

BIOSPECIFIC INTERACTIONS OF AFFINITY-LABELLED SURFACTANTS

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INTRODUCTION

This paper describes the synthesis and characterization of non-ionic and zwitterionic surfactants that bind specifically to proteins. We are interested in studying the binding properties of surface-active ligands to proteins and the physical properties of the resulting complexes. These protein-surfactant systems are of interest in the formation of vesicles and micelles and in the study of foams and emulsions involving biological molecules. Potential applications include new formulations of liposomes and oil-in water emulsion adjuvants for site-specific biomolecule delivery.

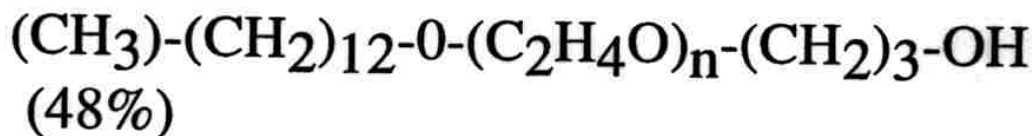
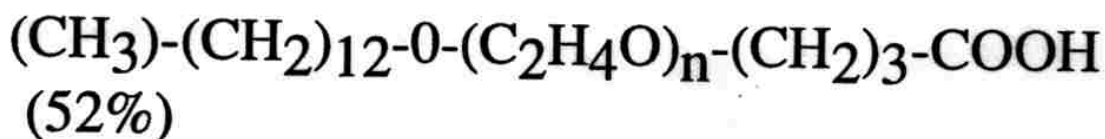
We have studied two types of surfactants. The first type consists of non-ionic ethoxylated alcohols and carboxylic acids with straight-chained hydrocarbon tails. These are very water soluble, and have a controllable range of critical micelle concentrations (CMC) depending on the ratio of the length of the hydrocarbon chain to the chain of ethoxy groups. The second type consists of zwitterionic saturated phospholipids which are essentially insoluble in water, but can be solubilized by the addition of the

above-mentioned non-ionic surfactants in micellar solution.

SYNTHESIS OF SURFACTANTS

- Non-ionic ethoxylated fatty acid

Sandopan JA-36 (Sandoz Chemicals) is a commercial surfactant consisting of 52% carboxylic acid and 48% alcohol. It has a thirteen-carbon saturated fatty acid chain and a Poisson distribution of ethoxy groups ranging from 1 to 100 with a mean value of 19:



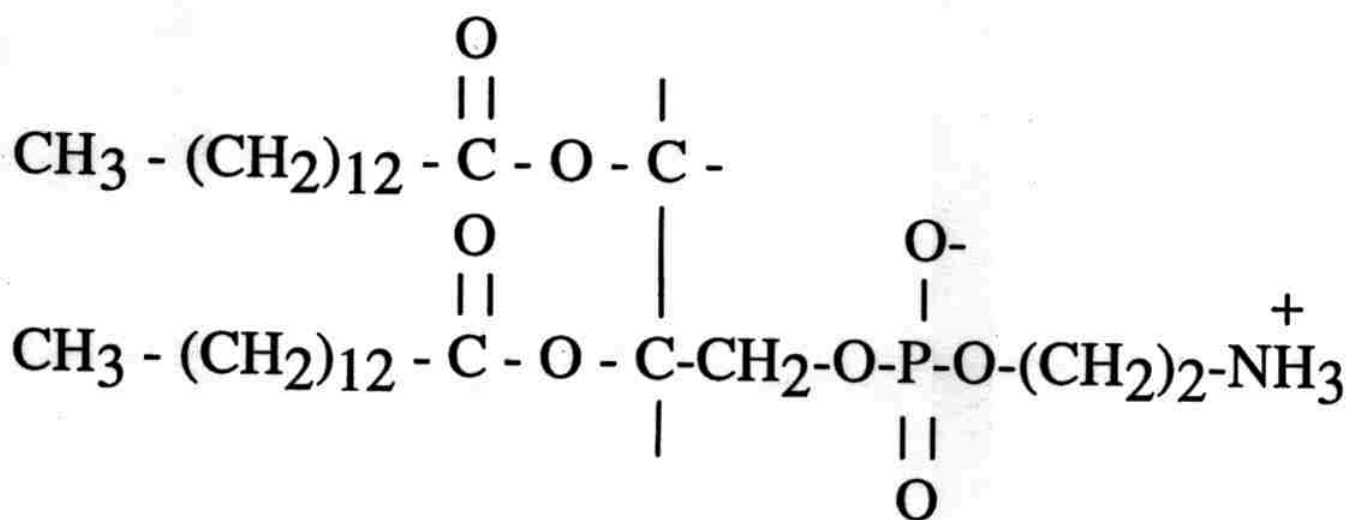
$$1 < n < 100, \bar{n} = 19.$$

The carboxylate group was reacted with the water-soluble carbodiimide EDC [ethyl

(dimethylaminopropyl) carbodiimide)] at pH 3.7 and 5°C. The intermediate was then reacted under the same conditions with a reversible inhibitor of β -galactosidase (EC 3.2.1. 23) and a reversible inhibitor for trypsin (EC 3.4.21.4). The inhibitor of β -galactosidase was p-aminophenylthio - galactopyranoside (APGP, $K_i = 10^{-3}M$) while that of trypsin was p-aminobenzamidine (PAB, $K_i = 8.5 \times 10^{-6}M$). The reactions were followed by the OPA test, and the product purified by foam fractionation in several stages. The products were identified by NMR spectroscopy and UV absorption spectra.

