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The Kinetic Mechanism and Properties of the Cytoplasmic Acetoacetyl-Coenzyme A Thiolase from Rat Liver

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1. Cytoplasmic acetoacetyl-CoA thiolase was highly purified in good yield from rat liver extracts. 2. Mg^{2+} inhibits the rate of acetoacetyl-CoA thiolysis but not the rate of synthesis of acetoacetyl-CoA. Measurement of the velocity of thiolysis at varying Mg^{2+} but fixed acetoacetyl-CoA concentrations gave evidence that the keto form of acetoacetyl-CoA is the true substrate. 3. Linear reciprocal plots of velocity of acetoacetyl-CoA synthesis against acetyl-CoA concentration in the presence or absence of desulpho-CoA (a competitive inhibitor) indicate that the kinetic mechanism is of the Ping Pong (Cleland, 1963) type involving an acetyl-enzyme covalent intermediate. In the presence of CoA the reciprocal plots are non-linear, becoming second order in acetyl-CoA (the Hill plot shows a slope of 1.7), but here this does not imply co-operative phenomena. 4. In the direction of acetoacetyl-CoA thiolysis CoA is a substrate inhibitor, competing with acetoacetyl-CoA, with a K_i of 67 μM. Linear reciprocal plots of initial velocity against concentration of mixtures of acetoacetyl-CoA plus CoA confirmed the Ping Pong mechanism for acetoacetyl-CoA thiolysis. This method of investigation also enabled the determination of all the kinetic constants without complication by substrate inhibition. When saturated with substrate the rate of acetoacetyl-CoA synthesis is 0.055 times the rate of acetoacetyl-CoA thiolysis. 5. Acetoacetyl-CoA thiolase was extremely susceptible to inhibition by an excess of iodoacetamide, but this inhibition was completely abolished after preincubation of the enzyme with a molar excess of acetoacetyl-CoA. This result was in keeping with the existence of an acetyl-enzyme. Acetyl-CoA, in whose presence the overall reaction could proceed, gave poor protection, presumably because of the continuous turnover of acetyl-enzyme in this case. 6. The kinetic mechanism of cytoplasmic thiolase is discussed in terms of its proposed role in steroid biosynthesis.

Rat liver contains three classes of oxoacyl-CoA thiolase, two of which are specific for acetoacetyl-CoA and will not catalyse the thiolysis of higher homologues (Middleton, 1973). These two acetoacetyl-CoA-specific thiolases have been shown (Middleton, 1973) to differ greatly in their intracellular location, their response to univalent cations and their simple kinetic properties. The present paper reports studies on the kinetic mechanism and properties of the cytoplasmic acetoacetyl-CoA thiolase of rat liver. This is the first detailed kinetic study of a purified mammalian cytoplasmic acetoacetyl-CoA thiolase although cytoplasmic thiolases have been purified from baker's yeast (Kornblatt & Rudney, 1971) and chicken liver (Clinkenbeard *et al.*, 1973).

Experimental

Materials

Cytochrome *c* (horse heart), chymotrypsinogen A (bovine pancreas), ovalbumin, serum albumin (bovine), aldolase (EC 4.1.2.13), catalase (EC 1.11.1.6), ferritin, phosphate acetyltransferase (EC 2.3.1.8), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), CoA (grade 1), NADH (grade 1) and acetylphosphate were purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. DEAE-cellulose (Whatman grade DE-32), cellulose phosphate and cellulose powder (standard grade) were purchased from W. and R. Balston (Modified Cellulose) Ltd., Maidstone, Kent, U.K. Sephadex G-25 (medium grade) and G-200 were obtained from Pharmacia, Uppsala, Sweden.

Methods

Calcium phosphate gel was prepared by the method of Keilin & Hartree (1938). Acetyl-CoA was prepared by the method of Simon & Shemin (1953), acetoacetyl-CoA by the method of Wieland & Rueff (1953) and desulpho-CoA by the method of Chase *et al.* (1966). All these CoA derivatives were purified by chromatography on DEAE-cellulose as described by Moffatt & Khorana (1961) for CoA. Bromoacetyl-CoA was

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prepared by the method of Chase & Tubbs (1969). Pantetheine and acetoacetyl-pantetheine were prepared as described by Gehring *et al.* (1968). CoA and pantetheine were assayed by their thiol content by the method of Ellman (1959); the purity of the CoA was checked by the use of phosphate acetyltransferase (Michal & Bergmeyer, 1963). Acetyl-CoA was assayed by the method of Chase (1967). Acetoacetyl-CoA and acetoacetyl-pantetheine were determined by the method of Decker (1963). Bromoacetyl-CoA was determined by the method of Chase & Tubbs (1969) and desulpho-CoA was assayed by its E_{260}, by using the extinction coefficient for adenine nucleotides of 16.4×10^3 litre·mol^{-1}cm^{-1} (Stadtman, 1957). Protein concentration was determined by the biuret method (Gornall *et al.*, 1949) or by the direct spectrophotometric method of Warburg & Christian (1941).

Assays of thiolase activity. The standard assay was that described by Middleton (1973). The enzyme activity was determined at 30°C by following the stimulation of acetoacetyl-CoA breakdown (measured at 303nm) caused by the addition of CoA (to a final concentration of 50μM) to a cuvette of 1cm path length containing 100mM-Tris–HCl, pH8.1, 25mM-$MgCl_2$, 50mM-KCl, 10μM-acetoacetyl-CoA and enzyme in a total volume of 2.0ml. The apparent extinction coefficient of acetoacetyl-CoA was 16.9×10^3 litre·mol^{-1}·cm^{-1}. The K^+ neither activates nor inhibits the cytoplasmic acetoacetyl-CoA thiolase.

During the purification cytoplasmic acetoacetyl-CoA was determined as a routine at 30°C by following the stimulation of acetoacetyl-pantetheine breakdown (measured at $\lambda_{max.}$ of acetoacetyl-pantetheine, 300nm) caused by the addition of pantetheine (to a final concentration of 300μM) to a cuvette of 1cm path length containing 100mM-Tris–HCl, pH8.1, 74μM-acetoacetyl-pantetheine and enzyme in a total volume of 2ml. Under these conditions the apparent extinction coefficient of acetoacetyl-pantetheine was 5.3×10^3 litre·mol^{-1}·cm^{-1}.

Kinetic studies of acetoacetyl-CoA thiolysis were carried out under the standard assay conditions (above) at the appropriate concentrations of substrates. Assays were started by the addition of CoA or enzyme. For studying the reaction in the direction of acetoacetyl-CoA synthesis the same buffer, pH and ionic constituents were used, but the sole substrate was acetyl-CoA and the reaction was started by the addition of enzyme. This assay could not be used for studying the effects of CoA on the rate of acetoacetyl-CoA synthesis, owing to the unfavourable equilibrium for the overall reaction (see the Results Section), and so the reaction was made irreversible by coupling to the 3-hydroxybutyryl-CoA dehydrogenase reaction (Lynen & Wieland, 1955):

Acetoacetyl-CoA + NADH + H^+ →
3-hydroxybutyryl-CoA + NAD^+

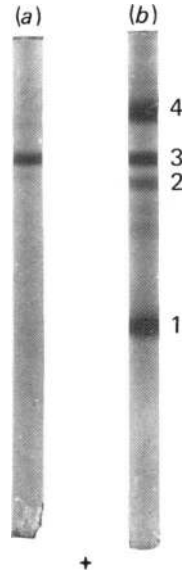
The reaction was followed at 340nm by the oxidation of NADH at 30°C in a cuvette of 1cm path-length containing 100mM-Tris–HCl, pH8.1, 50mM-KCl, 154μM-NADH, 10μg of purified 3-hydroxyacyl-CoA dehydrogenase and the required amounts of acetyl-CoA (and CoA or other CoA derivative if desired) in a 2ml total. The reaction was started by the addition of thiolase and the initial rate was proportional to enzyme concentration. With the exception of NADH and Mg^{2+} (for the latter see the Results section) the coupled assay system is identical in composition with the standard assay and the presence of NADH had no effect on thiolase activity when measured by acetoacetyl-CoA breakdown or synthesis in the standard assay. Further, the values of V and K_m for acetyl-CoA determined by the coupled assay and the direct assay were identical.

