matrix-matching is achieved, leads to the same results as the classical calibration curve method. This is true for all elements. This is in agreement with the measurements of Olejko (17) who stated that, regardless of oil type, no matrix-matching is necessary.

Generally, background stability is better for organic matrices. The apparatus works at the upper detectable limit for the determination of Fe and Zn by the char-ashing method. Less sensitive spectral lines for Fe (371.9 nm) and Zn (307.7 nm), in contrast to those for Na (330.3 nm) and K (404.4 nm), give unstable measuring results. By using the dilution method for the analysis of Cu and Ni, one reaches the lowest detection level.

Concentrating is excluded by using this analyzing technique. The 1% absorption sensitivity for Zn is considerably superior to that for Cu and Ni. A volatilization error of Zn is possible by using improper temperature programming of the carbon rod. The higher level for sodium by char ashing is attributed to contamination during char ashing or atomizing (7).

Relative standard deviations are more correlated to an element than to a method. This implies that metal content determinations by atomic absorptions spectrometry strongly depend on the relative sensitivity for each element with respect to absorption measurements.

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Study of Triglyceride-Protein Interaction Using a Microemulsion-Filtration Method[1]

LLOYD M. SMITH[a], PAOLO FANTOZZI[b,2] and RICHARD K. CREVELING[a],
Department[a] of Food Science and Technology, University of California, Davis, CA 95616, and Istituto[b] di Industrie Agrarie, Dipartimento di Scienze e Tecnologie Alimentari e della Nutrizione, Università degli Studi di Perugia, 06100 Perugia, Italy

ABSTRACT

Interactions between simple triglycerides and a variety of native and chemically modified proteins were studied. A new procedure was developed to evaluate hydrophobic binding of proteins at oil/water interfaces. The method involves emulsifying a triglyceride ultrasonically in water and equilibrating the microemulsion with a protein solution. The protein adsorbs to the surfaces of the globules as a stabilizing interfacial layer. Relative stability of different emulsions is evaluated under standard conditions of partition with a polycarbonate membrane filter. Either gas liquid chromatography or a radioactive technique is used to determine the amount of triglyceride stabilized by protein through hydrophobic binding. The procedure was used to compare interactions of six different triglycerides with bovine serum albumin (BSA) and interactions between trioctanoin and 26 different native and chemically modified proteins. In general, an increase in molecular weight of saturated triglycerides or an increase in double bonds of unsaturated triglycerides reduced hydrophobic binding. The order of binding of trioctanoin for the series of proteins (on a weight basis) compared to BSA taken as 100%, ranged from 160% for casein to 1% for lysozyme. Addition of nonpolar residues, including methyl, cyclohexyl, cyclopentyl and benzyl groups, to casein increased binding by 20-30%. Effects of protein mixtures, and changes in pH and ionic strength also were studied. The results demonstrate that interactions between a wide variety of triglycerides and proteins can be investigated by the microemulsion-filtration method.

INTRODUCTION

Interactions between lipids and proteins represent a major field of biological and technological interest (1). Different types of intermolecular forces may be involved: (a) covalent binding, (b) electrostatic binding, (c) polarization interaction, (d) dispersion interaction, and (e) hydrophobic binding (2,3). Hydrophobic lipid-protein interactions in aqueous systems are of special importance for the stability, conformation and function of biological macromolecules. However, hydrophobic interactions are difficult to study

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[2]Current address: Istituto di Tecnologie Alimentari, Università degli Studi di Udine, 03100 Udine, Italy.

because of the low reactivity of nonpolar groups and consequent difficulties in determining reactions involving such groups (4).

Various procedures have been proposed to evaluate hydrophobic interactions of proteins with other molecules. Feinstein and Feeney (5) employed colored dyes which show a perturbation of their absorption spectra upon interaction with the protein. Kato and Nakai (6) determined protein hydrophobicity using *cis*-parinaric acid as a fluorescence probe. Trumbetas et al. (7) demonstrated that pulsed nuclear magnetic resonance (NMR) measurements can be used to determine the degree of lipid-protein interaction in freeze-dried emulsions. Spector et al. (8) used a two-phase partition method to study binding of long-chain fatty acids to bovine serum albumin (BSA) and other proteins. Damodaran and Kinsella (9) employed a similar liquid-liquid partiation equilibrium method to investigate hydrophobic binding of carbonyls to BSA. Mohammadzadeh-K et al. (10) introduced a procedure that depends upon the direct determination of the amount of hydrophobic substances absorbed or dissolved in the protein solution by a gas liquid chromatographic (GLC) technique. This procedure was employed to study the relationships of hydrocarbon structure (11) and protein structure in hydrophobic binding (12). The hydrocarbons used included alkanes, aromatic compounds, alicyclic compounds and methyl esters of fatty acids. These hydrocarbons were comparatively small molecules which could be expected to fit into hydrophobic crevices of proteins.

