The Embryonic Rat Parietal Yolk Sac

THE ROLE OF THE PARIETAL ENDODERM IN THE BIOSYNTHESIS OF BASEMENT MEMBRANE COLLAGEN AND GLYCOPROTEIN IN VITRO*

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Basement membrane biosynthesis in vitro was studied in a rapidly growing embryonic tissue, the rat parietal yolk sac. This tissue consists of a thick, nonvascular basement membrane (Reichert’s membrane) separating two cellular layers (parietal endoderm and trophoblast). Morphologically, Reichert’s membrane appeared similar to other basement membranes. Previous analysis of the amino acid and carbohydrate composition of acellular Reichert’s membrane showed it to be typical of basement membranes isolated from other tissues and species.

Analysis of [14C]proline incorporation and hydroxy[14C]proline synthesis during the third quarter of gestation in vitro showed that basement membrane collagen synthesis in the parietal yolk sac was maximal around the 14th day of gestation. At this time, basement membrane collagen represented nearly 10% of the newly synthesized protein. The collagen synthesized in this system was characteristic of basement membrane collagen in that about 11% of the total hydroxy[14C]proline was present as the 3-isomer. In addition, after incubation in the presence of [14C]lysine, 83 to 94% of the hydroxy[14C]lysine was glycosylated, with the predominant form being glucosylgalactosylhydroxy[14C]lysine.

When the parietal endoderm and trophoblast were incubated separately with [15N]proline, it was determined that the former was solely responsible for the synthesis of basement membrane collagen since essentially all of the 4-hydroxy[14C]proline was associated with this cell type. Autoradiographic experiments with [3H]glucosamine also served to localize the synthesis of noncollagen basement membrane glycoprotein components to the parietal endoderm.

As with the results reported for basement membrane collagen secretion in embryonic chick lens cells, there appeared to be a 60-min delay between the incorporation of [14C]proline into protein and the secretion of collagen as measured by the appearance of 4-hydroxy[14C]proline in the culture medium. Experiments utilizing [3H]glucosamine to monitor glycoprotein synthesis did not show a delay between the incorporation of [3H]glucosamine and the secretion of nondialyzable 3H into the medium.

The results obtained using the parietal yolk sac system to study basement membrane biosynthesis were compared to those previously obtained using the kidney glomerular and embryonic chick lens systems. It was concluded that the parietal yolk sac system is superior for a number of reasons: (a) the extracellular matrix appeared to contain only basement membrane components; there was no contamination by acid mucopolysaccharides or other types of collagen; (b) only a single cell type appeared to be responsible for the synthesis of basement membrane components; and (c) a relatively large percentage of the newly synthesized protein was basement membrane collagen.

Basement membranes are ubiquitous extracellular matrices which are usually contiguous with an epithelial or endothelial cell layer, or both (3). Such cells are thought to be responsible for the elaboration of the basement membrane upon which they rest (4–7). All of the basement membranes examined thus far possess a characteristic amino acid and carbohydrate composition and are comprised of a collagen component in association with one or more noncollagen glycoprotein components. The relative proportions of these protein components...
vary with each type of basement membrane (for review, see Ref. 8).

Because changes in the morphology and function of basement membranes have been observed in a number of disease states, the basement membranes of the lens capsule of the eye and the glomerulus of the kidney have been the object of a number of physical, chemical, and immunological studies (8). Recently, we have begun investigating the chemistry and morphology of the basement membranes of the embryonic rat yolk sac because of their role in embryogenesis (9) and their implication in the production of congenital malformation, fetal growth retardation and embryonic death (10).

The parietal yolk sac of the rodent consists of two cellular layers (parietal endoderm and trophoblast) separated by a relatively thick nonvascular basement membrane (Reichert’s membrane). The histological and morphological characteristics of the rodent parietal yolk sac have been known for some time (4, 6, 11–16), but only recently have chemical analyses shown that the amino acid and carbohydrate composition of rat Reichert’s membrane is typical of basement membranes isolated from other tissues and species.1

A number of laboratories recently have begun to examine the biosynthesis of basement membranes in vitro. Because the basement membranes of the kidney glomerulus and lens capsule of the eye have been the best characterized, initial biosynthetic studies were performed using these systems (17–21). However, since the protein content of Reichert’s membrane continually increased during gestation (13, 14), it was suggested that the parietal yolk sac may be an active in vitro protein biosynthetic system. In this investigation, we therefore examined the efficacy of the parietal yolk sac system for studying the biosynthesis of basement membrane components in vitro and compared it to published reports for lens capsule and glomerular systems.

