On the Mode of Action of Lipid-Lowering Agents. II. *In Vitro* Inhibition of Acetyl Coenzyme A Carboxylase by a Hypolipidemic Drug*

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ABSTRACT: Acetyl coenzyme A carboxylase, a key enzyme in lipid biosynthesis, is being assessed as a possible locus of action for hypolipidemic agents. 2-Methyl-2-[*p*-(*p*-chlorophenyl)phenoxy]propionate, a newly described lipid-lowering agent, strongly inhibits hepatic acetyl coenzyme A carboxylase from chickens or rats. Kinetic analysis of the inhibition suggests that the inhibition is competitive for acetyl coenzyme A and isocitrate and noncompetitive for adenosine triphosphate and HCO_3^-. The values of the kinetic constants obtained are $K_m = 8 \times 10^{-5}$ M for acetyl coenzyme A and $K_i = 1.5 \times 10^{-4}$ M for acetyl coenzyme A as varying substrate; $K_m = 1.25 \times 10^{-3}$ M for isocitrate and $K_i = 7.9 \times 10^{-5}$ M for varying isocitrate concentrations; $K_m = 2.1 \times 10^{-3}$ M for adenosine triphosphate and $K_i = 2.8 \times 10^{-4}$ M for adenosine triphosphate as varying substrate; and $K_m = 1.5 \times 10^{-2}$ M for HCO_3^- and $K_i = 3.4 \times 10^{-4}$ M for varying HCO_3^- concentrations.

The hypolipidemic effect of CPIB[1] and TPIA in experimental animals as well as in the clinic has been well documented (Thorp and Waring, 1962; Hellman *et al.*, 1963; Oliver, 1963; Best and Duncan, 1963; Barret and Thorp, 1968; Hess and Bencze, 1968), but their mechanism of action is not understood.

Acetyl-CoA carboxylase plays a critical role in lipid biosynthesis (Wakil, 1962; Vagelos, 1964). It catalyzes the rate-limiting step in the overall process of fatty acid biosynthesis (Numa *et al.*, 1961) and is effectively controlled by a feedback control mechanism by the CoA esters of long-chain fatty acids (Numa *et al.*, 1965). This enzyme is activated by tricarboxylic acid cycle intermediates, most notably citrate and isocitrate (Martin and Vagelos, 1962; Waite and Wakil, 1962).

In an earlier paper (Maragoudakis, 1969) we have reported that CPIB and TPIA are effective inhibitors of avian and rat liver acetyl-CoA carboxylase, and have suggested that this inhibitory property can conceivably account for the hypolipidemic activity exhibited by these compounds. This hypothesis is currently under study in experiments aimed at demonstrating changes in lipogenesis at the cellular level and in whole animals.

The present paper extends the study to another hypolipidemic compound CDIB. This compound was reported by Leigh *et al.* (1968) to be an effective hypolipidemic at doses which do not cause liver enlargement in rats. CDIB was found to be a potent inhibitor of acetyl-CoA carboxylase purified from either avian or rat liver. The kinetics of the inhibition were studied and discussed in relation to the hypolipidemic potency of this compound.

Materials and Methods

ATP, acetyl-CoA, glutathione, and EDTA were obtained from Sigma Chemical Co. DL-Isocitric acid, trisodium salt, was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J., and [^{14}C]HCO_3^- was purchased from New England Nuclear Co., Boston, Mass.

CDIB and DIB are experimental compounds of Imperial Chemical Industries, England, and were synthesized in our laboratories by Dr. W. Bencze. The structural formulas of these compounds are shown in Table I and Table II.

Acetyl-CoA carboxylase was purified from chicken liver as described previously (Maragoudakis, 1969). The rat liver enzyme was extracted and purified by a method described by Majerus *et al.* (1968) with some modifications. Both enzyme preparations show an absolute requirement for acetyl-CoA, ATP, and Mg^{2+}. The activity is stimulated greatly by bovine albumin and glutathione or 2-mercaptoethanol. The rat liver enzyme needs preincubation with citrate for 30 min, at 37° for activation. With avian liver enzyme no preincubation with isocitrate is required.

