Inhibition of Monoacylglycerophosphate Formation by Chlorophenoxyisobutyrate and β-benzalbutyrate

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SUMMARY

Monoacylglycerophosphate is the major product formed when sn-glycerol-3-P is esterified by rat liver microsomes, palmitoyl-CoA, and albumin at pH 6.5. The effect of two hypolipidemic agents, chlorophenoxyisobutyrate and β-benzalbutyrate, on monoacylglycerophosphate synthesis was studied. Both agents (0.5 to 1.0 mM) inhibited monoacylglycerophosphate synthesis by 10 to 20%. Removal of albumin from the incubation mixture produced an effect similar to the addition of chlorophenoxyisobutyrate and β-benzalbutyrate. Palmitoyl-CoA is bound by albumin and microsomal protein. Addition of both drugs decreased binding of palmitoyl-CoA by albumin and increased microsomal-bound palmitoyl-CoA, without increasing the free palmitoyl-CoA. An increase in microsomal-bound palmitoyl-CoA above 20 nmoles per mg of protein resulted in reduced monoacylglycerophosphate formation. Therefore, both agents reduced monoacylglycerophosphate formation in part by raising the concentration of microsomal-bound palmitoyl-CoA. However, both agents inhibited the reaction in the absence of albumin and under conditions in which microsomal-bound palmitoyl-CoA was held constant. These agents had no effect on several other enzymes in the pathway of glycerolipid synthesis. Therefore, chlorophenoxyisobutyrate and β-benzalbutyrate appear to be direct inhibitors of monoacylglycerophosphate formation. This inhibition may explain their early hypolipidemic effect in vivo.

Chlorophenoxyisobutyrate and β-benzalbutyrate effectively reduce serum triglycerides and cholesterol in man (1-3) and experimental animals (4, 5). A variety of mechanisms have been proposed to explain the hypolipidemic effect of these agents (5-15), but the mode of action has remained uncertain.

Previous investigators concluded that chlorophenoxyisobutyrate did not reduce hepatic triglyceride formation (7, 10, 16). However, recent studies in this laboratory show that administration of chlorophenoxyisobutyrate reduces hepatic glyceride formation, measured in vivo, and also inhibits the conversion of sn-glycerol-3-P to diglyceride, triglyceride, and lecithin by rat liver homogenates (17). The decrease in triglyceride formation precedes many of the other metabolic alterations caused by chlorophenoxyisobutyrate and coincides with the initial fall in serum triglyceride. These results suggest that chlorophenoxyisobutyrate may inhibit hepatic glycerolipid synthesis at some step before the formation of diglyceride. The present work describes an inhibition by chlorophenoxyisobutyrate and β-benzalbutyrate of monoacylglycerophosphate synthesis from sn-glycerol-3-P in rat liver microsomal preparations. This inhibition is apparently direct and was not observed for other enzymes in the pathway.

METHODS

Materials—Palmitoyl-CoA was prepared from palmitoyl chloride (Hormel Institute) as described by Soubert (18), and [1-14C]palmitoyl-CoA was purchased from New England Nuclear. The palmitoyl-CoA purity was shown to be greater than 95% by techniques described previously (19).

Sodium salts of chlorophenoxyisobutyrate and β-benzalbutyrate were kindly supplied by Ayerst Laboratories and Istituto Biochimico Italiano (IBI), respectively. sn-[1,3-14C]Glycerol-3-P was prepared from [1,3-14C]glycerol (19) or purchased from Mallinckrodt.