EXPERIMENTAL PROCEDURE

Materials—Pregnant Wistar rats (150 to 200 g) were used for all experiments. L-[U-14C]Proline (200 mCi/mmol), L-[U-14C]lysine (260 mCi/mmol), D-[5-3H]glucosamine (5 to 15 Ci/mmol), and L-[3,4,6-3H]proline (90 to 50 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.); trypsin solution (250 units/ml) of bovine origin was purchased from Sigma Chemical Co. (St. Louis, Mo.); and collagenase (CLSPA) was purchased from Worthington Biochemical Corp. (Freehold, N.J.).

Preparation of Parietal Yolk Sac for Biosynthetic Studies—PEMT 1 were dissected and prepared as described elsewhere. Briefly, pregnant rats were killed 12 to 16 days after implantation and uteri removed. After uterine tissue was stripped from the isolated implantation sites, the PEMT was detached and separated from the choroidal placenta by surgical dissection. The resulting tissue consisted of a layer of trophoblast giant cells which in utero are in contact with decidual tissue and overlay Reichert’s membrane (13, 14). In order to separate T and PEM so that both tissues could be studied independently, PEMT were incubated in PBS containing hyaluronic acid (1 mg/ml) for 20 min at 37Ô with agitation. Under these conditions, the loss of PE from M was minimal.

The incubation mixture was transferred to a Petri dish and examined under a binocular dissecting microscope. One edge of the trophoblastic surface was grasped with fine forceps, and, at approximately the same point a free edge of Reichert’s membrane was grasped with a second pair of forceps. With careful pulling, it was possible to cleanly separate T from PEM. These tissues were washed separately in Krebs medium to remove the hyaluronidase and transferred to modified Krebs medium (usually containing 20% diazoyed fetal calf serum) and incubated as described above. The PBS in which the dissection was performed was centrifugated at 600 × g for 10 min to recover any parietal cells which were lost. These then were added to the appropriate PEM incubation sample.

RESULTS

Protein Biosynthesis by Intact Parietal Yolk Sac in Vitro—Previous experiments 1 have shown that in the developing rat parietal yolk sac, both the total protein content and the collagen content of Reichert’s membrane increase linearly between Days 12.5 and 17.5. In order to measure the capability of the parietal yolk sac system to synthesize basement membrane components in vitro, PEMT (12.5 to 14.5 days) or PEM (15.5 to 16.5 days) were incubated with [14C]proline and then assayed for total [14C] incorporated and for total 4-hydroxy[14C] proline synthesized. The ratio of 4-hydroxy[14C]proline to total [14C] was used as an index of basement membrane collagen biosynthesis. Our studies were restricted to the third quarter of gestation.

The abbreviations used are: PEMT, intact parietal yolk sac consisting of parietal endoderm (PE) plus basement membrane (M) plus trophoblast (T); PBS, phosphate-buffered saline, pH 7.2; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazoyl)-5-phenyloxazoyl]-benzene]
gestation since at earlier stages, uterine decidual tissue containing interstitial collagen contaminated preparations of PEMT.  

As shown in Fig. 1, the ratio of labeled 4-hydroxyproline to total \(^{14}\)C varied markedly between the 12th and 16th days of gestation, but was constant from litter to litter with embryos of a particular age group. The maximum ratio appeared to be attained between Days 13.5 and 14.5. A pattern qualitatively similar to that presented in Fig. 1 also was obtained when the content of 4-hydroxy\([^{14}\text{C}]\)proline was expressed either on the basis of endodermal cell number or endodermal cell DNA (data not presented). The inset to Fig. 1 shows further that the absolute values of total \(^{14}\)C (as a measure of protein synthesis) and 4-hydroxy\([^{14}\text{C}]\)proline (as a measure of collagen synthesis) reached a maximum during the 14th day. For this reason, 14.5-day tissues were used in subsequent experiments to study basement membrane protein biosynthesis in vitrō.

