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ISOLATION, SEPARATION, AND CHARACTERIZATION OF EPITHELIAL AND CONNECTIVE CELLS FROM RAT PALATE

by

VICTOR PAUL TERRANOVA

Submitted in Partial Fulfillment
of the
Requirements for the Degree
DOCTOR OF PHILOSOPHY

Supervised by Dr. John S. Brand

Department of Radiation Biology and Biophysics

University of Rochester
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DISCLAIMER

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VITAE

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ABSTRACT

The purpose of this research was to isolate, separate and characterize cells from rat palate in order to establish a model for the study of periodontal disease at the cellular level. Epithelial and connective tissue cells were isolated from rat palate by sequential collagenase, hyaluronidase and trypsin digestion of the extracellular matrix. Differences between the two populations were noted with respect to total cell protein, total cell water, proline uptake and incorporation, percent collagen synthesized, effects of parathyroid hormone, metabolism of D-valine and cell density.

Basal epithelial cells were subsequently separated from the heterogeneous epithelial cell population on shallow linear density gradients by velocity centrifugation.

The type of collagen synthesized by the basal epithelial cells was compared to the type of collagen synthesized by the connective tissue cells by means of labeled amino acid incorporation ratios.

In comparing cells isolated from the epithelial and connective tissue it was found that the epithelial cells: 1) have 235 $\pm$ 15 µg protein per 10^6 cells; 2) have 0.53 $\pm$ 0.1 µL cell water per 10^6 cells; 3) incorporate 860 pM proline into protein during a 4 hour incubation; 4) contain a D-amino acid oxidase; 5) have a density of 1.086 g/cc; and 6) the basal layer of epithelial cells synthesize base-

ment membrane or Type IV collagen. With the connective tissue cells it was found that they: 1) have 170 $\pm$ 10 µg protein per 10^6 cells; 2) have 0.23 $\pm$ 0.04 µL cell water per 10^6 cells; 3) incorporate 820 pM proline into protein during a 4 hour incubation; 4) contain a subpopulation of cells, periosteal cells, which respond to PTH; 5) have a density of 1.076 g/cc; and 6) synthesize Type I collagen.

From these studies it can be concluded that epithelial and connective tissue cells can be isolated from rat palate as viable and distinct populations with respect to the biochemical parameters examined. Furthermore, subpopulations can be separated and biochemically characterized.

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INTRODUCTION

Periodontal Disease.

Chronic inflammatory gingival and periodontal disease afflicts a major segment of human populations throughout the world. In the early stages of the disease, the tissues exhibit characteristic alterations including the accumulation of a dense infiltrate of inflammatory cells, marked alteration in the quality and quantity of connective tissue substance and pathologic pocket formation. Microbial dental plaque and its metabolic by-products are the main etiologic factors of gingivitis and periodontitis (Theilade et al. 1966; Lindhe, Hamp and Loe 1973; Socransky 1970). The microorganisms within dental plaque do not invade the periodontium (Freedman et al. 1968), but elaborate a variety of toxic products and enzymes which may contribute to tissue injury by direct action on the cells of the periodontium (Cowley and Levine 1972). Dental plaque may also indirectly contribute to tissue breakdown; that is, plaque bacteria contain certain substances chemotactic for neutrophil polymorphonuclear leukocytes (Tempel et al. 1969; Attstrom and Egelberg 1971; Lindhe and Helldin 1972; Helldin and Lindhe 1973). Neutrophil chemotactic activity is also generated by the interaction of endotoxins from dental plaque and the C_5 component of complement (Snyderman et al. 1969).