Enzyme units and expression of kinetic results. An amount of enzyme catalysing the cleavage of 1μmol of acetoacetyl-CoA/min under the standard assay conditions described constitutes one unit of activity, and caused the thiolysis of 0.7μmol of acetoacetyl-pantetheine/min in the routine assay (above) used for monitoring enzyme purification. Kinetic results in Figures and Tables are expressed with velocities corrected to 10munits of enzyme per cuvette.

Purification of the cytoplasmic acetoacetyl-CoA thiolase

Rat livers (freshly excised) were homogenized in a Waring Blender in 5–10vol. of cold 0.25M-sucrose (no EDTA) for 30s at the low-speed setting. The homogenate was then centrifuged at 12000*g* for 10min and the supernatant retained. Alternatively the 10000*g* supernatant of a preparation of rat liver mitochondria can be used as a starting material. The murky supernatant was adjusted to pH5.5 (at 4°C) with 1M-acetic acid and stirred for 5min, then centrifuged at 2000*g* for 10min. The clear supernatant was decanted off and rapidly adjusted with 1M-Tris base to pH7.2 (at 4°C). The neutralized clarified extract was then made 0.5mM with respect to dithiothreitol and stored frozen at −20°C. At this temperature the activity remained constant for at least 1 year. If loss of activity occurred it could be recovered by adding dithiothreitol (final concn. 1mM) and leaving at room temperature for 30min.

DEAE-cellulose chromatography. The neutralized pH5.5 supernatant was allowed to warm at 20°C and then adjusted to pH8.2 with 1M-Tris base. Sufficient 0.5M-Tris–HCl, pH8.2, was added to give a final concentration of 50mM. The extract was then applied to a short column (length:diameter ratio $\leq$ 2:1) of DEAE-cellulose, previously equilibrated at 20°C with 100mM-Tris–HCl, pH8.2, containing 0.5mM-dithiothreitol, at a loading ratio of 200mg of protein/10ml of packed DEAE-cellulose. After application of the extract the column was washed with at least 2



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of purified cytoplasmic acetoacetyl-CoA thiolase

Purified thiolase (*a*) (10–20 μg) and the marker polypeptides (*b*) (lysozyme, 1; pepsin, 2; carboxymethyl-aldolase, 3; bovine serum albumin, 4) were treated with sodium dodecyl sulphate and 2-mercaptoethanol, subjected to electrophoresis in polyacrylamide gels and stained for protein as described by Weber & Osborn (1969).

Table 1. *Purification of cytoplasmic acetoacetyl-CoA thiolase from rat liver*

This is a typical result starting from 132g of fresh rat liver. Experimental details are given in the text. Specific activity is expressed in μmol of acetoacetyl-pantetheine removed/min per mg of protein.

Procedure	Volume (ml)	Protein concn. (mg/ml)	Specific activity (μmol/min per mg of protein)	Yield (%)
12000*g* supernatant	1000	21	0.01	(100)
pH5.5 supernatant	900	6.2	0.035	90
DEAE-cellulose column	400	3.5	0.105	68
Calcium phosphate column	250	0.48	0.735	41
Cellulose phosphate column	4.2	0.45	37	32

column vol. of the equilibrating buffer. Cytoplasmic acetoacetyl-CoA thiolase was then eluted with 200mM-Tris–HCl, pH8.2, containing 0.5mM-dithiothreitol. This procedure gives complete separation from any contaminating mitochondrial thiolase activities (Middleton, 1973). The eluate could be stored overnight at room temperature without loss of activity.

Chromatography on calcium phosphate. This was carried out at room temperature. Calcium phosphate gel (880mg dry wt.) was mixed into a slurry with 10g of cellulose powder (Whatman standard grade, ashless) and poured to give a column of equal length and diameter. The DEAE-cellulose eluate was applied to this column such that loading of enzyme did not exceed an activity of 1μmol of acetoacetyl-pantetheine/min per ml of column volume. The column was then washed through with 0.5mM-dithiothreitol until no more protein was eluted and then the cytoplasmic thiolase was eluted with 10mM-sodium phosphate, pH6.6 containing 0.5mM-dithiothreitol. The eluate was stable for at least 1 day at room temperature.

Chromatography on cellulose phosphate. This was carried out at room temperature. The eluate from the previous column was adjusted to 50mM with strong sodium phosphate buffer, pH6.6, and was then applied to a short column (length:diameter ratio $\leqslant 2:1$) of cellulose phosphate previously equilibrated with 50mM-sodium phosphate, pH6.6, containing 0.5mM-dithiothreitol. At least 2mg of protein could be applied/ml of packed ion exchanger. The column was washed with 2vol. of the phosphate buffer before starting a linear gradient (4 column vol. total) for 50–250mM-sodium phosphate, pH6.6, containing 0.5mM-dithiothreitol. The purified cytoplasmic thiolase was eluted at a phosphate concentration of approx. 150mM. The enzyme at this stage had a specific activity of 40–60μmol of acetoacetyl-pantetheine removed/min per mg. The purification is summarized in Table 1. The enzyme could be concentrated by precipitation with 70% satd. $(NH_4)_2SO_4$ or by chromatography on a small DEAE-cellulose column under the conditions of pH and ionic strength used in the above purification. Solutions of the enzyme were made up to 50% (v/v) with glycerol and stored in the presence of 0.5mM-dithiothreitol at −25°C. Both these solutions and the $(NH_4)_2SO_4$ precipitates were stable when stored under these conditions.

Purity of the enzyme. After treatment with sodium dodecyl sulphate and 2-mercaptoethanol (Pringle, 1970) the cytoplasmic thiolase from the final stage of the purification was subjected to electrophoresis in polyacrylamide gels by the method of Shapiro *et al.* (1967) as modified by Weber & Osborn (1969). The following marker polypeptides were also run: lysozyme, pepsin, carboxymethyl-aldolase and bovine serum albumin. Cytoplasmic thiolase gave a single protein-staining band (Plate 1) with the same mobility as carboxymethylaldolase. The subunit molecular weight of the latter is 44000 (I. Gibbons, personal communication), which must also be the subunit molecular weight of the cytoplasmic thiolase.

The molecular weight of the native thiolase was determined by gel filtration on Sephadex G-200 (Andrews, 1965). The column (0.65cm×130cm, supplied by Cambridge Biolab, Cambridge, U.K.) was equilibrated with 100mM-Tris–HCl, pH8.0, containing 0.1mM-EDTA and was calibrated with cytochrome *c*, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase and ferritin. The molecular weight of purified cytoplasmic thiolase as determined from its elution volume was 170000± 20000.

