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Synthesis of Chloromethyl Ketone Derivatives of Fatty Acids

THEIR USE AS SPECIFIC INHIBITORS OF ACETOACETYL-COENZYME A THIOLASE, CHOLESTEROL BIOSYNTHESIS AND FATTY ACID SYNTHESIS

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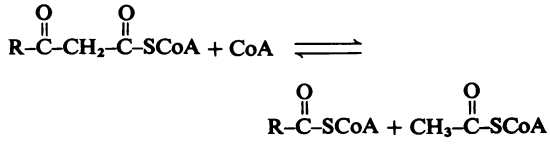
A general route for the synthesis of chloromethyl ketone derivatives of fatty acids is described. 5-Chloro-4-oxopentanoic acid, 7-chloro-6-oxoheptanoic acid, 9-chloro-8-oxononanoic acid and 11-chloro-10-oxoundecanoic acid were synthesized by this method and tested as covalent inhibitors of pig heart acetoacetyl-CoA thiolase. The K_i decreased by approx. 20-fold for each pair of methylenes added to the chain length, showing that the initial stage in inhibitor binding occurs at a non-polar region of the protein. This region is probably located at the enzyme active site, since inhibition was prevented by acetoacetyl-CoA or acetyl-CoA but not by CoA. The site of modification by chloromethyl ketone derivatives of fatty acids is restricted to a thiol group, since inactivation of the enzyme was prevented by reversible thiomethylation of the active-site thiol. In contrast, an amino-directed reagent, citraconic anhydride, still inactivated the enzyme, even when the active-site thiol was protected. Evidence that the enzyme thiol was particularly reactive came from studies on the pH-dependence of the alkylation reaction and thiol-competition experiments. Inhibition of the enzyme proceeded suprisingly well at acidic pH values and a 10^5 molar excess of external thiol over active-site thiol was required to prevent inhibition by 0.3 mM-9-chloro-8-oxononanoic acid. In addition to inhibiting isolated acetoacetyl-CoA thiolase, in hepatocytes the chloromethyl ketone derivatives of fatty acids also inhibited chloresterol synthesis, which uses this enzyme as an early step in the biosynthetic pathway. In isolated cells, the chloromethyl ketone derivatives of fatty acids were considerably less specific in their inhibitory action compared with 3-acetylenic derivatives of fatty acids, which act as suicide inhibitors of acetoacetyl-CoA thiolase. However, 9-chloro-8-oxononanoic acid was also an effective inhibitor of both hepatic cholesterol and fatty acid synthesis in mice *in vivo*, whereas the acetylenic fatty acid derivative, dec-3-ynoic acid, was completely ineffective. The effective inhibitory dose of 9-chloro-8-oxononanoic acid (2.5–5 mg/kg) was substantially lower than the estimated LD_{50} for the inhibitor (100 mg/kg).

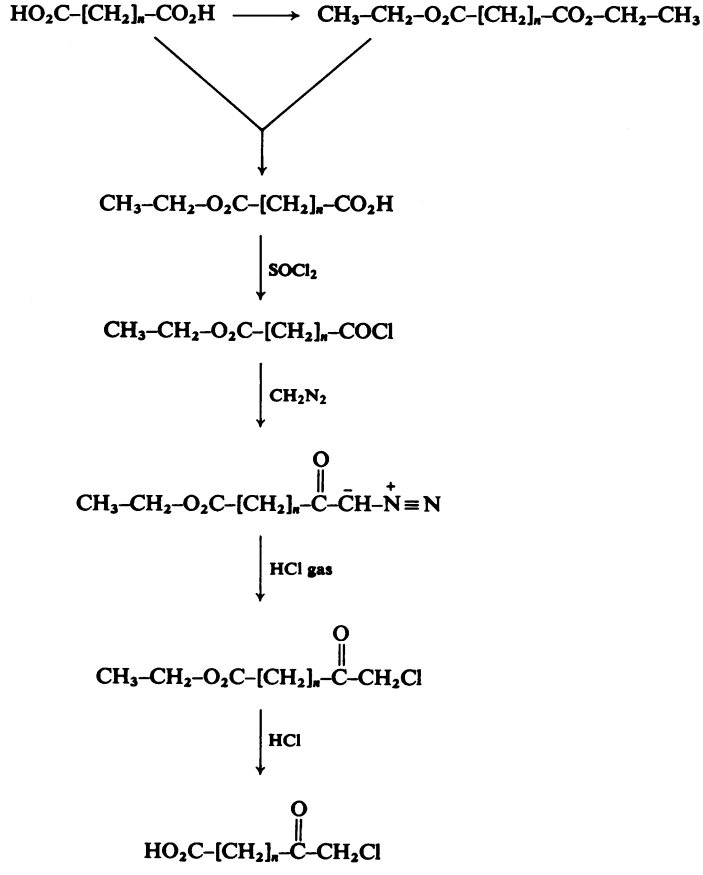
β-Oxoacyl-CoA thiolase (EC 2.3.1.16; acyl-CoA–acetyl-CoA *C*-acetyltransferase) catalyses the thiolytic cleavage of β-oxoacyl-CoA esters:



There are at least three isoenzymes of β-oxoacyl-CoA thiolase located in different fractions of the cell (Middleton, 1973). These isoenzymes differ in their substrate specificities and associated metabolic pathways. The cytosolic isoenzyme is highly specific for acetoacetyl-CoA and its role is restricted to cholesterol biosynthesis. An enzyme of similar specificity is found inside the mitochondrion, but its metabolic role is probably restricted to ketone-body synthesis (Huth *et al.*, 1975). This difference results from the cellular distribution of enzymes for the metabolism of β-hydroxymethylglutaryl-CoA. β-Hydroxymethylglutaryl-CoA reductase, essential for cholesterol synthesis, is located solely in the microsomal fraction (Bucher *et al.*, 1960; Dempsey, 1974; Clinkenbeard *et al.*, 1975*a*), whereas β-hydroxymethylglutaryl-CoA lyase, essential for acetoacetic acid synthesis, is located predominately in the mitochondria (Clinkenbeard *et al.*, 1975*b*). This means that acetoacetyl-CoA formed from acetyl-CoA by the thiolase reaction is probably committed to a single metabolic pathway. Thus the two isoenzymes of acetoacetyl-CoA thiolase can be considered as the first unique enzymes for cholesterol and ketone-body synthesis. It can be predicted that acetoacetyl-CoA thiolase could be a suitable target for site-specific enzyme inhibitors to regulate the activities of cholesterol or ketone-body synthesis.

Acetoacetyl-CoA thiolase is a particularly suitable enzyme for testing the design of specific inhibitors, because it contains a potentially reactive active-site cysteine thiol group, which is obligatorily required for catalysis by virtue of the formation of an acetyl-thioenzyme derivative (Gehring & Harris, 1968, 1970).

