The binding specificity of monoanionic spin label (1-aminobutyrate-5-N-[(1-oxyl-2,2,6,6-tetramethyl-4-aminopiperidinyl)-2,4-dinitrobenzene] to human serum albumin is used to probe the relationship between the albumin fatty acid and bilirubin binding sites and to study how fatty acid modulates albumin binding capacity.

This spin label is shown to bind to endogenous bilirubin binding sites of human serum albumin, as addition of bilirubin results in the increase of free spin label signal intensity and a concomitant decrease in bound spin label signal intensity.

The binding isotherm of the interaction of the spin label with human serum albumin in the form of a Scatchard plot shows that the binding of the first molar equivalent of bilirubin quantitatively displaces the spin label from its primary binding site. Graphical analysis of the binding isotherm shows that the high affinity bilirubin binding site has the capacity to bind 2 mol of spin label with different affinities. This binding isotherm at 2 molar equivalents of bilirubin shows that the spin label also binds specifically to the secondary bilirubin binding sites.

Previous studies (Soltys, B. J., and Hsia, J. C. (1977) J. Biol. Chem. 252, 4043-4048) have shown that the binding of palmitate to serum albumin allosterically enhances the spin label binding. The present study indicates that the fatty acid effect is chain length-dependent. Fatty acids of chain length of less than 10 carbon atoms competitively displace the binding of spin labels by serum albumin while fatty acids of longer chain length enhance the spin label binding allosterically.

The binding isotherm for the spin label albumin interaction in the presence of 1 to 4 molar equivalents of octanoate, laurate, and palmitate shows octanoate decreases the number of spin label binding sites, while laurate and palmitate enhance the affinity of the spin label to serum albumin.

The present results are consistent with the hypothesis that serum albumin has two distinct sets of endogenous binding sites for fatty acids of different chain length with a demarcation at approximately carbon 10. The view that endogenous bilirubin binding sites may overlap with those of fatty acid binding sites is substantiated by the present results. A further delineation of the extent of overlap of endogenous bilirubin and fatty acid binding sites is presented in the following paper (Soltys, B. J., and Hsia, J. C. (1978) J. Biol. Chem. 253, 3029-3034).

Albumin, the principal protein of plasma, is unique in its ability to bind a wide variety of substances, including fatty acids, bilirubin, hormones, drugs, etc. Plasma albumin binding functions in the transport and distribution of substances in the body, affecting their access to sites of action, of metabolism, and of excretion. In view of the multiple ligand binding properties of albumin, an important clinical question is whether the binding of certain drugs or physiological substances (or both) affect the binding of other drugs or physiological substances (or both). For a review on plasma albumin, see Ref. (1).

The binding of free fatty acids to albumin is highly specific and has recently been reviewed (2). Fatty acid levels in plasma fluctuate considerably, from a molar ratio of fatty acid to albumin of 0.5 to 1.5 normally (3) to greater than 4 following exercise (4, 5). Elevated fatty acid levels, furthermore, are characteristic of newborns (6) where fatty acid:albumin molar ratios of 1 to 4 are found. Previously, fatty acid levels within the physiological range had not been thought to affect the binding of other substances to albumin (2). The first definitive evidence of fatty acid induced conformation change was deduced from accessibility of tyrosine residues to iodination of bovine serum albumin in the presence and absence of stearic acid (7). However, there is now accumulating evidence that low fatty acid levels can allosterically affect albumin binding properties. Spector et al. (8) initially proposed an allosteric mechanism for the inhibition of chlorophenoxyisobutyrate binding by fatty acid. Spectroscopic evidence further shows that low fatty acid levels enhance the fluorescence of anilino-
Fatty acid binding affects the albumin conformational state and allosterically affects binding properties.

**EXPERIMENTAL PROCEDURES**

**Albumin**—Human albumin (Fraction V) was purchased from Sigma. The protein was defatted by charcoal treatment (15) and monomeric albumin purified as previously described (12). Defatted monomeric albumin at a physiological concentration of 0.5 mM has been used for all experiments. Protein concentration was determined by measurement of the peak-to-peak height of the high field line of the sharp three-line spectrum (18).

**Bilirubin**—Bilirubin was purchased from Sigma. All procedures involving bilirubin were carried out under minimal lighting conditions. To prepare bilirubin/albumin solutions, bilirubin was dissolved in methanol in the sodium salt form (checked by thin layer chromatography) and dried under nitrogen. The albumin solution was then added immediately and blended on a Vortex mixer. The bilirubin concentration was determined by measurement of the peak-to-peak height of the high field line of the sharp three-line spectrum (18).

