Binding of Long Chain Fatty Acids to β-Lactoglobulin[1]

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ABSTRACT

β-lactoglobulin (BLG), a bovine milk protein that is available commercially in crystalline form, binds long chain free fatty acids (FFA). The binding data were analyzed with a model containing one primary FFA binding site and a large number of weak secondary binding sites. At 37 C and pH 7.4, the apparent association constant for binding of FFA to the primary site was of the order of 10^5 M^{-1} and that for binding to the secondary sites was approximately 10^3 M^{-1}. The strength of binding was: palmitate>stearate>oleate>laurate. The affinity of BLG for palmitate increased as the pH of the incubation medium was raised from 6.5 to 8.7 and decreased as the ionic strength of the medium was raised. Palmitate binding was decreased in the presence of 6 M urea and when the protein either was exposed to elevated temperature or was acetylated prior to incubation. BLG took up methyl palmitate, cetyl alcohol, hexadecane and cholesterol to a lesser extent than FFA. Binding of FFA to BLG was associated with a small increase in the intensity of the fluorescent emission of the protein at 333 mμ. BLG can serve as an FFA acceptor or carrier in biological experiments. FFA released from adipose tissue during in vitro incubation was taken up by BLG. Net transfer of fatty acid to the incubation medium ceased when the molar ratio of FFA to BLG exceeded 1.1. ^{14}C-1-Palmitate bound to BLG was taken up by Ehrlich ascites tumor cells in vitro. At a given palmitate-protein molar ratio, much more labeled fatty acid was taken up by these cells from media containing BLG than from those containing bovine albumin, apparently because FFA is bound less firmly to BLG than to albumin.

INTRODUCTION

β-lactoglobulin (BLG), a protein present in bovine milk, can be obtained commercially in crystalline form. We have noted that this protein will bind long chain free fatty acids (FFA) in an aqueous solution (1). Therefore, it was reasonable to assume that BLG might serve as a substitute for serum albumin in incubations that require the presence of an FFA carrier or acceptor.

There are two properties of long chain fatty acids that make them difficult to work with in biological systems. First, they are poorly soluble in aqueous solutions. Second, unbound FFA are injurious to mammalian tissues even in relatively low concentrations, e.g., 10^{-4} M. Thus, for most biological work with FFA, it is necessary to employ a fatty acid carrier in order to introduce the required amounts of FFA into the incubation medium while, at the same time, preventing exposure of the tissue to an excessive amount of unbound fatty acid. Serum albumin, the physiological FFA transport protein, is used almost exclusively for this purpose. The availability of BLG as an alternative to albumin may provide a useful tool for the elucidation of several points concerning the mechanisms of FFA metabolism. For example, comparative studies of media containing either BLG or serum albumin may indicate whether albumin possesses some special property for transferring FFA to or into cells or for removing fatty acid from adipose cells.

The interaction of FFA with proteins also has been studied almost exclusively with serum albumin. Hence, it is of interest to determine whether the mechanism of FFA association with albumin is unique for that protein or is applicable in general to other proteins that can bind FFA. Study of FFA binding to BLG should help to clarify this point. In addition, our initial observations indicated that BLG possessed a much smaller capacity to take up FFA than serum albumin (1). Preliminary analyses of these data suggest that BLG contained only one binding site with high affinity for FFA. In contrast, serum albumin contains approximately six high energy FFA binding sites (1). For studies of the detailed mechanism of FFA binding to proteins and FFA transfer from a carrier to a cell, the use of a protein having only a single strong binding site is

[1] Special abbreviations used in this text: ν, average molar ratio of bound FFA to total protein; c, molar concentration of FFA in free solution and in equilibrium with that bound to protein; n, number of binding sites in a given class; k', apparent association constant for binding to a given class of sites.

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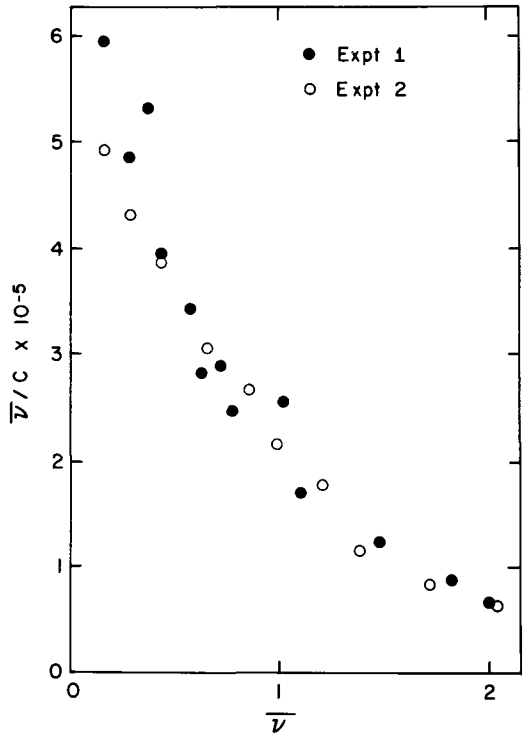

FIG. 1. Scatchard plot of data for binding of 1-^{14}C-palmitic acid to β-lactoglobulin at 37 C in phosphate-buffered salt solution, pH 7.4.

advantageous because it greatly simplifies the theoretical considerations and the mathematical analysis.

