Selective Inhibition of Cholesterol Synthesis by Cell-Free Preparations of Rat Liver by Using Inhibitors of Cytoplasmic Acetoacetyl-Coenzyme A Thiolase

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Cytoplasmic acetoacetyl-CoA thiolase (acetyl-CoA-acetyl-CoA C-acetyltransferase, EC 2.3.1.9) was partially purified from rat liver. The enzyme was irreversibly inactivated by 4-bromocrotonyl-CoA, but-3-ynoyl-CoA, pent-3-ynoyl-CoA and dec-3-ynoyl-CoA. In the case of the alk-3-ynoyl-CoA esters the potency as alkylating agents of acetoacetyl-CoA thiolase decreased with increased chain length of the alk-3-ynoyl moiety. Advantage was taken of the specific action of alk-3-ynoyl-CoA esters on acetoacetyl-CoA thiolase to show that in a postmitochondrial fraction from rat liver they are effective inhibitors of cholesterol synthesis from sodium [2-¹⁴C]acetate under conditions when mevalonate conversion into cholesterol and fatty acid synthesis are unaffected. Short-chain alk-3-ynoyl acids have little effect on sterol synthesis, although dec-3-ynoic acid is an effective inhibitor owing to its conversion into the CoA ester by the microsomal fatty acyl-CoA synthetase.

Atherosclerotic conditions are frequently associated with elevated cholesterol concentrations (Bach, 1970), and this has provided the impetus for developing agents which are capable of lowering the concentration of plasma cholesterol. This could be achieved by one of three means, namely (a) decreased dietary cholesterol intake, (b) stimulation of cholesterol degradation and (c) selective inhibition of cholesterol synthesis. All three methods have been tested with some degree of success. Although agents have been developed which inhibit specific enzymes involved in the biosynthesis of cholesterol, many have the disadvantage of causing the accumulation of other sterols, e.g. triparanol causes the accumulation of desmosterol (Herndon & Siperstein, 1963), which may be equally undesirable. Therefore it may be useful to develop compounds which inhibit cholesterol synthesis at an early stage in the biosynthetic route.

One of the early stages in the biosynthesis of cholesterol involves the condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA and CoA, which is catalysed by acetyl-CoA-acetyl-CoA C-acetyltransferase (EC 2.3.1.9; acetoacetyl-CoA thiolase). The mitochondrial enzyme has been studied in molecular detail (Gehring & Lynen, 1972) and is known to possess a reactive cysteine at the active site which can be alkylated by iodoacetamide (Lynen, 1953; Gehring *et al.*, 1968; Gehring & Harris, 1970), 2-bromoacetyl-CoA (Chase & Tubbs, 1966) and 4-bromocrotonyl-CoA (Bloxham *et al.*, 1973; Holland *et al.*, 1973a). Although all of these compounds would be expected to inhibit cholesterol

synthesis, they have the disadvantage common to many halogenated compounds of being too reactive, and this would lead to inhibition of numerous enzymes. Holland *et al.* (1973a) have developed the acetylenic compounds, alk-3-ynoyl-CoA esters, to alkylate the enzyme. The alk-3-ynoyl-CoA esters inactivate 3-ketoacyl-CoA thiolase by a mechanism which appears uniquely suitable for a specific alkylating agent. Thus alk-3-ynoyl-CoA esters are not highly reactive in their own right. Rather, they are converted by active 3-ketoacyl-CoA thiolase into the alka-2,3-dienoyl-CoA, which then readily inactivates the enzyme. Thus the enzyme catalyses its own inactivation. A number of acetylenic compounds have now been used as alkylating inhibitors of a variety of enzymes. The enzyme-catalysed isomerization of the acetylenic bond to an allene before inactivation appears to be a common feature of the inactivation mechanism (Endo *et al.*, 1970; Morisaki & Bloch, 1972; Abeles & Walsh, 1973; Hevey *et al.*, 1973). This feature suggests that suitably designed acetylenic compounds may have significant pharmacological applications. In the present work it is shown that alk-3-ynoyl-CoA esters are effective alkylating inhibitors of hepatic cytosolic acetoacetyl-CoA thiolase and that this results in a selective inhibition of cholesterol biosynthesis between acetate and mevalonate. A preliminary report of this work has been presented (Bloxham, 1974).