Triglycerides as a lipid class are highly apolar and when dispersed in water form either oil-in-water (O/W) or water-in-oil (W/O) emulsions. If proteins in aqueous solution are present, they may be diffused to the O/W interface, be adsorbed and unfolded to minimize hydrophobic free energy and stabilize the emulsion. The objective of the present study was to develop a procedure to assess hydrophobic interactions between triglyceride and protein surfaces. Each triglyceride type was first emulsified in water by sonication and then equilibrated with a protein solution allowing the protein molecules to adsorb to the surfaces of the much larger triglyceride globules as a stabilizing interfacial layer. Relative stability of each O/W emulsion was determined under standard conditions of partition with a polycarbonate membrane filter.

The method was used to evaluate interaction of a series of saturated and unsaturated simple triglycerides with BSA, which was selected as an example of a protein with appreciable hydrophobic properties. GLC was employed as the analytical procedure. Interactions were also studied between trioctanoin, a triglyceride with saturated fatty acid moieties that are nonpolar in nature, and a variety of native and chemically modified proteins of biological and technological interest. ^{14}C-Labeled trioctanoin was used in this phase of the research.

EXPERIMENTAL PROCEDURES

(A) Effect of Triglyceride Structure on Hydrophobic Interaction

Materials

Triglycerides used were: tributyrin, trihexanoin, trioctanoin, trinonanoin, tripalmitolein, triolein, trilinolein, trilinolenin (Sigma Chemical Co., London, 99%). BSA Fraction V was 96-99% pure. All other reagents were analytical grade from the same supplier.

Polycarbonate membrane filters (25 mm diameter) and filter holders were purchased from Nuclepore Corporation, Pleasanton, CA. Monojet disposable syringes were obtained from Sherwood Medical Industries, Deland, FL.

Method

Emulsion preparation. Each triglyceride suspension was obtained by emulsifying 0.25 g triglyceride in 50 mL distilled water with an Ultra Turrax TP 18/2 blender (Ika-Werk, Staufen, Germany) for 30 sec at a setting of 5. The suspension was sonicated with a Sonifier Cell Disrupter A180G equipped with a 1.27-cm diameter tip (Ultrasonics, Ltd., PBI, Italy). The sonifier was preheated by operating for 5 min in 50 mL water. The settings of the power and tune switches were at 8 and 2, respectively. Each suspension was sonicated for four periods of 30 sec duration separated by 30-sec intervals. The resulting microemulsion was passed through a No. 4 Whatman filter, discarding the last few milliliters along with the filter that retained nonemulsified triglyceride.

Equilibration and filtration of protein-triglyceride mixture. The buffer used to prepare 0.1% (w/v) BSA solution was 0.1 M sodium phosphate, pH 6.8. One volume of emulsion was mixed gently with six volumes of protein-buffer solution (sample) or with six volumes of buffer (blank). The blank was used to check the solubility of different triglycerides in buffer alone.

Following a 60-min quiescent equilibration at 20-22 C, 14 mL of the sample mixture or the blank was drawn into a 15-mL syringe. The filter holder with the "O" ring on the syringe side of the 0.4 μm pore size polycarbonate membrane filter was attached to the syringe. The first 3 mL of filtrate were discarded and the next 10 mL were collected for subsequent extraction. A 10-mL aliquot of unfiltered sample mixture was set aside as a control.

Extraction of triglycerides. Sample, control and buffer blank were heated with 15 mL of a mixture of ethanol-N HCl (15:1, v/v) in 50-mL capped tubes for 5 min at 60 C. Fifteen mL of hexane were added and the tubes were then shaken for 30 min in a mechanical shaker. Following phase separation, 10 mL of upper phase were removed and dried under nitrogen. Each residue was dissolved in 0.5 mL of hexane containing 1 mg/mL of tributyrin as internal standard (IS) for GLC.

Evaluation of triglyceride-protein interaction. Triglyceride-protein interaction or binding is considered to be related to the amount of triglyceride present before (control) and after (sample) passage of 10 g of protein-triglyceride mixture through the 0.4 μm Nuclepore filter. Percent binding is defined as the percentage of triglyceride in the sample compared to the unfiltered control.

The amounts of triglyceride were determined by GLC. The instrument used was a Varian Aerograph 3700 (Varian Associates, Palo Alto, CA) equipped with a flame ionization detector, a 67-cm glass column 3.2-mm id, packed with 3% OVl on Gas Chrom 100/120 mesh (Supelco, Inc., Bellefonte, PA). Conditions were: on-column injection at 350 C; helium flow rate, 100 mL/min; detector temperature, 350 C. For low molecular weight triglycerides, column

TABLE I

Relative Response Factors (RRF) of Triglycerides in Gas Liquid Chromatographic Analysis

Triglyceride	RRF[a]
Trihexanoin	0.90 ± 0.06
Trioctanoin	1.09 ± 0.10
Trinonanoin	1.60 ± 0.21
Tripalmitolein	1.03 ± 0.09
Triolein	1.07 ± 0.08
Trilinolein	2.22 ± 0.21
Trilinolenin	2.21 ± 0.23

[a]Mean ± SD of 8-14 replicate determinations. See text for calculation of RRF.

temperature was 120 C programmed to 250 C at 10 C/min. High molecular weight triglycerides (tripalmitolein and higher) were determined isothermally at 340 C. Four or more injections of ca. 2 μL were made and the results were averaged for each analysis.

