STUDIES ON SERUM ALBUMIN
BINDING OF VARIOUS LYSOLECITHINS

by

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STATEMENT OF THE PROBLEM

The purpose of this research was to determine the effect of variation of chain length of the saturated fatty acid moieties and that resulting from increase in unsaturation of the fatty acid moiety of lysolecithin on the enthalpy of its binding, in solution, with bovine serum albumin. Microcalorimetric techniques were used to disclose the thermodynamic relationships of this binding. Bovine serum albumin was chosen for this study since it is well characterized, available in a high degree of purity and for its known binding capacity for phospholipids. The lysolecithins were selected because of their known association with proteins, solubility in aqueous solutions at reasonable concentrations and since those used were readily available in satisfactory degree of purity. Further, they provided a homologous series of chain lengths for the saturated acyl moieties of lysolecithin and different degrees of unsaturation for a single chain length fatty acid in studying the effect of unsaturation on binding with bovine serum albumin.

This work is an extension of similar binding studies previously reported from these laboratories.

INTRODUCTION

Associations of lipids and proteins are found to be of major importance in the make-up of constituents of living organisms. Such complexes of these entities exist in biological membranes, in plasma systems and in sites such as alveoli surfaces, for example. In plasma, the composition with respect to quantities of lipids and proteins varies greatly within the lipoprotein complexes. These complexes serve requisite functions in transport of enzymes, hormones, vitamins and lipids throughout circulatory systems. The composition and nature of proteins and lipids, as well as their relationship to each other, in comprising a membrane lipoprotein structure may well determine its physical characteristics and biochemical functions. Membrane lipoproteins are affiliated with transport of materials across both cell walls and membranes of intracellular organelles. Thus membranes serve as important contributors to overall regulation of cell metabolism by their specific permeabilities and transport. Such possible differences in membrane properties permit preservation of cellular specificity and function. If the structures, functions and syntheses of membrane and plasma lipoproteins are to be delineated exactly, it will be necessary to first determine the fundamental associations of lipids with proteins. Therefore, it is felt results of this study may shed some light on properties and characteristics inherent in lipoproteins.

LITERATURE REVIEW

1. Historical Review of Investigations on Lipoprotein Interactions.

In view of the importance of lipoprotein complexes in biological systems reflected by their prominence in membranes, plasma and other tissues, a tremendous amount of endeavor has been expended to ascertain various aspects, characteristics, properties, compositions and associations of lipids and proteins in such tissues. For example, membrane models have been proposed by various investigators based upon their studies of lipid-protein interaction. Work on lipid films led Danielli and Davson to offer one such model (1). This hypothetical structure is comprised of a bimolecular phospholipid leaflet whose non-polar, acyl chains extend inward, perpendicular to the plane of the membrane surface, thereby producing an apolar region in the center of the membrane proper. Further, the protein portion of this model is considered to be ordered about the polar ends of the phospholipid leaflet through polar bonding forming the "outer and inner surfaces" or "layers" of such a membrane. Electron micrographs of myelin membranes have supplied evidence in support of this basic model. Robertson has suggested a similar membrane model and has coined the term "unit membrane" to describe his proposal (2). Predicated on the idea that all membranes paralleled the structure of the Danielli-Davson model investigators worked with renewed vigor toward elucidation of membrane lipoprotein association in an effort to explain any and all membrane and other lipoprotein structures and functions. Inadequacies in

this model were soon made evident as outlined by Korn, for one, and it fell into disfavor (3). Improvements were propounded by other workers leading to a more complicated system providing more sophisticated explanations for functional properties (4,5). In the Singer proposal, for example, the concept of a phospholipid bilayer is retained, essentially, but the protein portion not only is associated with the polar ends of phospholipid but also at certain sites extends partially through the width or thickness of the membrane to varying degree and at some regions extends completely through the membrane, i.e., extending from the outer protein surface through the membrane to its inner surface, with some segment or segments of the polypeptide chain being associated with the lipid bilayer portion of the structure.

Several chemical and physical techniques have been used to elucidate the characteristics of lipoprotein interactions. Some of the earlier investigations utilized precipitation and titration procedures leading to beliefs that these interactions were of ionic nature wherein protein associated with lipid micelles (6). The nonpolar lipids were not considered as interacting directly with proteins but only associated via polar lipids in micelles. More recent studies have indicated lipid-protein interactions to be more complex than the ionic type relationships proposed previously.

The fact that phospholipids possess both polar and apolar regions and will form a monomolecular layer at an air-water interface has promoted experimental examination of the properties of such films. Measurements of surface tension,

surface density and surface potential of such phospholipid monolayers before and after introduction of proteins under these films have been made. The changes noted in these parameters were a function of film penetration by the protein and its interaction with the phospholipid. These investigations have indicated specificity in binding, dependence upon lipid chain length and upon unsaturation of the fatty acids present, as well as a dependence upon the charge of the phospholipid (7,8,9,10).

Through use of a double dialysis technique Green and Fleischer (11) and Ji and Benson (12) have investigated the nature of lipid-protein interaction by reassociation of lipid with previously extracted membranes. Two types of system association, ionic and "hydrophobic" or apolar interactions, have been characterized in this manner.

A variety of physical methods have demonstrated merit in the study of lipoprotein relationships. Dimensions of natural lipoprotein systems have been determined through electron microscopy coupled with x-ray diffraction examination. Unfortunately, the rather severe sample preparation required for these techniques leads one to question the final physical state of the "natural" system being examined. However, the dimensions of the membrane structure so observed compare favorable with values predicted through use of "unit membrane" models. Ultracentrifuge techniques have been adapted to the study of properties of proteins associated with lipids as well as to classify serum lipoproteins by sedimentation values.

More recent examinations have probed the composition and structure of the proteins found in previously classified lipoprotein systems (13).

Nuclear magnetic resonance (NMR) has been used to elucidate changes in freedom of motion and degree of protection afforded characteristic portions of the molecules involved in associations between lipids and proteins in their complexes (13). Another physical tool for exploration of binding between protein and lipid systems is that of calorimetry. Steim has used differential scanning calorimetry (DSC) to study phase changes in lipids with change in temperature, including those changes in lipid components of membranes (14). Similarly, Ladbrooke and co-workers have used DSC to examine phase changes of phospholipids associated with cholesterol (15). Studies of lipoprotein complexes have been performed by Chapman through combining DSC and NMR techniques (16).

Heatburst microcalorimetry was developed by Kitzinger and Benzinger to determine changes in enthalpy, free energy and entropy of the enzymatic action of fumarase as found in the Krebs' cycle (17). This technique lends itself admirably towards determination of such parameters for many biochemical reactions and biological systems. Lovrien and Anderson have used this approach to study the binding of sodium dodecylsulfate by β-lactoglobin (18). Stoichiometric and thermodynamic properties of serum albumin binding of lysolecithin have been determined by Klopfenstein and by Morgan using heatburst microcalorimetry (19,20,21). This method was used in the investigation reported herein.

2. Nature of Lipoprotein Interaction.

As has been indicated previously, the interaction between lipid and protein may be polar or apolar, and in all probability, a combination thereof, and particularly so in the case of phospholipids depending upon the specific lipoprotein in question. The type and extent of association between moieties have been examined by several of the techniques presented above. For example, the ionic interactions between phospholipids and cytochrome C have been demonstrated by Shipley, et al., utilizing x-ray diffraction techniques (22). Evans and co-workers found upon extraction of low-density lipoproteins with diethyl ether that small quantities of lipid material were recovered. However, through denaturation of the lipoprotein prior to extraction considerably greater amounts of lipids were obtained by the ether treatment. Previous digestion with phospholipase C also increased the amount of lipid extracted with ether but exposure of the lipoprotein to phospholipase D failed to increase the yield of extractable lipid. These observations would suggest that the protein present provides some sort of "protective shielding" around the phospholipid with an apolar association of the two components (23). Infrared spectroscopy has been used to follow the extraction of lipid from endoplasmic reticulum and plasma membranes by chloroform-methanol solvent mixture, that portion of the spectrum contributed by the lipid being completely eliminated from the membrane spectrum upon extraction. This evidence indicates the presence of a weak or apolar binding in these systems (24).

The rate of binding of phospholipids to extracted mitochondrial membrane has been shown to be affected by their fatty acid composition. DePury and Collins have established that the rate of binding for lecithin lacking essential fatty acids with extracted mitochondrial membrane is some 2.5 to 3 times greater than the rate of binding for normal occurring lecithin (25). Their observations suggest that the apolar acyl chains of the phospholipid play a role in the binding mechanism of complexing in this system, at least.

The energy requirement or the nature of the driving force required for apolar binding has not been delineated clearly. Jencks has reviewed possible driving forces and their concepts with respect to biochemical interactions (26). The most probable entities: Van der Waals - London dispersion forces, energy used for creation of "solute cavities" in the solvent system, and entropy changes upon binding. Entropy changes as a driving force have been considered by Nemethy and Scheraga and by Kauzmann (27,28). In their viewpoint the lipid is surrounded by highly structured water having low entropy thereby providing low entropy to the system. Upon binding of the lipid to the protein some of this structured water is released consequently producing a net positive entropy change for the total system. Calorimetric studies by Klopfenstein on the binding of lysolecithin by serum albumin demonstrate production of negative enthalpy, free energy and entropy changes for this complex formation in aqueous solution (20). These results indicate that entropy changes are not the driving force in this

particular lipid-protein interaction. Other possible energy considerations for this type of system have not been evaluated to any appreciable extent.

3. Properties of Serum Albumin.

Serum albumin has the property of binding with many substances such as fatty acids, phospholipids, hormones, vitamins, enzymes and metallic ions, some of which are bound in rather substantial quantities. Bovine serum albumin is a protein whose properties have been investigated extensively and is, therefore, quite well characterized. It contains 147 titrable basic groups and 134 acidic groups and has a molecular weight of 69,000 (29,30). Alteration of the pH of an aqueous serum albumin solution has been shown to affect its properties to a considerable degree. Binding of alkane ligands by the protein is a function of pH. A low pH reduces the binding ability while subsequent increases in pH increase its binding capacity. Decreasing the pH of this protein solution produces an increase in viscosity of the system. A decrease in the sedimentation constant for the protein is encountered at a pH value below 4.0 (31).

In serum albumin the number of binding sites available to the various substances mentioned above has not been ascertained but in many cases it is known to be considerable. Phosphatidylserine is known to bind human serum albumin at two sites which differ in equilibrium constants. Some ions that bind to serum albumin alter its configuration such that additional binding sites become available for complexation.

The presence of residual fatty acid does not seem to affect the binding of anions having high affinity and has no effect on binding of uncharged ligands (32).

Electrophoretic examination of BSA near the region of its isoelectric point has demonstrated heterogeneity in the protein. Originally, these properties of microheterogeneity were considered to be due to "extrinsic contaminants" (33). It has been proposed, also, that this heterogeneity can result from imperfect disulfide bonding which could be the consequence of the protein having isomers of open configuration producing many possible combinations of disulfide pairs. This phenomenon is known to be influenced by reaction parameters such as temperature, pH, and bound ligands, e.g., fatty acids. Variations in tertiary structure through differences in disulfide bonding are not responsible entirely for microheterogeneity within the protein. It also may be of covalent origin totally intramolecularly with the dimer and polymer fractions remaining constant (34).

Calorimetric determinations show an endothermic condition occurring with the increase in acidity of BSA solution of pH 4.5 to 3.4, the reaction absorbing 3,100 calories per mole of BSA (35). This reaction is reversible, producing an equivalent exothermic reaction. This effect may be attributed to the unfolding and refolding of the protein.

4. Properties of Lysolecithin.

Lysolecithin, a phospholipid, occurs naturally in several biological tissues but its exact role is in doubt for the most part. The molecule is comprised of a polar region formed by the phosphoric acid - choline side chain ester of glycerol on number three carbon and an apolar region found in the acyl chain of the fatty acid ester of the number one carbon of the glycerol. The compound can be crystallized from hot pyridine yielding a white solid of fine needles. It is readily soluble in ether, acetone and in light petroleum hydrocarbon fractions and somewhat less so in water. Lysolecithins soften upon heating to approximately 100^{o}C and decompose around 260^{o}C. Optical activity has been reported in various solvents (36). The compound(s) dissolves in water to yield a clear solution and has a critical micelle concentration of 1-2 x 10^{-3} percent by weight. Above a 37% (w/w) solution its viscosity increases with increasing concentration, resulting in a thick, but clear, fluid at 50% concentration.

The amphipathic character of lysolecithin provides it with reasonably strong surface active properties. Its ability to lower the surface tension of water has been shown to be unaffected by acids, bases, or electrolytes (37). Indeed, this surface active property may well be the function it contributes in its biological role. The solubilizing effect of lysolecithin aids in dissolving homogenates of whole, fresh rat brain and in hemolizing erythrocytes, with time being

dependent upon lysolecithin concentration. It also demonstrates a great solubilizing effect towards other lipid materials (38).

