A Calorimetric Study of Peptide–Phospholipid Interactions: The Glucagon–Dimyristoylphosphatidylcholine Complex†

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ABSTRACT: The isothermal enthalpy of reaction of glucagon with dimyristoylphosphatidylcholine is found to be large and markedly temperature dependent, changing from −150 kcal/mol of glucagon at 25 °C to + 80 kcal/mol of glucagon at 23 °C. The observed enthalpy is shown to arise mainly from the glucagon-induced alteration of the distribution of the phospholipid between gel and liquid crystalline phases. The reaction is accompanied by a large change in the apparent heat capacity around the phase transition temperature of the pure lipid which explains the small variation of the free energy of binding with temperature despite the large changes in enthalpy. The equilibrium binding constant calculated from the enthalpy as a function of lipid–peptide ratio is in good agreement with that previously found from fluorescence titrations, but the number of lipid molecules bound per glucagon molecule at 25 °C is increased from 20 to 50, suggesting that glucagon can bind to 50 lipid molecules but that only 20 of these are close enough to affect the fluorescence of the hormone. Differential scanning calorimetry of dimyristoylphosphatidylcholine in the presence of glucagon shows a transition curve composed of two components. The major component, corresponding to 80% of the enthalpy change, is centered at 26.1 °C and has a cooperative unit of 45 lipid molecules. The total enthalpy change for the transition in the presence of glucagon is only +3.2 kcal/mol, compared with +4.8 kcal/mol for the pure lipid.

Proteins and phospholipids are two of the major constituents of biological membranes. A study of the nature of the interaction between these two classes of molecules is important to an understanding of the structure and function of biological membranes. One mechanism by which proteins can interact with lipids is through the formation of an amphipathic helix in which the hydrophobic residues of the protein are segregated on one face of the helix. Such protein structures are thought to be important in the formation of serum lipoprotein particles (Segrest et al., 1974). The hormone glucagon, a 29 amino acid polypeptide, also has a sequence which would enable it to form an amphipathic helix (Epand et al., 1977a,b). Glucagon is an appropriate model peptide for these studies because it has a relatively simple structure, it is available as a pure crystalline material, it reacts very rapidly with dimyristoylphosphatidylcholine (DMPC)[1] (Epand & Epand, 1980), and it forms a homogeneous reaction product with this phospholipid (Jones et al., 1978). The reaction product is apparently identical whether it is formed above or below the phase transition temperature of the pure phospholipid. This is shown by the similarity of the fluorescence emission spectrum of the glucagon–DMPC complex at 25 °C to those below this temperature and the invariance of the equilibrium binding constant and number of DMPC molecules bound per glucagon molecule below this temperature (Epand & Epand, 1980). In addition, freeze–fracture studies on this system demonstrate that samples quenched from temperatures either above or below the phase transition of the phospholipid both show similar discoidal particles along with increasing amounts of multilamellar structures at higher temperatures (T. P. Stewart, S. W. Hui, and R. M. Epand, unpublished observations). This study was therefore undertaken to explore an aspect of the thermodynamics of lipid–protein interactions using glucagon as a model system. However, although the physiological relevance of this interaction is uncertain, there are many processes in which it is of potential importance, such as storage, transport, or binding of glucagon to plasma membranes.

Materials and Methods

Materials. Crystalline bovine–porcine glucagon was purchased from the Elanco Corp. and used without further purification. The amino acid analysis obtained after acid hydrolysis of this material was that expected for glucagon. Ion-exchange chromatography on carboxymethylcellulose showed only a minor impurity representing about 5% of the total and probably corresponding to monodesamidoglucagon which has conformational and biological properties similiar to those of the native hormone (Bromer et al., 1972). The DMPC was purchased from Sigma Chemical Co. and its purity confirmed by differential scanning calorimetry.

Glucagon Solutions. Glucagon crystals were dissolved by gentle warming in a buffer of 20 mM Pipes, 1 mM EDTA, 150 mM NaCl, and 0.02 mg/mL NaN_3, pH 7.40. The concentration of glucagon was determined spectrophotometrically after clarification of the solution by centrifugation at 10 000 rpm in a JA-21 rotor of a Beckman centrifuge for 15 min. An absorption coefficient of 2.38 L g^{-1} cm^{-1} at 278 nm was used (Gratzer et al., 1967).