The gingival lesion is characterized by an altered topography and permeability of the crevicular blood vessels (Egelberg, 1967; Lindhe and Branemark, 1969; Soderholm and Egelberg, 1973), a dense infiltrate of leukocytes, and destruction of connective tissue matrix and fibers (Page, Davies and Allison, 1972; Schroeder and Page, 1972; Schroeder et al., 1973a, Schroeder, Munzel-Pedrazzoli and Page, 1973). Most of the inflammatory cells in the gingival crevice (Attstrom, 1971; Lindhe, Hamp, and Loe, 1973) and superficial portions of the junctional epithelium (Freedman, Listgarten and Taichman, 1968; Schroeder, 1972; Schroeder et al. 1973a, 1973b) have been characterized as neutrophil polymorphonuclear leukocytes. The basal portions of the junctional epithelium and the connective tissue, however, are infiltrated mainly by mononuclear leukocytes (Schroeder, 1972, 1973; Schroeder, 1973a, 1973b). Increased permeability of crevicular blood vessels in gingival inflammation results in an increased flow of gingival fluid (Egelberg, 1967), the composition of which is characteristic of an inflammatory exudate (Brandtzaeg, 1965; Shillitoe and Lehner, 1972).

The reduction of connective tissue, mainly collagen and ground substance, observed in gingivitis and periodontitis may be caused by proteolytic enzymes. These enzymes could be derived from neutrophil polymorphonuclear leukocytes, macrophages, from the cells of the epithelium, connective

tissue and alveolar bone, or a combination of these components. The occurrence, in the dentogingival region, of enzymes derived from PMN leukocytes has been extensively described (Attstrom 1971, Cowley 1972; Lange and Schroeder 1972; Page et al. 1972; Tynelius-Bratthal 1972; Tynelius-Bratthall and Sttstrom 1972, Attstrom et al. 1971; Ohlsson et al. 1973; Tynelius-Bratthall and Lindhe 1973). The possible existence of an inflammatory cell collagenase in diseased gingiva has been discussed by Beutner et al. (1966), who found that collagenase of inflammed gingiva was associated with inflammatory foci in the tissue. Fullmer et al. (1969b) demonstrated collagenase activity in gingival connective tissue free from epithelial cells, but containing infiltrates of inflammatory cells. Recently Wahl et al. (1974, 1975), have shown that macrophages when exposed to bacterial lipopolysaccharide (endotoxin) were stimulated to produce collagenase. Subsequently, they reported (1977) that the elevated levels of PGE_2 produced by endotoxin-stimulated macrophages have a regulatory role in the production of collagenase by these cells. Thus, the collagenolytic activity observed in gingival connective tissue might therefore involve enzymes orginating from inflammatory cells as well as from the tissues. Furthermore, the neutral proteases, collagenase (Lazarus et al. 1968a, 1968b, Ohlsson and Olsson 1973) and elastase (Janoff and Schere 1968; Ohlsson and Olsson 1973)

have been isolated and purified from lysosomes of human blood leukocytes.

Hopps and Prout (1972) suggested the hyaluronidase found in human saliva might be of leukocytic origin (PMN leukocytes from gingival fluid). Tynelius-Bratthall and Attstrom (1972) demonstrated significant hyaluronidase activity from gingival exudate fluid. In attempts to evaluate the in vivo action of hyaluronidase in gingival tissue destruction, enzyme preparations have been applied topically to the gingival margin. Cowley (1966) found that bacterial hyaluronidase had no effect on the permeability of the epithelium. Other investigators (Schultz-Haudt, Deward and Bibby 1953, Thilander 1963, Murphy and Sallard 1968) using commercially available hyaluronidase from bovine testes found a widening of the intercellular space in the junctional epithelium. The effect of hyaluronidase on the intrigity of the basement membrane which contains proteoglycan subunits has not been investigated. Repeated injections of testicular hyaluronidase into the interdental papillae of monkeys resulted in proliferation of the crevicular epithelium probably due to connective tissue break-down (Aisenberg and Aisenberg 1951).