Methods used for preparation of microsomes and to identify products of the reaction were the same as those described elsewhere (19, 20) except for the substitution of 0.05 mM sodium phosphate, pH 6.5, for Tris-HCl as the buffer used in the preparation of microsomes.

sn-Glycerol-3-P Acyltransferase—The usual assay contained 1.5 mM dithiothreitol, 90 μM palmitoyl-CoA, 70 mM sodium phosphate, pH 6.5, 1.25 mg of fatty acid-poor bovine albumin (Fraction V, Pentex, Inc.), 0.6 mM sn-[1,3-14C]glycerol-3-P (0.1 μCi) and 0.1 to 0.2 mg of microsomal protein in a total volume of 0.35 ml. The reaction was initiated by the addition of microsomes and stopped after 10 min by the addition of 10 ml of chloroform-methanol (2:1). Lipids were extracted by the method of Folch, Lees, and Sloane Stanley (21). The only product identified under these incubation conditions is monoacylglycerophosphate (19).

Determination of Palmitoyl-CoA Attachment to Protein—Isotopically labeled [1-14C]palmitoyl-CoA was used in all experiments for determination of palmitoyl-CoA binding. Several techniques were used to determine the proportion of palmitoyl-CoA bound to microsomes and albumin and the effects of chlorophenoxyisobutyrate and β-benzalbutyrate on this binding. Two of the techniques, sedimentation by ultracentrifugation and Millipore filtration, were adequate only for measuring microsomal-bound...
albumin and microsomes in mixtures. The usual assay combined to permit an estimation of the palmitoyl-CoA bound by previously heated for 15 min at 85° to remove deacylase and acylase in small polyethylene centrifuge tubes. Microsomes were pre-

minations.

corrections were made for quenching in all radioactive deter-

ternal mixture was taken as a measure of the relative binding of content of the exterior volume when proteins were added to the external reaction mixture. Differences in 

of toluene-methanol-Liquifluor (70:30:8.4) and radioactivity determined with a Beckman LS-250 spectrometer. In this manner, the distribution of [I-14C]palmitoyl-CoA between the internal volumes was placed in scintillation vials containing 10 ml

was determined with an S 40 rotor in the Spinco ultracentrifuge and the super-

bated for 10 min at 37°. The microsomes were separated from inactivated microsomes, and [I-14C]palmitoyl-CoA were incu-

tions. Monoacylglycerophosphate formation in the presence of drug was 5.5 and 6.0 mM, respectively. Monoacyl-

isobutyrate (CRIB, left) and p-benzalbutyrate (BBB, right) con-

Millipore filtration studies were performed by removing an aliquot of the incubation mixture and collecting the microsomes by filtration under reduced pressure on IIA filters. The filters were washed several times with water, dried, and placed in scintillation vials containing scintillation fluid. Radioactive palmitoyl-CoA associated with the filter was a measure of microsomal-bound palmitoyl-CoA.

Chlorophenoxyisobutyrate and β-benzalbutyrate were determined by a spectrophotometric method. Solutions containing either chlorophenoxyisobutyrate or β-benzalbutyrate were extracted by the addition of 0.1 ml of 3 N HCl and 2 ml of ethanolic isoheptane (5:95) (24). The upper phase was removed and the optical density determined at 226 nm on a Beckman spectrophotometer. Optical density was proportional to drug concentration in the various buffers used and in the presence or absence of protein. The ultraviolet spectrum of drugs isolated from albumin and microsomal mixtures was identical with that of the pure drug.

Drug binding to various protein fractions was usually deter-

ed by the ultrafiltration technique described above. An aliquot of the filtrate was removed and then the amount of drug present was determined by the aforementioned spectrophotometric procedure.

RESULTS

The effects of microsomal concentration, incubation time, and omission of individual assay components on the rate of monoacylglycerophosphate formation in the standard assay have been described previously (19).

