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AN ULTRASTRUCTURAL ANALYSIS OF THE PHYSICAL ORGANIZATION OF COLLAGENOUS (TYPE I) MATRICES: ONE DETERMINANT OF UROTHELIUM MAINTENANCE IN VITRO

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Abstract

Collagenous matrices, used as cell culture substrata, can be prepared from different collagen types in a variety of forms using a range of polymerization procedures. Type I collagen has been most frequently used either as dried collagen films or hydrated collagen gels. Sheets of isolated bladder urothelium, when plated onto such matrices prepared from type I collagen by different polymerization methods (eg. air-drying; NaOH; NaCl; NH_3; or NH_3 followed by glutaraldehyde crosslinking) demonstrate the capability of urothelial cells to attach to a variety of differently prepared matrices irrespective of polymerization procedure. In contrast, both cell proliferation and maintenance of the urothelium are markedly influenced by the polymerized form of the collagen matrix.

Comparative ultrastructural (scanning and transmission electron microscopy) analysis of these matrices demonstrates dissimilarities in their physical organization. The level of filamentous, fibrillar or fibrous reaggregation of solubilized collagen molecules varies in relation to the polymerization procedure used viz, a) air dried matrices form a dense meshwork of many forms of collagen fibrils and associated filaments with an irregular surface array of coarser collagen fibres; b) matrices prepared by NaOH, NaCl and NH_3 polymerization present no major differences and form a felt of interlocking collagen fibres with discrete filamentous networks associated with these fibres; and c) matrices polymerized by NH_3 and crosslinked with glutaraldehyde form a dense meshwork of filaments with a more occasional distribution of fibrils associated with filaments or dense "amorphous" aggregates.

The level of supramolecular reassemblage of solubilized collagen may be, therefore, a significant factor in determining urothelial cell growth and differentiation on collagen matrices.

Key Words: Stereo-transmission electron microscopy; scanning electron microscopy; collagen substrata; collagen ultrastructure; urothelium; tissue culture.

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Introduction

In recent years, collagen, a major component of the extracellular matrix (ECM) and found in some form in most tissues of the body, has become increasingly used as a substratum for tissue culture. A wealth of literature and the wide variety of uses clearly show the interest and the difficulties encountered in studying collagenous matrices as a substratum for cultured cells (reviewed recently by Bissell 1981; Hay 1981; Kleinman et al. 1981, 1982; Yang and Nandi 1983; Allen et al. 1984).

In vivo, collagen occurs in several structurally distinct forms (Bornstein & Sage 1980; Eyre 1980; Hay 1981; Linsenmayer 1981) which are products of separate genes and exist in tissues of the same animal associated with either bone, tendon, skin (Type I); cartilage, cornea vitreous body (Type II); skin, spleen, blood vessels, smooth muscle (Types I, III); basement membrane (Type IV); interstitial tissues (Types I, II, III); blood vessels, smooth muscle, skin, placenta (Type V). In regular association with the collagens are to be found various attachment proteins (such as fibronectin, chondronectin and laminin), proteoglycans and glycosaminoglycans. These and various other components of the extracellular matrix (including collagen) can have a significant influence on cell morphology and function and have been recognized as specific activators in cellular attachment or adhesion, contraction, proliferation, migration and, in some cases, differentiation as indicated in a selection of recent studies and reviews (Reid and Rojkind 1979; Schor and Court 1979; Schor 1980; Kleinman et al. 1981, 1982; Bellows et al. 1981; Chambard et al. 1981; Grinnell & Bennett 1981; Schor et al. 1981; Bissel et al. 1982; Yang and Nandi 1983).

This literature also establishes that collagenous matrices used as a cell culture substratum can be prepared from different collagen types in a variety of forms using a range of polymerization procedures. Collagen extracts containing primarily type I collagen have been most frequently used for tissue culture. This is true despite the fact that other types of collagen, genetically distinct from the collagen used in cell culture, may be part of the normal in vivo substratum for particular cell types; and, that evidence from in vitro studies indicates the possibility of marked differences in biological effect with collagen type (Murray et al. 1979; Salomon et al. 1981; Kleinman et al. 1982). Collagen has been mostly employed in the form of thin films of dried collagen and of attached or floating rafts of hydrated collagen gels to provide so-called two- or three-dimensional substrata for cell behavioural studies (Kleinman et al. 1982, Grinnell and Bennett 1982; Yang and Nandi 1983). A striking feature recorded in the literature is the marked influence of the physical form of the collagen substratum on the cell phenotype (Bissell et al. 1982; Grinnell and Bennett 1982; Lee et al. 1984). Furthermore, differences in the molecular organization of the collagen within the matrix, as a consequence of the method of preparation, may also markedly influence the biological effect of the substratum (Grinnell and Minter 1978; Schor and Court 1979; Hawrot 1980; Rubin et al. 1981; Yang and Nandi 1983).

Because of the possible importance of the physical state of the substratum as a parameter in directing the phenotypic expression of cells in culture this article explores the topographical nature of matrices prepared from type I collagen by different methodologies in order a) to appreciate the dissimilarity between matrices and, b) to propose possible mechanisms by which such matrices may modulate selective cellular response as specifically investigated with respect to the growth and differentiation of urothelial cells in vitro.

Preparation and ultrastructure of collagenous (Type I) matrices

Collagenous matrices may be prepared by a number of different procedures (outlined in Table 1) using extracts of type I collagen obtained from various sources including, most commonly, rat tail tendon and rat, mouse, guinea-pig or bovine skin (Reid and Rojkind 1979; Grinnell & Bennett 1982; Kleinman et al. 1982; Miller & Rhodes 1982). Until recently, it was assumed that these collagenous matrices, irrespective of their mode of preparation, were equivalent in terms of ultrastructure and capacity to promote attachment, growth and differentiation. But, present evidence now indicates that even minor changes in the preparation of collagen substrata can impose marked differences in their physical organization (summarized in Table 2) and in their promotion of cell growth and function (Iversen et al. 1981; Kleinman et al. 1981, 1982; Grinnell and Bennett 1982; Yang and Nandi 1983).

Other collagen types (Reid and Rojkind 1979), collagen-hydroxyethylmethacrylate hydrogels (Civerchia-Perez et al. 1980; Carbonetto et al. 1982), and various matrix-containing tissues such as demineralized bone matrix (Nogami and Urist 1970; Nathanson et al. 1978) or bone powder (Nakagawa & Urist 1977); frozen-killed lens, lens capsule or corneal stroma (Dodson & Hay 1974; Meier and Hay 1974) or embryonic dermis (Dodson 1967); salt-extracted devitalized cartilage (Pauli et al. 1981); and amniotic membrane (Liotta et al. 1980; Russo et al. 1983) have been also successfully used as substrata to culture cells and promote their differentiation, and reference should be made to this literature for specific methods of preparation.

In vivo, Type I collagen exists primarily as ropelike fibres consisting of bundles of fibrils: ordered assemblies of collagen molecules form the 4–5 nm diameter microfibrils which further associate to constitute the approximately 100 nm collagen fibril on which the regular cross-banding pattern of 67 nm periodicity can be seen. Under appropriate conditions in vitro, collagen molecules can be induced to aggregate into levels of structural organization similar to those which exist in vivo. The mode of molecular assembly and of fibril and fibre construction is dependent on a variety of factors such as pH, ionic strength, temperature and presence of various compounds including proteoglycans; under specific conditions collagen molecules can be caused to associate together in ways which do not occur naturally (for further discussion see Bornstein and Traub 1979; Bornstein and Sage 1980; Hay 1981; Linsenmayer 1981; Cheung and Nimni 1982).

Collagen as a substrate for urothelial growth and differentiation

Collagen, as indicated earlier, has been used frequently for establishing primary cultures of cells from a range of tissues including different epithelia. While various of these cell types have been found to require fibronectin or other extracellular matrix components for attachment to collagen substrata in vitro, some cell types have been shown capable of attachment in the absence of these matrix molecules (Kleinman et al. 1981; 1982; Schor et al. 1981; Yang and Nandi 1983).

