Water-phase palmitate concentrations in equilibrium with albumin-bound palmitate in a biological system

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Abstract The palmitate (PA) binding and transport capacity of human and bovine red cell membranes enables us to establish, in a biological system, the existence of a well-defined monomer concentration in equilibrium with PA bound to bovine serum albumin (BSA, 30 μM) inside the resealed red cell ghosts. Supernatants of suspensions of the [^{3}H]PA-labeled ghosts contain a tiny quantity of dissolved binding capacities besides the monomer PA. This is demonstrated by linear regression of supernatant tracer concentrations versus ghost concentrations in a dilution series. The extrapolated value, corresponding to zero ghost concentration, is the monomer PA concentration in equilibrium with PA bound to BSA within the ghosts in molar ratio (ν). Measurements have been carried out for ν between 0.1 and 1.5 and at 0°C, 10°C, 23°C and 38°C. The important ν-dependent binding of PA to the ghost membrane itself enables us to use preparations of BSA-free ghosts in the same way, whereas this is impossible in the case of arachidonic acid. Within the physiological range of ν the PA monomer concentrations are accounted for by an apparent dissociation equilibrium constant (K_d) 3.4 10^{-8} M at 38°C calculated on basis of three equivalent binding sites per mol BSA. K_d depends on temperature with a well-defined enthalpy of 38.4 kJ/mol.—**Bojesen, I. N., and E. Bojesen.** Water-phase palmitate concentrations in equilibrium with albumin-bound palmitate in a biological system. *J. Lipid Res.* 1992. **33:** 1327–1334.

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Albumin is the physiological carrier of long-chain fatty acids (FA) in blood. FA are tightly bound to albumin in the physiological range of FA/albumin molar ratio, ν, which ranges from 0.5 to 1.5. The ν value of PA is 0.2–0.3 whereas for some FA species, such as arachidonic acid, ν is as low as 0.02. The transfer of FA between albumin and cells and vice versa is presumably mediated by a water-phase shuttle of monomers. Therefore, information on water-phase concentrations are of great physiological importance. To date information concerning equilibrium water-phase concentrations of albumin-bound FA has been provided by the classical heptane–water-phase partition method of Goodman (1), particularly through the work of Spector et al. (2–4). However, the physiological relevance is questionable because of unpredictable dimer formation in both phases (4). Such aggregation in the water phase corroborates the observation by Patil, Matthews, and Cornwell (5) that diffusion coefficients of FA greater than lauric acid in vitro correspond to dimers or even higher orders of aggregations. The ambiguity of results obtained by means of phase partition makes the negative results of equilibrium dialysis (6, 7) in the case of palmitate critical.

In biological studies on FA uptake (8–12), the equilibrium concentrations of unbound FA in the media are calculated by a stepwise equilibrium constant method (3, 13) using the association constants for FA binding to albumin reported by Spector, John, and Fletcher (2). If the constants are wrong, then all these data must be interpreted differently.

We have shown recently (14) that PA concentrations in albumin-free supernatants of PA-loaded resealed human red cell ghosts were roughly in accordance with the data of Spector et al. (2), but we did not exclude the possibility that the results were artefacts. Further work has, however, shown that this indeed is the case. The supernatants contained in each preparation, besides unbound PA, a variable tiny amount of PA-binding components released from a few damaged ghosts. As shown in the present study this can be demonstrated by a simple dilution series. Corrections for this fraction enable us to determine, for the first time, the equilibrium water-phase monomer concentration of long-chain fatty acids with more than fourteen carbon atoms.

Abbreviations: FA, fatty acid.

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Ghost preparations are particularly qualified tools because the membrane in this system is a biological membrane and the results are therefore directly physiologically relevant. The system has the further merit that equilibria are established very rapidly because of the enormous surface area of the large number of small semipermeable bags. The ν dependency below 1.5 of PA binding to BSA can be accounted for by three identical binding sites and the linear van't Hoff isochor between 0°C and 38°C suggests that in this ghost system the equilibrium state of PA in water is indeed the monomer state.

