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Studies on the Specificity of Rabbit Hepatic Carbohydrate-Binding Protein Using Neoglycoproteins†

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ABSTRACT: The binding of amidinoneoglycoproteins of bovine serum albumin to rabbit liver membranes was measured. Derivatives of bovine serum albumin to which equivalent amounts of β-D-Gal, 6-*O*-Me-β-D-Gal, β-D-Fuc, α-L-Ara, β-D-Glc, β-D-Xyl, and β-D-GalNAc had been attached bound to the membranes equally well. The attachment of α-D-Man, β-L-Fuc, β-D-GlcNAc, β-D-allose, 3-*O*-Me-β-D-Glc, and 2-deoxy-β-D-Glc did not promote strong binding. The specificity of binding to the membranes was confirmed by measuring the binding of neoglycoproteins to the purified rabbit hepatic carbohydrate-binding protein immobilized on Sepharose 4B. The results indicate that, for binding, (1) neither the 6-OH (D-Fuc) nor the 5-CH_2OH (L-Ara; D-Xyl) is required, (2) the 4-OH can be axial (D-Gal; L-Ara) or equatorial (D-Glc; D-Xyl), (3) the 3-OH must be equatorial (D-Glc) not axial (D-All) nor may it be substituted (3-*O*-Me-D-Glc), (4) the 2-OH must be equatorial (D-Glc) not axial (D-Man) and must be present (2-deoxy-D-Glc), and (5) the 2-OH can be replaced by an equatorial acetamido group if the 4-OH is axial (D-GalNAc) but not if it is equatorial (D-GlcNAc).

The carbohydrate prosthetic group has long been implicated as having a role in the recognition and binding of many glycoproteins by cells (Roseman, 1970; Ashwell & Morell, 1974; Stahl et al., 1978; Kaplan et al., 1977; Neufeld et al., 1977). Of particular interest is the observation that the requirements for binding with respect to carbohydrate structure are quite stringent. In order to approach the general problem of defining the structural requirements for the binding of glycoproteins to cells and receptors, we have prepared a series of synthetic glycoconjugates (neoglycoproteins) by covalently attaching carbohydrates to proteins using the 2-imino-2-methoxyethyl 1-thioglycosides (Lee et al., 1976). In particular, we have examined the binding of these neoglycoproteins to the rabbit hepatic carbohydrate-binding protein (Krantz et al., 1976; Stowell & Lee, 1978) which has been extensively characterized (Ashwell & Morell, 1974; Hudgin et al., 1974; Kawasaki & Ashwell, 1976a,b; Sarkar et al., 1979). As would have been predicted on the basis of the behavior of deglycosylated serum-type glycoproteins (Ashwell & Morell, 1974), β-D-galactosylneoglycoproteins bound to the binding protein in rabbit liver membranes whereas 2-acetamido-2-deoxy-β-D-glucosyl- and α-D-mannosylneoglycoproteins did not (Krantz et al., 1976; Stowell & Lee, 1978). Interestingly, β-D-glucosylneoglycoproteins were also found to bind to the binding protein as strongly as β-D-galactosylneoglycoproteins or asialoorosmucoid whose oligosaccharide chains are terminated in β-D-galactosyl residues. The evidence of the somewhat relaxed specificity of this binding protein prompted us to examine the structural requirements for binding using some new amidinoneoglycoproteins (Stowell & Lee, 1980). The use of (neo)glycoproteins rather than simple oligosaccharides or glycosides in the present study is imperative because the binding of low molecular weight carbohydrates to the binding protein is many orders of magnitude weaker than the binding of macromolecules (Stowell & Lee, 1978; Sarkar et al., 1979) and may not accurately reflect the specificity of the binding site. In addition, the neoglycoproteins offer the advantage of providing large amounts of chemically homogeneous probes which are not readily available from natural sources, thereby permitting a detailed study of the binding site.