Materials and Methods

Sodium [2-¹⁴C]acetate and [RS-2-³H₂]mevalonate lactone were obtained from The Radiochemical

Centre, Amersham, Bucks., U.K. Dec-3-yn-1-ol, oct-3-yn-1-ol and hex-3-yn-1-ol were synthesized by the addition of ethylene oxide to the Grignard reagent of the appropriate 1-alkyne (Knight & Diamond, 1959). But-3-yn-1-ol was obtained from Ralph N. Emanuel Ltd., Wembley, Middx., U.K. The purity and structure of each acetylenic alcohol was confirmed by g.l.c. followed by mass spectrometry. In each case the alcohols were free of starting material and the expected molecular ion and fragmentation pattern were observed. The acetylenic alcohols were oxidized to their corresponding acids with CrO_3 in H_2SO_4 (Heilbron *et al.*, 1949). 4-Bromocrotonic acid was prepared by refluxing crotonic acid with *N*-bromosuccinimide in the presence of benzoyl peroxide (Bradshaw *et al.*, 1969). Oxalyl chloride was used to convert carboxylic acids into their acid chlorides, which were subsequently used to acylate CoA (Holland *et al.*, 1973a). Acyl thioester concentrations were determined by the ferric hydroxamate method of Lipmann & Tuttle (1945). Acetoacetyl-CoA was prepared by the treatment of CoA with diketene in 0.1 M- KHCO_3 . The concentration of acetoacetyl-CoA was assayed with pig heart β -hydroxybutyryl-CoA dehydrogenase obtained from Sigma (London) Chemical Co., London S.W.6, U.K. (Lynen & Wieland, 1955).

Hepatic cytoplasmic acetoacetyl-CoA thiolase

A partially purified preparation of hepatic cytoplasmic acetoacetyl-CoA thiolase was prepared from rat liver by modification of a procedure described by Middleton (1973). In this procedure, the cytosolic enzyme is eluted from a DEAE-cellulose column by a gradient of 0.1–0.5 M-Tris-HCl, pH 8.2, containing 10% (v/v) glycerol. Fractions containing enzyme activity were used directly.

For the inactivation experiments, 0.1 unit of acetoacetyl-CoA thiolase ($\approx 200 \mu\text{g}$ of protein) in $50 \mu\text{l}$ of 0.25 M-Tris-HCl, pH 8.2, was diluted with $150 \mu\text{l}$ of water. Inactivation was commenced by the addition of a small volume of the compound under test and $10 \mu\text{l}$ samples were removed at appropriate times for assay of enzyme activity. Incubations were done at room temperature. Four separate preparations of partially purified acetoacetyl-CoA thiolase (all about 1 unit/mg; 1 unit of enzyme transforms $1 \mu\text{mol}$ of substrate/min) were used to study the effects of the various inhibitors used in the present work. The response to the inhibitors was completely reproducible in all four enzyme preparations. All data points were confirmed on at least three separate occasions.

Acetoacetyl-CoA thiolase activity was determined, unless indicated otherwise, at 25°C in 1.0 ml of 0.1 M-Tris-HCl (pH 8.0) containing 10 mM-KCl, 2.5 mM- MgCl_2 , 25 μM -acetoacetyl-CoA and 50 μM -CoA. The

reaction was started by the addition of CoA and the cleavage of acetoacetyl-CoA was monitored by the subsequent decrease in extinction at 303 nm (Lynen *et al.*, 1952).

Incorporation of radioactive precursors into sterols and fatty acids

Postmitochondrial supernatants from rat liver were prepared in 0.25 M-sucrose containing 50 mM-potassium phosphate, pH 7.4, 25 mM-KCl and 10 mM- MgCl_2 . Postmitochondrial supernatant fractions (0.7 ml; 6–8 mg of protein) were incubated in a volume of 1 ml with 10 mM- KHCO_3 , 1 mM-NADP $^+$, 2.5 mM-ATP, 5 mM-glucose 6-phosphate and either sodium $[2\text{-}^{14}\text{C}]\text{acetate}$ (2 μCi) or $[2\text{-}^3\text{H}_2]\text{mevalonate}$ (1 μCi). Incubations were performed in 15 ml centrifuge tubes at 37°C for 15 min. The reaction was terminated by the addition of 2 ml of methanolic 20% (w/v) KOH. After being hydrolysed at 80°C for 5 min, the total sterols were extracted into light petroleum (b.p. $60\text{--}80^\circ\text{C}$)–diethyl ether (1:1, v/v) (Bloxham & Akhtar, 1971). For analysis of cholesterol synthesis, radioactive cholesterol was isolated as the 5,6-dibromide derivative (Akhtar *et al.*, 1969). To isolate fatty acids, the aqueous phase remaining after the sterol extraction was cooled on ice and carefully acidified with conc. HCl. Fatty acids were then extracted with $3 \times 3 \text{ ml}$ of light petroleum (b.p. $60\text{--}80^\circ\text{C}$)–diethyl ether (1:1, v/v). The organic extracts were pooled and their volume decreased to 1 ml. This was mixed thoroughly with 1 ml of 5% (w/v) KOH. The organic phase was discarded and the aqueous phase washed three times with diethyl ether. After acidification of the aqueous phase, the total fatty acids were extracted with $3 \times 2 \text{ ml}$ of light petroleum–diethyl ether (1:1, v/v), and the sample radioactivity was determined.

Liquid-scintillation counting of radioactive samples was performed as described previously (Bloxham *et al.*, 1971).

