MOLECULAR PHARMACOLOGY, 13, 224-231

Displacement of Free Fatty Acids from Albumin by Chlorophenoxyisobutyrate

HERMAN MEISNER

Department of Pharmacology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

(Received March 25, 1976)
(Accepted October 13, 1976)

SUMMARY

MEISNER, HERMAN (1977) Displacement of free fatty acids from albumin by chlorophenoxyisobutyrate. *Mol. Pharmacol.*, 13, 224-231.

A two-phase hexane-aqueous salts buffer, pH 7.45, has been used as a model system to study the displacement of strongly bound long-chain free fatty acids (FFA) from bovine serum albumin by a more weakly binding drug, chlorophenoxyisobutyrate, that is insoluble in the organic phase. The hydrophobic phase serves to trap unbound FFA, thereby reducing the bound ligand concentration, but does not solvate the more hydrophilic drug. At equilibrium chlorophenoxyisobutyrate displaces [^{14}C]FFA from albumin, in the order stearate > palmitate > myristate. A drug to albumin molar ratio of 2.2 reduces the binding of [^{14}C]palmitate to albumin by only 2%, but increases the organic concentration by 50%. The binding curve of [^{14}C]palmitate-albumin is shifted to the right by 1 mM chlorophenoxyisobutyrate, indicating competitive displacement. At a constant ratio of total drug to bound FFA, a greater fraction of FFA is displaced from albumin at higher FFA to albumin ratios, suggesting that FFA bound to lower-affinity sites are removed more readily. Thus, in a two-phase system, a ligand which binds weakly to albumin can competitively displace a tightly bound ligand that partitions favorably in the hydrophobic phase. The model may have general applications in explaining the mechanism of action of albumin-bound drugs that reduce circulating FFA levels.

INTRODUCTION

The association of amphiphilic ligands, such as long-chain fatty acids, with albumin has been extensively studied (1-6). This association is characterized by (a) a requirement for both electrostatic and hydrophobic interactions (7-9), (b) high equilibrium association constants, on the order of 10^6-10^8 (3, 6), (c) a constant increment in free energy of transfer ($\delta\Delta F_{l\to w}$) per methylene group from lipid to aqueous phase as a function of free fatty acid chain length (5), and (d) multiple albumin binding sites having progressively lower association constants as occupancy of the sites becomes greater. From the last characteristic, it follows that the binding of FFA[1] to albumin is best described in terms of a continuous range of equilibrium constants, rather than distinct classes of binding sites (6).

Serum albumin also binds many drugs, some of which show association constants between 10^4 and 10^6 (10). The displacement of these drugs from albumin by FFA has been examined in aqueous monophasic systems, with variable results. For example, Rudman *et al.* (11) found that palmi-

This work was supported by grants from the Diabetes Association of Greater Cleveland and the Northeastern Ohio Heart Association.

[1] The abbreviations used are: FFA, free fatty acid(s); CPIB, chlorophenoxyisobutyrate; BSA, bovine serum albumin.

ISSN 0026-895x

tate displaces salicylate, sulfadiazine, and diphenylhydantoin, but only at FFA to albumin ratios greater than 3.5, and concluded from this that physiological changes in FFA levels have little effect on drug binding. However, others have shown that thyroxine (12), indole analogues (13), salicylates (14), 2,4-(4'-hydroxyphenylazo)benzoic acid (15), diphenylhydantoin (16), and chlorophenoxyisobutyrate (17) are displaced at more physiological FFA to albumin molar ratios. Clinically, such displacement may be important only if the fraction of bound drug is quite high (more than 90%); otherwise the unbound concentration will not increase significantly (18, 19).

The possibility that more weakly bound drugs like chlorophenoxyisobutyrate (17) can displace stearate or other tightly bound hydrophobic ligands from albumin has not been seriously considered. However, by using a two-phase hexane-salts partitioning system, we have presented preliminary evidence (20) that CPIB can displace sizable amounts of [^{14}C]palmitate from albumin, despite the fact that the equilibrium association constant of palmitate-albumin is $10^3 > K_a$ of CPIB-albumin (14, 15). The ability of CPIB to displace FFA depends on the presence of a hydrophobic phase, which, by trapping unbound nonpolar ligands, reduces bound ligand concentration. Thus the effectiveness of the more weakly bound drug is enhanced, provided it is insoluble in the low dielectric phase.