- Biotin-conjugated phospholipid

Dimyristoyl phosphatidylethanolamine (DMPE) was reacted with NHS-Biotin in a 2:1 mixture of $CHCl_3$ and methanol in the presence of triethylamine for 30 minutes at room temperature.



The product was purified using preparative TLC on precoated silica gel plates using a mixture of CHCl_3 , methanol and water (74:24:2) as the developing solvent. The product was detected by a phosphate-specific spray (ammonium molybdate) and a biotin-specific spray (dimethylaminocinnamaldehyde). The product was recovered from the plate by a 2:1 CHCl_3 , methanol solution. The solvent was then evaporated under N_2 and stored at 0°C . Biotin is a potent ligand for the protein Avidin D with a binding constant of 10^{-15}M .

LIGAND BINDING STUDIES

The inhibition properties of the surfactant-inhibitors for β -galactosidase and trypsin were determined from kinetic assays. For β -galactosidase, the substrate o-nitrophenyl- β -D-galactoside (ONPG) was converted to o-nitrophenol in a 0.05 M Tris-HCl buffer at pH 7.5 with 0.01M $MgCl_2$ and 0.1M NaCl at 22°C. Figure 1 summarizes the effects of pure surfactant JA36, pure inhibitor, APGP, and the surfactant-inhibitor JA36-APGP on the ratio of initial reaction velocity in the presence of additive to that in the absence of any additive. The pure surfactant, because it is non-ionic, has little effect on the activity of the enzyme. The pure inhibitor APGP inhibits according to Michaelis-Menten kinetics for a classical reversible inhibitor ($K_i = 1.0mM$). The surfactant-inhibitor JA36-APGP inhibits about 8 times more strongly than pure APGP until a concentration of $10^{-4}M$ is reached. Beyond this value, which is close to the CMC, the inhibition remains constant with increasing surfactant-inhibitor concentration. This is probably due to the formation of micelles which remove the monomer from solution. It is evident that

the monomeric form of the surfactant is the more effective inhibitor.

For trypsin the substrate used was DL-BAPNA (DL-benzoylarginine-p-nitroanilide) in a 0.05M Tris-HCl buffer, pH 8.15 with 0.02M CaCl_2 . Figure 2 shows the effect of adding the surfactant JA36, the pure inhibitor (PAB) and the JA36-PAB surfactant inhibitor on the initial rate of reaction. Since the inhibition constant for this inhibitor is small ($K_i = 8.5 \times 10^{-6}\text{M}$) all of the enzyme is inhibited before the CMC of the surfactant is reached. This is in sharp contrast with the β -galactosidase inhibitor (Figure 1) which only inhibited about 30% of the protein prior to reaching the CMC. It is also evident from Figure 2 that JA36 by itself does not inhibit trypsin and that JA36-PAB follows Michaelis-Menten kinetics with the same K_i value as the unmodified inhibitor PAB. Since trypsin is a hydrophilic protein there is no tendency for the hydrocarbon tail of the surfactant to adhere to its surface. In the β -galactosidase case the hydrophobic portion of the surfactant can bind strongly to the surface of this large hydrophobic protein and thus increase the binding capacity of the inhibitor below its CMC. As discussed above,

Relative Enzymatic Reaction Rate with Added Inhibitor, Surfactant and Surfactant-Inhibitor