Preparation and assay of fatty acyl-CoA synthetase

Freeze-dried microsomal fractions from rat liver were prepared by the method of Bar-Tana *et al.* (1971). Microsomal fractions (3 mg) were incubated at 38°C in 0.35 ml of 0.1 M-Tris-HCl, pH 7.4, containing 0.2% (w/v) Triton X-100, 1.5 mM-EDTA, 35 mM- MgCl_2 , 10 mM-fatty acid under test and 1 mM-CoA. At appropriate times $50 \mu\text{l}$ samples were removed and the free thiol content was assayed by using 1 ml of 0.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) in 0.1 M-potassium phosphate buffer, pH 8.0. For the fatty acyl-CoA synthetase the activity was assumed to equal the rate of CoA disappearance.

Results

3-Ketoacyl-CoA thiolase from many tissues can be separated into distinct isoenzymes on the basis of either the physical properties of the enzyme or by its intracellular location (Middleton, 1973; Clinkenbeard *et al.*, 1973). The original studies on the alkylating properties of alk-3-ynoyl-CoA esters were performed with a mitochondrial^{*} isoenzyme of 3-ketoacyl-CoA thiolase isolated from pig heart (Holland *et al.*, 1973a). This is a distinct isoenzyme from the hepatic cytosolic isoenzyme (Middleton, 1973) which participates in the condensation of acetyl-CoA in cholesterol biosynthesis. If alk-3-ynoyl-CoA esters are to be effective inhibitors of cholesterol synthesis then they would be required to inactivate the cytosolic isoenzyme. Thus an initial part of this project was to evaluate the sensitivity of the cytosolic acetoacetyl-CoA thiolase to alk-3-ynoyl-CoA esters. For the alkylation studies, hepatic cytoplasmic acetoacetyl-CoA thiolase was separated from any contaminating mitochondrial 3-ketoacyl-CoA thiolase by chromatography on DEAE-cellulose (Middleton, 1973).

The catalytic mechanism of 3-ketoacyl-CoA thiolase involves the generation of an enzyme-bound

acetyl group which requires the participation of an essential thiol residue. Previous studies with heart mitochondrial 3-ketoacyl CoA thiolase (Lynen, 1953; Gehring *et al.*, 1968; Gehring & Harris, 1970) and liver cytosolic acetoacetyl-CoA thiolase (Middleton, 1974) have shown that the enzyme is readily inactivated by reaction of the active-site thiol with iodoacetamide. To test the reactivity of the active-site thiol in cytosolic acetoacetyl-CoA thiolase, the effect of 4-bromocrotonyl-CoA was tested. 4-Bromocrotonyl-CoA reacts very readily with thiol groups (Holland *et al.*, 1973a). Fig. 1 shows that the partially purified cytoplasmic acetoacetyl-CoA thiolase is rapidly inactivated. Inactivation follows first-order kinetics when the concentration of alkylating inhibitor is varied over a twentyfold range. A double-reciprocal plot of the apparent rate constant for inactivation versus the inhibitor concentration was found to be linear (Kitz & Wilson, 1962) and enabled the estimation of the dissociation constant for the enzyme-inhibitor complex (K_1 , 2.75×10^{-4} M) and a pseudo-first-order constant for inactivation (k_3 , 1.0 min^{-1}). Both of these values are somewhat higher than the comparable values for inactivation of heart mitochondrial 3-ketoacyl-CoA thiolase by 4-bromocrotonyl-CoA (K_1 , 1.25×10^{-5} M; k_3 , 0.50 min^{-1}). By dividing k_3 by K_1 it is possible to obtain an apparent value for a second-order rate constant for inactivation. For the alkylation of cytoplasmic acetoacetyl-CoA thiolase by 4-bromocrotonyl-CoA, a value of $60 \text{ M}^{-1} \cdot \text{s}^{-1}$ can be calculated. This value compares with a second-order rate constant of $96 \text{ M}^{-1} \cdot \text{s}^{-1}$ which can be calculated from data for the reaction of 4-bromocrotonyl thioester with the cysteine in glutathione (Holland *et al.*, 1973a). The similarity of these two values indicates that the active-site thiol in cytoplasmic acetoacetyl-CoA thiolase is not unusually reactive.