Recent studies have reported the short-term in vitro culture of isolated normal urothelial cells both on glass or on plastic (Elliott et al. 1975; Berky & Zolotor 1977; Bonar et al. 1977; Roszell et al. 1977; Chlapowski 1978; Herz et al. 1979; Pauli et al. 1980; Pauli & Weinstein 1981); and on collagenous substrata (Chlapowski & Haynes 1979; Pauli et al. 1980; Chlapowski et al. 1983; Leighton et al. 1984) while a range of continuous neoplastic urothelial cell lines derived from experimentally transformed urothelial cultures or from bladder tumours have been established on conventional tissue culture substrata (Summerhayes 1979; Hepburn & Masters 1983). It has been demonstrated from such studies that isolated normal urothelial cells will grow as monolayers or as incompletely differentiated bilayers on glass or plastic surfaces but, as more differentiated bi- to tri-layers on Type I collagenous matrices. There is evidence, however, to suggest that the method of collagen preparation may effect a differential urothelial response in that, whereas growth and some degree of differentiation has been obtained when urothelial cells are grown on a collagen substratum polymerized by NH_3 exposure and then glutaraldehyde cross-linked (see Table 1) (Chlapowski & Haynes 1979; Pauli et al. 1980; Chlapowski et al. 1983), only attachment and limited maintenance but no growth has been noted when isolated urothelial sheets are plated onto Type I air dried or sodium hydroxide polymerized collagen substrata (Norman 1982).

In that cell attachment and growth, and maintenance of a differentiated phenotype may be influenced by the chemical and physical properties of the substratum (for recent discussions see Klebe et al. 1977; Schor & Court 1979; Hay 1981; Schor et al. 1981; Kleinman et al. 1981, 1982; Bissell et al. 1982) then among significant questions to ask is whether the reaction of isolated urothelium to different collagenous matrices is due to modifications in the physical organization of these substrata, to the presence of specific mediator proteins complexed with the collagen or to a combination of these factors. We have approached these questions here a) by comparing, in preliminary experiments, the attachment and maintenance of isolated urothelial sheets to Type I collagenous matrices prepared in a variety of ways, with b) special emphasis placed on a combined scanning and transmission electron microscope (SEM & TEM) study of the structural characteristics of these matrices.

Materials and Methods

Preparation of collagen stock solutions

Type I collagen was extracted from rat tail tendon by a modification of Bornstein's method (1958) as described by Hallowes et al. (1980), and stored either as a clear viscous solution or as lyophilized aliquots at 4°C.

Purity and characterization of the collagen stock solution was determined by polyacrylamide gel electrophoresis (Laemmli 1970): this established the absence of significant amounts of non-collagenous protein in the collagen preparation (Howlett 1984).

The concentration of collagen in the stock solution was determined against reference Type I collagen (Calbiochem-Behring, C.P. Laboratories Ltd., Bishop's Stortford, Herts, England) by quantitative densitometric scanning analysis of the Coomassie blue-stained collagen chains resolved from the SDS-polyacrylamide gels, and then adjusted to 2–2.5 mgs/ml with 0.001% aq. glacial acetic acid.

Preparation of collagen substrata

All substrata were prepared either on membrane filters (MF-type composed of mixtures of cellulose acetate and cellulose nitrate, 3.0 μm pore dia, Millipore (UK) Ltd., Harrow, Middlesex, England) or, on discs of nylon 30 μm^2-mesh fabric (Henry Simon, Manchester, England) (Cereijido et al. 1978).

The substrata were prepared on the basis of a series of standard collagen polymerization methods to give two-dimensional or three-dimensional collagenous matrices (see Table 1).

Two-dimensional collagen matrices

i) Aliquots of non-lyophilized (NL) collagen solution (ca. 2 mg/ml) were applied to the support material and allowed to air dry (AD) at ambient temperature in a stream of sterile air for 2–3 h (termed AD (NL) collagen).

ii) Aliquots of non-lyophilized (NL) collagen solution (ca. 2 mg/ml) were polymerized by exposure to an NH_3 atmosphere (derived from a concentrated NH_4OH solution) for 3–4 min, at ambient temperature (termed NH_3 (NL) collagen).

iii) Aliquots of either non-lyophilized (NL) (ca. 2mg/ml) or lyophilized (L) (reconstituted to 5 mg/ml in 3% HoAc) collagen solution were polymerized as above ii) and the collagen matrix cross-linked by treatment with 4% aqueous glutaraldehyde for 90 min at ambient temperature (termed NH_3/glut (NL) or (L) collagen).

Three-dimensional collagen matrices

Aliquots of collagen solution (ca. 2 mg/ml) were brought to physiological ionic strength and pH at 4°C by incubation either:

iv) with a NaOH gelling solution (1:2 v/v 0.15M aq NaOH: 10x concentrated Dulbecco's modification of Eagle's Minimum Essential Medium (DMEM) (Flow Laboratories, Irvine, Scotland) containing 24.5 mg/ml $NaHCO_3$ and 0.6 mg/ml glutamine) viz: 2.5 ml samples of a 9:1 v/v collagen: NaOH gelling solution mix maintained at 4°C were gelled by incubation of the coated supports for 15–30 min in a humidified atmosphere at 37°C (termed NaOH collagen); or,

v) with a NaCl gelling solution (6% aq NaCl) viz: 2.5 ml samples of a 4:1 v/v collagen: NaCl gelling solution mix maintained at 4°C were gelled by incubation for 60 min in a humidified atmosphere at 37°C (termed NaCl collagen).

At the end of the respective polymerization procedures and after thorough washing in several changes of sterile 0.9% sodium chloride, the collagen substratum preparations were sterilized by exposure to ultraviolet (UV) irradiation, using a 15W UV light source (Philips G15 T8, Holland) at a distance of 35 cms for 90 min, and either processed immediately for TEM or SEM as described below, or used as culture substrate.

Culture of bladder urothelium

Sheets of urothelial tissue, devoid of stromal elements and basal lamina, were isolated from urinary bladders of young female Wistar rats (120–140g) by a trypsin-pancreatin dissociation procedure as previously described (Hodges et al. 1977; Norman 1982). The resulting urothelial pieces were plated onto the surfaces of the variously prepared collagenous matrices (conditioned overnight in DMEM medium supplemented with 10% newborn calf serum) and maintained in vitro using a previously established organ culture system (Hodges et al. 1977).

Electron Microscopy

For TEM, specimens were fixed at ambient temperature for 2h with 2.5% glutaraldehyde in 0.1M Sorensen's buffer pH7.2, post-fixed for 1h with 1% aqueous osmium tetroxide followed by two buffer washes and for 30 min with 1% aqueous uranyl acetate followed by two further buffer washes; dehydrated through graded methanols, and embedded in Araldite (Fluka AG, Buchs SG, Switzerland) using propylene oxide as the transitional medium. Sections were cut vertical to the plane of the specimen. Thick sections (1 μm) were stained with toluidine blue for light microscopy; thin silver/pale gold sections were stained with uranyl acetate and lead citrate for viewing in a Philips 301 G TEM equipped with a 60°C eucentric goniometer stage. Stereo pairs were chosen from groups of electron micrographs, taken at x9,800 or x36,000 with tilt angles of +6°, +3°, 0°, or −6°, of selected areas in the routine section. Prints (x3) were examined with an SB folding mirror stereoscope (Cartographic Engineering Ltd., Salisbury, Wilts, UK).

For SEM, specimens were fixed for a minimum period of 2h at ambient temperature with 2.5% glutaraldehyde in 0.1 M Sorensen's buffer pH 7.2. Specimens were stored in the fixative at 4°C until required for further processing which involved a modified thiocarbohydrazide-osmium procedure (Hodges et al. 1977), then dehydration through graded ethanols, critical point drying from liquid carbon dioxide and platinum coating (ca. 20 nm) for viewing in a JEOL JSM-35 SEM using an accelerating voltage of 25kV.

Observations

Behaviour of isolated urothelium on different collagenous (Type I) substrata

After 3 weeks of culture, the pieces of isolated urothelium plated onto AD, NH_3, NaOH, or NaCl-collagen substrata showed progressive degenerative changes. Scanning electron microscopy demonstrated generally fragmented tissues with individual cells often rounded, frequently blebbed, and cell surfaces grossly pitted (Figs. 1,2).

In contrast, SEM of urothelia plated onto NH_3/glut collagen substrata demonstrated expanded sheets of tissue composed of flat, polygonal cells (Figs 3,4) with surface configurations (Fig. 5) suggestive of either immature, early to late differentiating intermediate-type cells or benign hyperplastic cells (cf. Hodges et al. 1977). The growth and ultrastructural characteristics of the urothelial cultures on the different experimental series of collagenous substrata will be described in detail elsewhere (Howlett et al., in preparation; Norman and Hodges, in preparation.

