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EPITHELIAL BASEMENT MEMBRANES· THE ISOLATION AND IDENTIFICATION OF A SOLUBLE COMPONENT

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SUMMARY

A glycoprotein which carries an antigenic marker similar or identical to that of epithelial basement membranes in murine tissues has been isolated in a soluble form. This glycoprotein is synthesized and secreted by parietal yolk sac carcinoma cells grown in tissue culture, and was isolated by precipitation with $(NH_4)_2SO_4$ followed by column chromatography on agarose (Bio-gel A-5m). The protein which eluted with the void volume was characterized. Amino acid analysis revealed no hydroxyproline or hydroxylysine. Sugar analysis indicated the presence of fucose, mannose, galactose, glucose and hexosamines. Sialic acid was not detected The data obtained by immunodiffusion suggested the presence of a single protein species

The data indicate that one glycoprotein component of epithelial basement membrane can be obtained in a soluble form, thus simplifying subsequent characterization. Other components, including collagen, have not yet been isolated from this system.

INTRODUCTION

Basement membranes have been isolated from renal glomeruli[1-6], lens capsule[7,8] and parietal yolk sac carcinomas grown *in vivo*[9] for the purpose of biochemical characterization. These tissues yield relatively large amounts of insoluble material which after extensive washing appear free of cellular and other contamination when viewed with the electron microscope. Although morphologic methods including immunohistochemistry provide a ready means of identification, they are crude indicators of purity

The isolation of basement membranes has usually been by the method of Krakower and Greenspon[10]. In this method and its modifications, cells are disrupted by ultrasound, the preparation is centrifuged, and repeatedly washed in salt solutions and distilled water, and the insoluble residue is or contains the basement membrane. Electron microscopic examination of the pellet of insoluble material is considered the most reliable method for determining the absence of cellular contamination[11].

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The insoluble material has been solubilized in a number of ways including the use of denaturants, strong alkaline solutions or by enzymatic digestion. The biochemical analyses of solubilized material have yielded remarkably similar results and indicate the presence of a collagen-like component and a glycoprotein. The latter is believed to carry the antigenic site specific for the identification of the basement membrane molecule[12]. Minor compositional differences noted on comparison of the data obtained by various investigators are felt to reflect the analyses of material from different mammalian species, but, one cannot rule out the possibility that the molecules may have been altered during solubilization. Thus, in addition to the questionable degree of purity, the insolubility of the material creates important problems The purity of renal glomerular basement membrane preparations has been examined by Mohos and Skoza[13]. Their data suggest that the sialic acid attributed by others to basement membrane was instead contributed by cell membranes or glycocalyx which contaminated the preparations in significant amounts. Finally, the methods of isolation and preparation of basement membranes commonly employed has precluded the possibility of isolating a soluble component.

The purpose of this study was to isolate basement membrane components in a soluble form and free of contaminants This was achieved by growing parietal yolk sac carcinoma cells *in vitro* and isolating the newly synthesized soluble basement membrane antigens that had been secreted into the tissue culture medium[14,15].

METHODS

Tissue culture

Cell cultures were initiated from explants of a transplantable parietal yolk sac carcinoma of the mouse[16,17] termed PYS. This epithelial tumor has been shown to synthesize basement membrane antigens *in vivo* and in cell culture[17,18] The cultures were grown in Dulbecco and Vogt's modification of Eagle's medium supplemented with 10% fetal bovine serum and equilibrated with 10% CO_2. The cells do not form confluent monolayers but grow as separate colonies from which spherical masses of cells detach and float in the nutrient medium with subsequent additional growth. This characteristic suggested culture of the cells in a continuous suspension system. The one successfully employed is shown in Fig. 1. The upper reservoir bottle (A) was fitted with a Millipore filter through which fresh medium was periodically added. The medium continuously dripped, at a rate of 25–30 ml/h, into the culture flask (B) equipped with a magnetic stirring bar and agitation paddle. When the volume in the culture bottle (1000 ml) reached the level of the overflow arm, the effluent passed into the collection bottle (C) and was harvested for the isolation of soluble basement membrane glycoprotein. The number of cells which escaped from the culture bottle was minimized by lowering the agitation paddle in the culture bottle