Lysolecithin-lecithin sols exhibit very high viscosities under certain conditions which may make a significant contribution to membranal structures (38). One theory of cell membrane formation has been developed by Saunders based upon these observations (39).

Dissociation constants have been determined for lysolecithin resulting in values for $K_1 = 0.18$ and $K_2 = 1.26 \times 10^{-12}$ and it exhibits an isoelectric range between pH 2.75 and 9.90. It has been claimed that surface films can be formed at an air-water interface having a surface area/molecule of 108 $\mathring{A}^2$, which can be compressed to 65.5 $\mathring{A}^2$. Since lysolecithin is so highly soluble in water one might question these reports, and, in fact, the results given may be due to contamination by lecithin (38).

Light scattering experiments have produced a value for micellar weight of around 100,000 with a monomolecular weight of 515, resulting in the number of molecules per micelle as being approximately 190 (40). On the other hand, diffusion studies have given a value of 273 monomers per micelle (41).

5. Properties of Lysolecithin-Protein Complex.

Nuclear magnetic resonance studies have been made on lipoprotein complexes containing lysolecithin as a lipid moiety. The individual NMR spectra for both lysolecithin and BSA were first prepared and these then were compared to that of the lipoprotein complex. The lysolecithin spectrum ex-

hibited sharp methylene and choline peaks plus a broad methyl peak. Upon formation of the complex with BSA the spectrum indicated a lowering and broadening of the methylene peak while the peak representative of choline was unchanged relative to that found for lysolecithin alone (14). One would infer from this information that the methylene groups of the acyl chain of the lysolecithin are bound in some fashion in complexing with the protein; i.e., their movement is restricted by the BSA association. Similar results have been obtained by NMR examination of reaction between lysolecithin and erythrocyte membrane fragments (13). Again, the methylene signal was reduced in intensity and broadened with concurrent broadening to some degree occurring in the choline peak. These observations would support the concept that an association has taken place between the fatty acid hydrocarbon chain of lysolecithin and the protein with the possibility of some interaction on the part of the charged portion of the molecule.

MATERIALS AND METHODS

In order to familiarize this investigator with the procedures and techniques required for this study a portion of the work previously conducted in these laboratories was repeated (19,20,21). The calorimetric determinations of the enthalpy of interaction between aqueous solutions of various concentrations of lysolecithin and an aqueous solution of constant concentration of bovine serum albumin, the respective volumes 2.0 ml and 2.5 ml of reactants being held constant, were made.

1. Lysolecithins

The lysolecithin used in this phase of the study was obtained from Sigma Chemical Company (St. Louis, Mo.) and was from lot 110C-2440 with the major fatty acid content being palmitic acid plus a moderate amount of stearic acid. The material contained about 2.0% impurities. The synthetic lysolecithins containing only a single fatty acid moiety as used in the second phase of the investigation were purchased from Serdary Research Laboratories (London, Ontario, Canada). The company states these compounds to be 96-99% pure and they were used with no further purification being attempted.

For each experiment a nominal 50 milligrams was weighed accurately by an analytical balance, the lysolecithin then being dissolved in distilled deionized water, quantitatively transferred to a 25.0 ml volumetric flask and diluted to the volume index mark followed by thorough mixing. The synthetically prepared lysolecithin compounds were supplied as solutions in chloroform. In these instances, the solutions were transferred to small, tared erlenmeyer flasks, the chloroform evaporated at room temperature under a stream of nitrogen followed by storage in a vacuum desiccator until constant weight was attained. Then the compound, after final weighing, was dissolved in distilled deionized water and made to 25.0 ml volume as before. The solutions were stored at $2^{o}C$ when not in use. Occasionally a precipitate would form in the solution after some 48 hours of storage. This precipitate would redissolve readily upon gentle warming (35-

40°C) of the solution prior to use. The solutions were used within ten days after their initial preparation.

2. Bovine Serum Albumin

All bovine serum albumin (BSA) samples were secured from Pentex Biochemicals (Miles Laboratories, Kankakee, Illinois), Lot 21 and Lot 24 being used. This material is produced by the vendor from a purification process of its Fraction V Bovine Albumin. Its specifications require: 100% albumin electrophoretic purity by cellulose acetate, 4-6 polymer bands electrophoretic purity by acrylamide gel, globulins by precipitin test $<0.02\%$, protein $>98.0\%$. Further, the material is recommended for preparation of protein standards, for binding chloride ions in moving boundary methods, for binding organic ion ligands and for determination of protein bound compounds, such as atropine or iodine.

Crystalline BSA is known to contain fatty acids which are bound to the protein in an approximate molar ratio of two to one (42). One should consider the possibility that such binding of the fatty acid might interfere with that of lysolecithin through occupation of the same binding sites or through alteration of the protein conformation thereby influencing the binding, or lack of it, of the lysolecithin. Studies in these laboratories have concluded that no difference in lysolecithin binding is found between the crystalline BSA and the fatty acid - free material (21).

Solutions of the BSA were prepared by dissolving 1.6510 grams of the material in distilled deionized water using very gentle agitation to prevent formation of bubbles on the surface of the solution. The solution was transferred quantitatively to a 50.0 ml volumetric flask, again avoiding bubble formation that makes accurate dilution to the volume calibration mark exceedingly difficult. The BSA solution was then thoroughly mixed and stored, when not in use, at 2°C. The material was used within approximately four days after preparation. Based upon the molecular weight of BSA as 69,000, this solution provided a concentration of 0.48 μmoles/ml.

PROCEDURE

The enthalpy produced through binding of lysolecithin by serum albumin was measured by "heatburst" microcalorimetric technique. The equipment used for this study was fabricated from the design of the calorimeter presented by Benzinger and Kitzinger (17). The design of this instrument is such that heat produced at the reaction site is transferred rapidly through a thermopile comprised of a multitude (10,000) of copper-constantan couples in each of twin chambers. One chamber is provided for the reaction cell while the other contains the reference or "blank" cell with their respective thermopiles connected so that the signal produced by the reference cell is electrically subtracted from that generated in the reaction cell. The resultant signal is fed to a D.C. amplifier having a 50 to 2,000 microvolt stepwise range. The

amplifier's output, in turn, is registered on a millivolt-time scale recorder. The equipment is housed in a constant temperature room maintained at $20^{o}\pm1^{o}C$.

The calorimetry cells used here are of the type described by Benzinger and Kitzinger (17). They are fabricated of very thin-wall glass, are hollow cylinders with concentric walls joined together at each end and longitudinally bicompartmental, each side having its own charging port. The BSA and lysolecithin solutions are measured, by means of microburettes, into the respective compartments of the cell used for the binding reaction. The reference cell (blank) is charged with the same volume of BSA solution as used in the reaction cell with the second compartment being charged with a volume of distilled deionized water equivalent to and in lieu of the lysolecithin solution. After the cells are filled the ports are closed with silicone rubber stoppers to ready them for the calorimeter chambers. It should be noted here that for each series of dilutions of the lysolecithin used, the volume of BSA solution, i.e., 2.50 ml, and its concentration (0.48 μmoles/ml) remains the same while the volume of the lysolecithin solution, i.e., 2.0 ml, remains constant with its concentration being decreased stepwise. Thus, the total volume in the reaction and reference cells is equal and constant throughout calorimetric measurements of each set of experiments. In this manner, for each of the series studied, the total quantity of protein was kept constant with the lysolecithin concentration being varied to provide a range

of interaction ratios while the total system volume remained constant.

The charged cells next are coated with a thin, even coating of vaseline to insure good thermal contact with the aluminum tube which serves as the calorimeter chamber receptor for the cells. This operation is performed using either throw-away plastic or surgical rubber gloves to avoid contamination of the cells by one's fingers. The cells are now ready for insertion into the chamber followed by sealing the calorimeter with the heat sinks and insulating it by surrounding the heat sinks by two metal encased, large, glass dewars. Temperature equilibrium, as indicated by steady state reading of the circuit microvolt meter, is established by allowing the instrument to stand for the required period (30-120 minutes).

The reaction is initiated by mixing the solutions in each cell by rotation of the calorimeter within its framework through 360^{o} with an intermediate pause at 90^{o}, then rotation in the opposite direction to return to the original starting point. The calorimeter is then turned horizontally in its frame through 90^{o} such that it is now perpendicular to the axis of rotation of the framework. In this juxtaposition rotation of the frame through 90^{o} produces end for end mixing of the solution within the cells. Again, the instrument is returned to its original position. These maneuvers achieve thorough mixing of the solutions in the reaction and reference cells. The heat of reaction generated through binding of the lysolecithin by the BSA produces a voltage signal in the

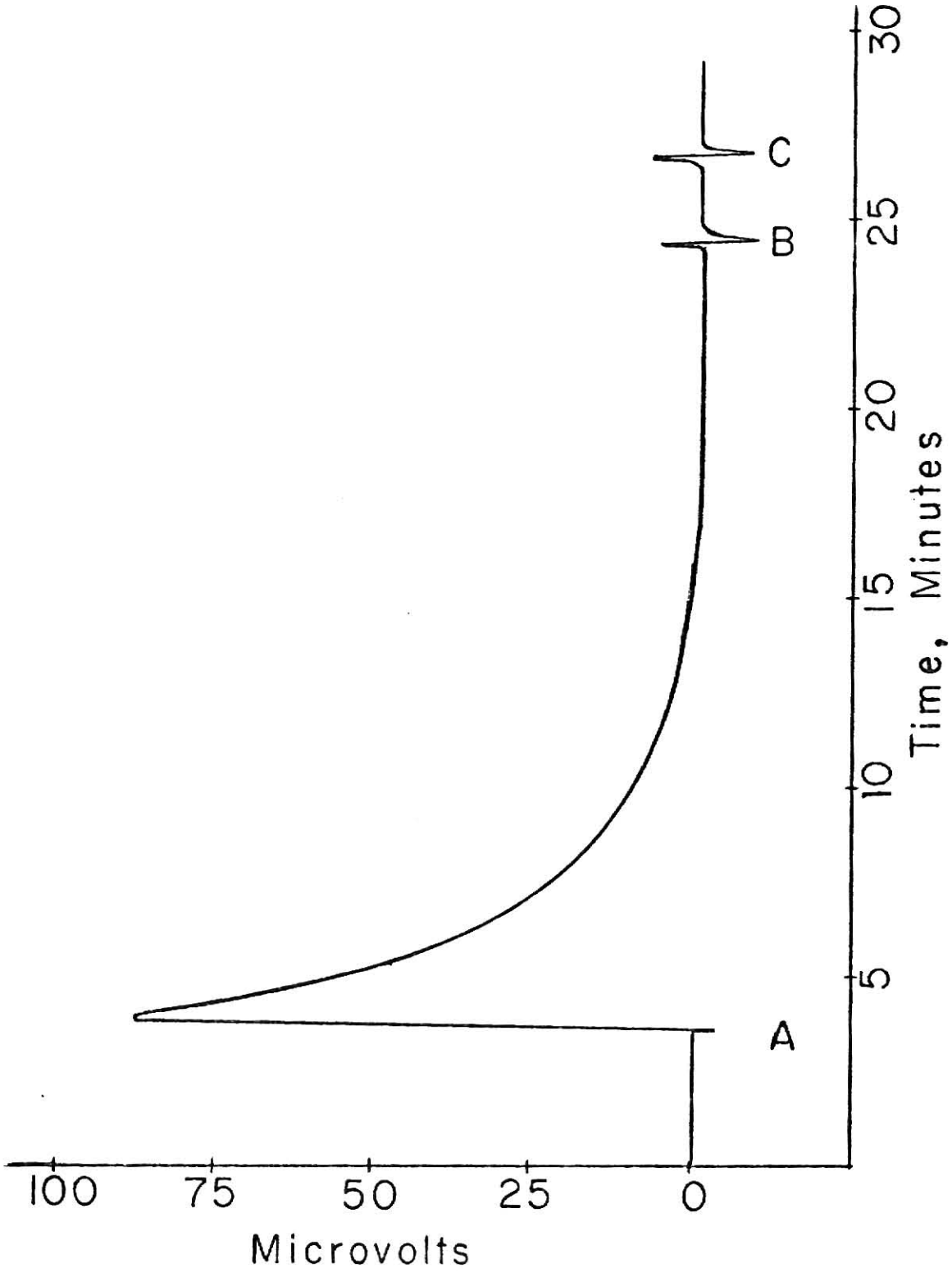
thermopile circuitry which is ultimately presented versus time on the recorder chart. The completion of the reaction output is noted when the recording instrument returns to its baseline. In order to insure complete reaction and to check "mixing spikes" produced by frictional forces occurring between the liquid and glass cell surfaces, electrical effects of the thermopile cutting a field of flux, agitation of solutions, imperfect mixing, etc., which may be applied as corrections to the measured enthalpy of reaction the procedure of mixing is repeated at suitable intervals at least two more times. All enthalpies of binding exhibited by association of the lysolecithin with BSA were determined to be exothermic by comparison with known exothermic and endothermic reactions. A recorder presentation of a typical reaction peak is shown in Figure 1.