Material from dental plaque may also mediate tissue damage by eliciting immune pathologic reactions. Hypersensitivity reactions with characteristics of both the

immediate (Rizzo and Mitchell 1966; Raney and Zander 1970; McDougall 1971,1972) and the delayed type (Okada et al. 1972) have been experimentally induced in gingival tissue. A variety of different studies support the hypothesis that hypersensitivity reactions contribute to tissue injury in periodontal disease (Mergenhagen, Temple & Snyderman 1970; Horton et al. 1971, 1972a; Horton et al. 1972b; Schroeder and Page 1972; Lehner 1972). In complement-mediated (Ward, Cochrane and Muller-Eberhard 1965, 1966) and in delayed hypersensitivity reactions (Ramseier 1967), neutrophil chemotactic factors are released. This indicates the lysosomal substances from neutrophils are possibly involved in extracellular tissue destruction in these reactions. The effects of these substances on the various subpopulations of cells that make up this tissue remains undetermined.

Although the case for a primary bacteriologic etiology for both gingivitis and periodontitis is well documented, the search for association of specific bacteria with the disease process has not yielded results. If a specific microorganism or group of microorganisms can be shown to be associated with the disease etiologically, then a means of controlling the disease by elimination or control of these specific organisms may emerge. However, this search is complicated by the fact that we do not yet know if actively metabolizing microorganisms, the metabolic by-

products elaborated by these microorganims, or the substances derived from them after death are the primary cause of the disease. Furthermore, there are no experiments reported relating (1) the effect of bacterial plaque or its metabolic by-products on the constituent cellular components of the periodontal unit and (2) the nature of the transport process of bacterial plaque by-products across the epidermal-dermal junction (basement membrane).

Thus, progress in our understanding of the etiology of chronic inflammatory periodontal disease requires consideration not only of (1), the role of microorganisms, but also of (2), local and systemic or constitutional factors that may alter the resistance or susceptability (possible alteration in the integrity of the basement membrane) of the tissues of the periodontium to bacterial plaque or its metabolic by-products. Therefore, in order to understand the abnormal viz., gingivitis and periodontitis, one must establish a base line for the normal. This base line must necessarily be a characterization of the normal tissues of the periodontium; namely, the gingival epithelium (basal cells, spinous cells, granular cells and keratinized cells), fibroblasts of the connective tissue layer and cells of osseous or preosseous origin (periosteal cells).

Cell Isolation

Furthermore, study of basement membrane should be

included in relation to its composition and function as a selective filtration barrier to bacterial plaque by-products. Ideally, any tissue characterization should begin with the study of each individual component population of cells. This necessitates taking oral tissue and dispersing from it cells of the epithelium segment and underlying connective tissue.

In 1953 Mandell et al. isolated and characterized collagenase from the bacteria Clostridium histolyticum. During 1959 Lasfargues described a procedure for obtaining epithelial cell suspensions from adult mouse mammary glands using bacterial collagenase in a concentration of 200 µg/ml of Simms solution, Hinz et al. in 1959, prepared cell suspensions from fetal human lung, fetal swine lung, adult rabbit lung and fetal monkey kidney using bacterial collagenase (100 µg/ml) in combination with trypsin (20 mg/ml) in GKN solution. Embryonic heart tissue was dissociated (Cavanaugh et al., 1963) in Grey's solution which contained crude bacterial collagenase at a concentration of 5 mg/ml. Isolation of bone cells from the frontal and parietal bones from the calvaria of rat fetuses using 100 µg/ml to 6 mg/ml crude collagenase in tris-buffered saline was first achieved by Peck et al. in 1964. Also, in 1964 Rodbell described a procedure for isolating fat cells from rat epididymal fat pads using 10 mg collagenase per ml bicarbonate buffer. The first successful isolation

of intact parenchymal cells from rat liver using 0.15% collagenase along with 0.15% hyaluronidase was described by Howard et al. in 1967.

A number of other cell isolation procedures which depend on treatment of intact tissue with various enzymes (trypsin, pancreatin, chymotrypsin, lysozyme, elastase, hyaluronidase) or with chelating agents (Na-citrate, EGTA, EDTA) were reported throughout the late 1960's and early 1970's.

Regardless of the methodology employed or the concentration of enzymes used to isolate cells from solid tissue, the primary criteria used to determine the success of the procedure is cell viability. Once viability is verified, by any number of biochemical parameters, the separation of a pure fraction of cells from a mixed population is an essential prerequisite if more than relatively gross and qualitative biochemical studies are to be made of the tissue in the normal and diseased states.