Structural characterisation of different collagenous (Type I) substrata

The physical organisation of the different collagen substrata were assessed by both scanning and transmission electron microscopy. Differences in the matrix pattern of these substrata could

Table 1

Preparation of Type I collagenous matrices: outline of procedures

Collagen		Matrix Preparation[1,2]	Selected References
Source	Solvent System		
A. Dried collagen films → 2-dimensional substrate[3]			
Rat tail Lathyritic rat skin Ascaris	Acetic acid or neutral salt	Either i) Aliquots of collagen or lyophilized purified collagen solutions (ca. 1–2 mg/ml) spread on tissue culture support. Air-dried at ambient temperature or 37°C–50°C for 1–40 h under normal atmospheric pressure or under vacuum.	Ehrmann & Gey 1956 Elsdale & Bard 1972 Grinnell & Minter 1978 Hallowes et al. 1980 Iversen et al. 1981. Jaros et al. 1975 Ketley et al. 1976 Kleinman et al. 1979 Michalopoulos & Pitot 1975 Murray et al. 1979
Bovine dermis (Vitrogen 100[4])			Hallowes et al. 1980
Chick embryonic muscle			Ketley et al. 1976
Foetal calf, rat, rabbit skin			Rubin et al. 1981
Rat tail Lathyritic rat skin Ascaris Bovine dermis (Vitrogen 100[5])	Acetic acid or neutral salt	ii)[5] Aliquots of collagen solution (ca. 2 mg/ml) polymerized at 22°C for 5–30 min in presence of NH_3 vapors. Collagen films then air-dried for up to 48h at 22°C.	Bornstein 1958 Ehrman & Gey 1956 Elsdale & Bard 1972 Grinnell & Bennett 1981, 1982 Grinnel & Minter 1978 Iversen et al. 1981 Kleinman et al. 1979 Linsenmayer et al. 1978 Schor & Court 1979
		iii)[5] As Aii) then collagen film treated with glutaraldehyde (4% aq) for 2–5 min at ambient temperature	Cereijido et al. 1978 Chlapowski & Haynes 1979 Chalpowski et al. 1983
		iv) As Aii) but collagen films treated with 8M urea for 10 min after NH_3 exposure	Klebe 1974 Schor & Court 1979
Rat tail, rat, rabbit skin, foetal calf, human skin		or v) Collagen solution incubated at temperatures between 37°C–80°C for 10 min–40 h before or after spreading on tissue culture support. Heat-denatured collagen solution used to prepare collagen film as Ai) or iv)	Delvos et al. 1982 Grinnell & Minter 1978 Haston et al. 1982 Kasai et al. 1983 Linsenmayer et al. 1978 Michalopoulos & Pitot 1975 Rubin et al. 1981 Schor & Court 1979

Table 1 continued on next page

Table 1 continued

Preparation of Type I collagenous matrices: outline of procedures

Collagen		Matrix Preparation^{1,2}	Selected References
Source	Solvent System		
B. Hydrated collagen gels → 2 and 3-dimensional substrates^6			
Rat tail Lathyritic rat skin Ascaris Bovine dermis (Vitrogen 100^5)	Acetic acid or neutral salt	Either i) Collagen solution (ca. 2 mg/ml) brought to physiological ionic strength and pH at 4°C by addition of 10x concentrated tissue culture medium and NaOH or $NaHCO_3$ Collagen polymerizes within 5–60 min at 22°–37°C	Bellows et al. 1981 Bornstein 1958 Elsdale & Bard 1972 Emerman & Pitelka 1977 Grinnell & Bennett 1981, 1982 Grinnell & Minter 1978 Hallowes et al. 1980 Iversen et al. 1981 Kleinman et al. 1979 Michalopoulos & Pitot 1975 Richards et al. 1983 Sanders & Prasad 1983 Schor & Court 1979
		ii) as Bi) Hydrated collagen gels then exposed to 8M urea for 10 min at ambient temperature	Elsdale & Bard 1972 Schor & Court 1979
		iii) Collagen solution (ca. 2 mg/ml) mixed with saline solution (eg. 6% aq NaCl) at 4°C. Collagen polymerized by incubation for 15–60 min or up to 18h at 22°C or 37°C	Haushka & Konigsberg 1966 Iversen et al. 1981 Jaros et al. 1975 Kleinman et al. 1979 Lillie et al. 1980 Reid & Rojkind 1979 Rubin et al. 1981
Rat tail Bovine dermis (Vitrogen 100^5)	Acetic acid	or iv) Collagen solution mixed with riboflavin –5'–phosphate (0.01–0.05% aq) and polymerized into gel by 5–30 min exposure to bright light (ultraviolet or incandescent)	Hanson & Partlow 1978 Iversen et al. 1981 Masurovsky & Peterson 1973

Notes: 1 Matrices may be sterilized by irradiation with UV light overnight (Kleinman et al. 1982).

2 Matrices may require conditioning by several changes of tissue culture medium.

3 Matrices on which cells are grown on the surface aspect only.

4 Vitrogen 100 – Collagen Corporation, Palo Alto, CA, USA.

5 Matrices used as dried collagen films or hydrated collagen gels.

6 Matrices on which cells are grown both on the surface aspect and within the internal structure.

be distinguished by SEM which provided a broad surface view (cf Figs. 7,8,10–12,15–17) but resolution of the TEM was required to distinguish the various modes of polymerisation of the collagen (cf Figs. 6,9,13,14,18,19).

These data showed the solubilized collagen molecules to reaggregate into structurally heterogenous assemblages with the level of fibrillar or fibrous aggregation varying in relation to the polymerization procedure used (Table 3). Within the range of structural forms identified were either smooth striated collagen fibrils in a wide range of diameters (0.1 μm to 15 nm) (although cross-striations could frequently be seen, their absence on occasion could be attributed to an unsatisfactory angle of viewing) or loose packing of filaments in the order of 2 nm diameter which were the thinnest linear structures observed. Fibrillar morphology was unusual in several respects in that collagen fibrils a) might suddenly end and splay out into the filamentous form; they might be curved or tapered; or they might divide and join another fibril; b) filaments might join the side of such collagen fibrils, and on occasion a bundle of fibrils in one direction would split and parts of some fibrils join others in a different bundle; and c) when the fibrils were associated in fibres, they frequently appeared to be linked by interconnecting filaments. The interrelations of these structures were confused in single TEM micrographs, and the finer details were best resolved in stereo pairs (Fig. 6a–e). In addition to the fibrillar structures, there were places where the filaments associated as networks. Rather amorphous granules about 5 to 25 nm in diameter occurred at their intersections (Fig. 6e).

Table 2

Reported Physical Organization of Collagenous Matrices: Effect of Polymerization Method

Matrix Preparation	Ultrastructure	Reference
A. Dried collagen films		
Air drying [see Table 1Ai]	Small number of fibrils; largely amorphous material	Iversen et al. 1981
Air drying + NH_3 treatment [see Table 1 Aii]	Primarily fibrillar: occasional amorphous areas.	Iversen et al. 1981.
Air drying + NH_3 treatment [see Table 1 Aii]	Predominantly amorphous, densely packed material; occasional collagen fibrils	Grinnell & Bennett 1981, 1982
B. Hydrated collagen gels		
Sodium chloride [see Table 1Biii]	Meshwork of collagen fibrils. Dense fibrillar structure, collagen fibrils 100–200 nm in dia.	Iversen et al. 1981 Lillie et al. 1980
UV light [see Table 1 Biv]	Globular, non fibrillar collagen aggregates	Iversen et al. 1981
Sodium hydroxide [see Table 1Bi]	Interlaced network of fine collagen fibrils, approx. 50–100 nm in dia.	Grinnell & Bennett 1981, 1982
Sodium bicarbonate [see Table 1Bi]	Dense meshwork of randomly oriented fibres, varying between 90–400 nm in dia.	Schor et al. 1980

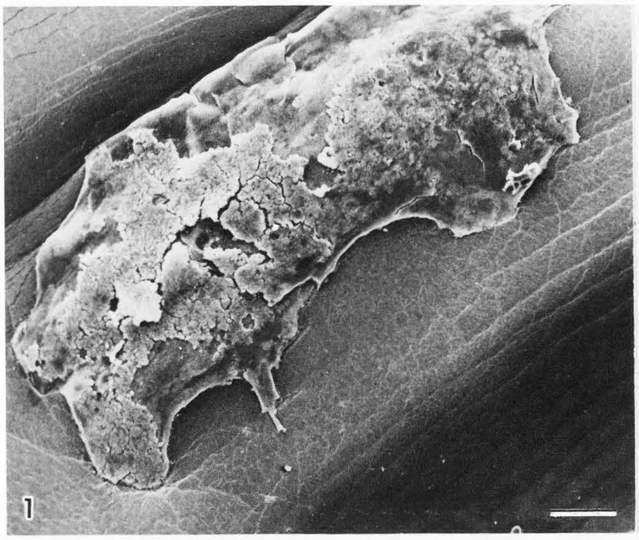
Table 3

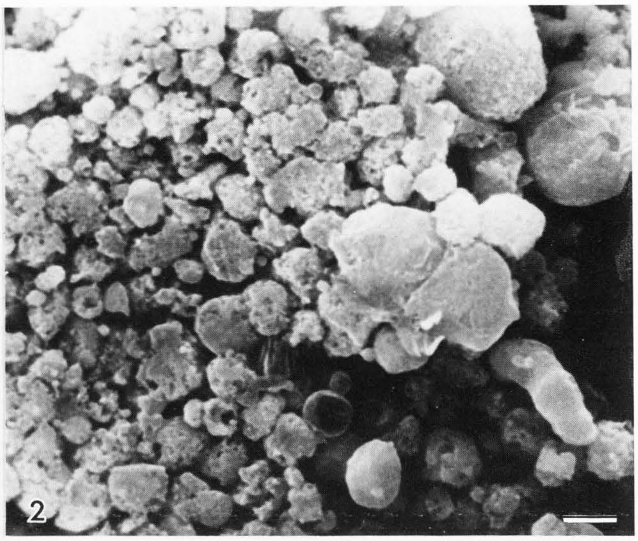
Summary of salient morphological features of collagenous (Type I) matrices prepared by different polymerization procedures