Under optimum reaction conditions, increasing concentrations of β-benzalbutyrate or chlorophenoxyisobutyrate inhibit the formation of monoacylglycerophosphate from sn-glycerol-3-P and palmitoyl-CoA as shown in Fig. 1. Addition of sodium butyrate in this same concentration range caused no inhibition of the reaction. The IC50 for chlorophenoxyisobutyrate and β-

Fig. 1. Incorporation of sn-[1,3-14C]glycerol-3-P (GP) into monoacylglycerol-3-P (MAGP) over a range of chlorophenoxy-

isobutyrate (CPIB, left) and β-benzalbutyrate (RRR, right) concen-

trations. Incubation conditions were the same as those for the standard mixture with the exception of adding 0.05 ml of 0.05 M phosphate buffer, pH 6.5, containing the appropriate amount of drug.

of water and the wash water added to the supernatant. The supernatant was placed in a boiling water bath for 3 min to precipitate albumin, cooled on ice for 10 min, and then centrifuged at 400 × g for 10 min to separate the albumin precipitate. The remaining protein-free supernatant was removed and the precipitate washed with 0.5 ml of water which was added to the supernatant. The albumin and microsomal pellets and an aliquot of the supernatant were placed in scintillation vials and radioactivity determined. The radioactivity recovered in the albumin and microsomal pellets, respectively, was taken as a measure of microsomal- and albumin-bound palmitoyl-CoA while the radioactivity present in the protein free supernatant was considered free palmitoyl-CoA. The binding of palmitoyl-CoA to albumin in the absence of microsomes was determined by this heat precipitation method and was equivalent to the estimation of albumin binding by dialysis techniques.

Dialysis and ultrafiltration studies were conducted in the following manner. Cellulose dialysis tubing, secured from Fisher Scientific Supply Corp., with flat diameter 0.39 inch, was filled with 0.7 ml of standard reaction mixture containing [1-14C]palmitoyl-CoA. Dialysis was conducted with agitation in test tubes containing 5.6 ml of water or 0.05 M sodium phosphate, pH 6.5, as the external phase for periods of 12 to 96 hours at 8°. The ultrafiltration method was based on the technique described by Toribara et al. (22). Filtration of the reaction mixture through cellulose dialysis tubing, secured from Fisher Scientific Supply Corp., with flat diameter 0.39 inch, was filled with 0.7 ml of standard reaction mixture containing [1-14C]palmitoyl-CoA.

Palmitoyl-CoA. Dialysis and ultrafiltration were methods used to measure both albumin and microsomal-bound palmitoyl-CoA. In some experiments protein was precipitated by heat. This was an alternative technique for measurement of albumin-bound palmitoyl-CoA.

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reaction rate in the absence of added albumin (Fig. 5). The data show that 5 mM chlorophenoxyisobutyrate inhibits monoacylglycerophosphate formation 40% in the presence of 3.75 mg per ml of albumin and 49% in the absence of albumin. Similar results to those shown in Fig. 2 were obtained with 5 mM benzalbutyrate.

The effect of variations in sn-glycerol-3-P concentration on the rate of monoacylglycerophosphate formation in the presence of 5 and 10 mM chlorophenoxyisobutyrate or benzalbutyrate is shown in Fig. 3. A double reciprocal plot of reaction velocity and substrate concentration for this data is shown in Fig. 4. The inhibition pattern is not typical of either competitive or noncompetitive type and suggests a mixed form of inhibition with relation to this substrate.

Increasing concentrations of the substrate, palmitoyl-CoA, was found to inhibit the reaction as shown in Fig. 5. The rate of monoacylglycerophosphate synthesis was measured over a range of palmitoyl-CoA concentrations and at several levels of albumin. In the absence of albumin, palmitoyl-CoA concentrations above 30 μM inhibit the reaction, while in the presence of 3.75 mg per ml of albumin, palmitoyl-CoA concentrations up to 90 μM were not inhibitory. The result of this effect is to shift the optimum palmitoyl-CoA concentration from 30 μM in the absence of albumin to 90 μM in the presence of optimum albumin levels. At low palmitoyl-CoA concentrations, a reduction in albumin level resulted in a greater reaction rate. At albumin concentrations above 30 mg per ml, a lower maximum reaction rate occurred despite increasing palmitoyl-CoA concentrations.