Because of the potential usefulness of BLG for these purposes we have investigated the binding of long chain FFA to this protein and the uptake and release of FFA by mammalian tissues in media containing BLG.

EXPERIMENTAL PROCEDURES

Materials

Crystalline BLG (lots 36 and 38) were purchased from Pentex, Inc. (Kankakee, Ill). These preparations contained less than 0.2 μeq FFA per μmole protein as measured by titration (2). BLG was dissolved in buffered salt solution and dialyzed against 4 liters of this solution for 24 to 48 hr at 4 C with mechanical stirring. The dialyzing solution was changed at least once during this time. Unless noted otherwise, the buffer solution contained 0.116 M NaCl, 0.004 M KCl, 0.0012 M $MgSO_4$ and 0.016 M sodium phosphate and was adjusted to pH 7.4 with 1.0 N HCl. This is referred to in the text as phosphate-buffered salt solution. Following dialysis, the BLG solution was adjusted to pH 7.4, and

TABLE I

Reversibility of Fatty Acid Binding to β-Lactoglobulin[a]

Time of incubation, hr	β-Lactoglobulin solution[b]		
	Radioactivity, cpm		Fatty acid,
	^{14}C	^{3}H	mμeq
0	3160	0	25
0.5	2250	565	55
1	1810	425	43
4	595	1340	94
7	440	1980	135
16	260	2280	154

[a]Incubation was done at 37 C. 1-^{14}C-palmitate was added to BLG dissolved in phosphate-buffered salt solution by exposure to palmitate-coated Celite particles. This solution contained 0.2 μmole/ml BLG and 0.025 μeq of 1-^{14}C-palmitate. A heptane solution containing palmitate-9,10-^{3}H (20 μeq/ml) was prepared, and 1 ml of it was incubated with 1 ml of the BLG solution containing 1-^{14}C-palmitate in a series of flasks. At each of the time intervals shown, two flasks were removed and the content of ^{14}C and ^{3}H in the heptane and aqueous phases was determined.

[b]Each value is the mean of two determinations.

the protein concentration was determined by the biuret method (3). Dried samples of BLG served as the standard for this procedure. The molecular weight of BLG was taken as 42,000. Freshly prepared solutions of the protein migrated as a single band when subjected to electrophoresis at pH 8.2 on glass microscope slides coated with Agarose (1). Additional tests to detect heterogeneity in these BLG preparations were not done (4).

BLG was acetylated at pH 8 according to the method of Fraenkel-Conrat et al. (5). Free amino groups were measured by the ninhydrin method (6).

The commercial sources and methods for purification of the radioactive lipids used in this study have been described (1). ^{14}C-4-Cholesterol that had been purified by thin layer chromatography (>99% radiopure) was a gift from Dr. W. E. Connor.

Measurement of Binding

Association of labeled FFA with BLG was measured by a modification of the equilibrium partition analysis method devised by Goodman (1,7). The reaction was carried out in specially constructed glass flasks (1). Incubation was done with shaking in a temperature-controlled water bath. Increasing amounts of labeled FFA of known specific radioactivity were dissolved in 1 ml of *n*-heptane and incubated with 1 ml of an aqueous solution containing BLG (2 x 10^{-4} M). The partition ratio of FFA between

heptane and the protein-free buffered salt solution was determined in a separate set of flasks (1,8). Equilibrium was reached in this system after 6-10 hr at 37 C and, for convenience, an overnight incubation usually lasting 16 hr was used. Both heptane and aqueous phases remained optically clear during incubation, and precipitates did not form at the interface.

Radioactivity was measured with a Packard Tri-Carb 3375 liquid scintillation spectrometer. The scintillator solution contained 0.3%, 2,5-diphenyloxazole and 0.01% 1,4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in toluene and methanol (7:3 v/v) (1). Quenching was monitored initially both with the external standard and by addition of ^{14}C-1-palmitate as an internal standard. Both methods indicated that the amount of quenching varied between 1% and 8%, and only the external standard was employed in subsequent experiments.