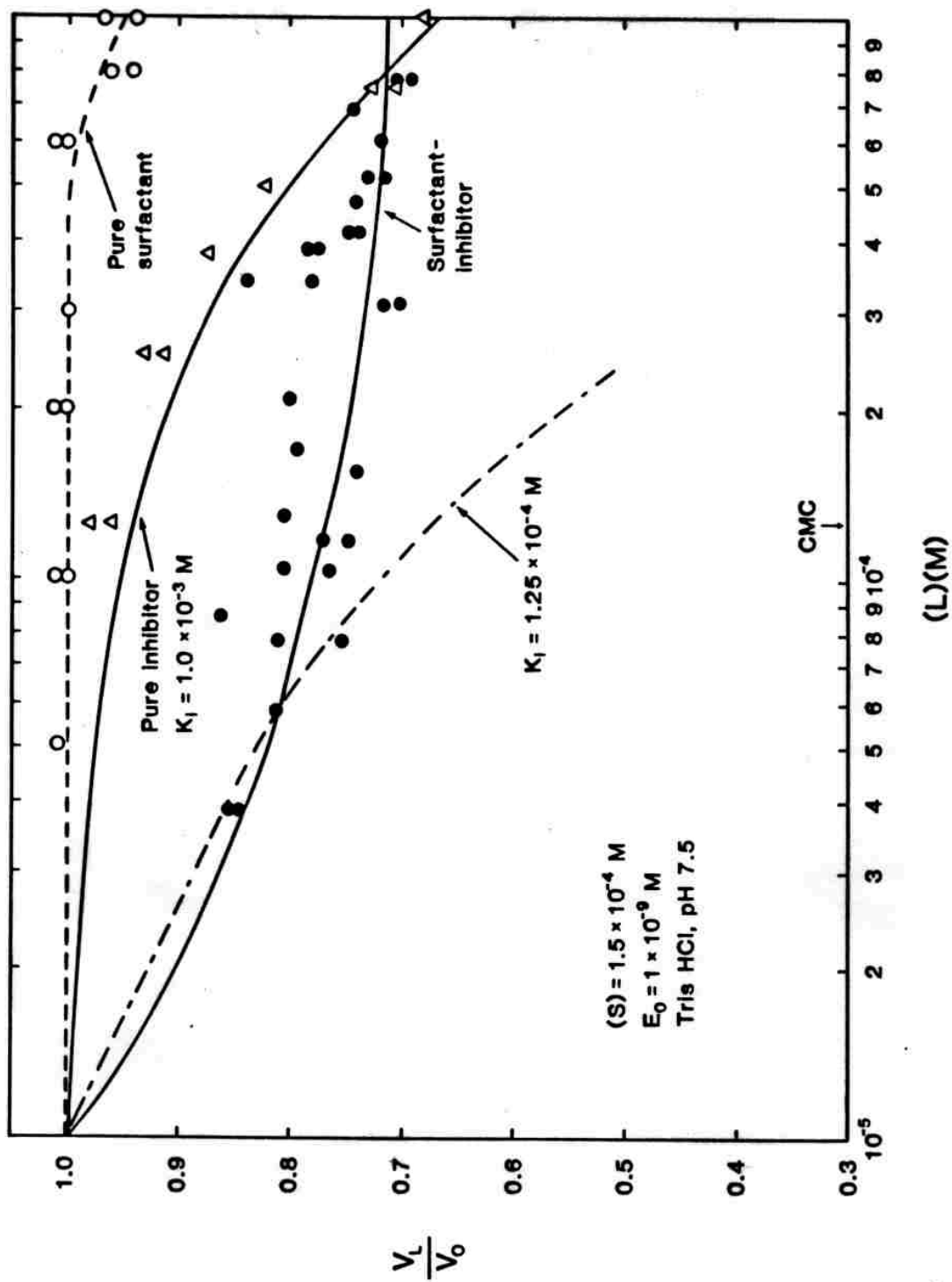


Figure 1: Binding studies with Beta-Galactosidase

Relative Enzymatic Reaction Rate Catalyzed by Trypsin
with Added Inhibitor, Surfactant, and Surfactant-Inhibitor

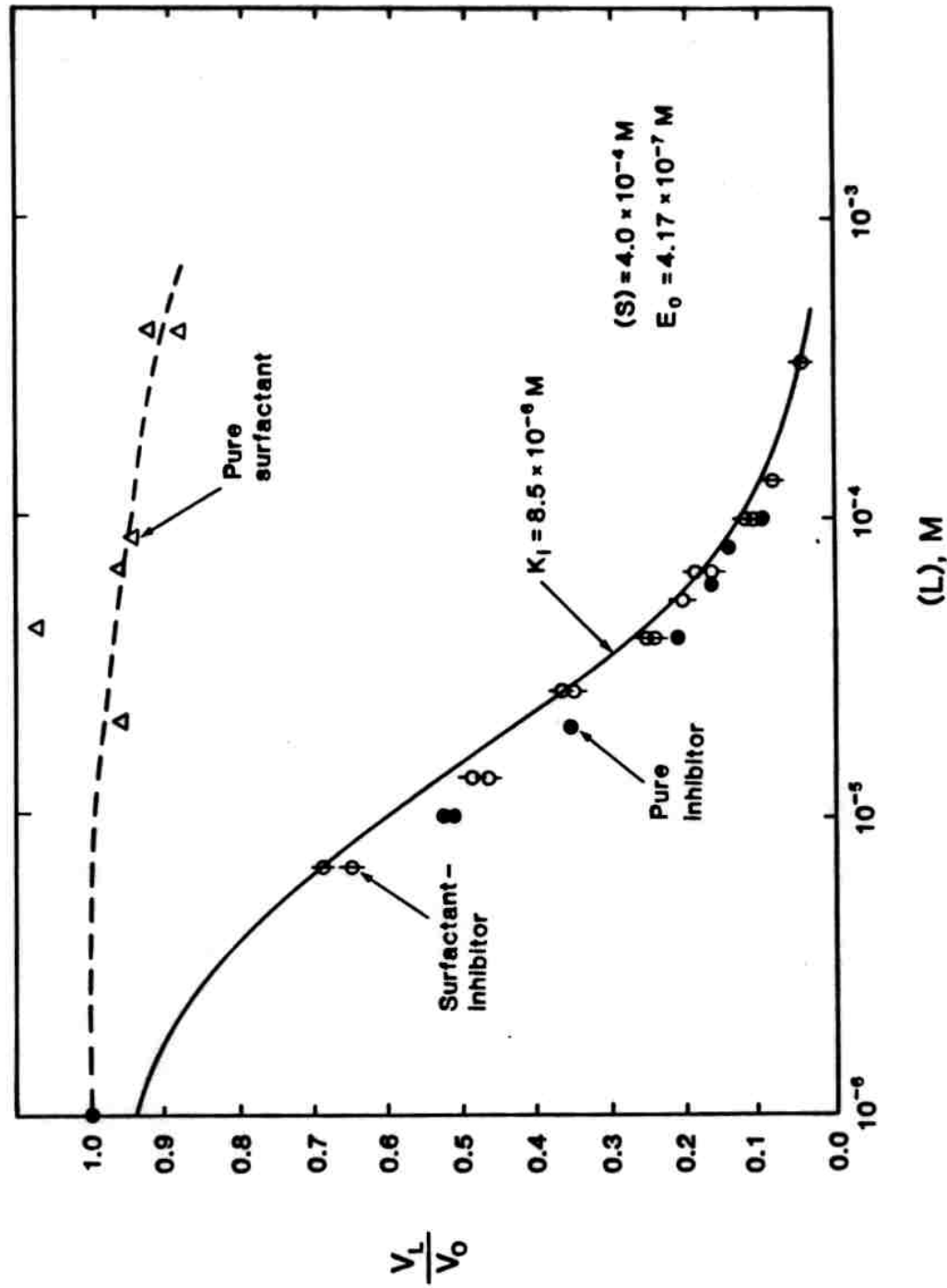
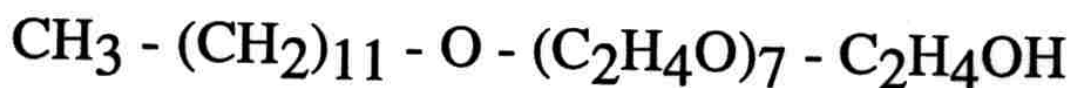