Phospholipid Suspensions. Solid samples of DMPC were suspended in the Pipes buffer by vigorous vortexing for 30 s at about 30 °C. Lipid concentrations in the final solutions were determined by the method of Bartlett (1959) after perchloric acid ashing of the sample.

Heats of Reaction. The isothermal enthalpy of reaction between glucagon solutions and suspensions of DMPC was measured by using an LKB 10700-2 batch microcalorimeter. The instrument was calibrated both electrically and by means of the reaction of NaOH with HCl (Grenthe et al., 1970). Each reaction mixture was taken through three mixing cycles, and the heat of reaction was corrected for the frictional heat

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[1] Abbreviations used: DMPC, 1,2-dimyristoyl-*sn*-3-glycerophosphocholine; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; DSC, differential scanning calorimetry.

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of mixing observed after completion of the reaction. Generally 4 mL of glucagon solution was used, and the final volume after mixing was 6 mL. Heats of dilution of glucagon and DMPC were measured separately. The heats of dilution were negligible compared to the heats of reaction, and either glucagon solution or buffer was used in the reference cells for the measurements of reaction enthalpies.

Differential Scanning Calorimetry (DSC). A scanning microcalorimeter designed by Privalov (1980) was employed. Scans of buffer vs. buffer resembled those of buffer vs. 50 μM glucagon in buffer, being linear and close to horizontal. Samples of glucagon–DMPC mixtures were in general obtained directly from the isothermal batch calorimeter. Care was taken to avoid allowing the temperature of the samples to rise during the transfer from the batch to the scanning calorimeter, scans were always begun at temperatures below that used in the batch calorimeter, and the system was rescanned at least once. Since in this method the amount of material used for scanning calorimetry is limited by the low solubility of glucagon at pH 7.4, more concentrated solutions were prepared from glucagon suspensions in order to obtain a higher signal to noise ratio at a low lipid to glucagon ratio. These samples were prepared by mixing, at 23 °C, 1 mL of glucagon suspension (5 mg/mL buffer) with 1 mL of a suspension of DMPC (20 mg/mL) which had been vortexed into buffer at 30 °C. The resulting suspension was warmed and cooled through the transition temperature several times until an optically clear solution resulted. This solution was used for the DSC studies. Scan rates of 0.5 °C/min were generally employed, but qualitatively similar curves were obtained at scan rates from 0.1 to 2 °C/min. Repeated scans of samples with a DMPC to glucagon ratio of less than 50 were identical, while repeated scans from samples containing more lipid showed an increase of a few percent in the peak corresponding to pure lipid at 24 °C after each scan.

Results

Heats of reaction of glucagon and DMPC at 23 and 25 °C are shown as a function of DMPC to glucagon molar ratio (Figure 1). The notable result from these experiments is the very large change in the enthalpy of reaction from −150 kcal/mol of glucagon at 25 °C to + 80 kcal/mol of glucagon at 23 °C at saturation of glucagon with lipid. The results at 25 °C were analyzed by assuming that the extent of reaction is proportional to the fractional change in the total enthalpy released per mole of glucagon, the binding sites are equivalent and independent, and the system is in equilibrium. The system appeared to be in equilibrium by several criteria. The heat changes observed in the batch calorimeter occurred over a period of only a few minutes; the DSC trace obtained after heating a sample containing DMPC and glucagon at a 30:1 molar ratio to 80 °C was very similar to that obtained when the sample had been heated to only 40 °C; incubation of the product of the reaction at 25 °C for 4 days at either 0 or 25 °C resulted in only minor alterations in the DSC curves. The observed data fit well to a calculated curve using 51 as the number of lipid molecules bound to protein, 7.8×10^4 M^{-1} as the equilibrium binding constant, and −155 kcal/mol of glucagon as the total enthalpy change at saturation with lipid. The results at 23 °C showed poorer precision. Factors contributing to this may include the much slower rate of reaction at 23 °C compared with 25 °C (Epand & Epand, 1980), resulting in part of the reaction being indistinguishable from base-line drift, incomplete reaction or small perturbations resulting in modification of either the product or the starting material.