The few cells which escaped into the collection bottle were removed by centrifugation as shown in Fig. 2. To ensure that this centrifugation removed all cells and cellular debris, the initial supernatant (S_1) obtained after removal of the cell button was centrifuged at 100000 × *g* for 24 h at 4 °C. No visible pellet was detected. The supernatant was decanted and buffered OsO_4 was added to the tube to fix microscopic amounts of material which might be present The bottom of the tube

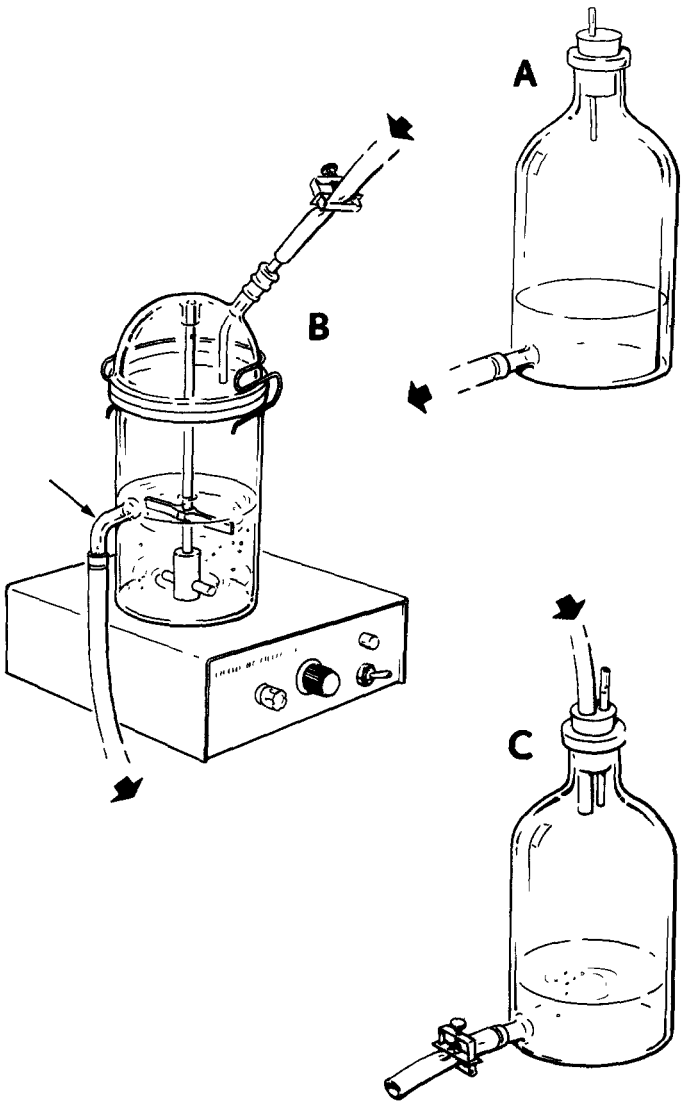

Fig 1 Continuous suspension tissue culture system The upper reservoir bottle (A) contained fresh medium which flowed into the culture bottle (B) by gravity The rate of flow was 25–30 ml/h. Depleted medium containing newly synthesized basement membrane glycoprotein passed from the culture bottle through the overflow arm (small arrow) and was collected in the lower bottle (C)

was carefully scraped although no material was visible. Inspection by electron microscopy confirmed that S_1 contained no cellular debris.

Isolation of soluble glycoprotein

The soluble glycoprotein was isolated by the fractionation procedure shown in Fig. 2 The harvested medium was centrifuged at 10000 × *g* for 10 min at 4 °C Solid $(NH_4)_2SO_4$ was added slowly to the supernatant (S_1) to 50 % saturation

