

STUDIES ON SERUM ALBUMIN BINDING OF LYSOLECITHIN

by

CANDICE ANN MORGAN

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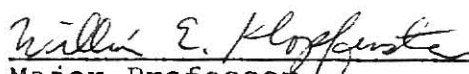
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Approved by:


Major Professor
Department of Biochemistry

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INTRODUCTION

Lipids and proteins are major constituents of living organisms. Their association in complexes occurs in the plasma, in biological membranes, and at various other sites such as at the alveoli surface.

The lipoprotein complexes found in the plasma vary greatly in their percent composition of lipid and protein. They are important in the transport of lipids, vitamins, hormones, and enzymes through the circulatory system.

Membrane lipoproteins are involved in the transport of substances across the cell wall and across the membranes of intracellular organelles. The species of protein and lipid present and their relationship determines the characteristics of transport and function in a particular membrane. Membrane structure and mode of action is a currently active area of research. The membrane is an extremely important component in the regulation of metabolism by its selective transport and permeability. The differences in membrane actions serve to maintain cellular specificity of function.

Before the structure and function of membranes and plasma lipoproteins can be determined the basic associations of lipid to protein must be characterized.

STATEMENT OF THE PROBLEM

The purpose of this research was to study the nature of lipid-protein binding, using lysolecithin and serum albumin as a model system. Microcalorimetric procedures were used to reveal the thermodynamic relationships of this binding. Lysolecithin was chosen because of its known association with proteins in various systems. Serum albumin was chosen for its binding capacity of phospholipids and its availability in a high degree of purity.

REVIEW OF THE LITERATURE

I. Historical Review of Studies on Lipoprotein Interactions

Interest in lipid-protein association was aroused with the work of Danielli and Davson with lipid films. They proposed a membrane model which consisted of a phospholipid bimolecular leaflet whose nonpolar, acyl chains were oriented inward, perpendicular to the plane of the membrane, forming a hydrophobic region. The protein was ordered around the lipid with polar binding (1). This basic membrane model has been supported by electron microscopic observations of myelin membranes. Robertson has proposed the term "unit membrane" to a similar model (2). This membrane model was theorized to be the basic organization of all membranes and was accepted as such until recently. This model is now recognized to be inadequate in many respects because of its inability to account for the tremendous physiological differences observed between the myelin membrane and more "active" membranes, and also because of unsubstantiated assumptions made with regard to the electron microscopic procedures. These inadequacies in the model have been pointed out by Korn (3).

The nature of lipoprotein interactions has been studied by a variety of physical and chemical methods. Early studies utilized precipitation and titration techniques. These observations led to the conclusion that all lipoprotein interactions were ionic with the protein interacting with lipid micelles. Nonpolar lipids were thought not to associate directly with proteins but only through their association with polar lipids in micelles (4).

Recent studies have shown lipid-protein interactions to be much more complex than the ionic association proposed in these early studies.

The fact that phospholipids contain both hydrophobic and hydrophilic regions and form a monolayer at an air-water interface has led to studies of these properties in relation to membrane properties. Measurements were made of surface tension, surface density, and surface potential, and changes in these were observed upon introduction of protein under the lipid film. The changes observed were a function of film penetration by the protein, and interaction with the lipid. These studies have shown specificity of binding (5), dependence upon chain length and unsaturation of the fatty acids (6), and dependence upon charge of the lipid (7,8).

Information has been obtained about the nature of lipid-protein interactions by reassociation of lipid to extracted membranes, using double dialysis techniques. Two types of interactions, ionic and "hydrophobic" or apolar have been characterized in this manner (9,10).

A variety of physical methods have proven valuable in studying lipoprotein relationships. X-ray diffraction studies of natural lipoprotein systems have been combined with electron microscopy studies to work out the dimensions of observed membrane structure. The width of the membrane was found to be in close agreement with the value predicted by the "unit membrane" model. X-ray diffraction studies alone have failed to give precise in-

formation about lipoprotein associations but have served to complement other methods (11).

Ultracentrifuge techniques have been adapted for the purpose of studying these interactions and were used initially to study the properties of the proteins associated with lipids and to classify the serum lipoproteins by their sedimentation values. Recent studies have explored the composition and structure of proteins in the previously classified lipoprotein systems (11).

Nuclear magnetic resonance (NMR) spectroscopy is another physical method which lends itself to this study by providing information about the changes in freedom of motion of characteristic portions of the lipids and proteins involved. In this procedure the spectra of the free proteins and lipids are obtained and compared to those of the lipoprotein complex (11).

Calorimetry is a new and useful method for the study of lipoprotein interactions. Differential scanning calorimetry (DSC) has been used by Steim (12), to study phase changes of membranes and their lipid components. Ladbroke, et al. (13), have used DSC to study phase changes of phospholipids when associated with cholesterol. Chapman (14) has combined DSC and NMR techniques in studies of lipid-protein interactions.

Heatburst calorimetry was utilized by Kitzinger and Benzinger (15) to determine the change in enthalpy, free energy, and entropy of the fumarase reaction of the Krebs' cycle. Lovrien and Anderson (16) have used this method to study the binding of sodium dodecyl sulfate by β -lactoglobulin. Stoichiometric and

thermodynamic functions of serum albumin binding of lysolecithin have been determined with this method by Klopfenstein (17,18).

II. Nature of Lipoprotein Interactions

Lipoprotein interactions can be either polar or apolar and depend upon the specific lipoprotein. A variety of techniques have been used to determine the type of interaction taking place. Examples of ionic interactions between phospholipids and cytochrome C have been demonstrated utilizing x-ray diffraction techniques (19).

Extraction of low density lipoproteins with diethyl ether yielded small amounts of lipid. Denaturation of the lipoprotein prior to the extraction, however, resulted in considerably greater quantities of lipid extracted. Digestion with phospholipase C also increased the lipid extracted by ether, but phospholipase D did not increase the lipid yield. This suggests that the protein forms a cover around the phospholipid with an apolar interaction (20). Chloroform-methanol extraction of endoplasmic reticulum and plasma membrane films completely eliminated the lipid contribution to the infrared spectra of the membranes which indicates a weak or apolar interaction(21).

Fatty acid composition of phospholipids has been shown to be important in the rate of their binding to extracted mitochondrial membranes. The rate of binding of lecithin, which lacked essential fatty acids, to extracted mitochondrial membrane, was 2.5 to 3 times greater than the rate of "normal" occurring lecithin (22).

This indicates that the apolar chain of the fatty acid was involved in the binding.

The driving force for apolar binding is highly speculative at this time. Possible driving forces and their concepts have been reviewed by Jencks (23). Those most probably involved in the interaction are: Van der Waals-London dispersion forces, entropy changes on binding, and energy used for the creation of solute "cavities" in the solvent. The possibility of entropy changes as the driving force has been described by Kauzmann (24), and Nemethy and Scheraga (25). The lipid is viewed as surrounded by highly structured water of low entropy which gives a low entropy to the system. Binding of the lipid to protein thus releases some of the structured water and results in a net positive entropy change for the system. Binding of lysolecithin by serum albumin has been shown by Klopfenstein (17,18) to yield negative enthalpy, free energy and entropy changes. These results suggest that entropy changes are not the driving force in this specific case. The other driving forces have not been studied to any extent.

III. Properties of Serum Albumin

Serum albumin is an exceptional protein because of its ability to bind many substances such as fatty acids, phospholipids, vitamins, hormones and enzymes in substantial quantities. These binding properties, with alkane ligands, are a function of pH, with a low pH reducing the binding ability and subsequent increase in pH resulting in an increase in binding ability. The number of

binding sites on the molecule has not been accurately determined but is known to be considerable. Phosphatidyl serine is known to bind human serum albumin at two sites which differ in equilibrium constants. Certain ions which bind to the serum albumin act to alter the configuration and render additional binding sites available. The presence of residual fatty acid, however, seems to have little effect upon the binding of anions of high affinity, and has no effect on the binding of uncharged compounds (26).

The physical properties of BSA have been studied extensively. It contains 147 titratable basic groups and 134 acidic groups (27) and has a molecular weight of 69,000 (28). Variation in pH has been shown to affect other properties of BSA as well as its binding ability. Decreasing pH results in increasing viscosity of the protein solution. A decrease in the sedimentation constant occurs below a value of pH 4 (29).

Electrophoretic heterogeneity of BSA has been observed in the region of the isoelectric point (30). These properties of microheterogeneity were first attributed to "extrinsic contaminants" (31). Imperfect disulfide pairing has been proposed as a possible explanation which could result from the equilibrium of the protein with isomers of open configuration, thereby giving rise to many possible combinations of disulfide pairs. This phenomenon is known to be influenced by many factors such as temperature, pH, and bound ligands, such as fatty acids. Microheterogeneity, however, is not entirely due to differences in tertiary structure resulting from disulfide variations, but is also of a covalent origin and is recognized to be entirely intra-

molecular with the dimer and polymer fractions remaining constant (32).

Calorimetric studies have shown an endothermic reaction to occur with the lowering of pH of BSA from 4.5 to 3.4. The reaction has a value of 3,100 calories/mole BSA and is reversible with an equal exothermic reaction. This reaction may be due to the unfolding of the protein molecule (33).

IV. Properties of Lysolecithin

Lysolecithin occurs in various biological systems but its exact role in many cases is unknown. The molecule is composed of a polar region formed by the phosphoric acid-choline side chain, and an apolar region formed from the acyl chain of the fatty acid.

Lysolecithin can be crystalized from hot pyridine into fine needles which form a pure white solid when dry, and it is readily soluble in ether, light petroleum and acetone. It softens on heating to 100°C and decomposes at 263°C. Its optical activity has been reported in various solvents (34). Lysolecithin dissolves in water to give a clear solution and has a critical micelle concentration of $1-2 \times 10^{-3}$ percent by weight. The viscosity, above a 37% (w/w) solution, increases with concentration, resulting in a thick but clear fluid at a 50% concentration. The amphipathic character of lysolecithin gives it strong surface active properties, similar to soaps. Its ability to lower the surface tension of water has been shown to not be affected by acids, bases or electrolytes (35).

Dissociation constants for lysolecithin have been determined to be: $K_1 = 0.18$ and $K_2 = 1.26 \times 10^{-12}$. It has an isoelectric range between pH 2.75 and 9.90. Surface films were claimed to have been formed at an air-water interface with a surface area/molecule of $108 \text{ \AA}^2$, which could be compressed to $65.5 \text{ \AA}^2$. These observations are questionable and may be due to lecithin contamination since lysolecithin is completely soluble in water (36).