Enzyme activity was followed, as described previously, (Maragoudakis, 1969) by measuring the acid-stable radioactivity incorporated into malonyl-CoA from [^{14}C]HCO_3^-.

Results

Inhibition of Acetyl-CoA Carboxylase by CDIB. Table I shows the activity of avian acetyl-CoA carboxylase in the presence of increasing concentrations of CDIB. Fifty per cent inhibition (I_{50}) is attained at 6.2×10^{-4} M of CDIB. Table II shows the same data using the rat liver enzyme. I_{50} for CDIB and rat liver enzyme is 2.5×10^{-4} M. For comparison, DIB was included in this experiment. DIB differs from CDIB in that it lacks the chlorine atom. The absence of the chlorine

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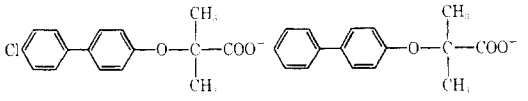
[1] The abbreviations used are: DIB, potassium 2-methyl-2-(*p*-phenylphenoxy)propionate; CDIB, potassium 2-methyl-2-[*p*-(*p*-chlorophenyl)-phenoxy]propionate; CPIB, potassium-2-(*p*-chlorophenoxy)-2-methylpropionate; TPIA, potassium 2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionate. This compound is also known under the code number Su-13437.

TABLE I: Inhibition of Avian Liver Acetyl-CoA Carboxylase by CDIB and DIB.

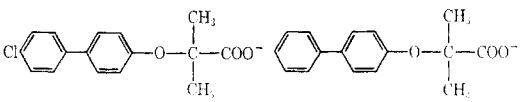


[CDIB] [DIB]

Drug Concentration (M)	CDIB Enzyme Activity[a] (cpm)	CDIB Inhibition (%)	DIB Enzyme Activity[a] (cpm)	DIB Inhibition (%)
0	4066		4000	
0	4032		3977	
5×10^{-5}			3837	4.1
10×10^{-5}	3848	4.9	3742	6.4
20×10^{-5}	3665	9.4	3781	5.5
30×10^{-5}	3253	19.6	3489	12.8
40×10^{-5}	2860	29.3	2962	26.0
50×10^{-5}	2400	40.7	2855	28.6
62.5×10^{-5}	2042	49.5	2986	25.4
75×10^{-5}	1523	52.4	2983	27.7
87.5×10^{-5}	1134	72.0	2616	34.6
100×10^{-5}	1105	72.7	2400	40.0

[a] Enzyme activity is expressed as cpm incorporated into malonyl-CoA per 5 min. The reaction mixture contained the following components in a total volume of 0.5 ml: Tris buffer (pH 7.5), 30 μmoles; ATP, 1 μmole; $MgCl_2$, 4 μmoles; [^{14}C]$KHCO_3^-$, 5 μmoles [4.2×10^5 cpm/μmole]; acetyl-CoA, 0.1 μmole; isocitrate, 10 μmoles; glutatione, 1.5 μmoles, bovine albumin, 0.3 mg; and chicken liver enzyme purified about 600-fold [diluted in 0.05 M potassium phosphate buffer (pH 7.0), containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol], 0.01–0.10 ml. After incubation at 37° for 5 min, the reaction was terminated with 0.2 ml of 6 N HCl. The activity follows zero-order kinetics with up to 4×10^{-3} unit of acetyl-CoA carboxylase for at least 8 min.

TABLE II: Inhibition of Rat Liver Acetyl-CoA Carboxylase by CDIB and DIB.