The purpose of the present study was to test the effects of CPIB on equilibrium partitioning of C_{18}, C_{16}, and C_{14} FFA, which have progressively increasing solubilities in the aqueous phase. The data show that as FFA chain length increases, displacement by CPIB becomes more pronounced, and that CPIB removes a particular FFA preferentially from lower-affinity binding sites on albumin. The possible pharmacological importance of the results is discussed.

METHODS

Defatted BSA (Sigma, fraction V, 96–99% pure) was prepared according to Chen (21). The amount of FFA remaining after dialysis was measured by the diethyldithiocarbamate method of Ducombe (22), and found to be less than 0.1 mole/mole of BSA.

[1-^{14}C]Palmitate, [1-^{14}C]myristate, and [1-^{14}C]stearate (New England Nuclear) were repurified by dissolving the salts in isooctane–acetic acid (1:1), adding 0.1 volume of water, and discarding the aqueous phase. Purity was 97–98%, determined by thin-layer chromatography in hexane–diethyl ether–acetic acid (70:30:1), followed by scintillation counting of 0.5-cm strips.

Different [^{14}C]FFA-BSA complexes were prepared by adding a hexane solution containing [^{14}C]FFA plus carrier to Celite resin (23), evaporating, and mixing the [^{14}C]FFA-Celite with an aqueous solution of BSA. Initial results showed that under these conditions the length of mixing time required to transfer defatted FFA from Celite to BSA increased with FFA chain length. Accordingly, [^{14}C]palmitate-Celite and [^{14}C]myristate-Celite complexes were routinely mixed with BSA for 3 hr, and the [^{14}C]stearate-Celite complex for 24 hr, before centrifugation and filtering. Final [^{14}C]FFA levels were calculated from initial counts per minute per nanomole of [^{14}C]FFA.

Binding of [^{14}C]FFA to defatted BSA was measured by equilibrium partitioning (2, 3), using acid-washed hexane (Fisher, "spectranalyzed") as the organic phase. Generally 1.0 ml of 0.1–0.2 mM BSA in 0.1 M NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 25 mM phosphate, pH 7.45, with and without drug, was incubated with 1.0 ml of hexane. The FFA was initially introduced into the aqueous phase as the [^{14}C]FFA-BSA complex, or into the organic phase as [^{14}C]FFA. The reaction was carried out in 5-ml-capacity capped glass minivials (No. 7460B, Rochester Scientific Company) at 37° and 108 oscillations/min. At the FFA concentrations employed, adsorption of radioactivity to the sides of the vials was insignificant. Samples were obtained by inserting a 200-μl micropipette through the organic phase while continually expelling air, and removing aliquots from the lower aqueous layer. For determination of partition ratios, an aliquot of the organic phase was first removed, the remaining

hexane was aspirated, and the aqueous phase was sampled. This procedure led to negligible amounts of radioactivity being trapped in the organic layer.

Scintillation counting was done in glass minivials containing 3.5 ml of a 2:1 toluene–Triton solution, 0.4% 2,5-diphenyloxazole, and 0.2 ml of 0.05 M HCl. Background was about 50 cpm, and efficiency, determined by the channels-ratio method, varied from 82% to 85%.

Chlorophenoxyisobutyrate (Na^+) was a gift from Ayerst Laboratories, and used without further purification.

RESULTS

The partitioning of FFA between hexane and the aqueous phase in the absence of albumin is depicted in Fig. 1. The partition ratios (concentration of FFA organic/FFA aqueous) found here are intermediate between those reported by others (5, 6, 24), all of whom used *n*-heptane as the organic phase. As described in Fig. 1, addition of 2 mM CPIB has no effect on partition ratios for the three FFA. Although not shown here, it should be pointed out that the drug is not soluble in the hexane phase.