The effect of 1, 5, and 10 mM chlorophenoxyisobutyrate and benzalbutyrate on the rate of monoacylglycerophosphate formation over a range of palmitoyl-CoA concentrations is shown in Fig. 6. These results were determined at an albumin concentration of 3.75 mg per ml. As shown in Fig. 6, both agents lowered the optimum palmitoyl-CoA level in direct proportion to drug concentration. At low concentrations of palmitoyl-CoA, reaction rate was increased in the presence of chlorophenoxyisobutyrate or benzalbutyrate. Moreover, increasing the palmitoyl-CoA level above the optimum reduced the reaction velocity in all cases. Thus, the effect of these drugs on optimum palmitoyl-CoA concentration is directly opposite to that caused by addition of albumin.

Effects of chlorophenoxyisobutyrate and benzalbutyrate on the binding of palmitoyl-CoA to albumin and microsomes might explain these results. Therefore, studies were undertaken to study palmitoyl-CoA binding by albumin and microsomes and the effect of chlorophenoxyisobutyrate and benzalbutyrate on the distribution of palmitoyl-CoA between albumin and microsomes. The effect of albumin on the microsomal binding of palmitoyl-CoA is shown in Table I. Incubation of 1 mg of microsomal protein and 30 nmoles of palmitoyl-CoA results in binding of 89.3% of the acyl-CoA to microsomes in the absence of albumin and only 23.5% in the presence of 3.75 mg per ml of albumin. These results show that the amount of microsomal-bound palmitoyl-CoA is markedly reduced by addition of albumin. Increasing chlorophenoxyisobutyrate concentrations did not displace palmitoyl-CoA from microsomes as shown in Table I.

The effects of increasing albumin concentration on the amount

![Fig. 3. Effect of 5 and 10 mM chlorophenoxyisobutyrate (CPIB) (A) and benzalbutyrate (BBB) (B) on monoacylglycerophosphate (MAGP) formation over a range of sn-glycerol-3-P (GP) concentrations. Standard assay conditions were followed except for the variable sn-glycerol-3-P concentrations and addition of drugs as described in the legend of Fig. 1.](http://www.jbc.org/)

![Fig. 4. A double reciprocal plot of the data shown in Fig. 3. Best straight lines were determined by the method of least squares. CPIB, chlorophenoxyisobutyrate; BBB, benzalbutyrate; GP, sn-glycerol-3-P.](http://www.jbc.org/)
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FIG. 5. The incorporation of sn-[1,3-14C]glycerol-3-P (GP) into monoacylglycerophosphate (MAGP) over a range of palmitoyl-CoA concentrations and at different albumin concentrations. The usual incubation conditions were followed except for the variable palmitoyl-CoA and albumin concentrations. CPIB, chlorophenoxyisobutyrate; BBB, β-benzalbutyrate.

FIG. 6. Effect of 1.0, 5.0, and 10.0 mM chlorophenoxyisobutyrate (CPIB) (A) and β-benzalbutyrate (BBB) (B) on monoacylglycerophosphate (MAGP) formation over a range of palmitoyl-CoA concentrations. Standard incubation conditions were used except for variable palmitoyl-CoA concentrations and drug additions as described in the legend of Fig. 1. GP, sn-glycerol-3-P.

TABLE I

Effect of chlorophenoxyisobutyrate, albumin, and microsomes on supernatant palmitoyl-CoA as studied by sedimentation techniques

Supernatant palmitoyl-CoA includes both free palmitoyl-CoA and palmitoyl-CoA bound to albumin. Free palmitoyl-CoA was always less than the critical micelle concentration of palmitoyl-CoA in the presence of albumin. Total palmitoyl-CoA concentration was 200 mM. The palmitoyl-CoA in supernatant in the presence of albumin and absence of microsomes was largely bound to albumin.