From the binding data, the unbound FFA concentration, c, at each molar ratio of bound FFA to protein, ν, was calculated (1,7). A computerized curve-fitting procedure (9) was employed to analyze these results in terms of number of classes of binding sites, number of sites within a given class, n, and the apparent association constant for binding to each class of sites, k'. Corrections for electrostatic interactions were not made, and the intrinsic association constants were not determined.

Incubation with Celite

Celite 545 was coated with labeled lipids of known specific radioactivity (10). Weighed amounts of these Celite preparations were incubated with BLG solutions or protein-free buffer solutions in a temperature-controlled water bath with shaking (1). The Celite was sedimented by centrifugation, and the uptake of radioactivity by the protein solution was determined (1).

Fluorescence Measurements

Fluorescence emission spectra of BLG and bovine albumin excited at 280 mμ were recorded between 280 and 450 mμ (11). All fluorescence measurements were made with the Aminco-Bowman spectrophotofluorometer calibrated by Chen (12).

Incubations with Mammalian Tissues

Epididymal fat pads obtained from fasted Osborne-Mendel rats were incubated at 37 C under air with shaking in 10 ml of phosphate-buffered salt solution containing protein and adrenocorticotropic hormone (11). One fat pad from each rat was incubated in medium containing BLG, the other in medium containing albumin. The FFA content of 1 ml aliquots of the incubation medium were measured by titration (2).

Isolated, washed Ehrlich ascites tumor cells were incubated with shaking under air at 37 C (13) with ^{14}C-1-palmitate bound to either BLG or bovine albumin. Incorporation of the labeled palmitate into the cell lipids or into CO_2 was measured (14).

RESULTS

FFA was taken up by BLG when an aqueous solution of the protein was incubated with a soap solution, fatty acid-coated Celite particles, a rat epididymal fat pad or fatty acid dissolved in heptane. If the BLG solution was exposed subsequently to heptane, fatty acid was released into the organic phase. As indicated by the data contained in Table I, this was due to the reversibility of FFA binding to BLG and not damage to the protein from exposure to heptane. ^{14}C-1-Palmitate was added to BLG, and the resulting solution was incubated with a large excess of palmitate-9,10-^{3}H in heptane. As the incubation proceeded, the ^{14}C content of the BLG solution decreased to less than 10% of the original amount while increasing amounts of ^{3}H appeared in the aqueous phase. This was accompanied by the appearance of increasing quantities of ^{14}C in heptane and a decrease in the ^{3}H content of the heptane. The total palmitate content of the BLG solution increased sixfold during the incubation. Thus, most of the ^{14}C-1-palmitate content of the BLG solution was exchangeable even under conditions that produced a large net increase in the total palmitate content of the protein solution.

Data obtained for palmitic acid binding to BLG at 37 C in phosphate buffered salt solution are shown in Figure 1. In this graph, ν is plotted against ν/c according to the method of Scatchard (15). These data were obtained in two separate experiments, each with a different commercial preparation of BLG. Note the excellent agreement of the two sets of data points. The data show a nonlinear correlation between ν/c and ν. This suggests that BLG, like the serum albumins (1,7), contains more than one class of palmitate binding sites. However, the possibility that the nonlinear correlation results at least in part from electrostatic effects that occur in the binding process cannot be excluded. Results similar to these were obtained with lauric, stearic and oleic acids.

Analyses of these data were made with a model consisting of two independent classes of

TABLE II

Constants for the Binding of FFA to β-Lactoglobulin as Derived From a Non-integer Model[a]

Fatty acid	Number of data points	Primary sites, M^{-1}			Secondary sites, M^{-1}			Root mean square error[c]
		$n_1k'_1 \times 10^{-5}$	$k'_1 \times 10^{-5}$	n_1[b]	$n_2k'_2 \times 10^{-5}$	$k'_2 \times 10^{-5}$	n_2	
Lauric	23	0.55±0.02[d]	0.41±0.01	1.34	0.06±0.08	0.58±3.8	0.11	0.042
Palmitic	23	5.17±0.26	3.91±0.38	1.32	3.12±0.29	0.76±0.17	4.11	0.033
Stearic	22	2.00±0.78	3.86±7.3	0.52	8.54±6.50	0.33±0.06	26.1	0.110
Oleic	23	0.39±0.09	0.41±0.01	0.95	32.35±0.49	1.34±0.35	24.1	0.018

[a]Incubation at 37 C in phosphate-buffered salt solution.
[b]Estimates for n. These were derived by dividing nk' by k'.
[c]This parameter is a measure of the average deviation of the data from the computed curve (1).
[d]Parameter uncertainty, i.e., asymptotic estimates of the standard error (9).