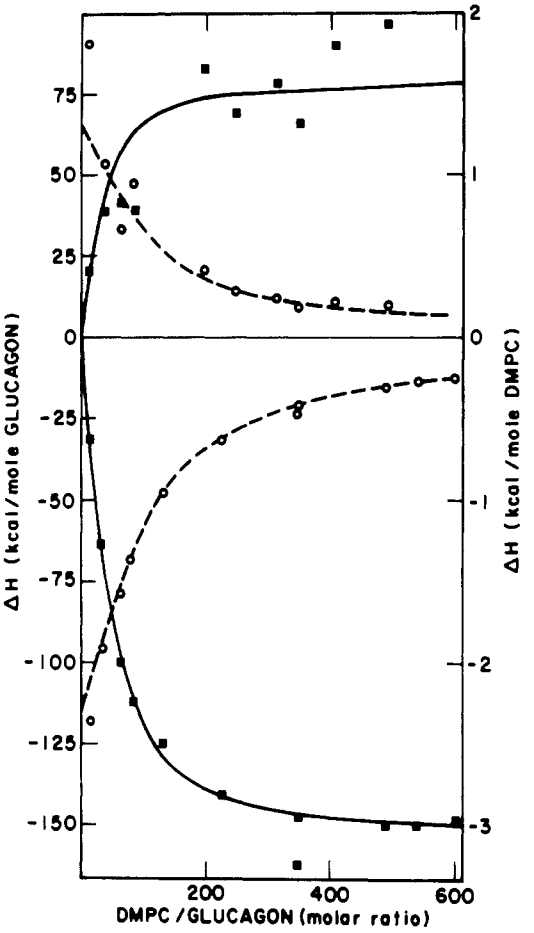

FIGURE 1: Isothermal enthalpy of the reaction of glucagon with DMPC (50 μM glucagon in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl, and 0.02 mg/mL NaN_3, pH 7.40, reacted with a vortexed suspension of DMPC). (○) Enthalpy per milliliter of DMPC; (■) enthalpy per mole of glucagon. Positive enthalpy values, data at 23 °C, curve calculated using 51 lipid molecules bound per glucagon, a binding constant of 1.2×10^5, and a total enthalpy change of 80 kcal/mol of glucagon. Negative enthalpy values, data at 25 °C, curve calculated using 51 lipid molecules per glucagon molecule, a binding constant of 7.8×10^4, and a total enthalpy change of −155 kcal/mol of glucagon. The binding constant at 23 °C was chosen to give a ratio of 1.5 for the constants at the two temperatures (see Discussion).

The products of several of the isothermal reactions were analyzed with DSC. After reaction at 25 °C with lipid to glucagon ratios below 50, no transition corresponding to free lipid could be observed, although it was present at higher lipid to peptide ratios and gave a small contribution to the 30:1 DMPC/glucagon sample prepared by reaction at 23 °C, confirming incomplete reaction at this temperature. At a lipid to glucagon ratio of 13:1 the DSC traces of samples reacted at 25 °C were identical with those of samples reacted at 23 °C (lipid concentration 0.35 mg/mL) as well as of a sample of 20:1 DMPC to glucagon (lipid concentration 10 mg/mL). The enthalpy of the transition is 3.2 ± 0.1 kcal/mol of DMPC, and the DSC curve can be resolved into two components by a trial and error curve-fitting procedure (Figure 2). The major component, comprising 80% of the total enthalpy, has a transition temperature of 26.1 °C and a van't Hoff enthalpy of 129 kcal/mol, corresponding to a cooperative unit of 40 lipid molecules. The lower transition is at 23 °C; it corresponds to 20% of the total enthalpy and has a cooperative unit of 83.

We have measured the isothermal heat of reaction of glucagon with a multilamellar suspension of DMPC at several temperatures. These observed values are compared in Figure 3 with those calculated on the basis of the enthalpy change

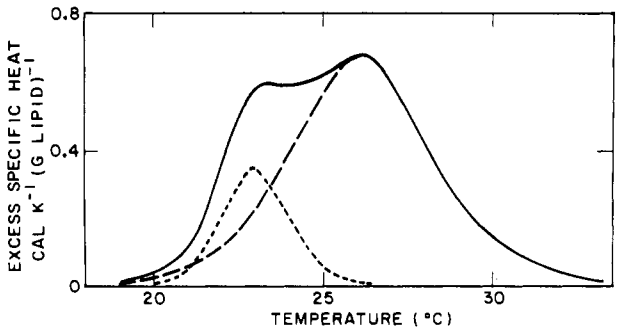

FIGURE 2: DSC of the transition region of the glucagon–DMPC complex at 1:13 molar ratio and its resolution into components.