MATERIALS AND METHODS

[(9, 10)-^{3}H]Palmitic acid, sp act 54 Ci/mmol, was obtained from Amersham International plc, Amersham, England. Unlabeled acid was obtained from Sigma. Labeled acid was purified every month by chromatography on a silicic column using 2% ethyl acetate in benzene as eluant. The scintillation fluid Opti-Fluor was purchased from Packard Instrument Company, Inc. (Downers Grove, IL). Bovine serum albumin (BSA) (Behring Institute, Germany) was defatted according to the method of Chen (15).

Preparation of albumin-free and albumin-filled ghosts was carried out as described previously (14). The resealed ghosts were isolated from the hemolysate by centrifugation and washed at 0°C with 165 mM KCl, 2 mM phosphate buffer, pH 7.3, containing 0.02 mM EDTA/EGTA. Albumin-free ghosts were stored in buffer and ghosts containing 0.2% BSA were stored in buffer containing 0.2% BSA.

Media for labeling of ghosts (16) (charge buffers) contained 165 mM KCl, 2 mM phosphate, 0.02 mM EDTA/EGTA 1:1, 0.2% defatted BSA (30 μM), 0.6–1 μCi/ml [^{3}H]palmitic acid, and suitable amounts of unlabeled acids giving the final molar ratios of acid to BSA (ν) from 0.08 to 1.44. Determinations of water-phase concentration of PA in equilibrium with PA bound by ghosts equilibrated with 0.2% (30 μM) BSA solution and corresponding ν were carried out using three modifications, all using BSA-free or 0.2% BSA-filled ghosts packed by centrifugation 5 min at 22,200 rpm (36,470 *g*) in a Cryofuge 6-4 (Heraeus Christ).

I. Four hundred mg packed ghosts (V1 ml) containing 0.2% BSA was incubated 50 min at 0°C, 10 min at 10°C, or 5 min at 23°C or 38°C with 600 μl charge buffer (W_b ml). Charge buffer was removed by washing 4 times with 50 volumes of the same buffer without BSA and palmitate. After the final washing, the ghosts were suspended in 4 ml buffer and 1 ml was distributed to each of four tubes containing 1 ml, 2 ml, 4 ml, and 7 or 8 ml buffer, respectively. Complete equilibration of ghosts with BSA-free buffer was accomplished by leaving the suspension for 50 min at 0°C, 10 min at 10°C, or 5 min at 23°C or 38°C. Ghost-free supernatants (2 × 200 μl) were taken from each tube after 10 min of centrifugation at 36,500 *g* for counting. Cell counts of supernatants (200 μl) taken from the top just after the analyzed fractions were the controls for the efficiency of the centrifugation.

Scintillation counting

Counting rates were determined in 10 μl charge buffer (C_b dpm/ml), in 10 μl charge buffer after equilibration with ghosts (C_a dpm/ml), in 2 × 200 μl ghost-free supernatants (C_s dpm/l), and in about 50 mg (V_2 μl, D dpm) ghosts weighed at the end of the experiments after lysis with 350 μl water, and after the addition of 3.9 ml Opti-Fluor scintillation fluid. Quench corrections were carried out according to a standard curve produced with variable amounts of water and ghosts added to the 3.9 ml scintillation fluid. PA concentrations in supernatants of the four tubes were calculated from the counting rates and the PA specific activity (S, dpm/nmol) as C_s/S. The data were subjected to a regression analysis with the reciprocal dilution factor as independent variable.

Unbound PA in charge buffer after use constitutes less than one-thousandth of total PA, therefore C_a is taken to represent the counting rate of BSA-bound PA and ν is calculated as:

$$\nu = C_a/(S\ 30),\ 30\ \text{nmol/ml equals 0.2\% BSA.} \quad \textit{Eq. 1)}$$

II. Four hundred mg packed BSA-free ghosts was equilibrated with 600 μl charge buffer, washed, and distributed on four tubes as described above (modification I). Virtually no PA bound by ghosts was lost during the washings. Therefore, the PA bound is to be regarded as in equilibrium with 0.2% BSA (see determinations a and b, Table 1). ν corresponding to membrane binding is now calculated as:

$$\nu = C_a\ (W_b + F1V1)/(S\ 30\ W_b) \quad \textit{Eq. 2)}$$

where F1 is the fraction of extracellular buffer in V1 ml packed ghosts before charge.

