Partition of Adsorbed and Nonadsorbed Bovine Serum Albumin in Dodecane-in-Water Emulsions Calculated from Front-Face Intrinsic Fluorescence Measurements

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Front-face fluorescence spectroscopy was used to estimate directly the extent of adsorption of bovine serum albumin (BSA) onto dodecane−water interface in dodecane-in-buffer (pH 7.6, 0.1 M) emulsions. The wavelength of the emission maximum (λ_{max}) of adsorbed BSA was blue-shifted (−15 nm) as compared to that of nonadsorbed BSA in phosphate buffer (Castelain and Genot, 1994). The concentrations of adsorbed and nonadsorbed protein in emulsions and the partition of BSA between cream and serum were calculated from volume, oil, and protein balances and λ_{max} measured on emulsions (oil volume fraction = 0.5 and 0.16; 0.1−36 g of BSA/L aqueous phase), creams, and serums. In emulsions containing 0.5 g of BSA/L, the protein was totally adsorbed at the interface and the serum phase did not contain any protein. When the protein concentration increased, the nonadsorbed BSA concentration increased more rapidly than the adsorbed one. However, serum phase contained adsorbed proteins at concentrations which cannot be neglected.

Keywords: *Front-face fluorescence; oil-in-water emulsions; bovine serum albumin; protein adsorption*

INTRODUCTION

The adsorption of proteins at interfaces is a subject of considerable interest, and applications can be found in many areas such as biology, medicine, biotechnology, food processing, and pharmacology. In these fields, one of the most widely used applications of protein adsorption is probably to stabilize emulsions or foams. Only part of the available protein adsorbs to the interface and is responsible for the stability of the dispersed system. In an emulsion, the amount of adsorbed proteins is generally estimated from the difference between the total amount of proteins in the emulsion and the amount of proteins in the separated serum phase (Elgersma *et al.*, 1990; Hunt *et al.*, 1993). Apart from the experimental difficulties, the method is invasive and time-consuming, and a major drawback of this procedure lies in the understatement that the serum phase does not contain adsorbed protein. However, some small oil droplets, whose density is very close to that of aqueous solution because they are covered with adsorbed proteins, can be present in the serum phase.

One characteristic of protein adsorption is that it involves conformational changes of the protein molecules. Structural changes of bovine serum albumin (BSA) upon its adsorption in model dodecane-in-water emulsions result in a blue-shift of the front-face fluorescence emission maximum (λ_{max}) and in an increase of the quantum yield of the protein (Castelain and Genot, 1994). The position of the emission maximum is directly related to the adsorbed/total BSA ratio in the system according to eq 1:

$$\text{adsorbed BSA/total BSA} = (335 - \lambda_{max})/(335 - 320) \quad (1)$$

This equation is based on the assumption that the fluorescence of BSA in emulsions, creams, or serums results from the fluorescence of nonadsorbed BSA molecules in the buffer (λ_{max} = 335 nm) and adsorbed BSA molecules (λ_{max} = 320 nm). To validate this equation, a concentrated emulsion, in which all the BSA was adsorbed, was diluted with BSA solutions having different protein concentrations and the emission maxima calculated with eq 1 were compared to the experimentally measured ones (Castelain and Genot, 1994). As the two sets of data were very well correlated, front-face fluorescence directly measured on emulsions can allow, with λ_{max} values and eq 1, to quantify adsorbed protein.

In this work, the changes of the position of the emission maximum of BSA upon its adsorption in dodecane-in-water emulsions were directly measured by front-face fluorescence. They were used to estimate, with eq 1, the amount of adsorbed BSA in emulsions and the protein partition between creams and serums.

EXPERIMENTAL SECTION

Materials. Dodecane (Merck; for synthesis, ref 820543), density 0.749−0.750, was used as the oil phase of emulsions. Phosphate buffer (0.1 M, pH 7.6) prepared with ultrapure water (18 MΩ, Millipore system) and per analysis chemical products was the aqueous phase of the emulsions. BSA (fraction V; ref A.4503, Sigma Chemical Co.) was used without further purification.