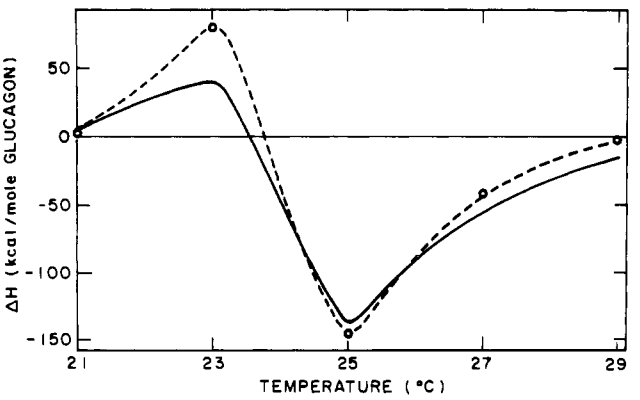

FIGURE 3: Effect of temperature on the isothermal enthalpy of reaction of glucagon with DMPC. (-O-) Observed enthalpy change at a DMPC/glucagon ratio of 350 or greater; (—) calculated enthalpy based on change in the state of the lipid, as described under Results.

expected from the change in the state of the lipid induced by glucagon. This change in state was estimated from the DSC curves of DMPC in the presence and absence of glucagon by measuring the fraction of the total area under the curves which occurred above the temperature in question. At 25 °C, for example, the pure lipid is completely in the liquid crystalline state while 57% of the glucagon–DMPC mixture has yet to undergo its transition. Therefore when glucagon reacts isothermally with DMPC at 25 °C, 57% of the lipid will be converted to the gel state. Since the conversion of DMPC from liquid crystal to gel state is an exothermic reaction of 4.8 kcal/mol (this work), the reaction of glucagon with DMPC at 25 °C would be expected to result in a release of (0.57)(–4.8 kcal/mol of DMPC)(50 mol of DMPC/mol of glucagon) = –137 kcal/mol of glucagon. Similar calculations made for other temperature are compared with the observed enthalpy change at these temperatures (Figure 3).

There are several assumptions required to make this comparison which may affect the quantitative agreement between observed and calculated values. It is assumed that the 350-fold molar excess of lipid is sufficient to shift the equilibrium largely toward the formation of the lipoprotein complex and that the reaction will proceed at a rapid enough rate to allow measurement. The reactions at 21 and 29 °C may not have gone to completion because of the much slower rate of reaction at these temperatures (Epand & Epand, 1980). It is also assumed that the number of lipid molecules bound per glucagon is invariant with temperature. The analysis also requires that the DSC curve in the presence of glucagon be taken as representing a two-state conversion of lipid from gel to liquid crystalline state despite its complex shape. The enthalpy determined for this transition was 3.2 kcal/mol of DMPC instead of 4.8 kcal/mol as observed in the absence of glucagon. Several factors may give rise to this lower enthalpy, including

Table I: Thermodynamic Parameters for the Reaction of Glucagon with DMPC at 23 and 25 °C

temp (°C)	23	25
ΔG° [kcal (mol of glucagon)$^{-1}$]	−6.9 ± 0.5	−6.7 ± 0.1
ΔH° [kcal (mol of glucagon)$^{-1}$]	80 ± 5	−150 ± 3
ΔS° [eu (mol of glucagon)$^{-1}$]	290 ± 20	−480 ± 10
ΔC_p [kcal K^{-1} (mol of glucagon)$^{-1}$]	−115 ± 4	

a perturbed gel state in the presence of glucagon (Tarashi & Mendelsohn, 1979) and the enthalpy of glucagon–lipid interaction (see Discussion). Despite these complexities, the calculated values of the enthalpy are in remarkably good agreement with the observed values, indicating that the enthalpy of the lipid transition is largely responsible for the observed enthalpy of interaction of lipid and peptide.