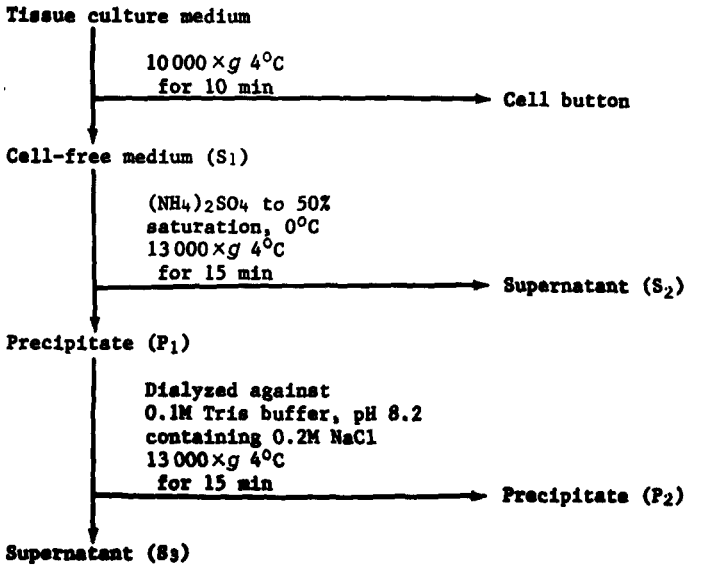

Fig. 2 Isolation of soluble protein The medium from the tissue culture system was fractionated as shown Basement membrane glycoprotein was isolated from the final supernatant (S_3) The fractions shown to the right were stored and analyzed for amino acid content

at 0 °C. The protein was allowed to precipitate overnight at 4 °C and was collected by centrifugation at 13000 × *g* for 15 min. The precipitate (P_1) from 1 l of medium was dissolved in 10–20 ml of 0.1 M Tris buffer, pH 8.2, containing 0.2 M NaCl and dialyzed extensively against the same buffer at 4 °C. The dialysate was centrifuged at 13000 × *g* for 15 min to remove all insoluble material The supernatant (S_3) was chromatographed on a Bio-gel A-5m column (2.5 cm × 40 cm) eluted with 0.1 M Tris buffer, pH 8.2, containing 0.2 M NaCl at 4 °C. The eluate was monitored at 280 nm on an LKB Uvicord and collected in 5-ml fractions. The cell button, S_2 and P_3 were stored at −20 °C and subsequently analyzed for amino acid content.

Tissue culture medium which had not been exposed to cells but was incubated and fractionated in a identical manner was also chromatographed on Bio-gel A-5m to determine the elution pattern of the proteins in fetal bovine serum.

Preparation of rabbit antiserum

Antiserum was prepared against a protein which eluted with the void volume on Bio-gel A-5m (Peak I on Fig. 3). For immunization, New Zealand white rabbits were anesthetized by continuous infusion of a 10% solution of pentobarbital sodium into an ear vein. The protein solution for injection was emulsified with an equal volume of Freund's complete adjuvant such that the final preparation contained 1.0 mg protein per ml. Injection of 0.4 ml of this preparation was made directly into the popliteal lymph nodes. An intramuscular injection of 400 μg of protein in sterile saline was given three weeks later Three weeks following the intramuscular booster, the rabbits were bled from an ear vein and the serum collected. Control serum was taken prior to the initiation of immunization.

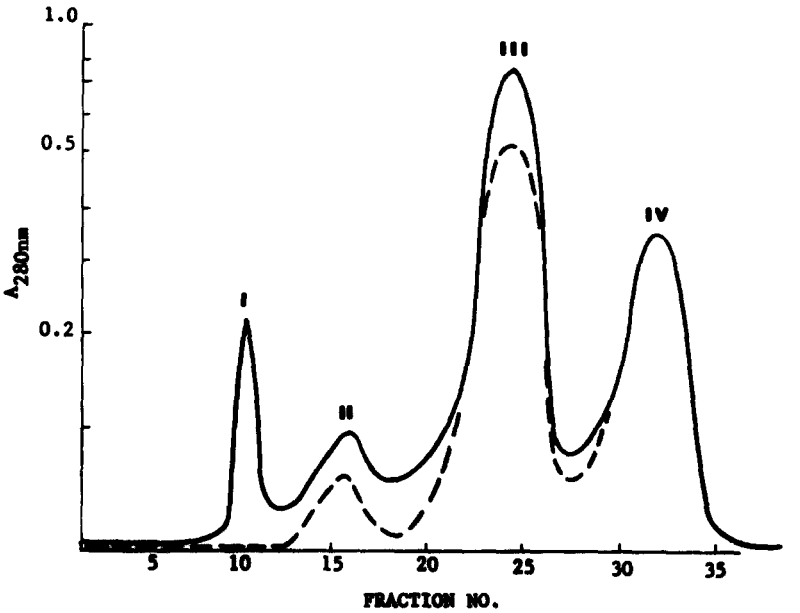

Fig 3 Elution pattern of (S_3) on Bio-gel A-5m The column was eluted with o 1 M Tris buffer, pH 8 2 containing o 2 M NaCl. The solid line denotes (S_3) and the broken line denotes medium not exposed to cells

Immunoassays

The antigen was identified as a component of epithelial basement membrane by utilizing the peroxidase-labeled antibody method[19]. Serial sections of frozen mouse tissues including kidney and spleen were cut at a thickness of 6 μm. Adjacent sections were reacted with the antiserum prepared above, or in the case of controls, with normal rabbit serum. The sections were then incubated with peroxidase-labeled goat anti-rabbit γ-globulin.

