Location and Identification of the Collagen Found in the 14.5-d Rat Embryo Visceral Yolk Sac

CHARLES C. CLARK, JANINE CROSSLAND, GARY KAPLAN, and ANTONIO MARTINEZ-HERNANDEZ

Connective Tissue Research Institute, University City Science Center; Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104; and Department of Pathology, Hahnemann Medical College, Philadelphia, Pennsylvania 19102

ABSTRACT The collagens associated with 14.5-d rat visceral yolk sacs were localized and identified by a variety of procedures. Morphological examination showed that both the visceral epithelium and mesothelium rested upon thin basement membranes, whereas the majority of the extracellular matrix consisted of a stroma containing occasional cells and abundant banded fibrils. Immunohistochemistry at the electron microscope level showed that the basement membranes specifically cross-reacted with antibodies directed against mouse basement membrane components, whereas the stroma specifically cross-reacted with antibodies directed against rat type I collagen. Extractions of acellular visceral yolk sacs and subsequent analyses showed that type I collagen components were prevalent. Furthermore, in vitro biosynthetic studies showed only the presence of type I procollagen components (or their conversion products) and α-fetoprotein. These findings, taken together with our previous studies on the 14.5-d rat parietal yolk sac, provide us with protein markers for studying the origin of cells in rat parietovisceral yolk sac carcinomas.

The yolk sac of the embryonic rat is composed of an outer, parietal wall (bilaminar omphalopleure) and an inner, visceral wall (vascular splanchnopleure). The parietal yolk sac (PYS) consists of two cellular layers (parietal endoderm and trophoblast) separated by a relatively thick nonvascular basement membrane (Reichert's membrane). To investigators using a variety of histological staining procedures, the PYS basement membrane appeared to be nearly identical to Descemet's membrane and lens capsule (47, 49). More recently, we confirmed that the amino acid and carbohydrate composition of this membrane was characteristic of basement membranes isolated from other tissues and species (9). In addition, in vitro biosynthetic experiments using isolated 14.5-d PYS showed that the collagenous protein synthesized was characteristic of basement membrane procollagen (6, 7, 10, 36, 37).

The visceral yolk sac (VYS) appears to be a more complex structure. The surface facing the yolk sac cavity consists of a sheet of visceral endodermal cells which, on the 11th d of gestation, begin to form villi. This epithelium rests upon the visceral basement membrane (VBM). The surface facing the exocoelom is lined by a single layer of flattened mesothelial cells which are supported by the serosal basement membrane (SBM). By histologic examination, the VBM and SBM differ markedly from each other as well as from the PYS basement membrane (49). In between these membranes is a vascularized mesenchymal layer (stroma) containing fibroblastlike cells (39).

In a recent study of collagen types in developing mouse extraembryonic tissues, Adamson and Ayers (1) reported that 10th- to 18th-d VYS endoderm (epithelium) synthesized primarily type I collagen, whereas the mesoderm (mesothelium) synthesized both types I and IV. In contrast, murine PYS endoderm synthesized only type IV collagen. In this study, we report on the distribution and identification of collagen types in the 14.5-d rat embryo VYS. Furthermore, we speculate about which cells are responsible for the deposition of the newly synthesized collagen.

MATERIALS AND METHODS

Materials

Timed-pregnancy Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). Materials were purchased from the following sources: L-[U-^{14}C]proline (250 mCi/mmol) from Amersham Corp. (Arlington Heights, IL); aprotinin, β-aminopropionitrile (BAPN), cycloheximide, α,α'-dipyridyl, *N*-ethylmaleimide, phenylmethylsulfonyl fluoride, α-chymotrypsin, and dithiothreitol (DTT) from Sigma Chemical Co. (St. Louis, MO); benzamidine/HCl from Calbiochem-Behring Corp. (La Jolla, CA); SDS from Pierce

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Chemical Co. (Rockford, IL); bacterial collagenase (CLSPA) and mercuripapain from Worthington Biochemical Corp. (Freehold, NJ); Enhance and [*methyl*-^{14}C]-labeled bovine serum albumin (BSA) from New England Nuclear (Boston, MA); PAGE reagents and Bio-Gel A-15m from Bio-Rad Laboratories (Richmond, CA); DEAE-cellulose (DE-52) from Whatman Inc. (Clifton, NJ); DEAE-Sephadex from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ).

Isolation of VYS

At a gestational age of 14.5 d, rats were killed by decapitation and the implantation sites were isolated. After removal of the PYS as described previously (9), the VYS was isolated by dissection under a microscope. Special care was taken to separate the VYS from the umbilical cord and from the underlying transparent amnion (27). The membranes were kept at 4°C in phosphate-buffered saline (PBS) until used (1–2 h).

Preparation of Acellular VYS

After isolation, VYS were washed with distilled water containing 0.01% sodium azide and frozen at −20°C for at least 12 h. The tissues were transferred to siliconized tubes containing fresh water and azide at 4°C, and the cells were removed by brief immersion (up to 1 min) in an ultrasonic bath (Cole-Parmer Instrument Co., Chicago, IL). The turbid solution was removed from the membranes with a Pasteur pipette and the membranes were rinsed with water several times. After overnight storage at 4°C in 1% EDTA and 0.01% azide, the above sonication procedure was repeated. At this time, by microscopy the membranes were >90% cell free. Any visible areas of cellular debris remaining were removed by microdissection.

Preparation of VYS for Electron Microscope Analysis

The extraembryonic membranes were dissected from 14.5-d rat embryos and fixed by immersion. For routine electron microscopy, tissues were fixed in glutaraldehyde-formaldehyde (Karnovsky's) fixative at 4°C for 2 h, postfixed in 1% OsO_4 for 1 h, dehydrated in ethanol, and embedded in Medcast (Pelco, Tustin, CA) via propylene oxide. Thick sections of 1 μm were stained with toluidine blue; ultrathin sections were stained with uranyl acetate and lead citrate.

Immunohistochemistry

Tissues were fixed in periodate-lysine-paraformaldehyde fixative (34, 35) at 4°C for 2 h and washed in multiple changes of PBS with 4% sucrose at 4° for 18 h. The two methods described below were used for immunohistochemical staining.

Frozen Section Staining

After washing extraembryonic membranes for one extra hour at 4°C in PBS containing 4% sucrose and 5% glycerol, they were placed in OCT and snap-frozen in methyl butane precooled to liquid nitrogen temperature. Thick sections (10 μm) were then incubated with appropriate antibodies according to previously described methods (19, 32). Briefly, the sections were incubated with primary antibody Fab or normal rabbit serum Fab overnight, washed in PBS, incubated with goat anti-rabbit IgG for 2 h, washed in PBS, incubated with rabbit Fab-peroxidase-antiperoxidase (PAP) for 2 h, washed in PBS, incubated in diaminobenzidine (DAB) solution (20) without H_2O_2 for 10 min followed by DAB with H_2O_2 for 15 min. The sections were poststained with 1% OsO_4, dehydrated in ethanol, and embedded in Medcast.

In-block Staining

Without sectioning, the fixed and washed extraembryonic membranes were immersed in the antibody solutions exactly as described for frozen sections.