Discussion

The large exothermic heat of reaction obtained with glucagon and DMPC, −150 kcal/mol of glucagon at 25 °C, is comparable to that found for the combination of serum apolipoprotein with phospholipids (Rosseneu et al., 1974, 1976, 1977; Pownall et al., 1977; Massey et al., 1979). The results with the serum apolipoproteins have been interepreted as arising from changes in the conformation or state of association of the protein caused by the presence of lipid. It is clear from our results that these large enthalpy effects can also occur as a result of a change in the state of the lipid. Thus only a 2 °C change, from 25 to 23 °C, results in the heat of reaction of glucagon with DMPC changing from highly exothermic to highly endothermic (Figure 3). The major lipid transition in DMPC–apolipoprotein recombinants, from DSC studies, resembles the results we have obtained with glucagon in that the lipid transition is broadened and shifted to slightly higher temperature (Andrews et al., 1976; Tall et al., 1977). Thus in the case of serum apolipoproteins, and indeed in the case of all substances that alter the phase distribution of phospholipids, a large contribution to the enthalpy of reaction is expected to arise from the change in the state of the lipid rather than as a reflection of the thermodynamics of the noncovalent bonding stabilizing the lipid–protein association or as a result of protein conformational changes.

Despite the sensitivity of the heat of reaction to changes in temperature, the standard free energy change appears relatively invariant with temperature [Table I of this work and Epand & Epand (1980)]. In order to explain this apparent anomaly, we can calculate an apparent ΔC_p for the reaction over the narrow temperature range 23–25 °C, arriving at an expression for the enthalpy

$$\Delta H = \Delta H_0 - 115T \quad (1)$$

where the constant ΔH_0 has the value 34137 kcal mol^{-1}. The large value of ΔC_p, −115 kcal K^{-1} mol^{-1}, results from the fact that all of the pure lipid melts over this temperature range while only a small fraction of the glucagon–DMPC complex melts between the same temperature limits. Integration of the van't Hoff equation

$$\mathrm{d} \ln K/\mathrm{d}T = \Delta H/(RT^2) \quad (2)$$

between the limits 23 and 25 °C, with ΔH given by eq 1, leads to the prediction that the ratio K_{23}/K_{25} should be very close to unity, in agreement with the experimental results. The near constancy of ΔG° means that the standard entropy change for the reaction changes markedly between 23 and 25 °C, to give the enthalpy–entropy compensation which is inevitable when ΔC_p is large.

Thus far we have considered the enthalpy changes only in terms of the change in the state of the lipid. However, lip-

id–protein interactions and protein conformational changes are also likely to make a contribution to the overall enthalpy change. Regarding the conformational change in the protein, we have used only dilute solutions of glucagon for the lipid-binding studies where the peptide is monomeric and largely devoid of helix content while it attains about 30% helix content when associated with lipid (Epand et al., 1977a). To estimate the magnitude of these enthalpy changes we can compare them to the enthalpy of association of glucagon which is accompanied by a similar change in helix content (Gratzer & Beaven, 1969) and involves hydrophobic interactions among subunits (Sasaki et al., 1975). The enthalpy of trimerization of glucagon is −10 kcal/mol of glucagon (Formisano et al., 1977; Johnson et al., 1979). This value is to be compared with that obtained from the transition of 50 lipid molecules, about −250 kcal/mol of glucagon. Thus, a priori, we would expect it to be difficult to observe the enthalpy contribution from lipid–protein interactions. The rate of dissociation of glucagon from DMPC is highly temperature dependent, with the energy of activation increasing markedly with decreasing temperature around the phase transition (Epand, 1978). The half-time of reaction at 35, 30, and 25 °C is about 5, 35, and 700 min, respectively. Thus even at a slow scan rate of 0.1 °C/min little glucagon will have dissociated by 30 °C, a temperature at which the lipid transition is essentially complete. By about 40 °C, however, even at faster scan rates, most of the glucagon should be dissociated. We have been unable to detect any transition in the DSC in the temperature range 30–40 °C that would correspond to the dissociation of glucagon from lipid, confirming that this process has a much lower enthalpy than that corresponding to the lipid transition. The results also demonstrate that the lipid-phase transition occurs independently of the dissociation of glucagon. Considering the reverse reaction, the formation of the glucagon–lipoprotein particle, both the enthalpy data (Figure 1) and the fluorescence titrations (Epand & Epand, 1980) indicate that a large fraction of the glucagon is binding to equivalent and independent sites. This process is not accompanied by a lipid-phase transition since we have shown that the binding of glucagon and the phase transition occur independently. Thus, in the series of reactions required to form the lipoprotein complex from glucagon and lipid there must be separate step(s) involving few or no glucagon molecules in which the lipid-phase transition takes place.