Immunodiffusion was carried out on 2 inch $\times$ 2 inch glass slides layered with 4 ml of 1 % agarose in Veronal buffer, pH 7.4, ionic strength 0.15. The antigen (Peak I, Fig. 3) was allowed to diffuse for 48 h at 25 °C prior to the addition of antiserum. An additional incubation of 24–48 h at 25 °C followed, the slides were thoroughly washed, examined for the presence of precipitin bands and stained with amido black.

Chemical determinations

Amino acid analysis of all fractions obtained during the isolation procedure were performed on a Beckman Model 116 Amino Acid Analyzer Fractions were hydrolyzed in constant boiling HCl for 24 h at 110 °C. Analyses were performed according the single column method of Starcher *et al.*[20].

The sugar composition of the glycoprotein was determined by gas chromatography. The heterosaccharides were cleaved and converted to monosaccharides according to the method of Reinhold *et al.*[21] The sugars were separated on a glass column, 5 5 ft in length and 0.125 inch in diameter, packed with Chromosorb HP coated with 2 % Lopol (Beckman Instruments, Inc , Fullerton, Calif) The column

was equilibrated at 140 °C in a Beckman model GC-45 gas chromatograph equipped with a flame ionization detector. After application of the sample, the temperature of the column was programmed to increase to 172 °C at a rate of 4 °C per min.

A standard mixture composed of the sugars commonly present in glycoproteins was also analyzed to determine the molar response of each sugar and the resolution of the column.

Acrylamide-gel electrophoresis was carried out in an Ortec electrophoresis system under the following conditions. Gels were prepared at 4 and 2 % with the lower concentration gel containing 0.5 % agarose to stiffen it. Glycoprotein samples were applied directly to the gel or were initially pretreated with a number of reagents. Aliquots of glycoprotein were incubated in 8 M urea and/or 1 % mercaptoethanol for 2 or 4 h at 37 °C prior to electrophoresis Additional aliquots were pretreated with 1 % sodium dodecyl sulfate or sodium deoxycholate and incubated for 2 h at 37 °C prior to electrophoresis.

RESULTS

The elution pattern of the fraction, S_3, following chromatography on Bio-gel A-5m, which has a molecular weight exclusion limit of $5 \cdot 10^6$, is shown in Fig. 3. The solid line represents the pattern obtained from medium incubated with PYS cells. The protein in Fractions 10–12 eluted with the void volume of the column as

