Structural Components and Characteristics of Reichert’s Membrane, an Extra-embryonic Basement Membrane*

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This paper presents an analysis of mouse and rat Reichert’s membrane, a thick basement membrane formed between the trophoblast and parietal endoderm cells of early mammalian embryos. When analyzed by polyacrylamide gel electrophoresis, Reichert’s membranes from rat and mouse conceptuses appear simple and consist primarily of collagen and noncollagenous glycoproteins of approximate Mr = 415,000, 245,000, 170,000, and 50,000. The proteins at 415,000 and 245,000 are similar in molecular weight to laminin and are immunoprecipitated by anti-laminin antiserum. The protein at 170,000 is co-precipitated with laminin and represents a novel form of a laminin-like protein, whereas the protein at 50,000 is unrelated to laminin. Using metabolic labeling experiments, it is shown that parietal endoderm cells synthesize these components and incorporate them into the matrix in organ cultures but do not degrade Reichert’s membrane in the conditions used over a 6-day culture period. Evidence is presented that the high molecular weight collagenase-sensitive proteins (greater than 420,000) observed on polyacrylamide gel electrophoresis are due to lysyl oxidase-derived cross-links between Reichert’s membrane components.

The parietal yolk sac is the outermost extra-embryonic membrane of midgestation rodent embryos. It is a three-layered structure composed of trophoblast cells on the uterine side and parietal endoderm cells on the embryonic side, separated by a basement membrane known as Reichert’s membrane (see Ref. 1). This extracellular matrix is a proteinaceous structure containing collagen (2, 3) and lacking proteoglycans (4, 5).

Situated as it is between the yolk sac cavity and the maternal blood sinuses of the trophoblast layer, RM is in an important position to act as a molecular sieve for the passage of macromolecules between the embryo and the mother. Many authors have observed that macromolecules pass from the mother to the embryo after removal of trophoblast cells, SDS, sodium dodecyl sulfate.

The chorioallantoic placenta (6–10). Furthermore, Jollie has proposed that the only barrier to filtration of macromolecules across the parietal yolk sac exists at RM (11). Permeability studies with tracer molecules suggest that RM has a rather large pore size, since γ-globulins (6), ferritin (11), and horseradish peroxidase (8) can all pass across RM. Structural characteristics which affect filtration properties are therefore likely to be important in the nutrition of the embryo.

Apart from its importance in embryogenesis, RM has many properties which make it useful for the study of basement membranes in general. First, it is bounded by cell monolayers on both sides, which makes the membrane easy to isolate by a simple dissection and to prepare in an acellular form. Second, since the parietal endoderm cells which synthesize RM (2, 12) can be maintained in culture for several days and remain biosynthetically active in basement membrane production (13, 14), this system can provide information about both the molecules present and the regulation of their biosynthesis. Third, RM expands as the embryo grows and can therefore be used for studying growth and restructuring of basement membranes during tissue remodeling. Clark et al. (15) have shown that the surface area of the capsular portion of rat RM increases from 100 to 1000 mm² in 6 days of gestation. The thickness also increases until day 15 of gestation when it starts to decrease markedly until the capsular parietal yolk sac ruptures on day 18 (15). The process of rupture itself is another example of tissue remodeling in this system.

RM is also of interest in developmental biology, since the parietal endoderm cells that synthesize this basement membrane differentiate from the inner cell mass very early after the development of the blastocyst (1). Likewise, teratocarcinoma stem cells can differentiate in culture into parietal endoderm-like cells spontaneously or after induction, and RM proteins have been used as a marker for this process (16–21).

In this work, various aspects of RM structure have been studied and characterized. First, the major glycoprotein components of mouse and rat RM are described. These fall into two classes of proteins, collagen and noncollagen glycoproteins. By metabolic labeling of RM, it is shown that the parietal endoderm cells synthesize the basement membrane components but do not degrade them once the basement membrane has been deposited. Finally, the existence of collagenous cross-linked aggregates of basement membrane proteins derived from the action of lysyl oxidase is described.