**Synthesis of 3- and 4-Hydroxy\([^{14}\text{C}]\)proline—PEMT were incubated for 4 hours in modified Krebs medium containing \([^{14}\text{C}]\)proline and were assayed for nondialyzable \([^{14}\text{C}]\)proline, and 3- and 4-hydroxy\([^{14}\text{C}]\)proline. A relatively large portion of the \([^{14}\text{C}]\)proline was incorporated into basement membrane collagen during this time since nearly 20% of the nondialyzable \([^{14}\text{C}]\)proline was recovered as hydroxy\([^{14}\text{C}]\)proline (Table I). Because purified basement membrane collagen contains an average of 70% of its imino acid as 3- plus 4-hydroxyproline (8), the results suggested that 20%/70%, or greater than one-quarter (Table I) of the \([^{14}\text{C}]\)proline incorporated was present in newly synthesized basement membrane collagen.

One characteristic feature of basement membrane collagen which distinguishes it from other types of collagen is its content of 3-hydroxyproline (3). Analysis (Table I) showed that the newly synthesized protein of the parietal yolk sac contained 11% of its hydroxyproline as the \(\alpha\)-isomer. This value was consistent with the amino acid composition of 14.5-day Reichert's membrane (1) as well as with the results reported by Grant et al. (17) for the lens system and Killen et al. (19) and Grant and Harwood (21) for the glomerular system.

**Synthesis of Glycosylhydroxy\([^{14}\text{C}]\)lysine—Another characteristic of basement membrane collagen is its content of glycosylated hydroxylysine (8). PEMT were incubated with \([^{14}\text{C}]\)lysine for periods ranging from 5 min to 4 hours and assayed for nondialyzable unsubstituted and glycosylated hydroxy\([^{14}\text{C}]\)lysine. Analysis (Table II) indicated that 83 to 94% of the hydroxy\([^{14}\text{C}]\)lysine synthesized by this system was glycosylated, and that the majority of the glycosylated hydroxy\([^{14}\text{C}]\)lysine was present as glucosylgalactosylhydroxy\([^{14}\text{C}]\)lysine. Similar values were reported by Grant et al. (17) and Grant and Harwood (21). Furthermore, it appeared that after only a 5-min incubation in the presence of \([^{14}\text{C}]\)lysine,

**Fig. 1.** Incorporation of \([^{14}\text{C}]\)proline into protein and synthesis of nondialyzable 4-hydroxy\([^{14}\text{C}]\)proline by intact parietal yolk sacs in vitrō. Tissues from seven rat embryos of each of the ages indicated were incubated at 37\(^\circ\) for 5 ml of modified Krebs medium containing 5 \(\mu\)Ci of \([^{14}\text{C}]\)proline for 2 ( ), 3 ( ), or 4 ( ) hours. The trophoblast was not removed from 12.5- to 14.5-day yolk sacs before incubation. After the incubation period, incorporation was stopped by addition of 0.4 ml of Krebs medium containing 0.54 mg of cycloheximide and 0.86 mg of \(\alpha,\alpha'-\)dipyridyl, the sample dialyzed against distilled water and assayed for total \([^{14}\text{C}]\) and 4-hydroxy\([^{14}\text{C}]\)proline after hydrolysis. Three separate experiments are represented. Inset, absolute amounts of total \([^{14}\text{C}]\) incorporated ( ) and 4-hydroxy\([^{14}\text{C}]\)proline synthesized ( ) in the 2-hour ( ) experiment.

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**TABLE I**

| Experiment | Total \([^{14}\text{C}]\)proline | 3-Hydroxy\([^{14}\text{C}]\)proline | 4-Hydroxy\([^{14}\text{C}]\)proline | 100 \* 3-hydroxy\([^{14}\text{C}]\)proline/total hydroxy\([^{14}\text{C}]\)proline | 100 \* total hydroxy\([^{14}\text{C}]\)proline | \([^{14}\text{C}]\)proline incorporated into \([^{14}\text{C}]\)BM collagen |
|------------|----------------------|---------------------|---------------------|---------------------------------|---------------------------------|---------------------------------|
|            | dpm x 10\(^3\)      | dpm                 | %                   |                                 |                                 |                                 |
| 1          | 2.41                 | 51,100              | 11.1                | 19.1                            | 27.3                            |
| 2          | 2.33                 | 49,400              | 10.8                | 19.5                            | 27.8                            |
| 3          | 2.51                 | 45,400              | 10.1                | 17.8                            | 25.4                            |
| 4          | 2.31                 | 53,900              | 11.4                | 20.5                            | 29.3                            |