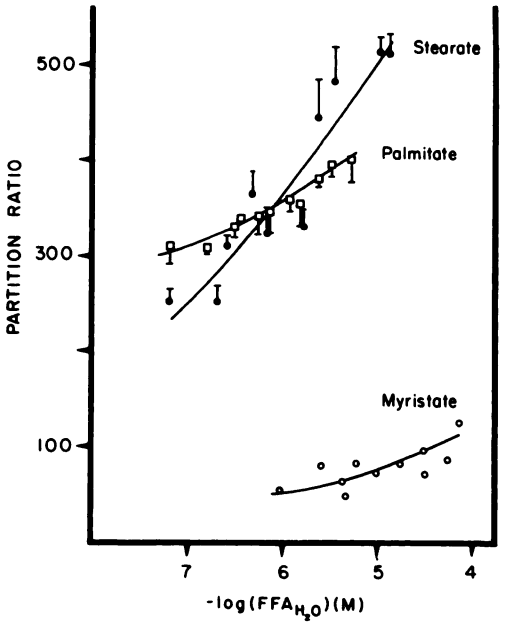

FIG. 1. *Partition ratios of FFA between hexane and phosphate buffer*

Partition ratio is defined as the ratio of the concentrations of fatty acid distributed between the organic and aqueous phases. Equilibration times were 16 hr (myristate) or 24 hr (palmitate and stearate). Symbols represent the means ± standard errors of three or four samples. For myristate, the standard errors are less than the areas of the symbols. The inclusion of 2 mM CPIB had no effect on partition ratios, and the data are included in the control samples.

The rate of approach to equilibrium in the two-phase system containing 0.2 mM BSA in phosphate buffer, pH 7.45, and 0.4 mM [^{14}C]FFA in the hexane phase is shown in Fig. 2. At 37° the rate of [^{14}C]FFA binding to BSA decreases in the order myristate > palmitate > stearate.

The ability of 2 mM CPIB to interfere with the rate at which [^{14}C]FFA added to the organic phase is bound to albumin is shown in Fig. 3. The data are plotted as a percentage of the FFA:BSA molar ratio ($\bar{V}$) in the aqueous phase obtained without drug, as in Fig. 1. In the case of [^{14}C]-myristate, the drug is most effective initially, but after 16 hr it reduces binding by only 1%. The binding of [^{14}C]palmitate is depressed to a relatively constant extent (15–20%), while that of [^{14}C]stearate is increasingly prevented as equilibrium is approached. Thus the pattern of long-chain FFA displacement by CPIB depends upon whether the measurements are performed

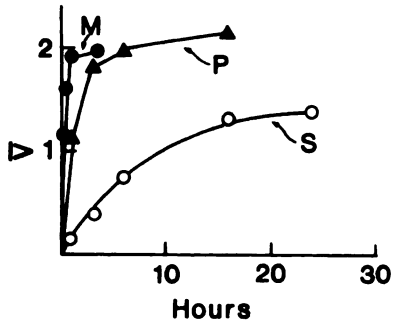

FIG. 2. *Rate of equilibrium partitioning of FFA to BSA*

The hexane phase contained 0.4 mM [^{14}C]FFA in 1 ml, and partitioning was initiated with 1 ml of 0.2 mM BSA in salts-phosphate buffer, pH 7.45. $\bar{V}$ is the molar ratio of FFA to BSA in the aqueous phase. The data represent means of four duplicate samples; standard errors are less than the areas of the symbols. Temperature, 37°, ●, myristate (M); ▲, palmitate (P); ○, stearate (S).