III: Four hundred mg packed BSA-free ghosts was suspended in 1 ml BSA-free buffer. The required radioactive acid was deposited on 200 mg glass beads (16) which were equilibrated with the ghost suspension directly by gentle shaking at an appropriate temperature. All the deposited PA was transferred to ghosts within minutes. Subsequently the ghost suspension was isolated and rinsed once with 20 ml buffer before the distribution into the four tubes described under modification I.

Determination of ν corresponding to the membrane binding in this modification requires calibration, i.e., knowledge of B (nmol PA bound per ml pure ghosts) dependency of ν (**Fig. 1**). This is obtained by modification II experiments and calculated according to:

$$B = [C_b W_b - (C_a (W_b + F1\ V1)]/(V1\ S\ (1 - F1)) \quad \textit{Eq. 3)}$$

or

$$B = D\ 1000/(V2\ S\ (1 - F2)) \quad \textit{Eq. 4)}$$

where F2 (smaller than F1) is the fraction of extracellular buffer in packed ghosts weighed at the end of experiments.

K_d determinations

K_d for the reaction: BSA:PA → PA + BSA is

$$K_d = (n[BSA] - [BSA:PA])\ [PA]/[BSA:PA]$$

or

$$K_d = ([PA]\ (n - \nu))/\nu \quad \textit{Eq. 5)}$$

where [PA] is the water phase concentration of monomer PA, n[BSA] is the total concentration of PA binding sites, and [BSA:PA] is the concentration of PA-albumin complex. On the basis of the determinations of the monomer PA water-phase concentrations and ν values, an equilibrium dissociation constant corresponding to n can be calculated.

RESULTS

The technique profits by the very strong binding of PA to BSA-filled as well as to BSA-free ghosts and by the fact that extensive washings with BSA-free buffer deplete the ghosts of bound ligand only insignificantly. The observations presented in **Fig. 2** demonstrate that we are dealing with a true and fast equilibrium between ghosts and buffer. Low temperature is required to obtain a supernatant concentration lower than the equilibrium value and the washing-centrifugation procedure has to be rapid within the limit that determines separation of supernatants and the low density ghosts (d ≈ 1.02 g/ml). Not all preparations show this phenomenon equally clear. It requires that the true PA water-phase concentration represents a considerable fraction of the supernatant PA. In some cases a PA-binding capacity (dissolved components or suspended small fragments of a few damaged ghosts) dominates. If only monomer PA is present in the supernatant, the concentration must decrease only slightly when the ghost suspension is diluted (see Discussion). **Fig. 3** shows that this is not the case. The supernatant PA concentration decreases linearly with the reciprocal of the dilution factor. This effect of dilution demonstrates that the supernatants contain a component that has a constant concentration, as expected of the water-phase equilibrium concentration, besides a certain amount of binding capacity that does not change by dilution. Therefore, the extrapolated concentration of an infinite dilution of the supernatant is the true water-phase concentration of PA monomer. The dilution effect, the slope, is in general smaller for BSA-free ghosts than for BSA-filled ghosts (Fig. 3).

The contact surface of the centrifugation tubes increases with the dilution factor in our procedure. The contribution of unspecific surface adsorption to the dilution effect has been excluded by using dilutions with constant buffer volumes, 7.4 ml, and varying the amount of

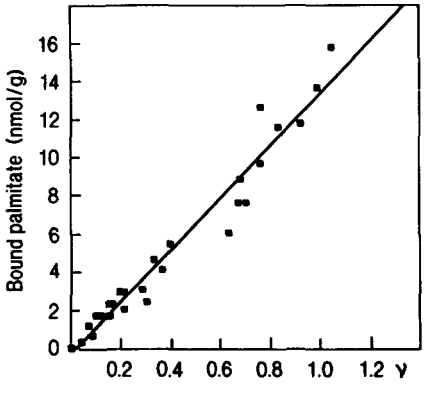

Fig. 1. The ν dependency of palmitate bound to the membrane of BSA-free ghosts (nmol/g). Ghosts were equilibrated with 0.2% BSA buffer solutions containing variable amounts of palmitate. Bound palmitate is calculated according to equation 2 (see Methods). The regression line is: Y = 13.61 (±0.58) X − 0.34 (±0.30); n = 28; r = 0.98; t = 24.