A number of useful active-site-directed inhibitors for this group have been developed, including iodoacetic acid, iodoacetamide (Gehring *et al.*, 1968), 2-bromoacetyl-CoA (Chase & Tubbs, 1966), 4-bromocrotonoyl-CoA and alk-3-ynoyl-CoA esters (Holland *et al.*, 1973). The alk-3-ynoyl-CoA esters act as suicide inhibitors for the enzyme (Bloxham, 1975). A common feature of all these inhibitors is that the best inhibitors are always derivatives of CoA esters. Thus although iodoacetic acid is an enzyme inhibitor, its K_i for the enzyme is several orders of magnitude greater than the CoA ester derivatives. Since the CoA ester is required for really effective inhibition, this minimizes the chance of developing a class of inhibitors that might be expected to function *in vivo*. Because of this we have synthesized a variety of potential inhibitors in an attempt to eliminate the requirement for a CoA ester. In the present work we report the synthesis of a new class of inhibitors, the chloromethyl ketone derivatives of fatty acids. Chain extension in this series markedly increased the affinity of the inhibitors for the enzyme active site and virtually eliminated the requirement for a CoA ester to produce an efficient inhibitor. A preliminary account of this work has been presented (Chalkley & Bloxham, 1976).



Scheme 1. *General route for the synthesis of chloromethyl ketone derivatives of fatty acids*
$n = 2$ for 5-chloro-4-oxopentanoic acid, $n = 4$ for 7-chloro-6-oxoheptanoic acid, $n = 6$ for 9-chloro-8-oxononanoic acid and $n = 8$ for 11-chloro-10-oxoundecanoic acid.

Materials and Methods

Synthesis of chloromethyl ketone derivatives of fatty acids

A general route for the synthesis of chloromethyl ketone derivatives of fatty acids is shown in Scheme 1. This synthesis started with a dicarboxylic acid, which was converted into a diethyl ester. Subsequent exchange between the two compounds yielded the monoester. The free carboxy group was then converted sequentially into the acid chloride, the diazo ketone and the chloromethyl ketone. Finally acid hydrolysis yielded the chloromethyl ketone derivative of a fatty acid. The synthesis of 5-chloro-4-oxopentanoic acid has been described in detail (Bloxham & Chalkley, 1976). The methods used in the synthesis of 7-chloro-6-oxoheptanoic acid, 9-chloro-8-oxononanoic acid and 11-chloro-10-oxoundecanoic acid are basically similar, except for the initial synthesis of the half-esters of the dicarboxylic acids. A general synthesis for these compounds is given by Swann *et al.* (1961). The final step in the synthesis of the chloromethyl ketone derivatives of fatty acids involves the hydrolysis of the ethyl ester derivative, and, since this was modified from the original synthesis, this is given in full here. For the remaining compounds, only the physical characteristics useful in identifying the compounds are included.

Hydrolysis of chloromethyl ketone fatty acid ethyl ester. The chloromethyl ketone fatty acid ethyl ester (15mmol) was dissolved in a minimum volume of 1,4-dioxan, and 10ml of 7M-HCl was added. The mixture was left to react for 16h in the dark at 38°C, then dried *in vacuo*. The residue was dissolved in water and dried again. This was repeated three times to remove traces of HCl. Water was removed from the product by dissolving in benzene and flash-evaporating. The product was finally crystallized from diethyl ether/light petroleum (b.p. 60–80°C).

Ethyl hydrogen adipic acid. This compound was obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A.

Ethyl adipoyl chloride. This had b.p. 84–86°C (172Pa); i.r. 1815cm^{-1} (–COCl) and 1750cm^{-1} ($-CO_2Et$).

7-Chloro-6-oxoheptanoyl ethyl ester. This had b.p. 118–120°C (40Pa); i.r. 1750cm^{-1} ($-CO_2Et$; C=O). N.m.r. showed δ 3.9–4.2p.p.m. (q, 2H, $-OCH_2-CH_3$); δ 4.05p.p.m. (s, 2H, $-CH_2Cl$); δ 2.45–2.7p.p.m. (t, 2H, $-CH_2-\overset{O}{\overset{\|}{C}}-CH_2Cl$); δ 2.15–2.4p.p.m. (t, 2H, $-CH_2-\overset{O}{\overset{\|}{C}}-O$); δ 1.5–1.7p.p.m. (m, 4H, $-CH_2,CH_2-$); δ 1.1–1.35p.p.m. (t, 3H, $-CH_2-CH_3$).

7-Chloro-6-oxoheptanoic acid. This had m.p. 74–76°C; i.r. 1700cm^{-1} ($-CO_2H$) and 1740cm^{-1} (C=O). The n.m.r. spectrum showed the loss of the resonances corresponding to the ethyl ester.

Ethyl hydrogen suberic acid. This had b.p. 156–162°C (27Pa). Mass spectrum showed *m/e* 185 (M^+-OH); *m/e* 157 ($M^+-C_2H_5O$); *m/e* 129 ($M^+-C_2H_5CO_2$).

Ethyl suberoyl chloride. This had b.p. 118–121°C (266Pa); i.r. 1750cm^{-1} ($-CO_2Et$) and 1815cm^{-1} (–COCl).

9-Chloro-8-oxononanoyl ethyl ester. This had b.p. 133–136°C (40Pa) and solidifies at room temperature. N.m.r. showed δ 3.9–4.15p.p.m. (q, 2H, $O-CH_2-CH_3$); δ 4.00p.p.m. (s, 2H, $-CH_2Cl$); δ 2.45–2.70p.p.m. (t, 2H, $-CH_2-\overset{O}{\overset{\|}{C}}-CH_2-Cl$); δ 2.15–2.4p.p.m. (t, 2H, $-CH_2-\overset{O}{\overset{\|}{C}}-OEt$); δ 1.25–1.7p.p.m. (m, 8H, $-[CH_2]_4-$) and δ 1.1–1.35p.p.m. (t, 3H, $-CH_2-CH_3$).

9-Chloro-8-oxononanoic acid. This had m.p. 73–74°C; i.r. 1700cm^{-1} ($-CO_2H$) and 1735cm^{-1} (C=O). The n.m.r. spectrum showed the loss of the ethyl ester.

Ethyl hydrogen sebacic acid. This had b.p. 140–144°C (20Pa) and solidifies at room temperature. Mass spectrum showed *m/e* 213 (M^+-OH); *m/e* 185 ($M^+-C_2H_5O$); *m/e* 157 ($M^+-C_2H_5CO_2$).

Ethyl sebacoyl chloride. This had b.p. 124–126°C (67Pa); i.r. 1815cm^{-1} (–COCl) and 1750cm^{-1} ($-CO_2Et$).

11-Chloro-10-oxoundecanoyl ethyl ester. This had b.p. 156–160°C (80Pa) which solidifies at room temperature. N.m.r. showed δ 3.8–4.15p.p.m. (q, 2H, $-CH_2-CH_3$); δ 4.05p.p.m. (s, 2H, CH_2-Cl); δ 3.7–3.95p.p.m. (t, 2H, $-CH_2-\overset{O}{\overset{\|}{C}}-CH_2-Cl$); δ 2.05–2.3p.p.m. (t, 2H, $-CH_2-\overset{O}{\overset{\|}{C}}-O$); δ 1.05–1.75p.p.m. (m, 17H, $[CH_2]_7-\overset{O}{\overset{\|}{C}}-C$ and $-CH_2-CH_3$).

11-Chloro-10-oxoundecanoic acid. This had m.p. 62–64°C; i.r. 1700cm^{-1} ($-CO_2H$) and 1735cm^{-1} (C=O). The n.m.r. spectrum showed the loss of the ethyl ester.

All n.m.r. measurements were made in [^{2}H]-chloroform and the reference standard was tetramethylsilane.