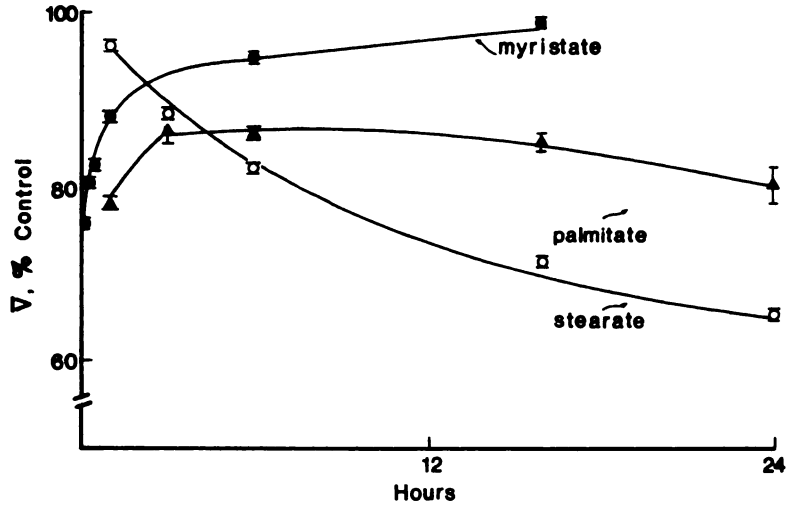

FIG. 3. *Effect of CPIB on rate of partitioning of FFA in the presence of BSA*
Controls are shown in Fig. 2. The concentration of CPIB was 2 mM.

under non- or equilibrium conditions. Consequently this variable was eliminated from further experiments by carrying them out at equilibrium.

The effect of CPIB on displacement of [^{14}C]FFA from high-affinity binding sites on BSA ($\bar{V} \cong 0.3$) is shown in Fig. 4. The drug is most effective against stearate, and causes a significant decrease in FFA binding at 0.4 mM CPIB. The drug is progressively less effective in removing FFA from the [^{14}C]palmitate-BSA and [^{14}C]myristate-BSA complexes.

It may be argued that FFA displacement by CPIB described in this study is of little importance *in vivo*, where CPIB:BSA ra-

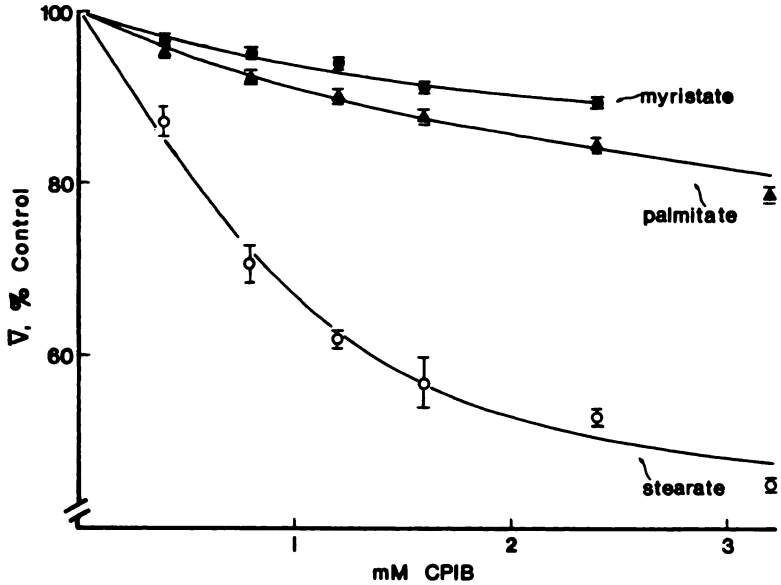

FIG. 4. *Displacement of FFA from high-affinity binding sites on BSA by CPIB*
Initially the aqueous phase contained 0.1 mM BSA as a [^{14}C]FFA-BSA complex, $\bar{V} = 0.35$ (myristate), 0.34 (palmitate), 0.25 (stearate). Incubation times were 7 hr (myristate), 16 hr (palmitate), and 24 hr (stearate). In the absence of CPIB, final $\bar{V}$ values were unchanged.