To obtain quantitative data for each triglyceride, a series of standard solutions was prepared with different concentrations of that triglyceride and a known amount of tributyrin as internal standard. A relative response factor (RRF) for each triglyceride (TG) was determined as follows:

$$RRF = \frac{\text{area IS}}{\text{area TG}} \times \frac{\text{weight TG}}{\text{weight IS}}$$

The weight in mg of each triglyceride was calculated:

$$\text{TG weight (mg)} = \frac{\text{area TG}}{\text{area IS}} \times \text{weight IS} \times RRF$$

RRF values for the triglycerides used in this study and their reproducibility are shown in Table I.

(B) Effect of Protein Structure on Hydrophobic Interaction

Materials

The triglyceride used was trioctanoin (Eastman Organic Chemicals, Rochester, NY) mixed with labeled [carboxy-^{14}C]-trioctanoin (ICN Chemical and Radioisotope Division, Irvine, CA) to give 11,356 cpm/mg. Aquasol scintillation fluid was obtained from New England Nuclear, Boston, MA.

BSA fraction V, ribonuclease A (bovine pancreas) type III-A, and lysozyme (crystallized 3 times) were purchased from Sigma Chemical Company, St. Louis, MO. Chymotrypsinogen A (crystallized 5 times) and α-chymotrypsin (crystallized twice) were from Worthington Biochemical Corporation, Freehold, NJ. Vitamin-free casein was purchased from Nutritional Biochemicals Corporation, Cleveland, OH. Cytochrome C (horse heart) was obtained from Boehringer and Soehne, Indianapolis, IN.

The following proteins were isolated and purified in the laboratory of R.E. Feeney, Dept. of Food Science and Technology, University of California, Davis, CA: chicken ovotransferrin and ovalbumin; chicken, penguin and turkey ovomucoids; fish serum "antifreeze" proteins; human albumin; α-amylase; β-amylase; γ-globulins; pepsin; and β-lactoglobulin. The following casein derivatives were synthesized and purified in the same laboratory: methyl casein, cyclopentyl casein, cyclohexyl casein and benzyl casein.

Method

Emulsion preparation and equilibration of protein-triglyceride mixture. These steps were conducted essentially as described above, but with a Virtis "23" blender (Virtis Research Equipment, Gardiner, NY) and a Branson Sonifier Cell Disrupter Model W-185E equipped with a 1.27-cm diameter tip (Branson Sonic Power Co., Danbury, CT). The trioctanoin emulsion consisted of 75 mg of the triglyceride and 15 mL of water blended for 30 sec at maximum speed in a 30-mL Virtis cup. This suspension was sonicated for three periods of 15 sec duration separated by 30-sec intervals. The tip was immersed halfway into the suspension and the Sonifier was operated at a setting of 4, which provided 75 watts of power at the tip. After filtration of the emulsion through the No. 4 Whatman filter, one volume of the microemulsion was gently mixed with six volumes of protein-buffer solution.

Evaluation of triglyceride-protein interaction. The amounts of trioctanoin bound by different proteins were determined after 60 min of quiescent equilibration at 22-24 C. Following equilibration, 9 mL of the mixture were drawn into a syringe and two aliquots of ca. 0.4000 g each were weighed into tared scintillation vials. These served as unfiltered controls. The syringe was attached to a filter holder containing a 0.4 μm pore size membrane filter and 3.0 g of the emulsion was passed through the filter and discarded. Then three filtrate aliquots of ca. 0.4000 g each were weighed into scintillation vials. To each vial, 1.0 mL of water followed by 10.0 mL of Aquasol was added. Each vial was counted in a Beckman CPM-100 Scintillation Counter using a window that was sensitive to ^{14}C emissions.

Interaction results were calculated as mg/g of trioctanoin in the filtrate (sample). Percent binding was expressed as percentage of trioctanoin in the sample as compared to trioctanoin in the unfiltered control.

RESULTS AND DISCUSSION

Development of the Method

Our procedure to study triglyceride-protein interaction depends upon the ability of a protein in aqueous solution to diffuse to an O/W interface of a microemulsion, be adsorbed chiefly by hydrophobic binding and unfold to stabilize the emulsion system. Protein molecules are believed to be adsorbed or bound to the triglyceride globules by van der Waals forces between hydrophobic amino acid residues and fatty acids of the surface triglyceride molecules (13). The greater the interaction between the protein and the triglyceride, the greater should be the physical stability of the emulsion globules or particles. This stability can then be evaluated by measuring the amount of triglyceride particles that have sufficient integrity to pass through a membrane filter.