**TABLE II**

Content of glycosylhydroxy\([^{14}\text{C}]\)lysine in protein synthesized by PEMT in vitrō in the presence of \([^{14}\text{C}]\)lysine for varying incubation periods

Five PEMT were incubated in 4 ml of modified Krebs medium containing 20 \(\mu\)Ci of \([^{14}\text{C}]\)lysine for the times indicated. Nondialyzable \([^{14}\text{C}]\)lysine, hydroxy\([^{14}\text{C}]\)lysine, galactosylhydroxy\([^{14}\text{C}]\)lysine, and glucosylgalactosylhydroxy\([^{14}\text{C}]\)lysine were measured on a Beckman amino acid analyzer (22). The data are the results of single experiments.

**Sample**

| Total glycosylated hydroxy\([^{14}\text{C}]\)lysine | Total glucosylgalactosylhydroxy\([^{14}\text{C}]\)lysine |
|---------------------------------|---------------------------------|
| Sample | % | % |
| Tissue |  | | |
| 5 min | 87 | 82 |
| 10 min | 88 | 85 |
| 30 min | 91 | 88 |
| 60 min | 90 | 92 |
| 75 min | 91 | 91 |
| 120 min | 83 | 95 |
| 240 min | 85 | 94 |
| Medium |  | | |
| 180 min | 91 | 97 |
| 240 min | 94 | 97 |
the newly synthesized hydroxy[14C]lysine was nearly completely glycosylated (Table II).

Localization of Basement Membrane Biosynthesis in Vitro—In order to determine the contribution of PE and T to the biosynthesis of Reichert's membrane, PEMT, PEM, and T were isolated as described under "Experimental Procedure." Microscopic examination of these preparations (see Figs. 2 and 4) confirmed their homogeneity. The various tissues then were incubated in the presence of [14C]proline for 4 hours and assayed for total [14C] incorporation and 4-hydroxy[14C]proline synthesis. Analysis (Table III) showed that whereas T contributed approximately one-third of the total [14C] incorporated by PEMT, it contributed less than 1% (0.11/1.88) of the total 4-hydroxy[14C]proline in PEM + T.

A similar experiment was performed in the presence of [3H]glucosamine. Analysis to determine the distribution of label (Table IV) showed that PE and T incorporated approximately equal amounts of total [3H] into glycoprotein. However, a further localization of the synthesis of basement membrane glycoprotein was obtained autoradiographically (Fig. 2). It can be seen that grains are rather evenly distributed over T and are not concentrated along the trophoblastic surface of M. By contrast, there appears to be a concentration of grains over PE, and particularly along the endodermal surface of M. The data in Tables III and IV further show that neither the hyaluronidase treatment nor the subsequent dissection had any deleterious effect on the system (compare PEMT with PEM + T). There does not appear to be a serum requirement for the PEMT system since experiments performed in the presence and absence of serum gave similar results (data not shown). However, experiments in which PEM were incubated in the absence of serum showed a marked loss of nondialyzable [14C]proline and 4-hydroxy[14C]proline relative to either PEMT or PEM + T. The cause of this is currently under investigation.

Delay in the Secretion of Basement Membrane Collagen—The time course of incorporation of [14C]proline into protein and collagen by PEMT in vitro was examined. In four separate experiments, [14C]proline incorporation and 4-hydroxy[14C]proline synthesis by the tissue was linear for several hours (Fig. 3, A and B). Identical to the observations of Grant et al. (17), there appeared to be a delay of at least 60 min before significant nondialyzable [14C]proline or 4-hydroxy[14C]proline was detected in the medium (Fig. 3, A and B). After this initial delay, the release of isotope into the medium increased linearly for the duration of the experiment.