Experimental Section

Materials. Amidinoneoglycoproteins of bovine serum albumin (BSA)[1] were prepared as described (Lee et al., 1976; Stowell & Lee, 1980). Orosomucoid (α_1-acid glycoprotein) was a gift of the American Red Cross National Fractionation Center, Bethesda, MD. Asialoorosomucoid (ASOR) was prepared by treatment with *Clostridium perfringens* neuraminidase (Boehringer Mannheim) and radioiodinated by using sodium [^{125}I]iodide (carrier free, in 0.1 M NaOH from New England Nuclear) and Chloramine T (Aldrich Chemical Co.) as described (Krantz et al., 1976).

Rabbit liver membranes were prepared by a modification (Morell & Scheinberg, 1972) of the method of Ray (1970) which does not include the final sucrose gradient centrifugation. Binding protein was isolated from Triton X-100 extracts

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[1] Abbreviations used: BSA, bovine serum albumin; ASOR, asialoorosomucoid; RIP, relative inhibitory power; $Glyc_n$-AI-BSA, neoglycoproteins of BSA to which *n* moles of thioglycosides had been attached by amidination using the 2-imino-2-methoxyethyl 1-thioglycosides (Krantz et al., 1976).

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of rabbit liver by affinity chromatography on columns of β-D-Gal-AI-BSA or β-D-Glc-AI-BSA attached to Sepharose 4B (Stowell & Lee, 1978). The purified binding protein itself was then attached to cyanogen bromide activated Sepharose as described (Sarkar et al., 1979).

Methods. (1) Assay of Neoglycoprotein Binding by Rabbit Liver Membranes. The efficiency of binding of neoglycoproteins by rabbit liver membranes was measured by a modification (Krantz et al., 1976) of the method of Van Lenten & Ashwell (1972). In brief, membranes were preincubated with the neoglycoprotein under consideration, followed by a second incubation with saturating amounts of [^{125}I]ASOR. The membranes with their bound ligands were collected by rapid filtration and counted in a γ counter. The ability of a neoglycoprotein to inhibit the binding of [^{125}I]ASOR was taken as a measure of its ability to bind to the membranes. For the purpose of normalizing the results, inhibition curves using unlabeled ASOR were always obtained along with the neoglycoprotein inhibition curves. As was done earlier (Krantz et al., 1976), the ratio of the 50% inhibition points of ASOR to neoglycoprotein was defined as the relative inhibitory power, RIP (nanograms of ASOR producing 50% inhibition per nanogram of neoglycoprotein producing 50% inhibition). Therefore, neoglycoproteins which bound to the membranes better than ASOR (i.e., were better inhibitors) would have RIP's greater than 1.

(2) Assay of Neoglycoprotein Binding by Immobilized Rabbit Hepatic Binding Protein. The ability of neoglycoproteins to bind to the purified rabbit hepatic binding protein was determined by using the method of Sarkar et al. (1979). In short, this method involves the coincubation of neoglycoprotein and [^{125}I]ASOR with purified binding protein which had been attached to cyanogen bromide activated Sepharose 4B. After a suitable incubation, the binding protein–Sepharose beads with their attached ligands are pelleted by centrifugation, and the supernatant is counted in a γ counter. Again, the ability of a neoglycoprotein to inhibit the binding of [^{125}I]-ASOR was taken as a measure of its ability to bind to the immobilized binding protein. The RIP was defined as in the membrane assay.

Results

Membrane Binding Assay. The ability of neoglycoproteins to bind to the glycoprotein-binding protein in rabbit liver membranes was assessed by using the inhibition assay described under Methods. The RIP's of various amidinoneoglycoproteins of BSA determined by this method were plotted as a function of the number of thioglycosides that had been attached. We observed earlier that the attachment of increasing numbers of β-D-galactopyranosides to BSA progressively enhanced its ability to bind to membranes (Krantz et al., 1976), as is shown in Figure 1.[2] At the highest levels of incorporation, β-D-Gal-AI-BSA bound even better than ASOR by ~2 orders of magnitude.

