Pre-eclampsia Is Associated with an Increase in Trophoblast Glycogen Content and Glycogen Synthase Activity, Similar to that Found in Hydatidiform Moles

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Abstract

Pre-eclampsia is a placental disorder, but until now, biochemical details of dysfunction have been lacking. During an analysis of the oligosaccharide content of syncytiotrophoblast microvesicles purified from the placental chorionic villi of 10 primigravid women with proteinuric pre-eclampsia, we found an excess of glycogen breakdown products. Further investigation revealed a 10-fold increase in glycogen content (223 ± 117 μg glycogen/mg protein), when compared with controls matched for gestational age at delivery (23 ± 18 μg glycogen/mg protein) ($P < 0.01$). This was confirmed by examination of electron micrographs of chorionic villous tissue stained for glycogen. The increase in glycogen content was associated with 16 times more glycogen synthase ($1,323\pm1,013$ relative to 83 ± 96 pmol glucose/mg protein per min) ($P < 0.001$), and a threefold increase in glycogen phosphorylase activity ($2,280\pm1,360$ relative to 700 ± 540 pmol glucose/mg protein per min; $P < 0.05$).

Similar changes in glycogen metabolism were found in trophoblast microvesicles derived from hydatidiform moles. Glycogen accumulation in villous syncytiotrophoblast may be a metabolic marker of immaturity of this cell which is unable to divide. The implications of these findings with regard to the pathogenesis of pre-eclampsia are discussed. (*J. Clin. Invest.* 1993. 91:2744–2753.) Key words: pregnancy toxemias • trophoblast • trophoblast tumor • maltose • glycogen

Introduction

The primary cause of pre-eclampsia is still unknown. The condition appears to originate in the placenta (1), as the disorder is always cured by emptying the uterus, and it may also develop without a fetus, as with hydatidiform mole (2).

The syncytiotrophoblast (STB)[1] covering the chorionic villi make up the major surface of direct contact between the placenta and maternal blood. Although it is not known where in the placenta the factor or factors that cause the maternal syndrome originate, in view of its position and size, the role of the syncytiotrophoblast in the pathogenesis of pre-eclampsia needs to be considered.

The little that is known about the STB in pre-eclampsia is derived more from histopathological, than biochemical studies. Changes in the STB include focal loss or clubbing of microvilli, and dilation of the rough and smooth endoplasmic reticulum, observed with electron microscopy (3, 4). These syncytial changes are associated with proliferation of underlying villous cytotrophoblast. Chorionic villi from normal placentae cultured under hypoxic conditions show similar histological changes, raising the possibility that placental ischemia has a role in the pathogenesis of pre-eclampsia (5, 6). The increased incidence of placental infarction in pre-eclampsia (3) provides further evidence for local placental ischemia in the condition.

The ultrastructural changes in the villous trophoblast morphology listed above are, however, not specific for pre-eclampsia and ischemia. They can also be seen with hydatidiform moles (7), and diabetes (3), both sometimes associated with pre-eclampsia, as well as in normal early first trimester tissue (3). A distinguishing feature between the conditions appears to be the accumulation of glycogen within the placental tissue from women with moles and diabetes (8–10), but not those with pre-eclampsia (11, 12). Normal early first trimester chorionic villous trophoblast also contain large amounts of glycogen, which rapidly disappears as the pregnancy advances (11, 13, 14).

In this study, we examined the glycocalyx of the STB apical membrane, known to show focal deformities of surface microvilli on electron microscopy in pre-eclampsia (3, 4). The glycocalyx consists of oligosaccharides of varying complexity linked to membrane-bound or associated lipids and proteins. The oligosaccharide chains of trophoblast glycolipids have been investigated, but only in normal pregnancy (15, 16). We have described the changes in protein-linked oligosaccharides of normal syncytiotrophoblast apical membranes, which occur with gestational maturity and parturition (17). Here we compare the protein-linked oligosaccharides of STB membranes from pregnancies complicated by pre-eclampsia with those from normal control pregnancies. We discovered an unexpectedly high content of glycogen in the STB membranes, sought possible reasons to explain the accumulation, and compared the findings with those in membrane preparations from molar and diabetic pregnancies.

Methods

Selection of patients, and collection of placentae. 10 primigravid, proteinuric (3.6 ± 1.0 g urinary protein/24 hours; mean±SD) pre-eclamptic women (PE) were studied, who had new hypertension at or above 160/110 mmHg. Neither the proteinuria nor hypertension were present in early pregnancy (before 20 wk of gestation), and both abnormalities had regressed by 6 wk after delivery. The average (±SD) age of the women was 23 ± 4 yr, and they delivered at 36 ± 3 wk gestation. The birth weights averaged $2,285\pm932$ g, and half were delivered by Cesar-

1. *Abbreviations used in this paper:* C-STB, control STB; PATO, periodic acid-thiocarbohydrazide osmium tetroxide; PE, pre-eclamptic; STB, syncytiotrophoblast.

ean section. 4 of the 10 patients had one or more features of the HELLP syndrome, a severe maternal consequence of pre-eclampsia, consisting of the triad of hemolysis, elevated liver enzymes (aspartate aminotransferase > 42 IU/liter), and thrombocytopenia (platelet count < 160 × 10⁹/liter) (18).

The 10 women of the control group were matched for gestational age 36±3 wk. The average maternal age was 29±6 yr, and the birth weight was 2,532±877 g. Four were delivered by Cesarean section. None of the control group had maternal infections or other medical disorders, and the neonates were normal. Premature delivery was due to spontaneous onset of labour, elective delivery for breech, or previous section in an otherwise normal pregnancy.

Seven women with diabetes (three with an onset before conception), who required insulin, were also studied. None had superadded pre-eclampsia. Their average age was 30±7 yr, and their newborns weighed 3,328±535 g at 38 wk. Two women were studied who had complete hydatidiform moles (17 and 10 wk gestation), confirmed by histology. Neither had superadded pre-eclampsia. The gestational ages at delivery were determined from the histories of the last menstrual period, and/or ultrasound scans performed in the first trimester.

Electron microscopy. Pieces of chorionic villous tissue were fixed as quickly as possible after delivery in 3% glutaraldehyde, and secondarily fixed in osmium tetroxide and embedded in Vestopal-W (Agar Scientific, Stansted, United Kingdom). Glycogen was stained using the periodic acid-thiocarbohydrazide-osmium tetroxide (PATO) method (19, 20), as modified by Ferguson (21). Embedded sections were initially treated with 1% periodic acid for 45 min in a dark room, washed with distilled water, transferred to 1% thiocarbohydrazide in 5% acetic acid at 60°C for 2 h, and then rewashed with water. The osmium tetroxide treatment was carried out for 2 h in a fume hood. Six replicates of each sample were processed in each experiment including controls, in which one of the reagents was omitted to exclude false positive reactions. Each series was carried out in duplicate and one grid was poststained with magnesium uranyl acetate and lead citrate. To allow easier identification of organelle structure, only micrographs obtained from poststained grids were used in Fig. 8.