Light scattering studies have revealed a micellar weight of around 100,000 with a monomer molecular weight of 515, giving the number of molecules per micelle as approximately 190 (37). Diffusion studies have given the value of 273 monomers per micelle (38).

The solubilizing effect of lysolecithin is an important property. It has the ability to solubilize homogenates of whole fresh rat brain and to hemolyze erythrocytes, with time being dependent upon concentration. It also has a great solubilizing effect on other lipid material (36).

Lysolecithin-lecithin sols possess a very high viscosity under certain conditions, which may be important in the structures of membranes (36). Saunders has proposed a theory of cell membrane formation based upon this (39).

V. Properties of the Lysolecithin-Protein Complex

Lipoprotein complexes containing lysolecithin have been studied with NMR techniques. The individual NMR spectra of lysolecithin and BSA were determined. The lysolecithin spectrum exhibited sharp choline and methylene peaks and a broad methyl peak.

Addition of BSA to the lysolecithin resulted in a lowering and broadening of the methylene peak of the lysolecithin. The choline peak of lysolecithin remained relatively unaffected by the addition of BSA (12). These spectra indicate that the methylene groups of the fatty acids of lysolecithin are being bound in some manner, i.e. their movement is restricted by the BSA.

Similar results have been obtained from NMR spectra of lysolecithin added to erythrocyte membrane fragments. The methylene signal was reduced and broadened, and some degree of broadening was observed for the choline signal. These results again indicate an association of the fatty acid of the lysolecithin with the protein (11).

PREPARATIONS AND ANALYSIS

I. Lysolecithin

The lysolecithin used in this study was obtained from Pierce Chemical Company (Rockford, Ill.) and was from Lot No. 1038-4. Solutions were made in distilled, deionized water and stored at 2°C. After approximately 48 hours a precipitate formed and it was necessary to warm the solutions slightly to obtain a clear solution. All solutions were used within ten days from the time they were initially prepared.

Analysis of the lysolecithin fatty acids by gas-liquid chromatography indicated the principal fatty acid was palmitic with smaller quantities of stearic and trace quantities of myristic, palmitoleic, oleic, and linoleic present (Table 1).

Table 1. Fatty acid composition of lysolecithin

Fatty Acid	% Composition
14:0	4.6
16:0	65.5
16:1	3.8
18:0	17.5
18:1	6.1
18:2	2.5

The lysolecithin used in this study was analyzed by thin layer chromatography with a chloroform, methanol, water (65:25:4, v/v/v) solvent system. Charring with chromic acid revealed the major component was lysolecithin with a trace quantity of lecithin and an unidentified contaminant at the solvent front. Densitometer scans of the TLC plate with a Densicord 542A densitometer indicated that 94% lysolecithin, 4% lecithin, and 2% unidentified contaminants were present.

II. Serum Albumin

All bovine serum albumin samples were obtained from Pentex Biochemicals, (Miles Laboratories, Kankakee, Ill.); crystalline BSA was from Lot No. 18, fatty-acid-free BSA was from Lot No. 12, and Fraction V BSA was from Lot No. 81. Analysis of Pentex Fraction V crystalline BSA from Lots No. 11, 14 and 15 by Hanson and Ballard (40), showed the presence of citrate as a contaminant. Lot No. 11 was reported to contain 52 μ moles citrate/mmole albumin, Lot No. 14 contained 253 μ moles/mmole and Lot No. 15 contained 252 μ moles/mmole. Information was not available as to the concentration of citrate in Lot No. 18. The concentration of pyruvate and lactate reported was less than ten μ moles/mmole except for lactate in Lot No. 11 which was reported to be 95 μ moles/mmole.

Solutions of BSA were prepared in distilled, deionized water, stored at 2°C, and used within 24 hours. A concentration of 0.7 grams/25 ml or 0.42 μ moles/ml, based on a molecular weight of 69,000 (28), was used throughout the study.

Fatty-acid-free BSA was reportedly prepared by removal of the fatty acids from Fraction V BSA by a modification of the low pH, charcoal method of Chen (42), which was followed by prolonged dialysis (41). It was specified to contain less than 0.1 equivalents of titratable acid per mole of albumin. Solutions were prepared under the same conditions as the crystalline BSA.

Monomer BSA was prepared according to the procedure of Pederson (42). Sephadex G-100 was swelled in buffer, 0.2 M NaCl plus 0.1 M Tris, pH 8.0 at 22°C (pH 8.4 at 3°C), and packed in a 2.5 x 100 cm column at 3°C to a column height of 90 to 95 cm. The packed column was washed with three void volumes of the above buffer prior to adding the samples. The samples consisted of one gram, either crystalline BSA or Fraction V BSA, dissolved in 5 ml buffer. Four ml fractions were collected and absorbance read at 278 nm with a Gilford 240 spectrophotometer. Figures 1 and 2 are typical separation curves.

Monomer fractions from four separations were pooled and subjected to dialysis against distilled water for a minimum of twelve hours at room temperature. The samples were then pooled and concentrated with Lyphogel (Gelman Instrument Company, Ann Arbor, Mich.). The pH of the resulting solution varied from 6.8 to 7.0 and was adjusted to 7.0 when necessary with dilute NaOH. The final concentration of the monomer BSA was adjusted to equal that of a known concentration (0.42 μ moles/ml) of crystalline BSA using absorbance readings at 278 nm (Gilford 240 spectrophotometer).

Figure 1. Sephadex separation of crystalline BSA

The dimer component of BSA was the first peak eluted from the Sephadex G-100 column with the monomer peak, the larger of the two, appearing last. Four ml fractions were collected and the absorbance read at 278 nm.

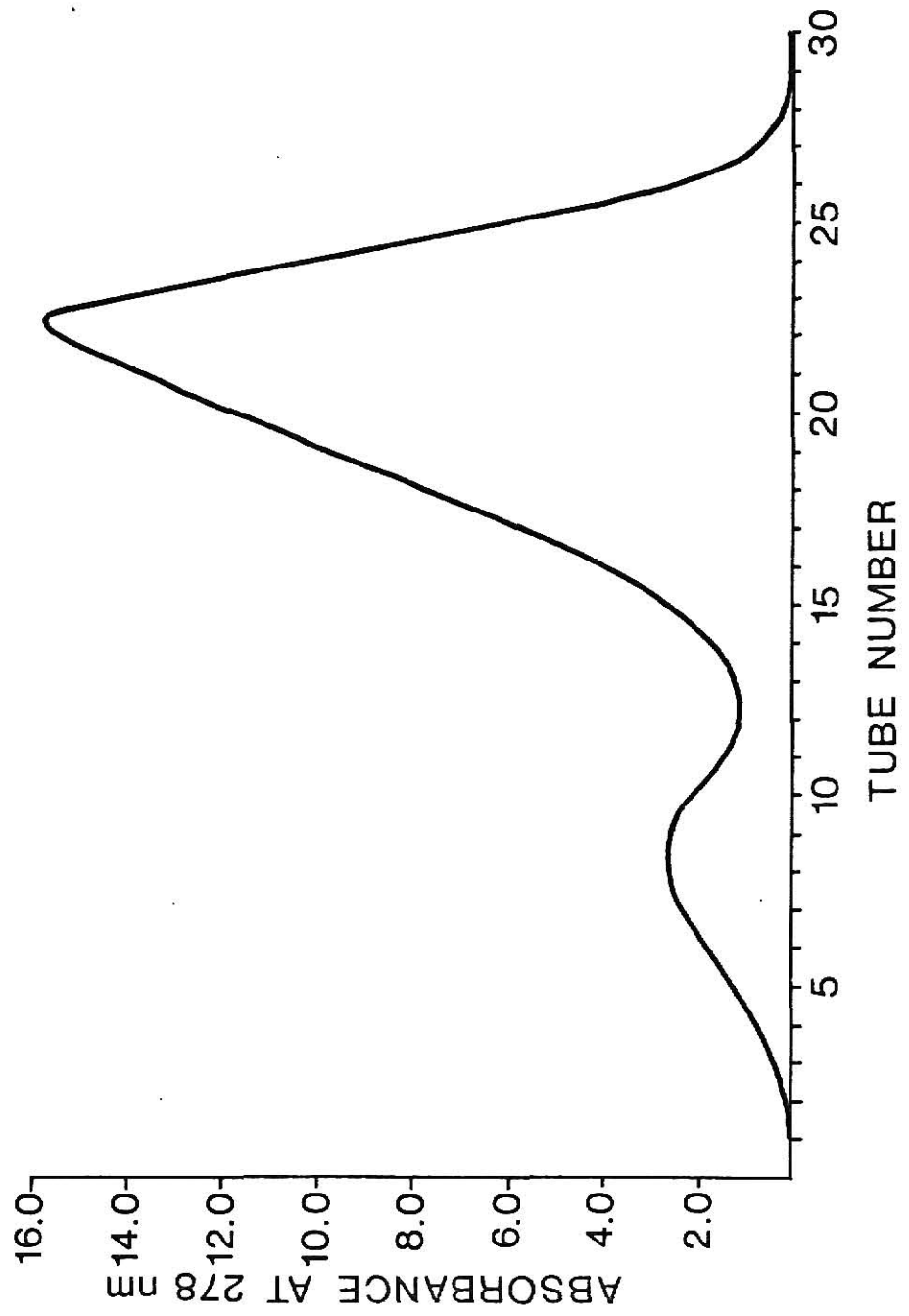
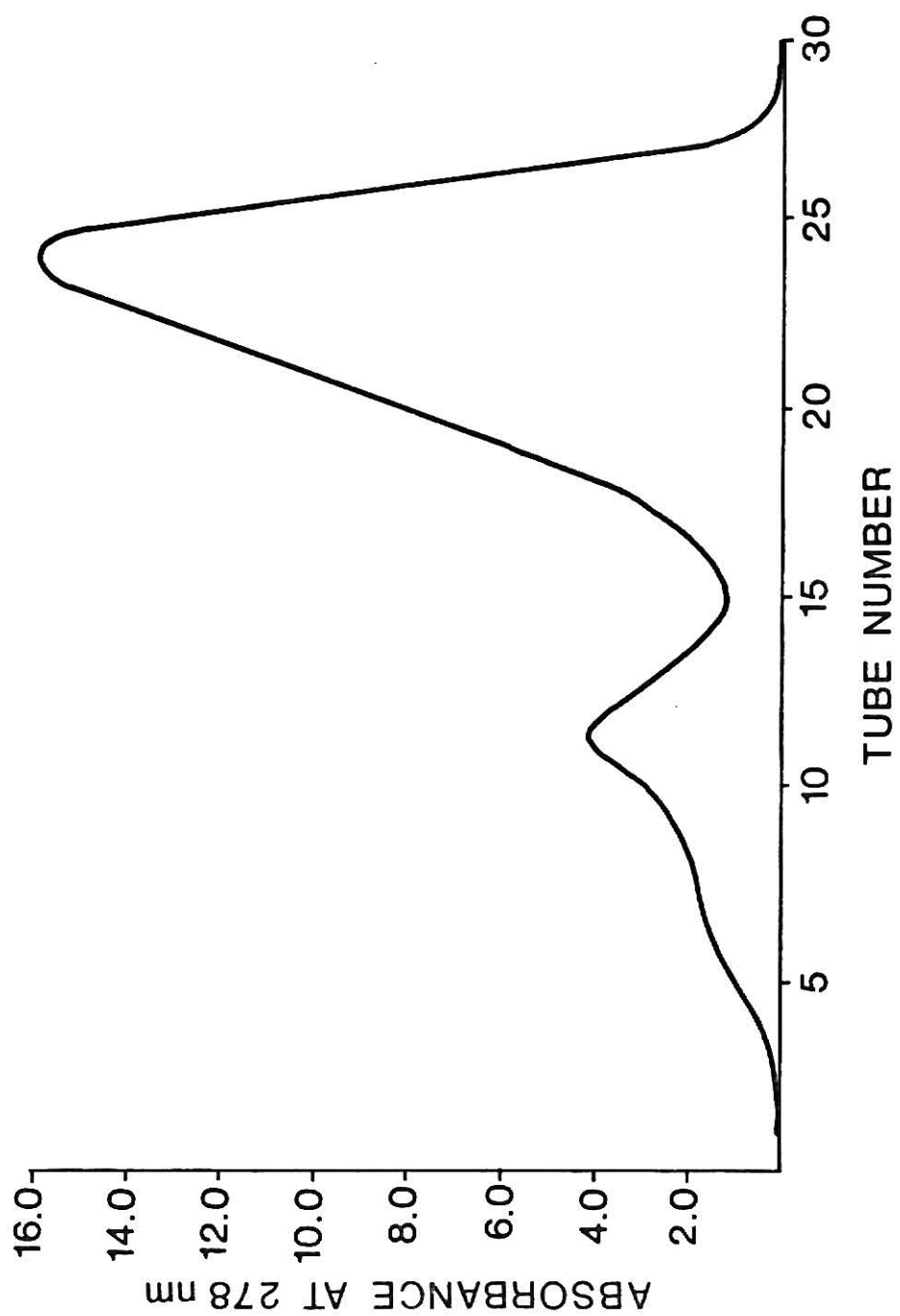