tios of about unity are found (25). In Fig. 5, drug to albumin ratios between 0.5 and 3.0 were employed, and the total amount of palmitate and myristate was examined in both phases at equilibrium. The decrease of FFA in the aqueous phase caused by CPIB is small, but statistically significant, at all drug concentrations. For example, at a drug to BSA molar ratio of 1.5, myristate is displaced 0.75% ± 0.015%, and palmitate, 1.57% ± 0.044%. When measured as a change of FFA in the organic phase, however, total myristate increases from 1.79 to 2.81 nmoles, and palmitate, from 3.94 to 6.28 nmoles, equivalent to a 50% increase at a drug to BSA ratio of 2.2. The greater percentage change of FFA in the organic phase, which reflects unbound aqueous FFA, is typical of ligands that are tightly bound to proteins. Thus, although the concentrations of CPIB used here are high, in order to observe significant shifts in FFA bound to albumin, it is clear that effects can be observed at low drug levels if unbound FFA is measured.

Figure 6 represents a curve for binding of [^{14}C]palmitate to BSA as a function of the molar concentration of free (unbound) palmitate in the aqueous phase. The drug was added either at 1 mM or at a constant molar ratio of total drug to bound palmitate of 10. Addition of 1 mM CPIB causes a parallel shift of the binding curve to the right, suggesting a competitive type of displacement of palmitate by the drug. This is verified in Fig. 7 by a double-reciprocal plot of the same data, showing that the drug competitively removes palmitate from the first two binding sites on albumin, i.e., $\bar{V}^{-1} \cong 0.5$. The equilibrium association constant (K_a) for palmitate is 2.4×10^7, a value similar to that generated by

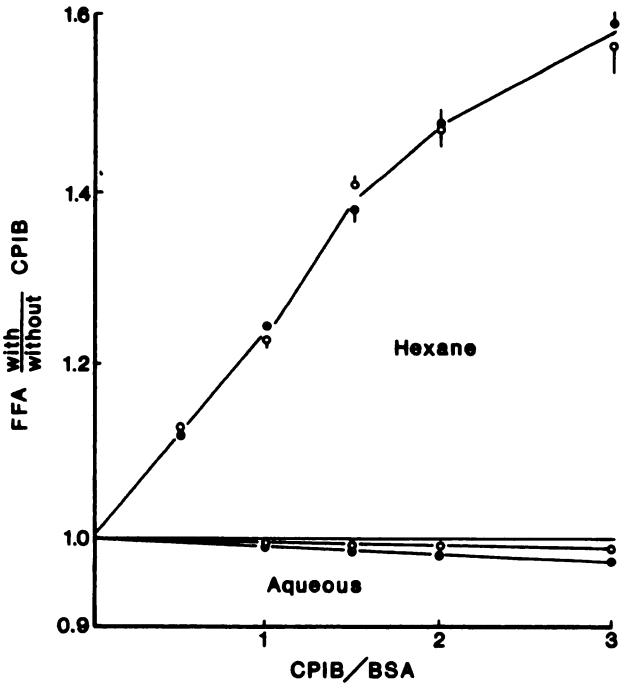

FIG. 5. *Effect of CPIB on partitioning of FFA*

Initially 200 nmoles of BSA were present in 1.6 ml of aqueous phase, plus CPIB as indicated. The organic phase contained 100 nmoles of [^{14}C]myristate or [^{14}C]palmitate in a total volume of 0.4 ml of hexane. The ordinate indicates the ratio of total FFA in the absence of CPIB to total FFA in the presence of CPIB. A ratio higher than 1.0 represents an increase of FFA in the organic phase, and less than 1.0 is a decrease in FFA in the aqueous phase by CPIB. Without drug, the aqueous phase contained, at equilibrium, 98.21 nmoles of myristate and 96.06 nmoles of palmitate. ○, myristate; ●, palmitate. In the aqueous phase, for six samples, the values for myristate and palmitate are significantly different ($p < 0.001$) at all drug levels.