Cell separation by gradient centrifugation according to their size (effective cell radius) and density is just one of several methods of sorting mammalian cells. Other methods include electronic cell sorting by volume (Fulwyler 1965, 1970), size filtration on glass-bead columns at 4°C (Shortman 1966, 1969), sedimentation at unit gravity (Mel 1963; Mel et al. 1965; Peterson and Evans 1967;

Miller and Phillips 1969), and centrifugal elutriation (counter current centrifugation)(Glick et al. 1971). All of these methods have their usefulness; however, cell separation by gradient centrifugation is by far the most important and widely used of the above techniques. The techniques of density gradient centrifugation are being applied to an increasingly widening spectrum with applications currently ranging from the separation of blood cell types (McEwen et al. 1971) through to the establishment of synchronous populations of dividing cells (Grabske et al. 1975). The most thorough study of the theoretical basis of the centrifugal separation of cells is presented in the work of Boone et al. (1968), and in some of the work that followed from that study (Pretlow and Boone 1968a, 1968b, 1969a, 1969b, 1970; Pretlow et al. 1969, Pretlow 1971).

In 1968 Boone et al. demonstrated that mixtures of viable cells could be resolved using density gradient centrifugation in gradients of Ficoll (synthetic sucrose polymer of average molecular weight 400,000) in tissue culture medium. The basic theoretical considerations which have been used in physically analyzing the sedimentation characteristic of subcellular particles may also be applied to intact mammalian cells. The ideal particle, as treated by classical sedimentation theory, is spherical, rigid, smooth, uncharged, unhydrated and constant in size and density

(therefore, in the case of cells, osmotically inactive). If a particle of diameter a (centimeters) and density Dp(g/cc), is suspended in a medium of density Dm and viscosity η (poises) and subjected to a centrifugation at an angular velocity ω (radians per second) it will assume a constant terminal velocity v (centimeters per second). Three forces will be acting on the particle all along the radius of sedimentation: F_c, a centrifugal force, F_b, a buoyant force, and F_s, Stoke's force of resistance to sedimentation. The dependence of these forces on the variables of the system are:

$$F_c = m\alpha$$
$$F_b = -(4\pi/3)(a/2)^3(Dm)\alpha$$
$$F_s = -3\pi a v \eta$$

where m is mass of the particle (grams), α is angular acceleration in centimeters per second squared (equal to $\omega^2 r$) and r is the radial distance of the particle from the center of rotation in centimeters. If the particle is sedimenting at a constant terminal velocity then the sum of the centrifugal, buoyant, and Stoke's forces equals zero, and the equation can be solved for sedimentation velocity v: (Boone et al., 1968)

$$v = \frac{dr}{dt} = \frac{a^2(D_p - Dm)\omega^2 r}{18\eta}$$

This basic differential sedimentation equation shows that the sedimentation velocity of the particle (cell) depends on

density and, above all, on diameter, since diameter occurs as a squared term. This sedimentation equation is also applicable to nonspherical particles if a shape factor (θ), or frictional ratio, is introduced (deDuve et al., 1959).

Isopycnic centrifugation depends solely on the buoyant densities of the cells, each cell sedimenting to a point where the density of the surrounding medium is equal to its own. In general, isopycnic sedimentation alone has not proved broadly useful in the separation of cells (Pretlow and Cassady, 1970). In some instances, isopycnic sedimentation has been a useful adjunct to rate-zonal (gradient differential) centrifugation on another gradient (Pretlow and Cassady, 1970; Haskell and Moore, 1970). In 1971 Pretlow described the construction of one gradient in which the sedimentation of most mammalian cells will closely approach being isokinetic. This method (velocity sedimentation) is more useful than isopycnic centrifugation, the major reason being that greater variation in cell diameter exists between cells, and that such differences are emphasized in velocity centrifugation.