<table>
<thead>
<tr>
<th>Chlorophenoxyisobutyrate</th>
<th>Albumin</th>
<th>Microsomes</th>
<th>Percentage of palmitoyl-CoA in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mg/ml</td>
<td>mg/ml</td>
<td>%</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.0</td>
<td>3.75</td>
<td>1.0</td>
<td>98.7</td>
</tr>
<tr>
<td>0.0</td>
<td>3.75</td>
<td>1.0</td>
<td>8.3</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>76.5</td>
</tr>
<tr>
<td>5.0</td>
<td>0.0</td>
<td>1.0</td>
<td>8.8</td>
</tr>
<tr>
<td>10.0</td>
<td>0.0</td>
<td>1.0</td>
<td>9.2</td>
</tr>
</tbody>
</table>

FIG. 7. Microsomes were sedimented by ultracentrifugation as described under “Methods” in the presence of several different albumin concentrations. Usual incubation conditions were followed except for the addition of [1,3-14C]palmitoyl-CoA, and variable albumin concentrations. Microsomal (MCSM) protein concentration was 0.60 mg per ml.

of microsomal-bound palmitoyl-CoA is shown in Fig. 7. An inverse relationship between albumin concentrations and the level of microsomal bound palmitoyl-CoA was noted. When albumin was added to a mixture of microsomes and palmitoyl-CoA, the redistribution of palmitoyl-CoA occurred in less than 60 sec at 37°. A comparison of the different techniques for determining protein-bound palmitoyl-CoA is shown in Table II. All the methods gave comparable results.

Thorp has indicated that chlorophenoxyisobutyrate is bound by albumin (25). Palmitoyl-CoA also is bound by albumin (Table I). In the present studies, bovine serum albumin and rat serum bound both chlorophenoxyisobutyrate and β-benzalbutyrate in direct proportion to protein concentration. At protein concentrations of 40 mg per ml, bovine serum albumin and rat serum bound 73 and 26%, respectively, of a chlorophenoxyisobutyrate solution containing 670 μg per ml.

The distribution of palmitoyl-CoA between microsomes and albumin was altered by the addition of chlorophenoxyisobutyrate and β-benzalbutyrate as shown in Table III. Increasing con-
TABLE II
Comparison of different techniques for determining protein-bound [1-14C]palmitoyl-CoA

The methodology for evaluation of these techniques is described under "Methods."

<table>
<thead>
<tr>
<th>Method</th>
<th>Percentage of palmitoyl-CoA bound ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal-bound</td>
<td></td>
</tr>
<tr>
<td>Sedimentation</td>
<td>88.55 ± 0.45</td>
</tr>
<tr>
<td>Millipore filtration</td>
<td>85.10 ± 4.20</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>91.00 ± 0.20</td>
</tr>
<tr>
<td>Albumin-bound</td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>97.85 ± 0.05</td>
</tr>
<tr>
<td>Heat precipitation</td>
<td>80.80 ± 0.20</td>
</tr>
</tbody>
</table>

TABLE III
Effect of chlorophenoxyisobutyrate and β-benzalbutyrate on free and bound palmitoyl-CoA

The [1-14C]palmitoyl-CoA was incubated with 0.2 mg of microsomal protein in the presence of the indicated drug concentration. The distribution between various components was measured as described under "Methods." Total volume of 0.35 ml containing 3.75 mg per ml of albumin.

<table>
<thead>
<tr>
<th>Amount of drug</th>
<th>Amount of palmitoyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
</tr>
<tr>
<td>β-Benzalbutyrate</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>5.0</td>
<td>1.7</td>
</tr>
<tr>
<td>10.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Chlorophenoxyisobutyrate</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>5.0</td>
<td>2.1</td>
</tr>
<tr>
<td>10.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

centrations of chlorophenoxyisobutyrate and β-benzalbutyrate displaced palmitoyl-CoA from albumin and resulted in increased microsomal-bound palmitoyl-CoA without affecting free acyl-CoA levels. Therefore, the inhibition produced by these drugs cannot be attributed simply to a displacement of palmitoyl-CoA from albumin into a free micelle phase with secondary inhibition of enzyme activity. However, the change in the level of microsomal-bound palmitoyl-CoA might affect reaction rate and this possibility was studied further.