Placental and syncytiotrophoblast tissue preparation. Placentae and hydatidiform moles were processed within 15 min of delivery. Pieces of chorionic villous tissue weighing ~ 1 g were snap frozen in liquid nitrogen, and stored at −70°C for estimations of glycogen content. Purified STB membrane microvesicles were prepared from whole placentae, the complete procedure being at 4°C (17, 22). The method is described briefly. Pieces of placental cotyledons were washed with 2 liters of ice cold 50 mM CaCl₂, then 500 ml of PBS to remove surface blood. The tissue was then minced, and STB microvesicles released by stirring in 350 ml of 0.15 M NaCl, pH 7.4 for 1 h. Large cell debris was removed by filtering through a nylon sieve, and the filtrate centrifuged at 800 *g,* and then at 10,000 *g,* each time for 10 min. The STB microvesicles were pelleted at 100,000 *g* for 45 min, and stored at −70°C until required. The validity of the method has been previously shown by electron microscopy of the STB before and after extraction of the microvilli, and of the membrane pellet (17, 23). Protein content was measured using the Bradford protein assay (24), with BSA as a standard.

Preparation of syncytiotrophoblast membrane glycan libraries. Total proteins were isolated from the STB after extraction of the lipids with acetone (20 ml/g STB) at −20°C (17). The insoluble protein was pelleted at 500 *g* for 10 min, and excess acetone was evaporated with N₂. The protein was resuspended in distilled water, and the suspension dialyzed with repeated (×5) changes of distilled water (a total of 10 liters of water per 2-ml sample) for 48 h, in tubing with an exclusion size of 6–8 K.

Oligosaccharides were prepared by hydrazinolysis of cryogenically dried 2-mg aliquots of the actone extracted STB (glycan library) as previously described (17, 25). The hydrazinolysates were N-acetylated by the addition of a 5-M excess of acetic anydride (0.5 M) in saturated NaHCO₃ at 4°C for 10 min, and then 60 min at 30°C, and desalted using Dowex AG50 X12 (H⁺) Bio-Rad, Hemel Hempstead, United Kingdom. Descending paper chromatography (27°C, 70% relative humidity) was subsequently performed for 48 h using *n*-butanol/ethanol/water (4:1:1 vol/vol) on samples applied to 3MM paper (Whatman Inc., Clifton, NJ).

Radiolabeling and analysis of reducing terminal saccharides. When oligosaccharides are cleaved from glycoproteins, the monosaccharide that was originally bound to the protein can be reduced, and is called the reducing terminal structure. Free oligosaccharides or monosaccharides can be reduced without prior treatment with hydrazine. The glycan library prepared from actone-extracted STB was reduced and radiolabeled with NaB³H₄ (Amersham International, Amersham, United Kingdom) (17, 25). During the reaction, tritium is bound stoichometrically in a nonexchangeable form, to the reducing terminal structure.

For analysis of reducing terminal structures, the oligosaccharides were then hydrolyzed with 1 M HCl for 3 h at 100°C, followed by re-N-acetylation with excess acetic anhydride, as described above. The acid hydrolysis and N-acetylation were then repeated once. To the resultant mixture of monosaccharides, reduced radiolabeled (³H) 2-deoxyribitol was added as an internal standard, before electrophoresing the samples on Whatman 1 paper for 4 h at 8.5 kV m⁻¹ in 15 mM sodium tetraborate, pH 9.5, using a custom-built 1.2-m flat-bed high-voltage electrophoresis unit (Locarte, London, United Kingdom). Radioactive regions detected by the linear analyser were eluted and the radioactivity in the glucitol, N-acetylglucosaminitol, and N-acetylgalactosaminitol peaks determined and compared, to determine the ratio of these three reducing terminal structures in the oligosaccharide pool.

To confirm the identity of the glucitol, the radiolabeled reducing terminal structures were also analyzed by gas chromatography. Monosaccharides prepared by acid hydrolysis were first derivatized to the alditol acetates by incubation with pyridine and acetic anhydride (1:5 vol/vol) at 120°C for 20 min. The per-acetylated sample was then dissolved in chloroform, and injected into a 3600 gas chromatography system (Varian, Surrey, United Kingdom) with a 25-m × 0.32-mm SP2380 capillary column (Supelco, Sawbridgenorth, United Kingdom). The sugars were eluted with helium (2.5 ml/min), at 90°C for 1 min. The temperature was raised at the rate of 30°C/min to 220°C, and maintained for 13 min, and finally increased to 230°C at 30°C/min.

Effluent from the gas chromatography column was mixed with hydrogen (3.5 ml/min), and passed into a catalytic reduction furnace (platinum catalyst/700°C) within the gas chromatography oven. The volatile reduction products were then mixed with counting gas (argon 90%, methane 10%, 120 ml/min) and radioactivity detected in a 10-ml counting tube (Rattest, Sheffield, United Kingdom). Unknown monosaccharides were identified by comparing their retention times with those of radiolabeled standards.

In addition, and using the same methods, the oligosaccharides were hydrolyzed, radiolabeled with tritiated borohydride, and analyzed by gas chromatography to determine their total sugar composition.

Separation of oligosaccharides by concanavalin A affinity chromatography. Oligosaccharide samples were desialylated with *Arthrobacter ureafaciens* neuraminidase (Cambridge Biosciences, Cambridge, United Kingdom) at 37°C for 18 h, before affinity chromatography to remove charged groups. Desialylation was confirmed by paper electrophoresis in pyridine/acetic acid/water (3:1:387 vol/vol/vol, pH 5.4) buffer for 45 min at 80 V cm⁻¹. After equilibration of a concanavalin A-agarose affinity column (Pharmacia, Uppsala, Sweden) (6 mm × 20 cm) with 0.01 M sodium acetate, pH 5.0, the desialylated STB glycan library was applied, and eluted first with 0.01 M sodium acetate/0.1 M NaCl, pH 5.0, and then with 0.1 M HCl at 10 ml/h. The fractions were desalted using Dowex AG-50 X12 (H⁺ form) and analyzed by Bio-Gel P4 (Bio-Rad) permeation chromatography as described below.

Analysis of hydrodynamic volumes of oligosaccharides. Radiolabeled STB oligosaccharides were mixed with isomalto-oligosaccharide standards, which were produced by partial acid hydrolysis of dextran, and applied to a high resolution gel filtration system comprising two columns of Bio-Gel P4 (−400 mesh), (Bio-Rad) (1.5 cm × 100 cm) in

series, maintained at 55°C and eluted with water. The effluent was monitored by a Berthold HPLC radioactivity flow monitor (model LB503; Berthold Instruments [UK] Ltd., St. Albans, UK) and an Erma refractive index monitor (model ERC7510; HPLC Technology Ltd., Macclesfield, United Kingdom) before collection. Elution positions of radiolabeled oligosaccharides were determined by comparing them with positions of glucose oligomers made up of known numbers of glucose residues or units.

All of the above procedures were performed on STB microvesicles extracted with acetone. The following were done without this initial extraction.

Enzymic labeling of syncytiotrophoblast membrane components using glycogen synthase. The maximum incorporation of radiolabeled glucose into control STB (C-STB) and STB derived from women with pre-eclampsia (PE-STB) was measured, after adding glycogen synthase and its cofactors. 20 mg/ml wet wt of STB was incubated with 20 mU/ml of glycogen synthase prepared from rabbit muscle (Sigma Immunochemicals, Poole, United Kingdom), 5 mM glucose-6-phosphate, and 250 μM UDP-glucose with 100 mCi tritiated UDP-glucose/mmol (Amersham International), in 50 mM Tris/HCl buffer, pH 6.5. The reactions were carried out at 37°C, in the presence or absence of 200 μg/ml *Triticum aestivum*–derived α-amylase inhibitor (type I) (Sigma Immunochemicals) (26, 27), and 5 mM EDTA, to determine the degree of breakdown of radiolabeled material by endogenous α-amylase. 10-μl aliquots of the 250-μl incubation mixture were removed at 0, 5, 10, 15, and 20 min. The reaction was terminated by adding 1% phosphotungstic acid/0.5 M HCl. Radiolabeled glycogen was precipitated and washed with ethanol onto glass fiber filter strips using a PHD cell harvester (Cambridge Technology Inc., Watertown, MA), before measuring the radioactivity on the punched out disks. All assays were done in duplicate.