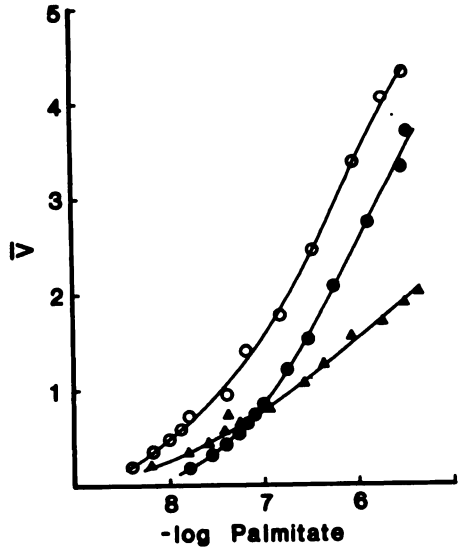

FIG. 6. *Extent of binding of palmitate to BSA as a function of concentration of unbound FFA.* The aqueous phase initially contained 200 nmoles of defatted BSA (O) plus, when indicated, CPIB at 1 mM (●) or at 10 times the equilibrium concentration of bound palmitate (▲). To the hexane phase was added 0.04–2.0 mM [^{14}C]palmitate. Ordinate, minus log free palmitate molar concentration.

Ashbrook *et al.* (6) for human serum albumin, assuming no aqueous phase dimerization. Beyond $\bar{V}$ = 2–2.5, however, both curves become steeper (not shown), as expected of lower-affinity FFA binding sites. If the drug to bound FFA molar ratio is not changed, while the concentration of palmitate is increased, a nonparallel shift of the [^{14}C]palmitate binding curve is obtained (Fig. 6, triangles). At $\bar{V}$ = 0.5, a 10-fold molar excess of drug to bound palmitate displaces 40% [^{14}C]palmitate, whereas at $\bar{V}$ = 4.0, 57% is removed. Similar data, not shown here, have been obtained with defatted human serum albumin. Thus the same ratio of CPIB to bound FFA displaces more palmitate from albumin as the amount of bound palmitate increases.

DISCUSSION

This study has demonstrated that a weakly bound but hydrophilic drug (chlorophenoxyisobutyrate) can displace from albumin a tightly bound hydrophobic ligand (long-chain FFA) that partitions favorably in the nonpolar phase. Although the experiments were carried out with chlorophenoxyisobutyrate, it is suggested that the model may be applicable to other drugs exhibiting similar partitioning characteristics and albumin binding affinities. For example, Dawkins *et al.* (14) have shown that salicylate, which is known to reduce circulating FFA *in vivo* (26, 27), displaces albumin-bound FFA in a two-phase partitioning system. Thus, in a monophasic system, only the more weakly bound ligand (drug) can be displaced, whereas in a biphasic system, the more tightly bound, hydrophobic ligand (FFA) can be removed as well.

The importance of FFA partitioning can be seen by comparing the actual results obtained in this study of drug effectiveness with known FFA-albumin association constants. Despite the linear increase in log K_a values with FFA chain length (5), CPIB is most effective against stearate, rather than myristate, at equilibrium. Only during the initial redistribution of FFA between both phases (Fig. 3) is the order of drug effectiveness proportional to the association constant. Therefore, at equilibrium, it is suggested that the dominant factor determining the relative effectiveness of CPIB in displacing FFA of different chain lengths must be the organic to aqueous FFA distribution ratio, rather

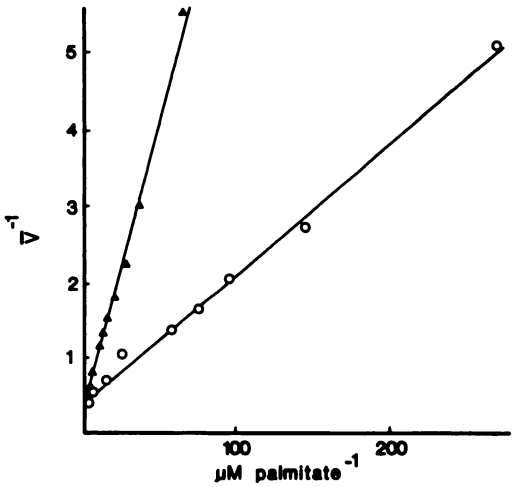

FIG. 7. *Double-reciprocal plot of palmitate binding vs. unbound palmitate concentration*