[CDIB] [DIB]

Drug Concentration (M)	CDIB Enzyme Activity[a] (cpm)	CDIB Inhibition (%)	DIB Enzyme Activity[a] (cpm)	DIB Inhibition (%)
0	7580		7157	
0	7226		7286	
3.8×10^{-5}	6124	17.2	5809	19.4
7.6×10^{-5}	5870	20.7	5669	21.4
15.1×10^{-5}	5222	29.4	5175	28.2
22.7×10^{-5}	3956	46.6	4977	31.0
30.3×10^{-5}	2502	66.2	4054	43.8
37.8×10^{-5}	1574	78.7	3991	44.6
45.4×10^{-5}	636	91.4	2989	58.6
52.9×10^{-5}	307	96.0	3044	57.8
60.5×10^{-5}	62	99.2	2292	68.2
76.0×10^{-5}	0	100.0	2051	71.6

[a] Activity is expressed as cpm incorporated into malonyl-CoA per 5 min. Incubation mixture contained 40 μmoles of Tris-HCl (pH 7.5), 12 μmoles of $MgCl_2$, 14 μmoles of potassium citrate, 0.8 μmole of 2 mercaptoethanol, 0.6 mg of bovine serum albumin, and rat enzyme, purified to the same extent as the avian liver enzyme, in a volume of 0.52 ml. After preincubation at 37° for 30 min, 1.4 μmoles of ATP, 0.1 μmole of acetyl-CoA, and 12 μmoles of [^{14}C]HCO_3^- [4.2×10^5 cpm/μmole] were added, yielding a total volume of 0.66 ml. After incubation for 5 min at 37°, 0.1 ml of 6 N HCl was added and the radioactivity was determined after drying.

causes a drop in the inhibitory potency to $I_{50} > 1 \times 10^{-3}$ M (Table I) for avian liver, and $I_{50} = 4.2 \times 10^{-4}$ M for rat liver enzyme (Table II). Moreover, the activity appears to decrease at high concentrations of DIB: even at 1 mM DIB, about 60% of the enzyme activity of the avian system remains measurable. The inhibitory properties of this class of compounds as related to their chemical structure and to their *in vivo* hypolipidemic properties are presently under study.

Kinetic Analysis of the Inhibition of the Avian Liver Enzyme by CDIB. Figures 1–4 show the Lineweaver–Burk plots of the effect of varying concentrations of acetyl-CoA, ATP, HCO_3^-, and isocitrate on the inhibition by CDIB of the purified avian liver enzyme (Lineweaver and Burk, 1934). Initial velocities of the enzyme activity were measured for the noninhibited system, and at two or three levels of inhibitor concentration. As shown, the inhibition is competitive for acetyl-CoA and isocitrate and noncompetitive for ATP and HCO_3^- as variable substrates. The kinetic constants calculated from the lines are summarized in Table III. For comparison, the ratios of the inhibition constants for CDIB to the inhibition constants for TPIA and CPIB (Maragoudakis, 1969) have been calculated. From these ratios it can be seen that as an inhibitor of acetyl-CoA carboxylase, CDIB is less active than TPIA but more active than the parent compound, CPIB.

Hill-Type Plots. The Hill equation (Hill, 1913) as modified by Changeux (1963), Atkinson *et al.* (1965), and Loftfield and Eigner (1969) can be used to calculate whether one or several inhibitor molecules are involved in the inhibition process. For both competitive and noncompetitive inhibition, when the expression $\log V_0 + \log (1/V - 1/V_0)$ is plotted against $\log I$, a straight line is obtained. The slope of this line is the interaction coefficient [n] and is numerically equal to the number of inhibitor binding sites, when the interactions are strong. This is an experimentally accessible expression of the inhibition, where V_0 is the rate of the reaction without inhibitor, V is the rate in the presence of inhibitor and I is the concentration of the inhibitor. For each acetyl-CoA level indicated (Figure 1) the values of n were determined from the V_0 and the V values for the three inhibitor concentrations