The number of DMPC molecules bound to 1 glucagon molecule is 50 (Figure 1). This is the same as the ratio of lipid to peptide found in the stable glucagon–DMPC lipoprotein particle (Jones et al., 1978) and is close to the size of the cooperative unit of the major component of the transition. The number, however, is much higher than the 20 molecules of DMPC found bound to glucagon by fluorescence titration (Epand & Epand, 1980). We therefore conclude that each glucagon molecule can alter the phase transition properties of 50 DMPC molecules to which it is bound but that only 20 of these 50 lipids are close enough to the peptide to affect its fluorescence properties.

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References

Andrews, A. L., Atkinson, D., Barratt, M. D., Finer, E. G., Hauser, H., Henty, R., Leslie, R. B., Owens, N. L., Phillips, M. C., & Robertson, R. N. (1976) *Eur. J. Biochem. 64*, 549–563.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Bromer, W. W., Boucher, M. E., Patterson, J. M., Pekar, A. H., & Frank, B. H. (1972) *J. Biol. Chem. 247*, 2581–2585.

Epand, R. M. (1978) *Biochim. Biophys. Acta 514*, 185–197.

Epand, R. M., & Epand, R. F. (1980) *Biochim. Biophys. Acta 602*, 600–609.

Epand, R. M., Jones, A. J. S., & Sayer, B. (1977a) *Biochemistry 16*, 4360–4368.

Epand, R. M., Jones, A. J. S., & Schreier, S. (1977b) *Biochim. Biophys. Acta 491*, 296–304.

Formisano, S., Johnson, M. L., & Edelhoch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3340–3344.

Gratzer, W. B., & Beaven, G. H. (1969) *J. Biol. Chem. 244*, 6675–6679.

Gratzer, W. B., Beaven, G. H., & Bailey, E. (1967) *Biochem. Biophys. Res. Commun. 28*, 914–919.

Grenthe, I., Ots, H., & Ginstrup, O. (1970) *Acta Chem. Scand. 24*, 1067–1079.

Johnson, R. E., Hruby, V. J., & Rupley, J. A. (1979) *Biochemistry 18*, 1176–1179.

Jones, A. J. S., Epand, R. M., Lin, K. F., Walton, D., & Vail, W. J. (1978) *Biochemistry 17*, 2301–2307.

Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1979) *J. Biol. Chem. 254*, 9359–9361.

Pownall, H. J., Hsu, F. J., Rosseneu, M., Peeters, H., Gotto, A. M., & Jackson, R. L. (1977) *Biochim. Biophys. Acta 488*, 190–197.

Privalov, P. L. (1980) *Pure Appl. Chem. 57*, 479–497.

Rosseneu, M. Y., Soetewey, F., Blaton, V., Lievens, J., & Peeters, H. (1974) *Chem. Phys. Lipids 13*, 203–214.

Rosseneu, M., Soetewey, F., Balton, V., Lievens, J., & Peeters, H. (1976) *Chem. Phys. Lipids 17*, 38–56.

Rosseneu, M., Blaton, V., Vercaemst, R., Soetewey, F., & Peeters, H. (1977) *Eur. J. Biochem. 74*, 83–88.

Sasaki, K., Dockerill, S., Adamiak, D. A., Tickle, I. J., & Blundell, T. (1975) *Nature (London) 257*, 751–757.

Segrest, J. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M. (1974) *FEBS Lett. 38*, 247–253.

Tall, A. R., Small, D. M., Deckelbaum, R. J., & Shipley, G. G. (1977) *J. Biol. Chem. 252*, 4701–4711.

Tarashi, T., & Mendelsohn, R. (1979) *J. Am. Chem. Soc. 101*, 1050–1052.