In order to evaluate the contribution of substituents at C-5 to the binding, we prepared series of neoglycoproteins of BSA to which various analogues of D-galactose had been attached. Neoglycoproteins of BSA to which 6-*O*-methyl-β-D-galactopyranosides, β-D-fucopyranosides, or α-L-arabinopyranosides had been attached bound to the membranes as well as or slightly better than β-D-Gal-AI-BSA at all levels of substitution (Figure 1). Neoglycoproteins containing ~20 mol of these

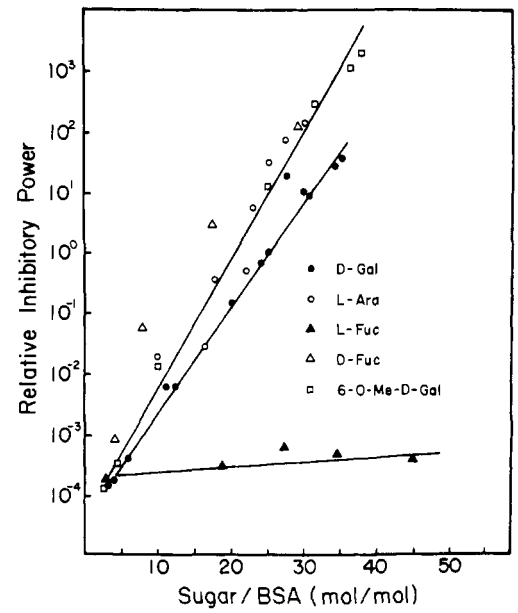

FIGURE 1: Relative inhibitory power of various neoglycoproteins. Effect of substitution on the C-5 position. Neoglycoproteins contained (●) β-D-galactopyranoside, (○) α-L-arabinopyranoside, (▲) β-L-fucopyranoside, (△) β-D-fucopyranoside, and (□) 6-*O*-methyl-β-D-galactopyranoside. The lines were drawn only for L-Fuc, D-Gal, and 6-*O*-Me-D-Gal.

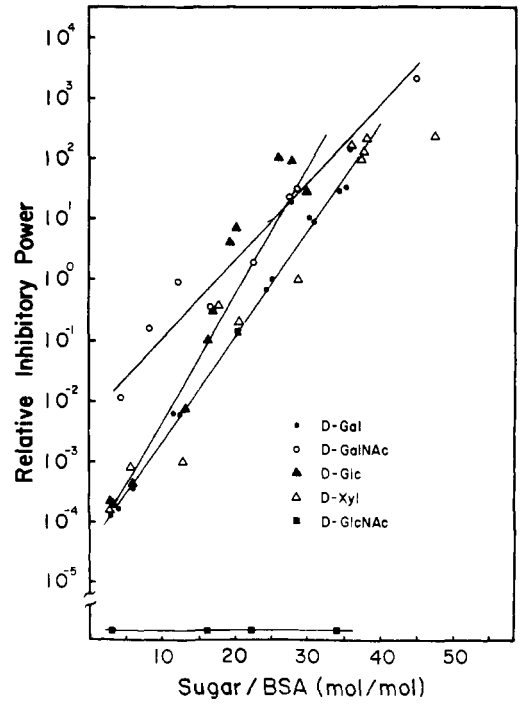

FIGURE 2: Relative inhibitory power of various neoglycoproteins. Neoglycoproteins contained (●) β-D-galactopyranoside, (○) 2-acetamido-2-deoxy-β-D-galactopyranoside, (▲) β-D-glucopyranoside, (△) β-D-xylopyranoside, and (■) 2-acetamido-2-deoxy-β-D-glucopyranoside. The lines were drawn only for D-Gal, D-GalNAc, and D-Glc.

glycosides/mol of BSA bound to the membranes about as well as ASOR. By contrast, neoglycoproteins bearing β-L-fucopyranosides, an enantiomorph of β-D-fucopyranoside, bound very poorly to the membranes.

The observation that the presence of the 5-hydroxymethyl group was not required for binding was confirmed by the finding that β-D-Xyl-AI-BSA bound to the membranes as well as β-D-Glc-AI-BSA (Figure 2). The results shown in this figure also illustrate the point that the configuration of the

[2] The same curve for β-D-Gal-AI-BSA is included in Figures 1–3 as a point of reference.