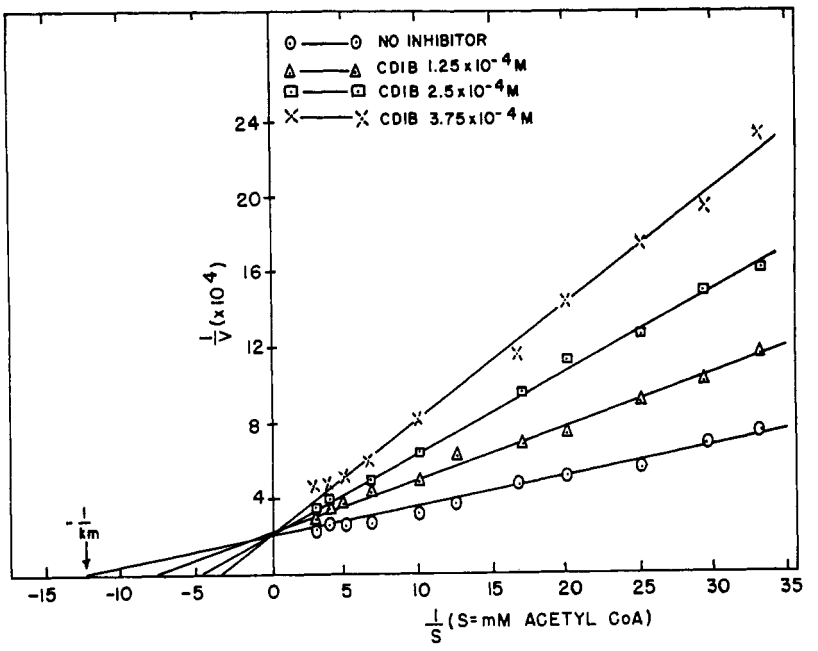


FIGURE 1: Effect of acetyl-CoA concentration on enzyme activity and CDIB inhibition. Conditions were the same as in the footnote to Table I. About 2×10^{-3} unit of acetyl-CoA carboxylase (purified after the third ammonium sulfate stage) was present in each tube. Initial velocity is expressed as counts per minute of $[^{14}C]HCO_3^-$ incorporated, in 5 min, per assay tube. Under the conditions of these experiments, the reaction was of zero order for at least 8 min.

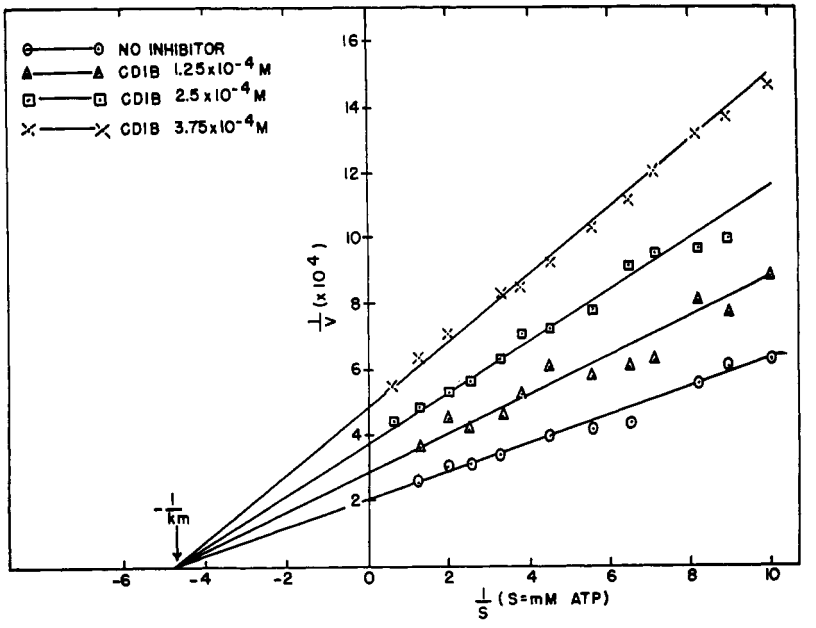


FIGURE 2: Lineweaver–Burk analysis of the inhibition of acetyl-CoA carboxylase by CDIB with ATP as the varied substrate. Experimental conditions as in Figure 1.

TABLE III: Kinetic Constants of the Inhibition.

Varied Substrate	Type of Inhibition	K_i for CDIB (M)	K_m (M)	Ratio I[a]	Ratio II[b]
Acetyl-CoA	Competitive	1.5×10^{-4}	8.00×10^{-5}	1.60	0.14
ATP	Noncompetitive	2.8×10^{-4}	2.10×10^{-3}	1.85	0.07
Isocitrate	Competitive	7.9×10^{-5}	1.25×10^{-3}	0.79	0.23
HCO_3^-	Noncompetitive	3.4×10^{-4}	1.54×10^{-2}	2.27	0.14

[a] Ratio I is the ratio of K_i for CDIB to K_i for TPIA. [b] Ratio II is the ratio of K_i for CDIB to K_i for CPIB.