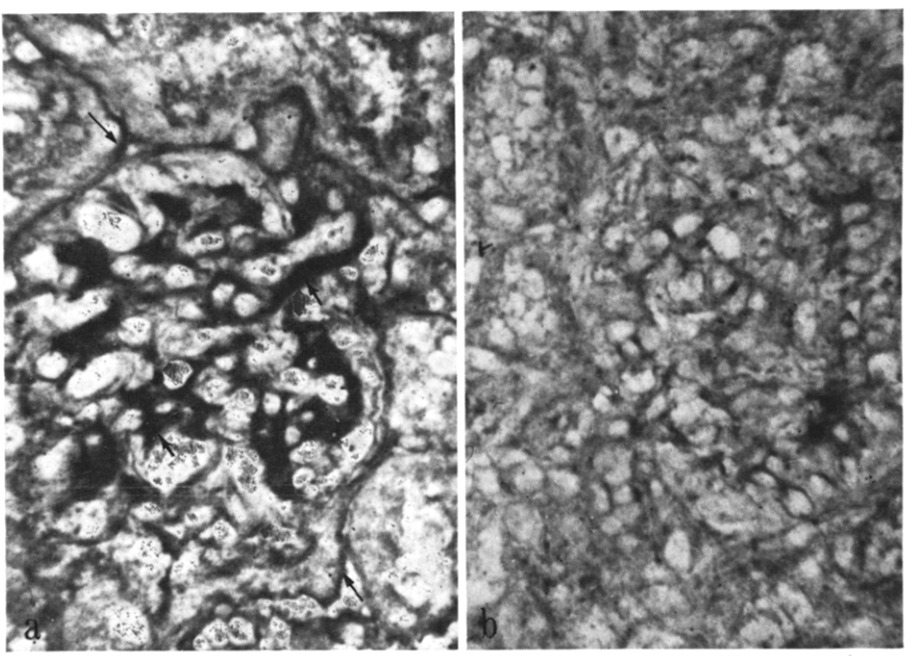

Fig 4 Reaction of antibody directed against Peak I protein with mouse kidney using the peroxidase-labeled antibody technique Reaction product is deposited specifically over the basement membrane, arrows in (a) In (b) the section of kidney was reacted with normal rabbit serum and is devoid of reaction product

determined by chromatographing T_4 DNA. By contrast, the elution pattern of medium not exposed to cells (broken line) contained no protein which eluted with the void volume The proteins which were retarded by the gel were primarily contributed by the fetal bovine serum.

When antiserum prepared against the Peak I protein was reacted with frozen sections of mouse kidney, specific localization of the antigen to the basement membrane was demonstrated (arrows in Fig. 4a). The control section (Fig. 4b) demonstrates the lack of staining in the area of the basement membrane when reacted with normal rabbit serum. Sections of spleen, which contains no epithelial basement membrane but does contain endothelial basement membrane, failed to stain, confirming the specificity observed previously for epithelial basement membrane antigen and antibody[22].

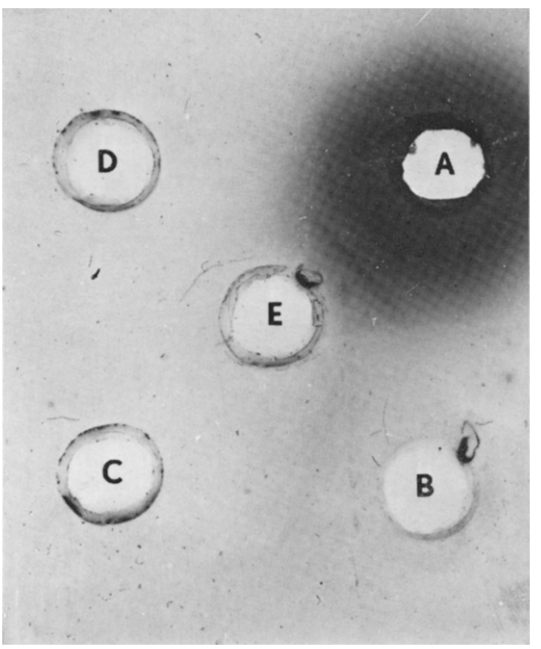

Fig 5 Immunodiffusion of basement membrane glycoprotein and fetal bovine serum in 1% agarose Well E contained antiserum directed against the protein which eluted in Peak I from Bio-gel A-5m Well A contained Peak I protein Wells B, C and D contained increasing concentrations of fetal bovine serum, respectively The precipitin lines were developed by staining with amido black

The results of immunodiffusion are shown in Fig. 5. A single precipitin line was present when rabbit antiserum (E) was reacted with the void volume protein (A). A large amount of precipitated protein was present around the antigen well, but did not obscure any additional precipitin lines as determined by examination of unstained slides Wells B, C and D contained fetal bovine serum at increasing concentrations, respectively, which did not react with the antiserum.

The amino acid composition of the fractions obtained during the isolation procedure are shown in Table I The values are expressed in residues of amino acid

TABLE I

AMINO ACID COMPOSITION OF THE BASEMENT MEMBRANE GLYCOPROTEIN AND OTHER FRACTIONS OBTAINED DURING THE ISOLATION PROCEDURE

The content of each amino acid is reported as residues per 100 residues of total amino acid