EXPERIMENTAL PROCEDURES

Materials

Mice used were ICR females from Flow Labs, McLean, VA and either SJL males from Jackson Labs, Bar Harbor, MA or NCR males from Rockefeller University. Females for mating were superovulated (22) using 2 units of pregnant mare serum gonadotropin and 2 units.
of human chorionic gonadotropin. Pregnant rats were purchased from Zivic Miller, Allison Park, PA. Rat PE Tissue X-100, phenylmethylsulfonyl fluoride, ethylenediaminetetraacetic acid, N-ethylmaleimide, α,α′-dipirydyl, iodoacetamide, NaBH₄, glyceral, periodic acid, bovine serum albumin (crystalline and lyophilized), basic Fucsin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, L-proline, trans-4-hydroxy-L-proline, glycine, dimethylamino-n-methane, trichloroacetic acid, putrescine, β-amino propionitrile, uric acid, tannic acid from Sigma; fluorescamine from Roche Diagnostics, Nutley, NJ; sodium dodecyl sulfate from GS-BDH, Carle Place, NY; dithiothreitol from Aldrich; hydrochloric acid, trichloroacetic acid, N-ethylmaleimide, α,α′-dipirydyl, iodoacetamide, NaBH₄, Biochemicals; tissue culture dishes from Falcon; chromatographically separated from the RM by dissection without the use of enzymes. This method removes most of the endoderm cells as judged by spotting a small aliquot on filter paper, drying, and counting in Liquifluor in a Packard scintillation counter 3385. Preparation of Reichert's Membranes with and without Parietal Endoderm Cells—Conceputuses of mice 12 days in gestation (the day of the seminal plug is designated day 1) or of rats 14 days in gestation (positive vaginal smear is day 1) were dissected in sterile phosphate-buffered saline (23) to obtain the parietal yolk sacs. Tropheoblasts were separated from the RM by dissection without the use of enzymes. RM with parietal endoderm attached is referred to as PE + RM. To obtain acellular RM, the PE + RM were washed in 2 ml of 0.1% Triton X-100 in water for 15 min in a 35-mm Petri dish, and then transferred with forceps to 1.5 M EDTA for 15 min. The following were added to all washes as protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 2.5 mM N-ethylmaleimide, 3.0 mM EDTA, and 0.3 mM α,α′-dipirydyl. The samples were shaken on a rotator for 15 min and washed several times during the procedure using a Pasteur pipette. This method removes most of the endoderm cells as judged by visual inspection. Although molecules extracted in the wash solutions were not analyzed, RM components exhibited a limited extractability in Triton, since RM metabolically labeled with [35S]methionine was not dissolved directly in 2.5% SDS, 0.1 M dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride (sample buffer). For collagenase digestions of RM shown in Figs. 1 and 2 (see following for a description of the collagenase), acellular RM was incubated with 8.5 µg/ml of purified bacterial collagenase, 2.5 M N-ethylmaleimide, 0.5 M NaOH with 50 µg/ml of phenylmethylsulfonyl fluoride, 0.05 M NaPO₄ buffer, pH 7.4, before the addition of solubilizing agents. Digestion was at 37 °C for 7 h.

Biosynthetically Labeled RM and Culture Methods—PE + RM were cultured in Dulbecco's modified Eagle's Medium supplemented with 5% dialyzed fetal bovine serum, 50 µg/ml of glycine, and 50 µg/ml of ascorbic acid. In the latter case DME minus methionine contained 40 µg/ml of proline was used. For studies on RM stability after deposition, PE + RM were incubated in 1 ml of DME containing 0.5 mg/ml of bovine serum albumin, 50 µg/ml of glycine, 50 µg/ml of ascorbic acid, and 10 µCi of [14C]putrescine for 12 h at 37 °C. RM were then washed in phosphate-buffered saline (23) containing 100 µg/ml of unlabeled putrescine to remove nonspecifically bound material. Membranes were frozen, thawed, and washed to remove parietal endoderm as described except that 100 µg/ml of putrescine were present in all washes. The sample was suspended in 200 µl of 0.05 M NaPO₄, pH 7.4, and treated with 4 pmol of NaBH₄ at room temperature for 1 h. RM were washed by centrifugation and resuspension, and then dissolved in sample buffer. Aliquots were removed for determination of protein content and of 14C-protein precipitable in trichloroacetic acid. For studies of β-aminoproprionitrile, 5 mouse PE + RM were incubated in each of two 16-mm cell culture wells containing 500 µl of DME minus methionine supplemented with 50 µg/ml of glycine, 50 µg/ml of ascorbic acid, 40 µg/ml of proline, and 10 µCi/ml of [35S]methionine; 5 µg of β-aminoproprionitrile was added to each well. Cells were incubated for 5 h at 37 °C before preparation of RM for gel analysis.