Having established the characteristic response of cytoplasmic acetoacetyl-CoA thiolase to a thiol-directed alkylating reagent, the effect of various alk-3-ynoyl-CoA esters was tested. The compounds used were but-3-ynoyl-CoA, pent-3-ynoyl-CoA and dec-3-ynoyl-CoA. Whereas the inactivation by pent-3-ynoyl-CoA and dec-3-ynoyl-CoA followed pseudo-first-order kinetics, the inactivation of hepatic cytoplasmic acetoacetyl-CoA thiolase by but-3-ynoyl-CoA could not be fitted to a first-order plot. Thus plots of log (percentage activity) versus time were curved once inactivation had proceeded beyond 50% inhibition of enzyme activity. This result indicates that the reaction follows a second-order process. Similar results have also been noted for the reaction of heart mitochondrial 3-ketoacyl-CoA thiolase with iodoacetamide (Gehring *et al.*, 1968), bromoacetyl-CoA (Holland *et al.*, 1973a) and but-3-ynoyl-CoA (Holland *et al.*, 1973a). It is generally assumed that an active-site-directed inhibitor

Fig. 1. Pseudo-first-order inactivation of cytoplasmic acetoacetyl-CoA thiolase by 4-bromocrotonyl-CoA

Acetoacetyl-CoA thiolase was incubated with the indicated concentration of 4-bromocrotonyl-CoA. At appropriate times a sample was removed and the enzyme activity was determined in the standard assay system (see the Materials and Methods section).

which follows first-order kinetics obeys the following general scheme (Kitz & Wilson, 1962):

In this scheme it is assumed that the rate of inactivation is low compared with the establishment of a steady-state concentration of EI which is essential if inactivation is to follow first-order kinetics. It must be presumed that for but-3-ynoyl-CoA, then k_3 must be appreciably faster than for pent-3-ynoyl-CoA, dec-3-ynoyl-CoA or 4-bromocrotonyl-CoA.

To illustrate the marked difference in the alkylating properties of the three alk-3-ynoyl-CoA esters under test, the enzyme inactivation produced in a fixed time was measured at a variety of inhibitor concentrations (Fig. 2). But-3-ynoyl-CoA is approximately a hundred times more potent than pent-3-ynoyl-CoA. This reflects the chain-length specificity of cytoplasmic acetoacetyl-CoA thiolase for 3-ketoacyl-CoA esters, since acetoacetyl-CoA thiolase is known to use acetoacetyl-CoA (C_4) in preference to longer-chain 3-ketoacyl-CoA esters (Middleton, 1973). The main decrease in the affinity of cytoplasmic acetoacetyl-CoA thiolase for alk-3-ynoyl-CoA esters occurs when the substrate chain length is extended by one methyl from C_4 to C_5 , and beyond this there is virtually no change. This is reflected in the fact that pent-3-ynoyl-CoA and dec-3-ynoyl-CoA have relatively similar potencies as inactivating agents of cytoplasmic acetoacetyl-CoA thiolase.

As will be shown below, dec-3-ynoyl-CoA appears to be a useful inhibitor of cholesterol synthesis, and its inhibitory properties were evaluated in slightly

Fig. 2. Inactivation of cytoplasmic acetoacetyl-CoA thiolase by alk-3-ynoyl-CoA esters

Cytoplasmic acetoacetyl-CoA thiolase was incubated for 2 min with various concentrations of alk-3-ynoyl-CoA esters, and then the residual enzyme activity was determined. The control reaction rate was measured by incubating acetoacetyl-CoA thiolase for 2 min in the absence of the inhibitor. ○, Dec-3-ynoyl-CoA; ●, pent-3-ynoyl-CoA; Δ, but-3-ynoyl-CoA.

Fig. 3. Dependence of the apparent first-order rate constant for inactivation ($k_{app.}$) of cytoplasmic acetoacetyl-CoA thiolase on dec-3-ynoyl-CoA concentration

The rate of inactivation of acetoacetyl-CoA thiolase was measured in the presence of several concentrations of dec-3-ynoyl-CoA by using the standard conditions described in the Materials and Methods section. $k_{app.}$ for each concentration of dec-3-ynoyl-CoA was then estimated from the slope of plots of log (percentage activity) versus time of inactivation.

Fig. 4. Competitive inhibition of cytoplasmic acetoacetyl-CoA thiolase with respect to acetoacetyl-CoA by dec-3-ynoyl-CoA

The standard assay conditions for acetoacetyl-CoA thiolase were modified so that irreversible inhibition was minimized. Reaction mixtures (1 ml) containing 0.1 M-Tris-HCl (pH 8.0), 10 mM-KCl, 2.5 mM-MgCl₂, 50 μM-CoA, various concentrations of acetoacetyl-CoA and the indicated concentration of dec-3-ynoyl-CoA were initially equilibrated, and the reaction was started by the addition of 0.01 unit of enzyme. Only the initial rate recorded in the first minute was used to estimate reaction velocity.