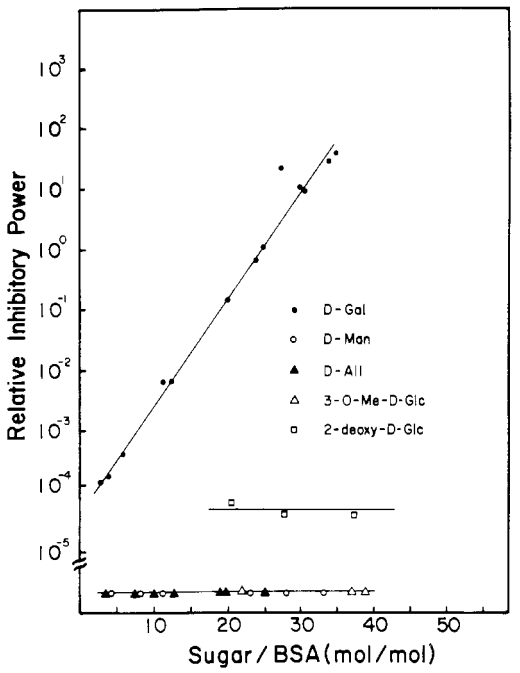

FIGURE 3: Relative inhibitory power of various neoglycoproteins. Effect of substitution on the C-2 and C-3 positions. Neoglycoproteins contained (●) β-D-galactopyranoside, (○) α-D-mannopyranoside, (▲) β-D-allopyranoside, (△) 3-*O*-methyl-β-D-glucopyranoside, and (□) 2-deoxy-β-D-glucopyranoside.

hydroxyl group at C-4 does not directly influence binding, since β-D-Gal-AI-BSA and β-D-Glc-AI-BSA bound to the membranes about equally as well, as was the case with α-L-Ara-AI-BSA and β-D-Xyl-AI-BSA.

The effect of structural modifications at C-2 and C-3 on the binding of neoglycoproteins to the membranes is shown in Figure 3. Attaching α-D-mannopyranosides, β-D-allopyranosides (the 2 and 3 epimers, respectively, of β-D-glucopyranoside), or 3-*O*-methyl-β-D-glucopyranoside to BSA did not result in detectable binding to the membranes (RIP < 10^{-5}), whereas 2-deoxy-β-D-Glc-AI-BSA had a barely measurable RIP value (10^{-4}–10^{-5}). From the results shown in Figure 2, it can be seen that substitution of an acetamido group at C-2 of β-D-glucopyranoside also eliminated binding since β-D-GlcNAc-AI-BSA did not bind to the membrane. However, substitution of an acetamido group at C-2 of β-D-galactopyranoside enhanced binding. β-D-GalNAc-AI-BSA bound to the membranes as well as β-D-Gal-AI-BSA and, at lower levels of incorporation, even better than β-D-Gal-AI-BSA.

Assay with Purified Binding Protein. The ability of neoglycoproteins to bind to the purified rabbit liver glycoprotein-binding protein immobilized on Sepharose 4B was determined by using the inhibition assay described under Methods. The RIP values determined in this and the membrane assay were compared for several neoglycoproteins, most of which contain ~17 mol of attached thioglycoside/mol (Table I). The RIP values for each neoglycoprotein determined by the two methods are similar. These results confirm that the neoglycoproteins do indeed bind to the purified binding protein and that the quantitative estimates obtained in the membrane assay are a reasonably accurate reflection of the specificity of the binding protein.