Figure 2. Sephadex separation of Fraction V BSA

Globulin is present in this BSA preparation and was eluted from the column slightly ahead of the dimer component (shoulder). The larger monomer peak appears last. Four ml fractions were collected and the absorbance read at 278 nm.



Disc gel electrophoresis of crystalline BSA exhibited a major monomer band, a minor dimer band and a faint trimer band. A scan of the gels with a Densicord 542A densitometer indicated the dimer band to be approximately 13 % and the monomer 87% with the trimer less than 1% (Figure 3). The prepared monomer exhibited a slight dimer peak but was calculated to be less than 3% from the density scans (Figure 4).

Figure 3. Densitometer scan of disc gel electrophoretic separation of crystalline BSA

The monomer is the larger peak and migrates faster toward the anode than the smaller dimer peak.

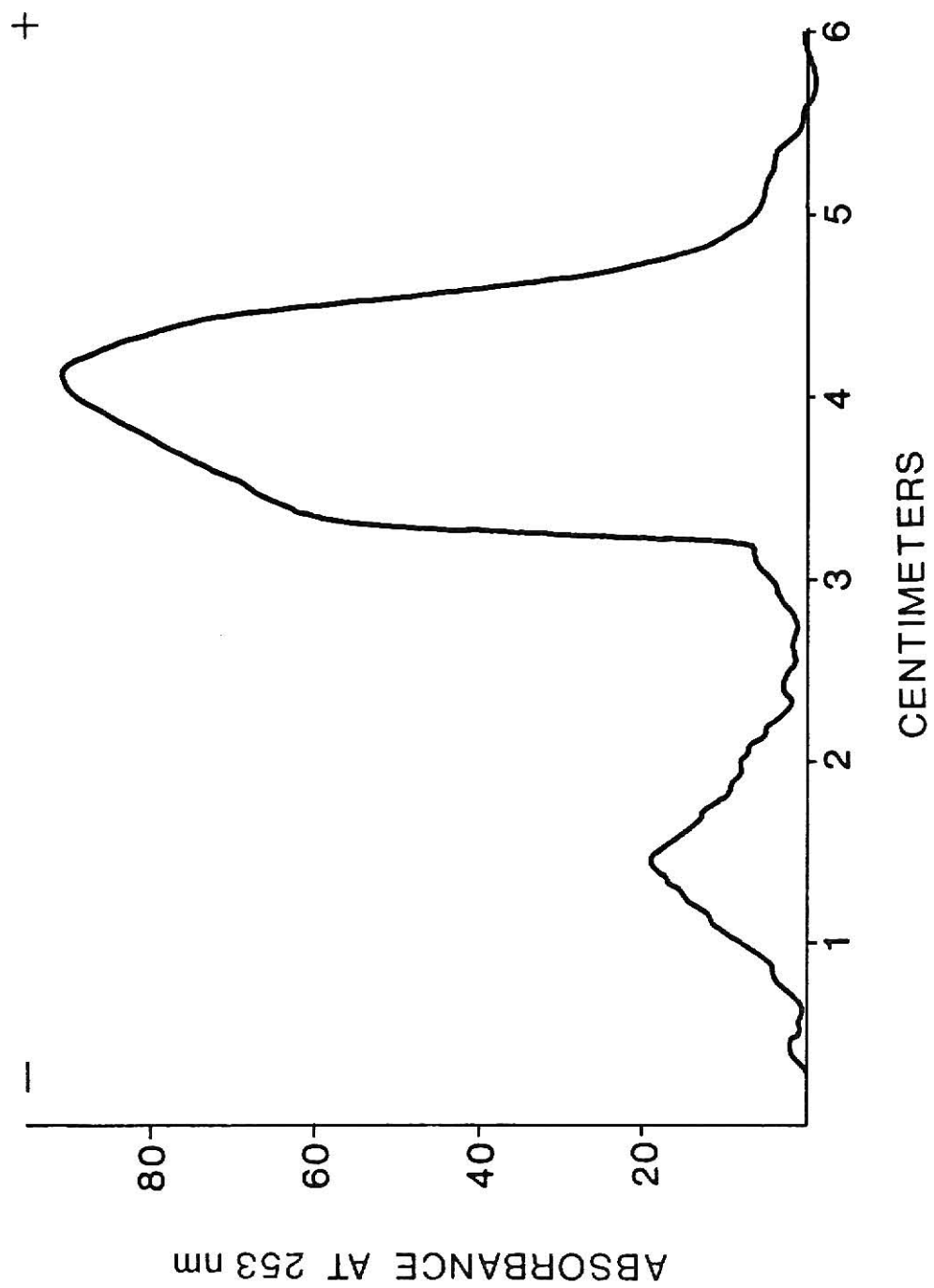
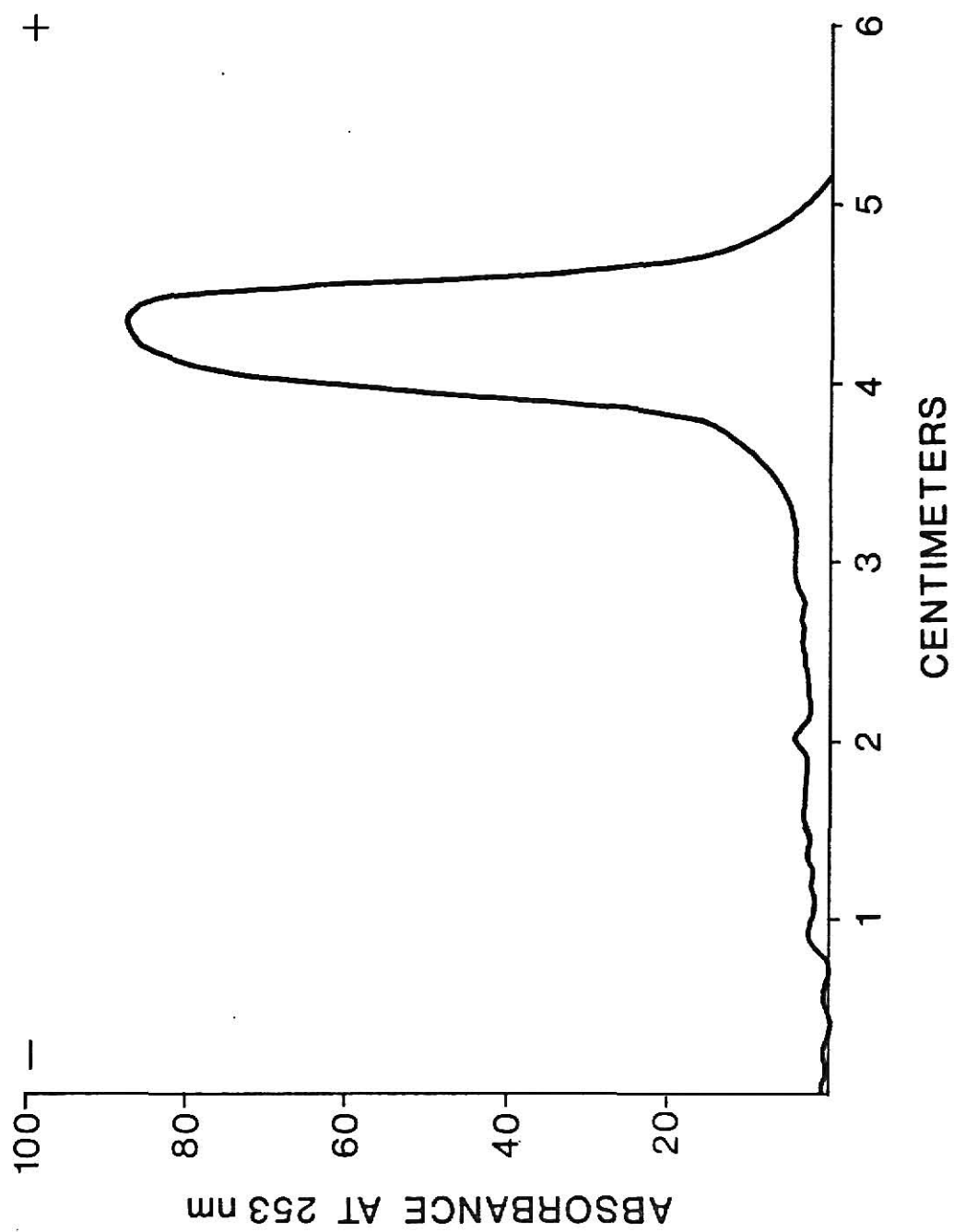


Figure 4. Densitometer scan of disc gel electrophoretic separation of previously prepared monomer component of BSA.