In order to calculate the value of the enthalpy produced by a given reaction the area under its reaction curve was computed by measurement with a Kueffel and Esser #4236 compensating polar planimeter. The area of negative "mixing peaks" with respect to the area of the reaction peaks was added to that of the reaction peak as a correction. Likewise, positive "mixing peak" areas were subtracted unless they were determined to reflect inadequate initial mixing of reactants, in which cases they were considered to be a second reaction peak. The areas so obtained were converted to microcalories by conversion factors currently and previously established by calibrations of the calorimeter both electrically and through the known

exothermic neutralization reaction of HCl by NaOH (43,44). Data for the area under the curves representing the reactions for one series of experiments and their equivalent number of millicalories plus the calculated change in enthalpy, ΔH, derived therefrom are given in Appendix I, page 49.

Figure 1. Heat change on binding of lauroyllysolecithin to BSA.

The curve is a replica of the recorder presentation of the reaction peak and is typical for the exothermic reactions produced by the phospholipid-BSA interactions studied. The point of initial mixing is designated by A while points B and C indicate additional mixing spikes of zero area. The area under the peak represents 20.5 mcal which is produced by interaction of 1.20 μmoles BSA and 4.4 umoles of lauroyllysophosphatidylcholine.


C
B
A
30
25
20
15
10
5
Time, Minutes
100
75
50
25
0
Microvolts

Results and Discussion

From the data obtained calorimetrically for reaction of the various ratios of the lysophosphatidylcholine to serum albumin one can plot the change in enthalpy, ΔH, produced by the interaction versus the mole ratio of reactants. Typical curves for a saturated fatty acid ester of lysolecithin binding with albumin are shown in Figure 2, page 21, while those for one of the unsaturated fatty acid esters of lysolecithin are given in Figure 3, page 23. The maximum theoretical heat change produced by the interaction would be that obtained through saturation of the BSA with lysophosphatidylcholine for the reaction in question. These values are derived from the extrapolation of the high ratio portion of the curve for the heat change per mole of serum albumin to zero reaction ratio as shown by the broken line in the figures. Similarly, extrapolation of the curve for lysolecithin to zero reaction ratio provides its ΔH value per mole which when compared to the maximum theoretical heat change for the system yields the molar ratio of the binding reactants occurring in the complexed product for the system under investigation. Since the initial concentrations for the protein and phospholipid are known, the determination of their binding ratios permits the calculation of the equilibrium concentrations for the complexed phospholipid-protein product and those for the reactants of the system. From this information apparent association constants for the interactions resulting from various experimental ratios of reactants can be established

and, subsequently, the free energy change, ΔG, for these reactions can be calculated. Then, knowing the maximum theoretical ΔH/mole for the albumin binding and the ΔG/mole for the interaction, plus having the temperature of the reaction, one may ascertain the change in entropy, ΔS, for the process.

In the two experiments wherein a 1:1 mole binding ratio was established, i.e., a single binding site was utilized on the protein, a valid association constant can be calculated with subsequent values determined for ΔG and ΔS. The information derived from these experiments only permits calculation of average association constants for the interactions occurring at multiple binding regions of the protein molecule and does not necessarily reflect the binding constants for the individual binding reactions.

Other experimental approaches would have to be employed to obtain required information to delineate individual association constants for multiple binding regions within the protein. Nevertheless, knowing from various works reported in literature that association constants vary for different binding sites encountered in protein systems, the assumption is made that the binding sites are of equivalent, or nearly so, binding affinity with respect to phospholipid where the BSA binds more than one molecule of lysophosphatidylcholine (45,46,47). Through this assumption some comparisons can be made among the systems studied using the average values calculated for various parameters.

Figure 2. Enthalpy curves for myristoyllysolecithin binding with BSA.

The enthalpy change was computed on a molar basis for ratios of moles of the lysolecithin to moles of BSA. These curves are typical for data obtained for saturated phospholipid compounds interacting with BSA.

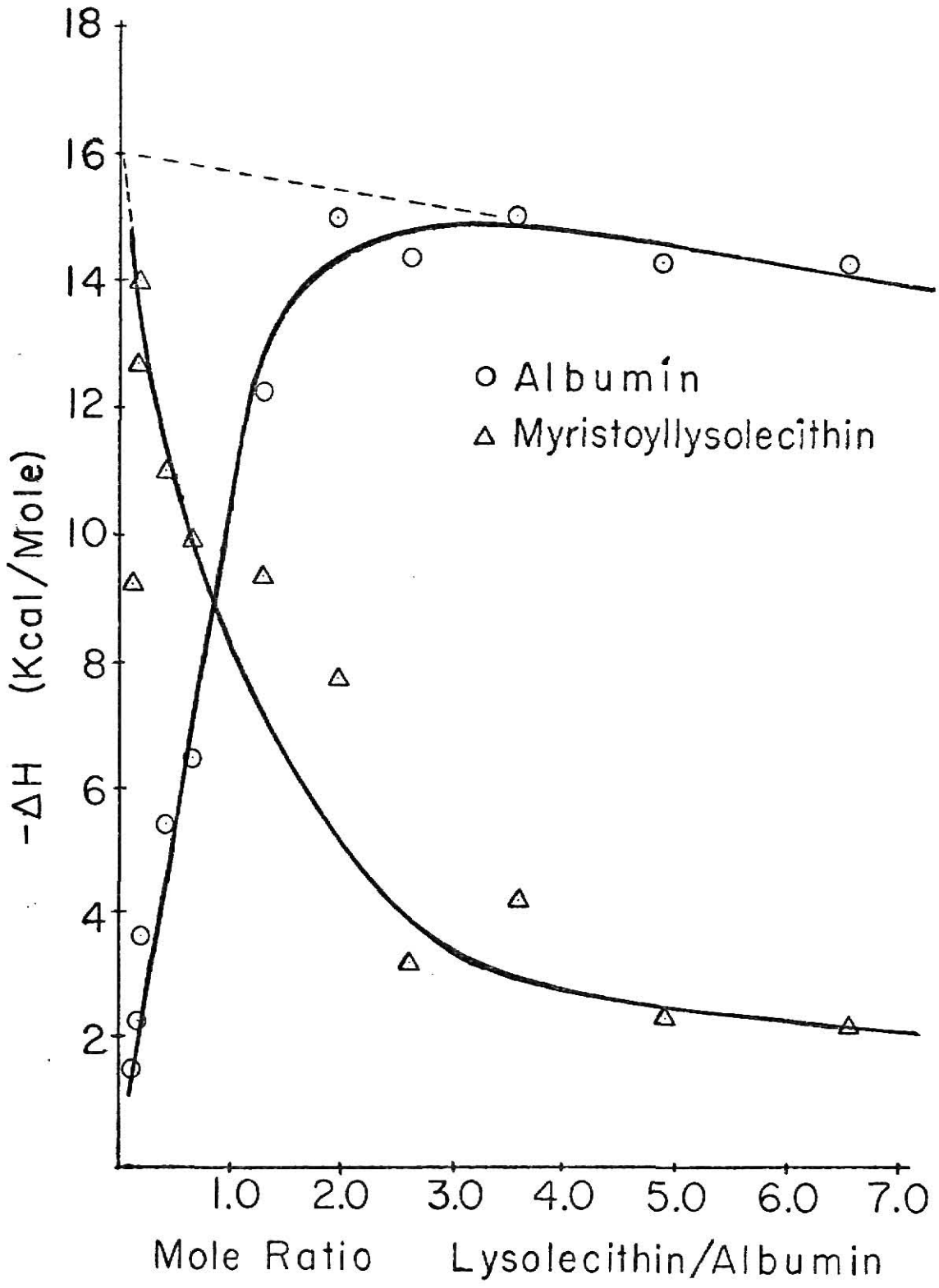
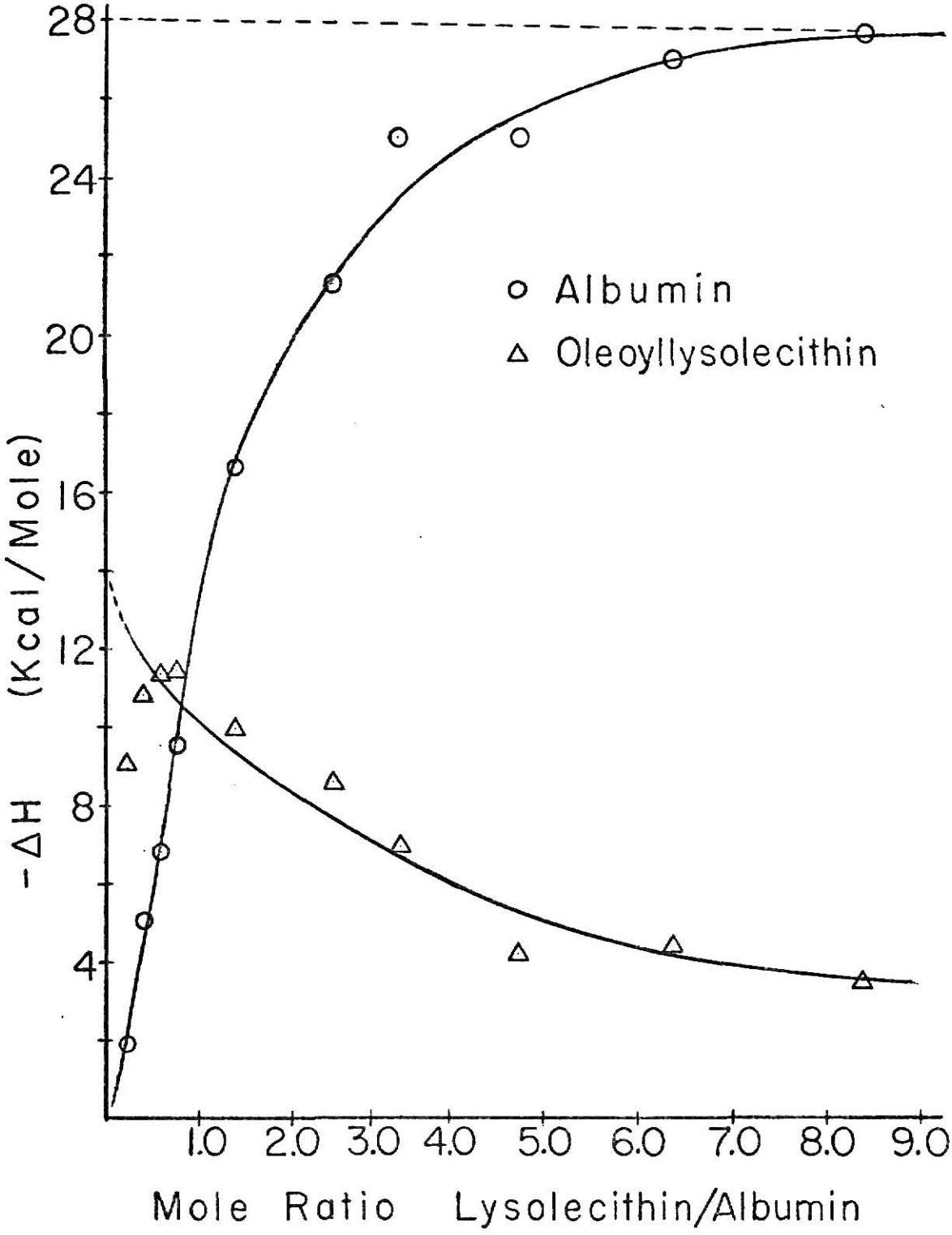

18
16
14
12
10
8
6
4
2
-ΔH (Kcal/Mole)
Albumin
Myristoyllysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Mole Ratio Lysolecithin/Albumin

Figure 3. Enthalpy curves for oleoyllysolecithin binding with BSA.

The presentation is the same as found in Figure 2, and is representative of data obtained for the unsaturated phospholipids interacting with BSA.


28
24
20
16
12
8
4
-ΔH (Kcal/Mole)
Albumin
Oleoyllysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Mole Ratio Lysolecithin/Albumin

Enthalpy measurements were made on a series of a minimum of 13 different concentrations of lysophosphatidylcholine reacted with a single concentration (1.20 mmoles in 2.50 ml of solution) of BSA for each of the eight lysolecithins whose binding properties were studied. The concentrations of phospholipid extended over a range of about 8.0 mmoles to approximately 0.12 mmoles, each in 2.0 ml of solution, in stepwise fashion. The lysolecithins investigated for the effect of acyl chain length on the binding properties with BSA were: caproyllysophosphatidylcholine, lauroyllysophosphatidylcholine, myristoyllysophosphatidylcholine, egg lysolecithin*, and stearoyllysophosphatidylcholine. Those selected for examination of the effect of unsaturation on the interaction between the albumin and phospholipid were the oleoyl-, linoleoyl-, and linolenoyl-lysophosphatidylcholines. A set of the plots representing the relationship between ΔH for the interaction and mole ratio of reactants is provided in Appendix II, page 50.

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*Lysolecithin obtained from egg yolk was used in lieu of the palmitoyllysophosphatidylcholine. Analyses showed this material to contain 65.5% palmitic acid and 17.5% stearic acid esters with minor amounts of other fatty acid moieties plus 4% of lecithin and 2% of other contaminants.