BLG binding sites. This treatment assumes that BLG contains a fixed number of total FFA binding sites and that the sites are not altered by the binding process. Starting estimates for fitting the experimental points to a binding curve were obtained with a system that did not constrain the number of binding sites, n, to integer values (1,9). These estimates are shown in Table II. The range of estimates for n, (0.52 to 1.34) suggested that the primary class of BLG sites consisted of only a single site. In marked contrast, the computed estimates for n_2 (0.1 to 26.1) suggested that a single integer value for the secondary class of sites may not be applicable to each of the fatty acids. Moreover, the large uncertainties in $n_2k'_2$ and k'_2 for lauric and stearic acids further suggested that different integer values of n_2 should be considered for each acid (9).

With the values listed in Table II as initial estimates, these data were fitted to models constrained so that n_1 and n_2 must be integers. Table III contains the most satisfactory integer models for binding of each fatty acid. The best fit for oleate was obtained when $n_1 = 1$ and $n_2 = 24$. A best fit for stearate also was obtained with this model, but other integer values produced equally good fits. This is consistent with the large uncertainty in $n_2k'_2$ with stearate (Table II). The best fits for lauric and palmitic acids were obtained with a 1,2 and 1,6 integer model, respectively. In order to compare more readily the binding of each of the four acids and to satisfy our initial assumption that BLG has a fixed number of pre-existing binding sites, we attempted to fit all of the data to a single model. The value of 24 was selected as most representative for the actual number of secondary binding sites because it was best for oleate, the ligand with which we obtained the highest experimental values of ν. The fits obtained with the 1,24 model for laurate and palmitate, although adequate, were not as good as the respective best case for these acids. With the 1,24 model, k'_1 was of the order of $10^5 M^{-1}$ and k'_2 was of the order of 10^3 to $10^2 M^{-1}$ for each of the four acids. Note that even when n_2 was varied over a wide range, there was very little change in the value obtained for k'_1. This indicates that the character of the single high energy BLG binding site is relatively insensitive to the assumptions made concerning the class of weaker secondary binding sites.

Figure 2 shows plots of the data for each acid and the binding curves derived from the values of n and k' for the 1,24 model listed in Table III. These are graphs of ν (the molar ratio of bound FFA to BLG) against the negative logarithm of c (the unbound FFA concen-

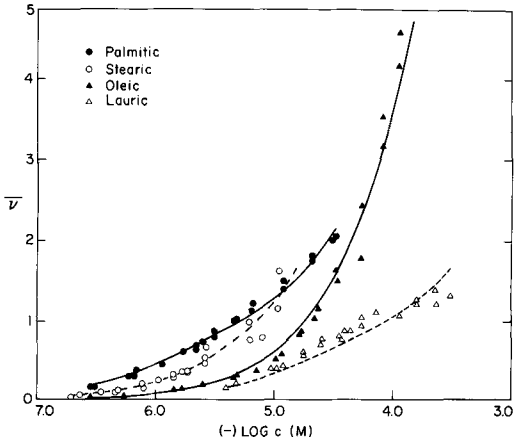

FIG. 2. Graphs for the binding of radioactive free fatty acids to β-lactoglobulin at 37 C in phosphate-buffered salt solution, pH 7.4, for the 1,24 model.

tration). The strength of association was palmitate>stearate>oleate>laurate. The maximum values of ν that were obtained in these experiments were: oleate, 4.6; palmitate, 2.0; stearate, 1.6; laurate, 1.3. Similar maximum ν values were obtained when FFA adsorbed on Celite were incubated with BLG. With each acid, the concentrations of c that occurred at the maximum ν values were equal to the maximum solubility of that particular fatty acid in the protein-free aqueous phase (1). A large excess of FFA was present in the heptane phase or on the Celite under conditions where the maximum ν values were obtained.

Effect on Binding of Changes in Lipid and Protein Structure

The maximum quantity of ligand that was taken up by BLG from Celite decreased when the palmitate carboxyl group was modified or removed. The highest ν values that were obtained were: palmitic acid, 1.9; cetyl alcohol, 0.67; methyl palmitate, 0.28; hexadecane, 0.92. Cholesterol also was taken up to a limited extent, the maximum ν being 0.40. In these systems, large excesses of ligand in the Celite phase also were present when the maximum ν values were reached.

Alterations in the structure of BLG by either physical or chemical means affected the binding of palmitate (Fig. 3). Both the maximum uptake and the strength of association were reduced when the incubation medium contained 6 M urea and when the protein was acetylated or exposed to elevated temperatures prior to incubation.

Effect on Binding of Changes in the Incubation Medium

Palmitate was bound more firmly by BLG at pH 7.4 in 0.05 M Tris-HCl than in phosphate-buffered salt solution (Table IV). In Tris buffer, palmitate was bound more tightly at pH 8.7 than at pH 7.4. In contrast, we did not observe these differences when palmitate binding to bovine albumin was studied (1). In phosphate buffer, palmitate was bound more tightly at pH 7.4 than at pH 6.9. Similar results were noted for FFA binding to both bovine and human serum albumin (1). This observation also is consistent with the finding that palmitate uptake by tumor cells from media containing BLG increased as the pH was decreased below 7.4 (16).