Figure 2: Binding studies with Trypsin

beyond the CMC the formation of micelles presents additional inhibition. These effects are illustrated in Figure 3. It is important to note that the surface activity of the surfactants is not changed significantly by modification of the head group. This is illustrated in Figure 4 where the surface tension of JA36 and JA36-APGP are plotted as a function of bulk concentration of surfactant.

DMPE-Biotin was solubilized by using a pure ethoxylated fatty acid alcohol from Nikkol, C₁₂E₈:



The C₁₂E₈ concentration was fixed at 10⁻³M in a 0.2M NH₄CO₃ buffer at pH 8.9 and we were able to achieve a 5x10⁻⁵M solution of the phospholipid.

The titration of Avidin proceeds by the addition of 40 μl aliquots of the DMPE-Biotin solution in C₁₂E₈ to 3 ml of a 0.06 mg/ml Avidin solution in the same NH₄CO₃ buffer mentioned previously. The reference cell of the UV spectrophotometer initially contained buffer only. Aliquots of the DMPE-Biotin solution were also added to the reference during the titration.

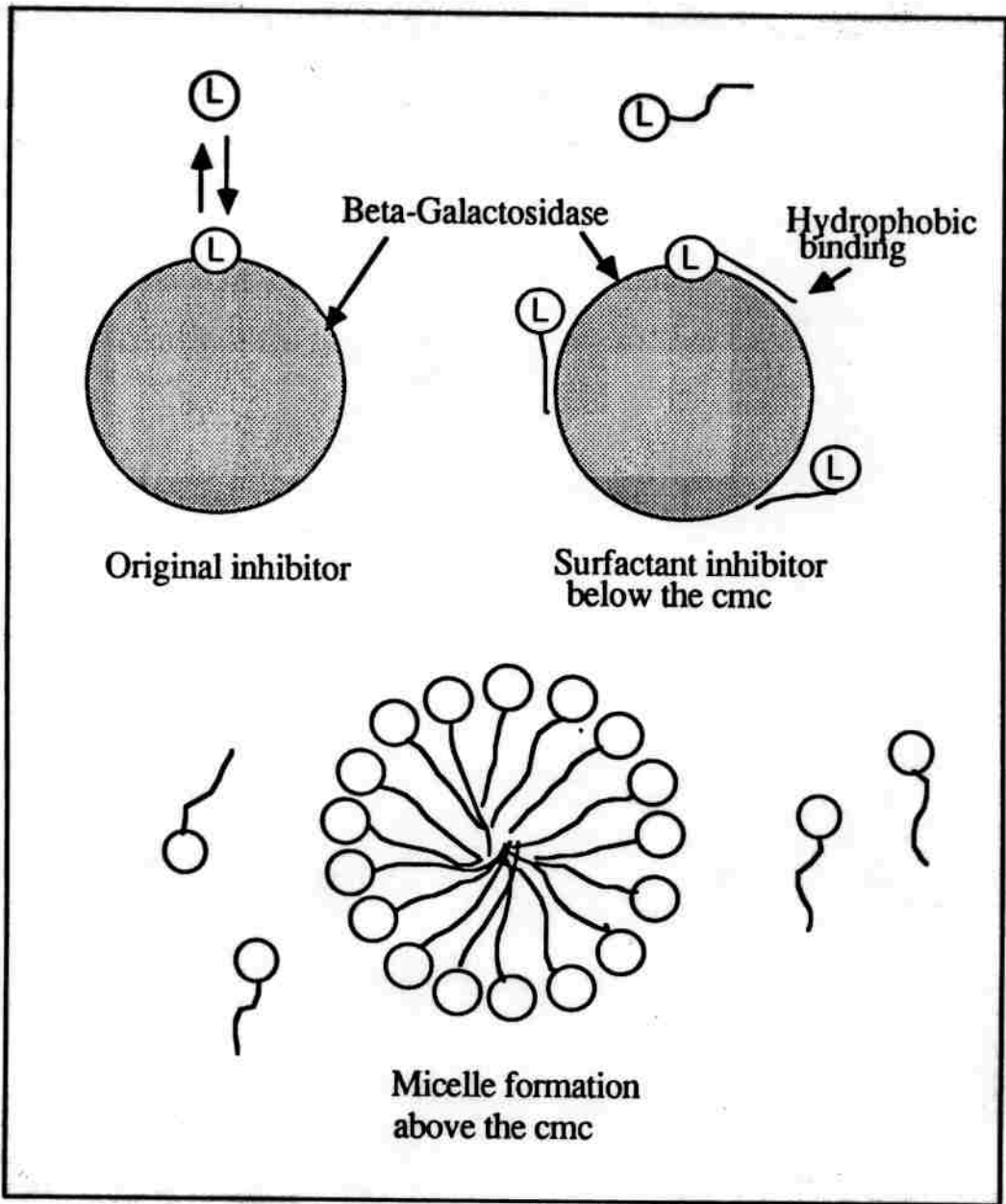
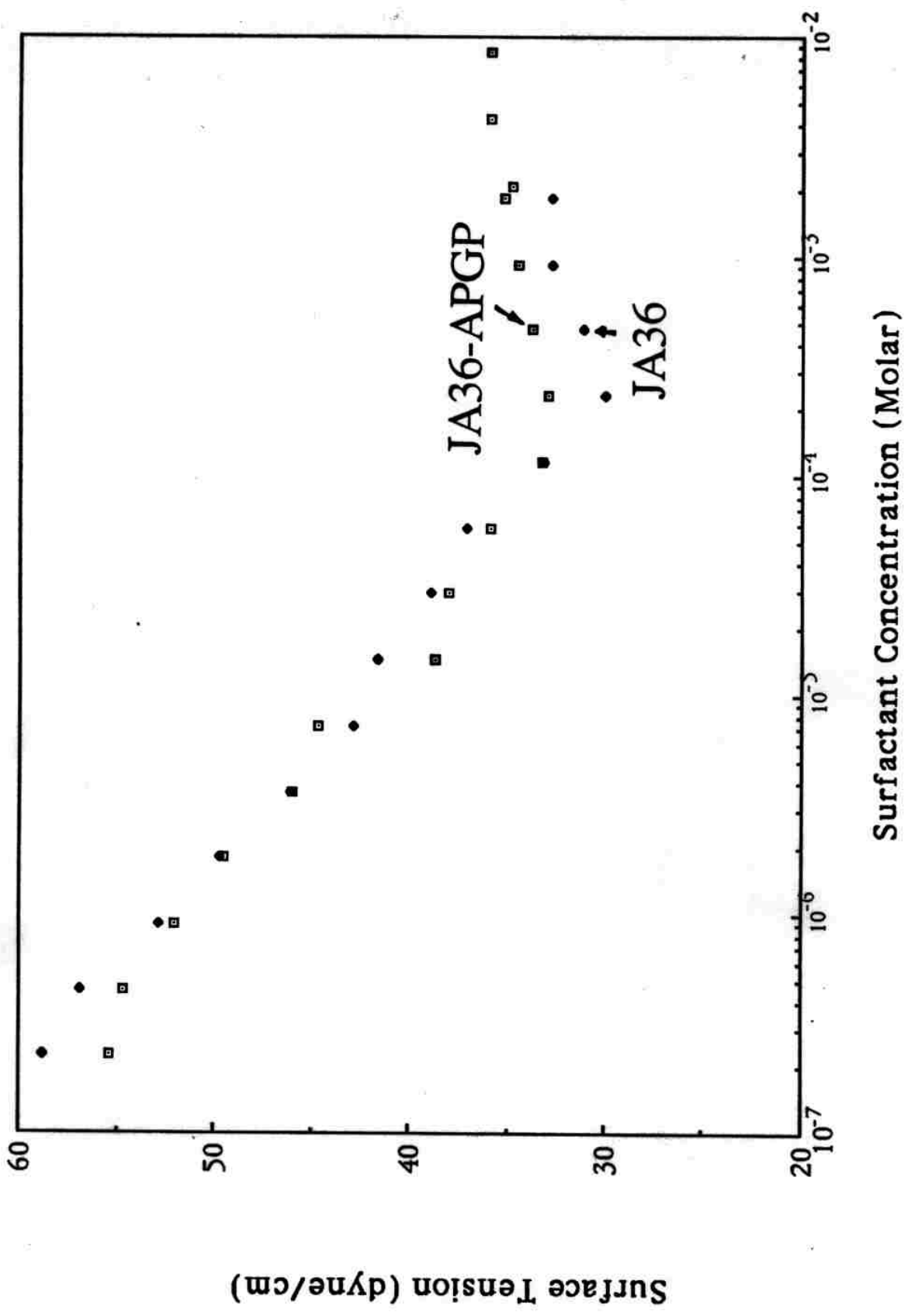