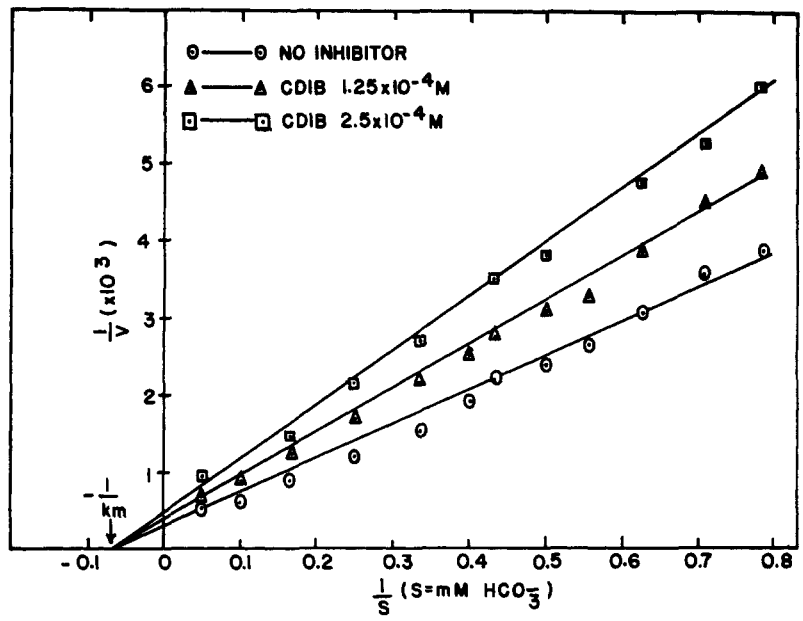


FIGURE 3: Lineweaver–Burk plots of the inhibition of acetyl-CoA by CDIB with HCO_3^- as the varied substrate. Other components of the reaction mixtures as in Figure 1.

[*I*] using program 310 on computer IBM-1130. Table IV summarizes the linear regression equations and the slopes [*n*]. Interactions of CPIB with the acetyl-CoA binding site of the enzyme are quite strong as indicated by the low dissociation constant, $K_i = 1.4 \times 10^{-4}$ M when acetyl-CoA is the varying substrate. Values of *n*, numerically approaching 1, can be interpreted to mean that one molecule of drug per acetyl-CoA binding site of enzyme is involved in the inhibition.

Discussion

As proposed previously, hypolipidemic agents of the nature of CPIB or TPIA may owe their *in vivo* effect to their ability to depress acetyl-CoA carboxylase activity. Thereby, they could control rate and extent of fatty acid and cholesterol synthesis. The most recently described member of this class of agents—CDIB—is active *in vivo* at much lower doses than is CPIB (Leigh *et al.*, 1968). It was to be expected that CDIB would also be a more potent inhibitor of the enzyme, acetyl-CoA carboxylase, and this prediction was fully borne out by the study reported here.

TABLE IV: Slopes of Hill-Type Plots with Acetyl-CoA as Varying Substrate.

Acetyl-CoA (mM)	Linear Regression Eq for *x vs. y*[a]	Slope ± Std Dev (*n*)
0.04	$y = 4.26 + 1.15\,x$	1.15 ± 0.08
0.06	$y = 3.06 + 0.85\,x$	0.85 ± 0.02
0.10	$y = 3.48 + 0.96\,x$	0.96 ± 0.01
0.30	$y = 4.75 + 1.39\,x$	1.39 ± 0.01

[a] $y = \log V_0 + \log(1/V - 1/V_0)$, $x = \log I$, n = interaction coefficient.

It seems likely that all three hypolipidemic drugs studied (TPIA, CDIB, and CPIB) have the same mechanism of interaction with the enzyme since they are all competitive for isocitrate and acetyl-CoA and noncompetitive for ATP and HCO_3^-.