Description of the Systems. A schematic description of the emulsion (volume fraction ϕ_v) and of the separation of emulsion in cream and serum phases (volume fractions ϕ_{cr} and ϕ_{se}) by creaming or centrifugation is shown in Figure 1. During emulsification, some proteins adsorb and cover the surface of the oil droplets created by mechanical treatment. The total available proteins (concentration C_t) separate in adsorbed (concentration C_a) and nonadsorbed (concentration C_s) proteins. Because the aqueous and oil phases have different densities, the emulsion creams under the force of gravity. The system separates into a cream layer (upper phase) and a serum layer (bottom phase). The cream layer (protein concentration C_{cr}) separates to a maximal volume fraction ($\phi_{cr} = \phi_{max}$) with most of the oil droplets covered with

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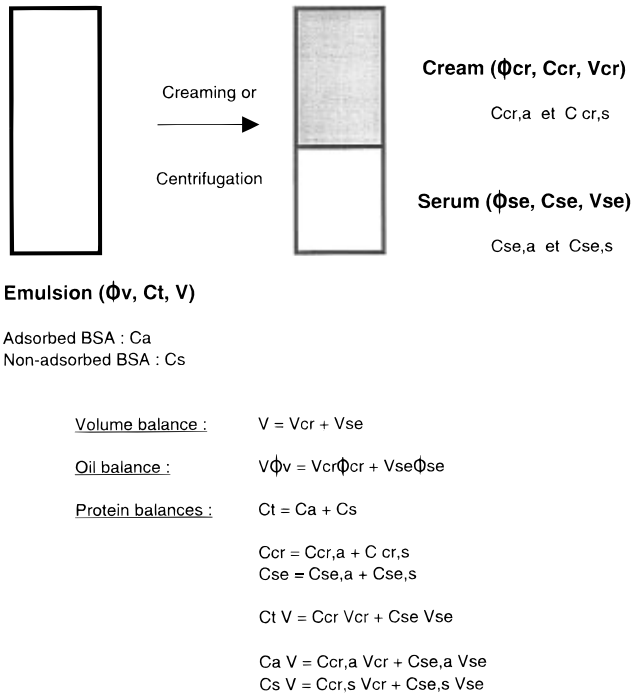

Figure 1. Schematic description of emulsion, cream, and serum with notations and volume and concentration balances.

adsorbed proteins ($C_{cr,a}$). The space between droplets is filled by a protein solution ($C_{cr,s}$). The serum layer (protein concentration C_{se}) is mainly constituted by a protein solution ($C_{se,s}$), but it can contain some small oil droplets covered with adsorbed protein ($C_{se,a}$) that do not cream. The creaming rate can be increased by centrifugation.

Thus, emulsion, cream, and serum are systems characterized by different adsorbed/total protein ratios. Volume, protein, and oil balances can be drawn according to the equations shown in Figure 1.

Preparation of the Samples. BSA solutions (0.1−36 g of BSA/L) were prepared by gentle stirring of powdered BSA in phosphate buffer to avoid protein foaming and denaturation, and the protein concentration was determined spectrophotometrically. Dodecane-in-water emulsions (50%, v/v) (20 mL of protein solution, 20 mL of dodecane) were prepared in a 100 mL beaker (diameter, 43 mm; height, 75 mm) kept in an ice bath, with a Polytron PT 45-28 shear agitator equipped with a 35 mm mobile head, at a speed of 20 000 rpm for 1 min.

In the first set of experiments (set 1), we studied concentrated emulsions (50%, v/v) whose total protein concentration in the aqueous phase varied from 1.3 to 19 g of BSA/L (concentration in emulsion: $C_t = 0.65-9.5$ g/L). The emulsions were stored at 20 °C for 24 h and gently stirred for homogeneity. Aliquots of emulsions (8 g) were centrifuged at 2000*g* for 30 min to separate creams and serums. Fluorescence of concentrated emulsions and corresponding creams and serums was investigated.