The strength of binding of palmitate to BLG in 0.02 M sodium phosphate, pH 7.4, decreased as the NaCl concentration was raised from 0-1.0 M. Similar results were noted for palmitate binding to bovine albumin (1).

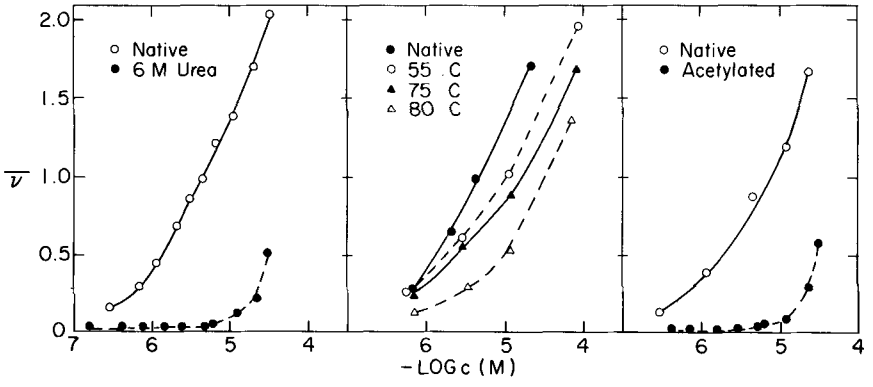

FIG. 3. Binding of 1-^{14}C-palmitate to physically or chemically altered β-lactoglobulin. The incubations were done in phosphate-buffered salt solution, pH 7.4, at 37 C. Approximately 90% of the free amino groups were modified in the acetylated BLG preparation.

TABLE III

Constants for the Binding of FFA to β-Lactoglobulin as Derived From Integer Models[a]

Fatty acid	Primary sites, M^{-1}		Secondary sites, M^{-1}		Root-mean-square root
	n_1	$k'_1 \times 10^{-5}$	n_2	$k'_2 \times 10^{-3}$	
Lauric	1	0.52±0.15	2	1.12±0.14	0.085
	1	0.40±0.06	24	0.10±0.02	0.130
Palmitic	1	6.61±0.39	6	7.09±0.14	0.052
	1	7.00±0.51	24	1.58±0.06	0.063
Stearic	1	1.61±0.39	2	4.22±5.50	0.110
	1	1.79±0.28	4	16.98±0.59	0.110
	1	1.56±0.24	6	11.87±0.21	0.110
	1	1.51±0.19	12	5.87±0.01	0.110
	1	1.87±0.24	24	2.48±0.18	0.110
Oleic	1	0.40±0.04	24	1.34±0.01	0.067

[a]The data are the same as those described in Table II.

The temperature dependence of binding of palmitate to BLG between 23 and 37 C was too small to be measured accurately by the present incubation techniques.

Uptake of FFA from Adipose Tissue

Like serum albumin, BLG took up FFA released from adipose tissue (Table V). However, when equimolar amounts of BLG and bovine albumin were present, the net release of fatty acids was greater in the media containing albumin (Experiments 1-3). In these incubations, the rate of FFA release was constant for 90 min when albumin was present but diminished gradually in the media containing BLG. When the BLG concentration was 3.7 times greater than that of albumin (Experiments 4-6), the rates of FFA release in media containing either BLG or albumin were approximately equal, and FFA release was linear during 90 min of incubation in both media. Six additional fat pad incubations were done with media containing low concentrations of protein in order to determine the maximum molar ratios of total FFA to BLG that could be reached in this system. A 4 hr incubation period was employed. The maximum molar ratio that was observed with BLG was 1.1. Under these conditions, there was very little increase in the FFA content of the BLG medium after 2 hr of incubation. In contrast, a molar ratio of 4.7 was reached after 4 hr of incubation when albumin was substituted for BLG, and FFA release continued at a linear rate over the entire 4 hr period. Taken together, these results indicate that BLG is less effective than bovine albumin as an acceptor for FFA released from adipose tissue, apparently because its binding sites have a weaker affinity for FFA than do those of albumin.

TABLE IV

Effect of pH on the Binding of Palmitate to β-Lactoglobulin[a]

$\bar{\nu}$	(−) log C[b], M			
	Phosphate buffer[c]		Tris buffer[d]	
	pH 6.9	pH 7.4	pH 7.4	pH 8.7
0.4	5.6	6.0	6.5	6.8
0.7	5.1	5.7	6.2	6.5
1.2	4.6	5.2	5.6	6.1
2.0	e	4.5	4.9	5.3

[a]Incubation was done at 37 C.