Purification of pig heart mitochondrial acetoacetyl-CoA thiolase

The following purification is suitable for a small-scale preparation from eight pig hearts. All stages in the purification were performed at 0°C. The first five

Table 1. *Purification of pig heart mitochondrial acetoacetyl-CoA thiolase*

Enzyme fraction	Volume (ml)	Activity (units)	Protein (mg/ml)	Specific activity (units/mg of protein)
1. Extract	3080	6522	18	0.118
2. First $(NH_4)_2SO_4$ precipitation	220	3900	6	2.95
3. Acetone precipitate	90	2200	6	4.07
4. Acid treatment	90	1450	3	5.37
5. Second $(NH_4)_2SO_4$ precipitation	68	1200	2	8.82
6. CM-Sephadex	27	944	1.4	25.0
7. Sepharose 6B	2.4	670	4.8	58.2

stages, involving extraction, $(NH_4)_2SO_4$ fractionation, acetone precipitation, acid treatment and a second $(NH_4)_2SO_4$ precipitation, followed the procedure of Stern (1955), except that 0.5mM-β-mercaptoacetic acid was included in all buffers. The second $(NH_4)_2SO_4$ precipitate was dissolved in 0.01M-potassium phosphate, pH6.9, containing 0.5mM-β-mercaptoacetic acid, and dialysed against the same buffer overnight. The enzyme was applied to a column (20cm × 5cm) of CM-Sephadex C50 equilibrated with the same buffer. The column was washed with 100ml of starting buffer, 500ml of 0.1M-potassium phosphate, pH6.9, containing 0.5mM-mercaptoacetic acid and a 400ml linear gradient between 0.2M-potassium phosphate, pH6.9, and 0.6M-potassium phosphate, pH6.9, containing 0.5mM-mercaptoacetic acid. Fractions (5ml) were collected and scanned at 280nm. Three main peaks of protein were eluted between 20 and 90ml, 100 and 250ml and 350 and 400ml. Only the second fraction contained enzyme activity. This was pooled, concentrated to 25ml and dialysed against 50mM-potassium phosphate, pH6.9, containing 25% (v/v) glycerol and 0.5mM-β-mercaptoacetic acid. The enzyme was divided into two portions and each was applied to a column (100cm × 2.5cm) of Sepharose 6B equilibrated in 50mM-potassium phosphate, pH6.9, containing 0.5mM-β-mercaptoacetic acid and the enzyme was eluted with the same buffer. Fractions containing enzyme activity were pooled, dialysed against the glycerol-containing buffer and the volume then decreased. The purification is summarized in Table 1. The final enzyme was judged to be pure by the technique of polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (Weber & Osborn, 1969).

General conditions for the inactivation of acetoacetyl-CoA thiolase

Acetoacetyl-CoA thiolase (10μl; 4.8mg/ml) was diluted into 1ml of 50mM-potassium phosphate, pH7.4, at 0°C. A 10μl sample was then removed and its activity was assayed by following the decrease in A_{303} in 1ml of 0.1M-Tris/HCl, pH8.2, containing 10mM-KCl, 2.5mM-$MgCl_2$, 40μM-acetoacetyl-CoA and 100μM-CoA. The molar extinction coefficient of acetoacetyl-CoA was measured as 7200 litre·mol^{-1}·cm^{-1} under the assay conditions. A small volume of the inhibitor in aqueous solution was then added and the time-dependent inactivation of the enzyme was followed. All apparent rate constants ($k_{app.}$) for inactivation were measured from the slopes of graphs of log(percentage activity) versus time. The maximum rate constants for inactivation ($k_{in.}$) were measured from double-reciprocal plots of $k_{app.}$ versus inhibitor concentration (Kitz & Wilson, 1962). When protecting reagents were present, these were always added before the inhibitor. Binding constants for ligands in protection experiments were measured from plots of $k_{app.}^{-1}$ versus ligand concentration (Mildvan & Leigh, 1964). All other experimental conditions used in this work have been described (Holland *et al.*, 1973; Bloxham, 1975). Most of the experiments in this work were performed with 9-chloro-8-oxononanoic acid. Although this inhibitor is inferior to 11-chloro-10-oxoundecanoic acid, certain features make its use preferable. First, since the rate constants for inactivation by 9-chloro-8-oxononanoic acid are lower, this means that time-dependent reaction rates are more easily followed. Secondly, 9-chloro-8-oxononanoic acid was the only inhibitor available in sufficient quantities for the administration programme *in vivo*.

All numerical values quoted for inhibition constants are presented ± S.E.M. for a minimum of four experiments.

Measurement of synthesis of cholesterol and fatty acid in rat hepatocytes and in vivo

Hepatocytes were prepared by collagenase digestion of rat livers (Zahlten & Stratman, 1974). The hepatocytes (2ml; 40mg of protein) were incubated at 38°C under O_2/CO_2 (19:1) in the presence of 5mM-glucose, 3mM-lithium [U-^{14}C]lactate (0.01μCi/μmol) and the appropriate concentration of inhibitor. The incorporation into sterols and fatty acids was measured after 1h incubation by the method of Bloxham *et al.* (1977).

Animals used in the current work were fed on a high-carbohydrate diet containing 67% (w/w) sucrose, 23% (w/w) casein, 8% (w/w) minerals and

2.7% (w/w) vitamins. Meal-fed animals were fed between 09:00 h and 13:00 h each day. Feeding regimes were established for at least 7 days before animals were used for experiments.

For measurements of lipogenesis *in vivo*, recently weaned mice were chosen for reasons of economy, because of the large number of animals involved, and to decrease the amount of inhibitor required to complete the experiment. Experimental animals were given an injection of inhibitor or 0.9% NaCl for 5 days at 10:00h. On the final day, animals were injected intraperitoneally with 1 mCi of ^3H_2O and the synthetic rate was estimated 2h later (Lowenstein, 1971). For this the animal was killed, the liver removed, weighed and homogenized in 10% (w/v) trichloroacetic acid (1g wet wt./10ml). The aqueous phase was used to measure specific radioactivity of hepatic water and the precipitate was used to measure fatty acid and cholesterol synthesis.

All inhibitors used in the present work were prepared as 0.1 M solutions in water, after neutralization with 1 M-$NaHCO_3$.

Results and Discussion

Inhibition of acetoacetyl-CoA thiolase by chloromethyl ketone derivatives of fatty acids

When acetoacetyl-CoA thiolase was incubated with 9-chloro-8-oxononanoic acid, the enzyme lost activity in a time-dependent and inhibitor-concentration-dependent reaction (Fig. 1). Under the conditions chosen for modifying the enzyme, the sample was diluted 100-fold for the spectrophotometric assay so that the concentration of inhibitor gave negligible direct inhibition of the enzyme. Therefore 9-chloro-8-oxononanoic acid produced its effect by virtue of a covalent modification of the enzyme. Plots of log(percentage enzyme activity) versus time were linear for the inactivation reaction, showing that inhibition follows pseudo-first-order kinetics. Presumably the inhibitor forms a binary complex with enzyme before the covalent reaction. Double-reciprocal plots of the apparent rate constant for inactivation versus inhibitor concentration were linear, and enabled K_i to be estimated as 0.49 ± 0.1 mM ($n = 4$) and the maximum rate constant for inactivation ($k_{in.}$) was $0.52 \pm 0.09 min^{-1}$ ($n = 4$) (Fig. 2). This experiment was now repeated with a series of chloromethyl ketone derivatives of fatty acids, which became progressively more hydrophobic as the length of the alkyl chain increased. Table 2 shows that the most pronounced effect of making the inhibitor progressively more hydrophobic was to decrease K_i, indicating that the affinity for the modified site was increased markedly. The value of K_i decreases by a factor of approx. 20 for every two methylene groups added to the inhibitor. In contrast with the large change in K_i (approx. 2000), there was only a small change in $k_{in.}$. Collectively these results show that K_i values are primarily a property of the inhibitor,