**Fatty Acid**—Fatty acids were purchased from Sigma. Fatty acids were dissolved in albumin solution essentially as previously described (12). Briefly, fatty acid (sodium salt form) in methanol was gently stirred with a magnetic bar until optically clear. In the case of stearic acid, prolonged stirring was necessary, from 6 to 8 h at room temperature, to effect solution. Stirring itself had no effect on albumin spin label binding properties. Serial dilutions of fatty acid/albumin solutions of 4:1 with the stock albumin solution gave similar results to the solutions of lowered fatty acid content prepared directly. Unsaturated fatty acid were maintained under nitrogen conditions at all times.

**Spin Label**—The synthesis of GABA-DNB-SL has been described previously (19). Spin label/albumin solutions were prepared by adding 50 or 100 μl of the appropriate albumin solution to culture tubes containing the dried sodium salt form of GABA-DNB-SL and mixed on a Vortex mixer until dissolved.

**Instrumentation**—ESR spectra were recorded with a Varian E-6 X-band spectrometer equipped with a variable temperature controller. The temperature of the cavity was maintained at 37 ± 0.5°C and monitored with a thermocouple. Samples were contained in glass disposable 25- or 50-μl pipettes. The field was calibrated with Fremy's salt.

**Quantitation of Spectral Lines**—Free spin label concentrations were determined by measurement of the peak-to-peak height of the high field line of the sharp three-line spectrum (18).

**RESULTS AND DISCUSSION**

In an attempt to localize the observed enhancement of GABA-DNB-SL binding in the presence of palmitate (12) to specific albumin binding sites, it has been determined that GABA-DNB-SL binds to the endogenous bilirubin binding sites of human albumin. Fig. 1 presents two representative spectra of the effect of bilirubin on GABA-DNB-SL binding.
Serum Albumin Binding Specificity and Mechanisms 3025

binding. In Fig. 1A, the intensity of the sharp three-line spectrum, representing free spin label, is shown to greatly increase in the presence of bilirubin. This is accompanied by a decreased intensity of the high and low field broad line extrema of the immobilized spectrum, representing protein-bound spin label. These changes in the immobilized spectrum, consistent with a direct displacement effect by bilirubin on GABA-DNB-SL, are more clearly seen in Fig. 1B.

The binding isotherm of the interaction of GABA-DNB-SL with albumin is presented in Fig. 2 in the form of a Scatchard plot. The binding of GABA-DNB-SL to albumin has been revealed to be complex and a detailed quantitative evaluation of the number of specific GABA-DNB-SL binding sites, their respective affinity constants, and changes in these parameters upon the formation of complexes with other ligands is outside the scope of the present study. Such an analysis will require adopting computerized fitting of binding data according to the stepwise equilibrium model (19, 20). Nevertheless, qualitative information will be drawn from the binding isotherms presented here to advance the arguments presented.

Fig. 2 also shows the effect of 1 to 2 molar equivalents of bilirubin on the GABA-DNB-SL binding isotherm. A major inhibitory effect is indicated. Bilirubin itself is known to have a single high affinity binding site \( K_a = 10^4 \) and a number of secondary sites \( (K_a = 10^3) \) (21). The effect of 1 molar equivalent of bilirubin is particularly striking. In view of the comparatively lower binding capacity of albumin for GABA-DNB-SL, as defined by the intercept on the ordinate axis \( K_a = 6 \times 10^4 \), the binding of the first molar equivalent of bilirubin will quantitatively displace spin label bound at the high affinity bilirubin binding site. Based on graphical properties (22), the binding isotherm in the presence of 1 molar equivalent of bilirubin has been subtracted from the control isotherm in order to construct the binding isotherm representing the binding of GABA-DNB-SL to the primary bilirubin binding site, presented in Fig. 2 as a dashed line. It is shown that the high affinity bilirubin binding site has a capacity to bind 2 molar equivalents of spin label \( (K_a = 4.5 \times 10^9) \). This suggests that the high affinity bilirubin binding site corresponds to the first two binding sites of GABA-DNB-SL. Further, these two sites are nonidentical, as evidenced by the curvature of the line. In that bilirubin is a dianionic ligand, it is proposed that the monoaonic structure of GABA-DNB-SL permits 2 molar equivalents of spin label to occupy the primary bilirubin binding site. This proposal is supported by the synthesis of a dianionic analogue of GABA-DNB-SL in which case only 1 mole of spin label occupies this site (23). It is interesting to note that the immobilized spectrum in the presence of 1 molar equivalents of GABA-DNB-SL shows no evidence of significant exchange or dipolar broadening (or both). Apparently, the nitroxides are not in close proximity within this binding site.