The monomer peak, which migrates the fastest, is the only major peak present. Trailing of the monomer peak may also contain small amounts of dimer.



PROCEDURE

The calorimeter used in this study was built according to the design of Benzinger and Kitzinger (43). This "heatburst" microcalorimeter rapidly transfers heat from the reaction area through thermocouples into the surrounding heat sinks. It is a twin system with one chamber containing a blank cell whose change in enthalpy is subtracted electrically from that of the reaction cell. The instrument was operated in a constant temperature room held at $20^{\circ} \pm 1^{\circ}\text{C}$.

The BSA and lysolecithin solutions previously described were measured into the individual compartments of bicompartmental calorimetry cells. The volume relationships were kept constant at 2.5 ml BSA and 1.25 ml lysolecithin, giving a total volume upon mixing of 3.75 ml. The protein concentration was kept constant at 0.42 umoles/ml and the lysolecithin concentration was varied to provide a range of reaction ratios. Blank cells contained 2.5 ml BSA and 1.25 ml deionized, distilled water in the individual compartments. The cells were then stoppered and placed in the calorimeter, the calorimeter was sealed with heat sinks, insulated, and allowed to come to equilibrium (30-120 min).

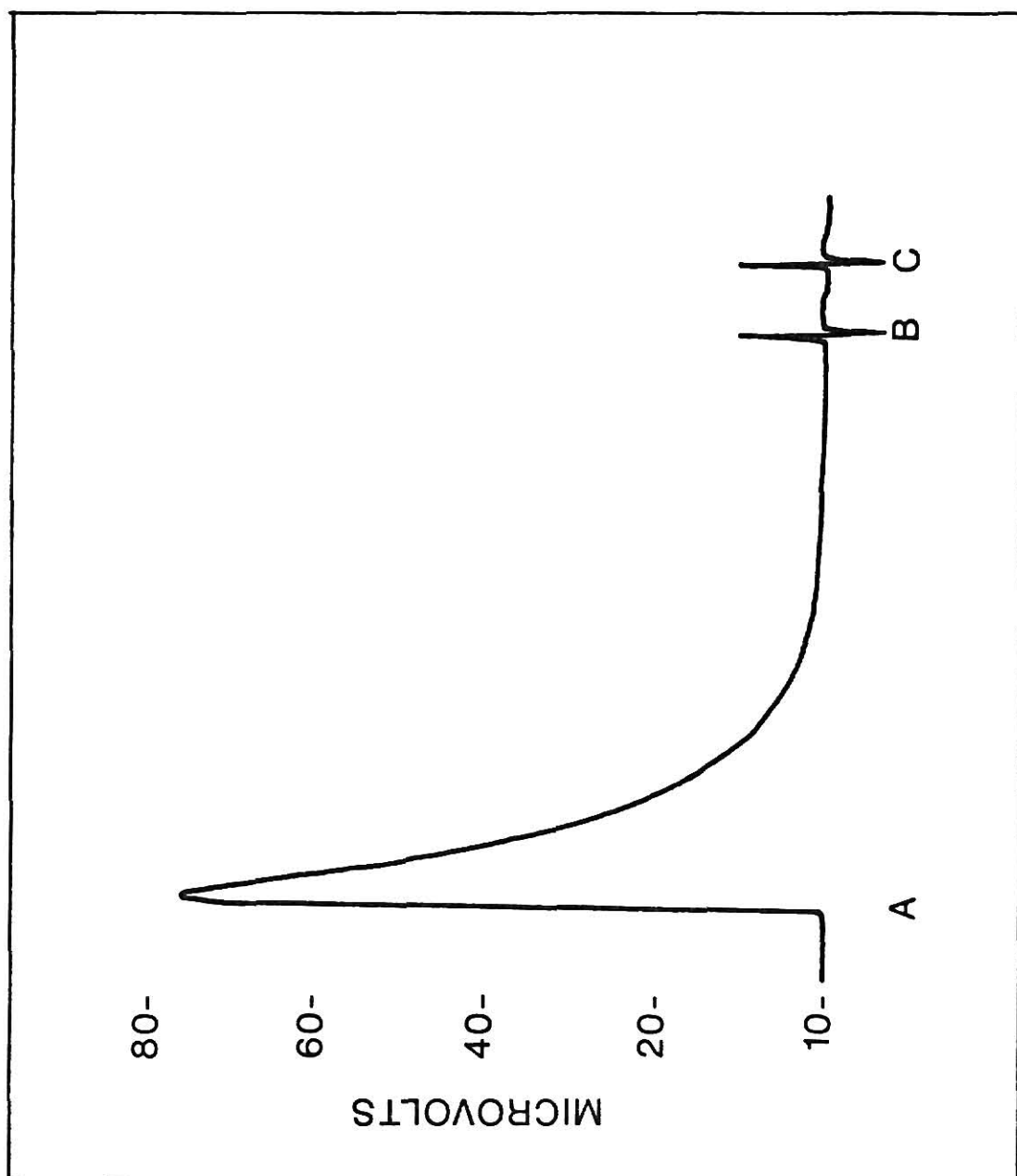
The reaction was initiated by rotation of the reaction chamber which allowed mixing of the reactants. When the reaction was complete, as determined by the instrument returning to the baseline, the calorimeter was again rotated a minimum of two additional times to insure complete reaction and to determine any peaks due to rotation or agitation of the fluids. Reactions

were determined to be exothermic by comparison with known reactions. A typical reaction peak is shown in Figure 5.

The areas under the curves were computed with a K&E #4236 compensating polar planimeter. The areas of negative mixing peaks, in relation to the reaction peak, were added to the area of the reaction peak. Positive peak areas for mixing were subtracted unless they appeared to be due to incomplete initial mixing of the reactants, in which case they were considered as a second reaction peak. The areas obtained were converted to millicalories by factors previously determined for the instrument (44).

Figure 5. Heat change on binding lysolecithin to BSA

The point of initial mixing is designated A. Points B and C are additional mixing spikes of zero area. The area under the reaction peak represents 17.53 mcal which resulted upon the mixing of 1.05 μ moles BSA and 2.88 μ moles lysolecithin.



RESULTS AND DISCUSSION

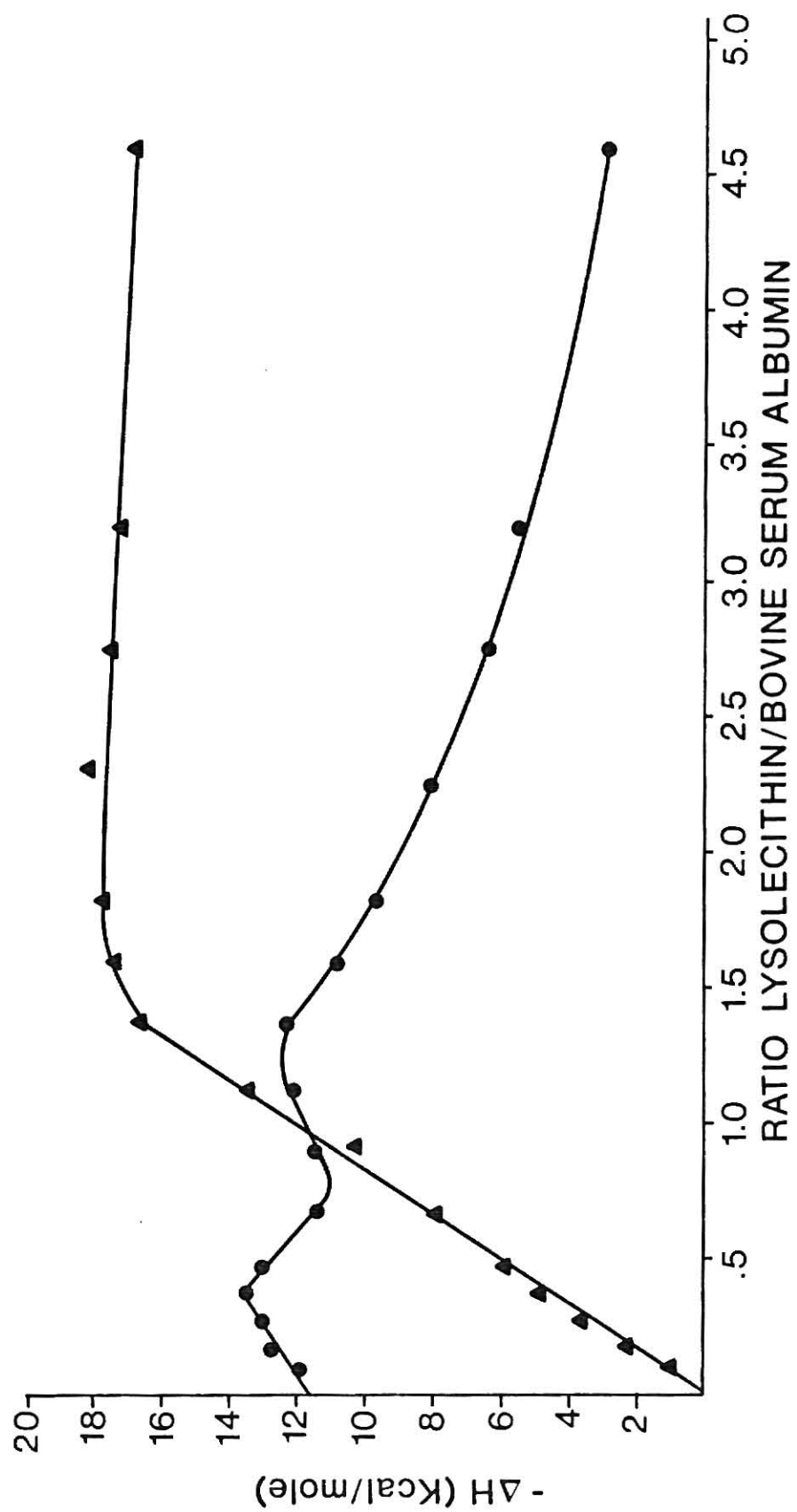
I. Crystalline BSA

The binding titration curves formed from lysolecithin binding to crystalline BSA are shown in Figure 6. A slight negative slope is observed for the enthalpy/mole BSA curve, above the saturation ratio of approximately 1.85. The predicted result would be a slope of zero above this ratio since the BSA is assumed to be saturated and subsequent addition of lysolecithin should not affect the maximum enthalpy thus obtained. This result may be due to the presence of small amounts of shorter chain fatty acids in the lysolecithin which have lower binding energies and compete with the longer chain fatty acids. This curve was extrapolated to the ordinate to obtain the maximum enthalpy/mole of BSA at saturation. The value determined was -18.2 kcal/mole BSA which was the same value as was obtained in a previous study by Klopfenstein (17).