Microemulsion Preparation

Ideally, equilibration of triglycerides with protein solutions should provide optimum contact between these two molecular species without protein denaturation occurring prior to contact. However, the triglycerides as a class are the most chemically inactive and apolar of the lipids and are not soluble in water. Chapman (2) reported that sonication of lecithin dispersions breaks up the aggregates to produce

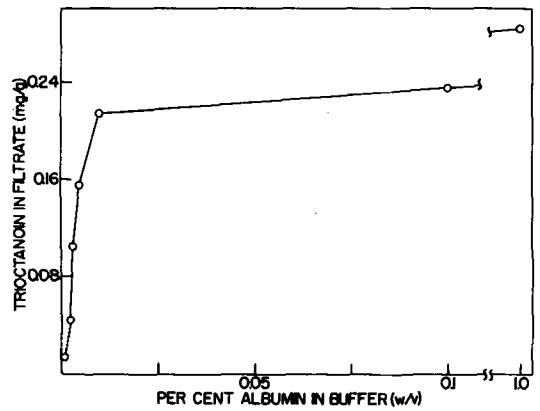

FIG. 1. Effect of concentration of bovine serum albumin (BSA) on binding of trioctanoin. BSA solutions were in 0.1 M sodium phosphate, pH 6.8. Experimental conditions are given in the text.

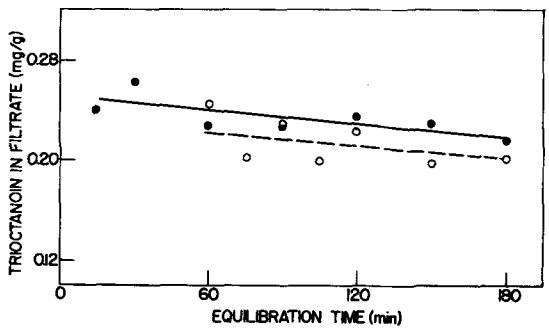

FIG. 2. Effect of equilibration time and temperature on binding of trioctanoin by 0.1% (w/v) BSA in 0.1 M sodium phosphate, pH 6.8. Temperatures were 22 C (solid circles) and 30 C (open circles).

small particles with diameters less than 1 μm. Phospholipases, which hardly attack coarse lecithin dispersions, readily hydrolyze sonicated lecithins. In the present study, sonication of triglycerides in water gave microemulsions with very small globules (<1 μm in diameter), enhancing the opportunity for triglyceride-protein interaction. To avoid protein denaturation as a result of sonication, the protein-buffer solutions were added to freshly sonicated triglyceride microemulsions.

Preliminary experiments established sonication conditions necessary to prepare emulsions containing reasonably reproducible concentrations of triglycerides. For example, 7 emulsions prepared as in Method of section A, were analyzed for trioctanoin by GLC. For 5-mL aliquots of emulsion, the mean was 13.5 mg with SD of 1.55 mg.

Equilibration Conditions

Concentration of protein. Figure 1 shows the effect of BSA concentration on amount of trioctanoin bound. The curve indicates that a plateau was reached essentially with concentrations between 0.01% and 1.0% BSA in the aqueous buffer. In the method adopted, the concentration of protein in the buffer was 0.1% (w/v) prior to equilibration with trioctanoin microemulsion. This amount assured that there was no depletion of protein from the protein-buffer solution.

Time and temperature. Figure 2 illustrates the influence of equilibration time at two temperatures on binding of trioctanoin by BSA. The data show that an increase in time from 60 min to 180 min had no marked effect. Slightly more trioctanoin was bound at 22 C than at 30 C. For convenience, we chose a 60-min equilibration time at 22 C for the method.

TABLE II

Relative Stability of Trioctanoin Emulsions Determined by Membrane Filtration and by Centrifugation Methods

Protein in buffer (%)[a]	Relative binding[b] Filtration[a] (%)	Centrifugation[c] (%)
0.100 Casein	160	115
0.100 β-Lactoglobulin	130	104
0.100 BSA	100	100
0.050 BSA	–	97
0.010 BSA	91	50
0.005 BSA	66	28
0.001 BSA	6	8
0.100 Lysozyme	1	3

[a]Equilibration and membrane filtration details in text.
[b]Binding is relative to bovine serum albumin taken as 100%.
[c]Following equilibration, emulsions were centrifuged at 29,000 RCF for 30 min at 22 C. Concentrations of trioctanoin in subnatant were determined by GLC.