more detail. The inactivation of cytoplasmic acetoacetyl-CoA thiolase was found to follow first-order kinetics for loss of at least 90% of the enzyme activity. Fig. 3 shows that the rate of inactivation of acetoacetyl-CoA thiolase was dependent on the dec-3-ynoyl-CoA concentration. From this plot, the rate constant for inactivation was estimated at 0.25 min^{-1} and the dissociation constant was $170 \mu\text{M}$. The initial process in the inactivation mechanism presumably involves the formation of a binary complex between acetoacetyl-CoA thiolase and the alkylating agent. For dec-3-ynoyl-CoA, where the loss of activity is relatively slow at low concentrations of inhibitor then the formation of the inhibitory complex may be studied on the basis of the ability to interfere with the binding of substrate. To assess this action, dec-3-ynoyl-CoA was incubated with the enzyme and substrates under conditions when alkylation of the enzyme was minimum. This was achieved by commencing assays by the addition of enzyme to the complete reaction mixture and by following only the initial reaction velocity in the first minute. Inactivation of the enzyme, as indicated by progressive slowing of the reaction rate, was only noted if the reaction was followed over longer periods of time. Fig. 4 shows that when the effect of dec-3-ynoyl-CoA on the activity of acetoacetyl-CoA thiolase is assayed directly then it is a competitive inhibitor with respect to acetoacetyl-CoA. The apparent dissociation constant for the binding of dec-3-ynoyl-CoA to acetoacetyl-CoA thiolase is $36 \mu\text{M}$. This value is somewhat lower than the dissociation constant ($170 \mu\text{M}$) determined in the inactivation studies. This may reflect the more complex phenomenon required for inactivation, which may involve isomerization of the acetylene to the allene. The competitive nature of the binding of dec-3-ynoyl-CoA with respect to acetoacetyl-CoA would be expected if the alkylating inhibitor binds to the enzyme active site. It is known that inclusion of acetoacetyl-CoA in the inactivation incubation medium prevents inactivation of 3-ketoacyl-CoA thiolase by alk-3-ynoyl-CoA esters (Holland *et al.*, 1973a).

Cholesterol synthesis

The experiments presented so far show that alk-3-ynoyl-CoA esters are effective inhibitors of cytoplasmic acetoacetyl-CoA thiolase. Inhibition can occur either through an irreversible inactivation or as a result of competitive binding at the active site.

The influence of alk-3-ynoyl-CoA esters on cholesterol synthesis was studied by using postmitochondrial preparations from rat liver. When sodium $[2-^{14}\text{C}]$ acetate was used as a precursor for sterol synthesis, the incorporation showed a lag for the first 5 min, and beyond this time the rate of incorporation was constant.

Fig. 5. Inhibition of sterol synthesis by alk-3-ynoyl-CoA esters

Total sterol synthesis from sodium $[2-^{14}\text{C}]$ acetate was measured in postmitochondrial supernatant fractions from rat liver. Appropriate concentrations of the inhibitor were incubated directly with the complete cell-free preparation for sterol synthesis and the incorporation of radioactivity into total sterols was measured after 15 min at 37°C (see the Materials and Methods section). The results are the average of three separate experiments. For each experimental point the standard error was never greater than 10% of the mean. ○, Dec-3-ynoyl-CoA; ●, pent-3-ynoyl-CoA; △, but-3-ynoyl-CoA.

When but-3-ynoyl-CoA, pent-3-ynoyl-CoA and dec-3-ynoyl-CoA were added directly to the incubation this resulted in an inhibition of incorporation. The demonstration of inhibition did not require a preliminary incubation of the postmitochondrial preparation with the inhibitor. The potency as inhibitors was a function of the chain length of the alk-3-ynoyl-CoA esters (Fig. 5). However, it is clear that there is not the same degree of difference in the sensitivity to the alk-3-ynoyl-CoA esters as is observed in the alkylation experiments on isolated acetoacetyl-CoA thiolase. The similarity in potency is due predominantly to a decrease in the effectiveness of but-3-ynoyl-CoA. But-3-ynoyl-CoA has only limited stability at alkaline pH (Holland *et al.*, 1973a), and it is presumed that the crude preparation required for sterol synthesis promotes its destruction. It was not possible to demonstrate directly that cholesterol synthesis was inhibited in an irreversible manner. However, when postmitochondrial fractions were incubated with alk-3-ynoyl-CoA esters then at the end of the incubation the demonstrable activity of acetoacetyl-CoA thiolase was decreased compared with the control.

As pointed out in the introduction, it is an aim of the development of specific inhibitors of cholesterol

synthesis that they should not result in the accumulation of unwanted sterols. This should not be the case with the alk-3-ynoyl-CoA esters, which affect cholesterol synthesis at an early stage and which inhibit the accumulation of total sterols (Fig. 5). To confirm that alk-3-ynoyl-CoA esters do not lead to an abnormal pattern of sterol synthesis, the total sterol synthesized from acetate in a control incubation was compared with the sterols synthesized in the presence of $50\mu\text{M}$ -dec-3-ynoyl-CoA. The addition of dec-3-ynoyl-CoA gave 60% inhibition of total sterol synthesis. After t.l.c. in light petroleum (b.p. $60\text{--}80^\circ\text{C}$)-acetone (14:3, v/v), three major sterols were identified, namely, cholesterol (R_F , 0.33; 30% of total radioactivity), lanosterol (R_F , 0.50; 10% of total radioactivity) and squalene (R_F , 0.90; 60% of total radioactivity). The relative distribution of radioactivity in these three fractions was not significantly altered by the presence of dec-3-ynoyl-CoA.

