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ISOLATION OF PLASMA MEMBRANE GLYCOPROTEIN FROM BOVINE THYMOCYTES

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Summary

Glycoprotein was isolated from a purified thymocyte membrane preparation by two methods: lithium diiodosalicylate-phenol extraction and hot 75% ethanol extraction. A higher yield of membrane sialic acid was obtained by the latter method. The preparations had similar apparent molecular weights on sodium dodecyl sulfate gel electrophoresis. Both had similar receptor activities against a panel of hemagglutinins, although the 75% ethanol extract was more active on a weight basis. However, there were significant differences in carbohydrate and amino acid compositions of the two thymocyte extracts. The lithium diiodosalicylate-extracted material had much more glucose, ribose, and glycine than the ethanol extract. The glycoprotein preparations from thymocytes were quite distinct from the glycoprotein of bovine erythrocytes in both composition and receptor properties.

Introduction

The glycoproteins of the plasma membrane have been demonstrated to possess important receptor properties, for example blood group M and N [1,2] and heterophile antigen [1] activity in the erythrocyte, histocompatibility antigenic activity [3], tumor-specific antigenic activity [4], virus receptor sites [5], tissue-specific antigenic activity [6], lectin receptor sites [7—9].

The characterization of plasma membrane glycoproteins is dependent upon the development of suitable isolation methods. The most studied glycoprotein membrane constituent is that of the human erythrocyte. Winzler [5] has suggested this glycoprotein as a model for other membrane glycoproteins. This glycoprotein was first extracted from erythrocyte ghosts by hot aqueous phenol [10]. Variations of this phenolic extraction have been described by other workers. Springer et al. [2] used buffered 45% aqueous phenol at 23°C

for extraction of a glycoprotein with blood group NN and virus receptor activities. Marchesi and Andres [11] described pre-solubilization of erythrocyte ghosts by means of the detergent-like action of 0.3 M lithium diiodosalicylate followed by extraction with 50% aqueous phenol at 4°C. Solubilization of erythrocyte membrane by detergents followed by affinity chromatography on lectin-Sepharose is reported to give yields of glycoprotein equal to that obtained by the lithium diiodosalicylcate-phenol method [12].

Aqueous pyridine extraction has also been successfully used for the preparation of erythrocyte glycoprotein [13] as has chloroform/methanol/water [14]. In this laboratory, a modification of the hot organic solvent extraction method originally described by Schwartzweiss and Tomscik [15] has been applied to extraction of glycoprotein from the erythrocytes of five mammalian species, horse, sheep, goat, bovine and human [1].

Most studies on cell types other than erythrocytes have been on soluble glycopeptides released by proteolysis of the surface of intact cells. Membrane glycoproteins from nucleated cells are difficult to isolate in quantity. Glycoproteins have been isolated from detergent-solubilized membranes by affinity chromatography [6,8,9]. The lithium diiodosalicylate-phenol method has been applied to several types of mammalian cell membranes [16—18]. The hot organic solvent method has not been applied to cells other than erythrocytes. The present study was undertaken to compare the utility of these latter two methods for extraction of glycoprotein from membranes of nucleated cells. The bovine thymocyte was chosen because of ease in obtaining the tissue and also because we are interested in glycoproteins which have receptor activity for the Paul-Bunnel heterophile antibody of infectious mononucleosis. It was previously reported that bovine tissue cells, including thymocytes, as well as the erythrocytes, have this specificity [19,20]. Also, the Paul-Bunnel antigen was reported to be a marker for murine T cells [21,22].

Materials and Methods

Preparation of the thymocyte membrane fraction. Thymus glands were obtained from young calves at a local slaughter house. The glands were transported to the laboratory packed in ice. Glands which were fibrous were discarded. The chilled, selected tissue was trimmed of all fat and fascia and frozen at −70°C. The frozen tissue was then shredded with a Kitchen Magic vegetable cutter (Popeil Brothers, Inc., Chicago, Ill.), fitted with an 8-inch plate with 3/32 × 3/8 inch cutting slits. The finely shredded, still-frozen tissue was then suspended in four volumes of cold 0.25 M sucrose containing 0.05 M Tris · HCl, 0.001 M EDTA, pH 7.5 and homogenized in an Oster blender for 5 s at low speed and 5 s at high speed. It was then filtered through cheese-cloth. This homogenate was processed exactly as described by Kornfeld and Seimers [23] to yield a crude membrane fraction. The crude membrane fraction was then fractionated through at 30—40% sucrose step gradient, also as described by Kornfeld and Seimers [23]. After centrifuging at 54450 × *g* for 4 h in a Spinco No. 30 rotor, each tube contained a milky band between the 30 and 40% sucrose layers. The contents of each tube were fractionated as follows: the upper 10 ml of slightly turbid supernatant was collected and called "top". The