The usual isolated mammalian cell approximates the ideal particle dealt with by the sedimentation in that it is essentially spherical once isolated and, provided the centrifugation medium is controlled so as to avoid detrimental osmotic effects, is of constant size and density (Pretlow and Boone, 1969). The approximation of isolated mammalian

cells to the ideal particle is borne out by the very reasonable correspondence between the computer - predicted sedimentation behavior of isolated cells and the results actually observed experimentally (Boone et al., 1968). Using this system, subpopulations of cells can be separated if the parameters of cell size and density remain constant, and above all, if the subpopulations can be identified.

Since there is very little data available relating to tissue inter-relationships in the periodontium or to the effect of plaque by-products on individual population of cells, the techniques of cell isolation and subsequent separation should prove extremely useful.

Type IV Collagen: Basement Membranes

The region between the epithelium and the underlying stroma of a given tissue is traditionally considered to be the site of the basement membrane. Concepts concerning the basement membrane have been modified progressively during the course of the past century. In 1842 Bowman described a simple, homogeneous, perfectly transparent tunic over the epithelium of the uniniferious tubule which expanded over the Malpighian tuft to form a capsule (later called Bowmans Capsule). He termed this coating the basement membrane. When the epidermal basement membrane was described (Herxheimer, 1916), it too was considered by some to be homogeneous and transparent. Questions such as the following were subsequently debated. Was there a boundary

layer between the dermis and epidermis? If so, was it a membrane? Was it structured or unstructured? Was it derived from the epithelium or the connective tissue? Was it fibrous or was it identical to ground substance? During the next period of development, the fibrous structure of the basement membrane was described and the question of epithelial and connective tissue fibers communicating across it was considered (Fireboer, 1920; Homma, 1922; Kogaj, 1923; Welti, 1923; Szodoray, 1931).

Early suggestions that the basement membrane was composed of ground substance was convincingly demonstrated by Gersh and Catchpol (1949) who showed the basement membrane to be a periodic acid-Schiff reagent positive zone. The earliest suggestion of the collagenous nature of the basement membrane came from the findings of Goodman et al. (1955) of significant amounts of hydroxyproline. Also, Kefalides and Winzler (1964) demonstrated that canine glomerular basement membrane contained high amounts of hydroxylysine. It became obvious over the years that basement membranes are complex structures composed of dissimilar protein subunits rich in carbohydrate. On the basis of compositional, structural, and biosynthetic studies, it is now clearly established that one of the protein components is a collagen (Referred to as Type IV or basement membrane collagen). Glycine accounts for one-third of its amino acid residues and proline plus hydroxyproline account for 22% of all the

amino acids. Physical studies indicate that Type IV collagen has a triple helical configuration. Compared to interstitial collagen, it contains unusually high amounts of hydroxylysine and hydroxyproline. About 10% of its total hydroxyproline is 3-hydroxyproline. Unlike most interstitial collagens, it has a low alanine and arginine content and high amounts of isoleucine, leucine and phenylalanine (Kefalides, 1973). Depending on the source of basement membrane, there are 4 to 8 residues per 1000 residues of half-cystine. Whether the half-cystine is located in the α-chain is not yet clear (Kefalides, 1972). One possibility would be that it is involved in disulfide formation linking the α-chain with the non-collagen polypeptide chain, or that it forms a disulfide bridge within the noncollagen polypeptide chain (Kefalides, 1972). Type IV collagens also contain 10-12% carbohydrate, almost exclusively glucose and galactose. The glucose and galactose are involved in the formation of the disaccharide glucosylgalactose which is distributed throughout the collagen α-chain (Kefalides and Wingler, 1966; Tanzer and Kefalides, 1973). Another interesting feature of the basement membrane collagen is the fact that its molecule is composed of three identical α-chains having the chromatographic properties on CM-cellulose of the α1 chain from interstitial collagens (Kefalides, 1971). Immunochemical studies also suggest that these structures possess at least three antigenic components which are shared by both the vascular and nonvascular types

of basement membranes (Kefalides, 1972).

