On the Mode of Action of Lipid-lowering Agents

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V. KINETICS OF THE INHIBITION IN VITRO OF RAT ACETYL COENZYME A CARBOXYLASE

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SUMMARY

Rat liver acetyl coenzyme A carboxylase was purified about 200-fold and the inhibition of this enzyme by certain hypolipidemic drugs was studied. The inhibition was more pronounced if the drugs were added before rather than after the citrate activation of the enzyme. Kinetic analysis revealed noncompetitive inhibition of the drugs with respect to the substrates acetyl-CoA, ATP, and HCO$_3^-$, and competitive inhibition with respect to the activator, citrate.

Sucrose density gradient centrifugations showed that the drugs reverse the aggregating effect of citrate to form the active polymeric forms of the enzyme from the inactive monomers. Arrhenius plots and heat-inactivation studies suggest gross conformational changes of the enzyme protein in the presence of the drugs.

Relative affinities of acetyl coenzyme A carboxylase for citrate and the drugs are expressed by the calculated dissociation constant for citrate and the inhibition constants of the drugs. Derangement of fatty acid synthesis in vivo is conceivable by competition of the drugs with the activation of acetyl-CoA carboxylase at low and physiologically possible concentrations of the drugs and citrate.

Acetyl-CoA carboxylase (acetyl-CoA-carbon dioxide ligase (ADP), EC 6.4.1.2) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is the first committed step in fatty acid biosynthesis and has been considered as the rate-limiting reaction in the over-all process (1, 2). The enzyme is thought to be regulated by a feedback mechanism involving the end products, palmitoyl-CoA and stearyl-CoA (3), and is activated by certain Krebs cycle intermediates such as citrate and isocitrate (4, 5). Under metabolic conditions, such as fat feeding or starvation, in which lipogenesis is depressed, acetyl-CoA carboxylase was found to be the locus of this depression (6, 7).

Our interest in acetyl-CoA carboxylase arose from our earlier findings that this enzyme is inhibited by certain hypolipidemic drugs (8–13). It was postulated that this inhibition might account, in part at least, for the lipid-lowering properties of these compounds. Derangement of lipid biosynthesis at the stage of acetyl-CoA carboxylase would be an ideal mode of attack because accumulated acetyl-CoA can be diverted to Krebs cycle and no undesirable intermediates are likely to accumulate. A correlation of elevated plasma lipids with atherosclerosis has found wide acceptance in recent years and hypolipidemic drugs such as 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionate and 2-(p-chlorophenoxy)-2-methyl propionate have attracted considerable interest as chemotherapeutic agents in the treatment of hyperlipoproteinemias. However, the mechanism of action of these agents is not understood, although many hypotheses have been advanced (14–20).

We have shown that a number of lipid-lowering agents inhibit acetyl-CoA carboxylase purified from avian (8, 10, 12), rat (9, 10), and monkey liver (13). TPIA-treated rats showed significantly lower levels of hepatic acetyl-CoA carboxylase than controls and on prolonged TPIA treatment and fat-free diet mice showed progressive reduction in carcass lipids and lipogenesis from acetate (11). This paper describes the isolation of rat acetyl-CoA carboxylase and the kinetics of the inhibition of this enzyme by TPIA and CPIB.

EXPERIMENTAL PROCEDURES

ATP, acetyl-CoA, glutathione, palmitoyl-CoA, and crystalline bovine serum albumin were obtained from Sigma Chemical Company; potassium citrate from J. T. Baker Chemical Company, Phillipsburg, New Jersey; and fat-free diet from Nutritional Biochemicals. NaH$_4$CO$_3$ and 1,3-14C-malonyl-CoA were purchased from New England Nuclear.

CPIB is a product of Imperial Chemical Industries, England, and TPIA is an experimental hypolipidemic compound synthesized in our laboratories by Dr. W. Benezet.

Purification of Acetyl-CoA Carboxylase—Acetyl-CoA carboxylase was extracted from fresh rat livers obtained from male white CF$_1$ strain rats, (Carworth Farms), which were fasted for 48 hours and refed a fat-free diet (Nutritional Biochemicals) for 48 hours prior to being killed in a CO$_2$ chamber. This dietary alteration is known to drastically increase the enzyme activity (21).

The enzyme purification was carried out as described by Matsushashi, Matsushashi, and Lynen et al. (22) with some modifications. Table I summarizes the purification steps involved to obtain 4.5 units of enzyme from 400 g of rat liver corresponding to a purification of about 200-fold. The specific activity of this enzyme is twice the one obtained by Matsushashi et al. (22) under similar conditions. The enzyme is highly unstable after the DEAE-cellulose column stage and can only be preserved

The abbreviations used are: TPIA, 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid. The compound is also known as Melipan. The corresponding potassium salt was used in this work. CPIB, 2-(p-chlorophenoxy)-2-methyl propionic acid as its potassium salt.
under liquid nitrogen in the presence of 20 mM citrate and 0.6 mg of albumin per ml. Under these conditions the enzyme is stable for at least 2 months. Enzyme at the second ammonium sulfate stage is relatively stable at -20° for about a month without any addition of citrate and albumin.