For studies on RM stability after deposition, PE + RM were dissected in sterile Simms balanced salt solution (25) and cultured in 15-mm wells (four 11-day mouse or three 13-day rat RM/well) in 150 µl of DME:Simms balanced salt solution/fetal bovine serum (2:2:1) with 5 µCi/ml of [14C]proline and 50 µg/ml of ascorbic acid. Cultures were labeled for 24 h, then washed for 1 h in DME:Simms balanced salt solution as above but without [14C]proline and with 0.28 mg/ml of proline, and incubated in this medium over the next 5 days with a medium change every 24 h. After the 1-h preincubation period and every 24 h thereafter, samples from 2 or 3 wells were removed, washed free of parietal endoderm cells as described above, and the acellular RM processed for the amount of collagen (see following).

PAGE Analysis—Gradient slab gels were made according to the methods described by Laemmli (26) (35-10%), and for molecular weight determinations gels prepared by the method of Neville (27) (4-10%); gels were also used. Electrophoresis was performed at 8 mA constant current overnight or 25 mA for 4.5 h. The gels were stained for protein with 0.25% Coomassie brilliant blue in an aqueous solution of 40% methanol/8% acetic acid and for glycoproteins with periodic acid-Schiff stain (28). Gels containing radioactively labeled samples were analyzed by autoradiography and visualized using the x-ray film (29). The gel in Fig. 4 was analyzed by fluorography at ~80 °C after impregnating the gel with En'Hance. The lengths of exposures of films are given in the figure legends. The gels in Figs. 3 and 4 were also analyzed using a position-sensitive β-detector (30) equipped with an Ophelion model 6220 pulse height analyzer.
The soluble extract was treated with collagenase at 37°C for 6 h. The soluble material was then precipitated in trichloroacetic acid to remove non-collagenous proteins. The precipitate was analyzed by two-dimensional thin layer chromatography on polyamide sheets (4 cm) exactly as described by Woods and Wang (55). Dansylated amino acids were detected by ultraviolet irradiation, and spots corresponding to the standards were cut out of the polyamide sheets, washed, dried, and counted in a toluene-based scintillation mixture in a Packard 3383 scintillation counter; results are expressed as the percentage of total radioactivity co-migrating with proline and hydroxypoline for 24 h, incubated + or - collagenase, and precipitated in trichloroacetic acid according to the procedures described for determining the fraction of collagen in RM (Experimental Procedures). The trichloroacetic acid-insoluble material was washed three times with ether and hydrolyzed in 6 N HCl in vacuo at 105°C. Amino acids were dansylated and analyzed by two-dimensional thin layer chromatography on polyamide sheets (4 cm) exactly as described by Woods and Wang (55). Positions of migration of proline and hydroxypoline were determined using dansylated [14C]proline (5 x 10^3 cpm/pg) and 4-hydroxy-[14C]proline (10^6 cpm/pg) standards. Dansylated amino acids (500-3000 cpm) from hydrolyzed [14C]RM were run on the polyamide sheets with 0.02 µg of dansylated [14C]proline for 4 days and fed every 24 h with [14C]proline as described under "Experimental Procedures" for the studies on RM stability. Acellular RM were then prepared and incubated in the absence or presence of collagenase for the times indicated, and then assayed for trichloroacetic acid-insoluble [14C]-protein, with reaction conditions exactly as described under "Experimental Procedures." The addition of fresh collagenase after 36 h did not affect the percentage of [14C]-protein solubilized.