Competitive inhibition for isocitrate and acetyl-CoA may indicate that the drugs interfere with the activity of acetyl-CoA carboxylase, either by competing with the substrate for the same active site on the enzyme protein, or by interfering with the activation process of the enzyme by competing with the activator, isocitrate. Our present data do not allow a choice between the two possibilities, and indeed, both of them may be functioning. In its ability to compete with acetyl-CoA, the new agent, CDIB, is weaker than TPIA (about 1.6-fold) and stronger than CPIB (about 7-fold). With regard to competition with isocitrate, CDIB and TPIA are comparable, and both of them are some four times more potent than CPIB.

Competitive inhibition for both acetyl-CoA and isocitrate may also indicate that the binding sites on the enzyme for the substrate acetyl-CoA and the activator isocitrate are either the same or sufficiently close together so that binding of the drugs to either of them effects the binding affinity for both acetyl-CoA and isocitrate.

Inhibition of acetyl-CoA carboxylase is not unique for avian liver. Rat liver enzyme is even more susceptible to the inhibitory effect of CDIB than the avian enzyme under our assay conditions.

An indication of the probable molecular order of participation of CDIB in the inhibition of avian liver acetyl-CoA carboxylase was obtained by the modified Hills plots (Loftfield and Eigner, 1969) for acetyl-CoA as varying substrate. Interaction coefficients [*n*] close to unity (Table V) make it plausible that the binding of one drug molecule per active site is sufficient for inactivation.

Inhibitory potency on acetyl-CoA carboxylase and hypolipidemic activity seem to be dependent on specific structural requirements of the drugs. DIB, the compound without the chlorine atom, shows completely different inhibitory patterns

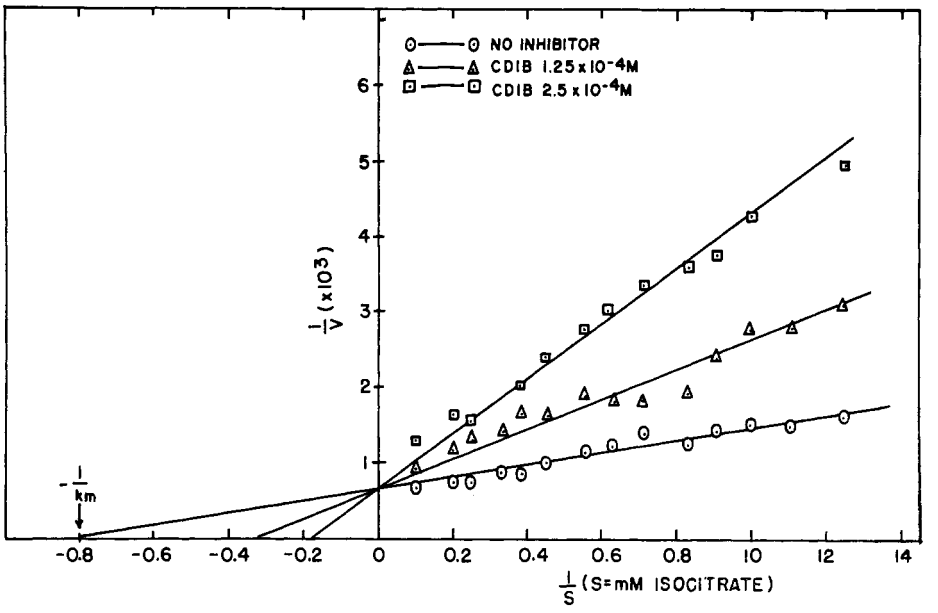


FIGURE 4: Lineweaver–Burk analysis of the inhibition of acetyl-CoA carboxylase by CDIB with varying isocitrate. Isocitrate and CDIB concentrations are as indicated other conditions as in Figure 1.

and hypolipidemic properties from CDIB. The structural features of hypolipidemic agents that permit interaction with acetyl-CoA carboxylase will be discussed in future reports.

Present results are consistent with the suggestion that CPIB, TPIA, and CDIB may act by controlling the level of malonyl-CoA available for lipogenesis. They are also in agreement with the growing body of evidence which indicates that the level of acetyl-CoA carboxylase play a critical role in the control of fatty acid synthesis.

Acknowledgment

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