As a result of these analyses we can deduce that the purification used here yields a homogeneous preparation of rat liver cytoplasmic acetoacetyl-CoA thiolase, the native molecule of which is composed of four subunits of molecular weight 44000.

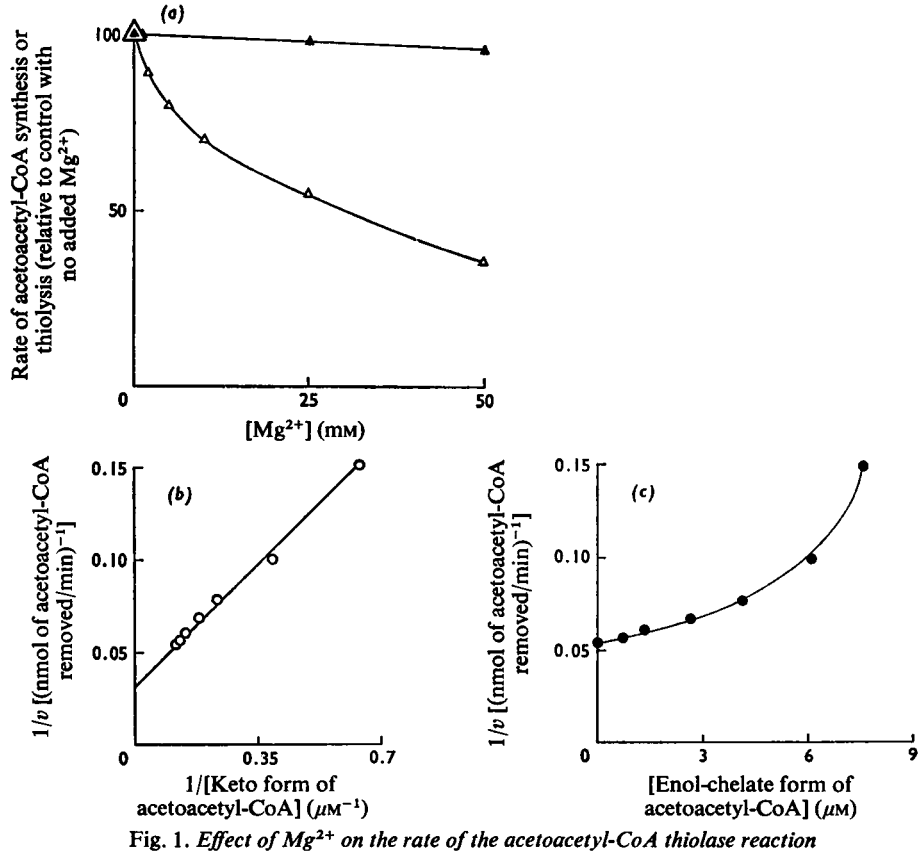


Fig. 1. *Effect of Mg^{2+} on the rate of the acetoacetyl-CoA thiolase reaction*

(*a*) Effect of Mg^{2+} concentration on the initial rates of acetoacetyl-CoA thiolysis (△) and acetoacetyl-CoA synthesis (▲). Results are corrected, where appropriate, for the change in apparent extinction coefficient of acetoacetyl-CoA. Acetoacetyl-CoA thiolysis was measured under standard assay conditions and the initial rate in the absence of Mg^{2+} was 18.3 nmol/min per 10 munit of enzyme. Acetoacetyl-CoA synthesis was measured at the same pH and ionic conditions by the coupled assay described in the Methods section with acetyl-CoA at 97 μM. The initial rate of acetoacetyl-CoA synthesis in the absence of Mg^{2+} was 1.75 μmol/min per 10 munit. (*b*) The reciprocal of the initial rate of acetoacetyl-CoA thiolysis (above) expressed as a function of the reciprocal calculated concentration of the keto form of acetoacetyl-CoA. (*c*) As (*b*) but the initial rates of acetoacetyl-CoA thiolysis are expressed as a function of the calculated concentration of the chelate form of acetoacetyl-CoA.

Results and Discussion

Effect of Mg^{2+} on the thiolase reaction rate

This was investigated in the directions of acetoacetyl-CoA synthesis and acetoacetyl-CoA thiolysis. The results (Fig. 1*a*) demonstrate the absence of any effect of Mg^{2+} on the rate of the condensation reaction (acetoacetyl-CoA synthesis), but there is considerable inhibition of the acetoacetyl-CoA-cleavage reaction. The lack of any effect on the condensation reaction suggests that the inhibition of thiolysis was not due to an interaction between enzyme and Mg^{2+} and that the decrease in velocity seen in Fig. 1(*a*) must be due to either the increase in concentration of a reversible inhibitor or the decrease in concentration of the true substrate, both these changes being linked to the change in Mg^{2+} concentration. As Stern (1956) has shown, at pH values greater than 7.5 Mg^{2+} interacts with the enol form of acetoacetyl-CoA to form a chelate, thus decreasing the amount of free keto form present. Stern (1956) has calculated the constants controlling this process and by using his values it was possible to calculate (Table 2) the concentrations of free keto, enol and chelate forms of acetoacetyl-CoA at the different Mg^{2+} concentrations used in Fig. 1(*a*). As the Mg^{2+} concentration rises the proportion of acetoacetyl-CoA found in the keto form falls steadily, that of the chelate form rises and that of the enol form rises slightly and then falls. The fall in rate of acetoacetyl-CoA thiolysis could be

Table 2. *Calculated composition of acetoacetyl-CoA solutions in the presence of increasing Mg^{2+} concentration*

The concentrations of free keto, free enol and chelate forms of acetoacetyl-CoA were calculated from the effect of Mg^{2+} on the absorbance of 10μM-acetoacetyl-CoA (under the standard assay conditions) by using the method of Stern (1956).

Concn. of Mg^{2+} (mM)	Concn. of various forms of acetoacetyl-CoA (μM)		
	Keto	Enol	Chelate
0	8.47	1.53	0
1	7.55	1.71	0.74
2	6.76	1.92	1.32
5	5.40	1.97	2.63
10	4.23	1.64	4.13
25	2.52	1.33	6.15
50	1.55	0.89	7.56

most simply explained if the keto form of acetoacetyl-CoA was the true substrate. This is confirmed by Fig. 1(*b*), which presents the velocity data of Fig. 1(*a*) replotted in reciprocal form against the reciprocal concentration of the free keto tautomer (calculated in Table 2) and yields the usual straight-line relationship between substrate concentration and enzyme rate. In contrast with this there is no simple relationship between the velocity and the calculated concentrations of free enol or chelate forms; Fig. 1(*c*) shows that the velocity decreases with increasing amounts of chelate, but not in the way expected for a simple reversible inhibitor. It can be concluded therefore that the true substrate form of acetoacetyl-CoA for the thiolase reaction is the keto tautomer and that the inhibitory effect of Mg^{2+} is due to the depletion of this form to give the enol-chelate.

Equilibrium constant for the thiolase reaction

The apparent equilibrium constant for this reaction varies with the pH and Mg^{2+} concentration (Goldman, 1954; Stern, 1956) and it was therefore determined under the conditions of the standard assay used in the present study. The equilibrium concentration of acetoacetyl-CoA was determined spectroscopically (Fig. 2) after enzymic synthesis from acetyl-CoA at three different initial concentrations, in both the presence and the absence of CoA and acetoacetyl-CoA. The summary of the results for K are shown in Table 3. The average value of K in the direction of acetoacetyl-CoA synthesis was 128×10^{-6} under the standard conditions used here.