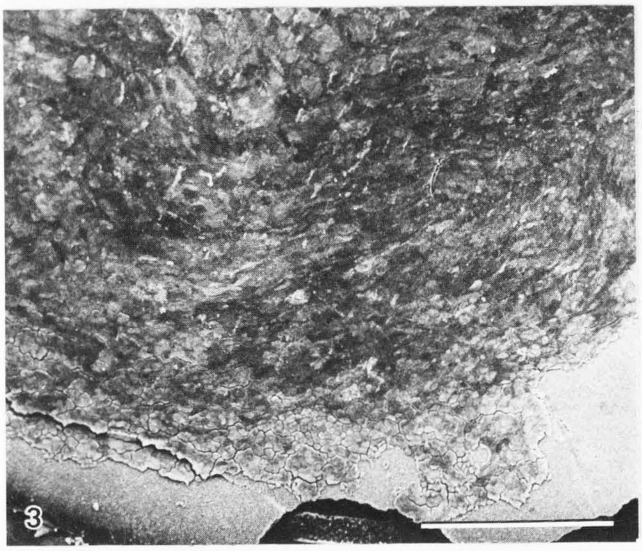
Preparation	Collagen cross-striations	Main features
AD collagen matrix	Present	–Spiral surface collagen fibres –Atypical range of fibrillar structures in a meshwork –Filaments
NaOH collagen matrix NaCl collagen matrix NH_3 collagen matrix	Present	–Felt of interlocking fibres –Discrete filamentous networks
NH_3/glut collagen matrix	Not observed	–Method needs rigorous control –Fine filament meshwork associated with globular granular masses or –Fibrils associated with filaments or granular accretions

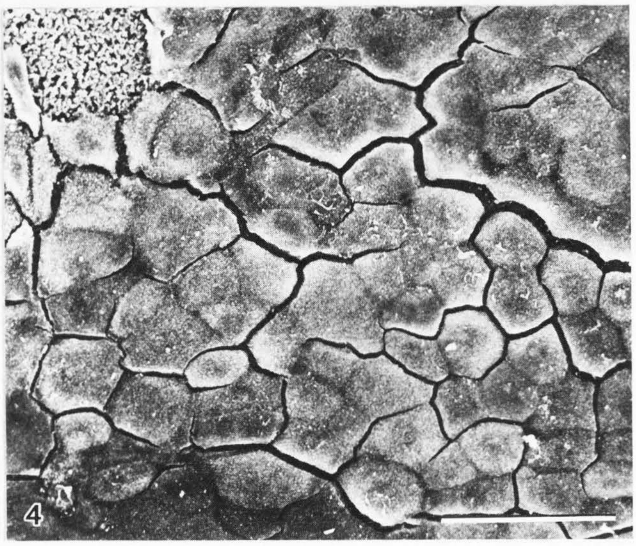
In the air-dried specimens SEM showed the collagen substratum to consist of a dense fibrillar meshwork with an irregular surface array of coarser fibres projecting from this meshwork (Figs. 7,8). These fibres consisted of twisted fibrils which divided to be inserted in the underlying meshwork. TEM (Fig. 9) showed that these ends became merged with the tangle of irregular fibrils and filaments which interlocked to form the superficial layer of the meshwork. TEM also demonstrated the collagen substratum to be stratified into a series of successive layers each layer containing the range of morphological forms of polymerised collagen described above.

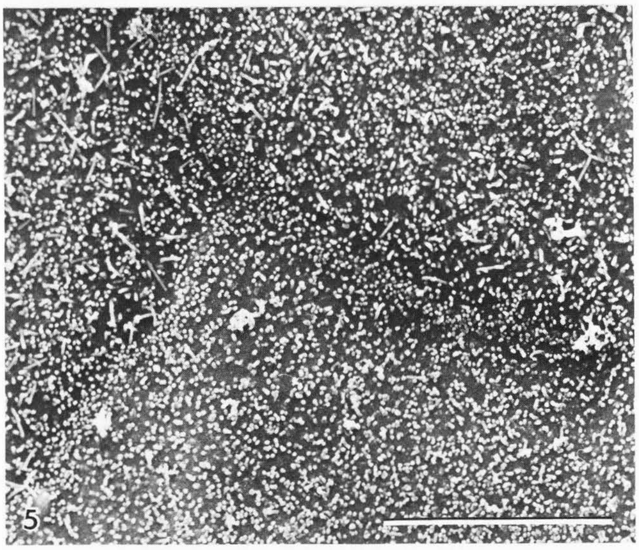
In the NH_3, NaOH and NaCl preparations, both SEM and TEM demonstrated the structural features of these collagen substrata to be broadly similar. By SEM, the substrata were shown to consist of a homogenous felt of interlocking fibres composed of twisted fibrils (Figs. 10–12). Small accretions of material were found to frequently decorate the twisted fibrils, an effect most pronounced in the NaCl specimen (Fig. 12). TEM (Figs. 13, 14) confirmed that the fibres were composed of twisted collagen fibrils; these were often interlinked by the finest filaments, and often divided to join a fibril in another fibre. In places and generally at the intersections between fibres there were circumscribed patches of filamentous networks which appeared to be the basis of the decorations seen by SEM. The felt arrangement











Figs. 1,2. SEM of urothelium on AD collagen, 3 weeks in culture showing continuing attachment but degenerative changes. Bar = 100 μm (Fig. 1); 10 μm (Fig. 2).

Fig. 3: SEM of part of a culture of urothelium on NH_3/glut collagen, 3 weeks in culture, showing attachment and extensive growth (cf. Fig. 1). Bar = 1000 μm.

Fig. 4. Detail of the specimen in Figure 3, showing flat polygonal cells. Bar = 100 μm.

Fig. 5. Cell surface configurations suggestive of early differentiating intermediate-type urothelial cells cultured on NH_3/glut collagen. Bar = 10 μm.