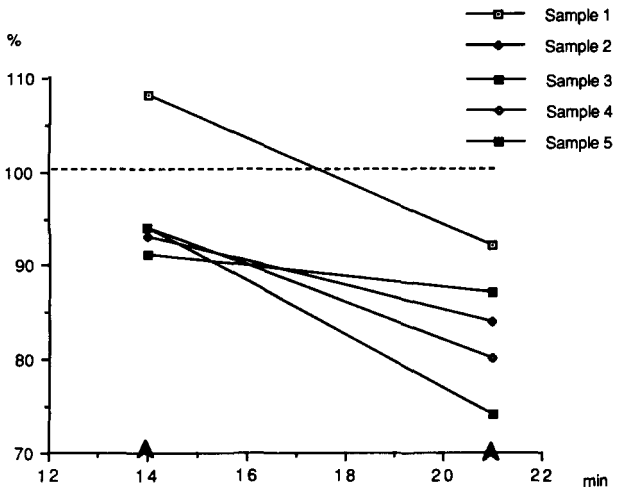

Fig. 2. Demonstration of rapidly obtained equilibrium concentrations of PA in supernatants of suspensions of PA-loaded ghosts, containing 0.2% BSA. Temperature is 0°C. The lines connect two data points from the same experiment. The arrows indicate the approximate time interval, between the second and the third supernatant sampling immediately after centrifugation of ghost suspensions (buffer-ghosts (50:1) v/v). The supernatant PA concentrations are presented as the percentages of the equilibrium value (---) measured in the fourth and final supernatant after 50 min incubation and centrifugation.

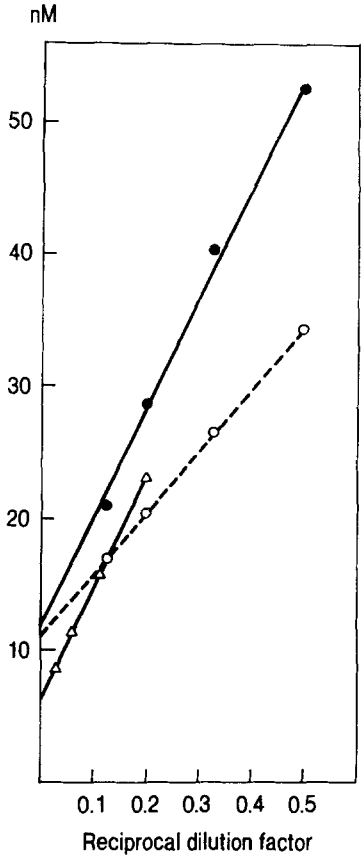

Fig. 3. Regression analyses of the dilution effects on equilibrium supernatant PA concentrations from suspensions of PA-loaded ghosts. The abscissa is the reciprocal dilution factor and the ordinate is PA concentration of the supernatants. The ordinate intercept reflects the monomer PA concentration, which is independent of the ghost concentrations. (●): Analysis at 23°C, ν 1.4 with ghosts containing 0.2% BSA. Dilution volumes 2, 3, 5, and 8 ml. (○): Analysis at 23°C, ν 1.44 with BSA-free ghosts. Dilution volumes 2, 3, 5, and 8 ml. (△): Analysis at 23°C, ν 0.96 with BSA-free ghosts. Constant dilution volume (7.4 ml).

ghosts from 200 mg to 1600 mg (Fig. 3). The regression is still linear and PA water-phase concentrations are not different from the values obtained by the generally used procedure with the same ν (Table 1, $P < 0.1$).

Although only two measurements are required to estimate the extrapolated concentration of infinite dilution, we prefer to use four dilutions in order to control linear regression and to evaluate the precision by which the intercept is estimated. The results obtained by the three modifications of the method are presented in **Table 1.**

With BSA-filled human and bovine ghosts, the average coefficient of variation is about 20%, whereas with BSA-free ghosts it is about 12%. A fairly large fractional uptake on ghosts implies, in the case of modifications I and II, that the final ν can be predicted only approximately. The error by which the final ν values are estimated (see Methods) is not presented because it is generally negligible compared with the uncertainty of the estimated water-phase concentration.