Figure 3: Interactions of the pure inhibitor and the surfactant-inhibitor with Beta-Galactosidase

Figure 4: Surface Tension of JA36 and JA36-APGP Solutions



The absorbance at 233 nm was monitored as a measure of the binding of Avidin to DMPE-Biotin. Very slow increases in absorbance and ultimately very high values were obtained. An end point to the titration was observed and it is illustrated in Figure 5. The end point corresponds to 3.6 moles of DMPE-Biotin bound per mole of Avidin, a value close to the theoretical value of 4. An identical solution of C₁₂E₈ showed no change in UV absorbance upon addition of Avidin. This indicates that the observed absorbance increase in time is the direct result of the binding of DMPE-Biotin to Avidin.

The large absorbance values at 233 nm are due to the aggregation of the phospholipid-Avidin complex in solution. In fact, the entire UV spectrum of Avidin shifts upwards in time as seen in Figure 6. There is evidence of Tyndall scattering at the large wavelengths and the solution becomes turbid. In fact, some sedimentation can occur, as evidenced by a gradual decrease in the entire UV spectrum over time. The slight absorbance decrease in the last two aliquots shown in Figure 5 are the result of this effect. However, large concentrations of C₁₂E₈ ($1.0 \times 10^{-3} \text{M}$) can solubilize these aggregates, giving rise to clear