To determine if the delay in the appearance of 14C-labeled basement membrane collagen in the medium was due to an extracellular accumulation of protein on M, PEMT were incubated with [14C]proline for either 30 or 180 min and the isolated tissue was treated with bacterial collagenase and trypsin (17). The results (Table V) showed that proteolytic digestion of the tissue during the delay period (e.g. 30 min) did not release 4-hydroxy[14C]proline in excess of the control, whereas prior lysis of the cells permitted the release of nearly all the 4-hydroxy[14C]proline synthesized. On the other hand, proteolytic digestion of the tissue after longer incubation periods (e.g. 180 min) showed that a significant portion of the 4-hydroxy[14C]proline was extracellular. This suggested that labeled basement membrane collagen was being deposited on M during the incubation period.
A similar conclusion can be drawn from the autoradiographs presented in Fig. 4. After a 30-min incubation period in the presence of [3H]proline (Fig. 4, A and C), grains are almost exclusively localized over PE. However, after a 180-min incubation period (Fig. 4, B and D), grains are also localized along the surface of M which is contiguous with PE. This region has been shown to contain newly deposited basement membrane (26).

**Table III**

Comparison of amounts of total ^14^C and 4-hydroxy[^14^C]proline synthesized by PEMT, PEM, and T after incubation in presence of [^14^C]proline

| Treatment of PEMT before incubation with [^14^C]proline | Tissue | Total ^14^C | Percentage of control | Total 4-hydroxy[^14^C]proline | Percentage of control |
|--------------------------------------------------------|--------|-------------|-----------------------|-------------------------------|-----------------------|
| PBS control; no dissection                              | PEMT   | 1.45        | 100                   | 1.48                          | 100                   |
| PBS + hyaluronidase, no dissection                      | PEMT   | 1.58        | 109                   | 1.93                          | 130                   |
| PBS + hyaluronidase; dissected but incubated together   | PEM    | 1.36        | 94                    | 1.59                          | 107                   |
| PBS + hyaluronidase; dissected and incubated separately | T      | 0.49        | 34                    | 0.11                          | 7                     |

A similar conclusion can be drawn from the autoradiographs presented in Fig. 4. After a 30-min incubation period in the presence of [^14^C]proline (Fig. 4, A and C), grains are almost exclusively localized over PE. However, after a 180-min incubation period (Fig. 4, B and D), grains are also localized along the surface of M which is contiguous with PE. This region has been shown to contain newly deposited basement membrane (26).

**Table IV**

Comparison of amount of [^3H]glucosamine incorporated by PEMT, PEM, and T

| Treatment of PEMT before incubation with [^3H]glucosamine | Tissue | Total [^3H] | Percentage of control | [^3H]glucosamine incorporated into tissue fraction and subsequent release into the medium appeared to be linear for at least 120 min (Fig. 5). In contrast to incubations with [^14^C]proline (Fig. 3), no delay period was observed in the appearance of [^3H] in the medium.

**DISCUSSION**

Basement membrane biosynthesis in vitro has recently been studied using either isolated kidney glomeruli (18-21) or whole embryonic chick lenses and isolated lens cells (17, 27, 28). While the basement membranes of both of these systems have been extensively characterized (for review, see Ref. 8), these systems appear to suffer from several disadvantages when it comes to studying basement membrane biosynthesis. Therefore, we have examined the efficacy of the rat embryo parietal yolk sac for this purpose. In the discussion which follows, we shall note the similarities in results found in all these in vitro systems, as well as emphasize the apparent advantages of the latter system.

Previous studies (4, 6, 11, 13, 14, 29) have shown that the rodent parietal yolk sac is a relatively homogeneous system consisting of a single morphologically identifiable extracellular

**Fig. 3.** A, incorporation of [^14^C]proline; and B, synthesis of 4-hydroxy[^14^C]proline by PEMT. See text for incubation conditions. ○ , nondialyzable radioactivity in tissue fraction; ◦ ◦ ◦ , nondialyzable radioactivity in medium. The results are the average of four experiments. 

by PEM in vitro was also examined. PEM was used rather than PEMT since it was previously shown that while T contributed approximately 50% of the total [^3H] incorporated by PEMT (Table IV), it probably does not contribute significantly to the synthesis of M (Fig. 2) (26). In two separate experiments, [^3H]glucosamine incorporation into the tissue fraction and subsequent release into the medium appeared to be linear for at least 120 min (Fig. 5). In contrast to incubations with [^14^C]proline (Fig. 3), no delay period was observed in the appearance of [^3H] in the medium.