As explained above, information from these curves was used to obtain the binding ratio on a molar basis between the lysolecithin and serum albumin and for calculation of an apparent association constant for each of a series of four mole ratios of reactants in each set of the eight experiments, again being mindful of the assumption of an average value for binding affinity where multiple binding regions are encountered. From the available information the thermodynamic parameters listed previously were calculated for each set of experiments performed. The values for these parameters, including average values where appropriate, are shown in Table A, below. Information and examples of data used are presented in Appendix III, page 63.

A sample set of calculations used is provided in Appendix IV, page 65.

Table A. Thermodynamic Parameters from the Binding Properties of Lysolecithin-Albumin.

Fatty Acid Chain	Lyso-lecithin Identity	Max. ΔH Kcal/Mol (Graph)	Molar Binding Ratio Lysolecithin/Albumin	Average ΔH/Mole Lyso-lecithin	Calc'd ΔG Kcal/Mol BSA	Calc'd ΔS e.u./Mol BSA
10:0	Caproyl	-16.0	2:1	-8.0	-9.0	-24.0
12:0	Lauroyl	-24.0	2:1	-12.0	-9.3	-50.0
14:0	Myristoyl	-16.0	1:1	-16.0	-5.5	-35.0
16:0&18:0	[a]Lysolecithin	-18.0	1:1	-18.0	-11.8	-40.0
18:0	Stearoyl	-19.0	3:1	-6.3	-14.7	-14.6
18:1	Oleoyl	-28.0	2:1	-14.0	-10.0	-61.4
18:2	Linoleoyl	-28.0	2:1	-14.0	-10.5	-59.7
18:3	Linolenoyl	-24.0	2:1	-12.0	-11.2	-48.1

[a]Lysolecithin isolated from egg yolk was used in lieu of the palmitoyl compound. See text above.

The results obtained from these experiments show an increase in the negative direction for binding enthalpy change of the interaction with increase in the hydrocarbon chain length of the fatty acid moiety of the phospholipid. Assuming equal affinity for the ligand at multiple binding sites on the protein the decrease in ΔH is particularly uniform (2.0 kcalories per additional carbon atom) on a per mole basis of the lysolecithin bound for the regular increases in chain length of the first three compounds studied. The fourth experiment using egg lysolecithin, rather than a synthetic palmitoyllysophosphatidylcholine, continued to show an increase in enthalpy change (decrease in enthalpy) in the negative direction but produced only 2.0 kcalories difference for the extension in chain length compared to the 4.0 kcalories change per two carbon atom extension in each of the first three experiments. Again, it should be pointed out that analyses of the egg lysolecithin used revealed that 65.5% of the fatty acids present was the palmitoyl ester and 17.5% was the stearoyl ester with minor amounts of other esters and impurities existing in the material. One might speculate, in view of the results obtained for the stearoyl compound, that had the pure palmitoyllysolecithin been used the uniformity of the change in ΔH with respect to extension of acyl chain length might have been maintained through the first four compounds in the series investigated.

Since nothing was changed experimentally except for the extension of the acyl chain of the phospholipid compounds in

the first five experiments of this study the resultant increase in the negative direction for the enthalpy change concomitant with increase in hydrocarbon chain length would indicate apolar interaction between the acyl chain of the lysophosphatidylcholine and nonpolar regions of the protein as a binding mechanism for the system. This finding supports the evidence provided by Steim and coworkers (48). Further, NMR peak assigned to the quaternary amino group of choline remained virtually unchanged after binding implying that the polar portion of the phospholipid was not appreciably involved in the complexing process. However, nothing in the investigation reported here rules out a role for the polar end of the molecule in the protein-phospholipid interaction.

Wishnia and Wishnia and Pinder have reported a series of studies of the interactions of tritium labeled, short chain alkanes with some proteins including BSA, hemoglobin, myoglobin, and β-lactoglobins A and B. These studies have covered the effect of pH, temperature, partial pressure of gas phase introduction of alkane ligands into buffered aqueous solutions of proteins, ionic strength of solutions, etc., upon the protein-ligand association (45,49,50,51,52). The conclusions offered, in part, are that the alkanes are bound directly to some of the hydrophobic regions in BSA, for example, and that binding sites are inside the BSA molecule; i.e., most if not all the apolar side chains of these regions are not in contact with the solvent. Further, the proteins, β-lactoglobulins A and B, bind apolar solutes (ligands) at a few discrete sites with no

essential differences in binding to monomers, to the normal 36,000 MW dimers, or to the β-lactoglobulin-A octamers indicating that: (a) conformation changes of the protein, if any, associated with aggregation, are not transmitted to the binding sites, and (b) the structures at the dimer and octamer joints being inhospitable to butane, pentane and iodobutane, cannot contain even modest sized, flexible, hydrophobic regions. For this protein system it is concluded that all three solutes compete for the same binding site and that the binding site on each monomer is a single hydrophobic region, near the surface of the protein and which can accommodate more than 200 but less than 230 ml/mole of additional non-polar substance. Each β-lactoglobin monomer can bind two butane molecules equally well, two pentanes unequally well, or one iodobutane. In particular, for β-lactoglobin A at pH 2.0 and 25°C the dissociation constants, free energy changes, enthalpies, and entropies (on a mol-fraction basis) are: Butane $K_1 = 0.98 \times 10^{-5}$, $K_2 = 3.76 \times 10^{-5}$, intrinsic $K_0 = 1.92 \times 10^{-5}$, $\Delta G = 6.44$ Kcal, $\Delta H = 1.1$ Kcal, $\Delta S = -17.8$ cal/degree; Pentane $K_1 = 0.30 \times 10^{-5}$, $\Delta G = 7.54$ Kcal, $\Delta H = 2.1$ Kcal, $\Delta S = -18.3$ e.u., $K_2 = 2.9 \times 10^{-5}$, $\Delta G = 6.19$ Kcal, $\Delta H = 0.9$ Kcal, $\Delta S = -17.7$ e.u.; Iodobutane $K_1 = 0.71 \times 10^{-5}$, $\Delta G = 7.03$ Kcal, $\Delta H = 4.0$ Kcal, and $\Delta S = -10.2$ e.u., $K_2 = \infty$.

Wishnia and Pinder propose that large hydrophobic clusters in the interior of BSA are responsible for observed behavior, in accordance with Foster's model (53,54,55). The most likely sites of alkane binding are structures involving the apolar

amino acid side chains directly. They do not consider electrostatic or polar bonding mechanisms seriously for this system type. Wetlaufer and Lovrein have proposed alternatives in which the binding of alkane ligands lowers the local dielectric constant thereby strengthening the interaction between ionic or dipolar groups (56). However, they concede this mechanism to be unlikely. In summary, Wishnia and Pinder explain the interaction of butane and pentane with native BSA in terms of binding within relatively large (or with less likelihood, relatively many) hydrophobic regions. Although several proteins fit such a model, this mechanism is not universal.

It has been noted by Jirgensons, using optical rotary dispersion techniques, that n-propanol, decyl-, dodecyl-, and tetradecyl-sodium sulfates introduced an increase in α-helix content (increased order of the system) on interaction with several proteins (57,58). The tetradecylsodiumsulfate exhibited greater effect than did the shorter chain compounds. Thus, through association of an organic ligand an increase in degree of ordering is induced in the protein.

Plasma albumin has the property for binding a wide variety of organic compounds, dyes, normal metabolites, commonly used drugs and so forth (59). Many of these bind competitively at the same primary site of the protein molecule. This binding property of albumin is believed to be of importance since it can serve as a plasma transport protein thereby regulating plasma level of various metabolites and drugs.

Through use of several techniques for fragmenting the serum albumin and determination of amino acid content as well as some sequencing of amino acids thereof, King and King and Spencer have elucidated some structural and conformational properties of the molecule (59,60). From these experiments coupled with binding of organic ligands by the protein and two of the protein fragments, these authors conclude that the single primary site of fragment A is very similar in its organic ligand binding properties to those found for albumin. This finding would suggest that it is the same primary binding site. The reasoning is further supported by the weak binding site found for fragment B for octanoate which is less than 1/40 that of fragment A. When binding studies of fragment A were carried out in the presence of fragment B the binding constants for the primary and secondary sites were doubled; the number of the primary sites remained the same but the number of secondary sites increased from two to ten. Differences in binding properties of fragment A in presence of fragment B probably result from the formation of a 1:1 complex of the fragments. A summary of the organic ligand binding data obtained by these investigators is given in the table below.

Table 8. Organic Ligand Binding Constants

Sample Type	Ligand*		
	Octanoate	L-Tryptophan	D-Tryptophan
BSA	$n_1=1$ $K_1=16.0\times10^4$	$n_1=1$ $K_1=2.1\times10^4$	$n_1=1$ $K_1=0.09\times10^4$
	$n_2=10$ $K_2=0.1\times10^4$	$n_2=2$ $K_2=0.05\times10^4$	
Tryptic Fragment	$n_1=1$ $K_1=5.0\times10^4$	$n_1=1$ $K_1=0.84\times10^4$	$n_1=1$ $K_1=0.028\times10^4$
	$n_2=3$ $K_2=0.02\times10^4$		
Peptic Fragment A	$n_1=1$ $K_1=2.0\times10^4$	$n_1=1$ $K_1=0.38\times10^4$	$n_1=1$ $K_1=0.014\times10^4$
	$n_2=2$ $K_2=0.02\times10^4$		
Peptic Fragment B	$n_1=2$ $K_1=0.05\times10^4$		
Peptic Fragments A & B	$n_1=1$ $K_1=3.8\times10^4$		
	$n_2=10$ $K_2=0.07\times10^4$		

n = type and number of binding sites.
* Number of secondary sites and their binding constants are only approximate due to insufficient data for accurate extrapolations.

Further studies by King and Spencer utilizing cyanogen bromide degradation and limited tryptic hydrolysis treatment on BSA and defatted BSA have provided some additional information.(61). These methods provided large fragments of peptide chains on which sequential and structural data have been obtained. Binding studies between the same ligands reported above and the BSA and the peptide fragments were made by means

of equilibrium dialysis. Their data show that the tryptic fragment and defatted albumin both possessed one primary site for octanoate and L-tryptophan. D-tryptophan competitively displaced either of these two ligands from the fragment and from albumin, thus suggesting all three ligands bind at the same site. The binding constants of the fragment for the three ligands were all about one-third of those for the albumin molecule. The ratio for the binding constants of the L- and D-tryptophan for the fragment peptide was nearly the same as for that with albumin. This was also true for the binding constant ratio for octanoate and L-tryptophan. These similarities point strongly toward the concept that the binding site in the fragment is the one present in the albumin molecule.

Fatty acid binding of albumin as well as some other proteins has been demonstrated by affinity chromatography wherein the fatty acid moiety is immobilized on agarose (62). Various oleyl and palmityl aminoalkyl-amino-agarose preparations bound about 10 mg of albumin per ml of agarose; other proteins were retained in smaller quantities and with lesser affinity. The fatty acid-agarose columns which had been exposed to human serum and then washed yielded essentially pure albumin upon elution with 50% aqueous alcohol at pH 3.0. Lengthening the aminoalkylamine "arm" from 2 to 10 carbon atoms had little effect on capacity for the albumin but increased, in some cases very slightly, the binding of other serum proteins. That the albumin binding was caused by the

immobilized fatty acid was shown by the use of control preparations without fatty acid bound to agarose, by reverse relation of albumin binding to fatty acid content of the albumin and by ability to elute albumin from the agarose with solutions of sodium oleate. In these studies, efforts were made to isolate the fatty acid binding region of the BSA molecule after tryptic digestion of albumin which was bound to the fatty acid - agarose. Two peptides with molecular weights of about 10,000 and 23,000 were obtained which were resistant to further digestion. The larger of these was purified and its amino acid composition determined. This tryptic peptide lacked cysteine and tryptophan, and apparently arises from the carboxy terminal portion of the albumin molecule. Relative to BSA, the larger peptide fragment is distinctive in lacking tryptophan and is lower in histidine and tyrosine content and higher in threonine, serine, proline and cystine. In labelling experiments it was shown that the originally labelled cysteinyl residue of BSA was not present in the tryptic peptide and it was concluded that the 14 half-cystine residues form seven intramolecular disulfide bonds.