[b]Negative logarithm of the unbound palmitate concentration.

[c]Phosphate-buffered salt solution.

[d]0.05 M Tris adjusted to the required pH with I N HCl.

[e]The maximum molar ratio of palmitate to β-lactoglobulin that could be achieved at pH 6.9 was 1.2.

TABLE V

FFA Release From Adipose Tissue[a]

Expt No.	Net FFA release, μeq/g x hr	
	β-Lactoglobulin	Bovine albumin
1	1.4	3.6
2	1.0	1.9
3	1.7	2.9
4	2.6	2.3
5	2.6	2.6
6	2.1	2.6

[a]Epididymal fat pads were taken from Osborne-Mendel rats. One fat pad was incubated under air at 37 C in 10 ml of phosphate-buffered salt solution containing BLG, the other in 10 ml of this solution containing Fraction V bovine albumin. Porcine adrenocorticotrophic hormone (5 units) was present in each of the media. In experiments 1-3, both the BLG and bovine albumin media contained 0.5 μmole/ml protein. In experiments 4-6, the BLG media contained 1.1 μmole/ml protein, but the albumin media contained only 0.3 μmole/ml protein. The FFA content before incubation and after 20, 40, 60 and 90 min of incubation was measured in duplicate by titration of an extract of 1 ml of the medium (2).

Uptake of FFA by Tumor Cells from Media Containing BLG or Albumin

Ehrlich ascites tumor cells took up ^{14}C-1-palmitate when BLG served as the FFA carrier (Table VI). This confirmed our previous preliminary observations (16). At a given FFA-protein molar ratio, much more ^{14}C-1-palmitate was bound, esterified and oxidized to CO_2 when the cells were incubated in media containing BLG than when they were incubated in media containing bovine albumin. We have shown that the magnitude of FFA uptake (13), esterification (14) and oxidation to CO_2 (14) increase as the unbound FFA concentration is raised. At a given ν, palmitate is bound less firmly by BLG than by bovine albumin. Hence, at a given ν, the unbound palmitate concentration is greater in a BLG medium than in an albumin medium. Therefore, one would expect palmitate uptake and utilization to be greater in the BLG medium, and this was observed experimentally. At each ν value, the ratio of FFA to lipid ester radioactivity in the cells was much higher when the medium contained BLG. This observation is compatible with our previous observations concerning FFA utilization (13,14). As the unbound concentration to which a cell is exposed is raised, FFA incorporation in unesterified form increases exponentially whereas fatty acid esterification and oxidation approach limiting values (14). Therefore, one would expect the ratio of FFA to lipid ester radioactivity in the cells to be much higher in the experiments shown in Table VI that were done in the BLG media, for the unbound palmitate concentration with BLG at ν 0.8 is 12 times greater than that present with bovine serum albumin at $\nu = 1.6$ (1).

Similar distributions of radioactivity in the cell lipid esters occurred when incubation was done in either a BLG or an albumin medium. Phospholipids contained from 1.5-2.0 times more radioactivity than glycerides and from 10-40 times more radioactivity than cholesterol esters.

Ultraviolet Fluorescence Spectra

The fluorescence emission spectrum of BLG excited at 280 mμ was altered slightly when FFA were added (Fig. 4). The wavelength of maximum fluorescence occurred at 333 mμ whether or not FFA were added to BLG. However, the presence of 1 μeq of palmitate per μmole BLG produced an 8% increase in fluorescence intensity. Addition of more palmitate resulted in very little further increase in fluorescence intensity. In contrast, the addi-

TABLE VI

Utilization of 1-^{14}C-Palmitate by Ehrlich Ascites Tumor Cells[a]

Fraction	1-^{14}C-Palmitate incorporated, mμeq/10^8 cells			
	$\bar{\nu} = 0.8$		$\bar{\nu} = 1.6$	
	β-Lactoglobulin	Albumin	β-Lactoglobulin	Albumin
FFA	200	10	600	19
Lipid esters	250	100	560	140
CO_2	32	23	40	28

[a]Cells were incubated for 1 hr at 37 C under air with 1-^{14}C-palmitate bound to either β-lactoglobulin or Fraction V bovine albumin. The medium contained 3.0 μeq 1-^{14}C-palmitate (670,000 cpm/μeq) and sufficient protein so that the molar ratio ($\bar{\nu}$) of total palmitate to total protein was that listed in the Table. In addition, the medium contained 0.058 M NaCl, 0.0025 M KCl, 0.0006 M $MgSO_4$ and 0.085 M sodium phosphate, pH 7.4.