The rat enzyme, unlike the avian enzyme, requires preliminary incubation at 37° with citrate for enhanced activity (1, 2). The purified enzyme (third ammonium sulfate stage, Table I) has an absolute requirement for citrate, Mg++, albumin, ATP, and acetyl-CoA for activity. Addition of the citrate at the carboxylation stage, without prior preliminary incubation, yields only 50% of the activity under standard assay conditions.

Albumin is apparently essential at the preliminary incubation stage for stabilizing the enzyme during the 30 min incubation. Addition of albumin at the carboxylation stage does not restore the activity. The time dependence of the citrate induced activation of acetyl-CoA carboxylase is a rather complex process as discussed under "Kinetics of citrate activation."

Acetyl-CoA Carboxylase Assay—The enzyme activity was assessed by measuring acid-stable radioactivity incorporated into malonyl-CoA from H14CO3 (21). The reaction mixtures contained 40 μmoles of Tris-HCl (pH 7.5), 12 μmoles of MgCl₂, 14 μmoles of potassium citrate, 0.8 μ moles of 2-mercaptoethanol, 0.6 mg of bovine serum albumin, and enzyme in a total volume of 500 μl. After preliminary incubation at 37° for 30 min, 1.4 μmoles of ATP, 0.1 μmoles of acetyl-CoA and 12 μmoles of KH14CO₃ (4.2 x 10⁶ cpm per μmole) were added, yielding a total volume of 660 μl. After incubation for 5 min at 37°, the reaction was terminated by the addition of 0.1 ml of 6 N HCl. The reaction mixture, routinely contained in a glass ampoule, was placed into a liquid scintillation vial, briefly exposed to gaseous HCl, and dried under a stream of warm air at 40-50°. The HCl treatment ensures complete release of all unreacted H14CO₃-.

FIG. 1. Inhibition of rat hepatic acetyl-CoA carboxylase by the hypolipidemic drugs TPIA and CPIB. The drugs were added before and after citrate activation, at the concentrations indicated. I₅₀ indicates 50% inhibition.

The specific activity of acetyl CoA carboxylase was measured by the H14CO₃- fixation assay in the presence of increasing concentra-
Effect of 2-methyl-6-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionate concentration on acetyl-CoA carboxylase activity under constant molar ratio of inhibitor to enzyme

Incubation mixtures contained the components of the assay as outlined under “Materials and Methods” and enzyme (16.4 mg per ml of protein, determined by the method of Lowry) diluted 1:20 with 0.04 M phosphate buffer in the control experiment and 1:20 with 0.5 mM of TPIA in 0.04 M phosphate buffer in the drug experiment.

| Enzyme activity | Inhibition |
|-----------------|-----------|
| Control         | Control plus TPIA |
| | | % |
| 0 | 0 | 0 |
| 5 | 312 | 179 | 43 |
| 10 | 814 | 546 | 33 |
| 20 | 2038 | 1474 | 30 |
| 30 | 3459 | 2420 | 30 |
| 40 | 4559 | 3019 | 35 |
| 50 | 5422 | 3648 | 32 |
| 60 | 6224 | 3813 | 43 |
| 70 | 7540 | 4450 | 44 |

In Fig. 1, the enzymatic activity falls with increasing drug concentrations and this is evident at all stages of purification of the enzyme.

TPIA is at least 10 times more potent as an inhibitor of acetyl-CoA carboxylase than CPIB. It is of interest that TPIA (as the potassium salt, an agent newly described as lo-fold more active than CPIB (10)) is hypolipidemic at a much lower dosage than is CPIB, and this is comparable in vivo as well as in the enzyme assay here discussed. Partially purified acetyl-CoA carboxylase from lactating mammary glands of rats (28) was used to test the susceptibility of the enzyme to inhibition by TPIA and CPIB. A preparation purified through the first ammonium sulfate stage with specific activity of 71 mmoles per min per mg of protein was inhibited by 50% when 3.8 \times 10^{-6} M TPIA or 5.1 \times 10^{-4} M CPIB was present in the standard assay mixture.

Rat adipose tissue acetyl-CoA carboxylase, obtained by the procedure employed by Dakshinamurti and Desjardins (29) is also inhibited to about the same extent as the mammary or the hepatic enzyme.