The curves were generated from data presented in Fig. 6. O, controls; Δ, 1 mM CPIB.

than the degree of FFA-albumin association. However, inspection of FFA partition ratios obtained here (Fig. 1) and elsewhere (5, 24) yields results that are not of sufficient magnitude to overcome the high FFA-albumin association constants, and therefore does not predict relative orders of drug effectiveness in displacing FFA from albumin. Provided that the monomer distribution determines the extent of association of FFA to albumin, a likely explanation is that partition ratios do not measure monomer distribution, and that other factors, such as aqueous phase dimerization, must be invoked. Such aqueous associations have been pointed out (5, 6), but only in a qualitative sense. Data of Schrier *et al.* (28) and the review of Leo *et al.* (29) suggest that oligomerization is considerably more important than generally realized. Thus the monomer FFA concentration in the aqueous phase may be considerably lower, particularly for long-chain FFA.

The weaker binding of FFA to albumin at higher $\bar{V}$ (3, 6) provides a satisfactory explanation for a corresponding increase in drug effectiveness with increasing FFA (Fig. 6) (14, 15, 17), provided that the CPIB:BSA molar ratio remains constant. Spector and Soboroff (30) have shown similar effects of CPIB in a model system involving the incorporation of FFA into Ehrlich ascites cells from the [^{14}C]palmitate-albumin complex. They concluded that palmitate was displaced more easily from weaker than from stronger binding sites on albumin. Failure to take into consideration the drug to FFA molar ratio, or drug to albumin molar ratio if FFA is constant, has contributed to conclusions dealing with the interaction of CPIB with albumin and tissues (31–33) that may be incorrect.

The mechanism by which CPIB, and presumably other amphiphilic anions used as hypolipidemic drugs, reduces serum FFA (31, 34, 35) and triglyceride (36, 37) levels remains controversial. Thorp originally proposed (38, 39) that the hypolipidemic effect of CPIB, which is 96% bound to serum albumin (40), could be explained by displacement of thyroxine from albumin binding sites, followed by subsequent redistribution of the hormone. A prevalent theory is that the drug decreases mobilization of FFA from adipose tissue (34, 41) either directly, by inhibiting cyclic AMP formation (32, 41, 42), or indirectly, through reduction in acetyl-CoA carboxylase (43) or *sn*-glycerol phosphate acyltransferase (44). However, it is thermodynamically possible, from the results presented here, that the initiating event involves FFA displacement from albumin by the drug, an idea originally proposed by Thorp (38).

ACKNOWLEDGMENTS

I wish to thank Mr. E. Brass and Dr. K. Neet for their many helpful discussions.

REFERENCES

1. Teresi, J. & Luck, J. (1952) *J. Biol. Chem.*, **194**, 823–833.
2. Goodman, D. (1958) *J. Am. Chem. Soc.*, **80**, 3887–3892.
3. Goodman, D. (1958) *J. Am. Chem. Soc.*, **80**, 3892–3898.
4. Spector, A., John, K. & Fletcher, J. (1969) *J. Lipid Res.*, **10**, 56–66.
5. Smith, R. & Tanford, C. (1973) *Proc. Natl. Acad. Sci. U. S. A.*, **70**, 289–293.
6. Ashbrook, J., Spector, A., Santos, E. & Fletcher, J. (1975) *J. Biol. Chem.*, **250**, 2333–2338.
7. Boyer, P., Lum, F., Ballou, G., Luck, J. & Rice, R. (1946) *J. Biol. Chem.*, **162**, 181–198.
8. Klotz, I. & Walker, F. (1947) *J. Am. Chem. Soc.*, **69**, 1609–1612.
9. Tanford, C. (1973) *The Hydrophobic Effect*, p. 195, Wiley, New York.
10. Meyer, M. & Guttman, D. (1968) *J. Pharm. Sci.*, **57**, 958.
11. Rudman, D., Bixler, T. & Del Rio, A. (1971) *J. Pharmacol. Exp. Ther.*, **176**, 261–272.
12. Tabachnick, M. (1964) *J. Biol. Chem.*, **239**, 1242–1249.
13. McMenamy, R. (1965) *J. Biol. Chem.*, **240**, 4235–4243.
14. Dawkins, P., McArthur, J. & Smith, M. (1970) *J. Pharm. Pharmacol.*, **22**, 405–410.
15. Spector, A. & Imig, B. (1971) *Mol. Pharmacol.*, **7**, 511–518.
16. Fredholm, B., Rane, A. & Persson, B. (1975) *Pediatr. Res.*, **9**, 26–30.
17. Spector, A., Santos, E., Ashbrook, J. & Fletcher, J. (1973) *Ann. N. Y. Acad. Sci.*, **226**, 247–258.
18. Starinsky, R. & Shafrir, E. (1970) *Clin. Chim. Acta*, **29**, 311–319.
19. Koch-Weser, J. & Sellers, E. (1976) *N. Engl. J. Med.*, **294**, 311–316.