Antibody Preparation

All primary antibodies were prepared in rabbits. Anti-neoplastic basement membrane antibody (anti-NBM) was prepared as previously described (19, 34). This antibody recognizes type IV collagen and laminin, but does not cross-react with fibronectin (4). Anti-rat tail tendon collagen antibody (anti-RTT) was prepared as described previously (4). For all primary antibodies as well as normal rabbit serum, IgG was purified by ammonium sulfate precipitation followed by DEAE-Sephadex chromatography (17). To prepare Fab, IgG was digested with mercuripapain followed by CM-cellulose chromatography (31). Rabbit-Fab-PAP was prepared according to described methods (42). The specificity of all primary antibodies was measured by ELISA (4).

Incubation of VYS in Suspension Culture

Isolated VYS were incubated in suspension in modified Krebs medium as described previously for PYS (10). A typical experiment consisted of 50 membranes in 50 ml of medium containing 50 μg/ml BAPN and 250 μl aprotinin. After preincubation at 37°C for 30 min, 200 μCi of [^{14}C]proline was added and the incubation was continued for 3–5 h. At the end of the incubation, stock solutions of cycloheximide and α,α′-dipyridyl were added to the medium to give final concentrations of 10 and 1 mM, respectively.

Tissues were removed with forceps, washed with distilled water, and frozen. The cells were subsequently removed from these tissues as described previously. The medium was immediately centrifuged (1,000 *g*, 10 min) to remove cellular matter, and the supernatant was dialyzed at 4°C against 0.4 M NaCl, 0.1 M Tris/HCl (pH 7.5) containing 10 mM EDTA, 10 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine/HCl as protease inhibitors.

Purification of Medium Proteins

All procedures were performed at 4°C. After dialysis of the medium (S_I) as described above, collagenous proteins were precipitated by the addition of ammonium sulfate to a final concentration of 176 mg/ml of medium followed by overnight stirring. The insoluble material was isolated by centrifugation (20,000 *g*, 20 min), the supernatant (S_{II}) was decanted, and the pellet (P_{II}) was resuspended in 10 ml of 50 mM Tris/HCl, 2 M urea, 0.1% Triton X-100 (pH 8.3) containing protease inhibitors as described above. Stirring was continued overnight and any insoluble material (P_{III}) was removed by centrifugation. The supernatant (S_{III}) was frozen until needed.

For DEAE-cellulose chromatography, S_{III} was dialyzed against the above buffer to remove the protease inhibitors and applied to the column (18). After washing with ~50 ml of starting buffer, isolation of material bound to the column was achieved by elution with starting buffer containing 0.3 M NaCl.

Extraction of Acellular VYS

Labeled and unlabeled membranes were treated in the same way. 10 acellular VYS were extracted by heating at 56°C for 2 h in 275 μl of SDS PAGE sample buffer (0.0625 M Tris/HCl (pH 6.8), 4 M urea, 2% SDS, 2 mM DTT) in a 1.5-ml polypropylene Eppendorf Micro Test Tube (Bio-Rad Laboratories). The sample was clarified in an Eppendorf Micro Centrifuge (12,800 *g*, 5 min) and the supernatant was frozen until needed.

SDS PAGE

PAGE was performed in a Tris-glycine buffer (25) containing 0.5 M urea (28) using a 1.5-mm-thick slab consisting of a 5% separating gel and a 3% stacking gel. The gels were stained with Coomassie Brilliant Blue, destained, and processed for fluorography by permeation with Enhance followed by exposure to sensitized Kodak x-ray film (26).

Preparation of Other Collagens and Procollagens

Other rat collagens or procollagens were prepared from the tissues of the same animals from which the VYS were isolated. Type I collagen was extracted from tail tendons in 0.5 M acetic acid and purified by salt precipitations at both acid and neutral pH (41). Basement membrane procollagen was prepared by incubating 14.5-d PYS with [^{14}C]proline as described previously (10); the medium was used without further purification. Type I procollagen was prepared from the medium of 17-d chick embryo tendon cells after incubation with [^{14}C]proline (14) and was purified by ammonium sulfate precipitation as described previously.

Other Analytical Procedures

For amino acid analysis, acellular VYS were hydrolyzed under vacuum in triple-distilled constant-boiling HCl at 110°C for 24 h and applied to a Beckman 121 MB automatic amino acid analyzer equipped with an integrator (Beckman Instruments, Inc., Palo Alto, CA). For colorimetric hydroxyproline analysis, the procedure of Switzer and Summer (44) was used; for 4-hydroxy[^{14}C]proline analysis, the procedure of Juva and Prockop (24) was used; for 3-hydroxy-[^{14}C]proline analysis, hydrolyzed samples were chromatographed on the long column of an amino acid analyzer and the effluent was collected and counted

(21). Digestions with α-chymotrypsin or bacterial collagenase were performed as described previously (8). SDS agarose gel filtration was performed as described by Jimenez et al. (23).

RESULTS

Morphology of 14.5-d VYS

By electron microscopy, the structures of the rat VYS which have been described by others (5, 27, 48, 49) were easily recognized: the visceral epithelial cells with their microvilli and attendant basement membrane (VBM), a vascularized, oligocellular layer of loose connective tissue (stroma), and a continuous layer of mesothelial cells that were separated from the stroma by a basement membrane (SBM) (Fig. 1).

In addition to the cells illustrated in Fig. 1, occasionally a second cell type was observed that was characterized by a clear polarized cytoplasm; the basal portion contained abundant free ribosomes and few cell organelles while the apical portion contained secretory granules, rough endoplasmic reticulum (RER) and a Golgi complex (Fig. 2). The nuclei of these cells had loose chromatin granules and lacked a distinct nucleolus in marked contrast to the more abundant epithelial cells (Fig. 1). Since the VYS epithelium represents extraembryonic endoderm, and adult endoderm (e.g., gastrointestinal tract) contains more than one cell type (40), it should not be surprising that more than one cell type is present in the VYS epithelium.