Wetlaufer and Lovrien have demonstrated interactions of hydrocarbons and proteins and that these associations are reversible (56). Such interactions have been shown with aqueous solutions of BSA and β-lactoglobulin and the hydrocarbons butane, isobutane, propane, cyclopropane, 1,3-butadiene and benzene. The binding has been demonstrated by the perturbation of an equilibrium between structurally different forms

of the same protein, with resultant changes in H^+ equilibrium, viscosity, optical rotation and U.V. absorption spectra. The investigators claim their results provide further evidence that nonpolar groups in proteins play an important role in the energetics of protein structure and offer an approach to the investigation of protein structure and stability. The authors suggest the following as a mechanism of protein-hydrocarbon interaction. From the work of others and from their work it seems likely that the nonpolar side chains of a protein are grouped together in regions in a manner that tends to minimize their water exposed surfaces (63,64). On the hypothesis that nonpolar side chains will be found in groups of several, if partly exposed to aqueous solvent, or will alternatively be found on the interior of the protein, they suggest two distinctly different modes of interaction between a protein and a small nonpolar molecule. First, a dissolved nonpolar molecule could attach itself to the accessible surface of a nonpolar cluster, perhaps with partial penetration of the protein. Second, the nonpolar molecule might penetrate into the relatively nonpolar interior of the protein and lodge there. It seems to the authors that if the second process were to occur a substantial molecular rearrangement would be required to accommodate the nonpolar molecule or molecules, since the pre-existence of holes in the protein interior is unlikely on energetic grounds. An interaction of the first sort, on the other hand, might occur with very minor structural changes. A protein which binds a hydrocarbon when it is not involved in a pH-controlled structural

change should show evidence (O.R., U.V. difference spectra, etc.) of structural change if the second but not the first process prevails. The writers also suggest a third mechanism wherein a nonpolar molecule could be bound to a protein in the immediate neighborhood of an ion pair so as to strengthen this ion pair interaction. A fourth proposal is made covering the possibility that hydrogen bonds and other polar groups may be facilitated by placing a nonpolar group close to the interacting entities. These latter two processes carry rather stringent requirements for positioning of the nonpolar molecule vis a vis the interacting groups on the protein, however (65). Wetlaufer and Lovrien present experimental data to indicate that hydrocarbons of different sizes and shapes show effects of the same general magnitude in reversing conformation changes in proteins; this lack of specificity also argues against the third and fourth proposals offered above. Further, these authors do not agree that Wishnia's evidence supports a particular binding mechanism since all four of the mechanisms proposed by themselves could be rendered ineffective by the swelling of the protein.

Lovrien has looked at interaction between dodecylsulfate anion and BSA in solutions at high pH (10.5 to 12.0 pH range) and ionic strength of 0.15, using low concentrations of the detergent. The systems and resultant changes within the systems were measured by a variety of experimental methods including spectrophotometric titrations, photofluorescence

measurements, kinematic viscosity determinations and potentiometric titrations with and without detergent. The mechanism for the binding process favored by the author from his observations is that the dodecylsulfate causes a major conformation change in the protein as part of the complexing. Here, Lovrien feels that a volume shrinkage is the more simple mechanism for the binding process with changes in ionization of titrable groups of amino acid residues occurring as the final steps in the process. He suggests that the initial steps are produced by shrinkage of the macromolecule induced by the detergent, followed by the ionization of appropriate amino acid groups forced by electrostatic interactions of the contracted macroanion with the solvent; the initial steps were produced by shrinkage of the macromolecule induced by the detergent. Most interpretations make the assumption that pre-existing binding sites on or within the protein molecule are involved in the process. This assumption in the absence of other information is as reasonable as any other, but is nevertheless an assumption. Lovrien's experiments show that binding of a detergent to an alkaline expanded serum albumin leads to a rather strong conformational change so it is quite possible that sites for binding interaction are formed as part of the binding process itself. He reports evidence in support of this mechanism. Karush has contributed the postulate that serum albumin possesses "configurational adaptability" in its interaction with ligands (conformation is now preferentially used when dealing with main chain

arrangements)(68). He presents in his discussion the strong possibility that some macromolecules might engage in the binding by allowing the binding site to be formed during the actual binding process.

A property of importance in any binding process is the free energy change. Providing a binding region can be formed as a part of the interaction process, rearrangement of the protein molecule might be fundamental to it, if such change would decrease the free energy. It certainly would seem reasonable that the given system would accommodate itself to the most "comfortable" situation possible to produce the most stable conformation within the environment extant, i.e., the lowest free energy state. Further, specificity of binding (even specificity to a small degree) might not derive merely from somewhat rigid preformed sites, but from the ways in which the protein molecule can rearrange, even in subtle ways, in forming complexes with some substrates. In all this, the ligand or substrate could nevertheless be bound finally in a precise manner.

Recently, the complete amino acid sequence for both bovine and human plasma albumin have been reported as well as structures for both molecules (67,68). In Brown's investigation of BSA the structure was delineated by tryptic, chymotryptic, peptic and CNBr digestions followed by sequential determinations of the resulting peptide fragments. The ambiguity of pairing of S-S at cysteine-systeine sequences was solved for the peptide fragment containing amino acid residues

504 to 581 by its partial acid hydrolysis. The BSA structure proposed by Brown reveals three repeat units or domains containing residues: (1-190), (191-382), (383-581). It is further delineated that each domain is comprised of two, large, filled loops and the sequences (59-82), (248-270), (441-464) form helices 20 to 25 residues long. These configurations of the chains consistent with the disulfide bridges of the cystine residues may produce an overall domain structure as a pentahedral cylinder formed by five parallel helices. It is suggested that the albumin binding region is the right side hole in the cylinder where several positive charged groups, histidine and/or lysine (e.g., residues 143-145) near the entrance to the region can bind the negative charge of a ligand such as fatty acid. The model proposed agrees with the helix content (50%) and reasonably with the helix potential (69). This model for BSA also demonstrates hydrophobic and/or nonpolar regions suitable for apolar binding of ligands when the loops of helical structure are properly folded with regard to the domains as proposed. The linear amino acid segments comprising the end loops of the structure provide the charged residues at the openings (ends) of the cavities formed by folding of the domains of the protein molecule (70).

The studies performed in our laboratories have shown several interesting facets of the binding process for lysophosphatidylcholines and BSA in aqueous solution. The enthalpies of these interactions were measured by microcalorimetry and all systems produced negative values for enthalpy

change indicating the interaction to be exothermic. Extension of the hydrocarbon chain length of the fatty acid ester moiety (C-10 to C-18) of the lysolecithin produced an increase in the change in enthalpy, in the negative direction, (a decrease in enthalpy for the system) for the interaction with the protein. Since increase in the hydrocarbon acyl chain length was the only parameter changed experimentally, these data provide additional evidence for nonpolar binding on BSA. Several of the reports reviewed in this section have offered similar information. The uniformity of the decrease in enthalpy with each additional regular increase in chain length is noteworthy (in the first three experiments) in that this would indicate the binding process, with respect to energy changes, occurs in a similar environment for the binding site or region. It should be pointed out that the shorter chain compounds (those having fatty acids 10:0, and 12:0) apparently bind two molecules of lysolecithin per molecule of albumin while the intermediate chain length compounds (14:0 and 16:0) bind in a 1:1 mole ratio with the protein. Other studies show a primary site for ligand binding on BSA with a few or several (2 to 10) secondary binding sites for organic ligand complexing, the former possessing a larger association constant and the latter having a much smaller value. On the basis of Brown's model for BSA structure the binding of two molecules of the shorter chain compounds may be explained by geometry in that the cavity formed by the domains containing α-helix of 20-25 amino acid residues would be about 30 to 38 Angstroms in length. This cavity physically can

readily accommodate two 10:0 or 12:0 fatty acid chains, being on the order of 10 to 13 Angstroms in length overall, in an end-to-end fashion or side-by-side fit with nonpolar binding occurring between the ligands and internally situated apolar regions on the protein. Also, there is possible polar bonding occurring between the positively charged amino acid residues of the protein molecule at the open ends of the cavity and the negatively charged phosphate groups of the phospholipid. It should be restated that the experimental examinations used here do not indicate the presence or absence of polar binding in the systems studied. The 14:0 and 16:0 compounds have an acyl chain length of about 15 to 18 Angstroms and it may well be the cavity of the albumin cannot adapt to binding of two molecules of the phospholipid in the proper juxtaposition with the binding regions within the cavity. In addition, it may be that protein conformational changes occur during the binding process for the first ligand such that there is no site available for binding of a second phospholipid molecule. The experimental results for the 18:0 fatty acid ester of the lysolecithin show this compound to bind in a 3:1 mole ratio with albumin. One can only postulate that the binding of the first ligand causes conformational change, not produced by binding of the 14:0 or 16:0 compounds, that permits binding of a second stearoyllysophosphatidylcholine, and further, the binding of the second ligand or a combination of complexation of the two ligands produces additional conformational changes whereby an apolar site becomes

available for binding a third ligand. Considering the total ΔH for the process of binding three molecules of the 18:0 compound compared to that obtained for the cases where a single ligand is bound one would hypothesize that at least one, if not two, of the three ligands is bound rather weakly. It would seem more realistic to assume one ligand would be bound with about the same release of energy as that found for a single ligand having a somewhat shorter acyl chain, e.g., palmitoyl lysolecithin, with the other two stearoyllysophosphatidylcholine molecules releasing smaller quantities of energy upon apolar complexing with the protein binding sites made available through conformational change induced by the interaction process. Such a process should better describe the interaction than using an average value of ΔH per mole of bound ligand, e.g., -6.3 kcal as presented in Table A, and calculation of an average association constant for the binding process in toto. (However, this is the only approach available for data obtained by these experiments.)

The values for the change in free energy, derived from the association constants calculated for the various interactions, were all negative indicating these exothermic reactions may occur spontaneously. With the exception of the value for the 14:0 fatty acid moiety the interactions show a decreasing trend in free energy change with increasing acyl chain length, disregarding the number of ligands bound. In those cases where a like number of ligands bound per molecule of albumin, one may make the following comparisons: for 10:0 and 12:0 with 2:1 mole ratio the longer chain compound indicates slightly

tighter binding than the shorter since the 12:0 has the somewhat greater change in free energy in the negative direction; the 14:0 and 16:0, 18:0 mixture with 1:1 mole ratio show decided differences in ΔG with the longer acyl chain mixture producing the lower free energy state by -6 kcal. showing it to be the more tightly bound. The greatest change in negative free energy change per molecule of albumin was obtained with its interaction of three molecules of the 18:0 ligand at -14.7 kcal. This could suggest that the longest chain compound studied had the greatest extent or affinity for apolar binding; the total accommodation by protein for the ligands was at its greatest for this particular system.

The data for entropy change occurring through interaction between the saturated acyl chain lysolecithin compounds and BSA obtained from these experiments show a significant but irregular negative ΔS. On a per mole of ligand bound per mole of protein the data show an irregular but definite trend toward greater ΔS change in the negative direction with increase in chain length of adduct ligand for the 10:0, 12:0, 14:0, and 16:0-18:0 mixture phospholipids. The entropy change found for these systems indicates that binding of the lysophosphatidylcholine ligands induces an ordering of these systems not found in the aqueous solutions of the reactant individually. This ordering of the system may be due in part to restriction of movement of the acyl chain of the complexed lysolecithin. The binding process also may be producing an ordering of the protein's amino acid residues with respect to

increase in α-helix content as suggested by Jirgensons' work discussed above.

In their discussions on hydrophobic binding Kauzmann, Scheraga and Neurath, and Nemethy and Scheraga have given consideration to entropy changes as a driving force for interactions between lipids and proteins and other systems (28, 71, 27). For example, it is proposed that the lipid is surrounded by highly structured water molecules having low entropy and as such yields a system of low entropy. Binding of the lipid to protein releases some of the structured water and produces a net positive entropy change for the system. Such is not the case found here for associations between lysolecithins and albumin since all entropy changes upon binding were rather strongly negative. Thus, the entropy change cannot be considered as the driving force for this system.

For the experiments involving the association of BSA with the unsaturated lysolecithins there are several major factors to consider: a) degree of unsaturation of the ligand, b) position of the double bond(s) with respect to the w-carbon atom of the acyl chain, c) bond angle alteration in the hydrocarbon chain induced by the cis isomer configuration of each double bond present, and d) the rotational rigidity introduced in the "carbon backbone" of the molecule by the presence of the double bond(s) with respect to spatial geometry required for interaction between the protein and phospholipid. The experimentally determined enthalpies for reaction of the three unsaturated compounds used (oleoyl, linoleoyl and

linoleonoyl ester moieties) vary a relatively small amount considering the increasing degree of unsaturation. The 18:1 and 18:2 compounds produced the same enthalpy change, $\Delta H_=$ -28 kcal, with the interaction of the 18:3 lysolecithin yield-somewhat less change, $\Delta H_=$-24 kcal. Two molecules of the phospholipid are bound per molecule of BSA in each case. The average values, -14 kcal for 18:1 and 18:2 and -12 kcal for the 18:3 compound are comparable to those values obtained for the saturated lysophosphatidylcholines. These results would dictate that the interaction with the protein is not significantly altered by the degree of unsaturation nor by the unsaturation itself. The change in molecular geometry induced by the presence of the double bond(s) does not produce obvious changes in the complexing process, i.e., production of different extent, of conformational change within the protein, for the interaction of the mono- and di-unsaturated compounds. Binding of the tri-unsaturated linolenoyllysolecithin appears to be somewhat different and will be discussed below. Even though having a longer chain length than that for the myristoyl compound and that for the average chain length of the lysolecithin itself, none of the unsaturated lysophosphatidyl compounds bind only a single molecule per molecule of BSA but rather, complex two molecules of the ligand. In the same vein, neither do the unsaturated 18 carbon chain compounds bind three molecules per albumin molecule as was found for the stearoyllysolecithin, the saturated 18 carbon chain molecule. It is probably only realistic to compare binding of the

unsaturated compounds to that of the saturated compounds 10:0 and 12:0 which also complexed two ligands per molecule of the protein. Irrespective of possessing more carbon atoms in the acyl chain of the unsaturated lysolecithin versus the 10:0 and 12:0 entities, the presence of the cis double bond configuration produces a decided bend (123^{o} bond angle versus 109^{o} for the saturated bond angle) in the backbone of the chain. This brings about an overall decrease in molecular length. Further, the rotational rigidity about the double bond carbons, compared to complete rotational freedom for the single bond situation, alters the geometry of the area, or perhaps better, the volume, swept by the molecular motion of the entity. These considerations can allow for the better binding affinity through a better spatial accommodation relative to the protein binding regions thereby producing a greater total and average (per molecule bound) enthalpy change than for any of the saturated systems investigated. The average values obtained for the free energy change, ΔG, for the unsaturated systems compared to the 10:0 and 12:0 saturated results show the former to be somewhat more negative indicating that the binding is tighter for the unsaturated lysolecithin-protein complex. This also supports the argument for better spatial fit for the apolar interaction between unsaturated lysolecithins and serum albumin.