**DISCUSSION**

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Previous studies (4, 6, 11, 13, 14, 29) have shown that the rodent parietal yolk sac is a relatively homogeneous system consisting of a single morphologically identifiable extracellular
Seven PEMT were incubated in 5 ml of modified Krebs medium containing 5 μCi of [14C]proline. At the indicated times, protein synthesis and proline hydroxylation were inhibited as described under "Experimental Procedure." The tissue was isolated by centrifugation and washed twice in 3.0 ml of Krebs medium. The tissue was resuspended in 2.5 ml of Krebs medium to which was added 1.1 mg of collagenase and 0.11 ml of trypsin solution in 0.5 ml of Krebs medium. After incubation for 30 min at 37°C, tissue and supernatant were separated by centrifugation and assayed for 4-hydroxy-[14C]proline.

| Experiment | Incubation time with [14C]proline | Treatment of tissue after [14C]proline incubation | 4-Hydroxy-[14C]proline | Total 4-hydroxy-[14C]proline released |
|------------|---------------------------------|--------------------------------------------------|------------------------|-----------------------------------|
|            |                                 |                                                  | Tissue                 | Supernatant                       |                                      |
| 1          | min 30                          | Control                                          | 26,040                 | 3,785                             | 13                                   |
|            |                                 | Collagenase + trypsin                            | 22,588                 | 3,033                             | 12                                   |
|            |                                 | Lysis + collagenase + trypsin                    | 3,272                  | 18,672                            | 85                                   |
| 2          | 180                             | Control                                          | 241,954                | 32,688                            | 12                                   |
|            |                                 | Collagenase + trypsin                            | 107,232                | 131,251                           | 55                                   |
|            |                                 | Lysis + collagenase + trypsin                    | 7,315                  | 249,101                           | 97                                   |

*Values calculated as 100 × 4-hydroxy[14C]proline in supernatant/total 4-hydroxy[14C]proline.

*Cells were lysed by sonication (three × 5-sec bursts) before incubation with collagenase and trypsin.

The collagen synthesized by PEMT was shown to be typical of basement membrane collagen in that it contained relatively large amounts of 3-hydroxyproline (Table I) and glycosylated hydroxylysinyl (Table II) as compared to interstitial collagens. The contents of 3-hydroxy[14C]proline and glycosylated hydroxy[14C]lysine are similar to the values obtained by Grant et al. (17) using cells derived from a mouse "parietal yolk sac carcinoma." From these ratios, it is possible to estimate the percentage of the newly synthesized protein represented by basement membrane collagen using a modification of the equation presented by Diegelmann and Peterkofsky (38). From such calculations, it appears that only 0.5% of the newly synthesized protein in the glomerular, lens, and "parietal yolk sac carcinoma" systems is basement membrane collagen compared to 6% in PEMT cultures. Moreover, since T contributes about one-third of the total 14C incorporated but essentially none of the 4-hydroxy[14C]proline synthesized by PEMT (Table III), the basement membrane collagen represents at least 9% (6%/0.67) of the total newly synthesized protein in PEM cultures.

The collagen synthesized by PEMT was shown to be typical of basement membrane collagen in that it contained relatively large amounts of 3-hydroxyproline (Table I) and glycosylated hydroxylysinyl (Table II) as compared to interstitial collagens. The contents of 3-hydroxy[14C]proline and glycosylated hydroxy[14C]lysine are similar to the values obtained by Grant et al. (17) using the lens system. As noted, a full complement of glycosylhydroxy[14C]lysine was present after only a 5-min incubation with [14C]lysine (Table II). Since 5 min approximates the time necessary for the translation and hydroxylation of a collagen α chain (39, 40), we can reasonably conclude that the glycosylation of hydroxylysine in basement membrane collagen occurs soon after hydroxylation of peptide-bound lysine.

Continuous labeling of PEMT with [14C]proline demon-

\[
100 \times \frac{100 \times \text{dpm 4-hydroxy[14C]proline}}{\text{dpm 4-hydroxy[14C]proline} \times 0.60 - (\text{total dpm } ^{14}\text{C} - \text{dpm } ^{14}\text{C}\text{collagen} \times 5.4) + \text{dpm } ^{14}\text{C}\text{collagen}}
\]
Fig. 4. Light microscopic autoradiographs of 14.5-day PEM which were pulsed with $^{3}$H proline for either 30 min (A, C) or 180 min (B, D). Tissues were washed and fixed as described under “Experimental Procedure.” Note the absence of extracellular labeling at 30 min (A, C) in contrast to the concentration of extracellular labeling along the endodermal surface of Reichert’s membrane at 180 min (B, D). A and B, x 750; C and D, x 2000. Bar represents 10 μm.