next 9 ml of contained the milky band and was called "middle". The lower region was poured off the pellet and called "bottom". The pellets were then washed out of the tubes. Each fraction was washed three times in 0.05 M Tris, pH 7.5, sedimenting at 45000 × *g* for 1 h between each wash. Aliquots were removed for determination of protein, sialic acid, enzyme activity and DNA and the rest stored at −20°C.

Extraction of glycoprotein from thymocyte membranes. Organic solvent extraction method: This method was developed for the extraction of glycoprotein from erythrocyte membranes and has been described in detail previously [1]. In brief, a portion of thymocyte membrane (top and middle fractions only from the sucrose step gradient) equivalent to 130 mg of protein was freeze-dried. It was then extracted with acetone for 3 h under reflux. The residue was washed three times with acetone, then air-dried. The residue was next extracted with 100% ethanol under reflux for 3 h. The residue was washed three times with ethanol and air-dried. This residue was next extracted with 75% ethanol for 3 h under reflux and it was this extract which contained the major acid glycoprotein. The residue was washed three times with 75% ethanol and discarded. The 75% ethanol extract and washes were combined and concentrated under reduced pressure at 40°C, dialysed exhaustively against distilled water and freeze-dried.

Lithium diiodosalicylate-phenol extraction method: The procedure followed was essentially that described by Marchesi and Andrews [11]. A portion of thymocyte membrane (top and middle fraction from sucrose density step gradient) equivalent to 175 mg of protein was centrifuged at 45000 × *g* for 1 h. The pellet was suspended in 6 ml of 0.3 M lithium diiodosalicylate in 0.05 M Tris pH 7.5 and stirred for 15 min at room temperature. Two volumes of cold, distilled water were added. The mixture was stirred for 10 min at 4°C and then centrifuged at 45000 × *g* for 90 min. The supernatant was removed and mixed with an equal volume of freshly prepared 50% phenol in distilled water and stirred at 4°C for 15 min. After centrifugation for 90 min at 4000 × *g* at 4°C, an upper aqueous layer was removed and dialysed against three changes of distilled water (2 l) for 36 h and freeze-dried. The pellet which remained after the first lithium diiodosalicylate extraction was also dialysed, then re-extracted with lithium diiodosalicylate followed by phenol as above.

Extraction of glycoprotein from bovine erythrocyte membranes. Bovine erythrocyte glycoprotein was extracted from hemoglobin-free stroma as previously reported [1]. The 75% ethanol extract was then further purified by phosphocellulose chromatography [24].

Analytical methods. Sialic acids were determined by the alkaline-Ehrlich method and by the thiobarbituric acid method, both as described by Aminoff [25], after hydrolysis in 0.05 M H_2SO_4 at 80°C for 1 h. *N*-Acetylneuraminic acid (grade III, Sigma) served as standard. Since the predominant sialic acid in bovine material is the *N*-glycolyl derivative, values obtained were corrected for the difference in molecular extinction coefficients of *N*-acetyl- and *N*-glycolylneuraminic acids. Neutral sugars and amino sugars were determined as alditol acetates by gas-liquid chromatography as described by Griggs et al. [26]. The column used was 3% ECNSS-M on Gas-Chrom Q100/120 mesh (Regis). The internal standard, perseitol, was added to each sample prior to hydrolysis in

sealed tubes under N_2 in 3 M HCl for 3 h at 100°C. Standard curves were prepared for each sugar in which molar ratios of the sugar to perseitol were compared to the peak area ratios. The molar response factors calculated from the slopes of these curves were then used to estimate monosaccharide concentrations in the unknown samples as suggested by Clamp et al. [27].

Total protein was measured as described by Lowry et al. [28] using bovine serum albumin as a standard.