The relation of microsomal-bound palmitoyl-CoA to reaction velocity was determined. Variations in microsomal-bound palmitoyl-CoA to reaction velocity was determined. Variations in microsomal-bound palmitoyl-CoA were created by adding 1.0, 5.0, and 10.0 mM chlorophenoxyisobutyrate and β-benzalbutyrate to the reaction mixture. The results are shown in Fig. 8. These data relate reaction velocity to the concentration of microsomal-bound palmitoyl-CoA rather than total palmitoyl-CoA levels. Maximum reaction velocity was achieved at approximately the same microsomal-bound concentration regardless of total palmitoyl-CoA levels. However, reaction velocity was reduced even at optimum microsomal-bound palmitoyl-CoA in the presence of increasing chlorophenoxyisobutyrate or β-benzalbutyrate levels. Thus, inhibition of monacetylglucosolphosphate synthesis by chlorophenoxyisobutyrate and β-benzalbutyrate cannot be attributed exclusively to an increase in the concentration of microsomal bound palmitoyl-CoA but likely results from a direct effect of the drugs on reaction rate. This interpretation is supported by the evidence that chlorophenoxyisobutyrate and β-benzalbutyrate both inhibit monacetylglucosolphosphate synthesis in the absence of albumin as shown in Fig. 2.

Palmitate, ATP, and CoA were substituted for palmitoyl-CoA and incubated with microsomes under the conditions described previously (19). Monacetylglucosolphosphate, diglyceride, and triglyceride are major products under these conditions, and 5 mM

Fig. 8. The data presented in Fig. 6 is replotted in this figure on the basis of the amount of palmitoyl-CoA bound to microsomes in the reaction mixture. The amount of [1-14C]palmitoyl-CoA bound to microsomes was determined by Millipore filtration as described under "Methods." The incubation conditions are the same as those described in the legend of Fig. 6. GP, sn-glycero-3-P; CPIB, chlorophenoxyisobutyrate; BB, β-benzalbutyrate; MAGP, monoacylglycerophosphate; Mccm, microsomal.

TABLE IV
Effect of chlorophenoxyisobutyrate and β-benzalbutyrate on monoacylglycerophosphate, diglyceride, and triglyceride synthesis

Values are mean percentage inhibition of sn-[1,3-14C]glycerol-3-P incorporation into the indicated lipid product. Sodium palmitate (0.7 mM), ATP (4.0 mM), and coenzyme A (0.6 mM) were substituted for palmitoyl-CoA; 70 mM Tris-HCl, pH 7.5, was substituted for the phosphate buffer, and this mixture plus microsomes was incubated for 30 min. The reaction was stopped by the addition of 10 ml of CHCl₃-MeOH (2:1) and lipids extracted and identified by procedures described under "Methods."

<table>
<thead>
<tr>
<th>Drug</th>
<th>Monoacylglycerophosphate</th>
<th>Diglyceride</th>
<th>Triglyceride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophenoxyisobutyrate, 5 mM</td>
<td>37.7</td>
<td>25.8</td>
<td>33.4</td>
</tr>
<tr>
<td>β-Benzalbutyrate, 5 mM</td>
<td>65.0</td>
<td>61.5</td>
<td>37.9</td>
</tr>
</tbody>
</table>
chlorophenoxyisobutyrate and &-benzalbutyrate markedly reduced the synthesis of these glycerolipids as shown in Table IV.

**DISCUSSION**

Several mechanisms have been proposed to explain the reduction in serum triglyceride levels by chlorophenoxyisobutyrate (5-15). These include: accelerated adipose tissue uptake of triglyceride (7); reduced adipose tissue lipolysis (6); displacement of thyroxine from serum into liver (15); reduction in hepatic fatty acid synthesis (13, 14); decreased hepatic triglyceride release (9-12); and decreased hepatic triglyceride formation (17). The present studies were based on the observation that chlorophenoxyisobutyrate administration results in a decrease in hepatic triglyceride formation, *in vivo*, and inhibition of triglyceride formation from sn-glycerol-3-P by liver homogenates (17).