The specificity of action of alk-3-ynoyl-CoA esters was examined by measuring their effect on the

incorporation of sodium $[2\text{-}^{14}\text{C}]$ acetate into total sterols, isolated cholesterol and fatty acids and on the incorporation of $[2\text{-}^3\text{H}_2]$ mevalonate into sterols and cholesterol. This experiment was performed with all three alk-3-ynoyl-CoA esters, but for convenience only the result with dec-3-ynoyl-CoA is presented (Fig. 6). It is apparent that dec-3-ynoyl-CoA caused a complete inhibition of sterol synthesis from acetate at concentrations which produced virtually no effect on mevalonate incorporation. This shows that the site of inhibition lies between acetate and mevalonate, which is the expected result for an inhibitor of acetoacetyl-CoA thiolase which is essential for the conversion of acetate into mevalonate. In accord with this proposal, Fig. 6 also shows that the inhibitory action of dec-3-ynoyl-CoA on the catalytic activity of acetoacetyl-CoA thiolase in the postmitochondrial supernatant closely parallels the inhibition of acetate incorporation into cholesterol. It may be noted that once alk-3-ynoyl-CoA ester concentrations exceed about $100\mu\text{M}$ there is an inhibition of mevalonate incorporation. This inhibition occurs with most of the parameters measured in this work and appears to be a non-specific action of the alk-3-ynoyl-CoA esters.

Further specificity of the action of alk-3-ynoyl-CoA esters is shown by the fact that when sterol synthesis from acetate is markedly inhibited, there is no equivalent inhibition of acetate incorporation into fatty acids (Fig. 6). Indeed, for dec-3-ynoyl-CoA at low concentrations there appears to be some stimulation of incorporation. This stimulation was most prevalent with dec-3-ynoyl-CoA and was minimal with but-3-ynoyl-CoA and pent-3-ynoyl-CoA. This may indicate that dec-3-ynoyl-CoA is a substrate for the microsomal elongation process for fatty acid synthesis (Guchhait *et al.*, 1966). The absence of an inhibitory effect of alk-3-ynoyl-CoA esters on fatty acid synthesis is expected, since acetate incorporation into fatty acids involves the intermediacy of malonyl-CoA (Wakil, 1958), and acetoacetyl-CoA thiolase is not involved in this process.

Experiments on pig heart 3-ketoacyl-CoA thiolase showed that only thioesters of alk-3-ynoic acids were effective alkylating inhibitors and that CoA esters were the most effective (Holland *et al.*, 1973a). If alk-3-ynoic acids are to inhibit cholesterol biosynthesis then they should first be activated to their CoA esters. The ability of a variety of alk-3-ynoic acids to inhibit sterol synthesis was tested (Fig. 7). In postmitochondrial preparations, but-3-ynoic acid and pent-3-ynoic acid were completely without effect. As the chain length was increased beyond C_5 then there was an increase in the inhibition of synthesis. Both hex-3-ynoic acid and oct-3-ynoic acid have similar effectiveness, but there is a marked increase in inhibitory potency with dec-3-ynoic acid. The apparent K_i for inhibition of sterol synthesis is

Fig. 6. Influence of dec-3-ynoyl-CoA on lipid synthesis

Postmitochondrial supernatants were incubated directly with various concentrations of dec-3-ynoyl-CoA and the activity of a number of biosynthetic pathways during the incubation was measured as follows. Incorporation of sodium $[2\text{-}^{14}\text{C}]$ acetate into total sterols (○), into cholesterol (●) and into total fatty acids (△). Incorporation of $[2\text{-}^3\text{H}_2]$ mevalonate into total sterols (▲). In addition, $5\mu\text{l}$ of postmitochondrial supernatant fraction was assayed for acetoacetyl-CoA thiolase activity by adding the preparation to the standard assay system (see the Materials and Methods section) containing the indicated concentration of dec-3-ynoyl-CoA (□). The results are the average of three separate experiments. For each experimental point the standard error was never greater than 10% of the mean.

Fig. 7. Inhibition of sterol synthesis by alk-3-ynoic acids

The incorporation of sodium $[2-^{14}\text{C}]$ acetate into total sterols by postmitochondrial supernatant fractions was measured. Incubations were performed in the presence of various concentrations of fatty acids as follows: □, but-3-ynoic acid; ■, pent-3-ynoic acid; △, hex-3-ynoic acid; ▲, oct-3-ynoic acid; ○, dec-3-ynoic acid; ●, decanoic acid. The results are the averages of four separate experiments. For each experimental point the standard error was never greater than 10% of the mean.

$40\text{ }\mu\text{M}$, which is comparable with but slightly higher than the value observed for dec-3-ynoyl-CoA ($K_1 = 30\text{ }\mu\text{M}$). This result shows that dec-3-ynoic acid must be efficiently converted into the CoA ester.