Graphical subtraction has not been extended to higher bilirubin ratios because of the complexity of the underlying interactions. Nevertheless, it is seen that addition of bilirubin beyond its molar equivalence does further significantly inhibit GABA-DNB-SL binding. These data suggest that GABA-DNB-SL binds specifically to the primary and secondary bilirubin binding sites of albumin.

Previous studies (12) have shown that the binding of palmitate to albumin allosterically enhances GABA-DNB-SL binding. Given the specificity of GABA-DNB-SL binding, this allostatic effect may be mediated on the endogenous bilirubin binding site(s) of albumin. Spectrophotometric data have in fact indicated that low fatty acid molar ratios perturb the molecular interaction of bilirubin with albumin (10).

We have further investigated the effects of fatty acids on GABA-DNB-SL binding. It has been found that, depending upon the fatty acid chain length, GABA-DNB-SL binding can either be inhibited or enhanced. Fig. 3 shows representative spectra of the effect of a medium chain length fatty acid, octanoate, on the interaction of GABA-DNB-SL with albumin. Octanoate binding increases the intensity of the sharp three-
line spectrum (Fig. 3A), representing an increase of free spin label. As in the case of bilirubin binding, the effect is accompanied by a decreased intensity of the outer broad line extrema of the immobilized spectrum (Fig. 3B), consistent with a competitive inhibition of spin label binding. These spectra contrast with the effect of the long chain fatty acid, laurate, on the ESR spectra presented in Fig. 4. As in the ESR spectra with palmitate previously presented (12), spin label binding is enhanced (Fig. 4A) and this enhanced binding is accompanied by an increased intensity of and splitting between the outer broad line extrema of the immobilized spectrum (Fig. 4B). In that protein-bound spin label is sensitive to the motional freedom and polarity of the binding site microenvironment (24, 25), the latter changes in the immobilized spectrum are consistent with an allosteric effect by fatty acid.

Fig. 5 presents the effect of 1 to 4 molar equivalents of fatty acids of varying chain length on the binding of the first molar equivalent of GABA-DNB-SL. The biphasic curves indicate that fatty acids of chain length less than 10 carbon atoms inhibit GABA-DNB-SL binding, whereas longer chain length fatty acids exert an enhancement effect on spin label binding. The accompanying changes observed in the immobilized spectra are consistent with a competitive displacement of spin label by fatty acids of chain length less than 10 carbon atoms whereas, as in the spectra for palmitate (12) and for laurate, longer chain length fatty acids appear to allosterically perturb albumin binding sites (fatty acid chain length effect on the immobilized spectrum not presented).

The effect of fatty acid unsaturation with fatty acids of 18 carbon atoms chain length has also been examined, the results of which are presented in Fig. 6. The little apparent difference between these fatty acids suggests that the fatty acid confor-

Fig. 5. Binding of the first molar equivalent of GABA-DNB-SL to human plasma albumin in the presence of 1 to 4 molar equivalents of saturated fatty acids of varying carbon atom chain length (n). The conditions were the same as in Fig. 1.

Fig. 6. Binding of the first molar equivalent of GABA-DNB-SL to human plasma albumin in the presence of 1 to 4 molar equivalents of 18-carbon atom fatty acids of varying degrees of unsaturation. The conditions were the same as in Fig. 1.
Serum Albumin Binding Specificity and Mechanisms

FIG. 7. Scatchard plots of the binding of GABA-DNB-SL to human plasma albumin in the presence of 1 to 4 molar equivalents of octanoate (A), laurate (B) and palmitate (C). The dashed line represents the binding of GABA-DNB-SL in the absence of fatty acid, as presented in Fig. 2. The conditions were the same as in Fig. 1. r, moles of GABA-DNB-SL bound per albumin. A, free GABA-DNB-SL concentration.

GABA-DNB-SL for available binding sites would be justified. The binding of 1 to 4 molar equivalents of octanoate (Fig. 7A) shows a parallel displacement of the GABA-DNB-SL binding isotherm to the left, indicative of competitive binding. The actual sites affected, however, cannot be drawn by visual examination.

In contrast, the binding of 1 to 4 molar equivalents of laurate (Fig. 7B) or palmitate (Fig. 7C) greatly increases the albumin binding capacity for GABA-DNB-SL, increasing 3- and 8-fold, respectively, in the presence of 3 to 4 molar equivalents of fatty acid. Both fatty acids show similar changes in the binding isotherm, although palmitate is clearly more effective in enhancing binding and this may be related to its increased binding energy. Graphical examination of these data suggests the tentative statement that with either fatty acid only a single GABA-DNB-SL binding site may show enhanced affinity.