Possible Detergent Effect

Palmitoyl-CoA has been implicated in the control of various enzymes, including acetyl-CoA carboxylase (5). Some authors have suggested that the effects of palmitoyl-CoA are not physiological, but are merely a strong detergent action, because the binding with these enzymes is not site specific. Thus, Dorsey and Porter (30) showed that inhibition of fatty acid synthetase by palmitoyl-CoA is dependent on both the critical micellar concentration of palmitoyl-CoA and the molar ratio of inhibitor to enzyme.

In view of the hydrophobic-hydrophilic nature of the hypolipidemic compounds under investigation, we have considered the inhibition of acetyl-CoA carboxylase by these compounds as a possible detergent effect.

Table II indicates that at constant molar ratio of acetyl-CoA carboxylase to TPIA no drastic change in the inhibition is evident with increasing drug concentration, suggesting no dependence of the extent of inhibition on critical micellar concentration of TPIA.

Furthermore, comparison of the inhibition by TPIA (Fig. 1) with that by lauryl sulfate (Fig. 2) shows an abrupt drop of acetyl-CoA carboxylase activity at the critical micellar concentration of lauryl sulfate (about 0.1 mM), which is not evident in the TPIA-inhibited system. Moreover, Table III indicates that
TABLE III

Mixed inhibition of acetyl-CoA carboxylase by 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionate and lauryl sulfate

Assays for enzyme activity contained 66.7 µg of protein; all other conditions, components, and their concentrations as reported under "Materials and Methods." Inhibitors were added before citrate activation.

| Inhibitor                | Enzyme activity (cpm) |
|--------------------------|-----------------------|
| Control                  | 6092                  |
| TPIA, 7.6 × 10^{-8} M    | 4212                  |
| Lauryl sulfate, 3.78 × 10^{-4} M | 5192               |
| TPIA, 7.6 × 10^{-8} M + lauryl sulfate, 3.78 × 10^{-4} M | 2391               |

the mode of inhibition by lauryl sulfate and by TPIA are different because mixed inhibition is cumulative and not additive. Kinetic data demonstrating a different mode of interaction of TPIA and palmitoyl-CoA carboxylase have already been presented (8).

Effect of 2-Methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionate and 2-(p-Chlorophenoxyl)-2-methyl-propionate on Other Enzymes

A number of liver enzymes have been checked for their susceptibility to inhibition by hypolipidemic drugs (8). At drug concentrations where acetyl-CoA carboxylase activity is virtually 0, all enzymes tested are unaffected by the presence of these compounds.

Fatty acid synthetase seems to be stimulated by TPIA and only at very high concentrations is there an abrupt drop in enzyme activity, probably because of a detergent or salting out effect (Table IV). The same results were obtained with CPIB (data not shown).

Kinetics of Acetyl-CoA Carboxylase Inhibition by 2-Methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxy]-propionate and 2-(p-Chlorophenoxyl)-2-methyl-propionate with Respect to Substrates Acetyl-CoA, ATP, and HCO^-3

Since the inhibitory effect of TPIA and CPIB on acetyl-CoA carboxylase depends on the state of activation of the enzyme by citrate, rate measurements were performed with and without preliminary incubation of the enzyme with citrate. The steady state kinetic parameters were obtained from the initial velocity data by means of a digital computer program written for the Lineweaver-Burk (31) form of the Michaelis-Menten rate equation. Data weighing and calculation of standard errors of the kinetic constants were done by the method of Wilkinson (32).

Table V shows the kinetic parameters from the double reciprocal plots for variable concentrations of acetyl-CoA, ATP, and HCO^-3 at two levels of TPIA and CPIB added before preliminary incubation of the enzyme with citrate. Table VI shows the same data for the drugs added after 30 min of citrate activation of the enzyme. Table VII summarizes the kinetic constants calculated from these data. The inhibition was found to be noncompetitive for all three substrates, acetyl-CoA, ATP, and HCO^-3.

Previous results with avian acetyl-CoA carboxylase showed a competitive inhibition of this enzyme by TPIA and CPIB with respect to acetyl-CoA (8). In order to confirm this specific difference of acetyl-CoA carboxylase, we have obtained the Dixon plots, 1/1Ve/ vs 1/Ve (1), for the rat enzyme by varying TPIA concentration from 0 to 9 × 10^{-4} M, added at the time of preliminary incubation, at four levels of acetyl-CoA (33). This analysis confirms the conclusion to be drawn from Tables V and VI; i.e., the inhibition is of the noncompetitive type with respect to...
acetyl-CoA with $K_c = 7.98 \times 10^{-4} \text{ M}$ for TPIA. Noncompetitive inhibition for acetyl-CoA was also revealed by plotting the data as $V$ versus $V/S$. Parallel lines with slopes numerically equal to $K_m$ were obtained.