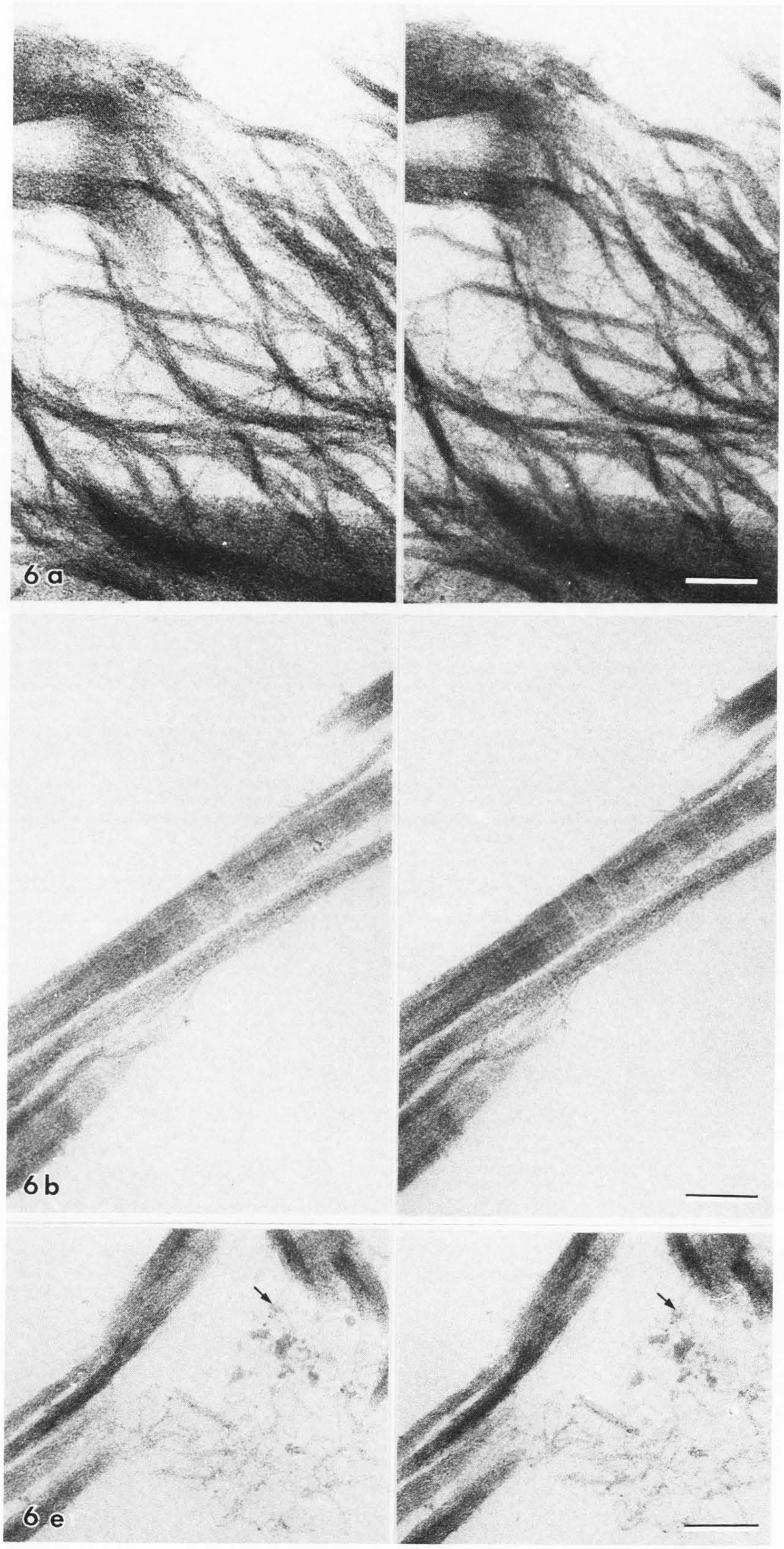
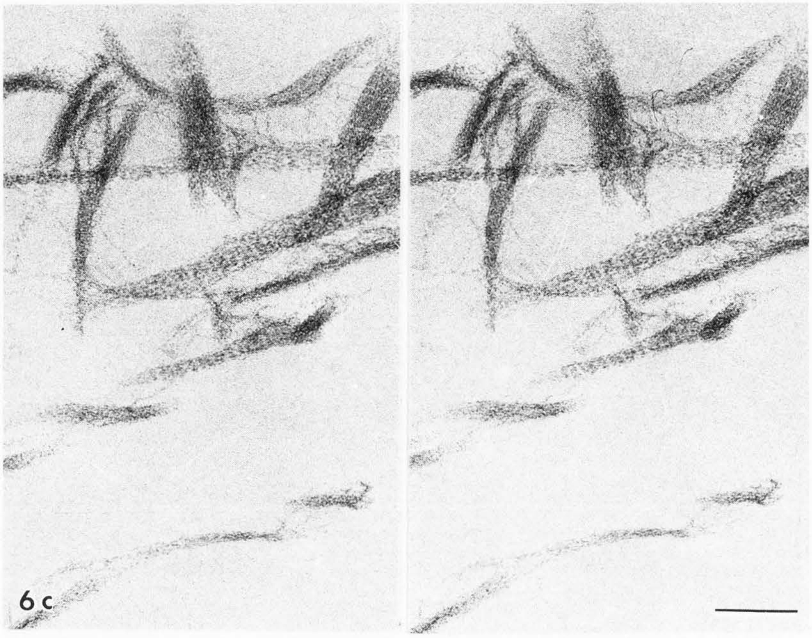


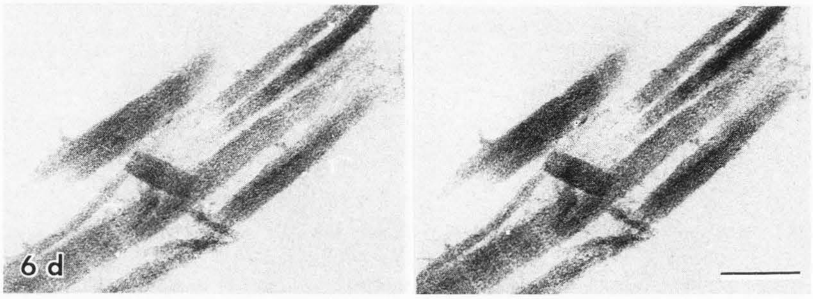
Fig. 6. **TEM stereo pairs to show various modes of collagen molecular assemblies. Bar = 0.1 μm.**

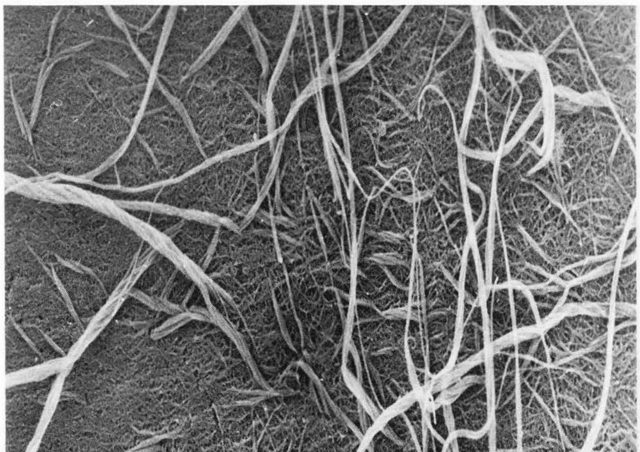
a. AD collagen +3°, −6°

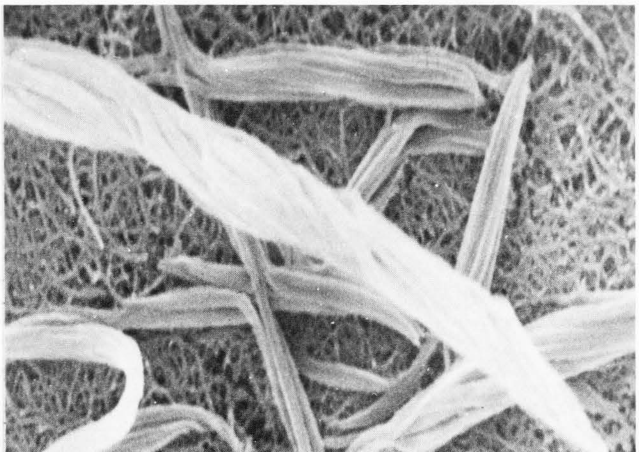
b. NaOH collagen 0°, −6°

c.d.&e. NH_3 collagen +6°, 0° e. shows filamentous net and amorphous granules (arrow).