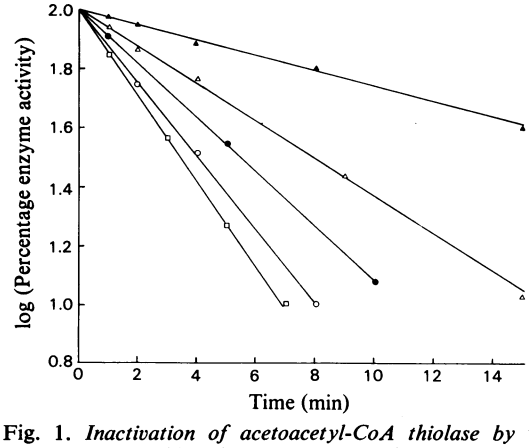


Fig. 1. *Inactivation of acetoacetyl-CoA thiolase by 9-chloro-8-oxononanoic acid*

Acetoacetyl-CoA thiolase (1 μM in subunits) was treated at 0°C in 50 mM-potassium phosphate, pH 7.4, and the time-dependent loss of enzyme activity was measured in the presence of various concentrations of 9-chloro-8-oxononanoic acid. The concentrations of 9-chloro-8-oxononanoic acid were: □, 1 mM; ○, 0.5 mM; ●, 0.3 mM; △, 0.2 mM; ▲, 0.1 mM.

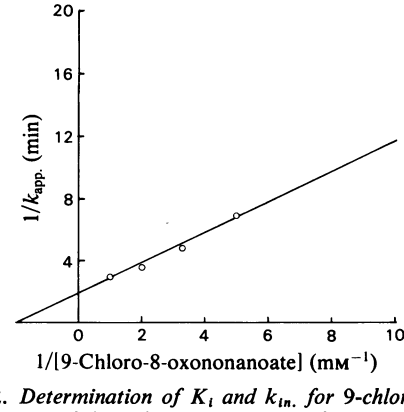


Fig. 2. *Determination of K_i and $k_{in.}$ for 9-chloro-8-oxononanoic acid-dependent inactivation of acetoacetyl-CoA thiolase*

Data from experiments shown in Fig. 1 were used to estimate $k_{app.}$ at various concentrations of 9-chloro-8-oxononanoic acid. K_i and $k_{in.}$ are related to $k_{app.}$ and inhibitor concentration [I] by the equation of Kitz & Wilson (1962):

$$k_{app.} = k_{in.}\left(1+\frac{[I]}{K_i}\right)$$

Table 2. *Estimation of K_i and $k_{in.}$ for the inactivation of acetoacetyl-CoA thiolase by a series of chloromethyl ketone derivatives of fatty acids and their CoA esters*

Apparent rate constants for inhibition ($k_{app.}$) were determined for at least six concentrations of each inhibitor. K_i and $k_{in.}$ were then measured from these data.

Inhibitor	K_i	$k_{in.}$
5-Chloro-4-oxopentanoic acid	150mM	$0.40\pm0.18\,min^{-1}$
7-Chloro-6-oxoheptanoic acid	11.04 ± 1.2mM	$0.53\pm0.3\,min^{-1}$
9-Chloro-8-oxononanoic acid	0.49 ± 0.1mM	$0.52\pm0.09\,min^{-1}$
11-Chloro-10-oxoundecanoic acid	0.028 ± 0.0058mM	$4.0\pm0.7\,min^{-1}$
5-Chloro-4-oxopentanoyl-CoA	$15\pm1.5\,\mu$M	$2.5\pm0.2\,min^{-1}$
7-Chloro-6-oxoheptanoyl-CoA	$2\pm0.2\,\mu$M	$2.7\pm0.4\,min^{-1}$
9-Chloro-8-oxononanoyl-CoA	$1.4\pm0.35\,\mu$M	$2.7\pm0.3\,min^{-1}$
11-Chloro-10-oxoundecanoyl-CoA	$2.5\pm0.4\,\mu$M	$3.0\pm0.5\,min^{-1}$

whereas the value for $k_{in.}$ is a function of the nucleophilic properties of the reactive group on the enzyme. Interestingly the increase in $k_{in.}$ occurs almost solely when the chain length of the inhibitor is extended from 9-chloro-8-oxononanoic acid ($k_{in.}$ $0.52\,min^{-1}$) to 11-chloro-10-oxoundecanoic acid ($k_{in.}$ $4.0\,min^{-1}$). The value of K_i for 11-chloro-10-oxoundecanoic acid is virtually identical with that observed with the CoA ester derivatives (Table 2), suggesting that once the inhibitor has reached a critical size then some of the binding energy between enzyme and inhibitor could be used to enhance the rate of chemical reaction (Jencks, 1975). As shown below, the reactive group at the enzyme active site is almost certainly a thiol group.

The wide divergence in the affinities of the inhibitors for the enzyme was decreased once they were converted into their CoA esters (Table 2). Now there was only an 8-fold difference in K_i values, and $k_{in.}$ was virtually constant. Presumably the enzyme reactive site is located in or near to a primary hydrophobic region of the protein, and making the inhibitor more hydrophobic in character makes it more accessible to the enzyme active site. Conversion of the inhibitors into their CoA esters may allow the same effect to be achieved, since the binding of the adenine ring of many nucleotides occurs in a primarily hydrophobic pocket on enzyme active sites (Rossman *et al.*, 1975).

The exact location of the non-polar region in relation to the active-site thiol is not clear. One possibility is that it could represent the binding site that normally accommodates the adenine ring of substrates. This region is generally hydrophobic and is a particularly dominant feature in the binding of nucleotide substrates (Blake & Evans, 1974; Rossman *et al.*, 1975). Whatever the exact location of the hydrophobic site, it seems probable that the chloromethyl ketone derivatives of fatty acids act firstly by binding in a non-polar region of the protein and subsequently the chloromethyl ketone reactive group must migrate within the active site to scavenge for the reactive thiol group. An initial tight binding would be expected for the pseudo-first-order kinetics for inhibition.

Inhibition of the enzyme by the CoA esters in Table 2 was completely dependent on the presence of a functional chloromethyl ketone group. This was shown by a control experiment where 5mg of CoA in 1ml of 1M-$KHCO_3$ was treated with 20mg of 5-chloro-4-oxopentanoate for 30min at 20°C to generate the non-hydrolysable CoA derivative shown below:

$$ClCH_2\text{–}\underset{\underset{O}{\|}}{C}\text{–}[CH_2]_2\text{–}CO_2H + CoASH \rightarrow HCl + CoAS\text{–}CH_2\text{–}\underset{\underset{O}{\|}}{C}\text{–}[CH_2]_2\text{–}CO_2H$$

Incubation of 2-oxo-4-carboxybutyl-CoA with acetoacetyl-CoA thiolase at concentrations up to $100\,\mu$M had no detectable action on enzyme activity. Under the same incubation conditions, $50\,\mu$M-5-chloro-4-oxopentanoyl-CoA completely inactivated the enzyme within 15min.