Mixed Inhibition Studies

The question of independent or identical sites of action for TPIA and CPIB has not been answered from the kinetic data of Tables V and VI. This question is of practical importance because the drugs might exert a synergistic effect in their effectiveness as lipid-lowering agents if they are bound at independent sites and if inhibition of acetyl-CoA carboxylase is, in fact, important in their mechanism of action.

Mixed inhibition studies were performed with acetyl-CoA as the varying substrate, in the absence of inhibitors, in the presence of each inhibitor separately, and then in the simultaneous presence of both TPIA and CPIB. The data were analyzed by Lineweaver-Burk plots and the slopes of each line, corresponding to $K_m/v$, were obtained. Theory predicts (34) that such slopes be numerically different for inhibitors acting upon one and the same or two separate sites. The experimentally found slope for the mixed inhibition with TPIA plus CPIB is in close agreement with the line calculated on the bases of identical sites of action of the inhibitors (data not shown). The same conclusion has been obtained with the avian liver enzyme (8).

Kinetics of Citrate Activation

The kinetics of the citrate activation of acetyl-CoA carboxylase are quite complex and not completely understood. As shown in Fig. 3, the enzyme has an absolute citrate requirement. Citrate catalyzes the formation of active aggregates of the enzyme from inactive monomers (4, 26). This process is time dependent, as

| Variable | Inhibitor | $V_{max}$ (± S.E.) | Apparent $K_m$ (± S.E.) |
|----------|-----------|-------------------|------------------------|
| Acetyl coenzyme A (0.03 to 0.30 mM) | | | |
| None | | | |
| TPIA | 15.2 | 4600 ± 471 | 0.253 ± 0.040 |
| TPIA | 22.8 | 3690 ± 193 | 0.291 ± 0.024 |
| CPIB | 152 | 5575 ± 360 | 0.208 ± 0.022 |
| CPIB | 304 | 4015 ± 114 | 0.324 ± 0.073 |
| ATP (0.1 to 3.0 mM) | | | |
| None | | | |
| TPIA | 15.2 | 1304 ± 38 | 0.266 ± 0.019 |
| TPIA | 22.8 | 937 ± 60 | 0.240 ± 0.037 |
| CPIB | 152 | 1638 ± 35 | 0.226 ± 0.013 |
| CPIB | 304 | 1133 ± 32 | 0.221 ± 0.017 |
| HCO$_3^-$ (1.2 to 15.0 mM) | | | |
| None | | | |
| TPIA | 15.2 | 6050 ± 117 | 5.756 ± 0.233 |
| TPIA | 22.8 | 2825 ± 537 | 3.938 ± 1.069 |
| CPIB | 152 | 1833 ± 194 | 3.221 ± 0.584 |
| CPIB | 304 | 3295 ± 169 | 4.135 ± 0.382 |

**Table VII**

Summary of kinetic constants

| Inhibitor with variables | Type of inhibition | Apparent* Michaelis constant | Inhibitor added before activation ($K_i$) | Inhibitor added after activation ($K_i$) |
|-------------------------|--------------------|------------------------------|------------------------------------------|----------------------------------------|
| TPIA                    | Acetyl-CoA         | Noncompetitive               | 2.30 × 10$^{-4}$                          | 7.85 × 10$^{-4}$                         |
|                         | ATP                | Noncompetitive               | 2.26 × 10$^{-4}$                          | 5.97 × 10$^{-4}$                         |
|                         | HCO$_3^-$          | Noncompetitive               | 5.03 × 10$^{-4}$                          | 7.06 × 10$^{-4}$                         |
| CPIB                    | Acetyl-CoA         | Noncompetitive               | 2.3 × 10$^{-4}$                           | 1.19 × 10$^{-4}$                         |
|                         | ATP                | Noncompetitive               | 2.26 × 10$^{-4}$                          | 7.25 × 10$^{-4}$                         |
|                         | HCO$_3^-$          | Noncompetitive               | 5.03 × 10$^{-4}$                          | 6.82 × 10$^{-4}$                         |

* Apparent $K_m$ and $K_i$ values are the average of the values obtained from the data of Tables V and VI.
shown in Fig. 4, and the inhibitor interferes with the activation rate of the enzyme. Citrate is also essential for the stability of acetyl-CoA carboxylase as stated above.

Under our preliminary incubation conditions (37° for 30 min), no appreciable inactivation of the enzyme occurs, even at suboptimal citrate levels i.e. below 16.7 mM. However, prolonged incubation at 37°, in the absence of enough citrate, leads to drastic reduction in enzyme activity (Table VIII). At the saturation level of citrate, the enzyme not only is stable at 37° for 6 hours, but also shows a slow increase in activity.

Table VIII shows that the presence of 7.5 × 10^{-5} M of TPIA in the preliminary incubation mixture does not seem to cause any time-dependent inactivation of the enzyme for at least 60 min. With longer periods of time, however, there is a definite inactivation of the enzyme due to TPIA, especially at low levels of citrate. This is probably a secondary effect due to competition of drug with citrate for the enzyme, which renders citrate stabilization ineffective.