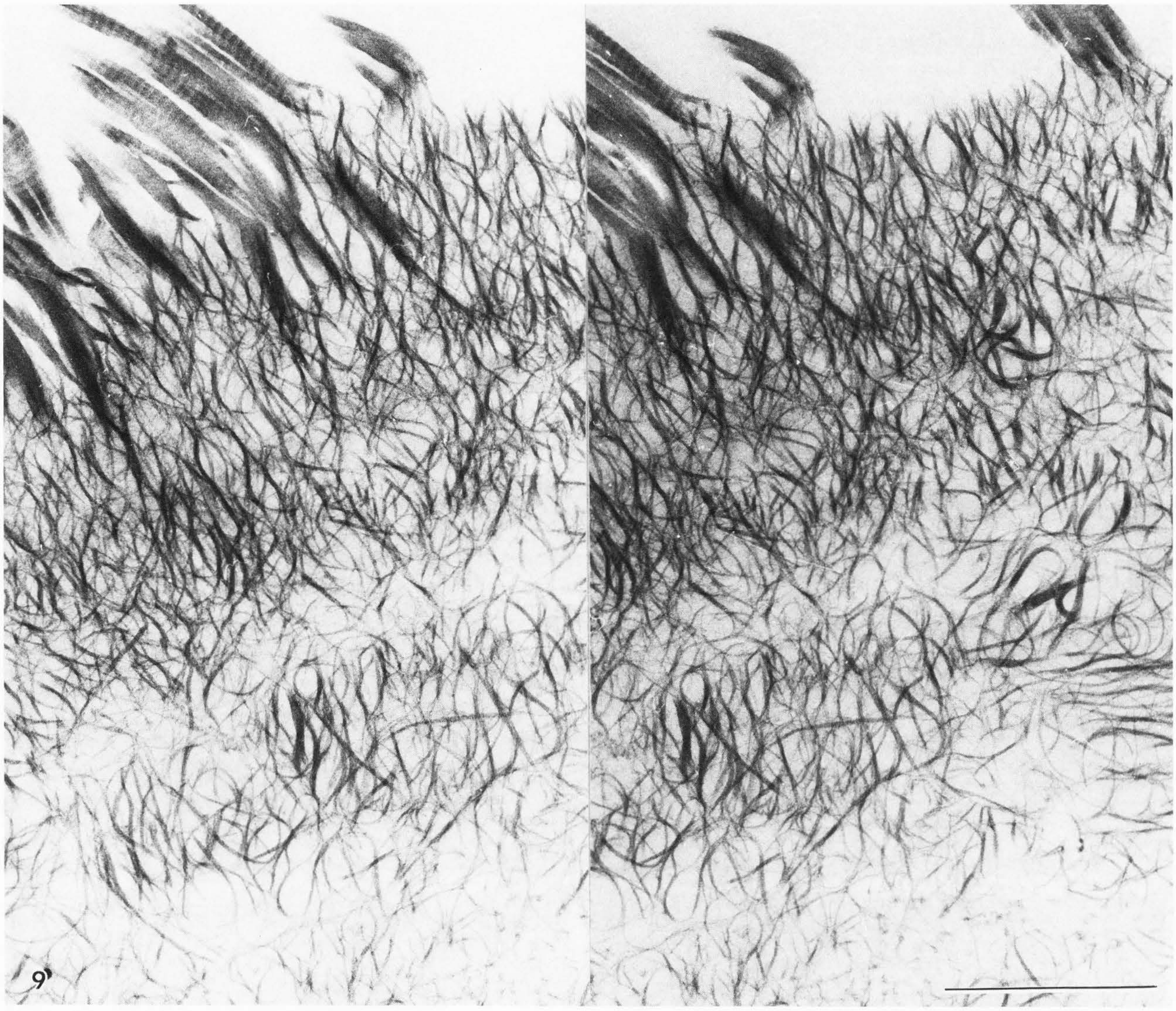




Figs. 7,8. SEM of AD collagen. Bar = 10μm (Fig. 7); 1 μm (Fig. 8).

extended through the full thickness of these preparations. TEM showed that any of the unusual forms described above could occur locally.

In contrast, the physical organization of NH_3/glut collagen preparations was markedly different when observed both by SEM (Figs. 15–17) and TEM (Figs. 18, 19). Although similar overall appearances could be identified between the substrata prepared from non-lyophilized and lyophilized collagen solutions, and between non-sterilized and UV-sterilized preparations



the SEM and TEM appearances varied between samples indicating potential inconsistencies in collagen molecular assemblage that may be imposed by this NH_3/glut method. In terms of surface structure, the substrata were composed to a varying extent of a crust of globular, granular masses (Figs. 15–19) analogous to that described for collagen substrata polymerized by exposure to ultraviolet light in the presence of a catalytic concentration of riboflavin (Iversen et al. 1981). In areas where the presence of these globular masses was minimal or where the surface crust had been disrupted a rather complex lacuna-like system (Figs. 15–17) was exposed. Four general appearances were seen by TEM (Figs. 18,19): a) a filamentous network of varying density compatible with the SEM appearance of pseudo sheet-like structures; b) fibrils with a much denser attachment of filaments than in the other collagen matrix preparations; c) fibrils with few filaments but covered with irregular dense aggregates; and d) morphological forms comparable to that seen in air dried samples.

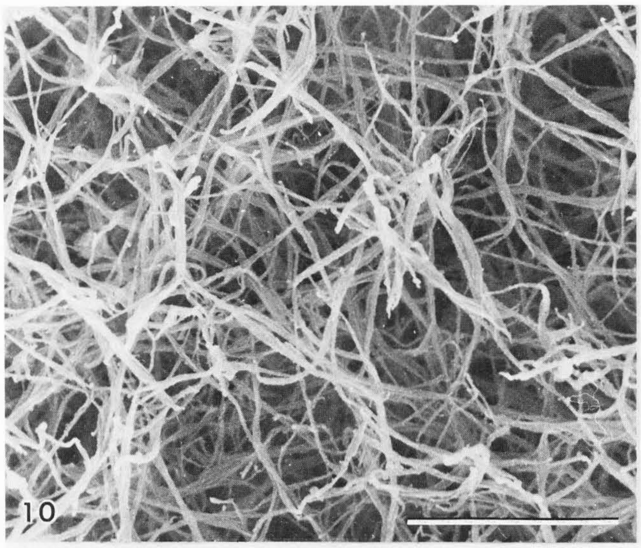
Fig. 9. TEM stereo pair of AD collagen to show the splayed insertion of the coarse projecting twisted fibrils and stratification of the various collagen forms. (Note: lateral fields are included to give more context) −3°, +6°. Bar = 1 μm.

Figs. 10,11: SEM of NaOH collagen. Bar = 10 μm (Fig. 10); 1 μm (Fig. 11).

Fig. 12. SEM of NaCl collagen to show the pronounced decoration on the fibrils. Bar = 1 μm.

Fig. 13. TEM of NaCl collagen showing substructure of the fibres and some accretions (this specimen, which is a negative result after ruthenium red staining shows heightened contrast). Bar = 1 μm.

Fig. 14. TEM stereo-pair of part of the specimen in Fig. 13 to show the filamentous net and its relations with fibrils +3°, −6°. Bar = 0.2 μm.



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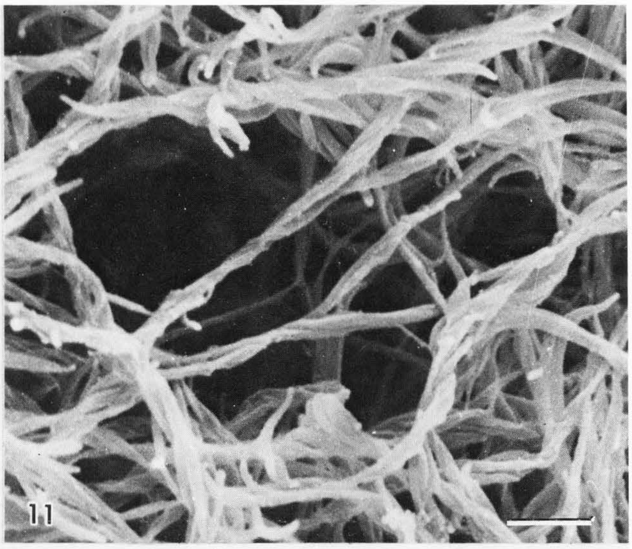

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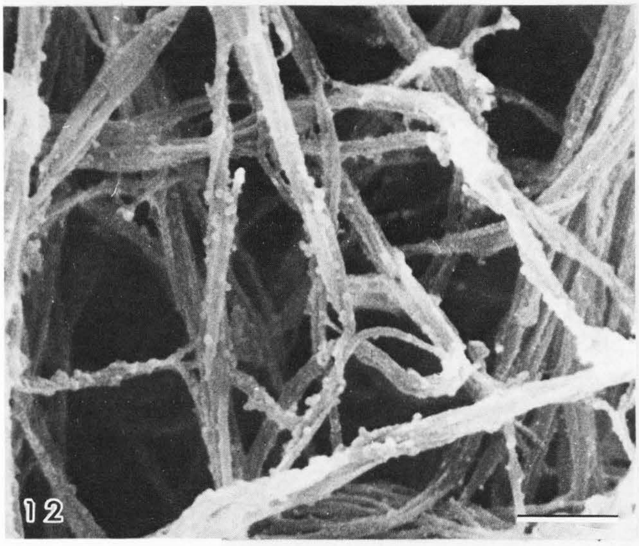

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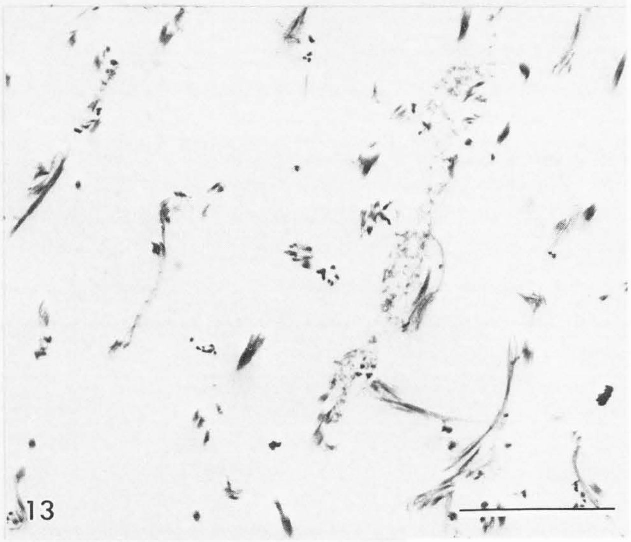