Discussion

In a previous publication (Krantz et al., 1976) we reported the use of neoglycoproteins in characterizing the binding site

Table I: Comparison of Relative Inhibitory Powers Determined by Two Assays

inhibitor	rel inhibitory power: membrane assay	rel inhibitory power: binding protein assay
ASOR	1	1
D-Gal_{17}-BSA	0.4	0.4
6-*O*-Me-D-Gal_{17}-BSA	0.5	0.4
D-Fuc_{17}-BSA	2	0.2
L-Ara_{16}-BSA	0.3	0.1
L-Fuc_{19}-BSA	4×10^{-4}	$<10^{-3}$
D-Glc_{18}-BSA	2	2
D-Xyl_{17}-BSA	0.4	0.3
D-All_{19}-BSA	$<10^{-4}$	$<10^{-3}$
D-$GalNAc_{16}$-BSA	0.4	1
D-$GlcNAc_{16}$-BSA	$<10^{-4}$	$<10^{-3}$

of the glycoprotein-binding protein of rabbit liver membranes. This binding protein appeared to recognize primarily the attached β-D-galactopyranosides since the attachment of other glycosides (e.g., β-D-GlcNAc) did not promote binding while the attachment of β-D-galactopyranosides, including both *O*- and *S*-galactosides to different proteins or using other types of linkages (Lee & Lee, 1980; Kawaguchi & Lee, 1979), always enhanced binding. Although the extent of binding varied depending on the protein, linkage type, and number of glycosides attached, the preference for D-galactose over D-mannose and D-GlcNAc was always patent.

The availability of a number of new amidinoneoglycoproteins (Stowell & Lee, 1980) has now made it possible to evaluate in more detail the contributions of various portions of the sugar to binding to the rabbit hepatic binding protein. The role of the 5-hydroxymethyl group in binding was examined since it had been observed earlier that galactose oxidase (EC 1.1.3.9) treatment of asialoceruloplasmin impaired its ability to be cleared from the circulation (Morell et al., 1968). More recently, it was found that very high levels of several monosaccharide analogues of D-galactose with alterations of the 5-hydroxymethyl group inhibited the binding of (neo)glycoproteins to rabbit liver membranes (Stowell & Lee, 1978) and to the purified rabbit heptatic binding protein (Sarkar et al., 1979). However, the concentrations of monosaccharides required to reach the 50% inhibition point were many orders of magnitude higher than the concentrations of (neo)glycoproteins required to achieve the same level of inhibition and therefore may not have been very reliable probes of binding specificity. For example, the monosaccharides L-arabinose and L-fucose inhibited the binding of [^{125}I]ASOR to membranes to the same extent (Stowell & Lee, 1978); however, derivatives of BSA to which comparable numbers of α-L-arabinopyranosides and β-L-fucopyranosides had been attached had very different RIP's (Figure 1).

By use of the amidinoneoglycoproteins of BSA, it appeared from the results of the present study that the 5-hydroxymethyl group was not required in binding since β-D-Fuc-AI-BSA and α-L-Ara-AI-BSA bound to the membranes as well as β-D-Gal-AI-AI-BSA, and β-D-Xyl-AI-BSA bound as well as β-D-Glc-AI-BSA. The nonbinding of the galactose oxidase treated asialoceruloplasmin observed by Morell et al. (1968) may be explained on the basis of the formation of the second ring through a hemiacetal linkage (Theander, 1961) which interferes with binding to the binding protein. The binding site must be quite loose in this region of the sugar since the substitution of a methyl group for the proton of the 6-hydroxyl group of β-D-galactopyranoside also did not interfere with binding. The results with these particular neoglycoproteins also indicate that the 4-hydroxyl group is not directly involved

in binding or that either an axial or an equatorial hydroxyl at this position permits interaction with the binding site.

The binding site apparently does interact with or is much more closely apposed to the substituents on C-2 and C-3 of the sugar. Both α-D-Man-AI-BSA and 2-deoxy-β-D-Glc-AI-BSA were not bound to any significant extent to the binding protein, indicating that the equatorial OH on C-2 is required for a strong binding. However, the fact that β-GalNAc-AI-BSA could be bound as well as Gal- or Glc-AI-BSA indicates that there is enough room to accommodate an acetyl group in this region of the binding protein. As to the 3 position, neither β-D-All-AI-BSA nor 3-*O*-Me-β-D-Glc-AI-BSA bound to the binding protein. Poor binding of 3-*O*-Me-β-D-Glc-AI-BSA suggests that either there is little room in this region of binding protein to accommodate a methyl group or the hydrogen atom of OH group is actively engaging in the binding process. It is obvious that an axial OH at the 2 or 3 position (D-Man and D-All, respectively) does not contribute to the binding. It is further possible that such a group would actually interfere with proper binding. A very small but measurable binding of 2-deoxy-β-D-Glc-AI-BSA to the binding protein suggests that this might be the case for the axial OH in the 2 position.