Substrate-protection experiments

The catalytic mechanism of acetoacetyl-CoA thiolase proceeds by a Ping Pong mechanism in which a covalent acetyl-thioenzyme is formed (Gehring *et al.*, 1968; Gehring & Lynen, 1972). The compulsory order of substrate addition or product formation means that only acetoacetyl-CoA or acetyl-CoA binds to the free enzyme, whereas CoASH binds to the acetyl-thioenzyme. This means that substrate-protection experiments should clearly demonstrate whether an inhibitor is directed specifically towards the active-site thiol group, since both acetoacetyl-CoA and acetyl-CoA should prevent inhibition, whereas CoA should be ineffective. Fig. 3 shows that this result was obtained with the chloromethyl ketone derivatives of fatty acids. Although

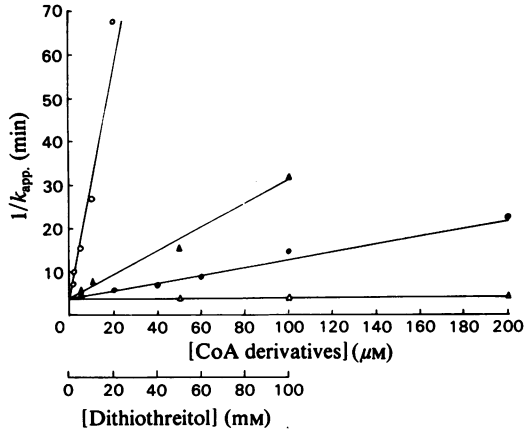


Fig. 3. *Protection against inactivation of acetoacetyl-CoA thiolase by 9-chloro-8-oxononanoic acid*

Acetoacetyl-CoA thiolase was inactivated under standard conditions in the presence of 0.3mM-9-chloro-8-oxononanoic acid and progressively increasing concentrations of acetoacetyl-CoA (○), acetyl-CoA (●), CoA (△) or dithiothreitol (▲). At each concentration of protecting ligand, $k_{app.}$ was measured from a first-order plot for the inactivation of the enzyme. The use of plots of $k_{app.}^{-1}$ versus protecting-ligand concentration to measure protection was developed by Mildvan & Leigh (1964).

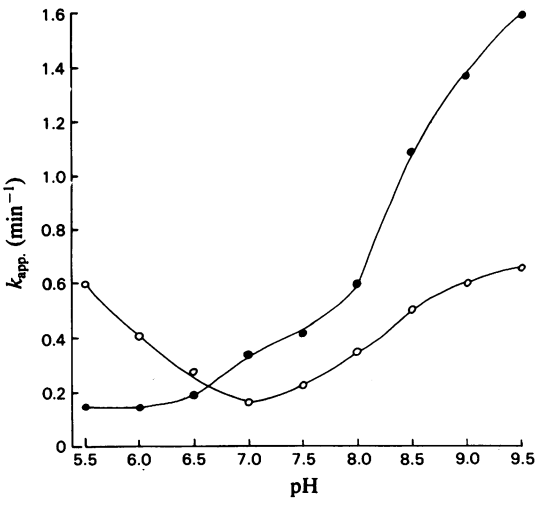


Fig. 4. *pH-dependent inactivation of acetoacetyl-CoA thiolase by 5-chloro-4-oxopentanoyl-CoA and 9-chloro-8-oxononanoic acid*

The apparent rate constants for the inactivation of acetoacetyl-CoA thiolase were measured at 0°C in 50mM-potassium phosphate solutions with either 50μM-5-chloro-4-oxopentanoyl-CoA (●) or 0.3mM-9-chloro-8-oxononanoic acid (○) as an inhibitor.

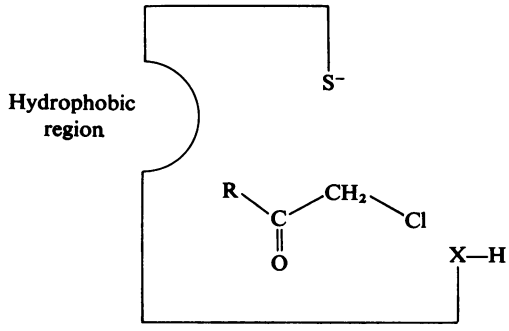
only the result with 9-chloro-8-oxononanoic acid is shown, identical experiments were performed with all of the inhibitors shown in Table 2 and in each case the results were the same. Fom the data in Fig. 3 the dissociation constants for acetoacetyl-CoA and acetyl-CoA were calculated as $2.5 \pm 0.5\,\mu M$ and $44 \pm 8\,\mu M$ respectively. These values are close to those for the kinetic constants for these two substrates (Huth *et al.*, 1975). This demonstrates that catalytically competent complexes provide protection against inhibition.

Inhibition of acetoacetyl-CoA thiolase by acetylenic fatty acid derivatives

Having observed that increasing the chain length of chloromethyl ketone derivatives of fatty acids makes then more effective inhibitors, it must be questioned whether this is a general effect or specifically related to the type of inhibitor tested. To answer this question, we studied the inhibitory effect of alk-3-ynoic acids of two different chain lengths. The CoA esters of alk-3-ynoic acids have been shown to act as suicide inhibitors (Holland *et al.*, 1973; Bloxham, 1975) for acetoacetyl-CoA thiolase. With a short-chain-length inhibitor, but-3-ynoic acid, it was not possible to detect any inhibition even at concentrations of 50mM (Holland *et al.*, 1973). In contrast, the longer-chain inhibitor dec-3-ynoic acid was an irreversible inhibitor, with a K_i of 10mM and $k_{in.}$ $0.033\,min^{-1}$. Clearly the increasing hydrophobic properties of the acetylenic fatty acid derivative have also improved its effectiveness as an inhibitor, although the free acetylenic acid is a poor inhibitor compared with its CoA ester. Since the improvement in inhibition by chain extension appears to be a general phenomenon, it seems reasonable to conclude that it is probably the consequence of a non-polar binding site on the enzyme.

Inactivation of hepatic acetoacetyl-CoA thiolase by chloromethyl ketone derivatives of fatty acids

Most of the work reported here uses pig heart acetoacetyl-CoA thiolase, which is the mitochondrial isoenzyme. Since in the latter parts of this work we studied the inhibitory effect on cholesterol biosynthesis, which requires the involvement of the cytosolic isoenzyme, the results were also checked with this isoenzyme. Cytosolic acetoacetyl-CoA thiolase was purified from rat liver by the technique of Middleton (1974). When this enzyme was tested with the chloromethyl ketone fatty acid derivatives, then it gave exactly the same decrease in K_i as the chain length of the inhibitor was extended. Apparently the binding characteristics of the inhibitors to the two types of enzyme were identical.