In the second set of experiments (set 2), 50% (v/v) dodecane-in-water emulsions prepared with 0.9−34 g/L BSA solutions were diluted with phosphate buffer to obtain 16% (v/v) emulsions (C_t = 0.14−5.6 g of BSA/L). During dilution procedure, the emulsions were carefully and gently stirred to prevent any new interface and new oil droplet formation by mechanical energy input. At 20 °C, the diluted emulsions creamed under the force of gravity over 5−6 h. Fluorescence of creams and serums was studied 24 h after preparation (storage temperature of 20 °C) without any further centrifugation.

Fluorescence Measurements. Fluorescence was measured in a temperature-controlled room (20 ± 1 °C) with a SLM 4800C (Aminco) spectrofluorometer equipped with a variable angle front-surface accessory set to 56° to lower reflected light, scattered radiation, and depolarization phenomena. The slit widths were 4 nm in both excitation and emission pathways. Samples were put in open quartz cells having a 1 mm optical path length. The fluorescence emission spectra (290−380 nm; resolution, 1 nm; averaging, 10) were recorded with the excitation wavelength set at 280 nm. All spectra were corrected for instrumental distortions in emission and excitation and smoothed when necessary.

Fluorescence intensity decreased by 1%/min for emulsions and creams and 0.2%/min for BSA solutions and serums when the samples were exposed to 280 nm of UV light. Therefore, each spectrum was performed in duplicate or triplicate with new aliquots.

Variation coefficients were about 2% for BSA solutions, creams, serums, or concentrated emulsions (50%, v/v). They increased to 6% for dilute emulsions (16%, v/v), probably because these emulsions creamed in the cell during measurement.

Determination of Droplet-Size Distribution. A separate set of concentrated (50%, v/v) emulsions prepared with 0.6−11 g of BSA/L and corresponding creams and serums separated by centrifugation had to be prepared to determine changes of droplet-size distribution as a function of protein concentration since available quantities were too low to allow both fluorescence measurements and determination of droplet-size distribution on the same samples. Droplet-size distribution was measured with a MALVERN MasterSizer 3600 apparatus, and volume/surface diameters and specific surface area were determined.

Calculations. Equation 1 was used to evaluate the proportions of adsorbed BSA in emulsions, creams, and serums prepared from concentrated 50% (set 1) and dilute 16% (set 2) emulsions. Next, the total concentrations of adsorbed and nonadsorbed protein in emulsions were easily obtained with C_t. Then, protein concentrations in creams and serums (C_{cr} and C_{se}) were calculated from protein, oil, and volume balances between emulsion, cream, and serum (equations in Figure 1), proportions of adsorbed BSA in each phase, and volume fractions (ϕ_v = 0.5 or 0.16) in emulsions and creams. It was assumed that the oil volume in serums can be neglected. Volumes of creams prepared from concentrated 50% (set 1) emulsions did not significantly change as a function of protein in emulsion resulting in $\phi_{cr} = \phi_{vmax} = 0.74$, corresponding to previous results on similar systems (Le Bihan, 1991). Finally, concentrations of adsorbed and nonadsorbed protein in each phase were calculated.

RESULTS AND DISCUSSION

The wavelength (λ_{max}) and the intensity (I_{max}) of fluorescence emission maximum of 50% (v/v) emulsions and BSA solutions are presented in Figure 2 as a function of protein concentration in the systems. The maximum emission of BSA in 0.1 M phosphate buffer was located at 334.9 ± 0.7 nm whatever the BSA concentration in the range studied. Fluorescence intensity first increased linearly with BSA concentration and then leveled off for a BSA concentration greater than 12 g/L. When BSA solutions were vigorously stirred with a shear agitator under conditions similar to that used for emulsification, their λ_{max} and I_{max} did not change at all. Nondefatted BSA was used in these experiments. Since residual fatty acids, pH, and ionic strength can interfere with BSA fluorescence properties, the emission spectra of defatted BSA with *n*-pentane in phosphate buffer and in purified water were recorded and compared to the spectrum of nondefatted BSA in phosphate buffer (data not shown). All spectra were similar in shape and intensity indicating that residual fatty acids and phosphate buffer did not modify the environment of BSA aromatic amino acids. Likewise, the fluorescence emission spectra of serum albumin were found unaltered upon defatting BSA by charcoal treatment (Chen, 1967).