Figure 5: Titration of Avidin D with DMPE-Biotin

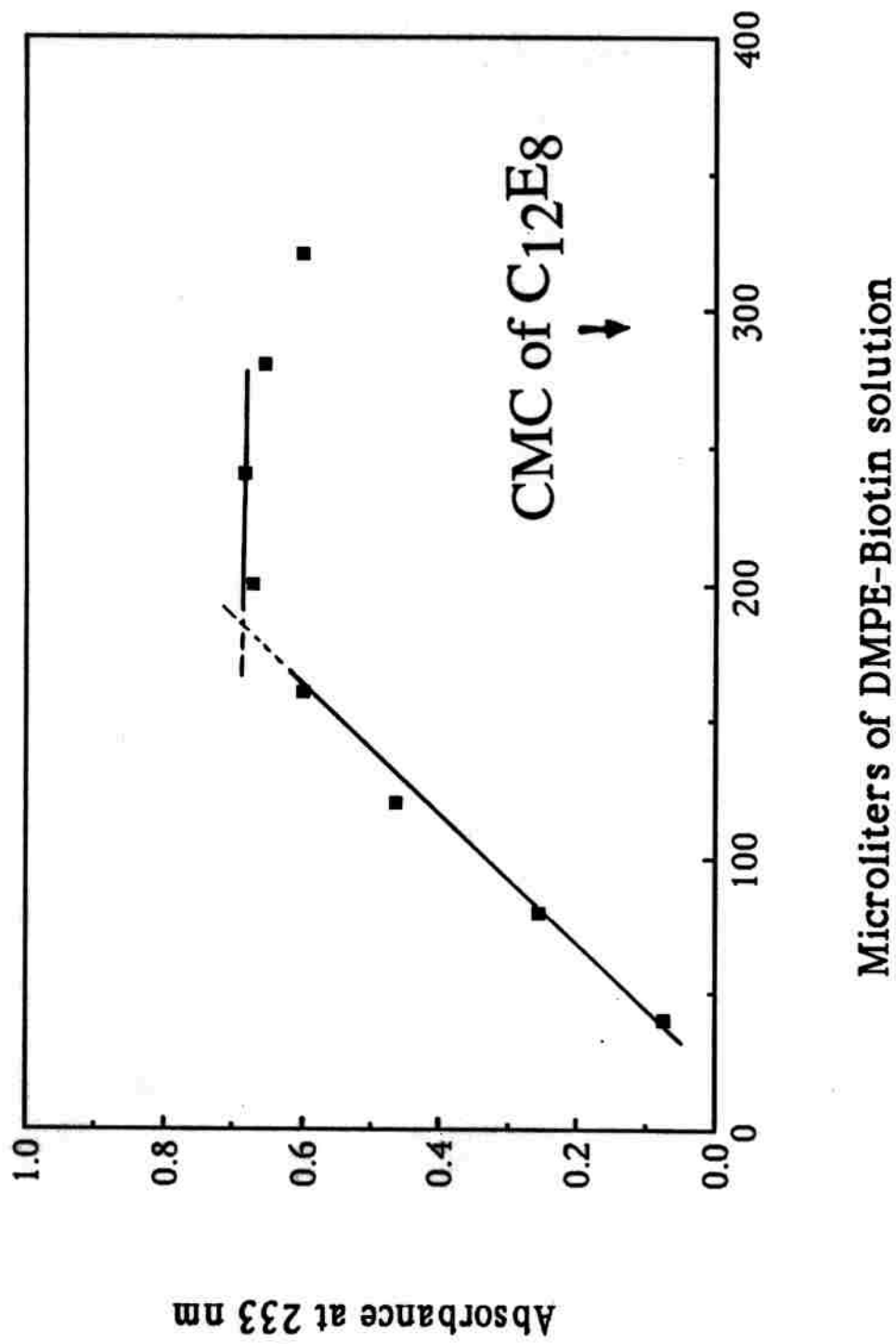
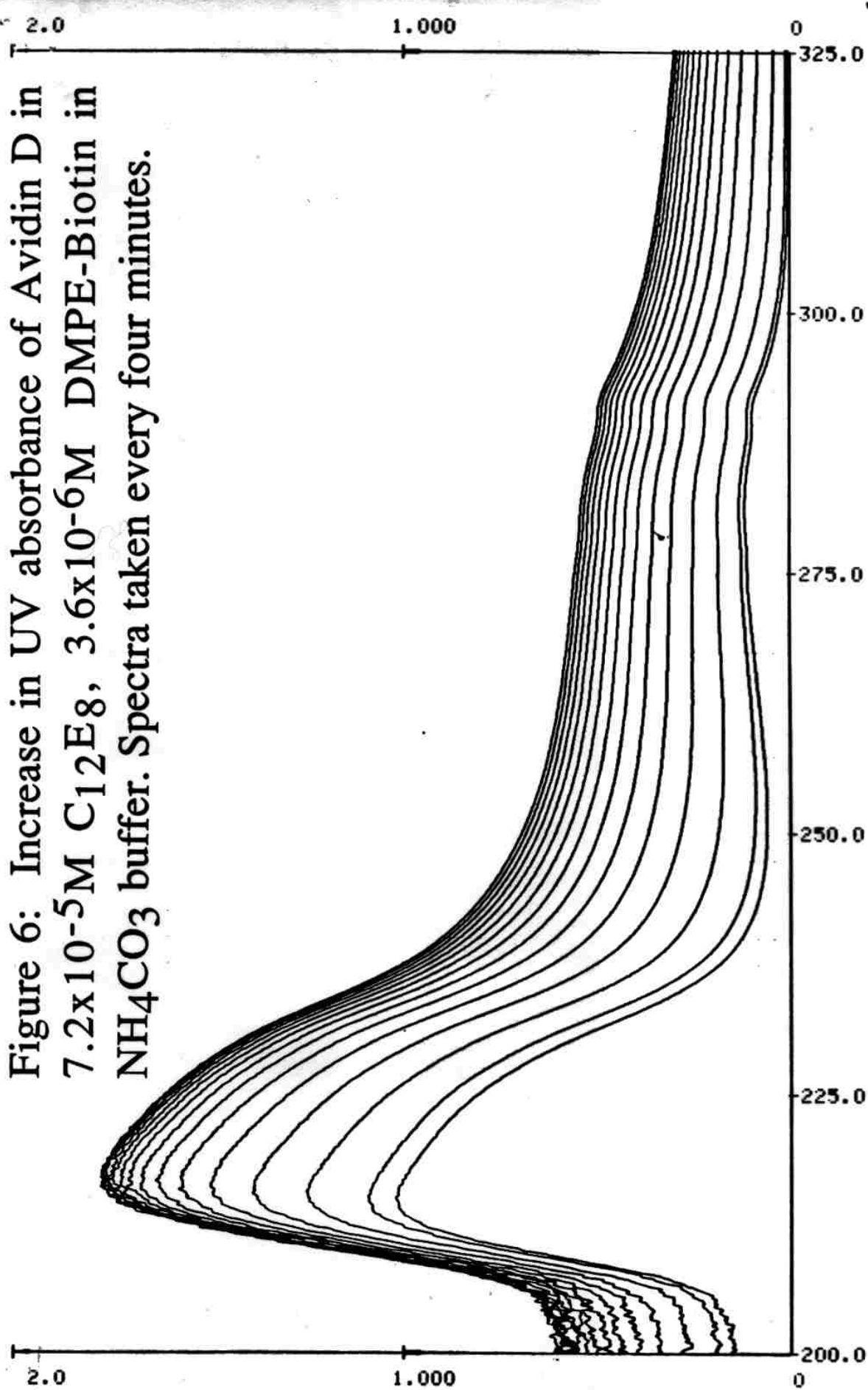


Figure 6: Increase in UV absorbance of Avidin D in $7.2 \times 10^{-5} \text{M}$ C12E8, $3.6 \times 10^{-6} \text{M}$ DMPE-Biotin in NH_4CO_3 buffer. Spectra taken every four minutes.



solutions and more typical Avidin spectra as shown in Figure 7.