In conclusion, the present study has shown evidence that GABA-DNB-SL binds to the endogenous bilirubin binding sites of albumin and that the high affinity bilirubin binding site has a capacity to bind 2 mol of spin label. It has further been shown that fatty acids can affect spin label binding in two ways. From 1 to 4 molar equivalents of fatty acids of chain length less than 10 carbon atoms competitively displace GABA-DNB-SL. This suggests that these fatty acids bind to the endogenous bilirubin binding site(s) of albumin. Specifically which site(s) are involved cannot be said at present. In contrast, the binding of longer chain length fatty acids markedly enhances the affinity of GABA-DNB-SL binding site(s). These fatty acids apparently perturb the albumin structure to varying degrees and in so doing, allosterically affect GABA-DNB-SL binding at the endogenous bilirubin binding sites. The identity of the long chain fatty acid binding site(s) (i.e. whether GABA-DNB-SL also binds to these sites) cannot be drawn from the present data. The data, nevertheless, suggest the presence of two distinct sets of primary binding sites for fatty acids. Furthermore, they suggest that long chain fatty acids would allosterically perturb the interaction of medium and short chain fatty acids and of bilirubin with albumin. The reader is referred to the second paper in this series (23) where further studies have permitted a more comprehensive discussion of the observed binding phenomena and their implications.

REFERENCES

1. Peters, T., Jr. (1975) in The Plasma Proteins (Putnam, F. W., ed) 2nd Ed, pp. 133-181, Academic Press, New York
2. Spector, A. A. (1975) J. Lipid Res. 16, 165-179
3. Fredrickson, D. S., and Gordon, R. S., Jr. (1958) J. Clin. Invest. 37, 1504-1515
4. Havel, R. J., Nairn, A., and Borahgrovink, C. F. (1963) J. Clin. Invest. 42, 1064-1063
5. Rodahl, K., Miller, H. I., and Isselsutz, B., Jr. (1964) J. Appl. Physiol. 19, 499-505
6. van Duyne, C. M., and Havel, R. (1969) Proc. Soc. Exp. Biol. Med. 162, 529
7. Glazer, A. N., and Sanger, F. (1963) J. Mol. Biol. 7, 453-457
8. Spector, A. A., Santos, E. C., Ashbrook, J. D., and Fletcher, J. E. (1973) Ann. N. Y. Acad. Sci. 226, 247-258
9. Santos, E. C., and Spector, A. A. (1974) Mol. Pharmacol. 10, 519-528
10. Woolley, P. V., and Hunter, M. J. (1970) Archi. Biochem. Biophys. 140, 197-209
11. Ryan, M. T., and Chopra, R. K. (1976) Biochim. Biophys. Acta 427, 337-339
12. Soltys, B. J., and Hsia, J. C. (1977) J. Biol. Chem. 252, 4043-4048
13. Soetewey, F., Rosseneu-Montreff, M., Lamote, R., and Peeters, H. (1972) J. Biochem (Tokyo) 71, 705-710
14. Scheider, W., Dintzis, H. M., and Oncley, J. L. (1976) Biophys. J. 16, 417-431
15. Chen, R. F. (1967) J. Biol. Chem. 242, 173-181
16. Clark, P., Rucinski, M. R., and Foster, I. F. (1962) J. Biol. Chem. 237, 2935-2940
17. Lee, K. S., and Gardner, L. W. (1973) Pediatr. Res. 10, 782-788
18. Hsia, J. C., Wong, L. T. L., and Kalow, W. (1973) J. Immunol. Methods 3, 17-24
19. Fletcher, J. E., Ashbrook, J. D., and Spector, A. A. (1973) Ann. N. Y. Acad. Sci. 226, 60-81
20. Fletcher, J. E., Spector, A. A., and Ashbrook, J. D. (1970) Biochemistry 9, 4560-4567
21. Jacobson, J. (1969) FEBS Lett. 5, 112-114
22. Rosenthal, H. E. (1967) Anal. Biochem. 20, 525-532
23. Soltys, B. J., and Hsia, J. C. (1978) J. Biol. Chem. 253, 3029-3034
24. Wong, L. T. L., Piette, L. H., Little, J. R., and Hsia, J. C. (1974) Immunochemistry 11, 377-379
25. Morrisett, J. D. (1976) in Spin Labeling: Theory and Applications (Berliner, L. J., ed) pp. 306-308, Academic Press, New York
26. Ashbrook, J. D., Spector, A. A., and Fletcher, J. E. (1972) J. Biol. Chem. 247, 7038-7042
Human serum albumin. I. On the relationship of fatty acid and bilirubin binding sites and the nature of fatty acid allosteric effects--a monoanionic spin label study.

B J Soltys and C Hsia

J. Biol. Chem. 1978, 253:3023-3028.

Access the most updated version of this article at http://www.jbc.org/content/253/9/3023.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/253/9/3023.citation.full.html#ref-list-1