In order to use these data to predict the water-phase concentration corresponding to a given ν value, it is convenient to convert the data to the corresponding apparent dissociation equilibrium constant (K_d) on basis of n equivalent binding sites. **Table 2** shows the weighted mean K_d values calculated according to equation 5 for different ranges of ν and n = 1, 2, 3 and 4. The coefficients of variation are shown to indicate the fit of calculation models to the data. Note that K_d values calculated for ν below 0.5 and for ν above 0.5 using n = 3 or 4 are not significantly different. This observation suggests that K_d is a true equilibrium constant for $\nu < 1.4$ and we have therefore presented the temperature effects by a van't Hoff plot. The temperature dependency of K_d is given by the van't Hoff equation ($d\ln K_d/d(1/T) = -\Delta H/R$). K_d values for n = 3 are used to describe the temperature effect and in the interval from 0°C to 38°C (**Fig. 4**) ΔH is determined to be 38 kJ/mol.

Investigations of the equilibrium binding of arachidonate to albumin require BSA-filled ghosts. BSA-free ghosts cannot be used as a reference binder because the affinity of arachidonate to the ghost membrane is much smaller than the affinity of PA. **Fig. 5** shows that at 23°C the affinity of arachidonate to BSA is indistinguishable from the PA binding.

DISCUSSION

As mentioned in the introduction, there is an urgent need for a method based upon principles other than phase partition. Such a one is the Lipidex binding assay procedure (17) suggested for PA binding to BSA. Unfortunately, the assay seems to work only at 0°C, as a high percentage of PA complex is also bound to Lipidex at higher temperatures.

The new principle

In principle, the ghost equilibrium method is expected to provide information of physiological interest because it is based upon the properties of physiologically relevant components. In other words, the assay almost simulates the physiological situation.

PA must leave its binding sites as a monomer and therefore the transfer of PA between albumin and cell membranes is mediated by this monomer, provided some secondary dimer formation in water does not occur. Under desorption from monolayers, PA, in contrast to laurate, diffuses as a dimer (11). However, dimer formation in water must depend greatly on concentration (18) and, in the desorption experiments, the water phase is not far from being saturated. According to the most probable theory on dimer formation, monomer concentrations in the albumin solution are about fourfold smaller than indicated by phase partition (4). Accordingly, the present

TABLE 1. Concentrations of unbound palmitate [PA] in 165 mM KCl, 2 mM phosphate buffer, pH 7.3, containing 0.02 mM EGTA:EDTA (1:1) in equilibrium with palmitate-bovine serum albumin (BSA), 0.2%, complexes of various molar ratios at four temperatures

PA/BSA Molar Ratio	[PA] at 0°C	[PA] at 10°C	[PA] at 23°C	[PA] at 37°C
ν	*nM*	*nM*	*nM*	*nM*
0.08			0.43 ± 0.01	1.30 ± 0.15
0.12	0.20 ± 0.04[a]	0.24 ± 0.02		
0.15			0.75 ± 0.05	2.48 ± 0.08
0.20	0.46 ± 0.30[a]	0.98 ± 0.10	1.23 ± 0.24[a]	3.25 ± 0.36[a]
0.20				4.24 ± 0.45[a]
0.25	0.37 ± 0.31			
0.26	0.39 ± 0.11	1.06 ± 0.12		
0.31		1.56 ± 0.06		
0.32			2.69 ± 0.10	2.99 ± 0.06
0.33				2.41 ± 0.75
0.35		1.63 ± 0.14[c]	2.61 ± 0.14[c]	
0.37	0.47 ± 0.06	1.62 ± 0.60		
0.38	0.53 ± 0.26	2.03 ± 0.04		3.53 ± 0.96[a]
0.39		1.57 ± 0.62		
0.43				3.69 ± 0.48
0.44			4.24 ± 0.67	5.78 ± 0.59
0.47	0.76 ± 0.60	2.17 ± 0.98		
0.48				5.09 ± 1.54[a]
0.49	1.07 ± 0.36[a]	1.18 ± 0.12[a]		
0.51			3.60 ± 0.73	5.63 ± 0.23
0.52				5.06 ± 0.79
0.55	1.06 ± 0.10	3.33 ± 0.28		
0.70				11.01 ± 2.41[a]
0.76		2.70 ± 0.25[c]	5.37 ± 0.48[c]	
0.77	1.42 ± 0.11[a]			
0.81				11.75 ± 2.61
0.83		2.07 ± 0.12[c]		
0.90	1.93 ± 0.22[b]			11.10 ± 1.16[b]
0.96			6.29 ± 0.26[b,d]	
0.97	1.34 ± 0.06[b]		7.11 ± 0.64[a]	17.89 ± 1.10[a]
1.00	3.17 ± 0.21[a]			20.64 ± 1.08
1.06				20.92 ± 3.39
1.11		3.18 ± 0.06[c]		
1.14	2.18 ± 0.36[b]	3.70 ± 0.16		
1.16			9.35 ± 1.09	19.00 ± 4.67
1.38	4.91 ± 1.88[a]	5.56 ± 0.71[a]	10.67 ± 2.34[a]	22.28 ± 2.01[a]
1.40			11.44 ± 1.49	26.68 ± 8.23
1.44		6.34 ± 0.55[c]	11.12 ± 0.22[c]	