SURFACE ACTIVITY OF SURFACTANT-PROTEIN COMPLEXES

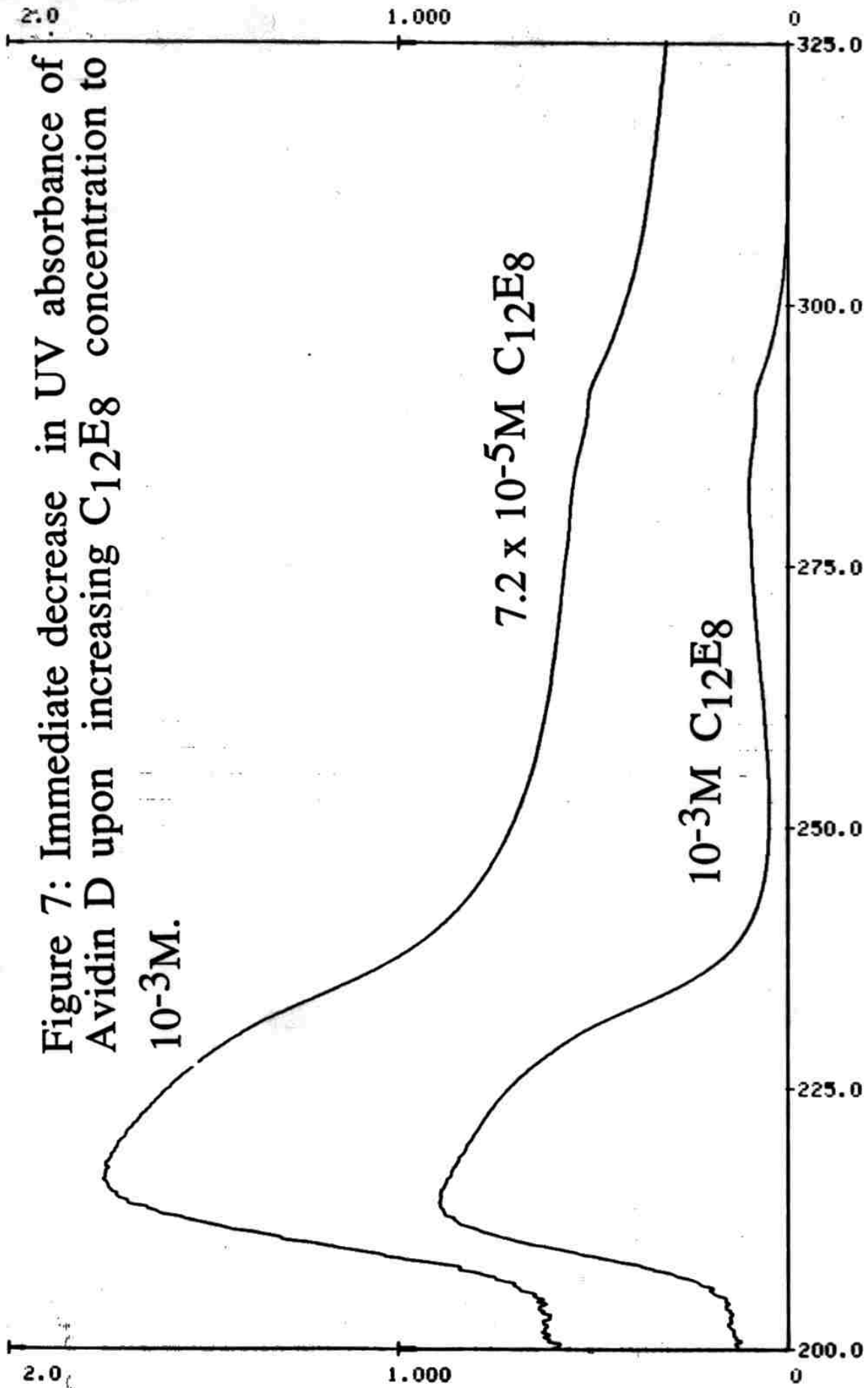
Solutions of $C_{12}E_8$ and DMPE-Biotin already bound to Avidin were placed in a flask and air was introduced through a sparger at the bottom, forming a foam. The foam rose up a column and the protein concentration in the foamate was measured by UV absorbance at 280 nm and 213 nm. It was found that the DMPE-Biotin-Avidin complex was not sufficiently surface active to compete effectively with the $C_{12}E_8$ which is at much higher concentration and was the primary component of the foam. At low $C_{12}E_8$ concentrations the aggregates of DMPE-Biotin-Avidin formed during complexation cannot diffuse sufficiently fast and do not have a sufficient driving force to break up and adsorb to the gas-liquid interface. At high $C_{12}E_8$ concentrations the DMPE-Biotin-Avidin complex is incorporated in micelles which are also not surface active. Similar foaming

Figure 7: Immediate decrease in UV absorbance of Avidin D upon increasing C₁₂E₈ concentration to

10⁻³M.

7.2 x 10⁻⁵M C₁₂E₈

10⁻³M C₁₂E₈



experiments done with Avidin not bound to DMPE caused denaturation and precipitation of the protein and large quantities of protein in the foam due primarily to flotation of the sedimented material.

CONCLUSIONS

Surfactant-inhibitors can bind more strongly than the original inhibitor to hydrophobic proteins as evidenced by the β -galactosidase results. If the inhibition constant is less than the CMC of the surfactant, the formation of micelles can prevent further inhibition. This indicates that the monomeric form of the surfactant inhibitor is more effective. With hydrophilic proteins such as trypsin the inhibition characteristics of the surfactant-inhibitor and the pure inhibitor can be identical. Phospholipids can be dissolved in water using non-ionic surfactants. The phospholipid-ligand in the solution can bind effectively to proteins as in the DMPE-Biotin-Avidin case. This can lead to sedimentation at low concentrations of the non-ionic surfactant. At high concentrations of the non-ionic it is possible to solubilize these aggregates. The protein-surfactant ligand complexes formed with DMPE-Biotin-Avidin

are not particularly surface active when compared to the smaller surfactant $C_{12}E_8$.