Two main functions have been ascribed to basement membranes: (1) support and (2) selective filtration (Briggaman and Wheeler, 1975). The first property is best exemplified by the lens capsule which completely envelopes the lens. Bowman's capsule which holds the capillary tuft of the glomerulus serves as another example. The filtration property of capillary basement membranes is determined primarily by the organization of their protein subunits and is best exemplified by the glomerulus. Molecules having the dimensions of ferritin (MW greater than 500,000) are completely retained by the glomerular basement membrane (Farguhar, 1960, 1964). Smaller molecules, such as horseradish peroxidase (MW 40,000), appear to traverse the basement membrane and be retained at the filtration slits between the foot processes of the epithelial cells (Graham and Karnovsky, 1966; Karnovsky, 1968). Lanthanum (MW 139) (Wolff and Schreiner, 1968) and rutheium red, (MW 552), (Hashimoto and Lever, 1970) cross the epidermal-dermal junction in skin and appear within the epidermis after subepidermal injection. Except for the studies mentioned above, little is known about the characteristics of the "junctional barrier" regarding molecular size, solubility properties, and other physical characteristics of the materials which are excluded by the basement membrane. Knowledge of this will indeed have practical significance for the pathogenesis of periodontal

disease, particularly in relation to bacterial plaque by-products.

The first chapter of this thesis presents a method of separating epithelial cells from connective tissue cells, and showing that these populations are viable and distinct with respect to proline uptake and incorporation, % collagen synthesized, effect of PTH and D-valine metabolism. In the following chapter a method of isolating the distinct sub-population of basal epithelial cells is presented along with initial studies on basement membrane collagen synthesis by these cells.

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CHAPTER I

ISOLATION AND CHARACTERIZATION OF EPITHELIAL AND CONNECTIVE TISSUE CELLS FROM RAT PALATE

INTRODUCTION

The study of periodontal tissue at the biochemical level can be significantly advanced by isolation and subsequent study of the separate population of cells that make up this tissue. There is much speculation as to which specific cell types are involved in periodontal disease. Characterization of the cells derived from normal tissue will provide the baseline for determination of the metabolic or biochemical aberrations which develop in periodontal disease.

The periodontal unit-epithelium, connective tissue, and underlying alveolar bone has been studied extensively by histological methods (1). Biochemical differences between fibroblasts and epithelial cells have been examined predominantly in tissue culture. It is now possible to further advance the study of the periodontium by isolation of the separate population of cells which make up this tissue.

Viable cells, both epithelial and those of mesenchymal origin, can now be dispersed from many mammalian tissues. The isolation technique which has proven to be most effective is based on the enzymatic digestion of the extracellular matrix. This type of procedure has been applied to the preparation of isolated hepatocytes (2), intestinal epithelial cells (3), adipocytes (4), and lymphocytes (5), as well as cells from bone (6), cartilage

(7), and other sources.

The present study was undertaken to isolate separate populations of viable connective tissue and epithelial cells from normal oral tissue and to begin a biochemical characterization of these populations of cells. The information obtained using normal tissue will provide a baseline for quantitating changes in cell subpopulations and determining the nature of the metabolic aberrations which develop in periodontal disease.

MATERIALS AND METHODS

1. Histology

Freshly excised palatal tissue (control) and palatal tissue removed at one hour intervals during a four hour incubation (test) were fixed in 10% neutral buffered formalin, dehydrated, embedded in parafin, sectioned (8 microns thick) and stained with haematoxylin and eosin.

2. Cell Isolation

The cell isolation technique is a modification of the method described by Dziak and Brand (6) for bone cells. Palatal tissue, freshly excised from decapitated young adult female rats was incubated at 37°C in a Hepes buffered isotonic salt solution, pH 7.4, containing crude collagenase[a] 3 mg/ml, crude hyaluronidase[a] 3 mg/ml and 0.5 mg/ml elastase[a].