The inhibitory effect of TPIA or CPIB depends on the stage of citrate activation of the enzyme. Since the activation is time dependent, so must be the inhibition of acetyl-CoA carboxylase by TPIA and CPIB. In Table IX are summarized the kinetic parameters of the TPIA-inhibited and noninhibited system at different times for activation (preliminary incubation) intervals. At zero time the inhibition by TPIA is of the mixed type with respect to the activator, citrate. After 60 min of preliminary incubation, the enzyme is almost fully activated and the inhibition is competitive for citrate with $K_i = 3.65 \times 10^{-4}$ M. Under similar conditions $K_i = 3.0 \times 10^{-4}$ for CPIB.

**Kinetic and Thermodynamic Parameters from Hill Plots**

The modified Hill equation as derived by Atkinson, Hathaway, and Smith (27) is a form of the Michaelis equation and as such it can be used advantageously in obtaining additional information from kinetic data.

Equation 1 is the empirical Hill equation as modified by Changeux (35).

$$\log \frac{V}{V_{max}} - \log (s) = n \log (\delta) - \log K$$

where $(s)$ is substrate concentration, $V$ is the reaction rate, $V_{max}$ is the rate attained when the enzyme is saturated with substrate, $n$ is moles of substrate per enzyme molecule, and $K$ is the product of the $n$ dissociation constants of the enzyme-substrate complexes (Equation 2).

$$K = \frac{(E)(S)^n}{(E_S)^n}$$
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Fig. 5. Hill plot of the citrate activation of acetyl-CoA carboxylase. Preliminary incubation time was 60 min; all other conditions are outlined in Table X. The regression equation describing the straight line is log \( V/V_{\text{max}} - V = 2.138 + 1.008 \log (S) \).

Table X

| Variable   | \( n \) | \( K \times 10^4 \) | \( K_m \times 10^4 \) |
|------------|--------|---------------------|---------------------|
| Acetyl-CoA | 0.986  | 0.225               | 0.200               |
| ATP        | 1.002  | 0.233               | 0.237               |
| HCO\(_3\)  | 0.984  | 4.45                | 4.21                |
| Citrate   | 1.010  | 7.27                | 7.53                |

* Data for varying citrate concentrations were obtained from Table IX (60-min citrate activation without drug). The same excellent correlation of \( K_m \) values was obtained for all time intervals of citrate activation. The data for varying acetyl-CoA, ATP, and HCO\(_3\) concentration were obtained from Table VI. In all plots the slope was significant at \( p < 0.001 \).

The assumption involved in deriving Equation 1 as a form of the Michaelis equation is that the concentrations of all complexes containing fewer than \( n \) molecules of \( S \) are negligible. Plots of \( \log V/V_{\text{max}} - V \) versus \( \log (S) \) are straight lines with slopes numerically equal to substrate-binding sites and intercepts equal to \(-\log K\).

Fig. 6. The effect of citrate and TPIA on the sedimentation pattern of acetyl-CoA carboxylase in sucrose density gradients. The enzyme, about \( 4 \times 10^{-2} \) units (purified after the second ammonium sulfate stage (see Table I)) was diluted to 0.1 ml with the same solution as the gradient medium (minus sucrose). The enzyme solution was incubated at 37\(^{\circ}\) for 10 min and then was applied to the top of the gradient, centrifuged, and analyzed for enzyme activity as described in "Experimental Procedures." All gradients contained 0.03 M phosphate buffer, pH 7.0, albumin, 0.6 mg per ml, manganese chloride, 0.5 mM, EDTA, 0.1 mM, and 2-mercaptoethanol, 0.5 mM. In addition, tube No. 2 contained 20 mM citrate and tube No. 3 contained 20 mM citrate plus 7.5 \( \times 10^{-4} \) M TPIA.

The enzyme-citrate complex is not dissociable to products; therefore, \( K_m \) is equal to \( K_{\text{eq}} \), a true thermodynamic constant. From this the free energy of binding of enzyme with citrate can be calculated and under the conditions of Fig. 4, \( \Delta F = 3018 \text{ cal per mole} \).