Eighteen mouse RM were biosynthetically labeled with [14C]proline for 24 h, incubated + or - collagenase, and precipitated in trichloroacetic acid according to the procedures described for determining the fraction of collagen in RM (Experimental Procedures). The trichloroacetic acid-insoluble material was washed three times with ether and hydrolyzed in 6 N HCl in vacuo at 105°C. Amino acids were dansylated and analyzed by two-dimensional thin layer chromatography on polyamide sheets (4 cm) exactly as described by Woods and Wang (55). Positions of migration of proline and hydroxypoline were determined using dansylated [14C]proline (5 x 10^3 cpm/pg) and 4-hydroxy-[14C]proline (10^6 cpm/pg) standards. Dansylated amino acids (500-3000 cpm) from hydrolyzed [14C]RM were run on the polyamide sheets with 0.02 µg of dansylated proline and 0.04 µg of dansylated 4-hydroxyproline standards. Dansylated amino acids were detected by ultraviolet irradiation, and spots corresponding to the standards were cut out of the polyamide sheets, dried, and counted in a toluene-based scintillation mixture in a Packard 3383 scintillation counter; results are expressed as the percentage of total radioactivity co-migrating with proline and hydroxypoline standards. The rest of the sheet was also counted but contained no cpm above background (10-25 cpm).

**Table II**

**Stability of labeled RM in organ culture**

<table>
<thead>
<tr>
<th>Days of chase</th>
<th>Collagen ± S.E.</th>
<th>Cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mg</td>
<td>%</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>23.8 ± 0.8 (2)*</td>
<td>1250 ± 230 (3)*</td>
</tr>
<tr>
<td>1</td>
<td>23.5 ± 0.4 (2)</td>
<td>1050 ± 140 (3)</td>
</tr>
<tr>
<td>2</td>
<td>26.8 ± 0.0 (2)</td>
<td>1350 ± 140 (3)</td>
</tr>
<tr>
<td>3</td>
<td>26.0 ± 3.0 (2)</td>
<td>1015 ± 315 (2)</td>
</tr>
<tr>
<td>4</td>
<td>25.2 ± 2.6 (2)</td>
<td>960 ± 435 (3)</td>
</tr>
<tr>
<td>5</td>
<td>25.1 ± 0.9 (2)</td>
<td>1250 ± 15 (2)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26.5 ± 2.0 (2)*</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>27.7 ± 0.7 (3)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.0 ± 1.5 (2)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>27.8 ± 1.0 (2)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>28.2 ± 0.6 (2)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent the number of samples used in the determination.
and washed free of cells as described. RM were suspended in 1 ml of 0.2 M NaPO₄, pH 7.4, and treated with 630 µg of [³H]NaBH₄ dissolved in dimethylformamide (previously dried over sodium sulfate) at 3 mg/ml. The [³H]NaBH₄ was added in 3 equal aliquots 3 min apart, with constant stirring. The solution was then adjusted to pH 3 with 10% acetic acid to terminate the reaction. The sample was rinsed exhaustively in 0.2 M NaPO₄, pH 7.4, and then twice in H₂O before being lyophilized. Dried RM were hydrolyzed in 3 N toluene sulfonic acid for 24 h at 100°C. The sample was then filtered through a sintered glass filter and applied to the amino acid analyzer developed by the method of Mechanic (38) for the separation of lysyl oxidase-derived cross-links. The effluent was monitored for radioactivity and the elution times of radioactive products were compared to those of known standards (38).

RESULTS

Protein Components of RM—The protein components of RM have been analyzed by a variety of methods. Initially, RM were dissected from embryos and studied directly by PAGE; Fig. 1 shows the proteins from rat and mouse RM soluble in denaturing and reducing conditions. Each sample was incubated in the absence (−) or presence (+) of collagenase to distinguish collagen from other proteins. The collagenase-insensitive molecules have Mₑ = approximately 410,000, 240,000, 165,000, and 50,000 in mouse and 420,000, 250,000, 170,000, and 50,000 in rat RM. There are two major collagenase-sensitive proteins, at 220,000 and 370,000. The collagen at 220,000 is resolved in various preparations as either a doublet or a triplet.