Of the systems examined, the unsaturated compounds demonstrated the greatest average entropy changes for the interaction. These results would suggest greater ordering (less

entropy for the system) which, in part, can be attributed to the postulated better fit of the complex formed by the two reactants. Additionally, the introduction of the double bond(s) with concomitant loss of motional freedom by the lipid moiety may through interaction with certain of the amino acid residues induce a degree of rigidity in the protein molecule. Such a mechanism could decrease the randomness of the system. Again, decrease in the entropy of the system may result from displacement of solvent molecules by ligand complexation.

Apparently the unsaturated compounds studied, although having the same number of carbon atoms in the acyl chain as the stearoyllysolecithin, do not cause the same conformational change, or extent of change, in the protein with respect to the binding process as that produced by the saturated 18:0 phospholipid. This is in sharp contrast to the concept postulated for the process occurring with the stearoyl compound wherein binding of three ligands per molecule of BSA takes place. This can be explained only upon geometrical differences between the phospholipids.

Change in position of the double bond along the acyl chain with respect to binding appears to have little effect on the process. In the monounsaturated compound the double bond is between carbons 9 and 10 from the w-carbon of the chain while in the 18:2 compound double bonds are located between carbons 6 and 7 and between 9 and 10 with respect to the end carbon. However, the same ΔH was measured for the interactions of the two compounds with serum albumin. This

observation does not provide unambiguous proof, however, since there was also the change from one to two double bonds which may have offset an effect produced by positional change of the double bond. A more definitive experiment could be examined wherein two mono-unsaturated acyl chains with the double bond occurring at different positions on each chain are used as ligands.

Introduction of a third double bond in the molecule (linolenoyllysophosphatidylcholine) causes a smaller enthalpy change for the protein interaction than that encountered with the 18:1 and 18:2 entities. This may be explained through consideration of the change in area (or perhaps, better, the volume) swept by the molecule in solution in its interaction in the system concomitant with addition of the third double bond to the ligand molecule. The "bending back" upon itself of the acyl chain brought about by the 123^{o} cis bond angle at the three uniformly spaced positions for unsaturation could be the factor involved. This configuration could result in some degree of steric hindrance affecting the binding efficiency between the molecules. The compound in its binding process, however, does not cause sufficient change, if any at all, in the conformation of the protein to prevent complexation at a second site; nor does it produce, by conformational change, availability of a third binding region as was found for stearoyllysolecithin interaction. In the same consideration, complexing of the first molecule of the 18:3 lysolecithin does not block, through steric hindrance alone, the entrance of a second ligand to its binding region.

The apparent association constants calculated for the equilibrium interactions encountered in these experiments vary quite widely even within concentration range selected for calculation for a given phospholipid. Of course, where more than one ligand molecule is bound per molecule of the serum protein only an average value for the different association constants may be calculated. The calorimetric method used here does not permit differentiation of the individual association constants for the interactions at different binding sites but only provides an overall value for the system. These association constants may be quite different as illustrated by the data obtained by King for octanoate, L-tryptophan and D-tryptophan and by Wishnia and Pinder for hydrocarbon interaction with proteins which were presented earlier (59,45). Values obtained in this study ranged from a $K_a = 1.2 \times 10^4$ for the complexing of the myristoyllysolecithin to a $K_a = 1.0 \times 10^{11}$ for the stearoyl derivative. The apparent association constants found here agree in part in order of magnitude with that found for the octanoate-BSA interaction, $K_1 = 16.0 \times 10^4$ and $K_2 = 0.1 \times 10^4$ by King (60). Also Klopfenstein obtained a range of 3.0 to 5.5 $\times 10^4$ for the constant of the lysolecithin-albumin reaction (20).

Conclusions

Microcalorimetric measurements have been made on the interactions between a number of different lysophosphatidylcholines and bovine serum albumin in aqueous solutions to determine the enthalpies of the exothermic reactions produced by these systems. Two series of experiments were performed. In the first set the chain length of the fatty acid ester of the phospholipid was varied uniformly from 10:0 through 18:0 to ascertain the effect of chain length on the binding properties of the interaction. The second set of experiments was concerned with the effect of unsaturation of the acyl chain of the lysolecithin on its binding properties with BSA using the interactions of 18:1, 18:2, and 18:3 compounds. From the experimental data obtained, apparent association constants were calculated and, subsequently, values for ΔG and ΔS for the reactions were derived. The values for ΔH, ΔG, and ΔS were all negative for the systems studied.

The effect of increasing the length of the acyl chain is to produce an increasing enthalpy change in the negative direction suggesting that the interaction between lysophosphatidylcholine and BSA in solution is nonpolar. These experiments do not preclude the possibility that polar interaction may be involved in the binding process of the system.

Increasing the degree of unsaturation of the fatty acid moiety of the phospholipid did not produce a measurable difference in interaction of the 18:1 and 18:2 compounds. The

18:3 compound produced somewhat less change in enthalpy than the other two which may indicate that steric hindrance causes less tight binding with the protein in this case.

The decrease in free energy found for all the interactions studied might suggest that rearrangement of nonpolar portions of the amino acid residues involved in the binding process, if such change would decrease the free energy of the system, could be a basic part of the complexing mechanism. Concomitant association of the phospholipid may well contribute to the lower energy level of the system.

The association or complexation process produced a decrease in entropy for all systems examined. This dictates that the system becomes more ordered through the interaction of the phospholipid ligand and the protein molecule. There is evidence reported to indicate that more ordered structure results through restriction of movement of the acyl chain as well as the lysolecithin ordering the protein molecule. If hydrophobicity played a significant role in this binding process the interaction would not produce the rather large negative entropy change observed.

The values obtained in these experiments for the thermodynamic parameters examined show that the enthalpy change is the driving force responsible for the reaction. It seems probable that London - van der Waals forces are involved in the association of the lysolecithin and BSA in aqueous solution.

This study provides some values for thermodynamic properties of the complexed lipid-protein system and contributes additional evidence for participation of nonpolar binding in this interaction. In this association the BSA molecule has or develops accommodation for one to three ligands depending upon the lysophosphatidylcholine being bound.

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Appendix I

Table C. Data for Interaction of Myristoyllysolecithin with BSA

The information presented below is typical for all the experiments performed during this study.

Expt. No.	Mol phospho-lipid x 10^{-6}	Mol BSA x 10^{-6}	Mol Ratio Lipid/BSA	Mcal produced	Kcal/Mol Lipid	Kcal/Mol BSA
1-M	7.68	1.20	6.42	17.1	2.23	14.3
2-M	5.76	1.20	4.81	13.8	2.40	11.5
3-M	4.23	1.20	3.53	18.1	4.28	15.1
4-M	3.07	1.20	2.57	17.6	5.73	14.7
5-M	2.31	1.20	1.93	18.2	7.88	15.2
6-M	1.54	1.20	1.28	14.7	9.57	12.3
7-M	0.77	1.20	0.64	7.8	10.2	6.51
8-M	0.58	1.20	0.48	6.5	11.3	5.43
9-M	0.42	1.20	0.35	4.7	11.1	3.93
10-M	0.31	1.20	0.27	4.4	14.3	3.67
11-M	0.23	1.20	0.19	3.9	15.9	3.26
12-M	0.31	1.68	0.18	3.9	12.7	2.33
13-M	0.38	2.40	0.16	3.6	9.02	1.50
14-M*	0.31	1.20	0.27	4.3	14.0	3.59

* Repeat of experiment 10-M.

Appendix II

Enthalpy Curves for the Various Lysolecithins Binding with BSA.

The curves for binding of the myristoyl and oleoyl compounds with BSA have been presented previously on pages 21 and 23, respectively.

Figure 4. Enthalpy curves for the binding of caproyl-lysolecithin with BSA.

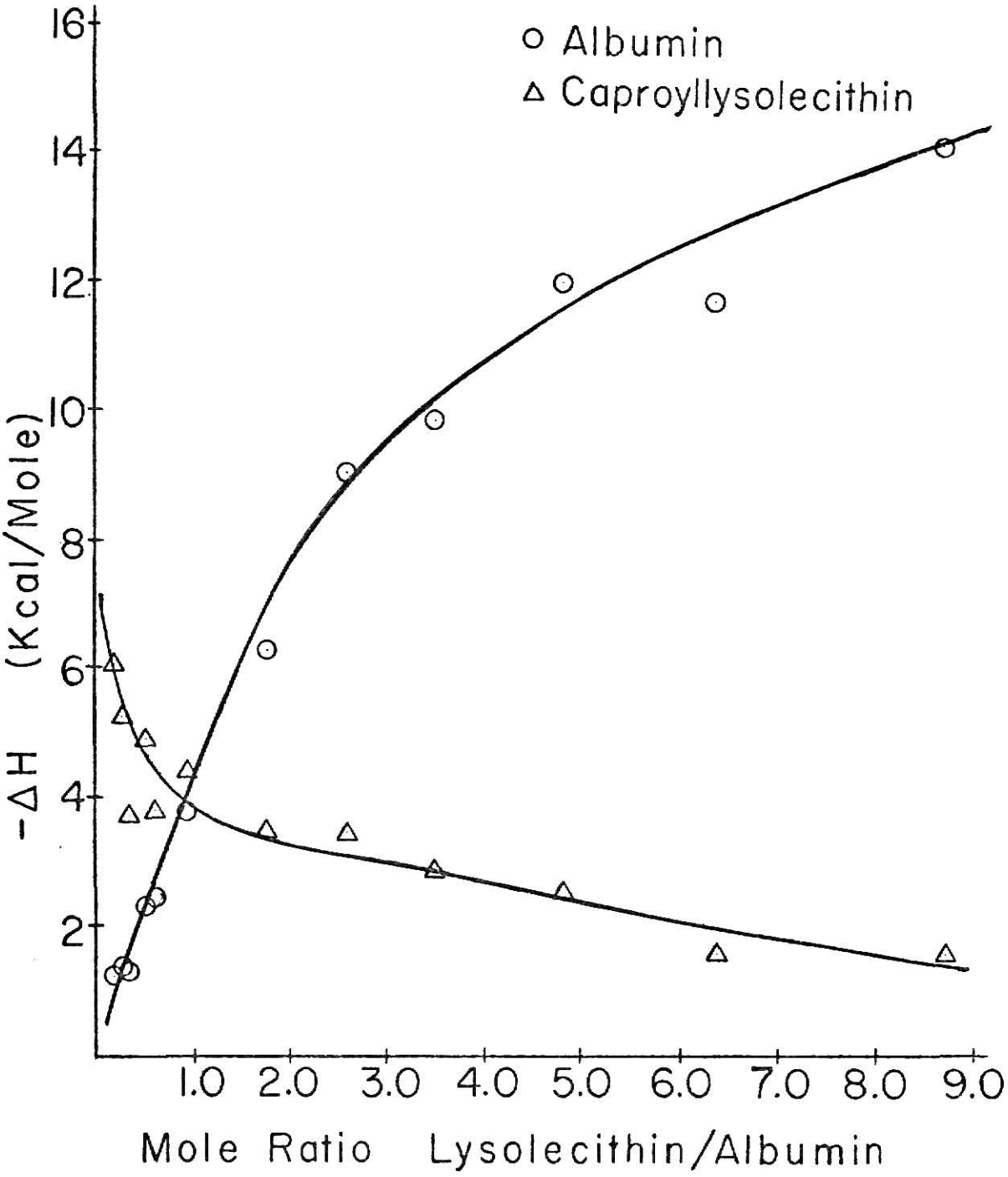

16
14
12
10
8
6
4
2
-ΔH (Kcal/Mole)
Albumin
Caproyllysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Mole Ratio Lysolecithin/Albumin

Figure 5. Enthalpy curves for the binding of lauroyl-lysolecithin with BSA.