When using the free acid to inhibit sterol synthesis from acetate, there is the possibility that the alk-3-ynoic acid inhibits by virtue of CoA sequestration (Williamson *et al.*, 1970). This does not appear to be the case here, since the simple fatty acid, decanoic acid, has virtually no effect on sterol synthesis (Fig. 7). Further, the inclusion of CoA ($50\text{ }\mu\text{M}$) was not essential for the incorporation of acetate into cholesterol and neither did it prevent the inhibition produced by either dec-3-ynoic acid or dec-3-ynoyl-CoA.

In a postmitochondrial preparation, fatty acids will be converted into their CoA esters primarily by the microsomal fatty acyl-CoA synthetase (Kornberg & Pricer, 1953). That this system is essential for the inhibitory action of alk-3-ynoic acids is reflected in the fact that the efficacy of the alk-3-ynoic acids in inhibiting sterol synthesis compares with the known chain-length specificity of the microsomal fatty acyl-CoA synthetase. The microsomal enzymes utilize C_{10} or C_{12} fatty acids preferentially and shorter-chain acids are very poor substrates (Kornberg & Pricer, 1953). These results were established for alkanolic acids and it appears that the activity of microsomal fatty acyl-CoA synthetase is similar with

Fig. 8. Activity of microsomal fatty acyl-CoA synthetase with dec-3-ynoic acid

Freeze-dried microsomal fraction from rat liver was incubated in the presence of either dec-3-ynoic acid (○), or dodecanoic acid (△) and in the absence of fatty acid (□). The esterification of CoA was measured on the basis of thiol disappearance. Duplicate analyses of thiol content agreed within 5%. The exact incubation conditions are described in the Materials and Methods section.

alk-3-ynoic acids. Thus but-3-ynoic acid is not a substrate, whereas dec-3-ynoic acid is a good substrate. Fig. 8 shows that the activity with dec-3-ynoic acid ($70\text{ nmol/min per mg}$ of microsomal protein) is approx. 30% of the activity observed with dodecanoic acid ($230\text{ nmol/min per mg}$ of microsomal protein).

In the 1 ml incubation system used for cholesterol synthesis there are 2–3 mg of microsomal protein. This means that at a concentration of dec-3-ynoic acid equal to its apparent K_1 ($40\text{ }\mu\text{M}$), the capacity of the microsomal-activating enzymes should be sufficient to achieve the rapid conversion of the acid into the CoA ester and this would produce the observed inhibition of synthesis.

Discussion

The results presented clearly indicate that alk-3-ynoyl-CoA esters are effective alkylating inhibitors of cytoplasmic acetoacetyl-CoA thiolase and that this action can lead to a selective inhibition of sterol synthesis *in vitro* between acetate and mevalonate. Although the inhibition of sterol synthesis can be correlated with the inhibition of acetoacetyl-CoA thiolase (cf. Fig. 6) it is not established whether inhibition of the enzyme in the postmitochondrial fraction is the result of alkylation or of direct inhibition of catalytic activity. In all probability inhibition results from a combination of the two processes, since it is found that the onset of inhibition is rapid

(competitive inhibition of catalytic activity) and that if a postmitochondrial fraction is incubated with alk-3-ynoyl-CoA esters then at the end of the incubation the demonstrable activity of 3-ketoacyl-CoA thiolase is decreased compared with the control (alkylation). It is possible that sterol synthesis may be additionally inhibited by the alk-3-ynoyl-CoA-dependent inhibition of β -hydroxy- β -methylglutaryl-CoA synthase (EC 4.1.3.5). This effect is feasible, since acetoacetyl-CoA thiolase and β -hydroxy- β -methylglutaryl-CoA synthase catalyse analogous reactions and β -hydroxy- β -methylglutaryl-CoA synthase also has an essential thiol which participates in the catalytic reaction (Middleton & Tubbs, 1974).

The experiments with alk-3-ynoyl-CoA esters also serve to establish the pathway for acetoacetyl-CoA formation in cholesterol biosynthesis. Occasionally, it has been suggested that this might occur through the condensation of acetyl-CoA with malonyl-CoA as in the early stages of fatty acid synthesis (Brodie *et al.*, 1963; Fimognari & Rodwell, 1970). The observation that alk-3-ynoyl-CoA esters do not inhibit fatty acid synthesis, although they are potent inhibitors of cholesterol synthesis, shows that the synthesis of acetoacetyl-CoA for cholesterol can only proceed through acetoacetyl-CoA thiolase. This result reinforces the conclusions drawn from the isotopic studies of Higgins & Kekwick (1973) and the studies with inhibitors of acetyl-CoA carboxylase (Barth *et al.*, 1973).