Kinetics of acetoacetyl-CoA synthesis

In this reaction two molecules of acetyl-CoA condense to give one molecule of acetoacetyl-CoA and one of CoA. The general rate equation for ordered two-substrate reactions

$$\frac{1}{v}=\frac{1}{V}\left(1+\frac{K_m^A}{[A]}+\frac{K_m^B}{[B]}+\frac{K^{AB}}{[A][B]}\right) \quad (1)$$

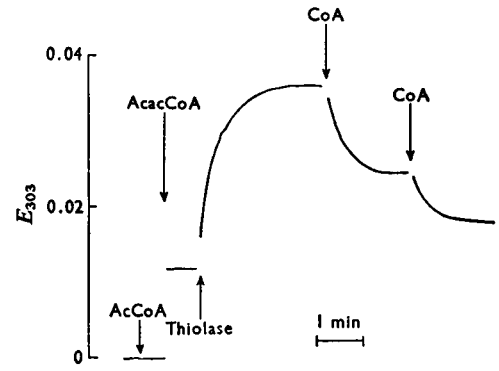


Fig. 2. *Determination of the equilibrium constant of the thiolase reaction under the standard assay conditions*

A 1cm path-length cell contained 194.6μM-purified acetyl-CoA (AcCoA) in 2ml of standard assay buffer (100mM-Tris–HCl, pH8.1, 25mM-$MgCl_2$, 50mM-KCl) at 30°C. After the addition of purified acetoacetyl-CoA (AcacCoA) (1.48nmol), thiolase was added as indicated to initiate acetoacetyl-CoA synthesis from the acetyl-CoA already present. When equilibrium was reached portions of CoA (5nmol) were added to reverse the reaction.

Table 3. *Determination of the equilibrium constant for the thiolase reaction*

This was determined under the conditions of the standard assay with purified reactants and enzyme. Measurements were made of the equilibrium concentration of the total acetoacetyl-CoA species present (determined from the absorbance change at 303nm) at different initial values of acetyl-CoA and acetoacetyl-CoA. After the equilibrium was reached a new equilibrium was then established by the addition of various amounts of CoA. The K values for acetoacetyl-CoA synthesis were calculated from the different equilibrium concentrations of the total acetoacetyl-CoA species present and are expressed as means ±S.D. with the numbers of determinations in parentheses.

Concn. of initial reactant (μM)			
Acetyl-CoA	CoA	Acetoacetyl-CoA (total species present)	$10^6\times K$
48.6	0	0	143
97.3	0–1.88	0–0.74	130±35 (10)
194.5	0–12	0–0.74	124±25 (12)
		Overall mean:	128±28 (23)

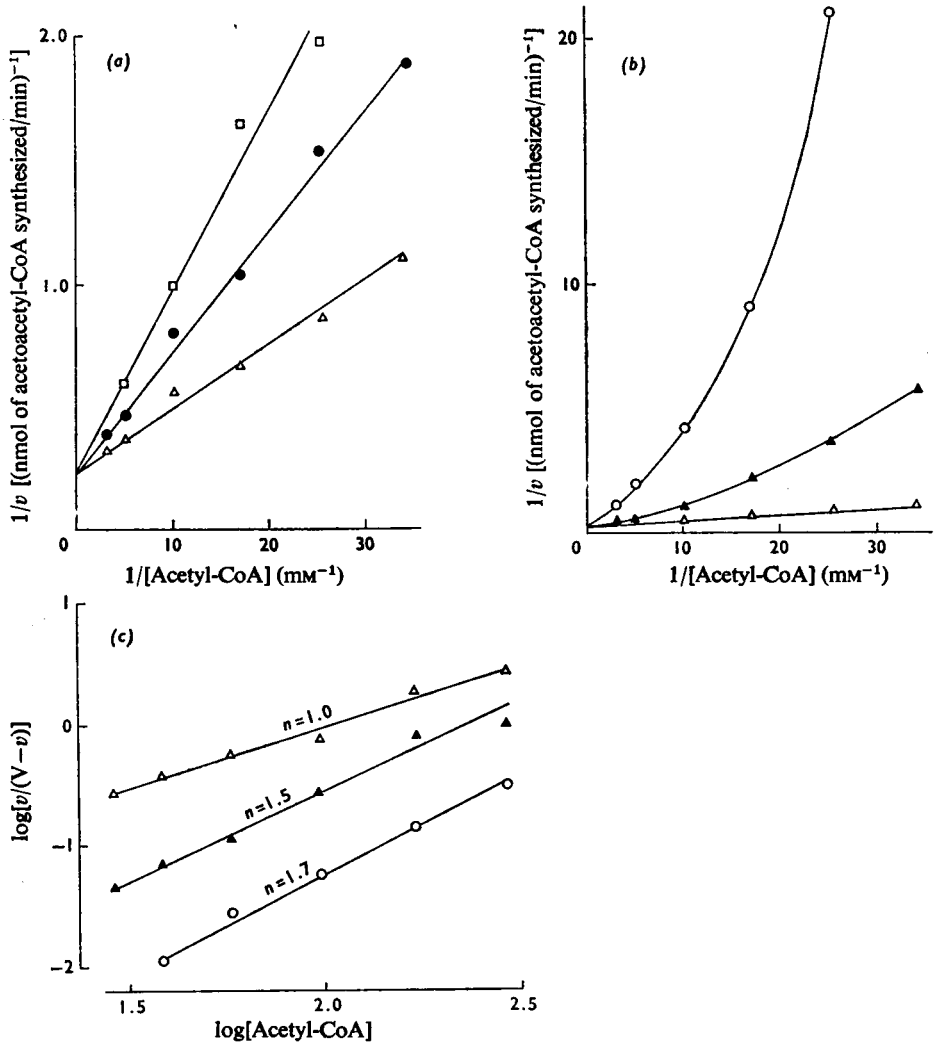


Fig. 3. *Effect of desulpho-CoA and CoA on the acetoacetyl-CoA thiolase-catalysed synthesis of acetoacetyl-CoA from acetyl-CoA*

Reciprocal velocity is expressed as a function of the reciprocal acetyl-CoA concentration. The coupled assay system (described in the text) was used to measure velocities. (*a*) Desulpho-CoA concentrations: (△) 0, (●) 300 μM; (□) 600 μM. (*b*) CoA concentrations: (△) 0; (▲) 5 μM; (○) 25 μM. (*c*) Data of (*b*) redrawn as a Hill plot with slope n_H.

includes a binary term $K^{AB}/[A][B]$, which becomes negligible only when substrate binding to enzyme (in the absence of products) can be regarded as irreversible (Cleland, 1970). In the case under study here A and B are identical molecules (acetyl-CoA) and therefore eqn. (1) should be rewritten

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_m^{A1}}{[A]} + \frac{K_m^{A2}}{[A]} + \frac{K^{AA}}{[A]^2}\right) \qquad (2)$$