Purification of the ethanol-precipitable, enzymically radiolabeled STB fraction by concanavalin A affinity chromatography. Material which radiolabeled using the above procedure, and was ethanol precipitable, could be purified by concanavalin A Sepharose (Pharmacia) affinity chromatography. The radiolabeled material from 1 g wet wt of PE-STB was dissolved in 1% Triton X-100, 5 mM EDTA, and 200 μg/ml α-amylase inhibitor, 50 mM Tris/HCl, pH 7.4, and applied to the concanavalin A affinity column, and eluted with 50 mM Tris/HCl, pH 7.4 containing 1% Triton, and then the same buffer without Triton to remove detergent from the column. The bound material was eluted with 0.1 M HCl, and HCl removed by evaporation after freezing.

Extraction of glycogen and measurement of glycogen content. Glycogen (micrograms glycogen per milligram protein) in chorionic villous tissue or STB microvilli was measured using the method of Van Handel (28). 4–10 mg of unwashed chorionic villi (wet wt) was extracted with 600 μl of 30% KOH at 95°C for 15 min. The solution was cooled, and the glycogen was precipitated by adding 500 μl of 2% Na$_2$SO$_4$ and 3 ml of ethanol. Glycogen was pelleted by centrifugation at 500 g for 5 min. The pellet was washed once with 3 ml of 50% ethanol, and then dissolved in water. Glycogen was measured at 620 nm, after heating in 0.12% anthrone/6.4 M H$_2$SO$_4$ at 90°C for 20 min, and then cooling for 15 min. Rabbit liver glycogen (Sigma Immunochemicals) was used as the standard.

Measurement of glycogen synthase activity. Glycogen synthase activity was quantified using a modification of the method of Thomas (29). In this assay, the incorporation of radiolabel from UDP-^{14}glucose into glycogen in the presence of the synthase enzyme is measured. 20 mg/ml (wet wt) of STB, glycogen 10 mg/ml (rabbit liver; Sigma Immunochemicals), 1 mg/ml α-amylase inhibitor, UDP-glucose 140 μM (Sigma Immunochemicals) containing 80 Ci/mol (Amersham International), and 40 mM Tris/8 mM EDTA, pH 7.5 were incubated together at 30°C. Aliquots were removed at 0, 4, 8, and 12 min, and the reaction stopped by adding 1% phosphotungstic acid/0.5 M HCl. Glycogen was precipitated with ethanol onto glass fiber filter strips and then washed with ethanol using a PHD cell harvester (Cambridge Technology Inc.), before measuring the radioactivity on the punched out disks. All assays were done in duplicate. Synthase activity (picomoles glucose per milligram mg protein per minute) was determined in the

presence (total), and absence (independent form) of the co-factor glucose-6-phosphate (8 mM). The dependent enzyme form can be calculated by subtracting the independent activity from the total.

Measurement of glycogen phosphorylase activity. A modification of the method of Tan and Nuttall (30) was used in which the incorporation of labeled ^{14}C-glucose-1-phosphate in glycogen was measured. The incubation mixture contained STB 20 mg/ml (wet wt), glycogen 10 mg/ml, 1 mg/ml α-amylase inhibitor, containing 670 mCi/mol (Amersham International), 50 mM 2-[N-morpholino]ethanesulfonic acid (MES), 100 mM potassium fluoride, and 5 mM EDTA, pH 6.1. The reaction proceeded at 30°C. Aliquots were removed at 0, 10, 20, and 30 min, and the reaction stopped by adding 1% phosphotungstic acid/0.5 M HCl. Glycogen was precipitated onto glass fiber filter strips with ethanol, and washed, as for the glycogen synthase assay described above. All assays were done in duplicate. Phosphorylase activity (picomoles glucose per milligram protein per minute) was determined in the presence (total), and absence (independent form) of the co-factor 5-AMP (2 mM). The dependent form can be calculated by subtracting the independent activity from the total.

Amino acid analysis. Samples were hydrolyzed in vapor phase HCl for 24 h at 112°C. Derivatization of amino acids was by the Picotag method, according to the manufacturer's instructions (Waters Associates, Millipore Corp., Watford, United Kingdom). Phenylthiocarbamyl derivatives were separated by reverse phase HPLC on a 5-μm Spherisorb ODS2 column (0.46 $\times$ 25 cm) (Waters Associates, Millipore Corp.) at 50°C using the Picotag solvent and gradient system (Waters Associates) and 510 pumps and M490 multiwavelength detector (Waters Associates). Pump control and data acquisition were by an 840 chromatography workstation (Waters Associates).

Statistics. Results in the text are always expressed as mean $\pm$ standard deviation, whereas results in the figures are given as means $\pm$ SEM. Paired t tests were used for comparing PE with matched controls. Unless otherwise stated, in all other cases, unpaired t tests were performed.

Results

Oligosaccharide content of glycan libraries, produced by hydrazinolysis of acetone-extracted STB membranes. The sizes of the labeled N-linked oligosaccharides in the tritiated glycan libraries were determined by Bio-Gel P4 (Bio-Rad) gel permeation chromatography. The distribution of structures ranging in size from 6 to 24 glucose units, was similar to that which we have documented previously (17). The glycan libraries from placentae of women with pre-eclampsia had similar distributions and relative proportions of N-linked oligosaccharides for structures of 6 glucose units and larger, but unlike the controls, in all 10 cases of pre-eclampsia, the largest peaks were smaller structures of between 2 and 4 glucose units in size (Fig. 1). The identity of these small oligosaccharides was therefore sought.

Glycan libraries were acid hydrolyzed, and the distribution of tritiated reducing terminal monosaccharides analyzed by borate buffer high voltage electrophoresis. The ratio of N-acetylglucosamine–reducing termini (classical N-linked oligosaccharides) to N-acetylgalactosamine–reducing termini (classical O-linked oligosaccharides) was similar in preparations from pre-eclamptic and normal pregnancies (Fig. 2 A). There was, however, a significant excess of reducing terminal glucose in pre-eclampsia (Fig. 2 B).