The buffer was 25 mM Hepes, 10 mM $NaHCO_3$, 100 mM NaCl, 3 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 60 mM mannitol, glucose 5 mg/ml, bovine serum albumin (Fraction V)[b] 1 mg/ml, 5 µg/ml Penicillin[c], 10 µg/ml Neomycin[c] and 5 µg/ml and 5 µg/ml Streptomycin[c]. Thirty milligrams of tissue per 1.0 ml buffer were incubated in 50 ml polypropylene beakers in a water bath shaking at 90 oscillations per minute.

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a. Worthington Biochemical Corp., Freehold, N.J.
b. Sigma Chem. Co., St. Louis, MO.
c. Gibco, Grand Isoland Biological Co., Grand Island, N.Y.

At one hour intervals up to four hours the cells were harvested as previously described (6) and tissue digestion resumed. Cell counts were made with a haemocytometer.

3. Cell Viability Studies

The viability of the cells isolated by the above technique was evaluated routinely using trypan blue dye exclusion. To 1 x 10^6 cells/100 λ buffer, 100 λ of 1% trypan blue was added. Cell amounts for inclusion of dye were made 15 minutes later.

Intracellular space was measured by a double label procedure. 50 x 10^6 isolated cells were incubated at 37°C for 5 minutes in 2.0 ml of buffer containing 1 mM ^{14}C polyethylene glycol (PEG)[a] (0.5 μCi/ml) and ^{3}H-H_2O[b] (0.3 μCi/ml). The cells were then sedimented for three minutes at 800 x g after which 100 λ of the supernatant was counted in 10 ml scintillation cocktail (9.67 ml Toluene-Omnifluor[c] + 0.33 ml BBS-3)[d]. The supernatant was completely removed with a micropipet and the side of the tube dryed with absorbent paper. The pellet was then resuspended in 1 ml fresh buffer equilibrated for 5 minutes at 37°C and centrifuged as before. 100 λ of the supernatant was then counted. Total H_2O was determined from the CPM tritium and extracellular fluid (ECF) was determined from the ^{14}C-PEG. Intracellular fluid

a. Amersham/Searle Corp., Arlington Heights, Ill.
b. Mallinckrodt, ST. Louis, MO.
c. New England Nuclear, Boston, Mass.
d. Beckman Instruments Inc., Fullerton, CA.

(ICF) per 10^6 cells was obtained by subtracting ECF from total H_2O and dividing by 50.

For an additional measurement of total H_2O, wet weights of cell samples were determined by sedimenting the cells at 800 x g for 3 minutes in a tared conical centrifuge tube. The supernatant was carefully decanted off and the sides of the tube dryed. Tubes were then dryed in a 37°C oven and weighed. This procedure was repeated until a constant weight was obtained. Total H_2O equals wet weight minus dry weight. Total cell protein was measured by the Toennier and Feng method (8) (a modified Lowry procedure).

Proline uptake was determined by incubating 2 x 10^6 isolated cells in 1.0 ml buffer with 0.2 mM ^{14}C proline (0.1 μc/ml)[a] for various time points up to four hours. The cells and incubation medium are then carefully transferred into small siliconized glass test tubes and centrifuged at 1500 x g for 2 minutes. The supernatant is removed and the resulting cell pellet washed with 1.0 ml cold buffer and recentrifuged. After discarding the supernatant the pellet is suspended in 200 λ distilled H_2O and placed in an oil bath at 100°C for 10 minutes to lyse the cells. 50 λ of 25% trichloroacetic acid (TCA) is then added and the sample centrifuged at 10,000 x g for 5 minutes. 100 λ of

a. Amersham/Searle Corp., Arlington Heights, Ill.