Scheme 2. *Potential participation of functional groups in the inactivation of acetoacetyl-CoA thiolase by chloromethyl ketone derivatives of fatty acids*

pH-dependence of alkylation reaction

If the reaction with the chloromethyl ketone analogues is related solely to the nucleophilic attack of the active-site cysteine thiol, then the pK_a for ionization of this group should determine the rate of the inactivation reaction. Fig. 4 shows that when acetoacetyl-CoA thiolase was treated with 50μM-5-chloro-4-oxopentanoyl-CoA, the rate of inactivation increased as the reaction pH increased. The pK_a value for this reaction was approx. 8.4, which is close to the value observed for alkylation reactions with acetoacetyl-CoA thiolase (Holland *et al.*, 1973). In contrast, if the pH-dependence of the reaction with 9-chloro-8-oxononanoic acid was studied, then the reaction increased at both acidic and alkaline pH values, showing that the reaction is described by at least two K_a values corresponding approximately to pK_a 5.9 and pK_a 8.2. Presumably the value for pK_a 8.2 represents the reaction with the enzyme thiol species, as with the CoA ester derivatives. Since the reaction also increases with decreasing pH values, this means that the presence of a protonated species in the reaction mixture also enhances the reaction. This might represent a protonated group on the enzyme facilitating displacement of Cl^- as shown in Scheme 2. However, it seems probable that this pK_a value corresponds to the pK_a for the carboxy group on the inhibitor. The pK_a value for 5-chloro-4-oxopentanoic acid by titration was shown to be 4.70 ± 0.05. Clearly the protonated species would be more hydrophobic, and this might be expected to enhance the binding to a non-polar region of the protein. The absence of the acidic pK_a value from the reaction of the enzyme with the CoA ester derivatives in which the carboxy group is masked is consistent with the acidic pK_a value corresponding to the carboxy group of the inhibitor. The enhanced reactivity at acidic pH values shows that if the inhibitor modifies an active-site thiol, then this thiol group must be considerably more reactive than would appear from the pK_a value of 8.4 estimated for the alkylation of the thiol. Thus titration of cysteine with 5-chloro-4-oxopentanoic acid also has a pK_a value of 8.9. This means that at pH6.5 the chemical reaction is only 1–2% of the reaction rate at pH8.9. Since inhibition does occur readily this implies that the active-site thiol group must be considerably more reactive than a normal cysteine group. This is demonstrated in the next section.

Thiol competition in the alkylation reaction

The preceding section suggested that the enzyme active-site thiol might be considerably more reactive than its apparent pK_a value would imply. This was verified by treating 1μM-acetoacetyl-CoA thiolase (molarity with respect to subunits) with 0.30mM-9-chloro-8-oxononanoic acid (K_i 0.49mM) in the presence of progressively increasing concentrations of a competing thiol (dithiothreitol, Fig. 3). At least 100mM-dithiothreitol was required in the reaction mixture before inactivation of the enzyme was decreased to a negligible rate. This means that the added thiol must be present at least in 10^5 molar excess over the active site of the enzyme to compete for all of the available inhibitor. This type of result was observed with a number of competing thiol reagents, including β-mercaptoethanol, cysteine and glutathione.

Dividing the apparent rate constant for inactivation of acetoacetyl-CoA thiolase at infinite inhibitor concentration ($0.52 min^{-1}$) by the molar enzyme active-site concentration (1μM) gives an apparent second-order rate constant for the reaction of $5.2 \times 10^5 M^{-1} \cdot min^{-1}$. When cysteine was treated with 5-chloro-4-oxopentanoate, the estimated second-order rate constant was $50 M^{-1} \cdot min^{-1}$, which also demonstrates the enhanced reactivity of the active-site thiol group.

Thiol-protection experiments to demonstrate the nature of the group reacting with chloromethyl ketone fatty acid derivatives

Acetoacetyl-CoA thiolase reacts with acetyl-CoA by the following reaction:

$$E\text{–}SH + CH_3\text{–}\overset{\overset{O}{\|}}{C}\text{–}SCoA \rightleftharpoons E\text{–}S\text{–}\overset{\overset{O}{\|}}{C}\text{–}CH_3 + CoASH$$

The formation of the acetyl-thioenzyme provides the basis for the chemical-protection experiments described above. Clearly an additional mode of protection would be to purify the acetyl-thioenzyme and show that this enzyme is not inhibited by the chloromethyl ketone fatty acid derivatives. Since this is technically a rather difficult process, we have chosen to study an analogous reversible reaction with methyl methanethiosulphonate:

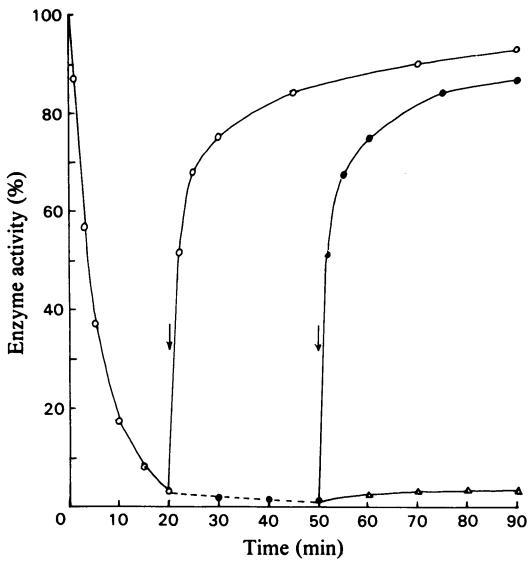
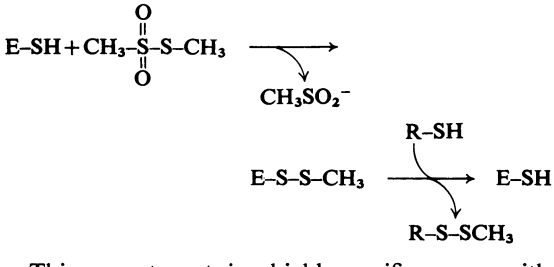


Fig. 5. *Use of thiomethyl acetoacetyl-CoA thiolase to investigate enzyme modification*

Acetoacetyl-CoA thiolase was treated under standard conditions with 50 μM-methyl methanethiosulphonate to generate the inactive thiomethyl-protected enzyme. Enzyme activity was regenerated directly if 50mM-dithiothreitol was added (○). The point of dithiothreitol addition is indicated by the arrow. In two subsequent experiments a second modification was performed after thiomethylation. The enzyme was treated with either 0.3mM-9-chloro-8-oxononanoic acid (●) or 500 μM-citraconic anhydride (△) for 30min. The second inhibitor was then removed by dialysis for 5h against 2×1 litre of 50mM-potassium phosphate, pH 7.0, before the effect of reduction with 50mM-dithiothreitol was measured.



This reagent reacts in a highly specific manner with cysteine thiol groups and the thiomethyl bond can be reversed by reduction (Smith *et al.*, 1975; Bloxham & Wilton, 1977; Bloxham *et al.*, 1978). Fig. 5 shows that thiomethylation of acetoacetyl-CoA thiolase with methyl methanethiosulphonate resulted in the complete loss of enzyme activity and that subsequent reduction of thiomethyl acetoacetyl-CoA thiolase with 50mM-dithiothreitol completely regenerated the active form of the enzyme. The thiomethyl group was inserted at the enzyme active site, since the reaction was completely prevented by acetoacetyl-CoA ($K^S_{AcAcCoA} = 15\,\mu$M) or acetyl-CoA ($K^S_{AcCoA} = 35\,\mu$M). In a parallel experiment the thiomethyl enzyme was treated with 0.3mM-9-chloro-8-oxononanoic acid for 30min, which would be sufficient time to inactivate the unprotected enzyme completely. This enzyme was then exposed to reducing agent and was found to be re-activated at exactly the same rate as the initial thiomethyl enzyme sample. In a control experiment, 9-chloro-8-oxononanoic acid-inactivated acetoacetyl-CoA thiolase was also reduced with 50mM-dithiothreitol and this did not lead to any recovery of enzyme activity, showing that the $-S-CH_2-\overset{\overset{O}{\|}}{C}-[CH_2]_6-CO_2H$ structure is stable to reduction. These results show clearly that the inactivation of acetoacetyl-CoA thiolase by the chloromethyl ketone fatty acid derivatives is restricted to modification of a cysteine thiol group.