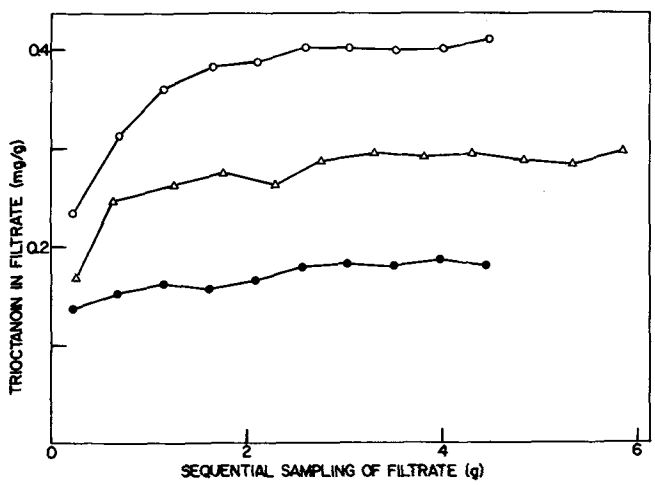

FIG. 3. Effect of sequential sampling of filtrate (after passage through Nuclepore membrane filter) on trioctanoin recovery in the filtrate. Filter pore sizes were 0.2 μm (solid circles), 0.4 μm (triangles) and 0.8 μm (open circles).

Nuclepore Filtration Step

In general, the greater the interaction of the protein with the triglyceride, the greater should be the physical stability of the emulsion. Our preliminary experiments established that triglyceride globules formed by sonication could be filtered through selected Nuclepore membranes readily only when a protein was associated with these emulsion droplets. Furthermore, comparison with a centrifugation method indicated that the relative binding and stabilities of different emulsion preparations were in essential agreement with results obtained by the membrane filtration method (Table II).

The effect of different filter pore sizes is shown in Figure 3. A series of ca. 0.5-g successive aliquots was col-

TABLE III

Interaction between Triglycerides and Bovine Serum Albumin (BSA)

Triglyceride	Molecular weight	Control[a]	Sample[a]	Binding (%)[b]
Trihexanoin	386	3.17	2.47	78
Trioctanoin	471	2.56	1.47	57
Trinonanoin	513	5.10	3.97	78
Tripalmitolein	801	0.59	0.29	49
Triolein	885	0.16	0.09	56
Trilinolein	879	0.49	0.23	47
Trilinolenin	873	0.13	0.05	38

[a]Results are expressed as mg of triglyceride in 10 g of emulsion as determined by GLC. Triglycerides were extracted from equilibrated mixture of emulsified triglyceride and BSA solution before (control) and after (sample) Nuclepore filtration.

[b]Percent binding is the percentage of triglyceride in the sample compared to control.

lected during filtration of trioctanoin microemulsion equilibrated with 0.1% BSA in buffer. Results for pore sizes ranging from 0.2 to 0.8 μm are given. A constant amount of trioctanoin was passed through each filter after ca. 2 mL (or g) filtrate had been collected. Therefore, in the method adopted, the first 3 g of filtrate were discarded before collecting each sample. The 0.4 μm pore size selected for the method allowed about one-half of the BSA-equilibrated trioctanoin to pass through under reasonable force, thus giving a convenient measure of relative emulsion stability.

Extraction Step

To test the efficiency of the solvent extraction step used in section A above, a series of seven trioctanoin microemulsions was extracted. Duplicate extracts were analyzed for trioctanoin by GLC. The amounts ranged from 10.9 to 13.3 mg in a 5.0-mL volume. The mean was 12.2 mg with an SD of 1.0 mg. These results and those for emulsion preparation (see above) were examined by the two-tailed dependent t-distribution ($t = 2.06$, 6 df, $P_{(f)} < 0.01$). Therefore the differences between the two series of results were not significant and the triglyceride recovery by extraction was 91% efficient.

Determination of Microemulsion Stability

All emulsions are considered unstable in a thermodynamic sense. Cante et al. (14) have reviewed several techniques to determine relative stability of emulsions. Accelerated destabilization methods such as centrifugation are commonly employed. The Nuclepore membrane filtration step used in our procedure can provide a convenient alternative. The actual mechanism of filtration has not been elucidated, but passage of microemulsion particles through the 0.4 μm membrane filter probably is affected by physical dimensions of the pores, as well as by charge effects. Electron microscopy showed that the pores in the membrane are round cylinders of uniform diameter. They are normal to the surface and evenly distributed over the surface. Our results are interpreted to indicate that more stable triglyceride emulsions pass through the filter more readily than the less stable emulsions.