20. Meisner, H. (1975) *Biochem. Biophys. Res. Commun.*, 66, 1134–1140.
21. Chen, R. (1967) *J. Biol. Chem.*, 242, 173–181.
22. Ducombe, W. (1963) *Biochem. J.*, 88, 7–10.
23. Spector, A. & Hoak, J. (1969) *Anal. Biochem.*, 32, 297–302.
24. Simpson, R., Ashbrook, J., Santos, E. & Spector, A. (1974) *J. Lipid Res.*, 15, 415–422.
25. Wolfe, B., Kane, J., Havel, R. & Brewster, H. (1973) *J. Clin. Invest.*, 52, 2146–2159.
26. Bizzi, A., Garattini, S. & Veneroni, E. (1965) *Br. J. Pharmacol. Chemother.*, 25, 187–196.
27. Stone, D., Brown, J. & Steele, A. (1969) *Metab. (Clin. Exp.)*, 18, 620–624.
28. Schrier, E., Pottle, M. & Scheraga, H. (1974) *J. Am. Chem. Soc.*, 86, 3444–3449.
29. Leo, A., Hansch, C. & Elkins, D. (1971) *Chem. Rev.*, 71, 525–554.
30. Spector, A. & Soboroff, J. (1971) *Proc. Soc. Exp. Biol. Med.*, 137, 945–947.
31. Hunninghake, C. & Azarnoff, D. (1968) *Metab. (Clin. Exp.)*, 17, 588–595.
32. D'Costa, M. & Angel, A. (1975) *J. Clin. Invest.*, 55, 138–148.
33. Homcy, L. & Margolis, S. (1974) *Atherosclerosis*, 19, 381–391.
34. Barrett, A. & Thorp, J. (1968) *Br. J. Pharmacol. Chemother.*, 32, 381–391.
35. Cenedella, R., Jarrell, J. & Saxe, L. (1968) *J. Atheroscler. Res.*, 8, 903–911.
36. Gould, R., Swyryd, E., Goan, B. & Avoy, D. (1966) *J. Atheroscler. Res.*, 6, 555–564.
37. Adams, L., Webb, W. & Fallon, H. (1971) *J. Clin. Invest.*, 50, 2339–2346.
38. Thorp, J. (1962) *Lancet*, 1323–1326.
39. Platt, D. & Thorp, J. (1966) *Biochem. Pharmacol.*, 15, 915–925.
40. Thorp, J. (1963) *J. Atheroscler. Res.*, 3, 351–360.
41. Carlson, L., Walldius, G. & Butcher, R. (1972) *Atherosclerosis*, 16, 349–357.
42. Weis, A., Tepperman, H. & Tepperman, J. (1973) *Endocrinology*, 93, 504–509.
43. Maragoudakis, M. & Hankin, H. (1971) *J. Biol. Chem.*, 246, 348–358.
44. Lamb, R. & Fallon, H. (1972) *J. Biol. Chem.*, 247, 1281–1287.