[where K_m^{A1} is the K_m value for the first molecule of acetyl-CoA (A) to bind to the enzyme and K_m^{A2} refers to the second molecule that binds]. Here we have a term in $1/[A]^2$ which (if it is of significant magnitude) will cause the plot of $1/v$ against 1/[acetyl-CoA] to become parabolic instead of linear. As shown in Fig 3(*a*) the reciprocal plot appears to be linear for 1/[acetyl-CoA] over an 11-fold range of substrate concentration and a 4-fold velocity range. Further, the presence of the chemically unreactive product analogue desulpho-CoA (Chase *et al.*, 1966), which acts as a competitive inhibitor and therefore increases the slope of the reciprocal plots (Fig. 3*a*), does not

cause any significant departure from linearity. The term in $1/[A]^2$ of eqn. (2) must therefore be negligible for this reaction and we can conclude that acetyl-CoA binding to the enzyme (in the absence of products, as here) is irreversible. In view of the established mechanism of action (Gehring *et al.*, 1968) of the pig heart acetoacetyl-CoA thiolase (a mitochondrial thiolase; Middleton, 1973) involving the formation of a stable acetyl-thiolase derivative, we might explain the apparent irreversibility of acetyl-CoA binding to the cytoplasmic acetoacetyl-CoA thiolase as being due to the formation of the same derivative:

$$\text{Acetyl-CoA} + \text{E} \rightleftharpoons \text{acetyl-E} + \text{CoA} \qquad (3)$$

$$\text{Acetyl-E} + \text{acetyl-CoA} \rightleftharpoons \text{E} + \text{acetoacetyl-CoA} \qquad (4)$$

In the absence of CoA or acetoacetyl-CoA reversal of reactions (3) and (4) would not occur and the binding of acetyl-CoA in eqn. (3) or (4) would appear to be irreversible. However, if CoA is present the partial reaction (3) can reverse, in which case the term in $1/[A]^2$ of eqn. (2) would become significant. This is illustrated in Fig. 3(*b*), where increasing concentrations of CoA cause increasing non-linearity of the reciprocal plot. When expressed as a Hill plot (Fig. 3*c*) the increasing value of n_H (the slope) with increasing [CoA] illustrates the growth in significance of the term in $1/[A]^2$. In this case therefore a value of n_H greater than unity does not imply any co-operative interactions between cytoplasmic thiolase and its substrates or products; the non-linearity of the reciprocal plot in the presence of CoA is a simple consequence of the reaction mechanism.

Because of the non-linearity caused by CoA, its K_i could not be determined but it is clearly an effective inhibitor of the cytoplasmic thiolase. The K_i value for desulpho-CoA (from Fig. 3*a*) was 300 μM and the apparent K_m for acetyl-CoA was 115 μM. It is worth noting that the apparent K_m for acetyl-CoA is the sum of two true K_m values, $K_m^{A1} + K_m^{A2}$ of eqn. (2).

Kinetics of acetoacetyl-CoA thiolysis

The mechanism suggested in eqn. (3) and (4) implies for the direction of acetoacetyl-CoA thiolysis a velocity equation of the form

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_m^A}{[A]} + \frac{K_m^B}{[B]}\right) \qquad (5)$$

where A and B are acetoacetyl-CoA and CoA respectively. There is no term here in 1/[A][B], and a kinetic investigation in which the concentrations of each substrate are varied independently should give a family of parallel lines when presented as a reciprocal plot. But the high substrate inhibition by CoA (Middleton, 1973) shown in Fig. 4(*a*) made this difficult to observe. The inhibition by CoA was competitive with respect to acetoacetyl-CoA (Fig. 4*b*). The K_i for the abortive binding of CoA calculated from Fig. 4(*b*) is 60 μM. Cleland (1970) has stated that the existence of such competitive substrate inhibition is itself characteristic of mechanisms (e.g. eqn. 5) in which terms in 1/[A][B] are absent. Based on this evidence the expression describing the initial velocity of acetoacetyl-CoA thiolysis should then be written:

$$\frac{1}{v} = \frac{1}{V}\left\{1 + \frac{K_m^A}{[A]}\left(1 + \frac{[B]}{K_i^B}\right) + \frac{K_m^B}{[B]}\right\} \qquad (6)$$

where K_i^B represents the dissociation constant of the abortive complex between enzyme and CoA (B) and all the other constants are as in eqn. (5). The data in

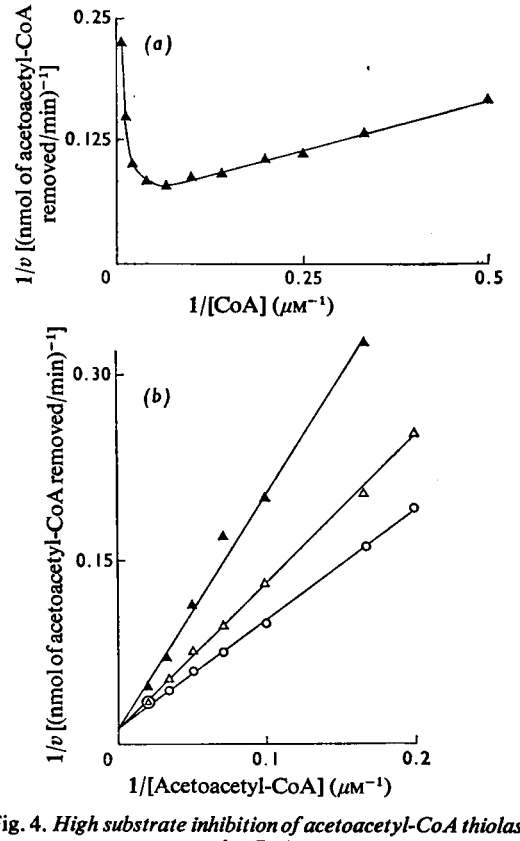


Fig. 4. *High substrate inhibition of acetoacetyl-CoA thiolase by CoA*

(*a*) Reciprocal rate of acetoacetyl-CoA thiolysis is a function of the reciprocal CoA concentration under standard assay conditions (10 μM-acetoacetyl-CoA). (*b*) Double-reciprocal plot of rate of acetoacetyl-CoA thiolysis against acetoacetyl-CoA concentration at different CoA concentrations: (○) 50 μM, (△) 100 μM, (▲) 200 μM.

Fig. 4(*b*) fit the situation, in eqn. (6), in which the term $K_m^B/[B]$ becomes insignificant owing to the high concentrations of B used. We can therefore calculate from Fig. 4(*b*) that $V = 76.5$ nmol of acetoacetyl-CoA removed/min per 10 munits of enzyme and that K_m^A (the K_m for acetoacetyl-CoA at infinite concentration of CoA) is 33 μM.

The absence of a binary term (i.e., in 1/[A][B]) from eqn. (6) can be tested by a method that will not be affected by the substrate inhibition. If substrates A and B are mixed in the ratio A/B = n and then the initial velocity is measured at different initial concentrations of A or B in this mixture, eqn. (6) can be rewritten with respect to 1/[A]:

$$\frac{1}{v} = \frac{1}{V}\left\{1 + \frac{K_A}{nK_i^B} + \frac{1}{[A]}(K_A + nK_B)\right\} \qquad (7)$$

or with respect to 1/[B]:

$$\frac{1}{v} = \frac{1}{V}\left\{1 + \frac{K_A}{nK_i^B} + \frac{1}{[B]}\left(\frac{K_A}{n} + K_B\right)\right\} \qquad (8)$$

These equations are both linear in reciprocal substrate. If a binary term were present in the original velocity equation then terms in $1/[A]^2$ or $1/[B]^2$ would be introduced and the reciprocal plots of velocity against each component of the substrate mixture would no longer be linear. Figs. 5 and 6 show that linearity is found over very wide ranges of substrate concentration and velocity for a very wide range of compositions of the substrate mixture ($n = 0.057$–4.0). This establishes the absence of any significant binary term in the velocity equation and is therefore consistent with the mechanism proposed in eqns. (3) and (4) and its kinetic formulation in eqn. (6).