The presence of reducing terminal glucose was confirmed using a second chromatographic procedure. After borate buffer high voltage electrophoresis, the radiolabeled monosaccharide from a PE sample, which co-migrated with the glucose standard, was eluted from the paper, and analyzed by radio-gas chromatography. The monosaccharide eluted in the same posi-
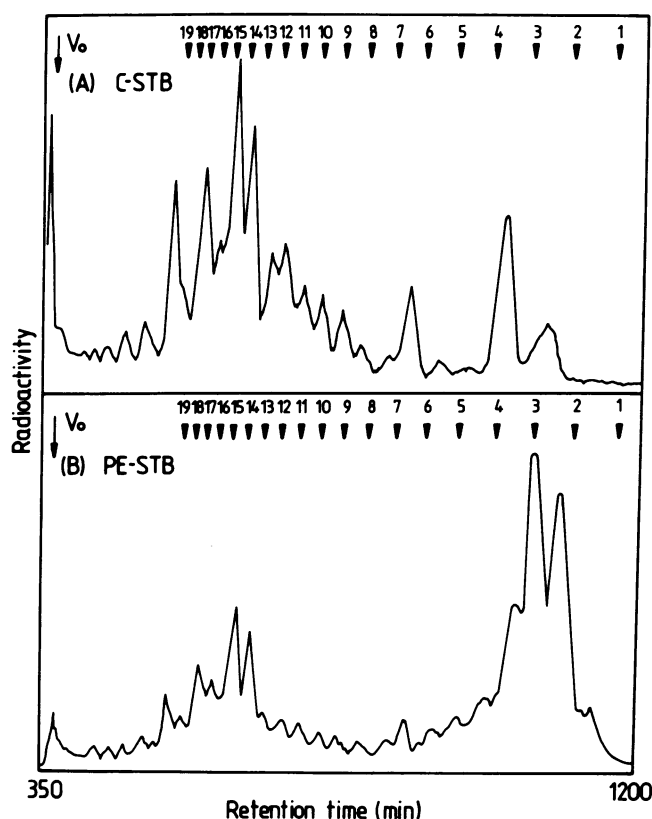
Figure 1. Bio-Gel P4 permeation chromatographs of glycan libraries from 1 of the 10 control (A) and PE-STB (B) samples. Tritiated oligosaccharides are detected as β radioactivity (cpm vertical axis). Individual oligosaccharides peaks are expressed in terms of glucose units (superscripts).

Figure 2. Distribution of reducing terminal structures of syncytiotrophoblast membrane glycan libraries prepared from the placentae of 10 pre-eclamptic (PE) and 10 matched control women, in terms of (A) the relative percentages of N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc); and (B) the percentage of total reducing termini which were glucose (Glc). Reducing terminal structures were determined by borate high voltage electrophoresis of acid-hydrolyzed oligosaccharides.

tion as the glucose standard (Fig. 3 B), and moreover, when it was spiked with the same quantity of glucose standard, the elution peak doubled in height, without any broadening (Fig. 3 C).

Oligosaccharides with reducing terminal glucose could be separated from the other oligosaccharides in the glycan library, using concanavalin A affinity chromatography. There was an increased proportion of labeled oligosaccharides in PE preparations, which were retarded by, but not bound to, the column (fraction II), when compared with the control (Fig. 4). Three oligosaccharide fractions could be defined (I–III), and their reducing terminal monosaccharides were determined by borate buffer high voltage paper electrophoresis of acid hydrolysates (inserts, Fig. 4). Reducing terminal glucose was found only in the retarded fractions of control and PE samples (fraction II). The acid hydrolysate of fraction II was subjected to a second reduction with tritiated borohydride, and found to consist solely of glucose by both borate buffer high voltage electrophoresis, and radio-gas chromatography (Fig. 3 D).

The sizes of the glucose oligomers in fraction II of a PE glycan library were determined by Bio-Gel P4 gel-permeation chromatography. There were two major structures of 2.5, 3.0 glucose units, and a minor one of 3.5 glucose units (data not shown), which corresponded to those identified in the P4 chromatogram of the unfractionated glycan library.

Glycoproteins with pure glucose oligosaccharide side chains are uncommon, glycogen and its precursor being one of
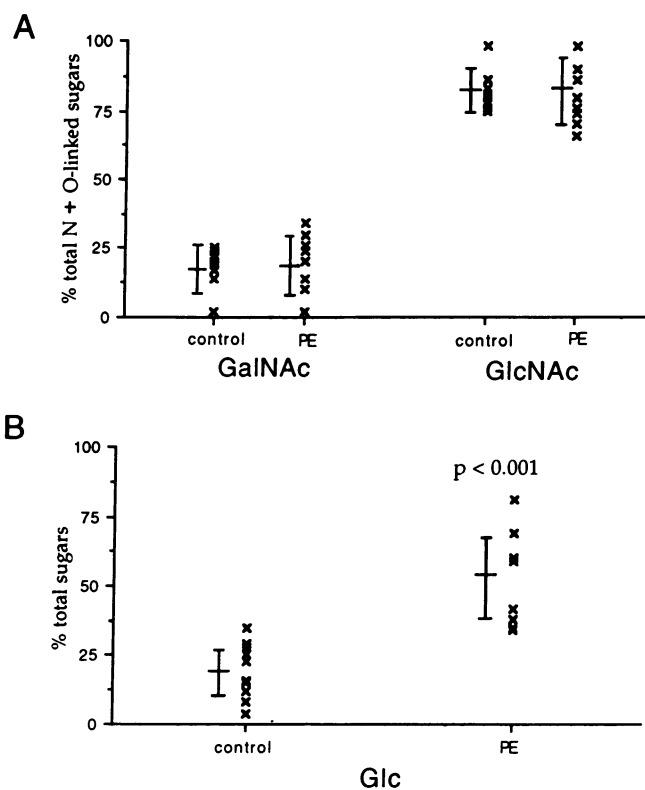
the few examples in mammals (31, 32). The possibility that the glucose oligomers seen in PE-STB were glycogen metabolites was therefore investigated.

Glucose oligomers in PE-STB membranes and glycogen metabolites. Pooled STB from the PE cases and controls were examined for the presence of glycogen precursors, initially through their ability to act as a substrate for glycogen synthase. A time course of the tritiated glucose incorporated into unprocessed STB microvesicles, using glycogen synthase and UDP-glucose, with and without EDTA and α-amylase inhibitor, is shown in Fig. 5 A.

Control membranes incorporated minimal amounts of glucose, in contrast to those from PE cases. In the latter, there was a large increase in glucose incorporation with time, which peaked within 5 min. The radiolabeled material was rapidly degraded unless an inhibitor of α-amylase was added to the incubation mixture.

The maximum incorporation of tritiated glucose into each of 10 preparations of individual PE-STB was 19-fold higher than that of control preparations, and there was no overlap between the two groups (Fig. 5 B). These experiments were done in the presence of EDTA and the specific α-amylase inhibitor.

The radiolabeled material was purified from PE-STB membranes by concanavalin A affinity chromatography, and its biochemical structure analyzed. It consisted solely of glucose, as
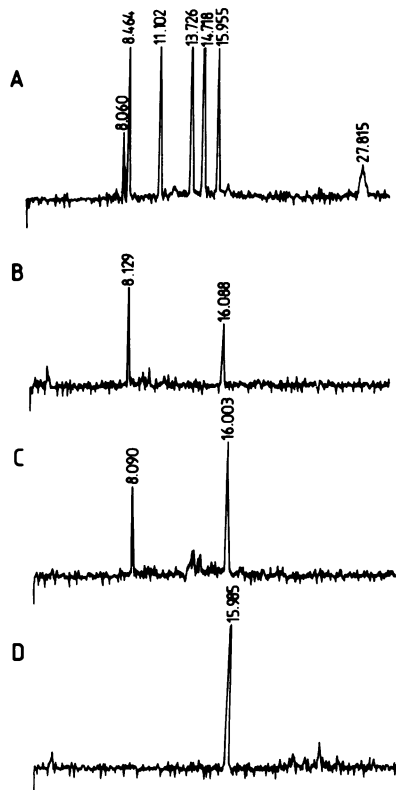
A

B

C

D

8.060

8.464
11.102
13.726
14.718
15.955

27.815

8.129

16.008

8.090

16.003

15.985

Figure 3. Radio gas chromatography. (*A*) Radiolabeled monosaccharide standards and elution times (min): 2-deoxyribitol (8.060), fucitol (8.464), xylitol (11.102), mannitol (13.726), galactitol (14.714), glucitol (15.955), *N*-acetylglucosaminitol (27.815); (*B*) reducing terminal monosaccharide from a PE-STB glycan library that co-migrated with glucitol on borate buffer high voltage electrophoresis (2-deoxyribitol added as a standard); (*C*) the same PE-STB–derived monosaccharide shown in *B*, spiked with an equal amount of radiolabeled glucitol and 2-deoxyribitol standards; and (*D*) compositional analysis of the concanavalin A–retarded fraction (fraction II) from a PE-STB sample by radiolabeling of an acid hydrolysate. All peaks are detected as β-radioactivity.