Another facet of the titration curves involves two inflections in the lysolecithin curve with peaks at ratios of 0.40 and 1.25. The positive slope observed at ratios less than 0.40 was extrapolated to -11.8 kcal/mole lysolecithin. These inflections were not observed in previous studies by Klopfenstein (17,18). A possible explanation for this difference could be the difference in the source of lysolecithin and subsequent variance in fatty acid composition and contaminants such as lecithin and metal ions. These phenomena will be discussed later. Extrapolation of the lysolecithin curve to the ordinate from higher ratios not

Figure 6. Enthalpy curves of lysolecithin binding by crystalline BSA.

Enthalpy change was computed on a molar basis for ratios of moles lysolecithin to moles crystalline BSA and was expressed as kcal/mole lysolecithin (o-o) and kcal/mole BSA (Δ - Δ).



involving the inflections, gave a value of -18.8 kcal/mole lysolecithin. This was comparable to the value obtained at the ordinate for the BSA curve (-18.2 kcal/mole BSA). The value obtained by Klopfenstein (17) was -18.0 kcal/mole.

Since both curves extrapolated to essentially the same value at the ordinate, and these changes represent enthalpy change per mole of each constituent, a one to one binding ratio was assumed.

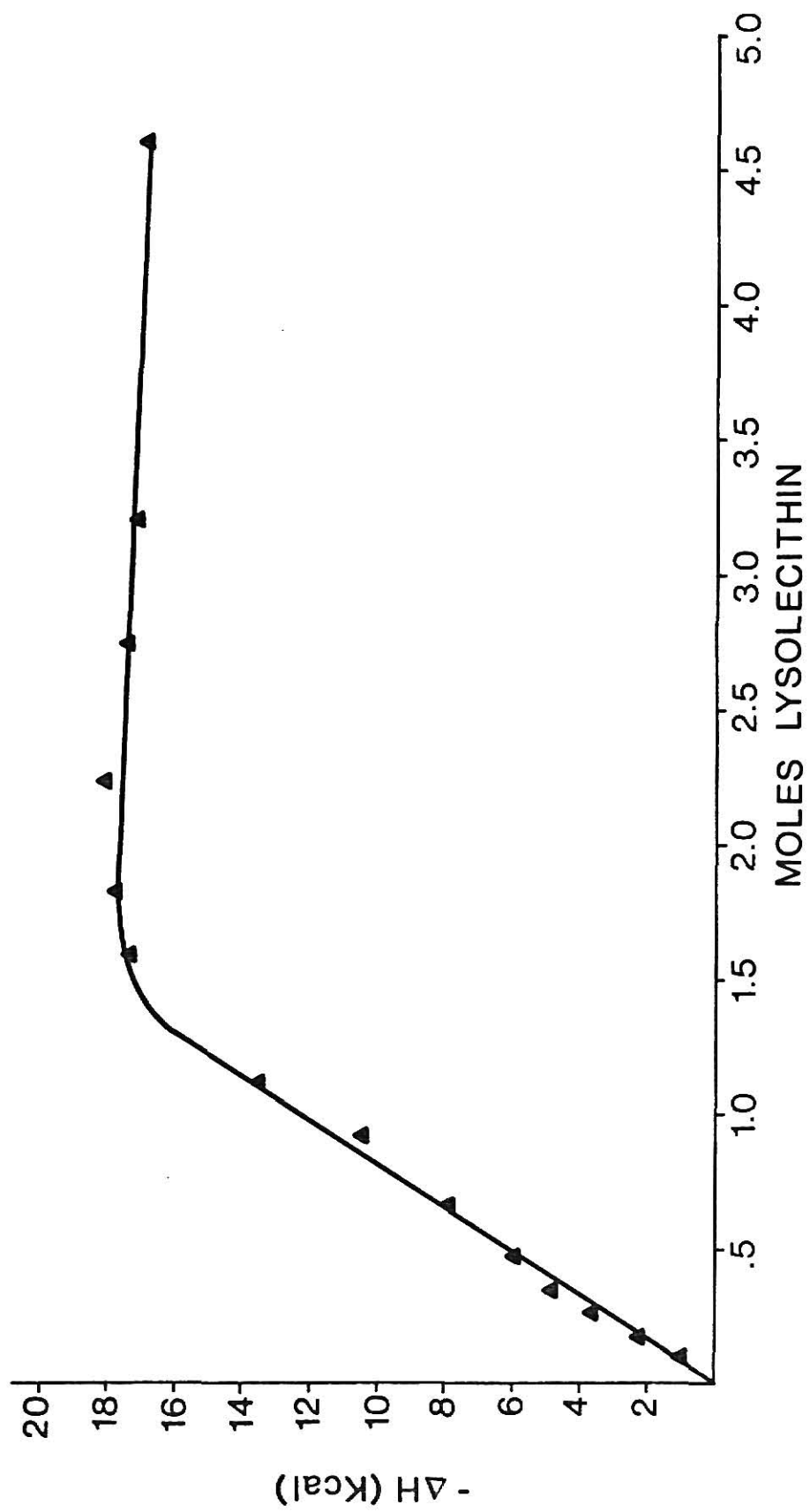
A variation of Figure 6 is shown in Figure 7. The ordinate is now kcal measured and the abscissa is moles lysolecithin added. The concentration of BSA remained constant and is expressed on a mole basis. This curve corresponds to the kcal/mole BSA curve in Figure 6.

The equilibrium constants at different concentrations of lysolecithin were calculated from Figure 7. The percent of the BSA bound by lysolecithin was calculated from the percent the enthalpy change was of maximum, assuming a binding ratio of one to one. The quantity of lysolecithin bound was therefore equal to the quantity of BSA bound and the free components were determined by subtraction of the quantity bound from the original quantity of each added. The volume factor for calculations of BSA and lysolecithin on a mole basis was 3570 liters.

Sample Calculation:

For the point of 0.5 moles lysolecithin added (Figure 7):

$$\frac{6.2 \text{ kcal measured}}{18.2 \text{ kcal maximum}} = 34.1\% \text{ of maximum binding}$$



Since 1 mole BSA is maximum binding:

$0.341 \times 1 = 0.341$ moles BSA bound = 0.341 moles complex

Free BSA: $1.0 \text{ mole} - 0.341 \text{ moles} = 0.659 \text{ moles}$

Free lysolecithin: $0.5 \text{ moles} - 0.341 \text{ moles} = 0.16 \text{ moles}$

Solvent volume/mole BSA = 3570 liters

$$K_{eq} = \frac{0.341 \text{ moles}/3570 \text{ liters}}{(0.16/3570)(0.659/3570)} = 1.15 \times 10^4$$

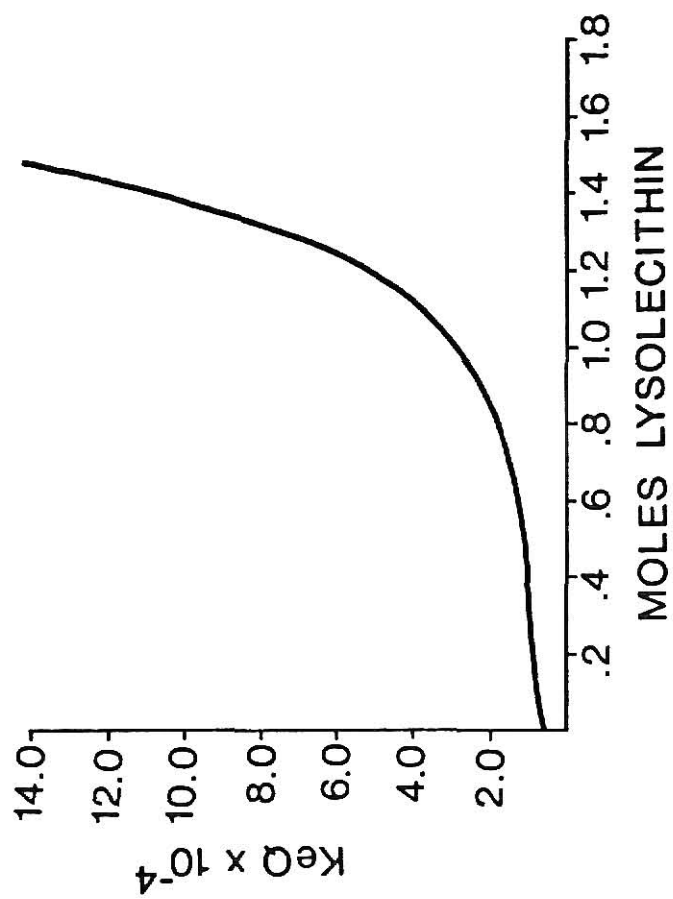
The equilibrium constants determined at each concentration of lysolecithin are shown in Table 2 and are represented graphically in Figure 8.

Table 2. Equilibrium constants at different moles lysolecithin added.

Moles Lysolecithin	% Saturation	K_{eq}
0.10	6.6	7.42×10^3
0.25	17.0	9.14×10^3
0.50	34.1	1.15×10^4
0.75	51.7	1.60×10^4
1.00	69.3	2.60×10^4
1.25	86.5	5.90×10^4
1.40	93.3	1.06×10^5
1.50	95.5	1.39×10^5
1.75	98.9	4.20×10^5
1.80	99.5	8.50×10^5

Figure 8. Variation of equilibrium constants.