Unbound PA concentration is the extrapolated supernatant PA concentration of ghost suspensions at infinite dilution, Values given as mean ± SD.

[a]Determinations carried out with human red cell ghosts containing 0.2% BSA.

[b]Determinations carried out with human red cell BSA-free ghosts used as a reference binder.

[c]Determinations carried out with human red cell BSA-free ghosts loaded directly with palmitate and used as a reference binder.

[d]A dilution process with constant volume is used (see Fig. 3).

All other determinations were carried out with bovine red cell ghosts containing 0.2% BSA.

method probably measures monomer concentrations since our values are about threefold smaller. This is apparent by comparing our data obtained with two ν values 0.5 and 1.0 at 38°C and 23°C (Table 1) with the corresponding values read from the published plots (Figs. 3 and 6 in ref. 2). A more precise comparison of equilibrium constants is unfortunately misleading, for reasons discussed later. The strikingly linear van't Hoff plot from 0°C to 38°C suggests that only one equilibrium is involved; in other words, that dimer formation is absent. This is strongly supported by the finding of a similar enthalpy for laurate and myristate when equilibrium constants are determined by dialysis and calculated for the high affinity sites (19), because dimer formation here is probably absent.

The limit of precision

Our estimates of extrapolated supernatant PA concentrations are obtained with widely different precisions (Table 1) mainly because the slopes of the regression lines

TABLE 2. Equilibrium dissociation constants (K_d) calculated according to equation 5 with the number of sites (n = 1, 2, 3, 4) and corresponding coefficients of variation at four different temperatures and at different ν intervals

Temp.	ν Interval	Dissociation Constant (mean ± SE) 1	2	3	4	Coefficient of Variation 1	2	3	4
				nM					
0°C	0.12–1.38		2.52 ± 0.18	4.11 ± 0.28	5.71 ± 0.43		0.26	0.25	0.28
	0.12–0.55	1.21 ± 0.11	2.80 ± 0.21	4.38 ± 0.32	5.97 ± 0.44	0.27	0.21	0.21	0.21
	0.55–1.38		1.95 ± 0.23	3.63 ± 0.46[a]	5.31 ± 0.74		0.29	0.31	0.34
10°C	0.11–1.44		5.18 ± 0.61	8.26 ± 0.83	11.33 ± 1.07		0.47	0.40	0.39
	0.11–0.55	2.36 ± 0.25	5.45 ± 0.56	8.53 ± 0.89	11.61 ± 1.23	0.31	0.31	0.31	0.32
	0.55–1.44		2.89 ± 0.87	5.87 ± 1.25[b]	8.85 ± 1.67		0.74	0.52	0.46
23°C	0.08–1.44		10.31 ± 0.91	15.72 ± 1.00	21.13 ± 1.18		0.32	0.23	0.20
	0.08–0.51	4.93 ± 0.28	10.33 ± 0.80	15.73 ± 1.41	21.13 ± 2.03	0.14	0.19	0.22	0.24
	0.51–1.44		5.84 ± 0.62	13.09 ± 0.53[b]	20.34 ± 0.51		0.26	0.10	0.06
38°C	0.08–1.40		21.88 ± 1.62	34.27 ± 2.23	46.67 ± 3.00		0.33	0.29	0.29
	0.08–0.52	9.54 ± 1.34	21.90 ± 2.53	34.27 ± 3.74	46.64 ± 4.95	0.47	0.38	0.36	0.35
	0.52–1.40		17.33 ± 1.32	34.42 ± 1.83	51.51 ± 2.55		0.22	0.15	0.14