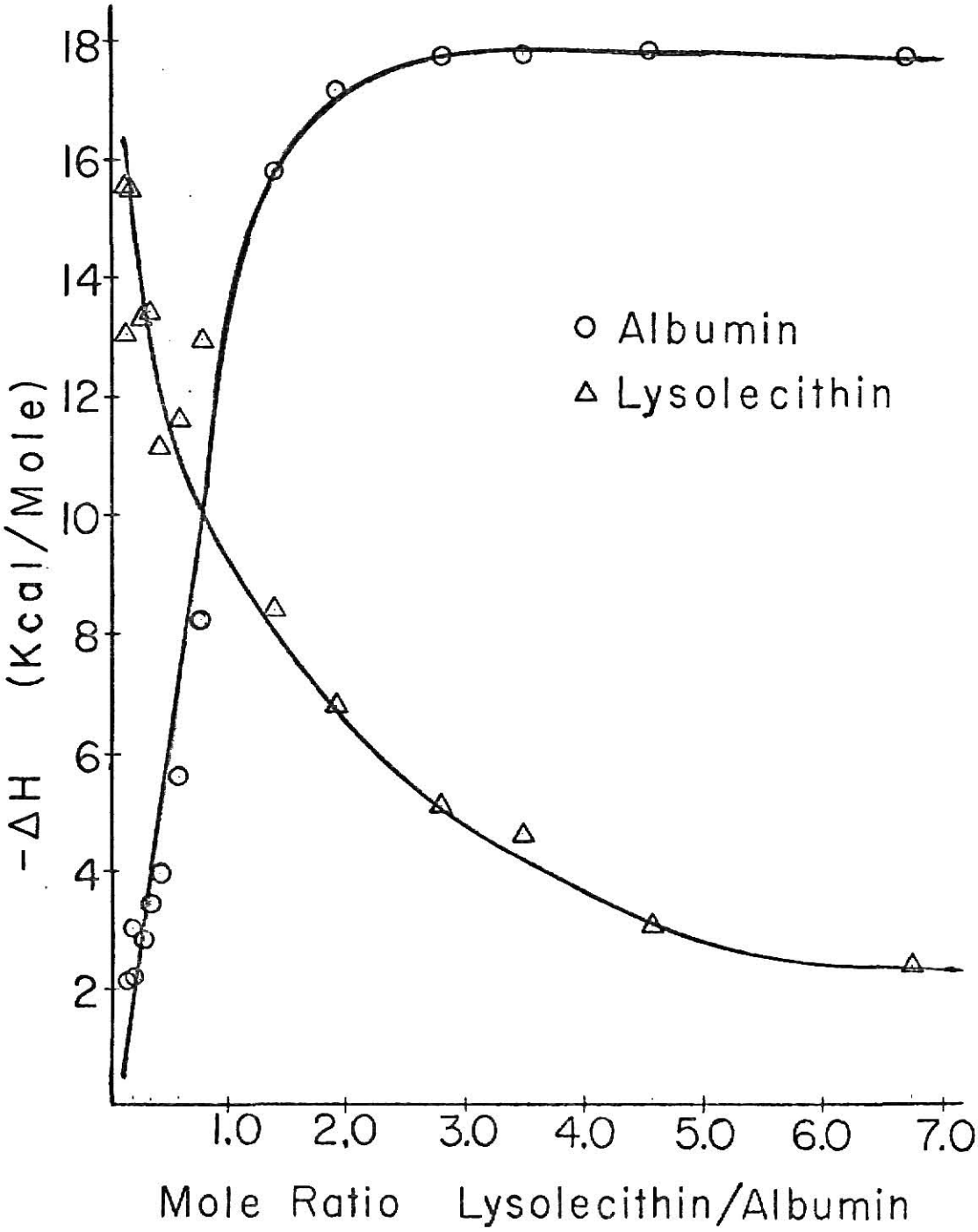

18
16
14
12
10
8
6
4
2
-ΔH (Kcal/Mole)
o Albumin
Δ Lysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Mole Ratio Lysolecithin/Albumin

Figure 6. Enthalpy curves for the binding of egg lysolecithin with BSA.

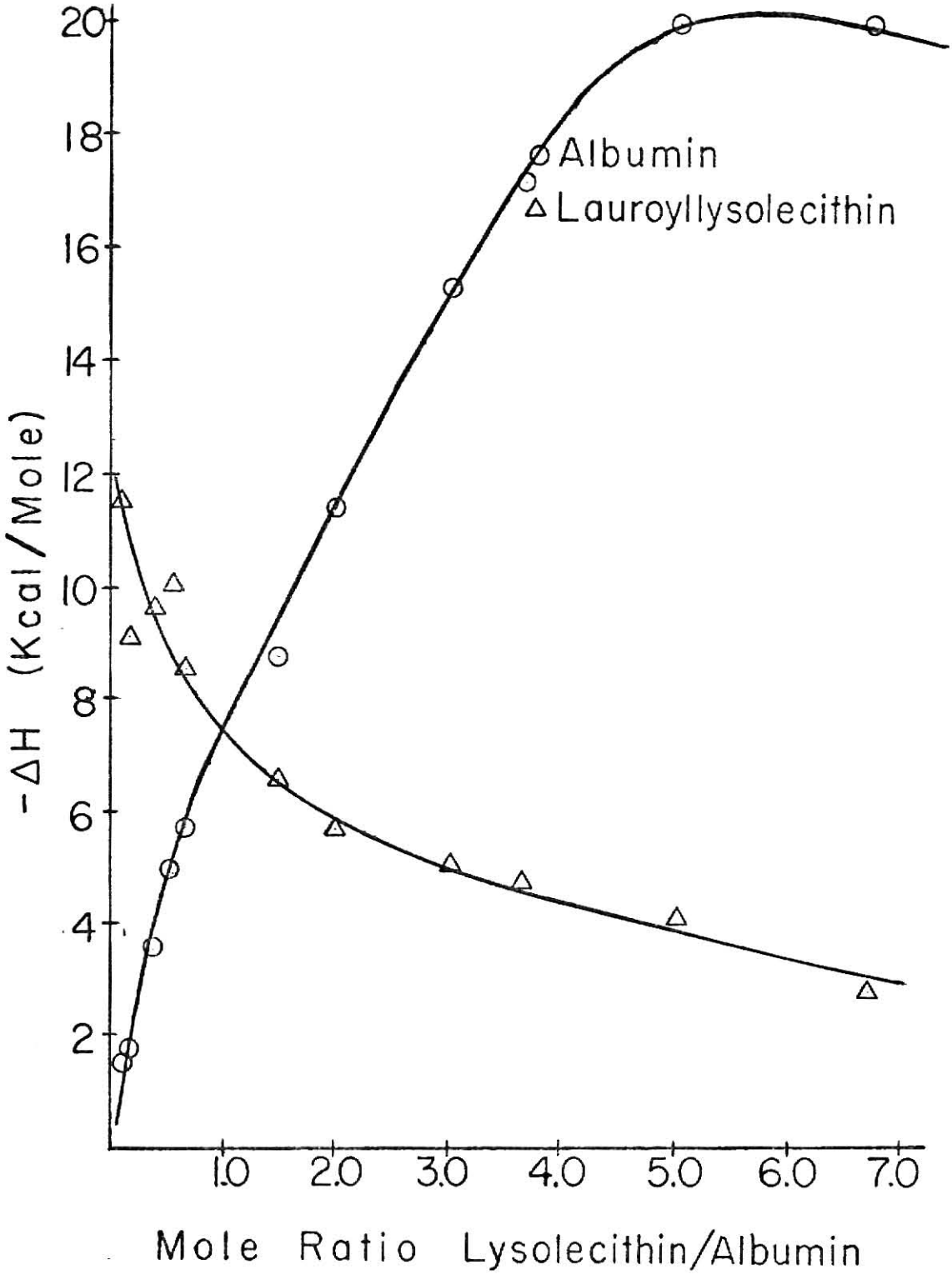

20
18
16
14
12
10
8
6
4
2
-ΔH (Kcal/Mole)
Albumin
Lauroyllysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Mole Ratio Lysolecithin/Albumin

Figure 7. Enthalpy curves for the binding of stearoyl-lysolecithin with BSA.

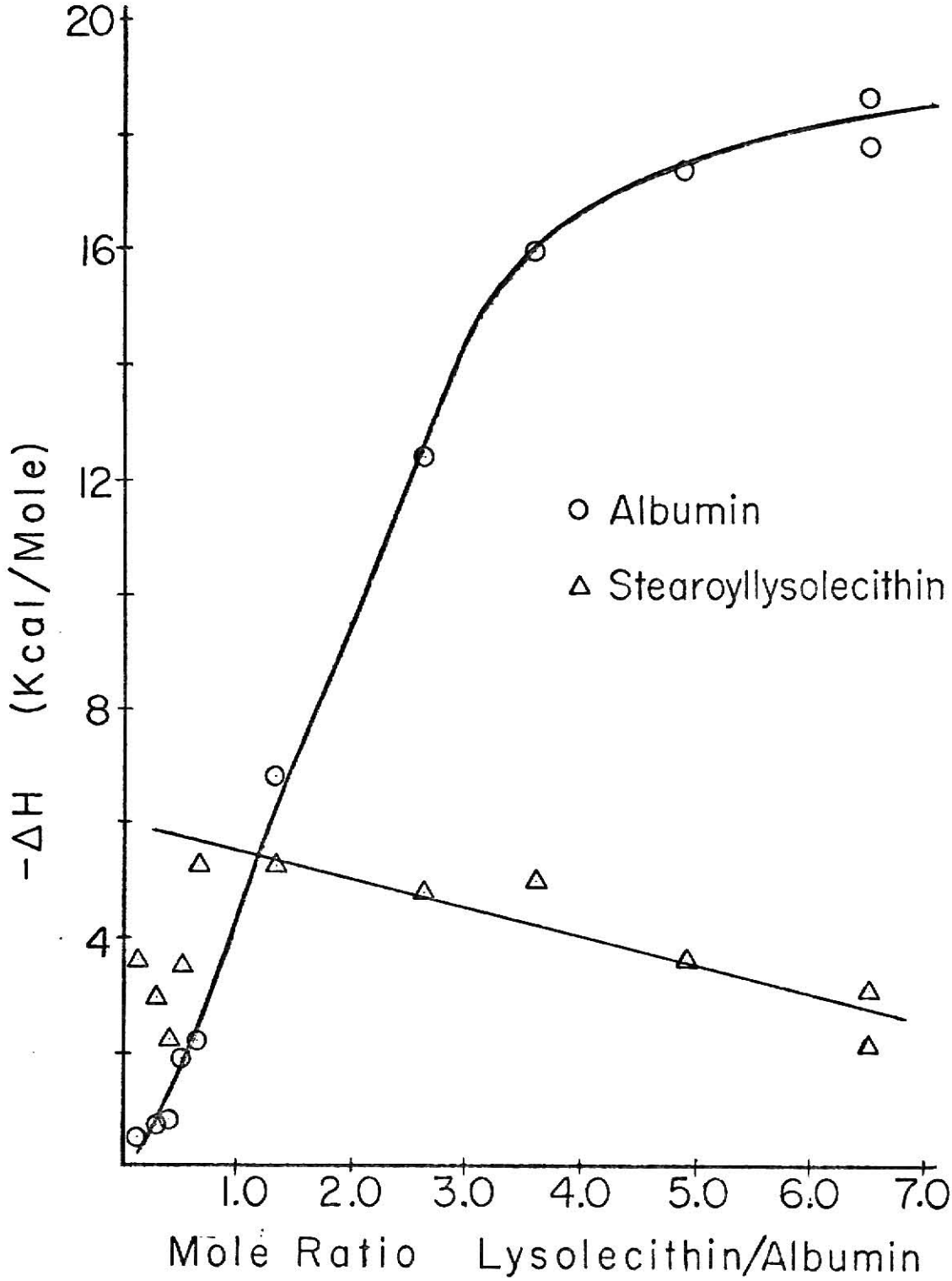

20
16
12
8
4
-ΔH (Kcal/Mole)
Albumin
Stearoyllysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Mole Ratio Lysolecithin/Albumin

Figure 8. Enthalpy curves for the binding of linoyl-lysolecithin with BSA.