The acetamido group at C-2 interferes with binding in the case of β-D-GlcNAc-AI-BSA but, paradoxically, not in the case of β-D-GalNAc-BSA. The ability of D-GalNAc- and D-GalNAc-containing glycoproteins to interact with the binding protein had been observed previously (Stockert et al., 1974; Sarkar et al., 1979). These results suggest that the contribution of various portions of the sugar to binding may not be completely independent. Although the binding protein appeared to be indifferent to the orientation of the 4-hydroxyl group, it is possible that axial and equatorial hydroxyl groups interact with the binding site with different consequences with respect to the interaction of the equatorial 2-acetamido group. Alternatively, if the interaction of the sugar with the binding site is hydrophobic in nature, as suggested by Lemieux et al. (1978) for the interaction of a carbohydrate antigen with antibody, then differences in intramolecular hydrogen bonding between β-D-GlcNAc and β-D-GalNAc may account for the differences in their ability to bind in the system studied here.

It had been observed that very high concentrations of L-fucose inhibited the agglutination of human erythrocytes mediated by the rabbit hepatic binding protein (Stockert et al., 1974) and inhibited the binding of (neo)glycoproteins to rabbit liver membranes (Stowell & Lee, 1978) and the purified binding protein (Sarkar et al., 1979). Neoglycoproteins containing β-L-fucopyranosides were found in the present study to bind weakly, though measurably, in the membrane assay. Whether this weak binding is due to some degree of tolerance by the β-D-galactopyranoside binding system or to a unique β-L-fucopyranoside-binding activity present in the membranes as well as in the purified binding protein is not clear at the present.

In order to confirm that the neoglycoprotein-binding activity in the membranes was the same as the ASOR-binding activity and validate the use of the membrane binding assay to study the binding site of the glycoprotein-binding protein, we previously characterized carefully the binding of one neoglycoprotein, β-D-Glc-AI-BSA (Stowell & Lee, 1978). The binding of β-D-Glc-AI-BSA to the membranes was shown to be identical with the binding of ASOR in three characteristic features: (1) requirement for calcium; (2) sensitivity to pretreatment of the membranes with neuraminidase; (3) inhibition by the same carbohydrate derivatives. Receptors isolated from rabbit liver on columns of β-D-Gal-AI-BSA–Sepharose and β-D-Glc-AI-BSA–Sepharose were shown to be identical with respect to several chemical and immunochemical criteria and in their binding specificities.

The results obtained in the membrane binding assay in the current study were further corroborated by measuring the binding of neoglycoproteins to the purified rabbit hepatic binding protein which had been immobilized on Sepharose 4B. These results confirm the results from the membrane assay both qualitatively and quantitatively.

In summary, the rabbit hepatic binding protein recognizes only a relatively small area of the sugar yet it binds (neo)-glycosides very strongly. The apparent strength of binding may be the result of the statistical effect of many sugars clustered together on the (neo)glycoprotein interacting with a multivalent receptor, an interpretation which is consistent with the observation that free sugars and small glycosides bind very weakly relative to (neo)glycoproteins. It should be emphasized that the ability of neoglycoproteins to bind increases exponentially as the number of appropriate glycosides attached increases.