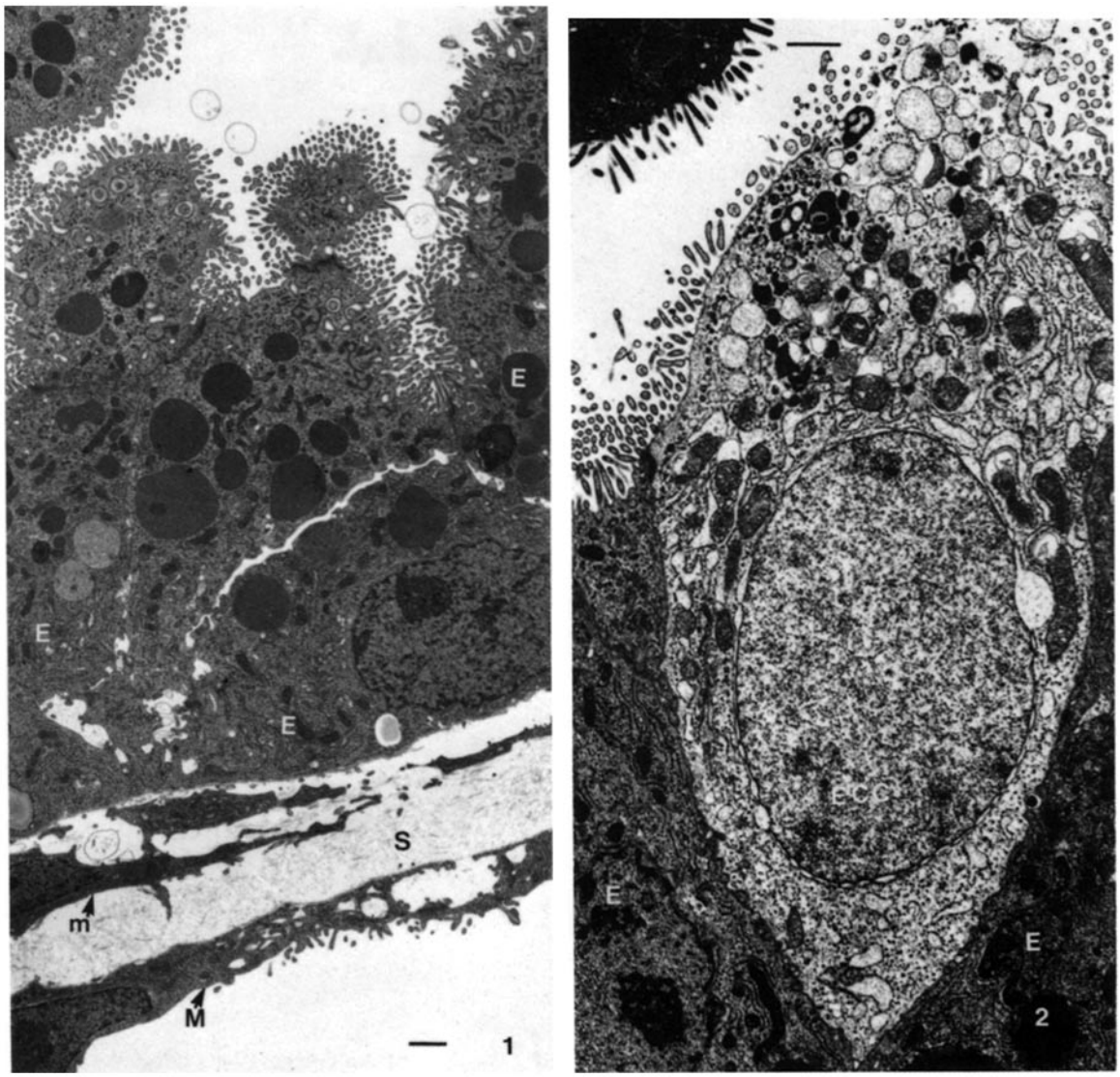


FIGURE 1 Electron micrograph of 14.5-d rat visceral yolk sac. Visceral epithelial cells (*E*) have microvilli on the luminal surface. The cytoplasm contains membrane bound granules, some of which are secretory, and a well-developed rough endoplasmic reticulum and Golgi complex. The nucleus contains a prominent nucleolus and peripherally clumped chromatin. The epithelial cells are separated from the stroma (*S*) by a delicate basement membrane (see also Figs. 3 *a* and *b* and 5). The stroma contains finely granular material and collagen fibers. Embedded in this matrix are occasional mesenchymal cells (*m*) which form a discontinuous layer. The innermost layer of the VYS contains the flattened mesothelial cells (*M*) which form a continuous layer separated from the stroma by a delicate basement membrane (see also Figs. 3 *a* and *b* and 5). Uranyl acetate and lead citrate staining. Bar, 1 μm. × 4,900.

FIGURE 2 Electron micrograph of a second type of epithelial cell. Clear cells (*ECC*) appear to represent <10% of all epithelial cells (*E*). The basal cytoplasm has sparse cell organelles but abundant free ribosomes, while the apical cytoplasm contains rough endoplasmic reticulum, mitochondria, secretory granules, lysosomes, and microvilli. The nucleus contains predominantly euchromatin. Uranyl acetate and lead citrate staining. Bar, 1 μm. × 7,000.

However, any possible functional differences between these cell types in the VYS is not known.

The VBM appeared as a thin (70-nm), delicate structure that followed the contours of the epithelial cells. The classical laminae rara and densa could easily be distinguished (Fig. 3).

The stroma was composed of individual cross-banded collagen fibers which were not associated in large bundles and which appeared to lack any specific orientation (Fig. 4). In between these fibers, the matrix consisted of granular or fibrillar material of variable electron density (Figs. 3 and 4). Also embedded in this matrix were individual cells, morphologically similar to the mesothelial cells, but lacking a basement membrane (Figs. 3 and 4). These cells had all the morphological features of mesenchymal cells including a prominent RER often distended with finely fibrillar material as well as a prominent Golgi apparatus. No membrane junctional complexes were observed in these cells in spite of their occasional apposition and convoluted infoldings.

The mesothelial cells also had well-developed cytoplasmic organelles (Figs. 1 and 3*b*). They formed a continuous layer, but although cell-to-cell contact was frequent, no junctional complexes were present. These cells were separated from the connective tissue stroma by a continuous, thin (70-nm), delicate SBM with its classical laminae rara and densa (Fig. 3).

Immunohistochemical Localization of Type I Collagen and Basement Membrane

The results obtained for both the in-block and frozen-section staining procedures were identical with respect to antigen localization. As expected, the in-block method gave better tissue preservation but poorer penetration.

Anti-NBM stained exclusively the VBM and SBM (Fig. 5). Lamina rara and lamina densa had similar staining intensities with no distinction between them. No reaction product was identified around the mesenchymal cells or on the banded collagen fibers. Conversely, anti-RTT stained exclusively the banded collagen fibers in the stroma (Fig. 6*a*). The reaction product was deposited at regular intervals of ~70 nm (Fig. 6*b*), producing a characteristic banding pattern identical to that seen on the interstitial collagen fibers of the adult rat kidney (33). The basement membranes and the ground substance surrounding these collagen fibers were negative (Fig. 6*a*).

Analysis of the Protein Extracted from Unlabeled Acellular VYS

Acellular membranes appeared as nearly transparent sheets of tissue (see Fig. 1 of reference 27). On the basis of five