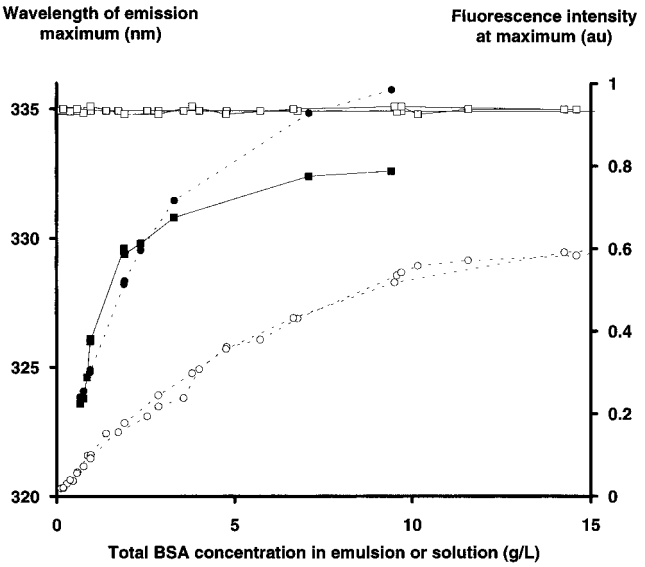

Figure 2. Wavelength of emission maximum (squares) and fluorescence intensity at maximum (circles) as a function of BSA concentration, for 50% emulsions (filled symbols) and BSA solutions (open symbols); temperature, 20 °C. Samples were studied 24 h after preparation; λ_{exc} = 280 nm. See the Experimental Section for fluorescence conditions.

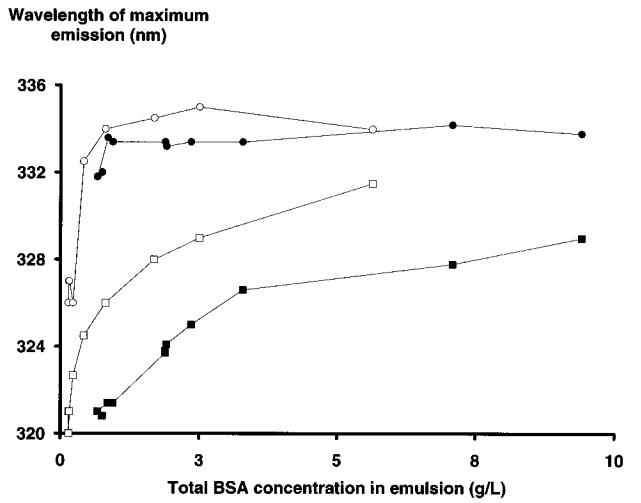

Figure 3. Wavelength of emission maximum as a function of BSA concentration in the emulsions for creams (squares) and serums (circles). Filled symbols represent creams and serums separated by centrifugation from concentrated 50% emulsions (set 1); open symbols represent creams and serums separated by creaming from 16% emulsions obtained by dilution with buffer of concentrated 50% emulsions (set 2); temperature, 20 °C. Samples were studied 24 h after preparation; λ_{exc} = 280 nm. See the Experimental Section for fluorescence conditions.

The emission spectra of BSA in 50% emulsions were shifted toward shorter wavelengths compared to those of BSA solutions; the maximum blue shift, about 12 nm, was observed for the lowest concentrations of BSA in emulsions (0.65 g / L). The shape of the I_{max}−BSA concentration curve was more or less similar to that of BSA solutions, but BSA in emulsions exhibited an enhanced fluorescence intensity when compared to BSA solutions at the same concentration.