PE + RM were also cultured in the presence of radioactive amino acids, and the metabolically labeled proteins of the acellular RM were subsequently analyzed by PAGE and autoradiography (Fig. 2). The pattern of major proteins generated (lanes 1 and 3) is seemingly more complex than that shown in Fig. 1; however, after collagenase treatment, the complex array of high molecular weight proteins (greater than 420,000) disappears, resulting in four principal collagenase-insensitive molecules designated A, B, C, and D (lanes 2 and 4), which correspond in molecular weight to those in Fig. 1. Collagenase-sensitive proteins are marked by arrows in Fig. 2 and also correspond in molecular weight in some instances to the collagen in unlabeled RM. Evidence is presented below that the high molecular weight, collagenase-sensitive proteins result from lysyl oxidase-derived cross-links. Hence, the major glycoproteins present in the basement membrane are all synthesized and deposited in the RM by cultured parietal endoderm cells, consistent with the reports that these cells synthesize RM (2, 12). In some cases there is also a diffuse protein migrating above glycoprotein D (see Fig. 2B). It is not considered a major component of RM because it is present in low amounts and is not detected in all preparations; it may represent a degradation product or cellular contamination. The intensities of bands below 50,000 are also variable.

Recently, the isolation and characterization of two high molecular weight proteins from basement membranes have been reported (42, 43). These proteins, called GP1 and 2 (42) and laminin (43), migrate with the same approximate molecular weights as glycoproteins A and B described previously. We performed immunoprecipitation of mouse RM with antilaminin antiserum to determine the immunological cross-reactivity of RM proteins with laminin. RM was first extracted with 0.5 M NaCl to solubilize the glycoproteins without denaturation or reduction, since the antiserum does not precipitate laminin which is reduced (43). The extract was treated with collagenase and immunoprecipitated with sheep antilaminin antiserum as described under "Experimental Procedures." Thirty-one µg (rat) and 40 µg (mouse) of protein were loaded/lane. Symbols under the lanes indicate incubation with (+) or without (−) collagenase for 7 h at 37°C. Gels A and B are Coomassie blue-stained, C is periodic acid-Schiff-stained to indicate glycoproteins. The minor Coomassie blue-stained band in A (+) and B (+) at Mₑ = 100,000 is due to the added collagenase. The molecular weight markers are, from high to low molecular weight, thyroglobulin, ferritin half-subunit, phosphorylase b, albumin, catalase, ovalbumin, and lactate dehydrogenase.

Studies on RM Stability in the Presence of Endoderm Cells—In light of the fact that parietal endoderm cells synthesize RM, and in order to investigate the processes by which RM expands and eventually ruptures, we were interested in studying the fate of the newly synthesized RM components. Of additional interest in this regard is the fact that in primary culture (44) and in organ culture,3 parietal endoderm cells secrete a protease, plasminogen activator, which is implicated in extracellular proteolysis in other systems (45). We have therefore performed pulse-chase experiments to determine whether RM is degraded following its deposition by parietal endoderm cells. PE + RM organ cultures were incubated for 24 h in the presence of [¹⁴C]proline to label RM and then cultured for 1 to 5 days in a medium without [¹⁴C]proline, with a medium change every 24 h (see "Experimental Procedures"). PE + RM were removed from culture throughout the chase and acellular RM were prepared and analyzed for the fraction of collagen present. In this study, the amount of collagen was determined using a collagenase assay which measures the amount of ¹⁴C-peptides released by bacterial collagenase from [¹⁴C]proline-labeled RM (36). The data in Table I and "Experimental Procedures" show that there is no contaminating proteolytic activity in the collagenase reaction.
and that the reaction goes to completion after 24 h of incubation with \( ^{14}\text{C}-\text{RM} \).

Our studies into the stability of RM components are similar to turnover experiments by Minor et al. (14) with the following modifications. One, measurement of the percentage of collagen in RM was performed on acellular RM rather than PE + RM to ensure that the measurements reflected events in the RM rather than the cells. Two, the culture method differed from that of Minor et al. in that the PE + RM was floated in medium as a monolayer rather than grown on agar as an organ culture.