A. NORMAL

GalNAc GlcNAc Glc

I   II   III

HCl

I   II   III

Elution volume

B. PRE-ECLAMPSIA

GalNAc GlcNAc Glc

I   II   III

HCl

I   II   III

Elution volume

Figure 4. Separation of (*A*) C-STB and (*B*) PE-STB membrane glycan libraries by concanavalin A affinity chromatography. Peaks I and II were eluted with 0.01 M Na acetate buffer, pH 5.0, and peak III was eluted with 0.1 M HCl. Upper panels (I–III) show reducing terminal analysis of fractions as determined by borate high voltage electrophoresis.

determined by radio-gas chromatography of an acid hydrolysate. The material was large and uncharged, as it remained at the origin after high voltage electrophoresis with either pyridine or borate buffer (data not shown), and eluted in the void on Bio-Gel P4 chromatography (Fig. 6 *A*). The void material was not degraded into smaller components by hydrazinolysis, and thus consisted of a larger polymer, rather than multiple small oligomers linked to a core structure (data not shown). When the purified void material from the P4 column, was reincubated with pooled PE-STB, extracted with acetone for 30 min at 37°C, without inhibitors of α-amylase, it was broken down into oligomers, 2–4 glucose units in size, with no radioactivity remaining in the void (Fig. 6 *A, insert*). The same P4 profile was obtained when the purified void material was incubated with pooled C-STB (data not shown). 1 mg dry wt contained < 10 pmol of protein, as did an equivalent amount of rabbit liver glycogen, used as a control. The radiolabeled material was therefore consistent with glycogen, and it was possible that the glucose oligomers seen in PE-STB hydazinolysates were breakdown products of α-amylase activity, either in, or contaminating, the STB preparations. This possibility was examined further.

When a pool of PE-STB, extracted with acetone, was washed with distilled water, the supernatant lyophylized, and then prepared for sugar analysis, without dialysis or hydrazinolysis, it contained 26 times more sugar than control STB, measured by reduction with tritiated borohydride. The ability to reduce oligosaccharides without prior hydrazinolysis shows
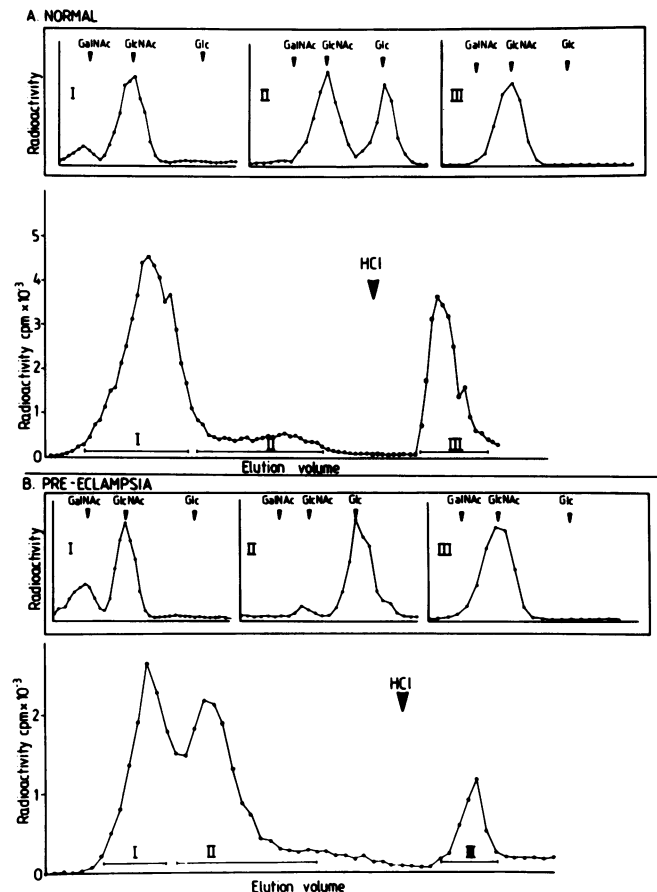
that they are not bound to protein. The radiolabeled oligomers had hydrodynamic volumes as assessed by Bio-Gel P4 chromatography (Fig. 6 *B*), similar to those of the glucose oligomers found in the hydrazinolyzed preparations of PE-STB extracted with acetone (Fig. 1 *B*), and to α-amylase degradation products of radiolabeled glycogen (Fig. 6 *A, insert*), suggesting that they could be the same structures.

Experiments were then done to quantitate glycogen metabolism in PE-STB.

Glycogen content of PE and control placental tissue. The chorionic villi from 8 of the 10 pre-eclamptic women contained significantly more glycogen per gram wet weight than that of controls matched for gestational age (Fig. 7 *A*). The difference was greater when the STB microvesicles of 10 PE (223±117 μg glycogen/mg protein) were compared with the microvesicles of 10 controls matched for gestational age (23±18 μg glycogen/mg protein) ($P < 0.01$). (Fig. 7 *B*).

The localization of glycogen in chorionic villi of pre-eclampsia and control cases was then studied using electron microscopy.

Distribution of glycogen in chorionic villi as determined by electron microscopy. Control chorionic villi did not stain for glycogen using the PATO technique (Fig. 8 *A*). No glycogen
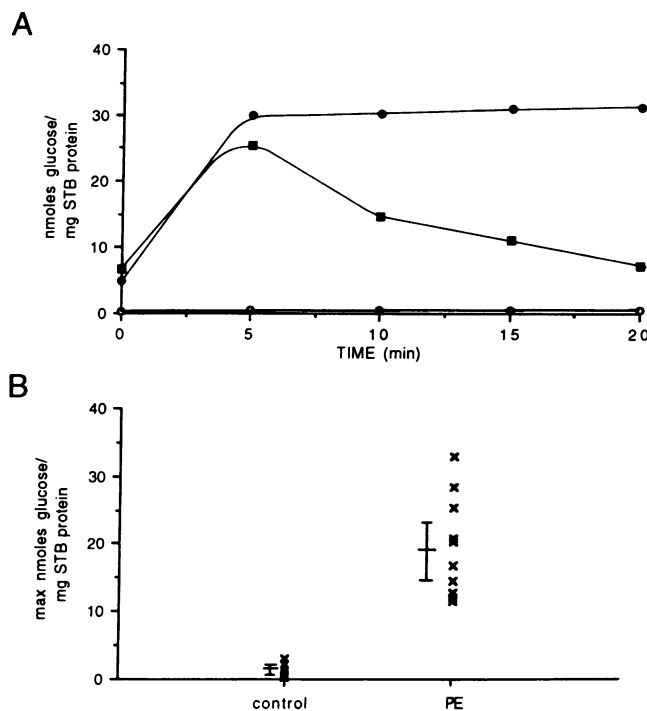
A

B

Figure 5. Glycogen synthase substrate (nmol glucose incorporated/ mg STB protein) in PE and control STB microvesicles. (*A*) Time course of glucose incorporation into STB pooled from 10 placentae: C-STB (○); PE-STB with (●) or without (■) α-amylase inhibitors (mean of duplicates). (*B*) Maximum glucose incorporation into control and PE-STB (mean±SEM; $P < 0.001$ for difference of means). EDTA and α-amylase inhibitor added.