The data in Fig. 5 yield a family of lines whose slope decreases with increasing values of n and which intersect to the left of the ordinate. When expressed with respect to the other substrate (acetoacetyl-CoA) (Fig. 6) the same data show no common intersection point (owing to overcrowding of data the results for only three values of n are shown in Fig. 6). This qualitative pattern is predicted by the mechanism given in eqns. (6), (7) and (8) (see the Appendix for further details). The intercepts of the lines shown in Figs. 5 and 6 should be linear functions of $1/n$, as shown in Fig. 7(*a*). The deviation from this prediction when $n = 4$ must represent the appearance of high substrate inhibition by acetoacetyl-CoA (see the Appendix for details), but this is clearly insignificant at lower values of n. The slopes (calculated in terms of the relevant substrate) from Figs. 5 and 6 are linearly related to n and $1/n$ (see Figs. 7*b* and 7*c*). From the secondary plots (Fig. 7) all the kinetic constants defining eqn. (6) can be calculated (see the Appendix for further details). The values of the

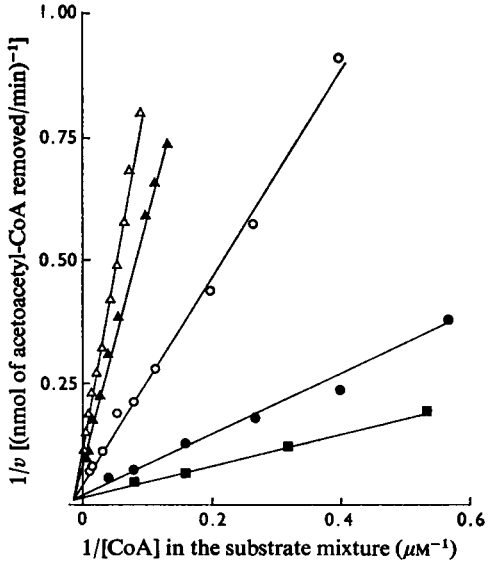


Fig. 5. *Rate of acetoacetyl-CoA thiolysis expressed as a function of the concentration of the CoA component of mixtures of acetoacetyl-CoA+CoA*

Reciprocal velocity is expressed as a function of the reciprocal CoA concentration in mixtures of acetoacetyl-CoA+CoA whose ratio of acetoacetyl-CoA/CoA (n) varies as follows: (■) $n = 2$; (●) $n = 1$; (○) $n = 0.2$; (▲) $n = 0.1$; (△) $n = 0.057$. Standard assay conditions of pH, temperature and ionic strength were used throughout.

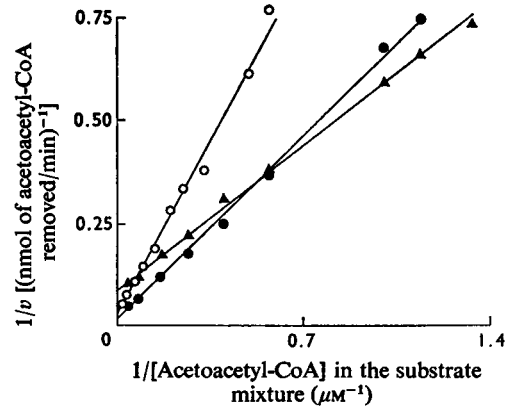


Fig. 6. *Rate of acetoacetyl-CoA thiolysis expressed as a function of the concentration of the acetoacetyl-CoA component of mixtures of acetoacetyl-CoA+CoA*

Reciprocal velocity is expressed as a function of the reciprocal acetoacetyl-CoA concentration in mixtures of acetoacetyl-CoA +CoA whose ratio of acetoacetyl-CoA/CoA (n) varies as follows: (▲) $n = 0.1$; (●) $n = 1$; (○) $n = 4$. Conditions were as in Fig. 5.

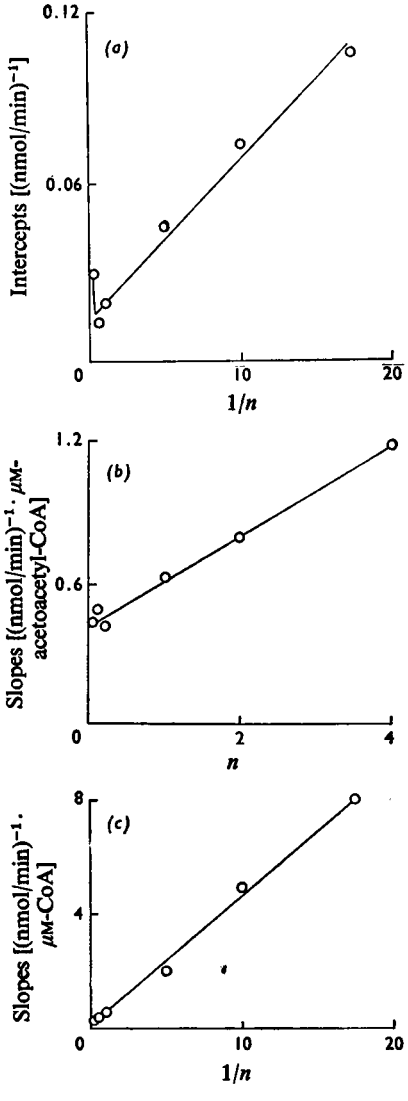


Fig. 7. *Secondary plots of slopes and intercepts from the results in Figs. 5 and 6*

(*a*) Intercepts plotted as a function of the reciprocal of the acetoacetyl-CoA/CoA ratio (*n*); (*b*) slopes (with respect to [acetoacetyl-CoA]) plotted as a function of *n*; (*c*) slopes (with respect to [CoA]) plotted as a function of the reciprocal of *n*.

constants are summarized in Table 4, and comparison with the values for the same constants independently derived from the data of Fig. 4(*b*) establishes the validity of the above approach. The complete kinetic mechanism proposed for the rat liver cytoplasmic acetoacetyl-CoA thiolase (omitting the possible abortive complex responsible for substrate inhibition by acetoacetyl-CoA) is shown in Scheme 1. The corresponding initial-rate equation for the direction of acetoacetyl-CoA thiolysis is given below:

$$v = \frac{V}{1 + \frac{K_m^{AcacCoA}}{[AcacCoA]}\left(1 + \frac{[CoA]}{K_i^{CoA}}\right) + \frac{K_m^{CoA}}{[CoA]}} \quad (9)$$

where AcacCoA is acetoacetyl-CoA, K_i^{CoA} is the dissociation constant of the abortive complex between enzyme and CoA responsible for competitive substrate inhibition by CoA, and the other constants have their usual significance.