Subsite modification in acetoacetyl-CoA thiolase with anhydride reagents

In the preceding sections we have suggested that the active-site thiol of acetoacetyl-CoA thiolase may be especially reactive. This can be achieved in a number of ways, but a reasonable possibility is that the presence of additional polar groups at the active site facilitates the reaction. As an example, an NH_3^+ group could enhance the reaction by protonating the displaced chloride group from the inhibitor. The problem with identifying subsite or secondary functional groups in an enzyme like acetoacetyl-CoA thiolase is that the thiol group is the most reactive group and would probably react with any reagent before modification of the second group. The availability of a method of specifically protecting thiol groups reversibly provides a method for examining the reaction of secondary functional groups. Methyl methanethiosulphonate is ideally suited for this purpose, since it allows the specific insertion of an $-SCH_3$ group into the enzyme active site, which can subsequently be removed by reduction. The thiosulphonate group is also substantially activated compared with a simple disulphide, which means that this reaction proceeds much more readily than with comparable reagents, such as hydroxyethyl disulphide (Lowe & Whitworth, 1974). By using this procedure we have demonstrated the involvement of an amino group at the enzyme active site. Fig. 5 shows that when thiomethyl acetoacetyl-CoA thiolase was treated with 500 μM-citraconic anhydride, an amino-modifying reagent (Dixon & Perham, 1968; Gibbons & Perham, 1970, 1974), for 30min (sufficient to inactivate unprotected acetoacetyl-CoA thiolase by

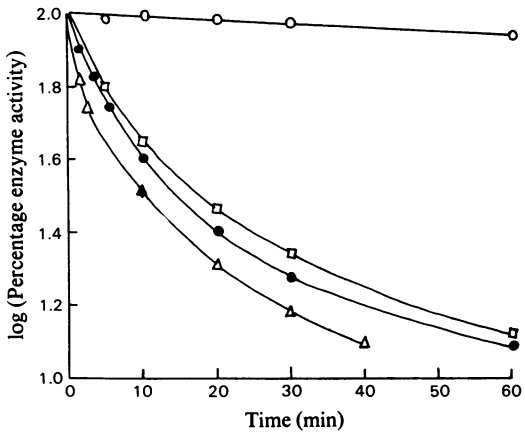
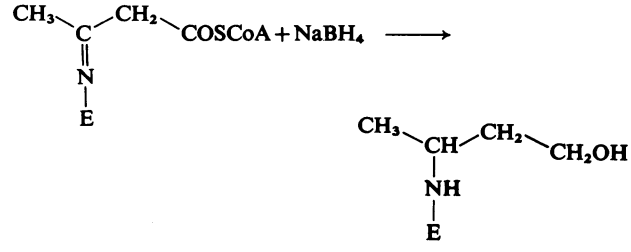


Fig. 6. *Substrate protection against inactivation of acetoacetyl-CoA thiolase by citraconic anhydride*
Acetoacetyl-CoA thiolase was inactivated under standard reaction conditions with 500μM-citraconic anhydride (●). The concentration of substrates added to estimate protection was 30μM-acetoacetyl-CoA (○), 75μM-CoA (△) and 100μM-acetyl-CoA (□).

85%), then subsequent reduction of the doubly modified enzyme with 50mM-dithiothreitol resulted in only a marginal recovery of enzyme activity. Clearly the citraconyl group alone modifies a second group on the enzyme, which results in inactivation of the enzyme even when the enzyme active-site thiol was free. Partial re-activation of the doubly modified enzyme was obtained if the enzyme was both reduced and exposed to acidic pH values. Reduction followed by 5h dialysis at pH5.0 (0°C) yielded 30–50% of control activity. The results from this experiment were limited by the fact that exposure of acetoacetyl-CoA thiolase to pH values below pH5.0 resulted in inactivation of the enzyme. However, the partial re-activation by acid is consistent with the formation of an amide derivative that hydrolyses at acidic pH (Dixon & Perham, 1968). This suggests that the enzyme may also possess an essential amino group. That this group may be located at the active site was shown by protection experiments on the direct modification of acetoacetyl-CoA thiolase by citraconic anhydride (Fig. 6). Acetoacetyl-CoA provided complete protection against inactivation. The inactivation of acetoacetyl-CoA thiolase by a number of anhydride reagents (citraconic anhydride, succinic anhydride and phthalic anhydride) differed from thiol-directed reagents in two important aspects. First, the time course of the reaction did not follow pseudo-first-order kinetics and secondly, inhibition was not prevented by acetyl-CoA (Fig. 6). Both of these observations emphasize that the anhydride reagents modify an alternative group to the enzyme active-site thiol and clearly this inhibitory process occurs through a different mode of inhibitor binding.

The possibility that an amino group of lysine could be functioning at the enzyme active site had been suggested in previous experiments where acetoacetyl-CoA thiolase was inactivated in a $NaBH_4$-dependent reduction in the presence of acetoacetyl-CoA (Kornblatt & Rudney, 1971; Holland *et al.*, 1973). Presumably the inhibition proceeds by reduction of an imine between the ε-NH_2 of lysine and the β-carbonyl of acetoacetyl-CoA as shown below:



It is doubtful that the postulated imine would be mechanistically important. Thus imines of the type formed above isomerize very readily to the conjugated alkene-amine (Glickman & Cope, 1945). This would result in the loss of a proton from the α-methylene of acetoacetyl-CoA. This pathway has been eliminated by studies showing that both α-methylene protons are retained (Willadsen & Eggerer, 1975). Rather than proposing a covalent bond between the substrate and an amino group, it seems more reasonable that the amine might enhance nucleophilic properties of the enzyme active-site thiol if it was suitably placed to abstract a proton ($-NH_2 + SH \rightleftharpoons NH_3^+ + S^-$). The enhancement by amino groups of the reactivity of thiol groups is well documented (Jocelyn, 1972). This work presents several pieces of evidence that the active-site thiol of acetoacetyl-CoA thiolase is highly activated compared with a simple thiol. The main evidence is (*a*) a higher apparent bimolecular rate constant for reaction of the active-site thiol with the chloromethyl ketone fatty acid derivatives, (*b*) the requirement for very high concentrations of externally added thiol to prevent inhibition by chloromethyl ketone fatty acid derivatives and (*c*) the unusually high rate of enzyme inactivation by chloromethyl ketone fatty acid derivatives at acidic pH values below the expected pK_a value of the cysteine thiol.

Inhibition of lipid synthesis in isolated hepatocytes

The development of specific active-site-directed inhibitors has the important consequence that this could lead to the production of useful agents to regulate the activity of metabolic pathways. Of all the approaches developed recently, the concept of suicide inhibitors appears to be the most interesting.

Table 3. *Inhibition of cholesterol and fatty acid biosynthesis in hepatocytes*

Synthesis of cholesterol and fatty acids was measured from [U-^{14}C]lactate as described in the Materials and Methods section. The control rates of synthesis were 0.1 and 2.0 μmol of acetyl units incorporated/h per g wet wt. for cholesterol and fatty acid synthesis respectively.