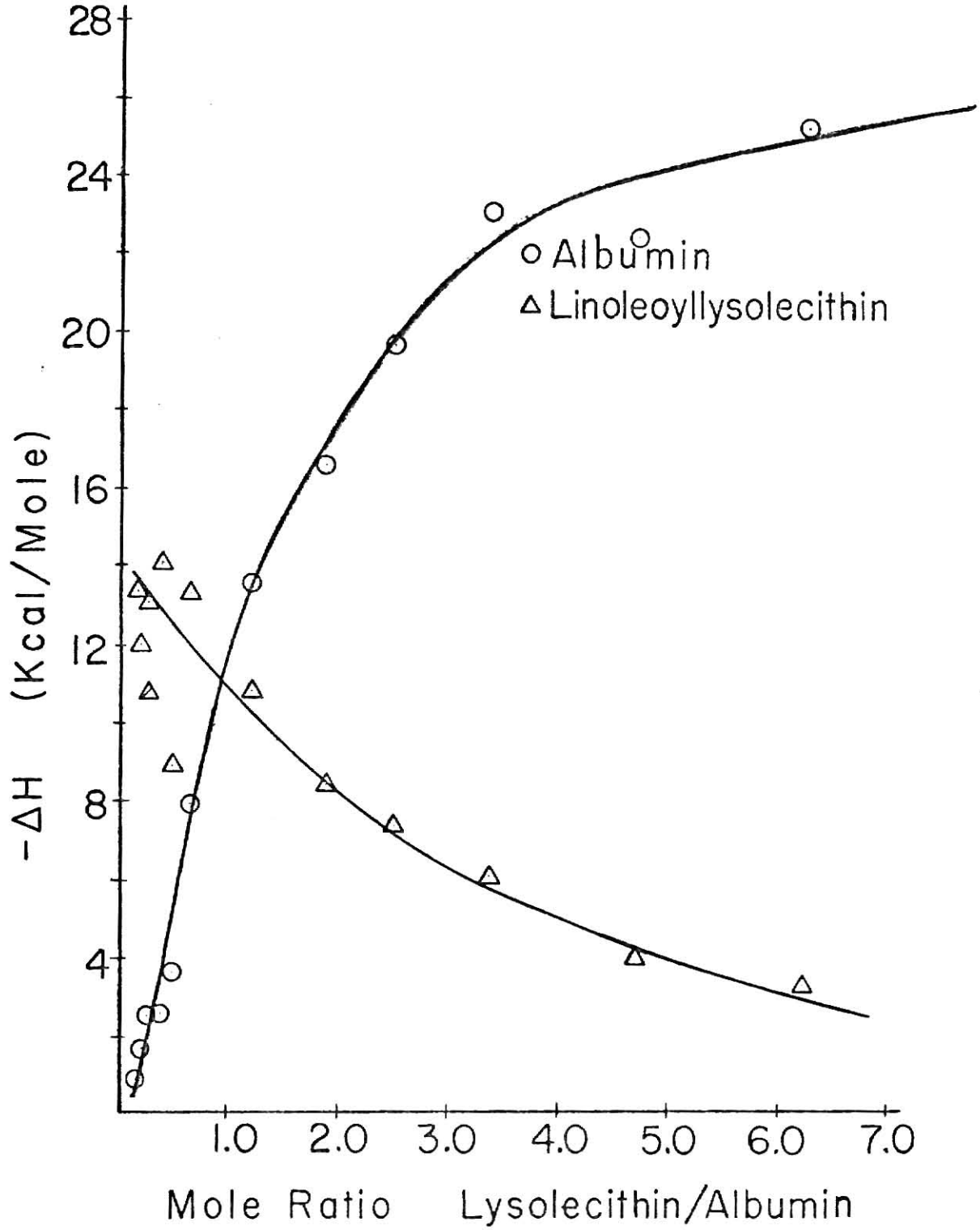
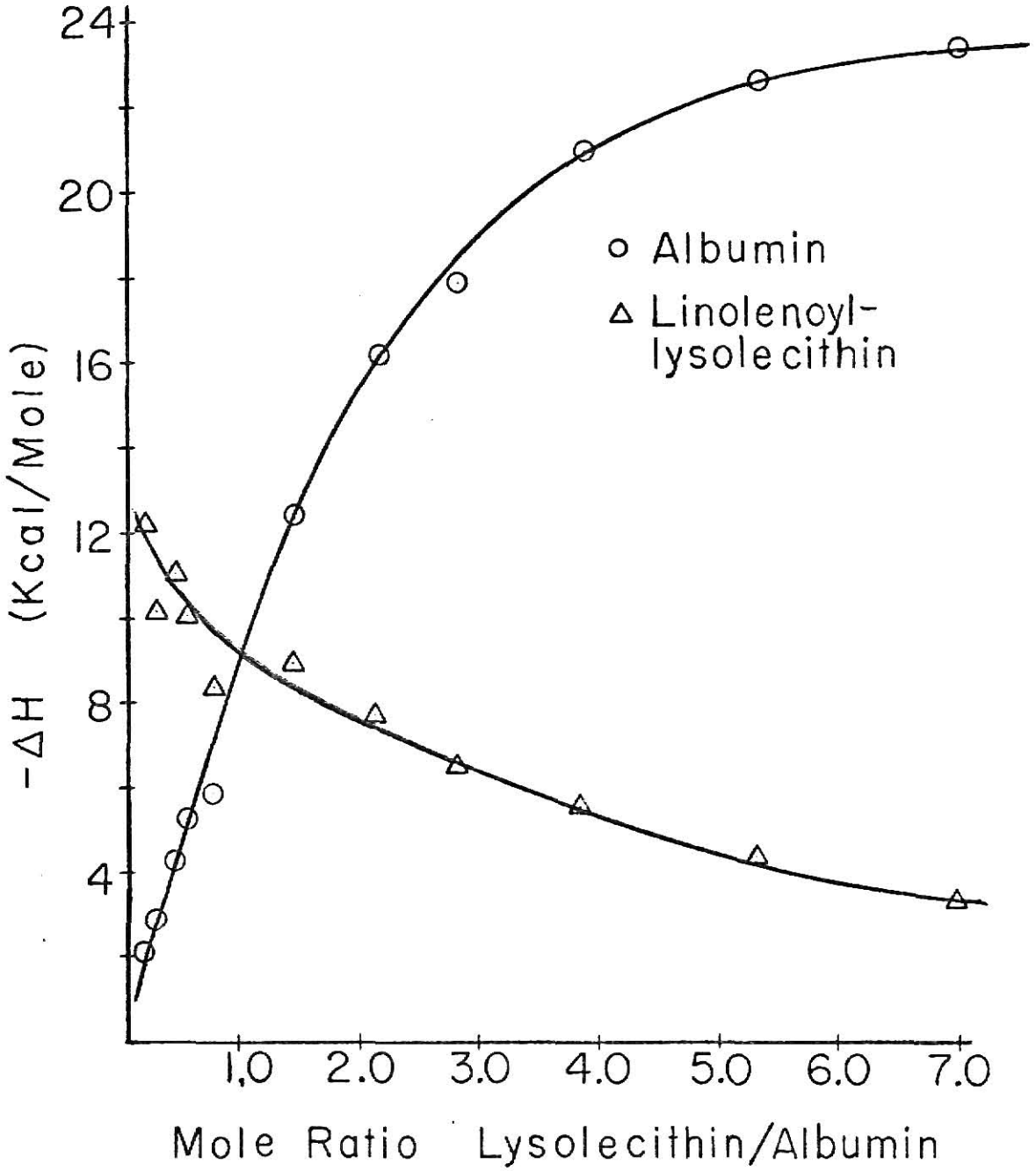

28
24
20
16
12
8
4
-ΔH (Kcal/Mole)
Albumin
Linoleoyllysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Mole Ratio Lysolecithin/Albumin

Figure 9. Enthalpy curves for the binding of linolenoyl-lysolecithin with BSA.


24
20
16
12
8
4
-ΔH (Kcal/Mole)
o Albumin
Δ Linolenoyl-
lysolecithin
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Mole Ratio Lysolecithin/Albumin

Appendix III

General Information and Data Used for Production of Table A, p. 25.

1. Conversion factors required for generating millicalories from area under the curve as measured by planimeter are given below (43).

μV Range	Conversion factor
50	4.866×10^{-2}
100	9.735×10^{-2}
200	0.1947

The above factors were supplied through the work of G. L. Dohm pursuant to a doctorate and this work is referenced above. During the course of this work it was necessary to overhaul the thermopile section of the calorimeter after which the instrument was recalibrated chemically by using the neutralization reaction of HCl by NaOH in solution. The average value thus obtained gave a calibration factor of 2.47 x 10^5 uV sec/cal or 2.47 x 10^2 uV sec/mcal. From this value new conversion factors were calculated for the various ranges, e.g., 200 uV range 1 planimeter unit is equivalent to 50.8 uV sec or 0.2056 mcal.

2. Extrapolation of the higher mole ratio portion of enthalpy curve for BSA, as shown by the broken line in Figure 2, p. 21, to zero mole ratio provides a value for the maximum theoretical heat change obtained by complete interaction of the ligand with one mole of BSA. Similarly, extrapolation of the enthalpy curve for the lysolecithin to zero mole ratio provides

the maximum enthalpy attributable to one mole of the ligand. The ratio of these two enthalpies provides, then, the molar binding ratio of ligand (the lysolecithin) to BSA for the system being examined.

3. Since the initial concentrations of reactants for a given experiment are known and the mole ratio in which the reactants combine to form the protein-phospholipid complex can be determined from the enthalpy curves, the equilibrium concentrations of reactants and complex can be calculated. From these values the association constants can be obtained. Values obtained are given below for myristoyllysolecithin interacting with BSA in aqueous solution at 20°C. to illustrate a typical set of data.

Mol ratio Lipid/BSA	K_a
0.5	6.73×10^3
1.0	1.19×10^4
2.0	1.52×10^4
3.0	1.59×10^4

4. From the molar binding ratio and the subsequent calculation of the association constants one can calculate the free energy change, ΔG, for the systems from the relationship, $\Delta G = -RT \ln K_a$. Having measured ΔH for the interaction and being able to calculate ΔG for the system at 20°C, one may then determine the change in entropy for the binding process for the lysolecithin complexing with BSA from the following equation:

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

. A complete set of sample calculations is provided in Appendix IV, p. 78.

Appendix IV

A Sample Set of Calculations

1. The binding of myristoyllysophosphatidylcholine with BSA does so in a 1:1 ratio as determined from the enthalpy curves presented in the body of the thesis, as Figure 2, p. 25, and is indicated by broken-line extrapolations of the curves. The maximum theoretical enthalpy is shown by the extrapolation of the BSA curve at the higher experimental mole ratios of the lysolecithin to BSA and is found to be, $\Delta H = -16.0$ kcal.

2. Selecting an experimental mole ratio of 2.0 for the reactants we find this represents an enthalpy of $\Delta H = -14.3$ kcal as obtained from the BSA enthalpy curve. The ratio of these values shows the extent of reaction occurring between the two species. Thus: $\frac{-14.3 \text{ kcal}}{-16.0 \text{ kcal}} = 0.89$ or a binding efficiency of 89% at a 2:1 mole ratio of the reactants.

Assuming 1 mole of BSA:

there is, 0.89 moles of complex formed
0.11 moles of free BSA
0.89 moles of myristoyllysolecithin complexed
1.11 moles of free myristoyl compound

therefore:

$$K_a = \frac{(0.89 \times 4.78 \times 10^{-4})}{(0.11 \times 4.78 \times 10^{-4})(1.11 \times 4.78 \times 10^{-4})}$$

$$K_a = 1.52 \times 10^{4}$$

3. At equilibrium, $\Delta G = -RT\ln K_a$

Reaction occurs at 20.0°C or 293.0°K

So, $\Delta G = -2.987 \times 293 \times 2.303 \times \log 1.52 \times 10^4$

$\Delta G = -5610$ cal $= -5.6$ kcal

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

Substituting the values above for ΔG and ΔH we have,

$$-5610 = -16{,}000 - T\Delta S$$

$$T\Delta S = -10{,}390 \text{ cal} = -10.4 \text{ kcal}$$

at 20°C. $\Delta S = -35.5$ e.u.

STUDIES ON SERUM ALBUMIN
BINDING OF VARIOUS LYSOLECITHINS

by

W. Mack Barlow

M.A., Kansas University, 1945

AN ABSTRACT OF

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Graduate Biochemistry Group

Kansas State University
Manhattan, Kansas

1976

Approved by:

William E. Kloppenstein
Major Professor
Department of Biochemistry

Abstract

Heatburst microcalorimetry has been used to measure the enthalpy of binding interaction of a number of lysolecithins with bovine serum albumin in aqueous systems. The effect of acyl chain length of the phospholipid and of degree of unsaturation of this chain of the compound upon the binding properties of the protein-lysolecithin interaction have been studied. By plotting the change in enthalpy of the system versus the mole ratio of the reactants one can determine the maximum theoretical heat change obtained from complete interaction of the ligand with one mole of BSA. Also, one can determine the molar binding ratio of the reactants from such plots. From these data one can determine the equilibrium constants for the interactions of the various systems. With this information the free energy change and entropy change may be calculated for the interactions studied.

All thermodynamic parameters calculated were in the negative direction; i.e., showed a decrease in value for the binding process. An increase in acyl chain length of the lysophosphatidylcholine exhibited an increase in enthalpy change for the complexing process with BSA. The results would indicate that non-polar bonding is an important factor in the interaction. The binding of lysolecithins having unsaturated acyl chains produced similar results found for saturated compounds binding in the same molar ratio but did not indicate much change with change in degree of unsaturatedness.