The results in Table II indicate that the percentage of collagen remained constant in rat and mouse RM and, furthermore, there was no significant turnover of protein in medium as a monolayer rather than grown on agar as an organ culture.

The experiments reported in Table II were performed on acellular RM prepared from PE + RM at the end of each chase period and therefore should reflect the fate of proteins laid down in RM following the 24-h labeling period. Our results indicate that this RM was stable in the presence of metabolically active parietal endoderm cells in the conditions of culture used. If degradation does exist as a mechanism for RM growth and restructuring, it may depend on the presence of other components of the embryo such as the trophectoderm.

Cross-links—The presence of a complex array of very high molecular weight (greater than 420,000) collagenase-sensitive proteins (see Fig. 2) suggested that the RM proteins were being covalently cross-linked after synthesis. There are at least two mechanisms by which this could occur. One is that active transglutaminase (Factor XIIIa) was present and was diffusing into the RM. However, transglutaminase from the fetal bovine serum in the culture medium was not responsible for the presence of the high molecular weight proteins since culture in the absence of serum resulted in the same protein profile (data not shown). The other possibility is that lysyl oxidase was present. Lysyl oxidase is found in connective tissue and is involved in collagen cross-linking (for a review see Ref. 46). This enzyme catalyzes the oxidative deamination of the \( \varepsilon \)-amino group of lysine yielding an aldehyde which is then available for reaction with amines (Schiff base formation) or with other aldehydes (aldol condensation).

Since both transglutaminase and lysyl oxidase could catalyze amine incorporation into RM, we incubated \( ^{14}\text{C} \)-putres-

![Fig. 2. Polyacrylamide gel electrophoretic analysis of biosynthetically labeled Reichert's membrane.](image)
Structural Components and Characteristics of Reichert's Membrane

Fig. 4 (left). Incorporation of \(^{14}\)C]putrescine into Reichert's membrane. Mouse PE + RM were incubated with \(^{14}\)C]putrescine as described under “Experimental Procedures.” Lane A is 1000 cpm of \(^{14}\)C]putrescine-labeled RM. Lane B is 3000 cpm of RM biosynthetically labeled with \(^{14}\)C]proline as in Fig. 2. The heavily labeled doublet is the 220,000-collagen doublet. The labeling pattern in lane B reflects the preferential uptake of proline into collagen and does not reflect the relative amounts of RM proteins present (compare the patterns of Fig. 1 with Fig. 2). Lane A was exposed for 1 month and lane B for 10 days.

Fig. 5 (right). The effects of \(\beta\)-aminoproprionitrile on the gel profile of \(^{35}\)S]methionine-labeled Reichert's membrane. RM + PE were incubated in serum-free conditions for 5 h in the presence or absence of \(\beta\)-aminoproprionitrile (\(\beta\)-APN) (10 \(\mu\)g/ml) and prepared for PAGE analysis as described under “Experimental Procedures.” A total of 10,000 trichloroacetic acid-precipitable cpm of protein was applied to each lane; the autoradiogram was exposed for 5 days. To quantitate the amounts of radioactivity in various regions of the gel, lanes A and B were analyzed on a \(\beta\)-sensitive detector (30) until approximately 30,000 \(\beta\) particles had been recorded and localized from each lane. The total number of events (\(\beta\) particles) in the peaks in regions of interest were determined by summing the events in the pulse height analyzer channels spanning the peaks. The values for each region of the two lanes are expressed as a ratio (A/B) to the left of the autoradiogram. The peak area was corrected for the amounts of radioactive background in nearby regions of the gel by subtracting the background from the number of events in each channel. There were two protein bands which were sensitive to the presence of \(\beta\)-aminoproprionitrile (marked by arrows to the right of the autoradiogram), whereas glycoproteins A and C had ratios of 1.0 and 0.96, respectively, and served as internal standards.