Individual amino acids were determined after hydrolysis in 6 M HCl at 100°C in sealed, evacuated tubes for 24 h using a Durrum model D-500 analyzer.

DNA was determined using the diphenylamine reaction as described by Burton [29] using calf thymus DNA as standard. Samples and standards were hydrolysed in 7.5% $HClO_4$ for 15 min at 70°C before addition of reagent. Polyacrylamide gel electrophoresis studies were carried out in the presence of 0.1% sodium dodecyl sulfate in 0.01 M phosphate buffer, pH 7.0, as previously described [24,30].

The 5'-nucleotidase activity, a plasma membrane marker enzyme, was assayed as described by Aronson and Touster [31]. The β-*N*-acetylglucosiminidase activity, a marker for lysosomes, was measured as described by Li and Li [32].

For electron microscopy of plasma membrane fractions, the material was first pelleted by centrifugation. The pellets were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, with 2% sucrose. This was followed by post-fixation in 1% OsO_4, dehydration in an ethanol series and embedding in araldite. Sections were cut on an LKB Ultratome III, stained in uranyl acetate and lead citrate and examined in a Phillips EM 300.

Immunologic and lectin receptor studies. Hemagglutination inhibition studies were performed as previously described [1]. Sources of reagents were as follows: (1) Sera were obtained from patients with clinically confirmed, heterophile antibody positive, infectious mononucleosis; (2) *Vicia graminea* hemagglutinin was extracted from the seeds as previously described [1]; (3) *Ricinus communis* hemagglutinin was extracted and purified as described by Nicolson and Blaustein [33]; (4) Concanavalin A was purchased from Pharmacia; (5) The lectin from *Phaseolus vulgaris* was purchased from Difco (Phytohemaglutinin M); (6) Wheat germ lectin was obtained from Miles Laboratories; (7) The lectin from *Limulus polyphemus* was purified as described by Roche and Monsigny [34]. Activities of inhibition were expressed in μg/ml which completely inhibited the agglutination of appropriate erythrocytes by four hemagglutinating doses of antiserum or lectin.

Results

The distribution of marker enzyme activities in the subcellular fractions of calf thymus are shown in Table I. Composition of the purified membrane fractions prior to extraction are shown in Table II. Only the top and middle fractions which were enriched in sialic acid and 5'-nucleotidase and contained only small amounts of DNA, were selected from extraction. Thin sections of these two fractions were examined in the electron microscope and the micrographs

TABLE I

MARKER ENZYME ACTIVITIES IN SUBCELLULAR FRACTIONS FROM CALF THYMUS

Total activities are expressed as μmol of substrate converted per h from fractions derived from 400 g of thymus. Specific activity is expressed as μmol per h per mg of protein.

Fraction	5′-Nucleotidase		β-*N*-Acetylglucosaminidase	
	Total activity	Specific activity	Total activity	Specific activity
Homogenate	4734	0.47	54 826	3.22
Nuclei	1198	0.16	10 002	0.68
Soluble	2282	0.94	30 836	6.35
Sucrose gradient membranes				
Top	346	4.44	270	1.73
Middle	151	2.34	258	2.00
Bottom	20	0.51	165	2.15
Pellet	2	0.01	512	1.54

are shown in Fig. 1. Both sections show primarily membrane profiles.

Table III presents the results obtained with lithium diiodosalicylate-phenol and 75% ethanol extraction of two aliquots of the same membrane preparation. It can be seen that, although the total amount of material solubilized by the two lithium diiodosalicylate-phenol extractions of the same aliquot of membrane is comparable to that in the 75% ethanol extract, the specific activity of sialic acid (μmol/mg dry weight of extract) is highest in the latter preparation. About 30% of the total membrane sialic acid was extracted by 75% ethanol as compared to only 10% by the two lithium diiodosalicylate extractions.

The serologic reactivities of the crude glycoprotein extracts are shown in Table IV and are compared to those of the bovine erythrocyte glycoprotein which was solubilized from hemoglobin-free red cell stroma by the organic solvent extraction method. Both of the lithium diiodosalicylate extracts of thymus were inactive with the IM antisera and the 75% ethanol extract had only a trace of activity as compared to the erythrocyte glycoprotein. All three thymocyte extracts inhibited the sialic acid-specific lectin from *L. polyphemus* but were not nearly as active as the erythrocyte glycoprotein. In contrast to the erythrocyte preparation, the thymocyte extracts had receptor activity for phy-

TABLE II

CHEMICAL COMPOSITION OF PURIFIED MEMBRANE FRACTIONS FROM SUCROSE DENSITY GRADIENT

From 400 g of thymus, wet weight.