The results obtained for creams and serums prepared by centrifugation from concentrated emulsions (set 1) or by creaming from diluted emulsions (set 2) are presented in Figure 3 as a function of BSA concentration in the corresponding emulsions. Blue shifts of emission spectra, as compared to BSA solutions, were also observed for creams and serums. For a given emulsion, the shift increased in the order serum < emulsion < cream. The blue shift was maximal (15 nm) for creams obtained from emulsions prepared with the lowest BSA concentrations. Fluorescence intensity in creams and serums increased with BSA concentration in corresponding emulsions (data not shown). As previously observed (Castelain and Genot, 1994), no fluorescence was detected in serums prepared from emulsions containing less than 0.5 g of BSA/L. Simultaneously, the minimal BSA concentration in 50% emulsion required for a stable emulsion to be prepared with the present procedure was observed to be about 0.5 g/L. At this concentration, the cream contains all the available protein, since no fluorescence was detected in serum phase of such emulsion, and the BSA concentrations in serum and cream can then be easily calculated as $C_{se} = 0$; $C_{cr} = 0.76$ g of BSA/L. When the BSA concentration is lower, the interfacial area created by mechanical energy input during emulsification process cannot be completely covered by the stabilizer, and consequently, the dodecane droplets are larger and hence coalesce. Thus, 50% emulsions prepared with BSA concentrations lower than 0.5 g of BSA/L were very unstable, which makes it impossible to obtain any reliable fluorescence measurement in these conditions. When concentration exceeds 0.5 g of BSA/L, fluorescence can be detected in serum phase and λ_{max} measured on creams and emulsions moves progressively from the value which is observed for 0.5 g of BSA/L emulsion to higher values (Figures 2 and 3). λ_{max} of the serum also shifted, very sharply, toward higher wavelengths, the highest value being reached for about 2 g of BSA/L emulsion.

BSA concentrations cannot be estimated from front-face fluorescence intensities for two reasons. First, in front-face geometry, fluorescence intensity is not proportional to the fluorophore concentration but to the ratio fluorophore absorbance to total absorbance (Genot *et al.*, 1992). Second, the contributions of adsorbed and nonadsorbed protein to steady-state fluorescence intensity cannot be dissociated to quantify the adsorbed and nonadsorbed protein proportions.

λ_{max} measurements, eq 1, and oil and protein balances were then used to calculate the partition of BSA between cream and serum. Figure 4 shows the partition of BSA between cream and serum as a function of BSA concentration in emulsion in the case of concentrated 50% emulsions. BSA concentrations in cream and serum increase with BSA concentration in emulsion, but the increase is markedly steeper in serum than in cream. A possible interpretation is that once the oil droplets are covered by adsorbed protein the major part of additional protein is not adsorbed and it is found in the serum phase. The nonadsorbed and adsorbed BSA concentrations in concentrated emulsions and in corresponding creams and serums as a function of the actual protein concentration in the sample were calculated, and they are shown in Figures 5 and 6, respectively. Both adsorbed and nonadsorbed BSA concentrations increase with BSA concentration in emulsion, cream, or serum, and in each phase nonadsorbed BSA increases more rapidly than adsorbed protein. However, as cream volumes are similar in a set of experiments, the continuous increase of adsorbed BSA concentration proves that a further adsorption of proteins takes place even when the oil droplets were covered by the emulsifier.