[a] $P < 0.2$; [b] $P < 0.1$; no significant difference from K_d calculated for $\nu < 0.5$.

differ, reflecting different amounts of the initial binding capacity in the supernatants. We have therefore tried to identify the sources. The density of ghosts is small (1.02 g/ml) but a suitable centrifugation procedure is nevertheless able to reduce the number of cells to insignificant levels. This has been controlled regularly by counting with the Coulter Multisizer. The number of cells was highest in the 1:1 dilution supernatant but never exceeded about 1 nl in the 200-μl aliquots sampled for tracer assay. This cell contamination accounts, at most, for about 2% of the tracer. Insufficient washing was excluded by the failure of further washings to reduce the slope. However, a variation among donors suggested that stability of the ghosts varies. Therefore we tried the readily available bovine erythrocytes, which differ considerably from human erythrocytes (20). The results were, however, almost the same. More successful was the introduction of ghosts without BSA using modifications II and III. The errors of estimates based upon the various ghost material appear in Table 1. We believe that the origin of the supernatant

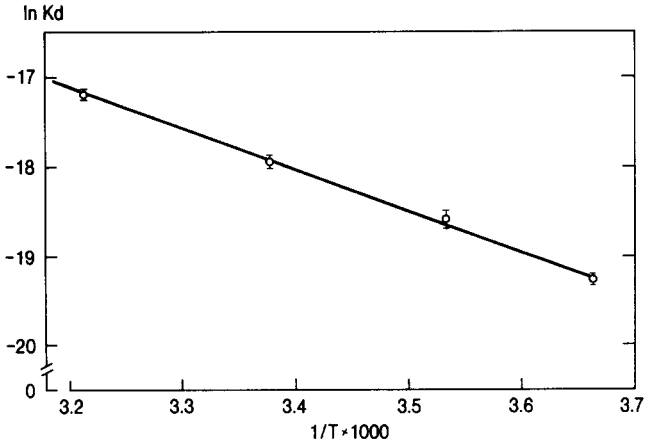

Fig. 4. Van't Hoff plot of the temperature effect on the apparent K_d calculated on the basis of three equivalent binding sites on BSA for PA.

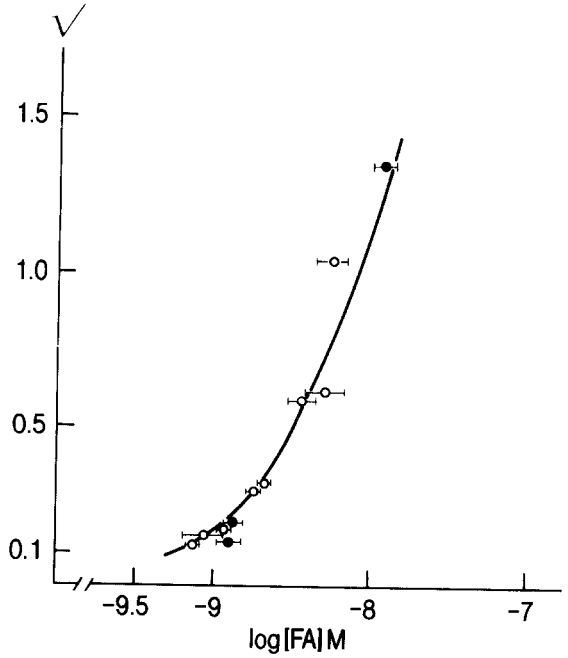

Fig. 5. Semilogarithmic plot of fatty acid/albumin molar ratio (ν) versus monomer concentrations (M) of fatty acids [FA]. The curve represents palmitate data at 23°C, where [PA] is calculated according to equation 5 using K_d = 15.72 nM. The points (± SE) represent single determinations of monomer arachidonate concentrations at 23°C obtained by the described method using BSA-filled ghosts (modification I). (●): Bovine erythrocyte ghosts; (○): human erythrocyte ghosts.

binding capacity is ghosts that are damaged by the washing procedure. Some ghosts are closely packed by centrifugation and the subsequent stirring may rupture cells adhering to each other. Some measures may eventually be devised that minimize this cell damage.