Fraction	Protein (total mg)	Sialic acid (total μmol)	DNA (total mg)
Top	60.00	2.55	0.43
Middle	86.70	3.05	0.67
Bottom	37.00	1.00	0.25
Pellet	178.25	3.08	1.95

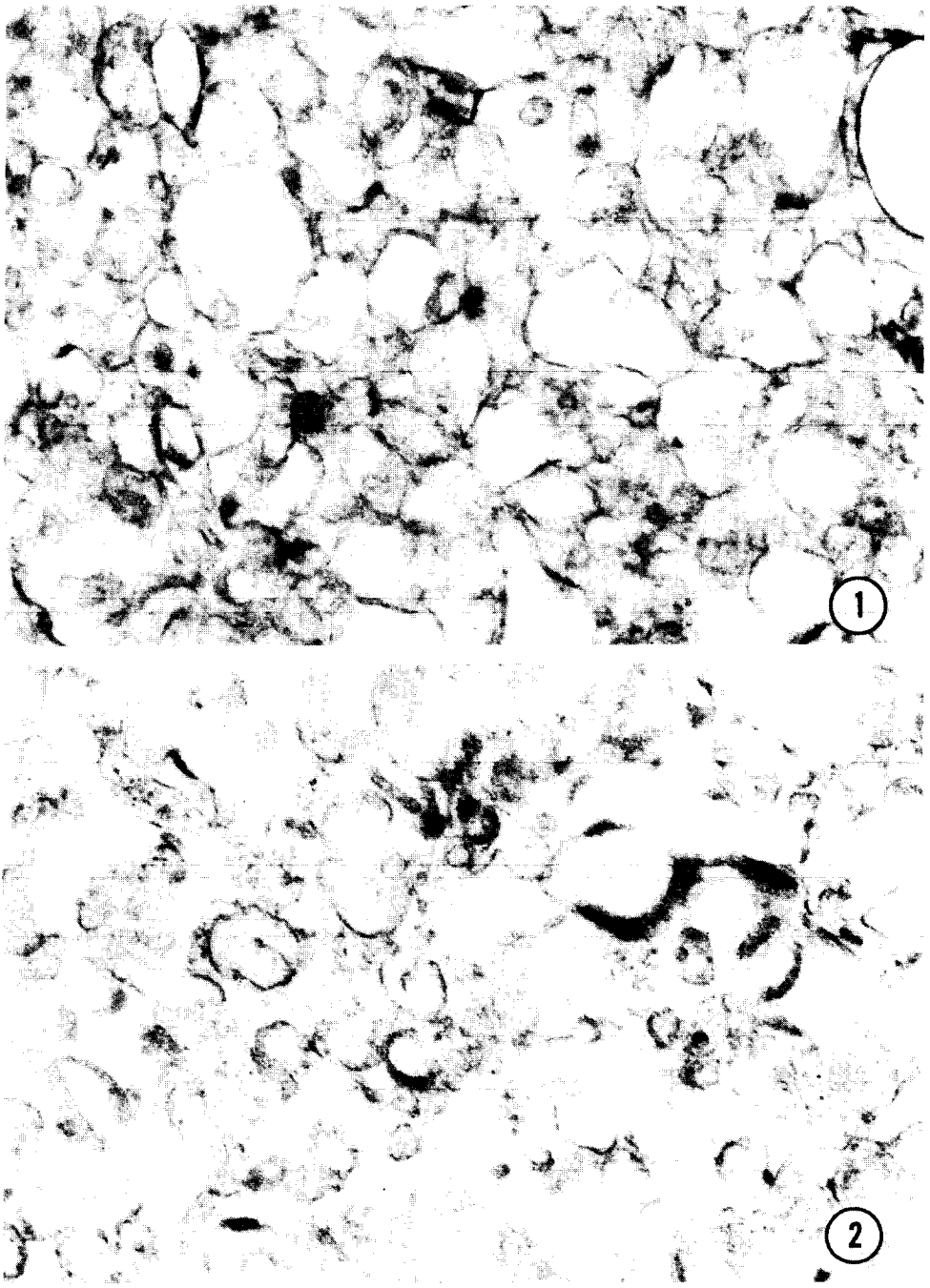

Fig. 1. Electron micrographs of plasma membrane fractions from sucrose gradient. Panel 1, top fraction; Panel 2, middle fraction. Magnification: ×33000.

