on two vicinal thiol groups. 3-Hydroxy-3-methylglutaryl-CoA synthase is extremely susceptible to inhibition by Cd^{2+} ions (Table 2) and it is also inhibited by arsenical compounds, though less readily. However, in the latter case, but not the former, the inclusion of low concentrations of CoA during the exposure to inhibitor greatly increases the resulting inhibition. This could be taken to indicate that there is only one susceptible thiol group at the active centre and that a stable enzyme–arsenical compound is not formed until CoA binds to the enzyme and provides a second thiol group adjacent to one in the enzyme.

The kinetic studies on the inhibition by *p*-chloromercuribenzoate, *N*-ethylmaleimide and bromoacetyl-CoA show a marked similarity in the ability of acetyl-CoA, unlike other acyl-CoA derivatives, to protect extremely well, even at very low concentrations. This behaviour is compatible with two simple hypotheses. Either acetyl-CoA binds extremely tightly to the active centre giving protection to the susceptible (thiol ?) group through steric shielding, or acetyl-CoA reacts with the enzyme to form a stable acetylated derivative of this group, thus giving protection by prior chemical reaction. This chemical protection would resemble that afforded by *p*-chloromercuribenzoate against irreversible alkylation of the enzyme by *N*-ethylmaleimide.

The following evidence favours the acetyl-enzyme hypothesis: (i) desulpho-CoA, which can bind to the free enzyme with K_s 30μM (Chase *et al.*, 1966; Middleton, 1972) does not protect at 122μM against inhibition by *p*-chloromercuribenzoate and *N*-ethylmaleimide. Therefore the bulky CoA moiety alone plays no part in shielding the active centre; (ii) propionyl-CoA, a close analogue of acetyl-CoA, but not a substrate for the enzyme, gives only weak protection at concentrations 10–30-fold higher than those at which protection by acetyl-CoA is total.

These experiments support the conclusions of Stewart & Rudney (1966*b*), who, using a less pure enzyme preparation, suggested that 3-hydroxy-3-methylglutaryl-CoA synthase catalysed an ordered reaction mechanism in which the first substrate, acetyl-CoA, acylated a susceptible group at the active centre, yielding a stable acetyl-enzyme. This would be the first step in the reaction, the next being the condensation with acetoacetyl-CoA.

The studies with bromoacetyl-CoA suggest that it behaves as an active-site-directed inhibitor. Thus inhibition by this compound might be expected to proceed in two phases; a rapid non-covalent

attachment to the acetyl-CoA binding site, followed by alkylation of some reactive group nearby. If this is the mechanism then any agent capable of binding reversibly at the acetyl-CoA-binding site should give some protection. This could explain the ability of desulpho-CoA to protect against bromoacetyl-CoA inhibition but not against *p*-chloromercuribenzoate and *N*-ethylmaleimide, since these latter agents presumably inactivate in a bimolecular process not involving prior non-covalent binding. It is relevant that CoA can bind to *p*-chloromercuribenzoate-inhibited enzyme, as is shown by the reactivation by very low concentrations of CoA (Fig. 2).

The finding that acyl-CoA compounds other than acetyl-CoA, when present at high enough concentration (e.g. 100μM), will give significant protection against various alkylating agents suggests that, although not capable of giving rise to a stable protected acyl-enzyme, they bind reversibly to the active centre and protect by non-covalent steric masking of the susceptible group. If, as proposed above, 3-hydroxy-3-methylglutaryl-CoA synthase catalyses an ordered addition reaction mechanism with acetoacetyl-CoA as the second substrate, it is clear from the partial protection obtained by preincubation with this substrate (Figs. 3 and 4) that acetoacetyl-CoA can form a non-covalent complex with the free enzyme. This may explain the high substrate inhibition by acetoacetyl-CoA observed by Stewart & Rudney (1966*a*).

The kinetic studies on 3-hydroxy-3-methylglutaryl-CoA synthase reported in the next paper (Middleton, 1972) have given more information about the reaction mechanism and support the above proposals.

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