Since the surface created during emulsification affects the extent of adsorption of the protein and the partition

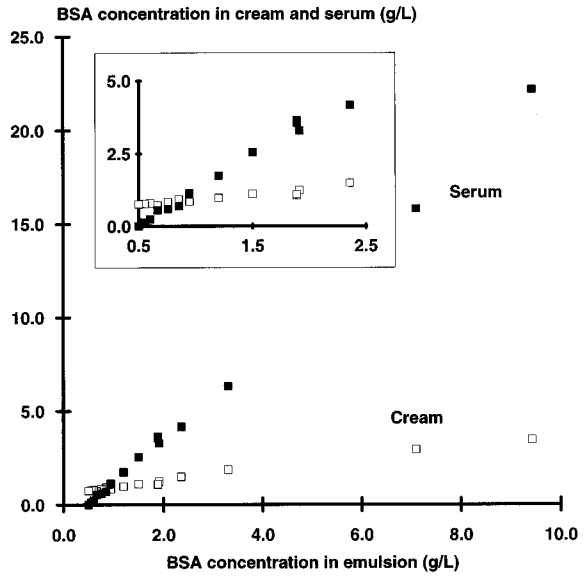

Figure 4. BSA concentrations in cream and serum as a function of total BSA concentration in 50% emulsion. Creams and serums were separated by centrifugation (2000*g* for 30 min).

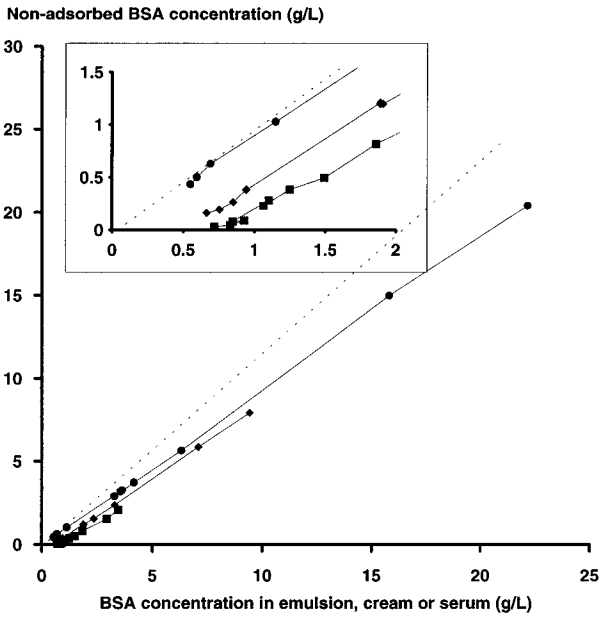

Figure 5. Nonadsorbed BSA concentrations in (◆) emulsion, (■) cream, and (●) serum. Creams and serums were separated by centrifugation (2000*g* for 30 min) from 50% emulsions.

between the cream and serum phases, the droplet-size distribution was measured on a separated set of 50% emulsions and on corresponding creams and serums. Specific surface area was calculated as a function of protein concentration in the emulsion (Figure 7). The more available protein during emulsification, the larger the surface is stabilized, that is, smaller droplets are created and the emulsifying process is not the limiting factor. This result correlates with the increase of the adsorbed BSA concentration (Figure 6) as the newly created surface is immediately covered by available protein. Such results could be used to calculate the protein surface coverage in the emulsions, creams, and serums, but separate sets of emulsions were used to determine fluorescence data and droplet-size distribution in this work.

The fact that in serums λ_{max} values differ from 335 nm (Figure 3) suggests that some oil droplets covered