...trated a time lag of about 60 min between the time at which the synthesis of hydroxy$[^{14}$C]proline became linear and the time at which hydroxy$[^{14}$C]proline secretion into the medium became linear (Fig. 3). This observation is identical to that reported by Grant et al. (17) and suggests that a 60-min lag period is characteristic of the secretion of basement membrane collagen. The absence of detectable $^{14}$C-labeled basement membrane collagen in the medium during this time did not appear to be due to an extracellular adsorption of the collagen on either PE or M since digestion of the tissue with trypsin and collagenase indicated that extracellular matrix was not labeled in excess of the control (Table V) (17). Autoradiographic
be the time required to synthesize the relatively large amount of hydroxyproline and hydroxylysine in this collagen. Indeed, Peterkofsky (41) has shown that when cultures of 3T3 fibroblasts are supplemented with ascorbate there was a 45-min lag in the secretion of collagen, while in the absence of the vitamin the lag period increased to 90 to 120 min. In addition, the preliminary experiments with matrix-free endodermal cells have shown an increase in the percentage of total 4-hydroxy[14C]proline recoverable in the medium at all times subsequent to the 60-min lag period. These data are consistent with the observation that in PEMT and PEM cultures in vitro, newly synthesized basement membrane collagen is being deposited on M.

It has been suggested (17) that one possible explanation for the delay in the secretion of basement membrane collagen may be the time required to synthesize the relatively large amount of hydroxyproline and hydroxylysine in this collagen. Indeed, Peterkofsky (41) has shown that when cultures of 3T3 fibroblasts are supplemented with ascorbate there was a 45-min lag in the secretion of collagen, while in the absence of the vitamin the lag period increased to 90 to 120 min. In addition, the collagen synthesized and secreted in the absence of ascorbate was extensively underhydroxylated (41). In PEMT cultures, however, supplementation of the incubation medium with 0.25 mM ascorbate had no effect on either the percent of the [14C]proline hydroxylated or the delay in the secretion of 14C-labeled basement membrane collagen (data not presented). The delay also does not appear to be due to time required for the extensive glycosylation of hydroxylysine since, after only a 5-min incubation with [14C]lysine, the newly synthesized basement membrane collagen appeared to be completely glycosylated (Table II). It is concluded, therefore, that at least for short term incubations this system does not require exogenous ascorbate for basement membrane collagen biosynthesis, and that the lag period is probably not due to a delay in the hydroxylation of either proline or lysine, or to a delay in the glycosylation of hydroxylysine.

Continuous labeling of PEMT for up to 480 min showed that the maximum ratio of 4-hydroxy[14C]proline to total 14C in the medium (21 to 28%) appeared to be attained between 120 to 240 min. This indicated that nearly 40% (24%/60%) of the [14C]proline in the medium at this time is present in newly synthesized basement membrane collagen. The ratio in the tissue fraction remained constant around 12 to 15% up to 240 min. The ratios in both fractions began to fall after longer incubation periods.

A time course experiment performed in the presence of [3H]glucosamine (Fig. 5) did not show a lag period for the release of nondialyzable 3H into the medium. While the precise nature of the glucosamine-containing material was not investigated, previous studies have shown that a relatively large amount of glucosamine was associated with protein components of M; mucopolysaccharide contamination was not detected. Experiments designed to determine either the presence or absence of glucosamine in newly synthesized basement membrane collagen are presently in progress.

From these considerations, it may be inferred that there exists both a temporal and a cellular compartmentalization of the collagen and glycoprotein components of M. That is, basement membrane glycoprotein may be present extracellularly before basement membrane collagen is secreted. Interaction between these components, perhaps through disulfide bonds, then would occur extracellularly to form a functional basement membrane. This interpretation is consistent with the observation in embryonic chick lens cells that in the absence of ascorbate, the extracellular hydroxy[14C]proline is present in large aggregates while the intracellular hydroxy[14C]proline is not aggregated.

Based on the results and comparisons presented, the parietal yolk sac system appears well suited for studying the synthesis and assembly of basement membrane components.

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