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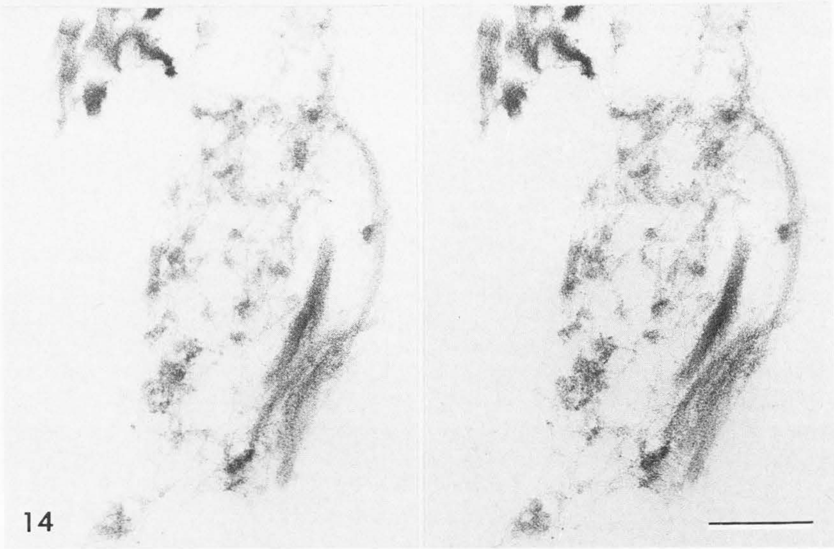

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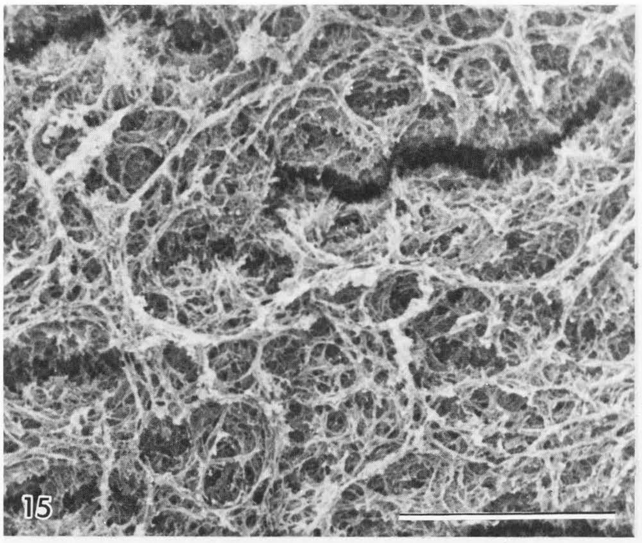
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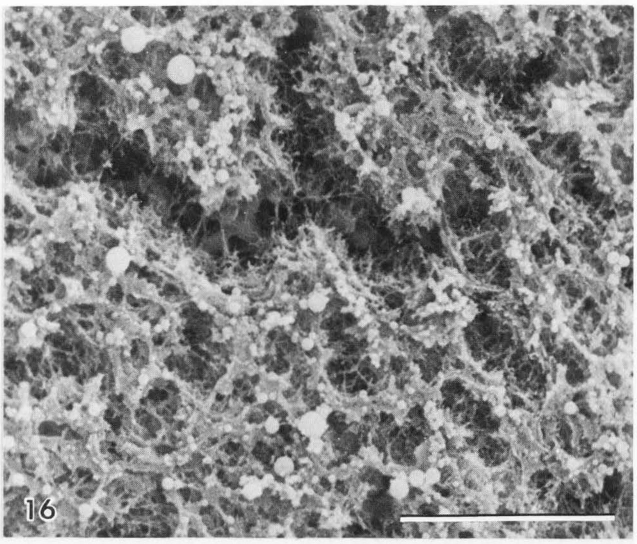
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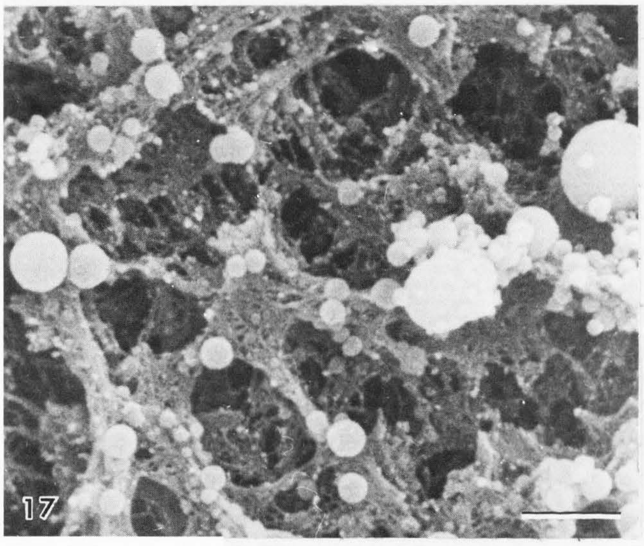
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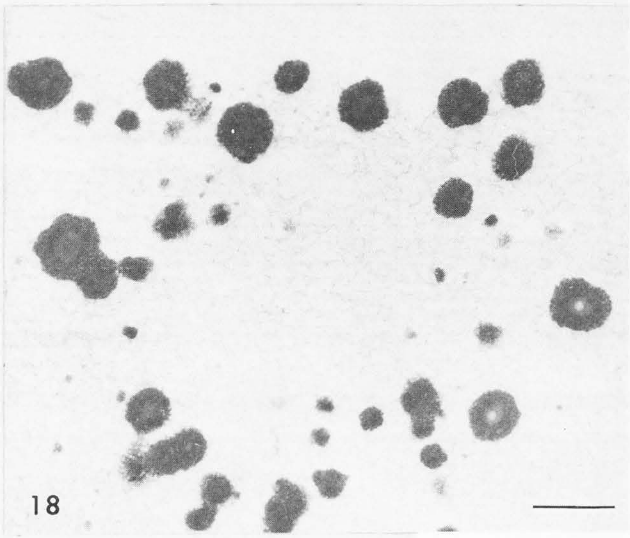
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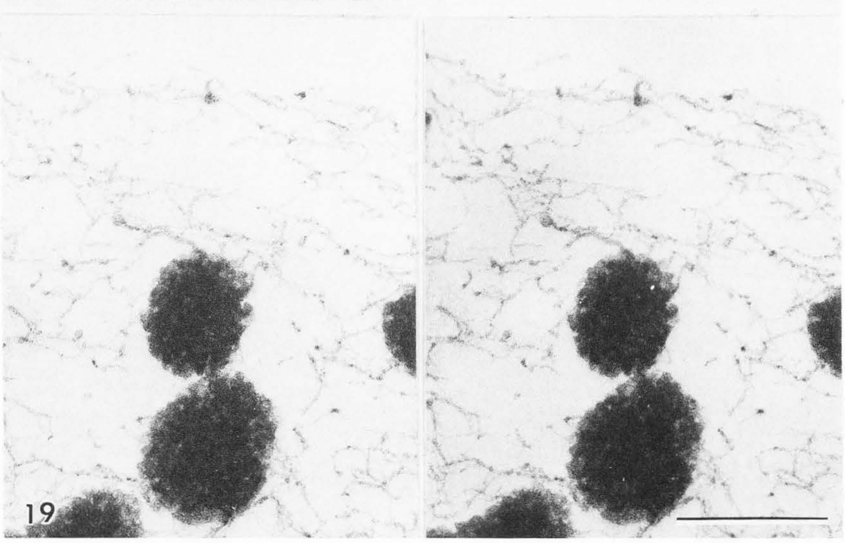
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Fig. 15–17. **SEM of NH_3/glut collagen to show pseudo-sheet like filamentous nets and globular masses. Bar = 10 μm (Figs, 15,16); 1 μm (Fig. 17).**

Fig. 18. **TEM of NH_3/glut collagen to show the filamentous nets and the granular globular masses. Bar = 1 μm.**

Fig. 19. **TEM stereo-pair of an adjacent field to Fig. 18 to show the topography of the fine filaments and their relation to the granular masses +3°, −6°. Bar = 0.2 μm.**

Discussion

Although several previous studies have employed Type I collagen as a substratum in the culture of urothelium with the resulting multicellular outgrowth in vitro showing some of the topographical and phenotypic features of urothelium in vivo (Chlapowski & Haynes 1979; Pauli et al. 1980; Chlapowski et al. 1983; Reznikoff et al. 1983; Leighton et al. 1984) none have critically evaluated the influence of different methods of collagen substratum preparation on the behaviour of the urothelial cells. The development of such an approach could gain further insight into possible collagen-cell interactive processes directing the growth and differentiation and the form and function of urothelial tissue.