A

B

Figure 6. (*A*) Bio-Gel P4 chromatographs of concanavalin A–bound material from pooled PE-STB radiolabeled using glycogen synthase and tritiated UDP-glucose in the presence of EDTA and α-amylase inhibitor. The insert is after treatment with PE-STB extracted with acetone for 30 min at 37°C without added inhibitors. (*B*) Bio-Gel P4 chromatograph of the supernatant from pooled PE-STB extracted with acetone, radiolabeled by reduction without prior dialysis or hydrazinolysis. Individual oligosaccharide peaks are identified in terms of their size in glucose units (superscripts). VVo, void volume.

was evident in the syncytiotrophoblast of unstained PE chorionic villi (Fig. 8 *B*), but there were other pathological features of pre-eclampsia, in particular, dilated rough endoplasmic reticulum and large juxtanuclear vacuoles. There were also large vacuoles in the underlying cytotrophoblast and fetal capillary endothelium, as well as focal loss of syncytial microvilli. When PE chorionic villi were stained for glycogen using the PATO technique, fine black granules of glycogen were found throughout the cytoplasm of both the superficial syncytiotrophoblast, and the underlying cytotrophoblast (Fig. 8, *C* and *D*). There were also fine particles of glycogen within the cytoplasm of the fetal capillary endothelium.

To explain the increase in glycogen content in the PE-STB, the relative activities of glycogen synthase and glycogen phosphorylase were investigated.

STB membrane glycogen synthase and phosphorylase activity in pre-eclampsia and matched control preparations. The activity of glycogen synthase was 16-fold higher in the microvesicles from cases with PE (1,323±1,013 pmol glucose/mg protein per min) compared to control preparations (83±96 pmol glucose/mg protein per min) ($P < 0.001$). The more marked biochemical changes were found in pre-eclamptic patients with more severe maternal, but not fetal, disease. Despite the small sample size, total synthase activity of patients with one or more feature of the HELLP syndrome (1,931±1,041 pmol/mg per min), was significantly higher than patients with no features of the syndrome (917±839 pmol/mg per min) (Mann-Whitney rank sum nonparametric statistic, $P < 0.05$). Pre-eclamptic patients with intrauterine growth retardation (fetal growth
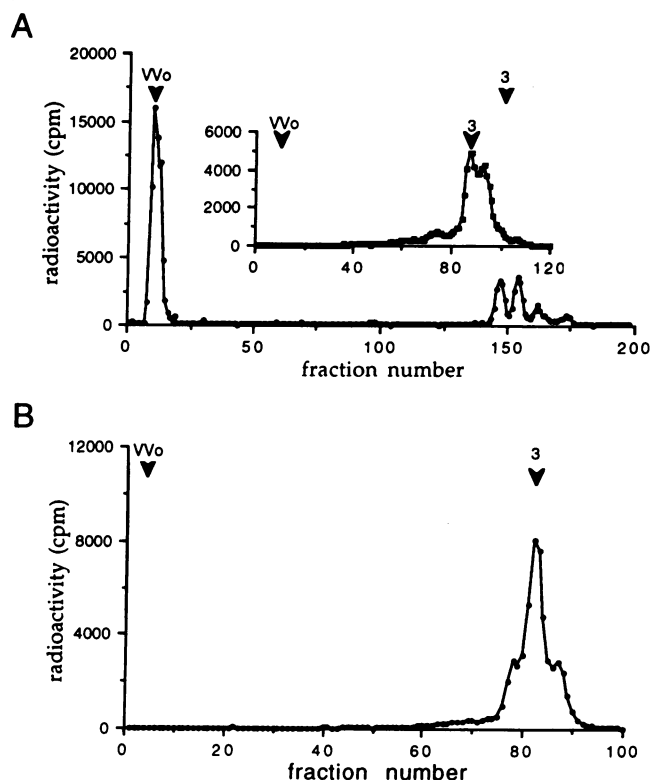
< third centile for gestational age), but not necessarily the HELLP syndrome, had the same glycogen synthase activity (1,337±1,370 pmol/mg per min; $n = 4$) as patients without intrauterine growth retardation (1,313±848 pmol/mg per min).

The activity of the glucose-6-phosphate independent and dependent forms of glycogen synthase were measured. The cofactor independent form of the enzyme was 17-fold higher in the PE-STB (447±391 pmol/mg per min) than the controls (27±17 pmol/mg per min) ($P < 0.01$) (Fig. 9 *A*). The activity of the co-factor–dependent form of the enzyme was 876 pmol/ mg per min in the PE-STB (66% of total activity), compared with 56 pmol/mg per min in the controls (67% of total activity [23-fold higher]). Thus the increase in activity of the two forms of the glycogen synthase enzyme occurred to an equal extent in pre-eclampsia.

Glycogen phosphorylase activity in the STB was also significantly increased in pre-eclampsia, but to a smaller extent. Total glycogen phosphorylase activity (in the presence of 5-AMP) was only threefold higher in pre-eclampsia (2,280±1,360 pmol glucose/mg protein per min) when compared with matched controls (700±540 pmol glucose/mg protein per min) ($P < 0.005$) (Fig. 9 *B, left panel*). The form of the enzyme independent of 5-AMP was only slightly higher in pre-eclampsia (Fig. 9
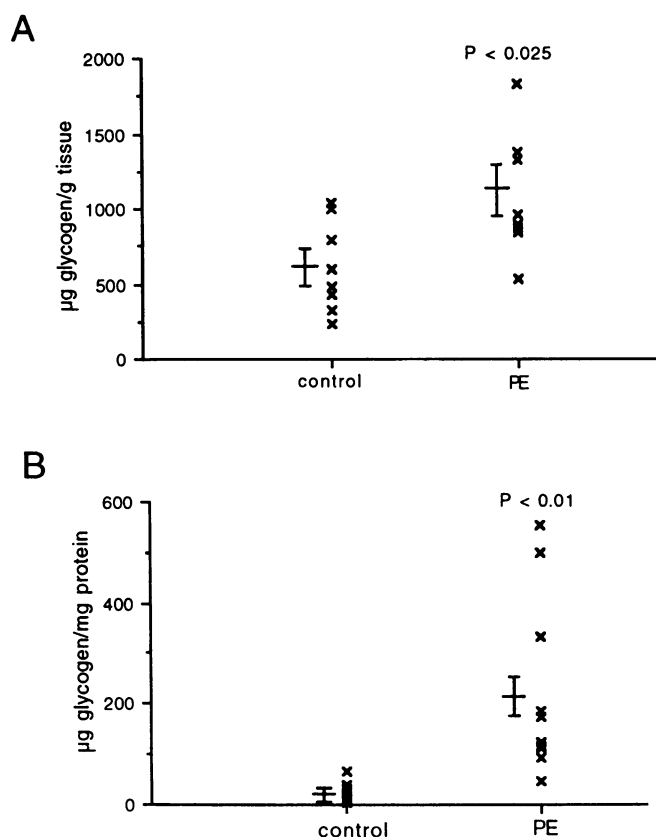
A

B

Figure 7. Glycogen content of (*A*) chorionic villi (8 pairs), and (*B*) syncytiotrophoblast microvesicles (10 pairs) from PE and matched controls. (Means±SEM).