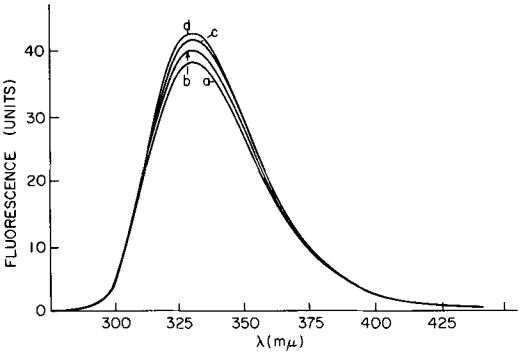

FIG. 4. Effect of palmitic acid on the ultraviolet fluorescence spectrum of β-lactoglobulin. The wavelength of excitation was 280 mμ. Spectra were recorded at room temperature in phosphate-buffered salt solution, pH 7.4. The BLG concentration was 10^{-4} M. The curves are labeled as follows: (a), no added FFA; (b), $\nu = 0.49$; (c), $\nu = 1.4$; (d), $\nu = 1.7$.

tion of FFA to bovine albumin produces a progressive decrease in fluorescence intensity and a blue shift in the wavelength of maximum emission of from 4-7 mμ (11).

Similar effects on ultraviolet fluorescence occurred when oleate, stearate or laurate were added to BLG. An increase in fluorescence intensity occurred independently of the method used for adding FFA to the protein, mixing with a soap solution, incubation with FFA-coated Celite, incubation with FFA dissolved in heptane, or incubation with rat epididymal fat pads in the presence of adrenocorticotrophin.

DISCUSSION

These results demonstrate that BLG can function as a long chain FFA carrier or acceptor in in vitro incubation systems. The affinity of BLG for FFA is considerably less than that of albumin (1,7). BLG contains only one high energy binding site for FFA. The association constant for FFA binding to this site ($k'_1 = 10^5 M^{-1}$) is one tenth that for FFA binding to the primary class of albumin sites (1); it is similar in magnitude to the association constant of the secondary class of albumin binding sites (1,7). Therefore, one can predict that at low values of ν, much more FFA should be available for uptake by tissues from media containing BLG than from those containing bovine albumin. This was confirmed experimentally by incubation with Ehrlich ascites tumor cells (Table VI). The weak secondary binding sites of BLG have an affinity for FFA (k'_2 of the order of $10^3 M^{-1}$) that is similar to the third class sites of the serum albumins (1,7). Hence, these sites are not effective in solubilizing much FFA in aqueous media. According to this interpretation, the factor that limits the quantity of fatty acid that can be taken up by BLG in these incubation systems is not saturation of available FFA binding sites. In fact, many unfilled FFA binding sites remain when the maximum ν values that can be attained experimentally are reached. We suggest that FFA uptake is limited by the solubility of the particular fatty acid in the aqueous incubation medium i.e., the maximum activity of unbound FFA anion that can exist in the aqueous phase.

Goodman has shown that the constant for association of palmitate with the binding sites of the human erythrocyte is in the range of $10^5 M^{-1}$ (17). Hence, one might predict that only the single primary binding site would be able to compete effectively with a mammalian cell membrane for FFA. This prediction was confirmed by the adipose tissue experiments (Table V) which demonstrated that BLG will remove net amounts of FFA only until the ν is in the range of 1.0. Under identical conditions of incubation, much more FFA was released from the fat pads when the medium contained an equimolar amount of bovine albumin. We conclude that the larger FFA release in the media containing albumin results from the greater ability of albumin to bind FFA; not from any special property that enables albumin to remove or enhance the release of FFA from the adipose cell.

FFA binding to serum albumin involves simultaneous electrostatic and nonpolar interactions (1,7,18,19). The present data concerning FFA binding to BLG are compatible with a similar interpretation. Palmitate analogues in which the carboxyl group is modified or removed were taken up to a lesser extent than the acid, and acetylated BLG bound palmitate very poorly. These observations are consistent with the presence of an electrostatic component in the binding process-attraction between the ionized FFA carboxyl group and a cationic protein site. In addition, the association constants varied with the structure of the FFA hydrocarbon chain, and binding was reduced when BLG conformation was altered by exposure to urea or heat. These findings indicate that nonpolar interactions also are involved in the binding process. In fact, binding of large organic ligands can occur in the absence of ionic interactions, for BLG took up small amounts of cholesterol, methyl palmitate, cetyl alcohol and hexadecane.