Fig. 6. Radioactive elution profile after cation exchange chromatography of \(p\)-toluenesulfonic acid hydrolysate of \([\text{H}]\text{NaBH}_4\)-treated rat RM. The \(\text{H}\)-hydrolysate was chromatographed on column 1 as described by Mechanic (38). The identity of hydroxyproline was established by its elution with dentin hydroxyproline on column 1. On rechromatography, the radioactive peak in fractions 150-160 eluted with the acid-hydrolysis breakdown product of the aldol. The peaks in fractions 250-300 elute in the region of reduced Schiff base cross-links. The presence of hydroxylysyluronic acid was established by rechromatography of a pool of peaks in this region on the basic column designed for resolution of Schiff base cross-links (38); a radioactive peak from rat RM eluted at the same position as hydroxylysyluronic acid from sponge collagen. These analyses were performed by Lila Graham and Dr. G. Mechanic.

cine (1,4-diaminobutane) with PE + RM in the absence of serum for 12 h, treated PE + RM with \(\text{NaBH}_4\) to reduce Schiff bases, and analyzed the RM for \(^{14}\)C]putrescine incorporation. The acellular RM contained a total of 3,900 cpm in 60 \(\mu\)g of protein, or 65 cpm/\(\mu\)g. The results in Fig. 4 of PAGE analysis of the labeled RM indicate that radioactivity was incorporated specifically into collagen. Since the RM was washed at each step in 100 \(\mu\)g/ml of unlabeled putrescine, and since the \(^{14}\)C]putrescine co-migrates with only collagen in the presence of SDS, the mobility probably represents covalent incorporation rather than nonspecific adsorption. If the covalent bond is a Schiff base, it could not have formed with the aldohexose of galactose or glucose, the carbohydrates of collagen, because it has been shown that the C-1 of each sugar in basement membrane collagen is involved in a glycosidic linkage (47). The fact that all of the detectable radioactivity was incorporated into collagen supports the possibility that the enzyme responsible was lysyl oxidase since that enzyme is specific for collagen.

We have tested the effect of \(\beta\)-aminoproprionitrile, a well described inhibitor of lysyl oxidase (see Ref. 46), on the gel profile of RM proteins. When PE + RM were incubated with 10 \(\mu\)g/ml of \(\beta\)-aminoproprionitrile for 5 h in the presence of \(^{35}\)S]methionine, the amount of radioactivity in the high molecular weight collagenase-sensitive proteins was decreased (Fig. 5). Thus the presence of an inhibitor of lysyl oxidase affected the fate of newly synthesized collagen to the extent that less was incorporated into the high molecular weight forms.

Rat RM was analyzed chemically to determine the presence of lysyl oxidase-derived aldehydes and aldehyde cross-links. Acellular rat RM treated with \([\text{H}]\text{NaBH}_4\), was prepared as described under “Experimental Procedures” and analyzed for amino acids and derivatives using a column system described by Mechanic (38). The elution profile of radioactive peaks is shown in Fig. 6 and supports the conclusion that RM proteins

\(^{4}\) A similar conclusion has been reported by others in abstract form (48).
were cross-linked by a mechanism involving lysyl oxidase. For example, hydroxynorleucine and Schiff base cross-links were present, and when a pool of peaks in the region of Schiff base cross-links was rechromatographed on a basic column (38), a radioactive peak eluted in the position of hydroxynorleucine. In addition there was radioactive material which co-migrated with the acid-hydrolysis breakdown product of the aldol\(^*\) formed between lysine-derived aldehydes, the identity of which was also confirmed by rechromatography. These derivatives all result from the action of lysyl oxidase on lysine. It cannot be determined from these experiments whether the lysyl oxidase was synthesized by parietal endoderm or was derived from another source.

**DISCUSSION**

The method described here is useful for preparing and solubilizing acellular RM in a form suitable for biochemical characterization. Since the conditions for dissolving the RM samples solubilizes almost all of the visible structure, and since the remaining insoluble pellet contains less than 2% of the RM proteins, it is likely that the proteins analyzed by this method include all the major species of basement membrane proteins. This technique provides the important advantage that no proteolysis is needed for preparation of the components.