Inhibition constants for the drugs are also true thermodynamic constants of dissociation of the enzyme-drug complex. The relative affinities, therefore, of the drugs and citrate to the enzyme can be calculated from the ratio of \( K_{\text{eq}}/K_i \) for the drugs. This ratio is \( K_{\text{cit}}/K_i = 7.27 \times 10^3/3.65 \times 10^{-4} = 198 \) for TPIA and \( K_{\text{cit}}/K_i = 7.27 \times 10^3/3 \times 10^{-4} = 24.1 \) for CPIB. From these affinity ratios the differences in the free energy of binding of citrate and drugs with the enzyme can be calculated. This difference in free energy of binding of citrate and TPIA is \(-3240\) cal per mole. The same parameter for CPIB is \(-1950\) cal per mole.
**Heat inactivation of acetyl-CoA carboxylase from rat liver in presence and absence of S-methyl-[p-(1,2,3,4-tetrahydro-1-naphthyl)-phenoxyl-propionate and 2-(p-chlorophenoxyl)-2-methyl propionate**

Reaction mixtures and assay conditions were as described in the HCO$_3^-$ fixation assay under "Experimental Procedures." Final concentrations of TPIA and CPIB were $7.6 \times 10^{-6}$ M and $2.56 \times 10^{-4}$ M, respectively, and were added before the citrate activation. At the stage of the assay indicated the reaction mixture was heat treated at $45^\circ$C for various times and the enzyme activity was measured. The inactivation pattern did not change materially from Experiment II if the drugs were added after the citrate activation.

| Heat treatment and inhibitor | 20% Inactivation | 90% Inactivation |
|-----------------------------|-----------------|-----------------|
| Experiment I                |                 |                 |
| Before citrate activation   |                 |                 |
| Control                     | 4               | 12.4            |
| TPIA                        | 2.8             | 9.4             |
| CPIB                        | 3.5             | 11.8            |
| Experiment II               |                 |                 |
| After citrate activation    |                 |                 |
| Control                     | 3.2             | 12.8            |
| TPIA                        | 2.0             | 7.0             |
| CPIB                        | 2.5             | 9.0             |
| Experiment III              |                 |                 |
| After addition of the carboxylation mixture | >15 | >15 |
| Control                     | 1.3             | >15             |
| TPIA                        | 2.4             | >15             |
| Experiment IV               |                 |                 |
| After addition of the carboxylation mixture minus acetyl-CoA | 1.0 | 7.0 |
| Control                     | 0.9             | 5.3             |
| TPIA                        | 0.8             | 6.1             |
| CPIB                        |                 |                 |

**Sucrose Density Gradient Centrifugation**

Variations in the molecular form of acetyl-CoA carboxylase in the presence and absence of citrate and presence of citrate plus TPIA are illustrated in Fig. 6, the sucrose gradient sedimentation patterns.

The enzyme is sedimenting in the large form in the presence of citrate as shown by the increase in sedimentation velocity. The simultaneous presence of TPIA reverses the aggregating effect of citrate.

Gregolin et al. (36) have shown conclusively in avian acetyl-CoA carboxylase that isocitrate-induced aggregation is associated with formation of an active polymeric form of the enzyme. We have shown earlier (8) that TPIA, a competitive inhibitor for the activator isocitrate, prevents the isocitrate-catalyzed aggregation of the avian enzyme. The same conclusions are supported from the present study on the rat liver enzyme.

**Arrhenius Plots**—Detailed studies of the temperature dependence of acetyl-CoA carboxylase activity in the TPIA-inhibited and noninhibited systems are shown in Fig. 7. Under our conditions the enzyme is not cold labile in either system (37) and the magnitude of the inhibitory effect does not change with temperature. The temperature at which the activity is determined, however, has a very pronounced influence on the reaction rate in both systems. Fig. 7 reveals that both plots are polyphasic with points of discontinuity at $22.5^\circ$, $30.5^\circ$, and $36.5^\circ$. Below $22.5^\circ$ there is an unusually high temperature dependence with temperature coefficient ($Q_10$) 7.2 and activation energy 34.2 kcal per mole. At $22.5^\circ$ an abrupt change in slopes occurs and in the region, $22.5-33.5^\circ$, the temperature coefficient ($Q_10$) is 2.9 and the activation energy is 18.6 kcal per mole. In the region, $30.5-36.5^\circ$, the temperature coefficient ($Q_10$) is 1.7 and the activation energy is 9.45 kcal per mole. Above $30.5^\circ$ there is a decrease in enzyme activity with temperature coefficient ($Q_10$) 2.2 and energy of activation $-15.4$ kcal per mole.

The same results were obtained when TPIA was added at the carboxylation stage. The slopes of the lines within the several temperature regions and the transition points were the same, but the inhibition was less pronounced at every temperature. Results obtained for CPIB were also the same (data not shown).