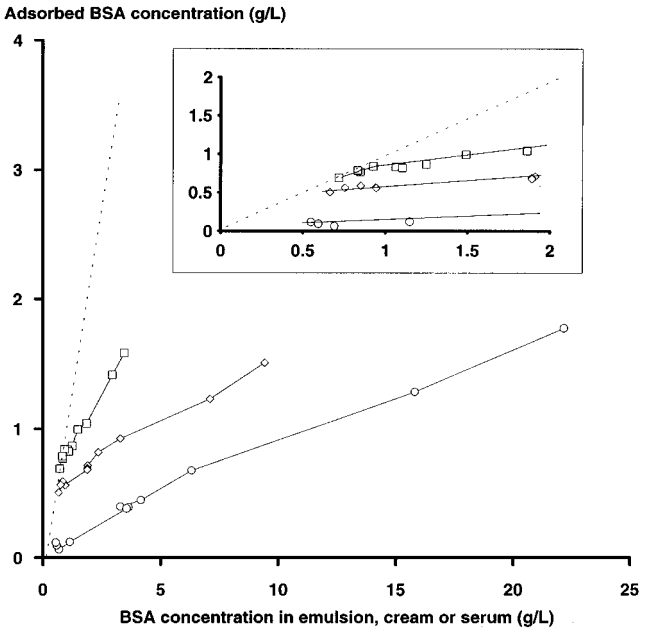

Figure 6. Adsorbed BSA concentrations in (◇) emulsion, (□) cream, and (○) serum. Creams and serums were separated by centrifugation (2000*g* for 30 min) from 50% emulsions.

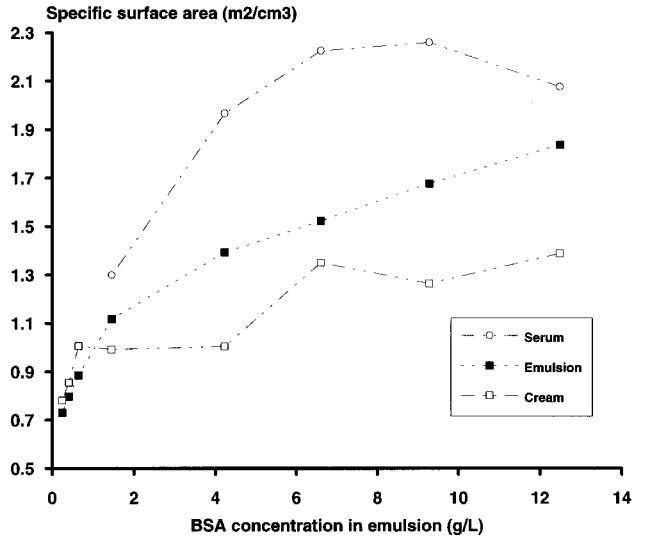

Figure 7. Specific surface area calculated from droplet-size distribution in emulsion, cream, and serum as a function of protein concentration. Creams and serums were separated by centrifugation (2000*g* for 30 min) from 50% emulsions.

with adsorbed proteins are present in serums. The adsorbed protein concentration in the serum reached 1.8 g of BSA/L when it was prepared from 50% emulsions containing 9.5 g of BSA/L (Figure 6). This concentration can hardly be neglected if compared to the total protein concentration in this serum phase (about 22 g of BSA/L). The presence of oil droplets in the serums was also evidenced by assaying for dodecane by GLC (data not shown) and determining size-droplet distribution and specific surface area (Figure 7). This figure also shows that upon centrifugation of an emulsion, the smallest droplets more quantitatively remained in the serum phase whereas the largest ones gathered in the cream phase. The evaluation of adsorbed protein concentration in an emulsion from the difference between the total protein concentration in emulsion and the concentration in serum phase considers that the nonadsorbed protein concentration is equal to the protein concentration in serum. This estimation

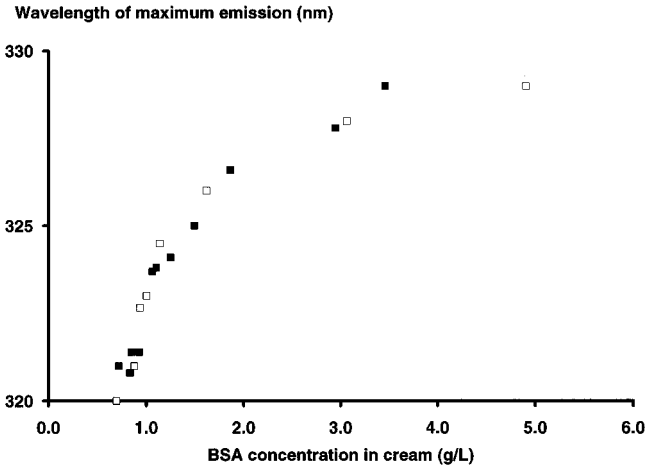

Figure 8. Wavelength of emission maximum as a function of BSA concentration in (■) creams separated by centrifugation from concentrated 50% emulsions (set 1) and (□) creams separated by creaming from 16% emulsions obtained by dilution with buffer of concentrated 50% emulsions (set 2).

is erroneous particularly at very low concentrations for which the decrease of λ_{max} values in the serum (Figure 3) corresponds to an increased adsorbed/total protein ratio.