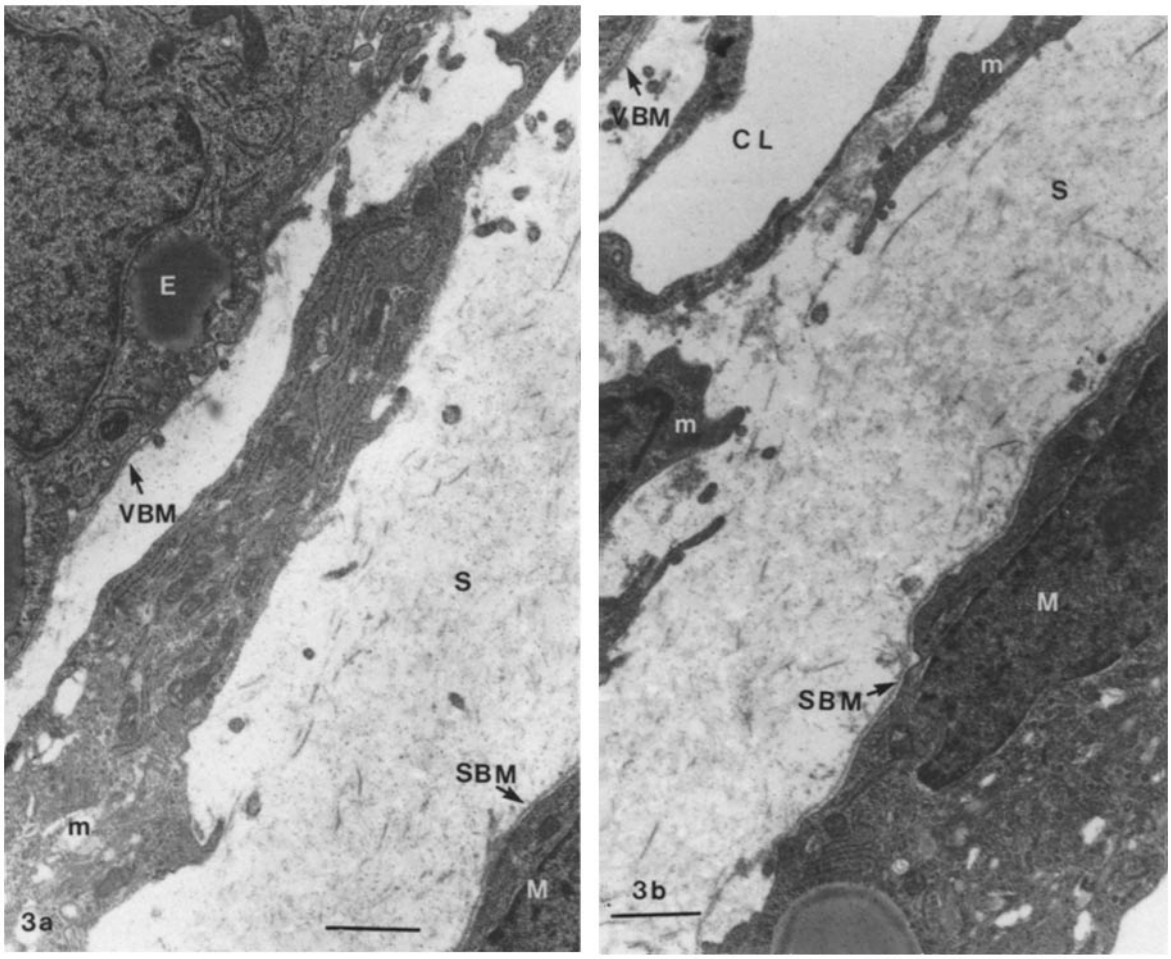


FIGURE 3 Detail of the yolk sac stroma and basement membranes. (*a*) The basement membrane (*VBM*) of the visceral epithelial cells (*E*) forms a continuous layer with the characteristic lamina rara and lamina densa. The mesenchymal cells (*m*) lack a basement membrane. The mesothelial cells (*M*) are separated from the stroma by a continuous serosal basement membrane (*SBM*) with the characteristic lamina rara and lamina densa. (*b*) The continuous basement membrane (*SBM*) of the mesothelial cell (*M*) is clearly seen. A portion of a capillary (*CL*) is present in the left upper corner. Uranyl acetate and lead citrate staining. Bars, 1 μm. × 11,400.

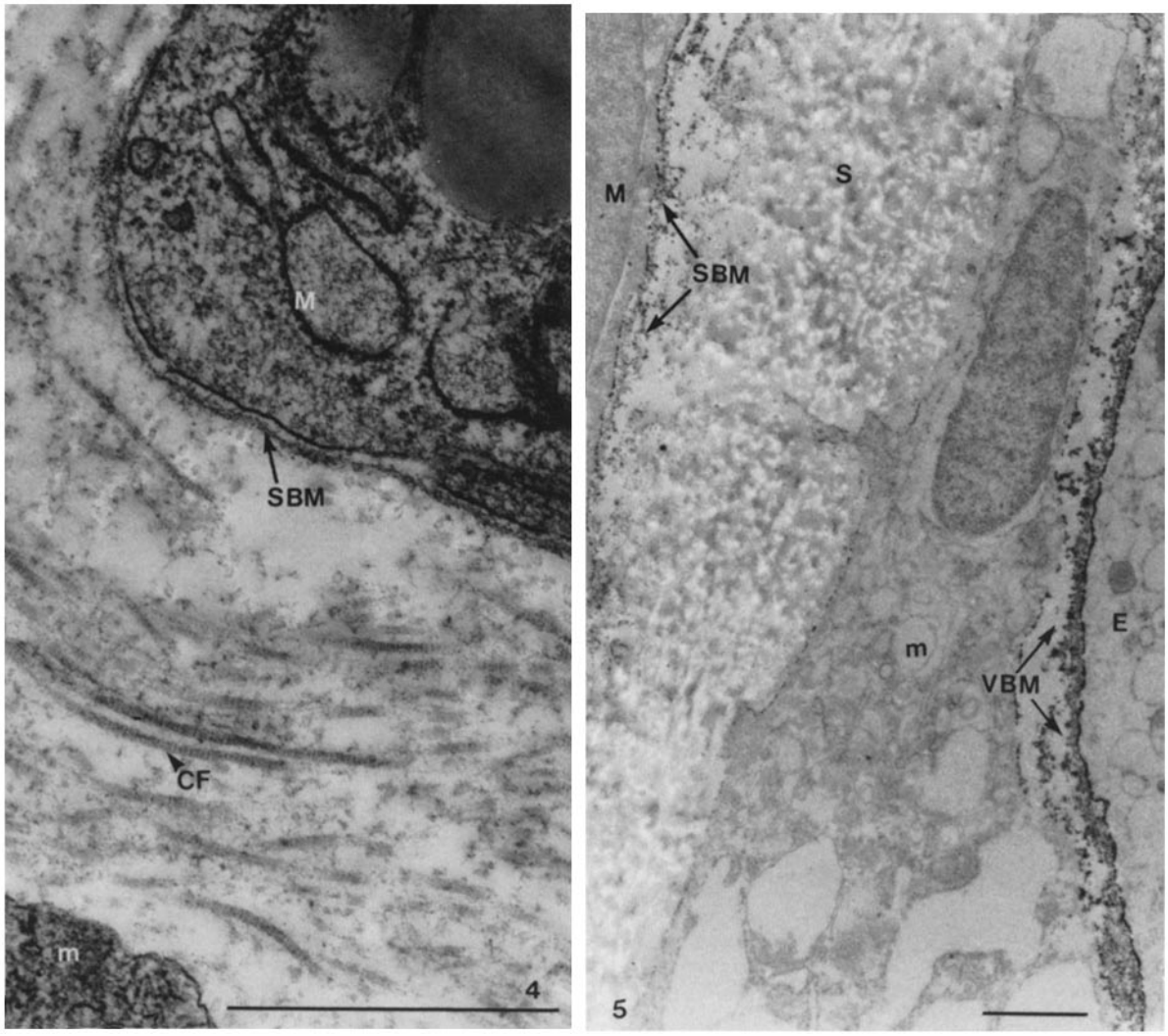


FIGURE 4 Higher magnification of the stroma demonstrating collagen fibers (*CF*) with the typical 68-nm periodicity and their relationship to a mesothelial cell (*M*), the serosal basement membrane (*SBM*), and a mesenchymal cell (*m*). Uranyl acetate and lead citrate staining. Bar, 1 μm. × 44,000.