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**Table XI**

| Heat treatment and inhibitor | Heat treatment and inhibitor |
|-----------------------------|-----------------------------|
|                             | 20% Inactivation | 90% Inactivation |
| Experiment I                |                 |                 |
| Before citrate activation   |                 |                 |
| Control                     | 4               | 12.4            |
| TPIA                        | 2.8             | 9.4             |
| CPIB                        | 3.5             | 11.8            |
| Experiment II               |                 |                 |
| After citrate activation    |                 |                 |
| Control                     | 3.2             | 12.8            |
| TPIA                        | 2.0             | 7.0             |
| CPIB                        | 2.5             | 9.0             |
| Experiment III              |                 |                 |
| After addition of the carboxylation mixture | >15 | >15 |
| Control                     | 1.3             | >15             |
| TPIA                        | 2.4             | >15             |
| Experiment IV               |                 |                 |
| After addition of the carboxylation mixture minus acetyl-CoA | 1.0 | 7.0 |
| Control                     | 0.9             | 5.3             |
| TPIA                        | 0.8             | 6.1             |

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**Fig. 7. Influence of temperature on acetyl-CoA carboxylase activity and inhibition by TPIA.** The reaction mixture is the standard HCO$_3^-$ fixation assay as outlined under "Experimental Procedures." Preliminary incubation temperature was $37^\circ$, and enzyme activity was measured at each temperature ($12-40^\circ$) in the absence and the presence of $7.5 \times 10^{-6}$ M TPIA. Initial velocity is expressed as counts per min fixed within 5 min in each assay tube.
Earlier results with the avian hepatic acetyl-CoA carboxylase (8) also revealed a biphasic character in the Arrhenius plots and nondependence of the magnitude of the inhibition on temperature. Below 22° the energy of activation was found 33.7 kcal per mole and above 22° 12.8 kcal per mole.

**Heat Inactivation**

The enzyme used in the heat treatment experiments was purified through the second ammonium sulfate stage. Table XI summarizes time required for 50% and 90% inactivation under different conditions of heat treatment at 45°. Figs. 8 and 9 are graphic illustrations of differences in rates of inactivation depending on the presence and absence of drugs and other conditions.

Stability of acetyl-CoA carboxylase at 45° is greatly increased when the carboxylation mixture is added prior to heat treatment (Experiments I and III, Table XI). The component of the carboxylation mixture, which has this protective effect on the enzyme, is acetyl-CoA, as shown in Experiments III and IV of Table XI. Protection by acetyl-CoA is drastically reduced when either CPIB or TPIA is present in the reaction mixture.

The drugs enhance the initial rate of heat inactivation; it appears that by competing with citrate they favor the formation of a protomeric, and more heat labile, form of the enzyme.

**DISCUSSION**

Acetyl-CoA carboxylase has been considered as the pace setter in lipid biosynthesis (1, 2). In this and previous reports (8–13) we have studied the inhibition of this key enzyme by a number of clinically important hypolipidemic drugs. As shown by kinetic analysis, the drugs interfere with the activation process of the enzyme, brought about by tricarboxylic acids. The activation has an apparent regulatory significance (4) and is associated with gross conformational changes of the enzyme protein (36). Kinetic evidence that the drug competes with the function of the activator to bring about these conformational changes is only circumstantial; rate variations are not directly referable to conformational changes.

More direct evidence on the competition of the activator, citrate, with TPIA were obtained from the sucrose gradient sedimentation experiments. The citrate-mediated aggregation of acetyl-CoA carboxylase does not take place when TPIA is present.

The inhibition of acetyl-CoA carboxylase is not unique for the hepatic enzyme. Acetyl-CoA carboxylases from rat adipose tissue and lactating mammary gland are equally susceptible to inhibition by hypolipidemic drugs.

The inhibitory effect on acetyl-CoA carboxylase appears to be quite specific. We are considering other enzymes as possible target molecules of these compounds, but at present no other enzyme tested is significantly affected by the drugs.

It seems unlikely that a detergent effect of the drugs in the assay mixture can account for the inhibitory effect on acetyl-CoA carboxylase. Lauryl sulfate, a strong detergent, causes 50% inhibition at a concentration of $8 \times 10^{-3}$ M. At the critical micellar concentration of lauryl sulfate, (about 0.1 mM), however, there is a precipitous drop in enzyme activity. Mixed inhibition studies of TPIA with both lauryl sulfate or palmitoyl-CoA show different modes of interaction with the activity of the enzyme.

The chemical nature of the interaction between TPIA or CPIB and the enzyme is very little understood. The drugs
presumably bind at the citrate binding site, or at a site near enough to account for competitive inhibition with citrate, thus preventing the activator from bringing about the aggregation of the inactive monomers of the enzyme.

The data contained in this paper lead us to believe that acetyl-CoA carboxylase inhibition by hypolipidemic drugs is a site-specific interaction and as such is probably physiologically important.