The equilibrium constants vary with the concentration of lysolecithin as outlined in Table 2.



The equilibrium constants were extrapolated to the ordinate to give the equilibrium constant for the initial reaction. This may be better termed the binding constant since it is for the initial binding reaction. The value obtained for the constant was 6.0×10^3 and was used for the determination of free energy change. Free energy change was calculated from the equation:

$$\Delta G^0 = -RT \ln K_{eq}$$

The free energy change was found to be -3.7 kcal/mole. Change in entropy was then determined using the following relationship:

$$\Delta G = \Delta H - T\Delta S$$

The value obtained was 49.5 entropy units with -14.5 kcal/mole entropy contribution to free energy. The negative entropy change indicates that hydrophobic bonding is not occurring since this requires an increase in entropy for the driving force. The negative entropy change indicates an increase in ordering of the system which could result from immobilization of the fatty acid chain by the protein which might also result in partial immobilization of the BSA.

Below the lysolecithin value of 1.25 (Figure 7) the slope of the curve is constant at -12.5 kcal/mole lysolecithin. This constant slope indicates that 70% (12.5kcal/18.2 kcal) of the lysolecithin added is being bound. A true equilibrium would not result in a constant slope but would increase in curvature until it approached saturation. With approximately 70% of every quantity of lysolecithin added being bound, the question arises as to the fate of the remaining 30% of the lysolecithin which was not bound. If the equilibrium constant were infinity, the slope

of the line would again be constant but would be -18.2 kcal/mole lysolecithin added or essentially 100% bound until saturation was reached. From the calculations of equilibrium constants as shown in Table 2 it can be seen that they are lower at zero moles lysolecithin added and approach infinity as greater quantities of lysolecithin are added. A possible explanation for this phenomenon would be the presence of a second binding site on the protein for lysolecithin. Its affinity for lysolecithin could be slightly less than the one under study having the affinity to bind 70% and the hypothetical one having the affinity to bind 30%. These observations would predict that the enthalpy change upon binding for the second site would have to be very near zero in order for its effect not to have been exhibited in the calorimetric measurements. For the reaction to be spontaneous, it would have to have a negative free energy change. With an essentially zero enthalpy change a positive entropy would result which would account for the driving force for the binding. If this were occurring the reaction could then be termed hydrophobic.

The presence of this proposed second binding site with a slight positive enthalpy change could also explain the slight decrease in absolute enthalpy change values measured at higher concentrations of lysolecithin.

Further exploration of this phenomenon utilizing additional physical and chemical methods will be necessary to firmly establish the existence and properties of a second binding site.

II. Fatty-Acid-Free BSA

Crystalline BSA is known to contain fatty acids bound in an approximate molar ratio of two to one (40). It was necessary to determine if the fatty acid present interfered with the binding of lysolecithin by occupying the same binding site or possibly influencing the conformation of the protein to alter the binding of lysolecithin.

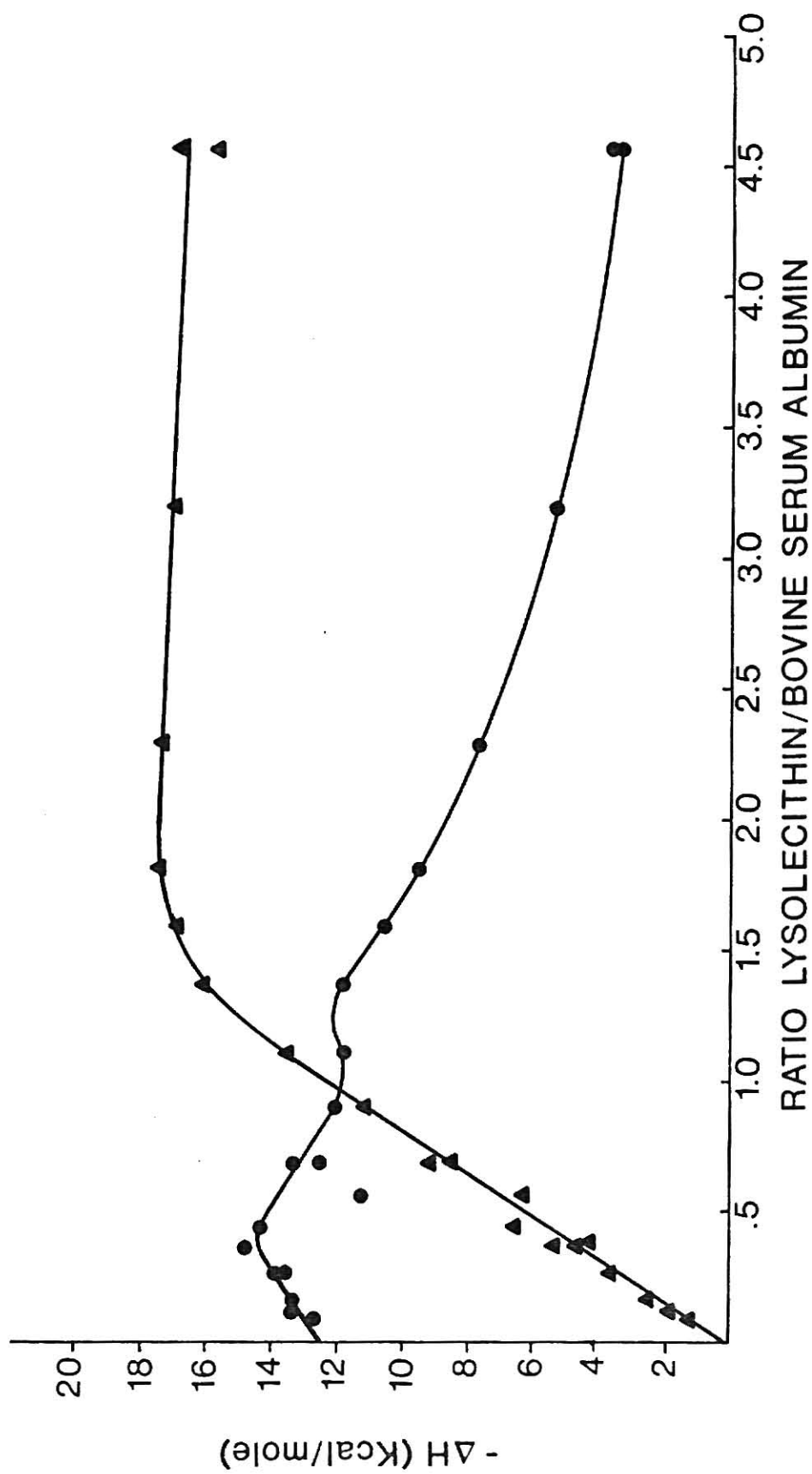
Fatty-acid-free BSA was obtained and its binding properties for lysolecithin compared to those of the crystalline BSA. The binding titration curves of lysolecithin to fatty-acid-free BSA are shown in Figure 9. They exhibited essentially the same properties as those of crystalline BSA. The value of -18.0 kcal/mole BSA was obtained upon extrapolation of the BSA curve to the ordinate. The corresponding lysolecithin enthalpy curve gave a predicted value of -18.8 kcal/mole lysolecithin when extrapolated from a ratio of 1.35 to the ordinate. This titration curve also exhibited inflections in the lysolecithin enthalpy curve at the same ratios as the crystalline BSA. The positive slope observed in this region was extrapolated to -12.4 kcal/mole lysolecithin at the ordinate, as compared to a value of -11.8 kcal/mole for the crystalline BSA.

III. Monomer BSA

An equilibrium exists between the monomer BSA and small quantities of dimer and trimer. The presence of approximately 13% dimer in the crystalline albumin used in this study could possibly affect the binding of lysolecithin, again, by occupying

Figure 9. Enthalpy curves of lysolecithin binding by fatty-acid-free BSA.

Enthalpy change was computed on a molar basis for ratios of moles lysolecithin to moles fatty-acid-free BSA. Enthalpy was expressed as kcal/mole lysolecithin (o-o) and kcal/mole fatty-acid-free BSA (Δ - Δ).



the binding site or altering the conformation. It was therefore necessary to prepare the pure monomer component.

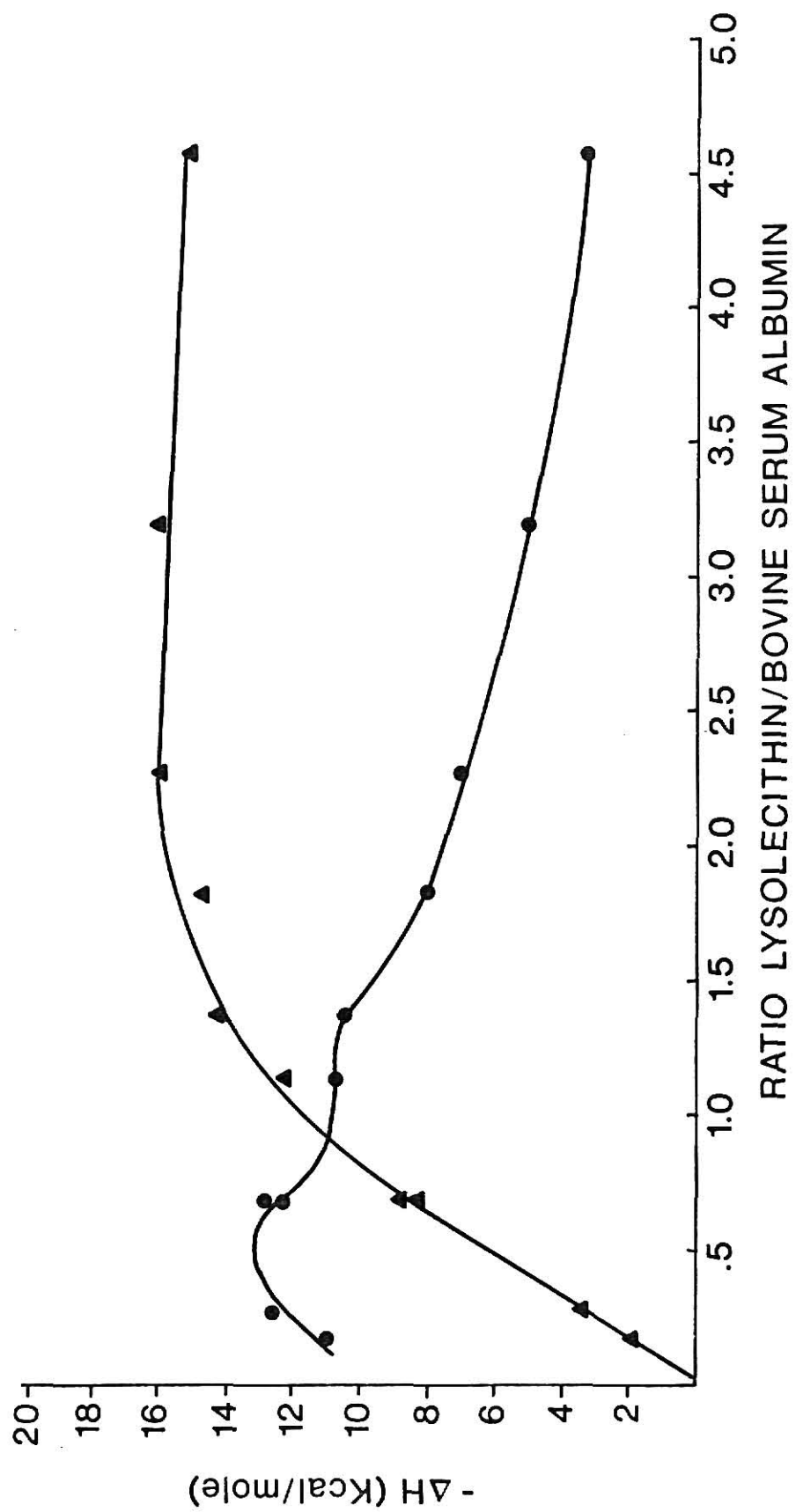
The binding titration curves for the monomer are shown in Figure 10. A decrease in maximum absolute enthalpy/mole BSA was observed for the monomer binding of lysolecithin. This curve extrapolated to an enthalpy value of -16.6 kcal/mole BSA at the ordinate. The slope of the lower ratios of the curve was identical to that of the crystalline BSA but a flattening of the inflection peaks was noted. Precise and extensive values in the lower ratios were not obtained due to a limited supply of the monomer.