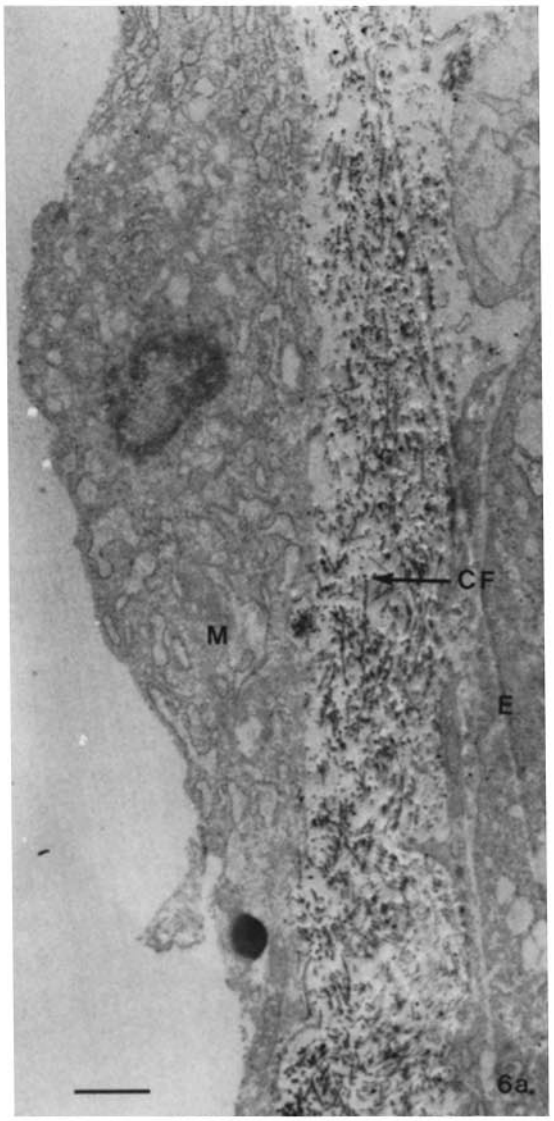
FIGURE 5 Anti-NBM antibody staining. The visceral (*VBM*) and serosal (*SBM*) basement membranes are positive. The mesenchymal cells (*m*) lack a basement membrane although the portion of the cell membrane immediately adjacent to the VBM has occasional reactivity. The stroma (*S*) is negative. Bar, 1 μm. × 1,300.

preparations, the dry weight per VYS ranged from 30 to 50 μg. The amino acid composition of acellular VYS was compared with that of acellular PYS and with purified rat tail tendon (RTT) collagen (Table I). Based on the glycine content, it appeared as if ~90% of the protein constituents of acellular VYS were represented by collagen. Compared with the composition of purified RTT collagen, the major differences in acellular VYS composition were the elevated amounts of 4-hydroxyproline and hydroxylysine (the latter is approximately twice that normally found in type I collagen), and the presence of ½ cystine, which is not found in type I collagen. In spite of these differences, the analysis of VYS was very similar to that of RTT collagen. On the other hand, it was apparent that the composition of acellular VYS was distinctly different from that of acellular PYS (Table I), which is known to consist primarily of basement membrane constituents (9). Preliminary carbohydrate analysis suggested that glucose plus galactose represented ~2% by weight of acellular VYS compared with <0.4% for RTT collagen (data not shown).

SDS extraction of acellular VYS resulted in the complete solubilization of the structure. Analysis of this extract by SDS PAGE showed that the major components appeared to be similar to the collagenous components of purified RTT collagen isolated from the same animals, but they consistently migrated slightly more slowly than their tendon counterparts (Fig. 7). No other collagen chains could be detected at this level of sensitivity.

Initial Characterization of [^{14}C]Proline-labeled VYS Medium and Tissue Proteins

VYS were incubated with [^{14}C]proline, and the medium and tissue fractions were isolated as described. Analysis showed that 75% of the total 4-hydroxy[^{14}C]proline synthesized was associated with the whole tissue fraction (tissue plus cells) while 25% was found in the medium. The ratio of 4-hydroxy-[^{14}C]proline/total ^{14}C for medium and acellular tissue samples was approximately 0.20 and 0.48, respectively. In addition, amino acid analysis showed that 3-hydroxy[^{14}C]proline represented <2% of the total hydroxy[^{14}C]proline (data not shown). Since rat type I collagen contains an average of 91 residues of



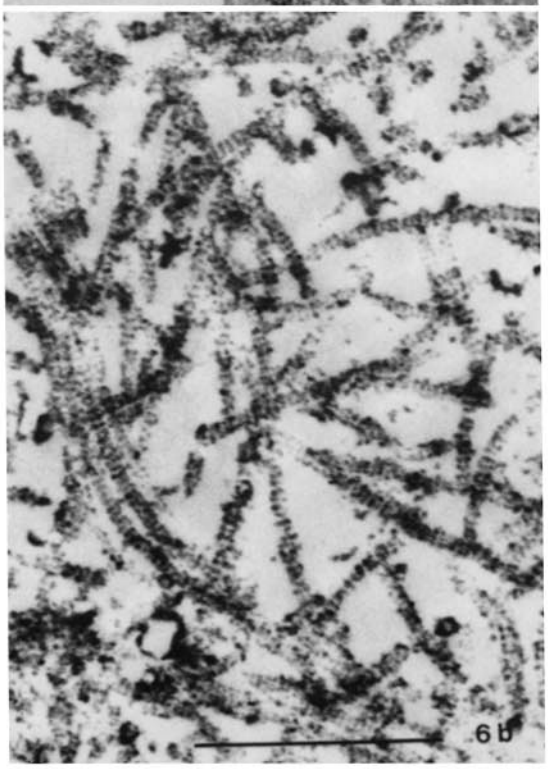


TABLE I

Amino Acid Composition of 14.5-d Rat VYS Compared with Rat Tail Tendon Collagen and 14.5-d Rat PYS

Amino acid residue	Acellular VYS	RTT collagen	Acellular PYS*
	residues/1,000 residues		
3-Hydroxyproline	1	tr‡	6.0
4-Hydroxyproline	100.4	85.9	46.2
Aspartic Acid	37	44	84
Threonine	22	20	48
Serine	47	42	67
Glutamic Acid	83	80	107
Proline	108.2	111.2	61.5
Glycine	300	336	179
Alanine	116	110	62
½ Cystine	2.9	—	18.8
Valine	17	17	44
Methionine	6.6	5.6	13.3
Isoleucine	10.0	10.2	30
Leucine	31	28	70
Tyrosine	5.7	3.1	18.4
Phenylalanine	15	14	31
Hydroxylysine	15.1	6.7	18.4
Lysine	24.6	29.2	33.0
Histidine	6.7	4.6	19.9
Arginine	52	53	45

* Clark et al. (9).
‡ *tr*, Trace.