This rather broad specificity range exhibited by the hepatic binding protein is not entirely unprecedented since several glycosidases recognize both the β-D-*galacto* and β-D-*gluco* configurations (Schwartz et al., 1970; Johnson et al., 1972; Glew et al., 1976) or show a lack of specificity for substituents on C-5 (Wallenfels & Malhotra, 1961; Glew et al., 1976; Chester et al., 1976). While the binding protein can discriminate accurately among the common sugar constituents of the serum-type glycoproteins and seems to be involved in the clearance and catabolism of D-galactose-terminated serum glycoproteins, its ability to bind β-D-Xyl, β-D-Glc, and β-D-GalNAc suggests that it may play a role in the metabolism of other classes of glycoconjugates as well.

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Thermotropic Behavior of Phosphatidylethanolamine–Cholesterol and Phosphatidylethanolamine–Phosphatidylcholine–Cholesterol Mixtures†

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ABSTRACT: The thermotropic behavior of aqueous dispersions of phosphatidylethanolamine–cholesterol and phosphatidylethanolamine–phosphatidylcholine–cholesterol mixtures has been studied by high-sensitivity differential scanning calorimetry. The gel to liquid-crystalline phase transition of phosphatidylethanolamines is broadened and shifted to lower temperature when cholesterol is incorporated into the bilayer. When the cholesterol content is below 25 mol %, the calorimetric endotherms seem to consist of two components, a broad one at considerably lower temperature than the original transition and another component at only slightly lower temperature. This thermotropic behavior can be explained by the assumption of a homogeneous distribution of cholesterol in phosphatidylethanolamine bilayers. Scanning calorimetry of equimolar mixtures of phosphatidylethanolamines with phosphatidylcholines, which show either ideal or nonideal mixing properties, reveals that when cholesterol is added to these mixtures it shows no preferential affinity for either of the phospholipids.

The membranes of eukaryotic cells usually contain large amounts of sterols, cholesterol being the dominant component (Rouser et al., 1968). The physicochemical behavior of phospholipid–cholesterol mixtures has therefore been a subject of considerable research (Demel & de Kruijff, 1976). Since the first experimental findings of Ladbrooke et al. (1968) that the addition of increasing amounts of cholesterol to phosphatidylcholine bilayers gradually diminishes the gel to liquid-crystalline phase transition of this phospholipid, controversial results have been published regarding the exact percentage of cholesterol at which the transition is abolished and concerning the existence of possible phosphatidylcholine–cholesterol complexes and their stoichiometry (Darke et al., 1972; Shimshick & McConnell, 1973a; Engelman & Rothman, 1972; Hinz & Sturtevant, 1972; de Kruijff et al., 1972, 1973, 1974; Phillips & Finer, 1974; Tsong, 1975). Very recently reexaminations of the phosphatidylcholine–cholesterol system by high-sensitivity differential scanning calorimetry revealed that indeed an endothermic transition can be observed at a cholesterol content above 33 mol %, which, however, is very broad and shifted to higher temperature as compared to the original unperturbed transition (Mabrey et al., 1978; Estep et al., 1978). At a cholesterol content below 20–25 mol % the calorimetric scans could be decomposed into two different peaks. These findings were interpreted as evidence for the coexistence of a cholesterol-rich and a cholesterol-poor or a pure phospholipid phase, respectively. Similar results were obtained with sphingomyelin–cholesterol mixtures (Estep et al., 1979; Calhoun & Shipley, 1979). Other workers, however, have proposed a homogeneous phase for mixtures with less than 25 mol % cholesterol (Owicki & McConnell, 1979; Rubinstein et al., 1979). The same suggestions were made before by Verkleij et al. (1974) on the basis of freeze-fracturing experiments. Recent deuterium NMR experiments with mixtures of specifically deuterated phosphatidylcholines with cholesterol seem to confirm this view (Haberkorn et al., 1977; Jacobs & Oldfield, 1979).

Another important aspect of phospholipid–cholesterol interactions is the differential affinity of cholesterol to certain phospholipids in a mixture. In PE–PC[1] mixtures, for instance, which show monotectic behavior, cholesterol shows a preferential association with PC, irrespective of whether PC is the higher or the lower melting component (van Dijck et al., 1976a). It could be shown that cholesterol has a decreased

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[1] Abbreviations used: DSC, differential scanning calorimetry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DLPE, dilauroylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine.

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