The first reaction in hepatic glyceride synthesis, sn-glycerol-3-P acyltransferase, results in the formation of monoacylglycerophosphate. Chlorophenoxyisobutyrate and &-benzalbutyrate were found to inhibit synthesis of monoacylglycerophosphate by liver microsomal preparations. Both agents inhibited this reaction by 10 to 20% when added in concentrations found in the serum of animals treated with chlorophenoxyisobutyrate (0.5 to 1.0 mm).

The type of inhibition produced by chlorophenoxyisobutyrate and &-benzalbutyrate was evaluated using a microsomal preparation as the enzyme source. The inhibition by these agents was not competitive with respect to sn-glycerol-3-P and was apparently of mixed type. The type of inhibition with respect to palmitoyl-CoA was not studied because the amount of this substrate exceeded the critical micelle concentration and was largely protein bound.

Albumin was added to the reaction mixture under the usual assay conditions because of the significantly increased sn-glycerol-3-P acyltransferase activity. Other investigators have noted the effect of albumin previously (20, 26, 27). The mechanisms of albumin activation are not entirely clear. Several hypotheses have been presented which might partially explain the effect of albumin. Microsomal acyl-CoA deacetylase activity is reduced in the presence of albumin resulting in more stable substrate levels during the reaction (20, 26). This effect is significant only when the incubation period is prolonged or the substrate concentration is low. Other possible effects of albumin include: binding of inhibitory fatty acids released during the period of incubation, or conversely, addition of potential activating factors to the reaction mixture (28). However, addition of palmitate in a concentration of 90 &mu;m or removal of fatty acids from albumin by the Chen method (29) did not reduce the activating effect of the albumin or alter the effect of chlorophenoxyisobutyrate and &-benzalbutyrate on monoacylglycerophosphate synthesis. Extensive dialysis of the albumin also did not reduce the activating effect (20). Tzur and Shapiro (30) suggested from their experiments that albumin activation was the result of enzyme stabilization.

An alternative explanation is that albumin binds the long chain fatty acyl-CoA substrate and lowers free acyl-CoA concentration below the critical micelle concentration. Palmitoyl-CoA has detergent properties in solution and has been shown to inhibit a variety of enzymes *in vitro* (31). Several investigators have shown that levels of palmitoyl-CoA which inhibit enzyme activity in the absence of albumin are not inhibitory in the presence of albumin (20, 27, 32). This has been attributed to binding of palmitoyl-CoA by albumin with reduction in the micellar phase.

Results presented here show that increasing albumin concentrations up to 3.75 mg per ml in the incubation mixture increases both the palmitoyl-CoA level which gives maximum reaction velocity and the activity of sn-glycerol-3-P acyltransferase at optimum substrate concentrations. Higher concentrations of albumin inhibited the reaction despite increases in the palmitoyl-CoA substrate level. This unusual effect was studied further by measuring the distribution of palmitoyl-CoA between microsomes, albumin, and the soluble phase under a variety of conditions. Albumin was shown to reduce the binding of palmitoyl-CoA by microsomes in proportion to the albumin concentration. The amount of microsomal-bound palmitoyl-CoA was estimated by sedimentation and Millipore filtration experiments. The results obtained show that the optimum reaction velocity was obtained at a relatively constant amount of microsomal-bound palmitoyl-CoA (15 to 20 nmoles per mg of protein). Also, the reaction was inhibited when microsomal bound palmitoyl-CoA exceeded the range of 20 to 25 nmoles per mg of protein. Thus, one effect of albumin is to maintain microsomal palmitoyl-CoA levels in a range which is optimum for monoacylglycerophosphate formation.