4-hydroxyproline and 121 residues of proline per 1,000 amino acid residues (16), the ratio of 4-hydroxy[^{14}C]proline to total ^{14}C in newly synthesized collagen should be ~43%. The data presented here suggest that in the medium 46% (20%/43%) and in the acellular tissue virtually 100% (48%/43%) of the incorporated radioactivity is in ^{14}C-labeled collagenous proteins.

SDS PAGE Analysis of Newly Synthesized Proteins in VYS Medium and Acellular VYS

Samples of medium (S_1) and acellular VYS were analyzed by SDS PAGE. For comparison, samples of PYS medium (basement membrane components) and chick embryo tendon cell medium (type I procollagen components) were also analyzed (Fig. 8, lanes *1-3* and *5*). There are several pertinent points to be made: (*a*) 14.5-d VYS does not synthesize detectable amounts of basement membrane components (compare lane *1* with *2* and *5*); (*b*) the slower migrating components from VYS medium migrated in a manner slightly different from that of the pro-α and pC-α components (see legend to Fig. 8 for definition of terminology) from chick embryo tendon cell medium (compare lane *3* with *5*), and the faster moving component from VYS (not present in either PYS or tendon cell medium) migrated slightly more slowly than BSA (lane *6*). It is also of interest to note that in tendon cell medium the major collagenous constituents were pro-α chains, whereas in the VYS medium the major collagenous constituents appeared to be p-α chains (compare lane *3* with *5*); and (*c*) the VYS membrane components appeared qualitatively similar to the medium components except for the absence in the VYS mem-

FIGURE 6 Anti-RTT collagen antibody staining. (*a*) The collagen fibers in the stroma (*CF*) are strongly positive. Both serosal and visceral basement membranes are negative. Bar, 1 μm. × 8,600. (*b*) Higher magnification demonstrating the binding of anti-RTT collagen on fibers at regular intervals. Bar, 1 μm. × 31,600.

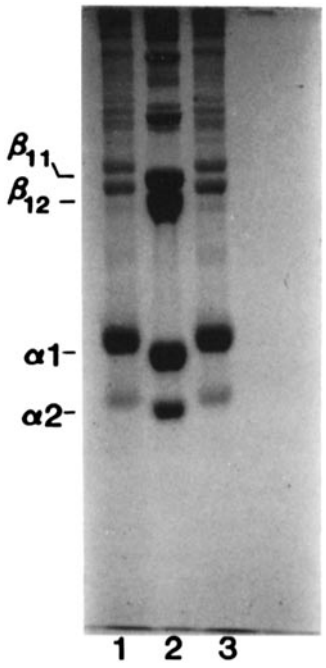


FIGURE 7 Analysis of an SDS extract of acellular 14.5-d rat visceral yolk sac. Coomassie Blue-stained SDS PAGE slab (5% separating gel) of an SDS extract of acellular 14.5-d rat VYS (lanes *1* and *3*) and purified acid-soluble RTT collagen (lane *2*). The identity of the RTT α and β components is indicated on the left.

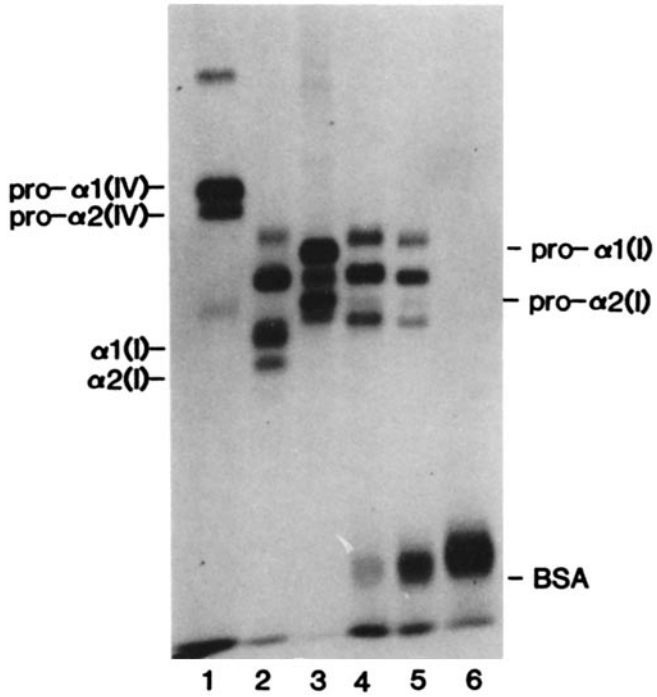


FIGURE 8 Comparison of newly synthesized proteins from 14.5-d rat parietal and visceral yolk sac medium. Fluorogram of an SDS PAGE slab of [^{14}C]proline-labeled samples from cultures of 14.5-d rat VYS and PYS, and chick embryo tendon cells. Samples were electrophoresed in a 5% gel slab under reducing conditions: lane *1*, PYS S_I; lane *2*, VYS acellular tissue extract; lane *3*, chick embryo tendon cell S_{III}; lane *4*, VYS S_{III}; lane *5*, VYS S_I; lane *6*, VYS S_{II}. The migration positions of basement membrane pro-α chains [pro-α1(IV) and pro-α2(IV)] and RTT α chains [α1(I) and α2(I)] are shown on the left, the migration positions of chick tendon cell pro-α chains [pro-α1(I) and pro-α2(I) in lane *3*] and bovine serum albumin (*BSA*) are shown on the right. [Terminology: procollagen refers to a collagen molecule containing both NH_2- and COOH-terminal propeptides; the constituent chains of type I procollagen are pro-α1(I) and pro-α2(I). Molecules which contain propeptides on only one end are called p-collagen; the constituent chains of type I p-collagen are p-α1(I) and p-α2(I). If, for example, it is known that the COOH-propeptide is present, then the molecule is called pC-collagen and the constituent chains pC-α chains.]

brane of the fastest migrating component (compare lane *2* with *5*) and the presence of components migrating slightly more slowly than RTT collagen α1 and α2 chains.

Because of the overall similarity between the VYS membrane fraction and medium, but primarily for ease of purification, medium samples (S_I or S_{III}) were used for the remaining experiments.

Partial Purification of VYS Medium Proteins

Ammonium sulfate precipitation resulted in a significant enrichment in collagenous proteins as measured by 4-hydroxy[^{14}C]proline analysis. Greater than 80% of the total 4-hydroxy[^{14}C]proline in S_I was precipitable, and the ratio of 4-hydroxy[^{14}C]proline/total ^{14}C in S_{III} was 0.33. Analysis of these fractions by SDS PAGE (Fig. 8) showed that S_{II} consists primarily of the component migrating slightly more slowly than BSA (lane *6*), while S_{III} consists primarily of the components migrating slightly more slowly than chick tendon pro-α and p-α chains (lane *4*).

Digestion of Medium Proteins by Bacterial Collagenase or Chymotrypsin

Samples of S_{III} were incubated under native conditions with either bacterial collagenase or α-chymotrypsin. After dialysis, approximately 55% and 70% of the total radioactivity, respectively, was recovered. The digests were then analyzed by SDS PAGE in the presence or absence of disulfide bond reducing agents (Fig. 9). Bacterial collagenase digestion removed all of the slower migrating bands, but not the faster migrating band (compare lane *1* with *3*). On the other hand, chymotrypsin digestion converted all of the slower migrating bands to material migrating similar to RTT α1 or α2 chains in an approximate ratio of 2:1 (compare lane *1* with *2*). When disulfide bonds were not reduced, all of the material appeared at the top of the separating gel (lane *4*). However, after chymotrypsin digestion, the pattern of the collagenous components was identical to that seen with reduction (compare lane *6* with *2*).