Irreversible inhibition of cytoplasmic thiolase

Under standard assay conditions low concentrations (5 μM) of iodoacetamide inactivate cytoplasmic thiolase with a $t_{\frac{1}{2}}$ of 3 min (Fig. 8). Neither desulpho-CoA (110 μM) nor 3-oxodecanoyl-CoA (10 μM) gave any significant protection, but the presence of acetyl-CoA (10 μM) increased the $t_{\frac{1}{2}}$ to 8 min. Higher concentrations of acetyl-CoA gave better protection. However, acetoacetyl-CoA at 2 or 10 μM gave extremely good protection, increasing the $t_{\frac{1}{2}}$ to more than 100 min. These results are good evidence in support of the proposed mechanism if one proposes that the group attacked by iodoacetamide is also that which is acetylated by acetoacetyl-CoA (eqn. 4) or acetyl-CoA (eqn. 3). Lack of protection by desulpho-CoA or a bulky acetoacetyl-CoA homologue indicates that neither CoA moiety nor acyl moiety alone is sufficient to prevent inactivation. The poor protection by acetyl-CoA (at 10 μM) represents the turnover of the acetyl-enzyme (eqn. 3 plus eqn. 4) continually exposing the susceptible group. By contrast the acetyl-enzyme derived from acetoacetyl-CoA cannot turn over in the absence of added second substrate (CoA) and protection must therefore be extremely efficient.

Significance of the kinetic mechanism in vivo

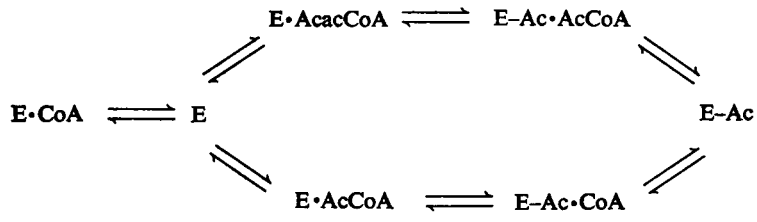
Middleton (1973) proposed that the mammalian cytoplasmic acetoacetyl-CoA thiolase is involved in the pathway of steroid biosynthesis, catalysing the synthesis of cytoplasmic acetoacetyl-CoA for subsequent conversion into 3-hydroxy-3-methylglutaryl-CoA etc. In the direction of acetoacetyl-CoA synthesis the equilibrium constant is unfavourable ($K = 128 \times 10^{-6}$ under the conditions of this study) and the maximum velocity of this enzyme for acetoacetyl-CoA synthesis is only 0.055 times the maximum velocity for thiolysis of acetoacetyl-CoA (Table 4). The published activity of the cytoplasmic thiolase in rat liver (Middleton, 1973) is 3.55 units/g fresh wt. (measured under the standard assay conditions in the direction of thiolysis). From the data of Table 4 the

Table 4. *Kinetic parameters for the cytoplasmic acetoacetyl-CoA thiolase from rat liver*

These were calculated from the data of Figs. 2 and 6 and were measured under the standard assay conditions of pH, temperature and ionic strength for both directions of the reaction (for further details see the Methods section). K_m values in the direction of acetoacetyl-CoA thiolysis are those that apply at an infinite concentration of the other substrate. K_m and K_i values are expressed in μM and those for acetoacetyl-CoA refer to the concentration of total acetoacetyl-CoA species present. The maximum velocity, V, is expressed in nmol of acetoacetyl-CoA synthesized or cleaved/min per 10munit of thiolase. See the text for further details.

		Acetoacetyl-CoA		CoA		Acetyl-CoA
Direction of reaction	V	K_m	K_i*	K_m	K_i*	K_m
Acetoacetyl-CoA synthesis	4.2	—	—	—	—	115
Acetoacetyl-CoA thiolysis	76.8	33	>133†	15	73	—

* Dissociation constants of abortive complexes causing substrate inhibition.
† See the Appendix for how this estimate was obtained.



Scheme 1. *Proposed kinetic mechanism for the cytoplasmic acetoacetyl-CoA thiolase from rat liver*

The following abbreviations are used: E, cytoplasmic thiolase; E–Ac, the acetyl-thiolase covalent intermediate; AcCoA, acetyl-CoA; AcacCoA, acetoacetyl-CoA.

maximum capacity for acetoacetyl-CoA synthesis (saturating concentrations of acetyl-CoA) can therefore be calculated to be 1.49 μmol/min per g fresh wt. of liver. This high activity (equivalent to an acetyl flux of almost 3 μmol/min per g of tissue) is far greater than the rates of acetyl incorporation into steroid *in vivo* or *in vitro* in perfused livers (23 nmol/min per g fresh wt. of liver; Brunengraber *et al.*, 1973), but it is unlikely that this capacity is normally achieved because of the kinetic properties of the cytoplasmic thiolase (see Fig. 3) and the probable concentrations of acetyl-CoA and CoA in cytoplasm. Williamson (1969) has calculated that in the cytoplasm of lactate-perfused rat livers the concentrations of acetyl-CoA and CoA are 62 μM and 104 μM respectively. It is possible to calculate, from the data of Fig. 3(*b*) and the value (above) for the maximum acetyl flux through the cytoplasmic thiolase step, that the rate of acetyl-CoA incorporation into acetoacetyl-CoA under these conditions is 42 nmol/min per g fresh wt. of liver. Brunengraber *et al.* (1973) have shown that sterol synthesis *in vivo* is depressed by 24 h starvation to 3.5 nmol of acetyl group incorporated/min per g fresh wt. of liver and that this must be due to an alteration in the supply of substrate to the pathway. Thus they find that the rate of steroid synthesis in perfused liver taken from these starved animals is normal. The approximate concentrations of acetyl-CoA and CoA in liver cytoplasm under starvation conditions *in vivo* can be estimated from the work of Williamson (1969), who calculated these quantities to be 15 μM and 47 μM respectively in the cytoplasm of rat livers perfused with oleate. The resultant acetyl flux through the cytoplasmic thiolase reaction for these concentrations can be calculated from the data of Fig. 3(*b*) to be 15 nmol of acetyl groups/min per g fresh wt. of liver.

These estimates of the flux *in vivo* through the cytoplasmic thiolase step give values that are far below the measured maximum tissue capacity for the reaction and approach the observed rates of steroid biosynthesis *in vivo*. This low thiolase activity is entirely due to the fact that the velocity at low concentrations of acetyl-CoA and high ratios of CoA/acetyl-CoA becomes proportional to [acetyl-CoA]^2, as seen from the slopes of the Hill plots (Fig. 3*c*). It is particularly noteworthy that this apparent allosteric behaviour in the presence of CoA finds a simple kinetic explanation in the fact that CoA is the product of a reversible condensation between two identical molecules, hence the tendency to second-order Hill plots in the presence of CoA. Dugan *et al.* (1972) have shown that the low

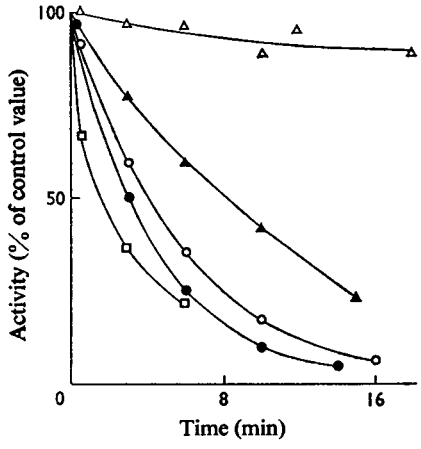


Fig. 8. *Time-course of inhibition of cytoplasmic acetoacetyl-CoA thiolase by iodoacetamide*