TABLE III

YIELD OF CRUDE GLYCOPROTEIN FROM THYMOCYTE MEMBRANES

Extracts I and II were obtained by sequential extraction of a single sample of thymocyte membrane.

Extraction method	mg	μmol sialic acid
Lithium diiodosalicylate-phenol-I	6.5	0.316
Lithium diiodosalicylate-phenol-II	7.1	0.299
75% ethanol	11.5	1.449

tohemagglutinin and *R. communis* agglutinin I. None of the extracts inhibited *Vicia*, concanavalin A, or wheat germ agglutinins.

Prior to determination of carbohydrate and amino acid composition and gel electrophoresis studies, the extracts of thymus membrane were further purified. Also, in order to have sufficient sample for analysis, the two lithium diiodosalicylate extracts and the 75% ethanol extract were each dissolved in distilled water and nine volumes of ethanol were added. After 4 h at 4°C, the precipitates were collected and washed with two portions of 90% ethanol. The precipitates were then extracted twice with chloroform/methanol (2 : 1, v/v) and freeze-dried.

The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 2. Both the lithium diiodosalicylate and 75% ethanol extracts of thymocyte membrane had a major band staining with both periodic acid-Schiff reagent and Coomassie blue stain with a mobility slightly faster than that of the erythrocyte glycoprotein preparation. The mobilities relative to standard molecular weight marker proteins indicated apparent molecular weights of 18500 for the two thymus preparations and 25000 for the glycoprotein from erythrocytes. No correction was made for the well-described anomalous behavior of glycoprotein on sodium dodecyl sulfate gels [30]. The 75% ethanol extract of thymus membrane showed an additional minor band which stained more heavily with periodic acid-Schiff than with Coomasie blue reagent and

TABLE IV

HEMAGGLUTINATION INHIBITORY ACTIVITY OF GLYCOPROTEINS

Source of hemagglutinin	Minimum amounts completely inhibiting four hemagglutinating doses (μg/ml)			
	Crude glycoproteins from thymocytes			Glycoprotein from bovine erythrocytes
	LIS I *	LIS II *	75% ethanol	
IM serum	>5000	>5000	1250	0.1
Limulus polyphemus	900	900	300	0.4
Phaseolus vulgaris	300	625	150	>2000
Ricinus communis	2500	>5000	625	>2000
Vicia graminea	>5000	>5000	>5000	>5000
Canavalin enisformis	>5000	>5000	>5000	>5000
Triticum vulgaris	>5000	>5000	>5000	>2000

* LIS I and LIS II were obtained by sequential extraction of a single sample of thymocyte membrane with lithium diiodosalicylate-phenol.

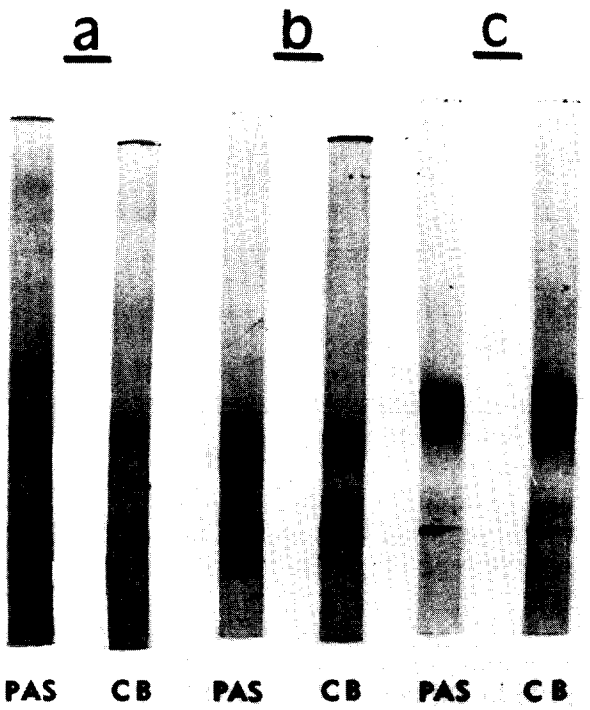

Fig. 2. Electrophoretic patterns in sodium dodecyl sulfate-polyacrylamide (7.5%) gels. PAS, periodic acid-Schiff stain; CB, Coomassie blue stain. 50 μg of protein applied to each gel. (a) lithium diiodosalycylate-phenol extract of bovine thymocyte membrane. (b) 75% ethanol extract of bovine thymocyte membrane. (c) 75% ethanol extract of bovine erythrocyte membrane.