Chromatographic Analysis of VYS Medium Proteins

When S_{III} was chromatographed on DEAE-cellulose under native conditions, all of the radioactivity bound to the column and could be eluted with salt (Fig. 10). Under these conditions, PYS basement membrane constituents did not bind to the column (Clark, C. C., and N. A. Kefalides, manuscript in preparation). After denaturation and disulfide bond reduction, S_{III} eluted essentially as a single peak on gel filtration (Fig. 11). Using the elution positions of type I collagen components as molecular weight standards, this material had a M_r ~130,000–135,000. The peak of radioactivity coeluted with chick type I pro-α chains, but eluted significantly after basement membrane pro-α chains (Fig. 11). Analysis of individual fractions showed that all of the 4-hydroxy[^{14}C]proline was associated with this peak, and the ratio of 4-hydroxy[^{14}C]proline/total ^{14}C in the peak fractions was on the order of 0.45.

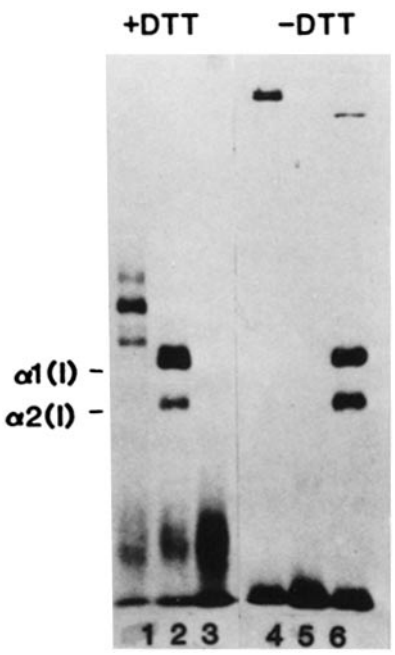


FIGURE 9 Analysis of chymotrypsin or bacterial collagenase digestion products of 14.5-d rat visceral yolk sac medium. Fluorogram of an SDS PAGE slab of [^{14}C]proline-labeled VYS S_{III} before and after chymotrypsin or bacterial collagenase digestion. Samples were electrophoresed in a 5% gel slab under reducing (*+DTT*) or nonreducing (*−DTT*) conditions: lane *1*, reduced control; lane *2*, reduced chymotrypsin digest; lane *3*, reduced bacterial collagenase digest; lane *4*, unreduced control; lane *5*, unreduced bacterial collagenase digest; lane *6*, unreduced chymotrypsin digest.

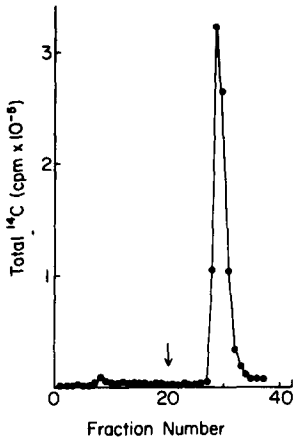


FIGURE 10 DEAE-cellulose chromatography of 14.5-d rat visceral yolk sac medium under native conditions. An aliquot of S_{III} was dialyzed against DEAE-cellulose starting buffer (see Materials and Methods) and applied to a 1.5 × 15-cm column of DEAE-cellulose at 4°C. The column was eluted with 50 ml of starting buffer followed by elution with this buffer containing 0.3 M NaCl (arrow) until the radioactivity returned to baseline. 2.5-ml fractions were collected. The recovery of radioactivity was >90%.

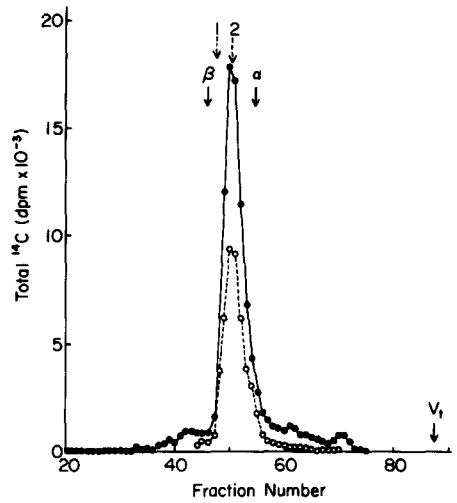


FIGURE 11 SDS agarose gel filtration of 14.5-d rat visceral yolk sac medium under reducing and denaturing conditions. An aliquot of S_{III} was reduced with mercaptoethanol, denatured in SDS (see Materials and Methods), and applied to a 1.5 × 90-cm column of Bio-Gel A-15m (200–400 mesh). 2 mg of rat-tail tendon collagen was included as an internal standard to estimate molecular weights. Aliquots of collected fractions were taken for determination of total ^{14}C and total 4-hydroxy[^{14}C]proline. The elution positions of β components (M_r ~196,000) and α chains (M_r ~98,000) were determined by a colorimetric hydroxyproline assay and are indicated by the solid arrows. The elution positions of rat PYS basement membrane pro-α(IV) chains (*1*) and chick embryo tendon pro-α(I) chains (*2*) are indicated by the dashed arrows. ●, Total ^{14}C; ○, total 4-hydroxy[^{14}C]proline; V_t, total included volume measured by ^3H_2O.

DISCUSSION

Localization of Collagen Types by Immunohistochemistry

Antibodies directed against basement membrane (anti-NBM) stained exclusively VBM and SBM (Figs. 5 and 6). Although the connective tissue stroma which represents the bulk of the extracellular matrix in the 14.5-d VYS (Fig. 1) was included as part of the SBM in morphological descriptions by Wislocki and Dempsey (48) and Padykula et al. (39), it is unreactive toward anti-NBM (Fig. 5). Other morphological descriptions have distinguished the SBM from the collagen fiber–containing matrix (5, 27). In corroboration of the latter observations, the stroma was strongly positive when stained with anti-RTT collagen, whereas the VBM and SBM were negative (Fig. 6). These results suggested that the majority of the collagen in the 14.5-d VYS was type I that was localized to the stroma.

Characterization of the Collagen in VYS Extracts

The amino acid composition of the total acellular VYS showed significant similarities to that of purified RTT collagen isolated from the same animals, but was distinctly different from the composition of total acellular PYS (Table I). However, there were significant variances in the amounts of 4-hydroxyproline and hydroxylysine between VYS and RTT collagens. In addition, on SDS PAGE the mobilities of the VYS components were significantly slower than the collagen components from RTT (Fig. 7). These results are similar to those described by others. Crouch and Bornstein (11) described a fetal human collagen chain (AF2) which was similar to α1(I) but which migrated more slowly on SDS PAGE. They attributed this behavior to an increased hydroxylation of peptidyl proline and lysine and glycosylation of hydroxylysine (11). Similarly, Little et al. (30) and Little and Church (29) described a collagen chain synthesized by embryonic mouse cells (TSD4 and MB4) which was most similar to mouse α1(I), but which contained increased levels of 4-hydroxyproline and hydroxylysine. According to these authors (30), MB4 cells closely resembled VYS endoderm. On the basis of CNBr peptide maps, however, it was concluded that this collagen represented a previously undescribed type (29). In general, it seems that increased hydroxylation (and glycosylation) may be a characteristic of embryonic collagens (3).