Amino acid	*Fraction analyzed*			
	Peak I	*Cell button*	S_2	P_3
Hydroxyproline	0	0	0	0
Aspartic acid	10 25	10 28	11 54	10 29
Threonine	8 05	5 00	5 85	8 01
Serine	5 91	6 93	8 04	5 91
Glutamic acid	11 40	12 28	11 94	11 62
Proline	5 42	5 43	5 28	5 29
Glycine	8 45	8 58	7 72	8 49
Alanine	7 76	8 24	8 45	7 72
Half-cystine	1 20	1 04	1 95	1 29
Valine	5 75	3 96	3 66	5 66
Methionine	0 25	1 84	1 14	0 29
Isoleucine	2 91	2 31	2 11	2 87
Leucine	8 70	8 66	9 42	8 78
Tyrosine	2 84	3 54	4 55	2 84
Phenylalanine	3 85	4 93	5 93	3 76
Hydroxylysine	0	0	0	0
Lysine	7 05	7 35	6 66	7 25
Histidine	6 95	4 66	2 44	6 92
Arginine	4 40	4 97	3 33	4 32

TABLE II

SUGAR COMPOSITION OF BASEMENT MEMBRANE GLYCOPROTEIN

The quantity of each sugar is reported in μg/mg of lyophilized glycoprotein as determined by gas chromatography.

Sugar	*μg/mg lyophilized Peak I glycoprotein*
Fucose	16 1
Mannose	17 5
Galactose	31 0
Glucose	8 9
N-Acetylgalactosamine	22 3
N-Acetylglucosamine	38 9
Sialic acids	0

per 100 residues of total amino acid. The analysis of Peak I protein showed neither hydroxyproline nor hydroxylysine. The high content of acidic amino acids, aspartic and glutamic acids in particular, suggest an acidic protein. Analysis of the cell button, S_2 and P_3 also failed to demonstrate hydroxyproline or hydroxylysine.

The results of sugar analysis are shown in Table II. The total sugar content of the glycoprotein was approximately 13.5 % which consisted of 43 % hexoses, 46 % hexosamines and 11 % fucose. No sialic acids were detected even when acid catalyzed methanolysis was limited to 10 min as suggested by Reinhold *et al.*[21].

DISCUSSION

An epithelial tumor grown *in vitro* synthesizes a soluble glycoprotein component of basement membrane which has been identified by the presence of an antigenic marker specific for epithelial basement membrane The advantages of this system over *in vivo* models are (1) the ability to isolate the basement membrane component in a soluble form and (2) the unequivocal elimination of contaminating cellular material

Because of the reliance on the use of morphologic examination for the identification of basement membranes and extensive washing during their isolation, soluble components have not been previously obtained. In addition, the methods of isolation do not provide adequate criteria for purity. Cell membrane contaminants account in part for the sialic acid attributed to basement membrane[13,23]. Likewise, a portion of the protein present is probably derived from the same source of contamination.

The immunologic data presented indicate that the glycoprotein isolated from this system carries an antigenic marker quite similar, if not identical, to that carried by the epithelial basement membranes in mouse tissues. The absence of staining in the spleen demonstrated that no component of endothelial basement membrane cross-reacted with the antigen and that no connective tissue proteins contaminated the preparation

The failure to detect hydroxyproline and hydroxylysine by amino acid analysis suggests that the collagen component of epithelial basement membrane constitutes only a small percentage of the total protein secreted by these cells This finding is not surprising in the face of recent data obtained by Grant *et al* [24,25]. Their studies with lens epithelium revealed that only 2% of the total protein synthesized by these cells was basement membrane collagen. As shown previously by Priest[26], parietal yolk sac carcinoma cells incorporate proline into protein as both proline and hydroxyproline as determined by the method of Juva and Prockop[27]

The sugar composition of this glycoprotein differs from that of the non-collagenous glycoprotein isolated from glomerular basement membrane[28] The glucose present in glomerular basement membrane has previously been associated only with the collagen-like component The absence of sialic acid is in agreement with the findings of Mohos and Skoza[13,23] Their data suggests that the sialic acid in glomerular basement membrane preparations is due to contamination with cell membranes.

A major difficulty which arose during attempts to further characterize the glycoprotein appeared to be due to the formation of large molecular weight aggregates, as suggested by the elution pattern of Peak I from Bio-gel A-5m and the results of immunodiffusion. In addition, the attempts to disrupt the aggregates by the use of denaturants and reducing agents (urea and mercaptoethanol) or detergents (sodium dodecyl sulfate or sodium deoxycholate) were unsuccessful In all cases, no protein penetrated acrylamide gels following pretreatment with these reagents.

In conclusion, parietal yolk sac carcinoma cells grown *in vitro* synthesize large amounts of a glycoprotein component of epithelial basement membrane. This glycoprotein has been isolated in a soluble form and partially characterized.

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