Studies on the dependence of carboxylation rate on temperature in the absence and presence of the drugs reveal that the inhibitory pattern does not change materially with temperature. The discontinuities of slope (Fig. 6) indicate that there are changes from one value of the activation energy to another at the transition temperatures. Since the over-all carboxylation reaction of acetyl-CoA involves several successive reactions, such as citrate activation of the enzyme, carboxylation of the biotin enzyme, and others, it is conceivable that these have different temperature coefficients. As the temperature increases, the reaction with the high coefficient will become the more rapid of the two and the over-all process will then be limited by the reaction with a temperature coefficient. The activation energy of the latter will then be the observed value. Other explanations, such as reversible inactivation at the higher temperatures, or the possible existence of the enzyme in more than one form with different activation energies, can be considered. This type of discontinuity in Arrhenius plots has been theoretically discussed by Stearn (38).

Arrhenius plots (Fig. 6) make it quite clear that the inhibitor does not materially affect the enthalpy of activation (\( \Delta H^* \)). Therefore, the rate decrease brought about by the inhibitor must be reflected in the entropy of activation (\( \Delta S^* = \Delta H^*/T \)).

The appreciable increase in the entropy term can be visualized as a conformational change in the protein when it becomes associated with the inhibitor. Entropy changes may further result from changes in hydration when the ionic inhibitors interact with the enzyme protein. The sedimentation data (Fig. 5) are evidence in support of gross structural changes of the enzyme in the presence of the inhibitory agent.

Contrary to the avian enzyme, the enzyme from rat liver is protected against heat inactivation by acetyl-CoA rather than by the activator, citrate. In the presence of the drug this protective effect is altered. This is difficult to explain in view of the noncompetitive kinetic behavior of acetyl-CoA and CPIB or TPIA. Acetyl-CoA probably can protect the enzyme by means of an enzyme-acetyl-CoA complex with a stable conformation that is less susceptible to heat inactivation than either the protomeric or the polymeric form of the enzyme.

Citrate-activated enzyme shows a biphasic rate of inactivation with a rapid initial rate, accelerated further by the presence of the drugs; this suggests a rapid denaturation of the nonaggregated fraction of the enzyme (Fig. 8).

From the kinetic, physical, and thermodynamic data available, it is suggested that TPIA and CPIB compete with citrate for the same or mutually interacting sites of the enzyme protein. The relative affinities of binding of citrate and TPIA or CPIB to the enzyme indicate that both drugs can displace citrate from the enzyme citrate complex at equimolar concentrations with a free energy gain of about 3240 cal per mole for TPIA and 1950 cal per mole for CPIB.

At the cellular level the contribution of enzyme-citrate and enzyme-drug complexes in the over-all carboxylation reaction will depend on the the relative concentrations of enzyme, citrate, and drug. The levels of citrate in the cell are not known with certainty. Cleland (39) has suggested that as a general rule enzymes operate in the cell at substrate levels about 0.5 their \( K_m \) value. Herrera and Freinkel (40) have found citrate levels in the rat liver, under normal feeding conditions, to be 0.38 \( \mu \)moles per g of fresh liver. On the basis of probable citrate concentration in the liver cells and the relative affinities of citrate and TPIA for the enzyme, we suggest that very low intracellular concentrations of free drug would be required for the inhibition to become manifest, by interference with the activation of acetyl-CoA carboxylase. Thus, hypolipidemic drugs may act as auxiliary feedback inhibitors applying a temporary and reversible block in the over-all sequence of fatty acid synthesis by inhibiting the rate-limiting carboxylation reaction. No undesirable intermediates are expected to accumulate since acetyl-CoA can be diverted to the Krebs cycle. Granzer and Schachter (41) have found an increase of acetyl-CoA of about 2-fold and a 2- to 3-fold increase in acetoacetate and pyruvate in the livers of TPIA-treated rats over that of controls.

TPIA or CPIB have no inhibitory effect on the activity levels of enzymes other than acetyl-CoA carboxylase, which is significantly lower in drug-treated animals (11). If no other enzymes are affected by these drugs, only the fatty acids synthesis will be deranged by inhibition of acetyl-CoA carboxylase while the degradation would proceed unaffected, since different enzymes and intermediates are involved in these unidirectional processes.

Inhibition of acetyl-CoA carboxylase by TPIA or CPIB should therefore result not only in inhibition of lipogenesis but also, on limited fat-intake, in reduction of lipid stores in the tissues (11). In agreement with that thought, long term treatment with CPIB has achieved certain regressions of lipid deposits (xanthomas) in humans (41). Koksnur and Malcom (42) have observed little or no hypolipidemic effect of CPIB in the serum of rats receiving a high fat regimen.

In summary, the effectiveness of a lipid-lowering agent, such as TPIA or CPIB, may be related to its inhibitory potency on acetyl-CoA carboxylase. Inhibition of this critical enzyme makes malonyl-CoA unavailable for lipid biosynthesis while degradation proceeds unaffected. This may lead to a decrease not only of circulating lipids, but also of lipid stores in the tissues. It is, therefore, conceivable that treatment with this type of hypolipidemic agents can prevent, arrest, or reverse the atherosclerotic processes resulting from high levels of circulating lipids.

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