B, right panel). Thus the increase in glycogen phosphorylase activity in PE-STB was largely due to the 5-AMP dependent form.

The changes in pre-eclampsia were then compared with those in maternal diabetes and hydatidiform moles, where alterations in glycogen metabolism had been previously documented (8–10).

Comparison of trophoblast glycogen metabolism in pre-eclampsia, diabetes, and hydatidiform moles. STB microvesicles from women with diabetes contained six times more glycogen (134 ± 114 μg glycogen/mg protein) ($P < 0.01$), and microvesicles from women with hydatidiform moles contained 24 times more glycogen (545 ± 58 μg glycogen/mg protein) than controls (23 ± 18 μg glycogen/mg protein).

Total glycogen synthase activity, when compared with controls (83 ± 96 pmol/mg per min), was threefold higher in diabetes (240 ± 171 pmol/mg per min) ($P < 0.05$), and 34-fold higher in hydatidiform moles ($2,832\pm189$ pmol/mg per min). Total glycogen phosphorylase activity was sixfold higher in moles ($4,209\pm864$ pmol/mg per min, 86% being co-factor dependent), and threefold higher in diabetes ($2,174\pm1,519$ pmol/mg per min; $P < 0.025$; 93% being co-factor dependent) compared with controls (700 ± 540 pmol/mg per min).

Discussion

We initially set out to analyze the protein-linked oligosaccharides of syncytiotrophoblast membrane glycoproteins in preg-

nancies complicated by pre-eclampsia. The N-linked structures were found to be unaltered, but an unanticipated and outstanding feature of the glycan libraries, obtained by hydrazinolysis of extensively dialyzed, acetone-extracted glycoproteins from the syncytiotrophoblast apical membranes was a significant increase in oligosaccharides with glucose-reducing termini. This was confirmed by the independent chromatographic method of radio gas chromatography.

It is improbable that differences in maternal age, gestational age at delivery, or mode of delivery could account for the observed changes, as the two groups were comparable for all these variables.

Concanavalin A affinity chromatography provided a one-step purification procedure for separating oligosaccharides with reducing terminal glucose from classical N- and O-linked structures (Fig. 4), thus allowing further analysis of these oligomers. They were proved to consist solely of glucose, with at least three different hydrodynamic volumes. The 2.5- and 3.5-glucose unit structures are consistent with maltose ($Glc\alpha4Glc_{ol}$) and maltotriose ($Glc\alpha4Glc\alpha4Glc_{ol}$), respectively. A reduced monosaccharide will elute at the "half" unit (i.e., glucitol, 1.5 glucose units) when compared with dextran ($[Glc\beta4]_nGlc$) hydrolysate. Oligosaccharides with 1,6 linkages have a hydrodynamic volume which is 0.5 glucose unit larger than normal (33). Thus the 3.0 glucose unit structure is consistent with isomaltose, $Glc\alpha6Glc_{ol}$. The fact that the oligomers were retarded by concanavalin A suggests that the nonreducing termini were α- rather than β-linked (34).

The saccharide that anchors N-linked oligosaccharides to asparagine is almost invariably N-acetylglucosamine, and that which anchors O-linked oligosaccharides is usually N-acetylgalactosamine. There are only one or two mammalian glycoproteins where the saccharide is linked by glucose. One is glycogen, where the glucose polymer is linked to a 332-amino acid core protein glycogenin (31, 32). The STB from pre-eclamptic women was a much better substrate for glycogen synthase than control membranes, into which little radioactive glucose could be incorporated. The radiolabeled product consisted solely of glucose, as determined by borate buffer high voltage electrophoresis and radio gas chromatography. No amino acids could be detected by picotag analysis. It was uncharged, as it remained at the origin on pyridine buffer high voltage electrophoresis, and it was a large polymer, as it eluted in the void on Bio-Gel P4 chromatography. The radiolabeled material thus had the biochemical properties of glycogen. Although glycogen is now known to be a glycoprotein rather than a polysaccharide, the protein is a small part of the molecule, making up 0.0025% by mass of rabbit liver glycogen, and 0.37% of rabbit muscle glycogen (32). Thus it is usually undetected unless gram amounts of glycogen are analyzed.

The relationship between PE-STB glycogen and the small glucose oligomers found in the glycan library was then sought. The STB membrane preparation contained α-amylase activity. α-Amylase has previously been reported as a constitutive enzyme of the microsomal fraction of placental homogenates (35). Levels increase until 20 wk gestation, after which time they decrease to a nadir at term. STB associated α-amylase rapidly degraded endogenous glycogen if appropriate inhibitors were not added. When radiolabeled PE-STB glycogen was degraded by the α-amylase contained in acetone extracts of STB microvesicles, the products mimicked those found in the native STB from pre-eclamptic patients. Furthermore, the su-
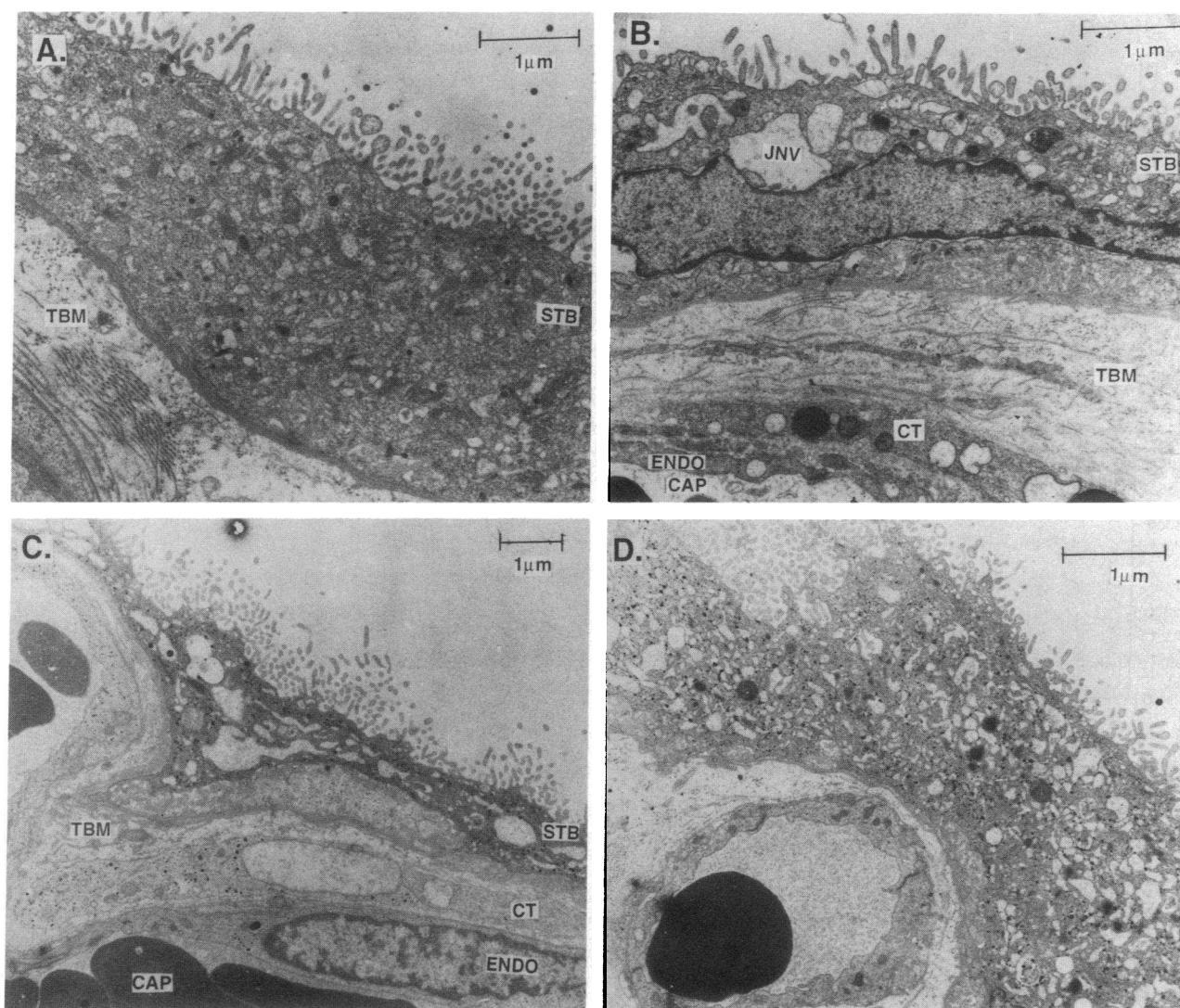
Figure 8. Electron micrographs of chorionic villi from normal and pre-eclamptic women. (*A*) Control chorionic villi stained for glycogen using the PATO method. (*B*) Chorionic villus from a case with PE, unstained. (*C*) and (*D*) Chorionic villi from the same placenta, stained for glycogen using the PATO method. *STB,* syncytiotrophoblast; *CT,* cytotrophoblast; *TBM,* trophoblast basement membrane; *ENDO,* endothelium; *CAP,* capillary lumen; *JNV,* juxtanuclear vacuoles. Magnification: bar = 1 μm.