Interaction of Different Triglycerides with BSA

Table III is a comparison of the binding of selected saturated, monounsaturated and polyunsaturated triglycerides. Selection of triglycerides was limited to simple triglycerides liquid at 22 C. Tributyrin was not used because of its slight solubility in aqueous solutions. The other triglycerides were not soluble in buffer alone, as confirmed by the blank determinations. The control column data reflect differences in the degree of emulsification of the various triglycerides by sonication, although reproducible emulsions were obtained for each individual triglyceride (see Emulsion Preparation). It is also possible that variations in the degree of triglyceride-protein interaction during the 60-min equilibration could influence the amount of triglyceride found in the controls. The data in the sample column represent the amount of each triglyceride sufficiently stabilized by interaction with BSA to pass through the membrane filter.

The binding (%) column presents a comparison of the relative binding of different triglycerides by BSA. In general, an increase in molecular weight of saturated triglycerides (trihexanoin to trioctanoin) or an increase in double bonds of unsaturated triglycerides (triolein to trilinolenin) reduced the percentage of interaction. The results for trinonanoin suggest that further research is needed to explore relative effects of odd-carbon chain fatty acids on binding of triglycerides.

Despite the importance of triglycerides, very few reports have dealt with their interaction with proteins because it has proven difficult to demonstrate (1). Phillips (13) has recently reviewed the adsorptive behavior of three types of proteins, including BSA, in stabilizing O/W emulsions. He postulated adsorbed films containing "trains" of amino acids in contact with the globule surface with loops and tails of residues protruding into either the triglyceride or aqueous bulk phases. Hydrophobic surface residues on proteins are deemed to interact with hydrophobic acyl chains of triglycerides at the surface of the triglyceride particles. Unfolding of protein molecules at the surface thus enhances exposure of hydrophobic groups and consequent interaction or binding. This results in lowering the interfacial tension and increasing physical stability of the emulsion particles.

Shanbhag and Axelsson (15) described a method for measuring hydrophobic interactions between aliphatic hydrocarbon chains and proteins in aqueous environments based on the partition of proteins in an aqueous two-phase system containing dextran and poly(ethylene glycol) and different fatty acid esters of poly(ethylene glycol). Keshavarz and Nakai (16) compared this method with hydrophobic affinity chromatography and found a close correlation. Interfacial tensions measured at 0.2 protein solution/corn oil interface showed lower interfacial tension with more hydrophobic proteins. Kato and Nakai (6) developed a fluorescence method using *cis*-parinaric acid which also correlated with interfacial tensions and emulsifying activity of the proteins studied. It is reasonable to assume, therefore, that emulsifying activity and emulsion stability, as used in the present study, can be used to assess hydrophobic interactions between triglycerides and proteins.

Compared to many proteins, BSA has a structure that permits appreciable binding with hydrocarbons (4,5). It is a relatively easily unfoldable globular protein. Although many hydrophobic residues may be buried in its interior, some hydrophobic groups may be exposed at the molecular surface or in crevices. Additional groups would be exposed

TABLE IV

Interaction between Trioctanoin and Various Proteins

Protein	Molecular weight	Binding		
		Trioctanoin (%)[a]	Trioctanoin relative (%)[b]	Heptane relative (%)[b,c]
Bovine caseins	27,000 (average)	64	160	
Bovine β-lactoglobulin	36,000	52	130	100
Turkey ovomucoid	28,000	51	128	42
Chicken ovotransferrin	76,000	47	118	29
Human albumin	69,000	46	115	
Penguin ovomucoid	28,000	44	110	
Chicken ovomucoid	28,000	43	108	24
Bovine serum albumin	69,000	40	100	100
Bovine γ-globulins	169,000	38	95	7.3
Pepsin	35,000	37	95	
Hemoglobin	65,000	8	20	
β-Amylase	206,000	7	18	
Nagarase	27,500	7	18	
"Antifreeze" TB-DM	25,000 (average)	6	15	
Cytochrome C	13,700	3	8	
α-Chymotrypsin	23,800	1	3	8.5
Chicken ovalbumin	46,000	1	3	2.4
α-Amylase	52,500 (average)	1	3	
Chymotrypsinogen A	25,000	0.7	2	7.1
Ribonuclease A	13,600	0.7	2	17
Lysozyme	14,500	0.3	1	0.49

[a]Percent binding is the percentage of trioctanoin in the sample compared to control.

[b]Binding is relative to bovine serum albumin taken as 100%.

[c]Data from Mohammadzadeh-K et al. (12).

with partial unfolding of the BSA molecules at the surfaces of the triglyceride particles of the microemulsion. The degree of interaction between hydrophobic regions of BSA and fatty acid chains of the triglycerides would also depend on the type of constituent fatty acid.