The decrease in the absolute enthalpy values observed is the opposite of what would be expected if the dimer altered or occupied the binding site. The pure monomer would be expected to exhibit maximum enthalpy of binding with the presence of the dimer acting to decrease this potential. However it would be conceivable for the dimer to alter conformation or influence the site to increase the binding of lysolecithin by the protein.

The separation, subsequent treatment, and binding of the monomer required in excess of two weeks to complete. The possibility of denaturation of the protein occurring, and leading to the observed results, therefore was considerable. This possibility led to the study of the effects of binding of lysolecithin by denatured BSA.

Figure 10. Enthalpy curves of lysolecithin binding by the monomer component of BSA.

Enthalpy change was computed on a molar basis for ratios of moles lysolecithin to moles denatured BSA. Enthalpy was expressed as kcal/mole lysolecithin (o-o), and kcal/mole BSA (Δ - Δ).



IV. Denatured BSA

Lyophilization was first tried as a means of denaturing the protein. Five percent (w/v) and one percent solutions were lyophilized. It was assumed that the more dilute would be the more denatured of the two. Subsequent binding of each by lysolecithin revealed little difference from that observed with the crystalline BSA. This was rejected as a controlled method of denaturation.

Urea was then used as a denaturing agent on the BSA. The lysolecithin was bound to BSA in varying concentrations of urea. The results obtained were varied and not reproducible. The heat of dilution of a urea solution is quite considerable, therefore the slightest difference in concentration of urea between the two reactant solutions would mask the enthalpy of the binding reaction. Denaturation by this method was therefore rejected.

The next method to be attempted was heat denaturation. Heating a reaction solution of crystalline BSA at 50°C for twenty minutes resulted in a colloid suspension. The solution was cooled immediately and binding experiments performed. The suspension gave values which fell on the same curves as the crystalline BSA. Subsequent denaturation of the crystalline BSA resulted in a precipitate forming even when heated at 40°C for two hours. The formation of the precipitate was quite rapid, once initiated, and it became apparent that the crystalline BSA could not be denatured and remain in solution.

Fatty-acid-free BSA was then denatured for the purpose of comparing the solution obtained when a colloid began to appear.

The fatty-acid-free BSA did not form a colloidal suspension after similar treatment (50°C , 20 min). The heating time and temperature were increased until a total heating time of four hours and a maximum temperature of 90°C were reached. This solution was used for binding and the results are shown in Figure 11. Denaturation reduced the binding capacity, but the BSA still retained a residual capacity for binding of the lysolecithin. Further attempts were made to form a colloidal suspension of the fatty-acid-free BSA but without success. It was therefore concluded that heat denaturation would precipitate the crystalline BSA and would not precipitate the fatty-acid-free BSA.

Fatty-acid-free BSA was then heated for 2.5 hours at 50°C and then bound by lysolecithin immediately after equilibrating the solution to 20°C . Enthalpy of binding at ratios of 0.5 to 3.2 were then determined. The maximum observed enthalpy of the BSA curve was approximately -13.6 kcal/mole which is considerably lower than the -17.6 kcal/mole observed for the crystalline BSA (Figure 12). Extrapolated values were not compared because of the limited number of points in the denatured BSA curve.

A tentative explanation for the two areas of decreased absolute enthalpy values near the ratios of 0.40 and 1.25 of the lysolecithin curve would involve conformational changes of the BSA. These conformational changes may be initiated at; 1) initial introduction of lysolecithin to the protein solution or 2) at a critical lysolecithin concentration which is reached at a lysolecithin/BSA ratio of 1. This does not mean that 100% binding

Figure 11. Enthalpy curves of lysolecithin binding by heat denatured BSA (4 hours).

Enthalpy change was computed on a molar basis for ratios of moles lysolecithin to moles denatured BSA. Enthalpy was expressed as kcal/mole lysolecithin (o-o), and kcal/mole BSA (Δ - Δ).

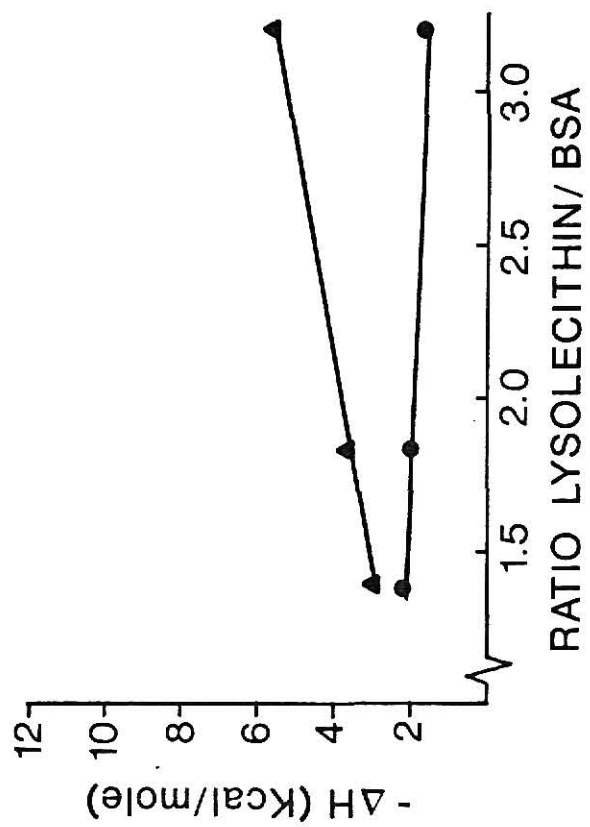
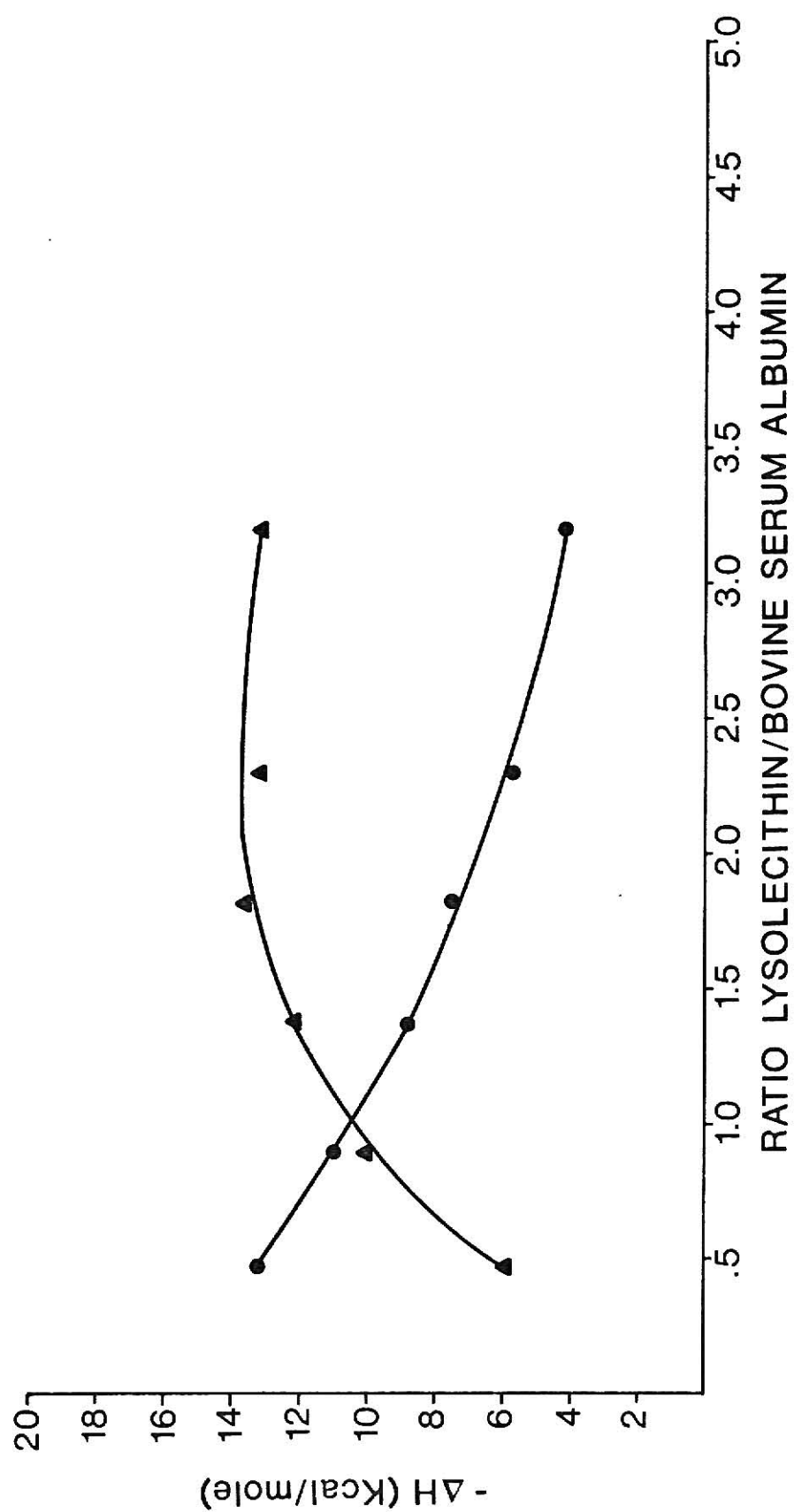


Figure 12. Enthalpy curves of lysolecithin binding by heat denatured BSA (2.5 hours).