Acetoacetyl-CoA thiolase was incubated at 30°C in the standard assay medium (100mM-Tris–HCl, pH8.1, 25mM-$MgCl_2$ and 50mM-KCl), at a concentration of 0.1 unit/ml. CoA compounds, if present, were added 0.5min before addition of iodoacetamide. Samples (25μl) were removed at the indicated times after the addition of inhibitor and immediately assayed for enzyme activity. Additions were: iodoacetamide, 5μM (○); iodoacetamide+acetoacetyl-CoA, 2 or 10μM (△); iodoacetamide+acetyl-CoA, 10μM (▲); iodoacetamide+desulpho-CoA, 110μM (●); iodoacetamide+3-oxodecanoyl-CoA, 10μM (□). All concentrations are final values. Results are expressed as percentages of the appropriate controls from which the inhibitor was omitted.

steroid-biosynthesis activity *in vivo* after 24h starvation is not due to lowered activity of 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34) or any enzymic step subsequent to this, and the estimates (above) of cytoplasmic thiolase activity under conditions *in vivo* show that this enzyme cannot be ruled out as rate-limiting while uncertainties about cytoplasmic levels of acetyl-CoA and CoA continue.

I am indebted to Dr. M. R. Edwards for the determination of the subunit molecular weight of the cytoplasmic thiolase.

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APPENDIX

The following rate equations represent the simple Ping Pong type mechanism (Cleland, 1963) and three modifications of this mechanism in which substrates act as competitive inhibitors.

Case I, no inhibition:

$$\frac{1}{v}=\frac{1}{V}\left(1+\frac{K_A}{[A]}+\frac{K_B}{[B]}\right) \quad \text{(i)}$$

Case II, competitive inhibition by A:

$$\frac{1}{v}=\frac{1}{V}\left\{1+\frac{K_A}{[A]}+\frac{K_B}{[B]}\left(1+\frac{[A]}{K_i^A}\right)\right\} \quad \text{(ii)}$$

Case III, competitive inhibition by B:

$$\frac{1}{v}=\frac{1}{V}\left\{1+\frac{K_A}{[A]}\left(1+\frac{[B]}{K_i^B}\right)+\frac{K_B}{[B]}\right\} \quad \text{(iii)}$$

Case IV, competitive inhibition by A and B,

$$\frac{1}{v}=\frac{1}{V}\left\{1+\frac{K_A}{[A]}\left(1+\frac{[B]}{K_i^B}\right)+\frac{K_B}{[B]}\left(1+\frac{[A]}{K_i^A}\right)\right\} \quad \text{(iv)}$$

Here K_i^A and K_i^B are the dissociation constants of abortive complexes between enzyme and the relevant substrate.

If the initial rate is measured as a function of A or B when A or B are components of a mixture (concentration ratio A/B = n) then in all cases above reciprocal velocity will be linearly related to reciprocal concentration of A or B. This is because no term in 1/[A][B] is present. If the composition (n) of the mixture of A+B is altered and the experiment carried out for several different values of n then reciprocal plots yield a family of straight lines. The appearance of these families of lines (Table 1) is peculiar to each separate case. Further, as shown below, quantitative deductions can be made from the variation of the slopes and intercepts of these primary reciprocal plots with the value of n.

Slopes of primary plots

In all cases (I to IV) the slopes with respect to 1/[A] have the value $(K_A+nK_B)/V$ and the slopes with respect to 1/[B] have the value $[(K_A/n)+K_B]/V$. This is illustrated below for the most general case (IV) of competitive inhibition by substrates. Substituting [B] = [A]/n in eqn. (iv):

$$\frac{1}{v}=\frac{1}{V}\left\{1+\frac{K_A}{nK_i^B}+\frac{nK_B}{K_i^A}+\frac{1}{[A]}(K_A+nK_B)\right\} \quad \text{(v)}$$

and substituting [A] = [B]n:

$$\frac{1}{v}=\frac{1}{V}\left\{1+\frac{K_A}{nK_i^B}+\frac{nK_B}{K_i^A}+\frac{1}{[B]}\left(\frac{K_A}{n}+K_B\right)\right\} \quad \text{(vi)}$$

A secondary plot of the slope of eqn. (v) is linear with respect to n, yielding K_B/V and K_A/V as its slope and intercept respectively. A secondary plot of the slope of eqn. (vi) is linear with respect to $1/n$, yielding K_A/V and K_B/V as its slope and intercept respectively. In all cases considered here these quantities can therefore be obtained from plots of slopes against n or $1/n$.

Intercepts of primary plots

The values of the intercepts in terms of the kinetic constants describing the four different cases are as follows:

Case I, $\frac{1}{V}$

Case II, $\frac{1}{V}\left(1+\frac{nK_B}{K_i^A}\right)$

Case III, $\frac{1}{V}\left(1+\frac{K_A}{nK_i^B}\right)$

Case IV, $\frac{1}{V}\left(1+\frac{K_A}{nK_i^B}+\frac{nK_B}{K_i^A}\right)$

Thus if no substrate inhibits (case I) then the intercept ($1/V$) is independent of the composition (n) of the substrate mixture, whereas in all other cases the intercepts vary with respect to n. In case II the intercept is a linear function of n, yielding $1/V$ and K_B/K_i^A as its intercept and slope respectively. The intercept in case III is linear with respect to $1/n$, yielding $1/V$ and K_A/K_B as its intercept and slope

Table 1. *Effect of substrate mixture composition on primary reciprocal plots*

Relevant kinetic situation	Nature of reciprocal plot when ratio of [A]/[B] is varied	
	$1/v$ against $1/[A]$	$1/v$ against $1/[B]$
Case I	Intersecting at $\frac{1}{[A]}=0$	Intersecting at $\frac{1}{[B]}=0$
Case II	Intersecting at $\frac{1}{[A]}=-\frac{1}{K_i^A}$	No common intersection point
Case III	No common intersection point	Intersecting at $\frac{1}{[B]}=-\frac{1}{K_i^B}$
Case IV	In general no common intersection point*	

* But if K_i^A and K_i^B differ appreciably then these primary plots will resemble the pattern seen for II or III depending on which K_i is lower.

respectively. Therefore in cases I, II and III a combination of the data obtained from secondary plots of intercepts and slopes will enable the calculation of all the relevant kinetic constants. Case IV, however, although giving linear primary plots and secondary plots of slopes, will not in general give a linear secondary plot of the intercepts with respect to n or $1/n$. The behaviour of this intercept plot will depend on the relative magnitude of K_i^A and K_i^B and on the range of n used. This is illustrated in Fig. 7(*a*) of the main paper, where intercepts are linear with respect to $1/n$ up to $n = 2$ (thereby confirming that only B inhibits here), but become non-linear for higher values of n, showing that inhibition by A is now significant. These data enable the value of K_i^A to be estimated from Fig. 7(*a*) of the main paper. Thus the expression for the intercept for case IV must, over the range $n \leqslant 2$, simplify to that for the intercept of case III. Therefore for $n \leqslant 2$

$$\frac{K_A}{nK_i^B} \geqslant \frac{nK_B}{K_i^A}$$

Substituting $n = 2$ and (from Table 4 of the main paper) $K_A = 43\,\mu M$, $K_B = 15\,\mu M$, $K_i^B = 73\,\mu M$, we find that $K_i^A > 133\,\mu M$.

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