Mohammadzadeh-K et al. (10,11) found protein interactions with completely nonpolar compounds such as aliphatic hydrocarbons. The amounts of homologous alkanes bound by BSA decreased as chain length was increased from C_7 to C_{13}. Similarly, the amounts of methyl esters of fatty acids bound by BSA decreased as the alkyl chain was increased from C_6 to C_{12}. The present results with triglycerides are in general agreement with the trends reported by these authors for simpler hydrocarbons. The results are also consistent with the principles of hydrophobic chromatography introduced by Shaltiel and coworkers (17) for the resolution, purification and probing of proteins. This technique makes use of differing strengths of hydrophobic interactions with an uncharged bed material which contains hydrocarbon chains. The hydrophobic alkyl side chains or "arms" interact with accessible hydrophobic regions in various proteins and retain only some proteins in a mixture. The length of the hydrocarbon chains is mainly responsible for the gradation observed in the retention power of the columns. Hydrocarbon chains ranging in length from C_1 to C_{10} differ in their capacity to retain a given protein.

The results for triglycerides reported in this paper are of an exploratory nature. More work is required to clarify the question of fatty acid specificity and to determine to what extent BSA possesses specific binding preferences for specific natural mixed triglycerides.

Interaction of Trioctanoin with Various Proteins

The amounts of trioctanoin interacting with different proteins as measured by this technique varied as much as 200-fold on a weight basis (Table IV). The order of binding for the series of proteins, compared to bovine serum albumin taken as 100%, varied from bovine caseins, 160, to less than 1 for lysozyme. Molecular size did not materially influence interaction. Ovotransferrin, serum albumins and bovine γ-globulin, among the larger proteins, were good binders, whereas α-amylase and ovalbumin, also larger proteins, were among the very poor binders. The presence of large amounts of carbohydrates in turkey, penguin and chicken ovomucoids (glycoproteins having up to 80% carbohydrate groups by weight) were among the better binders. In general, globular proteins with hydrophobic regions interior in their molecular structure, such as cytochrome C, α-chymotrypsin, ribonuclease A and lysozyme, were found to be less effective binders.

The data presented in Table IV generally confirm and extend results in the literature. Mohammadzadeh-K et al. (12) studied hydrophobic binding of heptane by several of the same proteins. Bovine β-lactoglobulin and serum albumin were the best binders, while turkey and chicken ovomucoids and ovotransferrin were intermediate binders. Lysozyme was the poorest binder of both heptane and trioctanoin. In general, proteins that bound heptane also bound trioctanoin. However, γ-globulins bound trioctanoin much better than heptane, and ribonuclease A bound heptane better than trioctanoin. Kato and Nakai (6) determined hydrophobic parameters of several proteins by fluorescense and partition techniques and found BSA, β-lactoglobulin and κ-casein to be highly hydrophobic while ovalbumin, α-chymotrypsin and lysozyme were much less hydrophobic.

Effect of Hydrophobic Substitution of Amino Groups of Casein on Interaction

The vitamin-free casein preparation used in this research was a mixture of caseins with an average estimated molec-

TABLE V

Effect of Hydrophobic Substitution of Amino Groups of Casein on Interaction with Trioctanoin

	Protein		Binding	
	Molecular weight	Number of residues[a]	Trioctanoin (%)[b]	Relative (%)[c]
Bovine serum albumin	69,000	0	40	100
Casein	27,000	0	64	160
Methyl casein	27,000	10.7	77	192
Cyclohexyl casein	27,000	10.1	78	195
Cyclopentyl casein	27,000	7.3	83	207
Benzyl casein	27,000	10.7	82	205

[a]Number of hydrocarbon residues added chemically to casein has little effect on molecular weight of the protein.

[b]Percent binding is the percentage of trioctanoin in the sample compared to control.

[c]Binding is relative to bovine serum albumin taken as 100%.

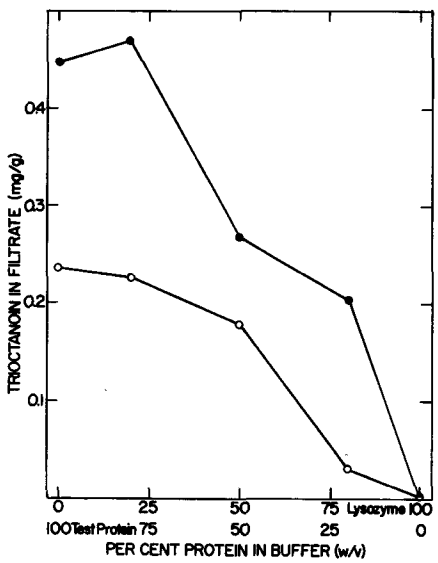

FIG. 4. Effect of lysozyme on binding of trioctanoin by BSA (open circles) or cyclopentyl casein (solid circles). The protein concentration was 0.1% (w/v) in 0.1 M sodium phosphate buffer, pH 6.8.