TABLE V

COMPOSITION OF BOVINE MEMBRANE GLYCOPROTEINS

No correction made for destruction of sugars during acid hydrolysis. Results are expressed in mol/10000 g protein.

	Thymocytes		Erythrocytes 75% ethanol extract
	Lithium diiodosalicylate extract	75% ethanol extract	
Fucose	2.5	2.5	trace
Ribose	5.8	0	0
Mannose	6.7	8.4	0.9
Galactose	9.5	7.8	5.7
Glucose	8.2	2.4	0
GlcNAc	10.9	7.2	2.0
GalNAc	2.0	0.9	2.0
Sialic acid	5.5	5.3	4.6
Protein (%) *	58	62	70
Carbohyrate (%) *	42	38	30

* Values based on sum of protein and carbohydrate (excluding glucose and ribose).

moved with the marker dye. When purified thymus membranes were solubilized directly in sodium dodecyl sulfate and electrophoresed, at least five bands staining with periodic acid-Schiff were seen. Apparent molecular weights were 100000—200000.

The chemical compositions of the three preparations are shown in Table V. The two thymocyte extracts had mannose, galactose, *N*-acetylglucosamine and sialic acid as main carbohydrate components. The lithium diiodosalicylate extract also had a high glucose concentration, four times more than was present in the 75% ethanol extract and also ribose which was not found in the 75% ethanol extract. Lesser but significant amounts of fucose and *N*-acetylgalactosamine were found in both thymocyte preparations. Deoxyribose was not detected in either preparation. The carbohydrate pattern of the major erythrocyte glycoprotein was quite different. Galactose and sialic acid were the principal sugars. Equimolar amounts of the two amino sugars were present along with a small amount of mannose. Fucose and glucose were absent. Table V indicates a carbohydrate content (excluding glucose and ribose) of 40% for the thymocyte extracts and 30% for the erythrocyte glycoprotein. It should be noted that the sum of protein and carbohydrate was 50% of the dry weight for the 75% ethanol extract of thymus membrane and only 25% (including glucose and ribose) of the lithium diiodosalicylate extract.

The amino acid compositions are shown in Table VI. The two thymocyte preparations had high proportions of threonine-serine residues and aspartic acid-glutamic acid residues which together accounted for approx. 40% of all amino acids. The major difference was the finding of 30% glycine in the lithium

TABLE VI

AMINO ACID COMPOSITION OF BOVINE MEMBRANE GLYCOPROTEINS

No correction made for loss on hydrolysis; tryptophan not determined. The results are expressed as mol/100 mol amino acid.

Amino acid	Thymocytes		Erythrocytes 75% ethanol extract
	Lithium diiodosalicylate extract	75% ethanol extract	
Cys/2	0.1	0.4	2.3
Asp	9.1	9.7	15.8
Thr	7.4	6.5	7.0
Ser	11.5	12.4	6.2
Glu	10.7	10.3	15.8
Pro	4.3	6.4	12.1
Gly	29.5	7.1	8.9
Ala	4.6	6.5	6.8
Val	2.3	5.4	6.6
Met	—	1.0	—
Ile	3.2	5.1	7.2
Leu	3.3	8.1	8.7
Tyr	1.8	2.2	0.8
Phe	2.0	4.7	4.3
His	1.8	1.8	1.6
Lys	4.7	6.3	2.9
Arg	3.4	6.0	3.0

diiodosalicylate extract as compared to 7% in the ethanol extract. This level of glycine was confirmed by running four separate hydrolysates on two different analyzers with different column size and buffer ionic strength. Also, the peak identified as glycine co-chromatographed with authenic glycine. The amino acid composition of the erythrocyte glycoprotein was distinct from those of thymocyte extracts. Less threonine and serine, only 13%, and more of the acidic amino acids, 32%, were present. The erythrocyte glycoprotein also had a small but significant amount of half-cystein residues.