At present, the action of the alk-3-ynoyl-CoA esters is limited to the system *in vitro*, and their potential as inhibitors of cholesterol synthesis *in vivo* is unknown. It seems obligatory that if these compounds are to have any effect *in vivo* then they will be administered as the free acid and that conversion into the reactive CoA ester will depend on intracellular activation. Within this context it seems unlikely that short-chain alk-3-ynoic acids will be effective on cytoplasmic acetoacetyl-CoA thiolase, since they are poor substrates for the microsomal fatty acyl-CoA synthetase. They have been tested with bovine liver mitochondrial short-chain fatty acyl-CoA synthetase (P. C. Holland & D. P. Bloxham, unpublished work) and found to be moderate substrates compared with hexadi-2,4-enoic acid (sorbic acid), which suggests that they might exert intramitochondrial effects. The longer-chain alk-3-ynoic acid, dec-3-ynoic acid, seems to be more promising, since it is a reasonable substrate for the microsomal fatty acyl-CoA synthetase and the CoA ester is an effective inhibitor of the enzyme. The observation that dec-3-ynoyl-CoA inhibits cytoplasmic acetoacetyl-CoA thiolase was unexpected, since this enzyme is known to use acetoacetyl-CoA preferentially and the catalytic activity with longer-chain 3-ketoacyl-CoA esters decreases markedly (Stern & Ochoa, 1956; Middleton, 1973). The fact that inhibition is obtained with

dec-3-ynoyl-CoA probably reflects the decreased restrictions for the formation of an enzyme-inhibitor complex compared with the formation of the catalytic enzyme-substrate complex.

On a general note, it is worthwhile considering why an enzyme such as cytoplasmic acetoacetyl-CoA thiolase should be chosen as a potential target for inhibition of cholesterol synthesis. Certainly, acetoacetyl-CoA thiolase is readily inhibited by suitably designed affinity labels, which is a distinct advantage; however, generally it is considered that the site of action should be the first unique enzyme in cholesterol synthesis. Conventionally this step is considered to be β -hydroxy- β -methylglutaryl-CoA reductase, but it is quite possible that acetyl-CoA is committed to cholesterol synthesis at the stage of cytoplasmic acetoacetyl-CoA thiolase. Thus 3-ketoacyl-CoA thiolase participates in two biosynthetic routes, namely cholesterol synthesis and ketone-body formation. Both of these synthetic routes involve the formation of β -hydroxy- β -methylglutaryl-CoA from acetyl-CoA, which requires 3-ketoacyl-CoA thiolase and β -hydroxy- β -methylglutaryl-CoA synthase (Dempsey, 1974). At this point the pathways diverge, and cholesterol synthesis requires β -hydroxy- β -methylglutaryl-CoA reductase and ketone-body formation requires β -hydroxy- β -methylglutaryl-CoA lyase. Since β -hydroxy- β -methylglutaryl-CoA reductase is a microsomal enzyme (Siperstein, 1970) and β -hydroxy- β -methylglutaryl-CoA lyase is an intra-mitochondrial enzyme (Bucher *et al.*, 1960; Dempsey, 1974), this implies that β -hydroxy- β -methylglutaryl-CoA formation for cholesterol synthesis uses cytosolic acetoacetyl-CoA thiolase whereas for ketone-body formation mitochondrial 3-ketoacyl-CoA thiolase is required (Dempsey, 1974). This means that in principle, an inhibitor which is specific for cytoplasmic acetoacetyl-CoA thiolase might only inhibit cholesterol synthesis and an inhibitor specific for mitochondrial 3-ketoacyl-CoA thiolase might only inhibit ketone-body formation. Whether this degree of specificity can be achieved with the alk-3-ynoyl-CoA esters is debatable and presumably will depend on (1) the susceptibility of the various isoenzymes of 3-ketoacyl-CoA thiolase to different inhibitor chain length, (2) the ability of the inhibitor to be converted into the CoA ester and (3) the distribution of the resultant CoA ester within the cell.

One reservation to the possibility of using acetoacetyl-CoA thiolase as a target enzyme for inhibition of cholesterol synthesis is that mitochondrial 3-ketoacyl-CoA thiolase is an obligatory enzyme in β -oxidation. This raises the possibility that alk-3-ynoic acids may have the disadvantage of inhibiting fatty acid oxidation. A closely related inhibitor of 3-ketoacyl-CoA thiolase, penta-2,4-dienoyl-CoA, has been shown to inhibit mitochondrial oxidation (Holland & Sherratt, 1973; Holland *et al.*, 1973b).

The consequences of an effect on fatty acid oxidation can only be ascertained by whole-animal studies.

In conclusion, suitably chosen acetylenic fatty acids act as selective inhibitors of cholesterol synthesis *in vitro* and this may be of potential pharmacological importance if these effects can be produced *in vivo*. Certainly it is known that olefinic fatty acids have useful actions as hypocholesterolaemic agents and it will be interesting to establish whether the extra degree of unsaturation in acetylenic fatty acids will have any additional effects.

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