A major difference between the embryonic collagens described by Crouch and Bornstein (11), Little et al. (30), and Little and Church (29) and the collagen isolated from 14.5-d VYS is that the former molecules were composed of three identical α chains, whereas the latter appeared to contain chains similar to both α1(I) and α2(I) (Figs. 7–9).

It was noted previously (see Results) that the collagen fibers (~20 nm in diameter) observed in the VYS stroma did not appear to associate into large bundles (Fig. 4). This may be a reflection of the composition of VYS collagen, since an inverse

relatic nship between the degree of fibrillar structure and the amount of hydroxylysine glycosides has been postulated (38, 43). Whether these fine fibers are responsible for the transparency of the VYS is not known.

Even though basement membranes could be clearly identified morphologically (Figs. 3 and 4) and immunohistochemically (Fig. 5) in the VYS, the amino acid composition (Table I) and SDS-PAGE analysis of VYS extracts (Fig. 7) did not show the presence of detectable amounts of basement membrane constituents. This finding was most likely due to the relatively small quantity of basement membrane in VYS. For the same reason, we cannot rule out the likelihood that, as in some other tissues (45), small amounts of other collagen types (e.g., type V) may be present in VYS. Experiments are in progress to examine this possibility.

Characterization of the Collagen Synthesized by VYS in Vitro

A variety of experiments were performed to determine which collagen type (or types) was being synthesized by 14.5-d VYS. The results were consistent with its being type I procollagen. These results are summarized briefly below.

Analysis of hydroxy[^{14}C]proline showed only a small amount (~2%) of 3-hydroxy[^{14}C]proline and a maximum ratio of 4-hydroxy[^{14}C]proline/total ^{14}C of 0.45 (Fig. 11). The mobility of newly synthesized components determined by SDS gel filtration (Fig. 11) or by SDS PAGE (Figs. 8 and 9) appeared most similar to those of type I collagen, p-collagen, or procollagen. Specifically, in VYS medium the migration of bacterial collagenase-sensitive components was similar to that of the pro-α1, pC-α1, pro-α2, and pC-α2 chains of chick type I procollagen (Fig. 9). In addition to the slight differences in mobility as noted previously (Fig. 7), there were distinct quantitative differences between these two samples, suggesting that the conversion of procollagen to pC-collagen in VYS medium was faster than that for chick tendon cell medium (Fig. 8, lanes *3* and *4*). This conversion was even more evident in VYS tissue samples where chains similar in mobility to RTT collagen α1 and α2 appeared (Fig. 8, lane *2*). Similar components could also be generated in an approximate 2:1 ratio after chymotrypsin digestion of VYS medium; the mobility of these components was not affected by electrophoresis in the absence of disulfide bond reducing agents (Fig. 9, compare lane *2* with *6*).

These results showed that the only detectable newly synthesized collagen in incubations of 14.5-d rat VYS was type I. This collagen was initially synthesized as a procollagen that was relatively quickly processed to pC-collagen and then to collagen. The absence of disulfide bonds in the chymotrypsin digest ruled out the presence of newly synthesized type III collagen, and the lack of any similarity to 14.5-d PYS components ruled out the presence of newly synthesized type IV procollagen or other basement membrane components (Figs. 8, 10, and 11).

In general, these findings are similar to those described by Adamson and Ayers (1) and Adamson et al. (2) for mouse VYS and PYS at similar gestational ages. For example, they reported that 13- to 16-d mouse PYS synthesized only type IV collagen, whereas mouse VYS synthesized primarily type I collagen. These authors, however, found no evidence of type I pro-α or p-α chains, but primarily α1(I) and α2(I). Furthermore, they suggested the presence of a small proportion of newly synthesized α chains from collagen types IV and V in VYS mesoderm (1). These collagens were not detected in our VYS cultures. It is possible, however, that these collagens are being synthesized in very small amounts in rat VYS or that they are synthesized at different gestational ages.

Speculation on the Cell Responsible for Type I Collagen Synthesis

Adamson and Ayers (1) enzymically dissected the mouse VYS nominally into endoderm and mesoderm. By SDS PAGE they showed that type I collagen was the major constituent synthesized by both fragments, but they did not identify the cellular elements that were present in each one. In particular, they did not note the presence of mesenchymal cells in their preparations. Since we did not separate the VYS elements from each other, we cannot say which cells are active in the synthesis of the type I procollagen that we detected. However, it seems unlikely to us that either the visceral epithelium or the mesothelium would be synthesizing type I procollagen and secreting it into the stroma across their respective basement membranes. In this regard, mesenchymal (fibroblastlike) cells have been previously observed in the rat VYS stroma (39). This situation is similar to that of the cornea, for example, where Descemet's membrane (DM) separates an epithelial cell (corneal endothelium) from the connective tissue stroma. In this case, the stroma is synthesized by fibroblasts in the matrix, whereas DM is elaborated by the corneal endothelium (22). We would like to propose, therefore, that the VYS stroma contains a fibroblast-like (mesenchymal) cell whose origin is unknown and which is responsible for the collagen synthesis which was observed. We have noted the presence of a cell embedded in the stromal matrix (Figs. 1 and 3 *a*), but we cannot yet say if this is the cell responsible for type I collagen synthesis. This situation, however, could explain why the majority of the newly synthesized protein remained associated with the tissue during incubation. If the peripheral cells (epithelium and mesothelium) were responsible for the collagen synthesis, one might expect the medium to contain a large proportion of the newly synthesized protein as was the case for the PYS (10). Further studies must be done to resolve this issue.

Preliminary Identification of α-Fetoprotein Synthesized by VYS in Vitro

Although no specific experiments were performed to identify the bacterial collagenase-resistant component (Fig. 9, lane *3*), it was presumed to be α-fetoprotein (AFP). This is not unreasonable, since the visceral epithelial cells of the rodent yolk sac are known to synthesize and secrete this protein (15, 46). Note that this component, a product of a peripheral cell, was found only in the medium samples (Fig. 8, compare lane *2* with *5*), and was not precipitable by 30% saturated ammonium sulfate (Fig. 8, lane *6*). These are characteristics which one would attribute to a circulating albuminlike protein.

Recently, it has been suggested that human yolk sac carcinomas resemble the normal rodent yolk sac in that they synthesize large amounts of AFP and epithelial basement membrane components (12). As a model of the human disease, parietovisceral yolk sac (PVYS) carcinomas have been produced in inbred rats (13). These tumors contain the cellular elements of both the PYS and VYS, and appear to synthesize both AFP and basement membrane components. Since this rat tumor is a good model of the human disease, a knowledge of the biosynthetic capacity of normal rat yolk sac constituents

may be helpful for an understanding of the biology of PVYS carcinomas in humans.

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