Chlorophenoxyisobutyrate is protein bound (25) and occupies anionic (8, 33) binding sites on albumin. Results presented here confirm the binding of both chlorophenoxyisobutyrate and &-benzalbutyrate to albumin. If binding of these drugs by albumin interferes with the binding affinity for palmitoyl-CoA, chlorophenoxyisobutyrate and &-benzalbutyrate would displace palmitoyl-CoA from albumin. The data in Table III show that both agents do displace palmitoyl-CoA from albumin and result in an increase in microsomal-bound palmitoyl-CoA. There is no increase in free palmitoyl-CoA under these conditions. Thus, inhibition by chlorophenoxyisobutyrate and &-benzalbutyrate cannot result from increased formation of palmitoyl-CoA micelles inhibiting the reaction in a nonspecific manner. The data would indicate that a part of the inhibitory effect of these drugs relates to an increase in microsomal-bound palmitoyl-CoA above the level of 20 nmoles per mg of microsomal protein.

However, other evidence indicates that chlorophenoxyisobutyrate and &-benzalbutyrate also are direct inhibitors of monoacylglycerophosphate formation. Both agents inhibit the acyltransferase reaction in the absence of albumin (Fig. 1) and under conditions in which the amount of microsomal-bound palmitoyl-CoA is unchanged. When reaction velocity was plotted against microsomal-bound palmitoyl-CoA at several different drug concentrations, the optimum microsomal-bound palmitoyl-CoA concentration was the same for each level of drug. The results in the presence or absence of drug suggest that the level of microsomal-bound palmitoyl-CoA is important in determining reaction velocity.

The significance of the inhibition of sn-glycerol-3-P acyltransferase by chlorophenoxyisobutyrate and &-benzalbutyrate in the reduction of triglyceride formation *in vitro* is supported by measurements of glycerolipid formation at pH 7.5. Palmitate, ATP, and CoA were substituted for palmitoyl-CoA and the mixture incubated for 30 min. Diglyceride and triglyceride are major products under these conditions and both are markedly reduced by chlorophenoxyisobutyrate or &-benzalbutyrate addition. Monoacylglycerophosphate synthesis was also reduced under...
these conditions. These results support the hypothesis that chlorophenoxyisobutyrate and β-benzalbutyrate inhibit an early step in glycerolipid synthesis.

The effect of these agents on other reactions of hepatic glycerolipid synthesis was studied. Chlorophenoxyisobutyrate and β-benzalbutyrate in a concentration of 1.0 to 10.0 mM did not alter diglyceride acyltransferase, monoacylglycerophosphate-acyltransferase, phosphatidic acid phosphatase, or palmitoyl-CoA synthetase. Thus, the inhibition of glycerolipid synthesis by these drugs appears to be specifically related to the inhibition of monoacylglycerophosphate formation. Inhibition of acetyl-CoA carbamoylase by chlorophenoxoisobutyrate also has been described (13, 14). Reduction in hepatic fatty acid synthesis would present an additional mechanism for reduction in triglyceride formation by this agent.

The inhibition of sn-glycerol-3-P acyltransferase by chlorophenoxyisobutyrate and P-benzalbutyrate was virtually identical. Possibly the structural similarity of these two agents accounts for their similar behavior. However, these two drugs differ in effects on hepatic size and microsomal enzyme function (5), indicating important differences in their metabolic effects.

The liver is a major source of serum triglyceride, especially in the postabsorptive state. Agents which reduce hepatic triglyceride formation should reduce serum triglyceride concentrations if no reduction in triglyceride removal from plasma occurs. Therefore, the inhibition of hepatic glycerolipid synthesis by chlorophenoxyisobutyrate and β-benzalbutyrate could explain their early hypolipidemic effects. Inhibition of sn-glycerol-3-P acyltransferase by these agents may account for this reduction in triglyceride formation.

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REFERENCES
Inhibition of Monoacylglycerophosphate Formation by Chlorophenoxyisobutyrate and β-benzalbutyrate
Robert G. Lamb and Harold J. Fallon


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