Current studies indicate that different cell types appear to use different mechanisms in their attachment to collagen and that the effect of collagen on cell adhesion, growth and differentiation could be direct or be mediated through collagen-bound cell attachment proteins such as fibronectin, laminin or chondronectin (Bissell 1981; Hay 1981; Bissell et al. 1982; Kleinman et al. 1981, 1982). Where the process does not require fibronectin, it has been suggested that the cell-collagen interaction may be mediated by receptors that recognize multiple repeating sites (for example, Gly-Pro-Hyp) along the collagen molecule (Rubin et al. 1981). Various studies also indicate that the tertiary structure of the collagen molecule may be of consequence in the cell-collagen interaction. Where cell attachment can occur without the aid of fibronectin, native collagen substrates have been shown to promote attachment of certain cell types better than denatured or chemically modified collagens. In contrast, cell attachment on such modified collagens show a partial or absolute dependence on fibronectin (Rubin et al. 1981; Schor et al. 1981; Kasai et al. 1983).

As judged by cellular topography, the studies here and elsewhere (Norman 1982) demonstrate the capability of sheets of isolated rat bladder urothelium to attach to a variety of differently prepared Type I collagen substrata (viz. those prepared by air-drying, NH_3, NH_3/glutaraldehyde, NaOH and NaCl polymerization procedures). The result of all these preliminary in vitro culture experiments indicate, however, that proliferation and maintenance of these urothelial cells are markedly affected by the polymerized form of the collagen matrix. Only collagen matrices polymerized by NH_3 vapour and cross-linked by glutaraldehyde have proved able, within the present series of substrata studied, to support urothelial growth and differentiation. A possible reason for the different patterns of urothelial response to different collagenous (Type I) matrices could be that it is a reflection of modifications in matrix organization: marked differences between hydrated and dried collagen substratum have been already reported (Lillie et al. 1980; Iversen et al. 1981; Grinnell and Bennett 1981, 1982). The further and more detailed investigation presented here, identifies several ultrastructural patterns of collagen molecular assembly (Table 3). The presence and the level of filamentous, fibrillar or fibrous reaggregation within the different collagenous matrices are shown to vary significantly according to the collagen polymerization method employed.

While the nature of the attachment of urothelial cells to collagen and of the specific interaction which may direct growth and differentiation is still obscure, data presented here provides an insight into the process. Although the results are preliminary it is feasible to consider that while urothelial cell attachment to collagenous (Type I) matrices prepared by different polymerization procedures is not determined by a particular matrix organization, urothelial cell spreading and proliferation may be correlated to the level of supramolecular assemblage of the collagen. It is interesting to note that basement membranes, which serve as a substratum for both epithelia and endothelia, are composed of a dense collagenous mat of fine fibrils embedded within a finely granular matrix. This raises the possibility that the physical organization of the NH_3/glut collagen substratum as a fine filamentous meshwork may be a significant factor in determining the unique capability of this particular substratum, within the matrix series investigated in this study, to support urothelial growth and differentiation. Furthermore, while the nature of the granular globular masses remains to be characterized, this type of globular substratum has been successfully used for growth of both sympathetic neurons and dorsal root ganglia (Iversen et al., 1981).

Additional information about the mechanism of urothelial cell attachment to collagen and of substratum modulation of the urothelial differentiation programme is now being sought through immunocytochemical analysis of possible collagen-mediator protein complexes and of unique collagen-cell surface determinants. This may provide further insight into the chemical and physical constraints of native and modified forms of collagen in the expression of different cellular processes.

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Discussion with Reviewers

E.D. Hay: The statement is often made (eg. Introduction) that different kinds and arrangements of collagen have markedly different effects on growth and differentiation of cells. This is an overgeneralization. Sugrue and Hay (J. Cell Biol (1981) **91**:45) and Meier and Hay (1974) report that the form and type of collagen are immaterial to its stimulatory effect on corneal epithelial differentiation. Have you ever presented your urothelium on Millipore filters with soluble collagen?

Authors: Certainly, as you correctly point out, your short-term studies of corneal epithelium (cultured for periods of up to 24–48h in vitro) demonstrate that, at least over these time periods, the form and type of collagen appear immaterial to its stimulatory effect on cornea epithileal differentiation (as based on criteria of glycosaminoglycans and collagen synthesis, and on basal cell surface topography and cytoskeletal organization). Nevertheless, the literature (see Introduction) clearly establishes both from short (few hours)–to longer (few days to weeks)–term in vitro studies that the kind and physical organization of collagen can have marked, and different, effects on the attachment, maintenance, growth and differentiation of a variety of cells including urothelial cells as reported in this study. Urothelium on Millipore filters has not been presented with soluble collagen.

P.B. Bell: What is the source of the spaces between the cells shown in Figure 4? Are they the result of shrinkage during dehydration and/or drying?

Authors: Spaces between individual cells or groups of cells can occur initially and to a varying extent during dehydration as a differential response of the biological material and the substratum to the dehydrating sequence.

P.B. Bell: Would you discuss briefly why you use methanol as the dehydrating agent for electron microscopy?

Authors: Methanol is a convenient, cheap, easily obtainable alternative to ethanol and has the further advantage of not being subject to statutory restrictions (in the U.K.).

P.B. Bell: Are there striated collagen fibrils in the NH_3/glutaraldehyde-prepared matrices? Could you include a figure of these fibrils or any other fibrils that you observed in the NH_3/glut matrices?

Authors: As discussed in the Results section, NH_3/glut-prepared matrices showed considerable variation between samples in both SEM and TEM appearances. One of the more variable factors in the NH_3/glut method is the probability of the atmosphere being less than totally NH_3 vapour-saturated. As a consequence, the possibility exists that even over the brief 3–4 min exposure of the collagen preparation to this atmosphere air-drying may occur leading to the range of fibrillar structures identified in the air dried (AD) specimens including striated undecorated collagen fibrils (cf. Fig. 9).

A.F. Brown: In Figs. 7 and 8 you show the structure of air dried collagen substrata to consist of a dense fibrillar meshwork underlying coarse fibres. Is the dense mat of fibrils the result of collapse of the mesh during air-drying, and does rehydration (and a possible change in structure) occur when medium is added with cells?

Authors: Yes, obviously some collapse of the meshwork must be expected as a consequence of the air-drying schedule used in this study. However, AD collagen preparations rehydrated by incubation in medium, utilized as a cell culture substratum, and then reprocessed for TEM present an ultrastructure comparable to that discussed in the text. A feature of the AD collagen preparations is the wide range of fibrillar morphology of varying diameters which occur within definite strata (cf. Fig. 9). Our hypothesis to explain this is that rather than purely a collapse of the meshwork by surface tension, gradients of collagen concentration occur during the drying process.

A.F. Brown: You say that you find accretions on fibres polymerized with NaOH and particularly with NaCl. Since the NaOH gelling solution contains DMEM and therefore NaCl (at a final concentration when mixed with collagen of about one third that in the NaCl gelling solution), could the accretions be due exclusively to NaCl polymerization?

Authors: The final sodium chloride concentration is in fact higher in the NaOH gelling solution than in the NaCl gelling solution. However, it is possible that the NaOH collagen preparation schedule results in a polymerization end-point not attained when using the NaCl protocol.

T.D. Allen: Can you envisage any mechanism whereby the globular masses of collagen found in the NH_3/glut polymerized gels might be directly involved in promoting urothelial attachment and proliferation; or are they merely indicative of an extra level of retention of collagen components not found in other polymerizations, presumably due to a lack of crosslinking in the absence of glutaraldehyde?

Authors: Stereo-TEM suggests that the globular masses are composed of aggregates of crinkly and not so fine filamentous substructure; these filaments insert irregularly within the globular masses rolling up to form these globular entities (comparable to a ball of wool). This may arise from glutaraldehyde-induced crosslinking of closely apposed collagen molecules causing a bending of these particular molecular assemblies. As indicated in the Discussion one of the possible factors involved in promoting urothelial attachment and proliferation may be the level of filamentous reaggregation attained in given polymerization procedures; here this may be such as to provide a collagenous mat of a density similar to that of basement membranes notwithstanding the role of specific collagen-extracellular matrix receptor sides.

A.F. Brown: The terms microfibril, fibril and fibre were defined in the Introduction, but the term filament is used in the Results. It would be useful to retain consistency of terminology or else to define "filament".

Authors: The term "filament" has been introduced as a morphological description when the structure has lain outside the term defined in the literature for morphologically established biomolecular aggregates of collagen.