pernatants from PE-STB but not C-STB when processed in the same way as for protein-linked oligosaccharide analysis, but without dialysis and hydrazinolysis, resulted in large peaks of the same small oligomers (Fig. 6 *B*).

Unbound glucose, or small glucose oligomers associated with, but not covalently attached to, the STB membrane were not initially expected in the preparations, as structures smaller than 8 kD should have been removed by the exhaustive dialysis of the acetone extract before hydrazinolysis. However α-amylase activity associated with the STB was not destroyed by acetone extraction, so that partial degradation of the PE-STB glycogen after dialysis and before hydrazinolysis provides the best explanation for the presence of the glycogen breakdown products.

Thus villous syncytiotrophoblast in pre-eclampsia contains an excess of glycogen when compared with normal. The difference is much clearer if purified preparation are used, rather than tissue homogenates, where there is, as expected, greater overlap between C-STB and PE-STB groups (compare Fig. 7, *A*

and *B*), and differences could thus be missed (12). In most cell types, an increase in glycogen content is associated with cellular immaturity or malignant transformation (36), the major exceptions being differentiated hepatocytes and myocytes, which also express large amounts of glycogen.

Villous trophoblast follows this general pattern, the more immature or malignant the phenotype, the higher the glycogen content. In normal pregnancies, glycogen accumulation is largely confined to the earliest stages of trophoblast development during the first trimester of pregnancy, an observation initially made by Claude Bernard in 1859 (13), and confirmed by others since (11, 14). Molar trophoblast also accumulates excess glycogen (8), but choriocarcinoma cells contain the most (0.4 mg glycogen/mg of protein) (37). A striking feature of this study is the similarity between the findings in PE-STB, and that of hydatidiform moles. The increase in syncytiotrophoblast glycogen in pre-eclampsia thus may indicate a regression to a more immature trophoblast phenotype.

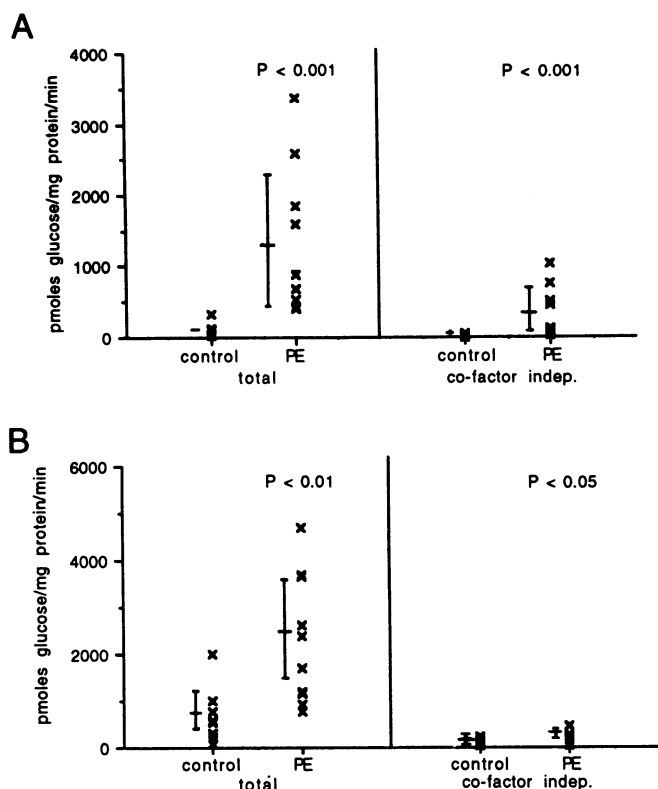Syncytiotrophoblast cells are the differentiated end-prod-

Figure 9. (*A*) Glycogen synthase and (*B*) glycogen phosphorylase activities of syncytiotrophoblast membranes from cases with PE and matched controls (10 pairs). Both total activity (with co-factor [glucose-6-phosphate for synthase, or 5-AMP for phosphorylase]) (*left panels*), and the activity of the independent form of the enzymes (no co-factor added) (*right panels*) are shown. Enzyme activity of each sample is displayed as well as means + SEM.

ucts of proliferating villous cytotrophoblast (3). In pre-eclampsia there is increased villous cytotrophoblast proliferation, the intensity of the changes being related to the severity and duration of the disorder (3, 4). There is also increased loss of villous trophoblast in pre-eclampsia. Chua et al. (38) have recently confirmed that these cells are shed in increased amounts into the maternal circulation (trophoblast deportation). We hypothesize that these two processes, cytotrophoblast proliferation and trophoblast deportation, result in an increased rate of villous trophoblast turnover, a decrease in the average age and level of maturity of these cells, and thus an increase in their glycogen content.

If trophoblast glycogen metabolism were a marker of rate of trophoblast turnover, then it may also correlate with the degree to which deported trophoblast might perturb the maternal circulation. Despite the small sample size and in keeping with this hypothesis, preliminary analysis showed that pre-eclamptic women who have serious maternal sequelae (features of HELLP syndrome), but not necessarily serious fetal sequelae (intrauterine growth retardation), had significantly higher STB glycogen synthase activity than women with less severe disease. However, severe maternal or fetal disease was not associated with significantly more PE-STB glycogen, or higher glycogen phosphorylase activity. Studies with larger sample sizes are necessary to confirm the correlation between severity of maternal disease and the degree to which STB total glycogen

synthase activity exceeds the norm. Finally, investigation of the nature of the stimulus causing the changes in trophoblast glycogen metabolism also warrants further investigation, as it is likely to lead to the elucidation of the underlying cause of the placental pathology in pre-eclampsia.

Acknowledgments

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