The composition of rodent RM is very simple, consisting after reduction of two major and several minor collagen molecules and four noncollagen glycoproteins (Figs. 1 and 2). Parietal endoderm cells synthesize (Refs. 2 and 12; Fig. 2) but do not degrade (Table I) RM components in the culture conditions used. The composition of rat and mouse RM is remarkably similar. The collagen molecules migrate in SDS gels as a doublet or triplet at \(M_\text{s} = 200,000-220,000\), a singlet at 370,000, and several high molecular weight proteins extending up to the top of the gel (Fig. 2). The collagen has not been characterized as type IV in this study, but this has been done by other investigators who have examined both rat (2, 3, 5) and mouse (39, 40) parietal yolk sac.

The high molecular weight RM glycoproteins A, B, and C of \(M_\text{s} = \) approximately 415,000, 245,000, and 165,000 on reduced PAGE are immunoprecipitated by anti-laminin antibodies (Fig. 3). Laminin has been shown by immunofluorescence to exist in skin, glomerulus, capillary, and embryonic basement membranes including RM (42, 43, 49, 50). On the basis of this accumulated evidence, laminin is a general basement membrane protein which is synthesized and deposited into RM by parietal endoderm cells. Our results support the suggestion (51) that RM proteins which co-migrate with laminin are related to it chemically. The synthesis of laminin or a molecule with very similar mobility characteristics has been observed in the culture medium of mouse parietal endoderm primary cultures (19-21, 51), parietal yolk sac carcinoma cells (19), and F9 cells induced to differentiate (20, 21). The results of this study suggest that these soluble extracellular products are destined for incorporation into basement membranes.

The properties of glycoprotein C and D are unknown. Although glycoprotein C is precipitated by anti-laminin antisera (Fig. 3), it is not the same molecular weight as that reported for laminin in the literature (43). Glycoprotein D has a molecular weight very close to those of two of the major structural proteins of the cell, actin and tubulin; however, glycoprotein D does not co-migrate with actin or tubulin on polyacrylamide gels (data not shown). We have also observed that there is a protein in RM which migrates between glycoproteins A and B at the same molecular weight as fibronectin synthesized by F9 teratocarcinoma stem cells.\(^4\) Fibronectin has been detected in mouse RM by immunofluorescent techniques (52, 53). However, the amounts of this protein in biosynthetically labeled RM are variable and usually minor, which is consistent with reports that parietal endoderm cells do not secrete significant amounts of fibronectin in culture (19, 53). Fibronectin in RM may derive from maternal plasma in the sinuses of the trophoblast; it is also possible that it is synthesized by trophoblast cells and its minor presence in biosynthetically labeled RM is due to variable contamination of PE + RM with trophoblast cells. Based on the observations presented here, however, fibronectin is not a major component of RM.

The presence of the very high molecular weight collagenase-sensitive proteins in RM (see arrows in Fig. 2) has been investigated.\(^5\) On the basis of the evidence presented, there exists a mechanism for the generation of covalent bonds between RM proteins which is due to lysyl oxidase. Lysyl oxidase-derived aldehyde cross-links have been reported in other basement membranes (54) and have been postulated to exist in RM (48, 51). The distribution of aldehyde-derived cross-links in RM is unusual in the relative abundance of aldehyde precursors and aldehydes compared to Schiff base derivatives (compare for example the profile in Fig. 6 with that of insoluble bovine collagen fibrils in Ref. 38). The significance of this difference is unknown, although it may reflect differences in the conditions governing cross-link formation in basement membranes and in interstitial collagen. In any case, it is likely that lysyl oxidase-derived aldehyde cross-links are important in stabilizing the insoluble matrix structure; they may also affect the pore size characteristics of RM.

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\(^*\) Aldol is the aldol condensation product of two residues of peptidyl-\(\alpha\)-amino-adipoyl-6'-semialdehyde to form the interchain, intramolecular cross-link.

\(^4\) Unpublished observations.

\(^5\) The large number and the molecular weight range of very high molecular weight (> 420,000) collagenase-sensitive proteins suggests that cross-links may exist between collagen and other RM components. Although this has not been studied in detail, the amount of glycoproteins A and B appears to increase after collagenase digestion, suggesting their release from high molecular weight forms complexed with collagen.
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