The possibility of erroneous results

The dilution principle may fail to eliminate contributions of bound PA, only if a palmitate-binding component, just as PA itself, is rapidly released from ghosts until the water-phase concentration reaches an equilibrium value. This possibility can, however, be dismissed because it implies a rate of release of a membrane component comparable with that of the ligand, PA. Another kind of error is the theoretical effect of the dilution on the monomer concentration, caused by PA depletion of ghosts and thereby a reduction of ν. In reality, this depletion appears to be of little, if any, importance. The effect of a significant depletion would be reflected in a deviation of the point representing PA concentration of the most diluted suspension from the regression line defined by the three less diluted suspensions. In these latter suspensions the effect of dilution is smaller and the concentration of bound PA much higher. The effects are predicted to be largest for analyses with BSA-free ghosts and increase with temperature. It is readily calculated that K_d is too small at 0°C and 10°C to permit any significant effect of the dilution, but at 23°C the monomer concentration is lowered by about 5% in assays with BSA-free ghosts. The corresponding two graphs of Fig. 3 indicate only hints of deviations of the data of the most diluted suspensions from the regression lines defined mainly by the data of the less diluted suspensions. Expected deviation of the total concentration is only about 3%, which is about the error of measurements. At 38°C the calculated decrease of monomer concentration is about the same in assays using BSA-filled ghosts as in those using BSA-free ghosts at 23°C. Therefore, we conclude that the effect of the dilution on the monomer concentrations results in a slight but insignificant bias on the regression line by which it is defined.

The equilibrium constant

The apparent equilibrium constants of Table 2 summarize the measured monomer-ν relations and define the monomer concentration corresponding to a given ν value within the investigated range. The calculations show that no information is lost by using a single constant as the values are not different for $\nu < 0.5$ and for $0.5 < \nu < 1.4$ (Table 2). It is, in fact, the first time that sufficient data have been available which show that K_d calculated for three identical sites is independent of ν below 1.5, the normal physiological range. To the obvious objection against a description of fatty acid binding to albumin in terms of sites, when no saturation can be demonstrated (21), the answer is that three distinct binding domains have been identified (22) and it is likely that unspecific adsorbing regions are exposed by conformation changes induced by occupation of the three domains. This will account for "unlimited" binding with low affinity. It is readily calculated that only about 12% of the albumin molecules have three similar sites occupied when ν is 1.5. As this fraction increases with ν, the role of unspecific low affinity binding becomes increasingly important. We believe that this speculation gains credence by the linear van't Hoff plot between 0°C and 38°C with an enthalpy of binding of −38 kJ/mol, which makes up for nearly all the free energy ($\Delta G = R T \ln K_d$) of about −43 kJ/mol. Accordingly, the binding of PA is only to a small degree "hydrophobic" since the entropy term is small. A trivial entropy term of the free energy has also been reported by Pedersen, Honore, and Brodersen (19) for laurate and myristate binding to human serum albumin at low ν. This suggests that albumin binding to long-chain fatty acids in the low ν range is accompanied by an effect on the conformation corresponding to "cave formation" in water which accounts for the poor water solubility of such hydrophobic amphiphiles (23). Without such change in conformation, the K_d would have been very much smaller. At higher ν the binding seems to proceed independently of temperature and is therefore pure "hydrophobic" (19).

Our K_d is not directly comparable with the constants that are used to characterize the data obtained by phase partition (2–4). The reason is the overlapping importance of a series of constants. Thus we note that our K_d at 38°C (Table 2) is more than fourfold smaller than K_d reported for three high affinity sites (2–4) for BSA and PA, whereas our water-phase concentration in the low range of ν is only about threefold smaller, as discussed above.

In conclusion, we have demonstrated for the first time that the water phase of dissolved albumin-bound PA in a biological system contains PA in the monomer state. The monomer equilibrium concentrations within the physiological range of ν are about 3- to 4-fold smaller than those measured by the phase partition method. This study suggests that albumin-filled red cell ghosts are a generally applicable, convenient tool to investigate binding to albumin and other proteins of compounds that are poorly water-soluble and penetrate the membrane. The very large surface area ascertains rapid equilibria. Albumin has no affinity to the surface of ghosts and albumin-free ghosts can therefore be useful as a disperse reference binder in studies of albumin binding of compounds which, like PA, are bound strongly by the membrane.

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