Discussion

In this study we have compared the utility of hot 75% ethanol extraction and lithium diiodosalicylate-phenol extraction for the isolation of glycoprotein from purified bovine thymocyte membranes. We have successfully isolated glycoprotein by both of these methods. In terms of yield, the hot solvent method extract gave three times as much sialoglycoprotein as did the lithium diiodosalicylate-phenol method. When tested for receptor activities against a panel of hemagglutinins, both preparations strongly inhibited phytohemagglutinin. This lectin reacts with glycoproteins having complex oligosaccharide chains containing mannose and *N*-acetylglucosamine as "core" constituents and terminating in the sequence galactose $\overset{\beta}{\rightarrow}$ *N*-acetylglucosamine [35]. Both extracts inhibited the sialic acid-binding lectin from the horseshoe crab. Neither inhibited *V. graminea* lectin, indicating that the sequence galactose $\overset{\beta}{\rightarrow}$ *N*-acetylgalactosamine → serine (threonine) [36] is absent or cryptic. The 75% ethanol extract had a trace of reactivity with Paul-Bunnel heterophile antibody, 10000-fold less than the erythrocyte extract. This may well represent trace contamination with red cell material. The major difference between the two thymus extracts in sugar composition was the much larger amount of glucose in the lithium diiodosalicylate extract and the presence of ribose. These sugars are probably not constituents of the glycoprotein. The glucose may indicate lipid contamination. Ribose indicates RNA contamination. The lithium diiodosalicylate extract differed from the 75% ethanol extract in having 30 mol% glycine. It is of interest that a glycoprotein fraction isolated by Jansons and Burger [37] from L 1210 cells by hypotonic buffer extraction followed by pyridine extraction had a large amount of glycine. Aspartic acid, gluamic acid, serine, threonine and glycine accounted for 85% of total amino acids. These five amino acids represent 70% of the total amino acids of the lithium diiodosalicylate extract reported in the present study. This extraction procedure must solubilize a fraction of the thymus very rich in glycine which is not extracted by the hot 75% ethanol. In fact, the compositional differences suggest that different glycoproteins are extracted by the two procedures. On sodium dodecyl sulfate gel electrophoresis, both glycoprotein preparations from cell thymus had the same apparent molecular weight, 18500, a value unexpectedly small for membrane glycoprotein. The 75% ethanol extract had, in addition, periodic acid-Schiff-reactive material migrating with the marker dye and probably representing glycolipid. Schmidt-Ullrich et al. [38] reported that calf thymus plasma membrane had at least five glycoprotein bands on sodium dodecyl sulfate gel

electrophoresis with apparent molecular weights ranging from 55000 to 280000.

Using different conditions for the sodium dodecyl sulfate gel electrophoresis we also observed at least five periodic acid-Schiff staining bands in our preparation of calf thymus membrane. Apparent molecular weights ranged from 100000 to 200000. We have previously reported that prior to purification, the glycoprotein of bovine erythrocyte stroma has an apparent molecular weight of >200000 [30]. After isolation with 75% ethanol, the apparent molecular weight is 25000 but glycoprotein isolated by the lithium diiodosalicylate-phenol method has an apparent molecular weight of 280000 [39]. It is well established that under certain conditions, membrane glycoproteins may not be completely disaggregated by sodium dodecyl sulfate. The conditions shown to affect the aggregation state include: the concentration of dodecyl sulfate [24, 40,41], buffer salts [24,40,41], protein concentration [41], duration and temperature of incubation [40,41], and presence or absence of associated peptides [41].

When compared to the thymus membrane glycoproteins, the bovine erythrocyte glycoprotein was distinct in carbohydrate and amino acid composition. This difference was reflected in its receptor properties. The erythrocyte glycoprotein was a potent inhibitor of infectious mononucleosis heterophile antibody and of *Limulus* lectin but did not inhibit the other lectins tested.

This study shows that compared to the lithium diiodosalicylate-phenol method the hot organic solvent method is a relatively efficient one for the extraction of sialoglycoproteins from the membranes of thymocytes as well as erythrocytes. We are presently extending these studies to other cell types.

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