Enthalpy change was computed on a molar basis for ratios of moles lysolecithin to moles denatured BSA. Enthalpy was expressed as kcal/mole lysolecithin (o-o), and kcal/mole BSA (Δ - Δ).



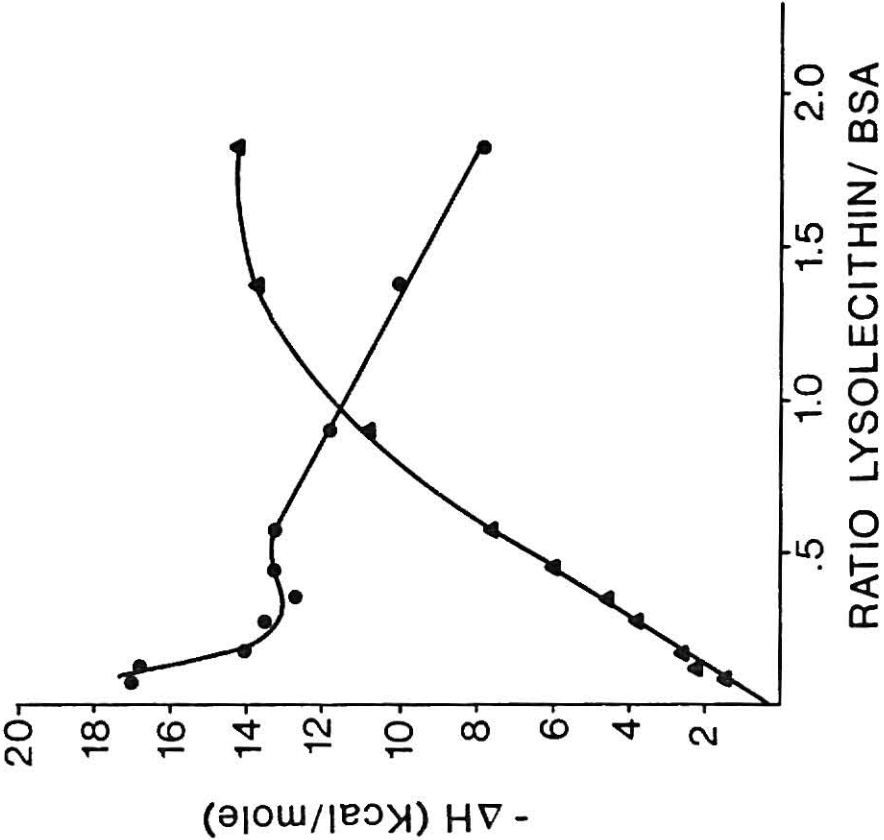
is occurring at a ratio of 1, but simply that the solution contains equimolar quantities of each component in an equilibrium state.

Conformational changes are often endothermic (45) and if combined with an exothermic binding reaction would result in a net decrease in the absolute value of the negative enthalpy which is measured. This would lead to the results observed in the lysolecithin curve.

A possible way of determining if this were occurring was again the observation of lysolecithin binding by BSA. If the conformational change were an unfolding of the protein molecule, denaturation would be expected to have achieved this prior to the introduction of the lysolecithin and hence the endothermic reaction would not occur. Observation of the lysolecithin binding curve of denatured BSA shown in Figure 12 did not reveal a inflection at a ratio of 1.25. Another BSA solution (fatty-acid-free) was then denatured for 1.5 hours at 50°C. The reason for the shorter heating period from that in Figure 12 was to denature the protein enough to result in the desired conformational change and to reduce the maximum enthalpy as little as possible. The binding curve thus obtained is shown in Figure 13. The maximum enthalpy measured was reduced to -14.2 kcal/mole BSA as compared to -17.6 kcal/mole BSA for the native protein. However, the inflection at a ratio of 1.25 is no longer observed and the initial slope is now negative as was observed originally by Klopfenstein (17,18) and as would be predicted from extrapolation of the lysolecithin curve from higher ratios to a zero ratio.

Figure 13. Enthalpy curves of lysolecithin binding by heat denatured BSA (1.5 hours).

Enthalpy change was computed on a molar basis for ratios of moles lysolecithin to moles denatured BSA. Enthalpy was expressed as kcal/mole lysolecithin (o-o), and kcal/mole BSA (Δ - Δ).



These results are not conclusive support for the theory that the two areas of decreased absolute enthalpy are due to conformational changes but they tend to strongly support this explanation. Additional studies of the phenomena will be necessary for a complete explanation.

The absorbance of the denatured BSA was run on the Cary (Model 14) recording spectrophotometer in an attempt to determine the exact percent denaturation. These results were rejected on the basis that a 1 to 21 dilution was necessary to bring the concentration of the protein solutions into the range of the instrument. The small differences in concentration resulting from the dilution could easily mask any small changes in absorbance from the denaturation. The only measure of denaturation therefore was the time and temperature of the heating.

CONCLUSION

Microcalorimetric measurements have revealed that crystalline BSA contains a binding site for lysolecithin, which when bound results in an enthalpy change of approximately -18 kcal/mole for both BSA and lysolecithin. This has led to the conclusion that this site on the BSA molecule binds one molecule of lysolecithin.

Two inflections in the kcal/mole lysolecithin curve appear at lower ratios (0.40 and 1.25 lysolecithin/BSA) in the lysolecithin curve. Slight denaturation leads to an increase in the absolute enthalpy values in these two regions and more closely approximates the enthalpy curve which would be obtained by extrapolation from higher ratios and which also was previously reported by Klopfenstein (17,18). This suggests that a conformational change occurs in the protein when the lysolecithin is added initially and at a slightly higher ratio approaching 1.25. These conformational changes could result in positive enthalpy and thus cause a decrease in the absolute enthalpy values observed for the binding reaction.

The equilibrium constant for the reaction was determined to be 6.0×10^3 with a free energy change of -3.7 kcal/mole and an entropy contribution to free energy of -14.5 kcal/mole. The enthalpy curve, measured on a molar basis, when lysolecithin was added to a fixed quantity of BSA, did not represent a true equilibrium. A constant amount of lysolecithin added (70%) appeared to be bound by the BSA until saturation was approached. This suggests that 30% of the lysolecithin added is acted upon in some other

manner. The possibility of the presence of an additional binding site therefore exists. This additional site, in order not to have been observed by the microcalorimetric measurements, would have to yield a small enthalpy change upon binding of lysolecithin. Other phenomena, however, could be occurring which would result in the constant fraction of lysolecithin being bound by the site under study.

Additional binding studies with fatty-acid-free BSA and the monomer fraction of BSA produced no significant difference in binding of lysolecithin. The monomer fraction exhibited a decrease in maximum absolute enthalpy which is quite probably due to denaturation of the protein during the preparation procedure. However, the overall pattern remained the same as for the crystalline BSA. Binding studies on denatured BSA exhibited the same decrease in maximum absolute enthalpy as was observed with the monomer binding of lysolecithin.

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STUDIES ON SERUM ALBUMIN BINDING OF LYSOLECITHIN

by

CANDICE ANN MORGAN

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AN ABSTRACT OF A MASTER'S THESIS

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The properties of bovine serum albumin (BSA), binding of lysolecithin were studied by microcalorimetric measurements.

Enthalpy changes were measured upon the binding of varied quantities of lysolecithin to a constant quantity of BSA, with both volumes of reactants remaining constant. Titration curves were determined with enthalpy, (kcal/mole BSA and kcal/mole lysolecithin), versus ratio lysolecithin to BSA. Ratios from zero to 4.6 were studied. Maximum enthalpy was determined by extrapolation of the curves to the ordinate. Calculations of equilibrium constants were made from this maximum enthalpy value and from calculations of percent bound obtained from the curves.

The equilibrium constant for the reaction was determined to be 6.0×10^3 with a free energy change of -3.7 kcal/mole and an entropy contribution to free energy of -14.5 kcal/mole. The enthalpy curve, measured on a molar basis, when lysolecithin was added to a constant quantity of BSA, did not represent a true equilibrium. A constant amount of lysolecithin (70%) was bound by the BSA until saturation was approached. This suggests that 30% of the lysolecithin added is acted upon in some other manner. The possibility of the presence of an additional binding site on the BSA therefore exists. This additional site, in order not to have been observed by the microcalorimetric measurements, would have to yield a small enthalpy change upon binding of lysolecithin. Other phenomena however could be occurring, which would result in the constant fraction of lysolecithin which was bound by the site under study.

Two deviations in the kcal/mole lysolecithin curve appear at low ratios, (0.40 and 1.25 ratios lysolecithin/BSA), in the lysolecithin curve. Slight denaturation leads to an increase in negative enthalpy in these two regions and more closely approximates the enthalpy curve which would be obtained by extrapolation from higher ratios. This suggests that a conformational change occurs in the protein when the lysolecithin is added initially and at a ratio approaching 1.25. These conformational changes could result in positive enthalpy and thus cause a decrease in total negative enthalpy observed for the binding reaction.

The enthalpy of binding curves for fatty acid free BSA by lysolecithin were determined for comparison with the enthalpy of binding curves obtained with crystalline BSA. The fatty acid free BSA exhibited no significant difference in binding of lysolecithin.

The enthalpy of binding curves for the monomer of BSA were also determined. The monomer in this study was prepared by Sephadex (G100) chromatography. Binding of the monomer fraction exhibited decreased maximum negative enthalpy from that exhibited by the crystalline BSA, which is quite probably due to denaturation of the protein during the preparation procedure. However, the overall pattern remained the same as that for the curves of the crystalline BSA. Binding studies on denatured BSA exhibited the same decrease in maximum negative enthalpy as was observed with the monomer fraction binding of lysolecithin.