Equation 1 and the subsequent calculations of protein concentrations are based on the hypothesis that no desorption or further adsorption of the protein takes place during the experiment (Castelain and Genot, 1994), *i.e.*, during dilution procedure, storage, creaming, or centrifugation. Such an assumption will not be valid if another surface-active molecule was present in the emulsion. Adsorption studies at solid−liquid interfaces have shown that the concept of reversible or irreversible adsorption depends on the means used to perturb the adsorbed protein. Proteins that are seemingly undesorbable by dilution become desorbable in the following cases: (i) when the pH is changed, (ii) when low molecular weight substances, including electrolytes, detergents or surfactants, are present, and (iii) when the absorbed proteins are exchanged for other dissolved proteins (Norde *et al.*, 1986; Bohnert *et al.*, 1986). On surfaces exposed to blood plasma, a complex hierarchy of adsorbed proteins was observed depending on time and surface composition, called the Vroman effect (Andrade and Hlady, 1991). Similarly, in emulsions containing milk proteins, proteins adsorbed at the oil−water interface can be exchanged for other proteins or surfactants (de Feijter *et al.*, 1987; Dickinson, 1991). Thus, the assumption that no further adsorption or desorption occurred storage or during the dilution procedure was tested in two ways. First, emission maxima of two concentrated emulsions did not change when measured just after emulsification and regularly over the next 7 h at 20 °C. Thus the adsorption of BSA in emulsion seems to be a very rapid phenomenon which occurs during the emulsification step as opposed to steady-state adsorption of proteins on planar interfaces where tensiometric measurements suggest modifications for many hours (Graham and Phillips, 1979; Le Bihan, 1991). Second, when the wavelength of emission maximum of the creams of the two sets was plotted as a function of actual protein concentration in the phase (Figure 8), it appeared that this wavelength did not depend on the volume fraction of the emulsion from which the cream develops but only on the actual BSA concentration in the cream. Protein partition between adsorbed and nonadsorbed BSA in the cream was similar when cream was obtained by centrifugation (2000*g* for 30 min) or by creaming under the force of gravity after dilution of the emulsion. Therefore, we estimated that no modification in the states of BSA adsorption occurred during our experimental procedure.

We present here a direct method to quantify adsorbed proteins in a complex system. The ratio of adsorbed to total protein as well as protein concentrations can be estimated in emulsions, creams, and serums from λ_{max}, with prior determination of λ_{max} of the adsorbed protein.

The accuracy of the determination of the protein partition with the present method depends on two main parameters: the difference between the emission maximum wavelengths of the nonadsorbed and adsorbed states of the protein and the accuracy of λ_{max} determination. This difference is a function of the studied protein, that is, of its fluorescence properties, which depend on the location of tryptophanyl residues in adsorbed and nonadsorbed states of the protein, and the characteristics of the phases. The accuracy of λ_{max} determination is related to the stability of the samples during measurements and the fluorescence experimental conditions which have to be optimized.

Finally, the reliability of this method depends on the purity of components to avoid interferences with protein fluorescence. For example, some components such as pigments and carotenoids, which are found in edible commercial oils, also absorb and fluoresce and therefore can interfere with protein fluorescent properties. With these limitations, this method can be used along with the determination of droplet-size distribution to investigate interfacial concentration isotherms of proteins in model emulsions.

ABBREVIATIONS USED

BSA, bovine serum albumin; I_{max}, intensity of emission maximum; λ_{max}, wavelength of emission maximum; ϕ, oil volume fraction.

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