Inhibitor	Inhibitor concentration for 50% inhibition (mM)	
	Cholesterol synthesis	Fatty acid synthesis
5-Chloro-4-oxopentanoic acid	0.5 ±0.2	0.25±0.1
7-Chloro-6-oxoheptanoic acid	0.5 ±0.15	0.5 ±0.3
9-Chloro-8-oxononanoic acid	0.35±0.05	0.6 ±0.15
11-Chloro-10-oxoundecanoic acid	0.10±0.02	0.75±0.30
But-3-ynoic acid	1±0.4	2±0.70
Pent-3-ynoic acid	0.1 ±0.05	0.75±0.25
Dec-3-ynoic acid	0.02±0.01	0.5 ±0.15

Suicide inhibition of an enzyme relies on a highly specific generation of a reactive species at the enzyme active site (Helmkamp & Bloch, 1969; Endo *et al.*, 1970; Morisaki & Bloch, 1972). Acetylenic inhibitors are classic examples of suicide inhibitors and the inihibition of acetoacetyl-CoA thiolase by dec-3-ynoic acid falls into this class of inhibition (Bloxham, 1975). The availability of two inhibitors, dec-3-ynoic acid and 9-chloro-8-oxononanoic acid, which are similar in physical properties and inhibit the same enzyme, but by different mechanisms, enabled us to contrast the inhibitory properties of a directly reactive reagent (chloromethyl ketone) with the suicide inhibitor.

To assess the inhibitory properties of the chloromethyl ketone derivatives of fatty acids, lipid synthesis was measured in isolated hepatocytes obtained from rats fed on a high-carbohydrate fat-free diet. This diet was chosen to induce maximum rates of lipogenesis (Numa & Yamashita, 1974). [U^{14}-C]Lactate was used as the biosynthetic precursor for these experiments, as it is the major precursor of both fatty acid and cholesterol biosynthesis (Clark *et al.*, 1974; Brunengraber *et al.*, 1973; Salmon *et al.*, 1974). The results with chloromethyl ketone fatty acid derivatives were also compared with 3-acetylenic suicide inhibitors for acetoacetyl-CoA thiolase. Chloromethyl ketone fatty acid derivatives were effective inhibitors of both fatty acid synthesis and cholesterol synthesis in hepatocytes (Table 3). For the inhibition of cholesterol synthesis the potency of the inhibitors increased with extension of the inhibitor chain length; however, the apparent K_i only varied by a factor of 5 for the cellular studies compared with 2000-fold for the inhibition studies on the purified enzyme. Further, there was little difference in the sensitivity of fatty acid synthesis and cholesterol synthesis to inhibition by chloromethyl ketone derivatives of fatty acids. Clearly, although the chloromethyl ketone group may confer reaction specificity in isolated enzyme systems, there are multiple enzymes that are sensitive to inhibition by chloromethyl ketone derivatives of fatty acids in the cell. These results are in contrast with the observations for the inhibition by the acetylenic inhibitors (Table 3). Here the longer-chain inhibitor, dec-3-ynoic acid, was a substantially better inhibitor (50-fold) than but-3-ynoic acid. Further, although dec-3-ynoic acid inhibited both cholesterol and fatty acid synthesis, the apparent K_i for inhibition of cholesterol synthesis (0.02mM) was substantially lower than the K_i for inhibition of fatty acid synthesis (0.5mM). This demonstrates a much improved specificity of inhibition with suicide inhibitors. This experiment also provides an excellent contrast between closely related directly reactive chloromethyl ketone inhibitors and suicide inhibitors, since it appears to fulfil the principal prediction about these suicide inhibitors, namely that they should be more specific in their inhibitory action. Presumably this is brought about by the requirement for enzyme-catalysed activation of the acetylenic fatty acid derivative to the allenic fatty acid derivative to generate a reactive inhibitor (Morisaki & Bloch, 1972; Holland *et al.*, 1973; Bloxham, 1975).

Inhibition in vivo of cholesterol and fatty acid synthesis

To develop a successful inhibitor, the agent must be delivered to the target tissue *in vivo*. At this level the susceptibility of the inhibitor to degradation by further metabolism will influence the effectiveness of the inhibitor. To assess lipogenesis *in vivo*, young mice were weaned on to a high-carboydrate diet to induce maximum lipogenesis. Table 4 shows that 9-chloro-8-oxononanoic acid inhibited cholesterol and fatty synthesis in both freely fed and meal-fed mice. The agent is generally hypolipoemic, since both cholesterol and fatty acid synthesis are equally inhibited. Inhibition of cholesterol synthesis was obtained with injections of 2.5 mg/kg, which compares reasonably with the effective dose of other hypocholes-

Table 4. *Inhibition of cholesterol and fatty acid synthesis in vivo by 9-chloro-8-oxononanoic acid*

Synthesis rates were measured by the incorporation of ^3H_2O into mouse liver fatty acids and cholesterol *in vivo*. The synthesis rates are expressed as μmol of ^3H_2O incorporated/2h per g wet wt. The numbers in parentheses show the percentage inhibition of synthesis rates by 9-chloro-8-oxononanoic acid.

		Fed *ad libitum*		Meal-fed	
Inhibitor	Injection (mg/kg)	Cholesterol synthesis	Fatty acid synthesis	Cholesterol synthesis	Fatty acid synthesis
9-Chloro-8-oxononanoic acid	0	33±3	298±17	8.77±0.52	300±17
	2.5	22.8±2.2 (30.9)	250±11 (16.1)	6.14±0.31 (30)	180±15 (40)
	10	17±1.5 (48.5)	142±14 (52.4)	4.65±0.45 (47)	60±7 (80)
	25	9.3±0.6 (71.9)	101±9 (66.1)	3.43±0.01 (60.9)	48±9 (84)
	50	4.5±0.3 (86.4)	80±7 (73.2)	2.00±0.27 (77.2)	49±5 (84)
Dec-3-ynoic acid	100	32±2.7	275±12	8.65±0.37	310±25

terolemic agents, e.g. clofenapic acid (0.25mg/kg), *N*-(α-methylbenzyl)linoleamide (10mg/kg) and bis-(hydroxyethylthio)-1,10-decane (20mg/kg) (Bencze, 1975).

The effective dose of 9-chloro-8-oxononanoic acid for inhibition of either cholesterol or fatty acid synthesis was limited by other side effects of the inhibitor. Thus at injections of 50mg/kg it was noticed that food intake in a large number of animals was considerably decreased (approx. 50–60%) and the animals failed to gain weight normally. The control animals gained 2.5±0.4g during the course of the experiment, whereas the injected animals only gained 1.0±0.4g. Further, if the dose of 9-chloro-8-oxononanoic acid was increased above 100mg/kg then its effects were lethal. A daily dose of 100mg/kg resulted in death in 60–70% of all animals injected within 3 days, although the animals were able to survive a single dose.

In contrast with the chloromethyl ketone derivative of the fatty acid, the acetylenic fatty acid derivative was virtually without effect on either hepatic cholesterol or fatty acid biosynthesis *in vivo*. Even daily injections of 100mg/kg were only marginally inhibitory, whereas 9-chloro-8-oxononanoic acid was toxic at this concentration. This seems to point out a drawback in the philosophy for the use of suicide inhibitors, namely that although lack of inherent reactivity creates a substantial degree of specificity (as in the hepatocyte experiments) this also makes the inhibitor more susceptible to degradation and hence renders the agent more difficult to administer to the target tissue *in vivo*.

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