The binding constants listed in Tables II and III were obtained by making the following

assumptions: (a) that FFA anions interact with BLG; (b) that the measured total unbound FFA concentration is an accurate approximation of the unbound FFA anion activity (1,8); (c) FFA bind to pre-existing BLG sites, and binding sites are neither formed nor altered in the binding process; and (d) each class of binding sites competes independently for available FFA. Recent evidence indicates that some dimerization of FFA occurs in sodium phosphate solutions even when the FFA concentration is low (20). If subsequent work should confirm these results, then our assumption concerning total unbound FFA concentration would not be valid and small corrections will have to be made in these binding constants. In addition, studies on the interaction of organic ligands with serum albumin led Karush to suggest that proteins display conformational adaptability, that is, binding is associated with conformational changes in which binding sites are either formed or altered (21). Recent work by Lovrien also demonstrated that the association of dodecyl sulfate with alkaline bovine serum albumin produces considerable conformational change in the protein (22). Our inability to obtain a single value for n_2 that was suitable for each of the four acids may be considered as further support for the conformational adaptability hypothesis. If the secondary BLG sites are formed or altered when the first FFA molecule is bound, then each acid may induce a somewhat different change. Therefore, n_2 may be different for each acid, and our attempt to fit all of the data to a single model may be an oversimplification.

Another possible source of error stems from the fact that small quantities of heptane were present in the aqueous phase in the equilibrium partition incubations (8). If heptane binds to BLG, then the binding of FFA might be altered. Since small amounts of hexadecane were taken up by BLG, it is likely that some heptane also was bound to the protein. In spite of this potential difficulty, we employed the partition method for the following reasons. Other procedures that are used to study protein-ligand association also present serious difficulties when long chain FFA serve as the ligand. Long chain FFA are very poorly soluble in aqueous solutions of neutral pH, they do not pass through ordinary dialysis tubing, and they adsorb onto many types of solid phases. Furthermore, our data suggest that incubation in the presence of heptane did not grossly alter the capacity of BLG to bind FFA. The maximum ν value that was obtained with palmitate was almost identical in heptane partition and Celite incubations. In addition, others have shown that the apparent association constant for binding of testosterone to bovine albumin is of similar magnitude when measured by either equilibrium partition or dialysis (23).

FFA binding was associated with a small change in the ultraviolet fluorescence intensity of BLG. Hence, it should be possible to monitor reactions involving FFA uptake or release from this protein by spectrophotofluorometric techniques. However, this can be done more readily with bovine albumin, too, because the FFA-induced fluorescence changes are larger (11). These effects upon fluorescence result from alterations in the environment of one or more protein tryptophan residues. This could occur because FFA interacts directly with a segment of the peptide chain that contains tryptophan. Alternatively, this could result from a small conformational change in the region of one or more tryptophan residues that is secondary to binding of FFA at a distant region of the protein.

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REFERENCES

1. Spector, A. A., K. John and J. E. Fletcher, J. Lipid Res. 10:56-67 (1969).
2. Chen, R. F., J. Biol. Chem. 242:173-181 (1967).
3. Gornall, A. G., C. J. Bardwill and M. M. David, Ibid. 177:751-766 (1949).
4. Aschaffenburg, R., and J. Drewry, Nature 176:218-219 (1955).
5. Fraenkel-Conrat, H., R. C. Bean and H. Lineweaver, J. Biol. Chem. 177:385-403 (1949).
6. Moore, S., and W. H. Stein, Ibid. 176:367-388 (1948).
7. Goodman, DeW. S., J. Am. Chem. Soc. 80:3892-3898 (1958).
8. Goodman, DeW. S., Ibid. 80:3887-3892 (1958).
9. Fletcher, J. E., and A. A. Spector, Computers and Biomed. Res. 2:164-175 (1968).
10. Avigan, J., J. Biol. Chem. 234:787-790 (1959).
11. Spector, A. A., and K. M. John, Arch. Biochem. Biophys. 127:65-71 (1968).
12. Chen, R. F., Anal. Biochem. 20:339-357 (1967).
13. Spector, A. A., D. Steinberg and A. Tanaka, J. Biol. Chem. 240:1032-1041 (1965).
14. Spector, A. A., and D. Steinberg, Cancer Res. 27:1587-1594 (1967).
15. Scatchard, G., Ann. N.Y. Acad. Sci. 51:660-672 (1949).
16. Spector, A. A., J. Lipid Res. 10:207-215 (1969).
17. Goodman, DeW. S., J. Clin. Invest. 37:1729-1735 (1958).
18. Boyer, P. D., G. A. Ballou and J. M. Luck, J. Biol. Chem. 162:199-208 (1946).
19. Teresi, J. D., J. Am. Chem. Soc. 72:3972-3978 (1950).
20. Mukerjee, P., J. Phys. Chem. 69:2821-2827 (1965).
21. Karush, F., J. Am. Chem. Soc. 72:2705-2713 (1950).
22. Lovrien, R., Ibid. 85:3677-3682 (1963).
23. Schellman, J. A., R. Lumey and L. T. Samuels, Ibid. 76:2808-2813 (1954).

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