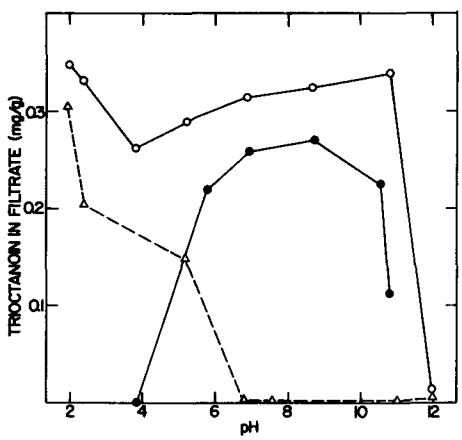

FIG. 5. Effect of pH on binding of trioctanoin by BSA (open circles) and lysozyme (triangles). The protein concentration was 0.1% (w/v) in orthophosphate buffers whose ionic strength was 0.6 M. Effect of increased ionic stength (1.6 M) on binding of BSA is also shown (solid circles).

ular weight of 27,000. It had the greatest binding ability of the proteins listed in Table IV. This may be because the casein molecules have a flexible structure approximating a disordered random coil with many hydrophobic amino acids exposed and available for binding to the trioctanoin molecules (18). As shown in Table V, addition of nonpolar residues to ϵ-aminolysyl groups of casein increased interaction capacity. There was little difference in the improved binding caused by different residues but the casein modified by substitution of cyclopentyl residues gave the greatest degree of binding, even though it had the smallest number of substituted residues. It also is interesting that methyl groups resulted in nearly the same increase in binding as the larger cyclic hydrocarbon residues.

Interaction of Trioctanoin with Mixtures of Proteins

Mixtures of a poor binder, lysozyme, and two good binders, BSA or cyclopentyl casein, were equilibrated with trioctanoin microemulsions (Fig. 4). With cyclopentyl casein, the binding curve shows that the presence of 50% lysozyme decreased the amount of trioctanoin bound by ca. 40%. However, with BSA the decrease was only ca. 25%, indicating that lysozyme interferes more with the availability of the hydrophobic binding sites of cyclopentyl casein than those of BSA. In similar experiments, when BSA and chicken ovomucoid were mixed in different proportions with the total protein concentration (w/v) kept constant, there was no change in total interaction indicating that these good binders do not interfere with each other.

Effect of Charge on Interaction

Effect of pH. Figure 5 shows binding of trioctanoin at different pH values by 0.1% solutions of BSA and lysozyme at a constant salt concentration of 0.1 M $-PO_4^{3-}$ (approximately ionic strength of 0.6 M). There was a slight reduction in binding by BSA near its isoelectric point of pH 4.9. However, the marked reduction above pH 11 suggests that a conformational change occurs in this region. Greater difficulty was encountered in forcing the microemulsion through the Nuclepore membrane at this pH, possibly due to coagulation.

Lysozyme, which was the poorest binder of all proteins at pH 6.8 (Table IV), exhibited increased interaction with trioctanoin as the pH was decreased to pH 2. This effect was similar to the binding of heptane by lysozyme (12). These results are suggestive of a pH-dependent conforma-

tional change exposing normally sequestered hydrophobic groups.

The above results indicate that the relative order of binding of trioctanoin and other triglycerides may be influenced by the pH of the test system. Proteins with relatively high isoelectric points (pH 9.5-10.6), such as cytochrome C, chymotrypsinogen A and ribonuclease A, might exhibit enhanced binding of triglycerides compared to the values found at pH 6.8 (Table IV), if tested under more acidic conditions.

Effect of ionic strength. Figure 5 also illustrates the effect on interaction of increasing the ionic strength of the test system from 0.6 M to 1.6 M in the case of bovine serum albumin. Binding is reduced at all pH values but the effect is especially great below pH 6 and above pH 10.

Results of the present investigation indicate that the microemulsion-filtration method is useful for studying hydrophobic interactions between triglycerides and proteins. The extent of these interactions depends on the type of fatty acid chains of the triglycerides and the nature and accessibility of hydrophobic regions of proteins. Binding of different triglycerides by a specific protein, as illustrated by BSA, can be evaluated. The same general technique can be used to compare the hydrophobic binding of a specific triglyceride, as illustrated by trioctanoin, by a variety of native and chemically modified proteins under different environmental conditions. Radioactive trioctanoin provides a simpler and more precise method than GLC for determining the amounts of triglyceride before and after partition by the membrane filter. However, the radioactive method is limited to available labeled triglycerides.

The data presented show that proteins vary widely in their ability to interact with triglycerides. Furthermore, changes in environment or chemical structure also markedly influence the binding capacity of specific proteins. The results obtained are in accord with the general interpretations that (a) most of the hydrophobic areas of proteins are buried in the interior of the protein, and (b) the native structure of the protein can be unfolded to expose these areas at an O/W interface. The techniques employed in the present study should be valuable to assess the effects of different parameters, including pH, temperature and denaturation, on the interaction of proteins with water-insoluble molecules such as sterols, carotenoids and oil-soluble food dyes. It also is possible that polycarbonate membrane filters of different pore sizes could be useful in characterizing globule size distributions in certain emulsions.

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