the resulting supernatant is then counted in 9.67 ml Toluene-Amnifluor[a] and 0.33 ml BBS-C[b]. (This is free ^{14}C-proline.) To the resulting cell pellet 200 λ of 5% TCA is added and the tube vortexed vigorously. After 10 minutes, the mixture is spun at 10,000 x g for 5 minutes and the supernatant discarded. To the pellet is added 0.5 ml 6 N HCl. This reaction mixture is hydrolyzed in glass tubes sealed with teflon-coated rubber-lined caps for 16 hours at 110°C and then dried under vacuum to remove the HCl. The hydrolysate was then dissolved in 25 λ distilled H_2O to which 50 λ 0.1M $NaHCO_3$ and 150 λ dansyl chloride (9 mg dansyl chloride per ml acetone) are added. The reaction is stopped after 10 minutes by the addition of 25 λ 0.4M acetic acid. 25 λ of the reaction mixture is spotted on 500 micron thick silica gel plates and developed in a benzene, pyridine, acetic acid buffer (80 ml:20 ml:2 ml)(9). Standards of proline and hydroxyproline (10 mM ^{14}C -proline and 10 mM ^{3}H-hydroxyproline)are run simultaneously and corresponding areas on the thin layer plates scraped and counted in scintillation fluid to determine 1) the amount of proline incorporated into protein and 2) the amount of protein bound proline hydroxylated. To calculate per cent recovery 25 λ of the dansylation mixture is spotted at the

a. New England Nuclear, Boston, Mass.
b. Beckman Instruments Inc., Fullerton, Ca.

top of the plate and subsequently scraped and counted. Thus total proline incorporated equals proline incorporated plus hydroxylated protein bound proline corrected to 100% recovery.

To study the effect of PTH on proline uptake and incorporation into protein, prior to isolation the tissue is preincubated with PTH at a concentration of 1 unit/ml buffer (without enzyme) for one half hour; controls had no PTH. Enzymes are then added and the cells are harvested from the first and last two hours of digestion. Cell protein hydrolysates are again assayed for content of labeled proline and hydroxyproline.

4. ^{14}C-D-Valine Metabolism

In order to insure no epithelial cell contamination in the connective tissue cell population for these experiments, about three fourths of the connective tissue cell layer was dissected away from the palates before incubation with enzyme. The remaining tissue was then treated as described above, discarding the cells from the first 2 hours of digestion. Thus, two preparations of cells were obtained; connective tissue incubated for two hours and epithelium incubated for four hours with the first two hour cells removed. Whole palates which were not dissected were also used in separate experiments.

Connective tissue cells and epithelial cells are incubated at 37^{o}C at a concentration of 4 x 10^{6} cells per ml

buffer with 1.0 Ci carrier free ^{14}C-D-valine (specific activity 9.8 mCi/mM)[a]. At two hour intervals 1.0 ml of cell suspension is removed from the incubator, placed in glass test tubes on ice and immediately precipitated with 0.5 ml 25% ice cold TCA. The test tubes are then centrifuged at 15,000 x g for ten minutes. 25 λ of the supernatant is reacted with dansyl chloride as described above and 50 λ samples of this reaction mixture are counted, with and without a solubilizer added, in a liquid scintillation counter.

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a. Calbiochem., Los Angeles, CA.

RESULTS

1. Histology

Histologically we can demonstrate a sequential removal of cells from the rat palate. The connective tissue layer closest to the palatal bone is the first area from which cells are liberated. Figure 1 shows a cross section through normal rat palate before incubation with enzymes. In Figure 2, after one hour of incubation with enzymes, the connective tissue layer closest to the palatal bone appears looser and a small percentage of cells are dispersed (app. 10% of the total cell yield). Figure 3 shows a cross section of rat palate after 1.5 hours of incubation with enzymes. Fifty percent or the connective tissue layer has been digested. After two hours of digestion 90% to 100% of the connective tissue cell layer has been removed (Fig. 4). During the next two hours of incubation the epithelial cells are liberated and all that remains of the palate is the cornified epithelial cell layer which does not digest further (Fig. 5).

This procedure yielded approximately 9×10^6 cells per palate, 40% of which was connective tissue cells and 60% epithelial cells.

2. ICF and Protein Measurements

Intracellular fluid space (ICF) measurements for 10^6 connective tissue cells gave 0.23 ± 0.04 µl. Epithelial cells had an ICF of 0.53 ± 0.10 µl/10^6 cells. Total cell

FIGURE 1

Cross section through rat palate before being placed in buffer containing enzymes (100 X).

PHOTOMICROGRAPH OF UNDIGESTED RAT PALATE

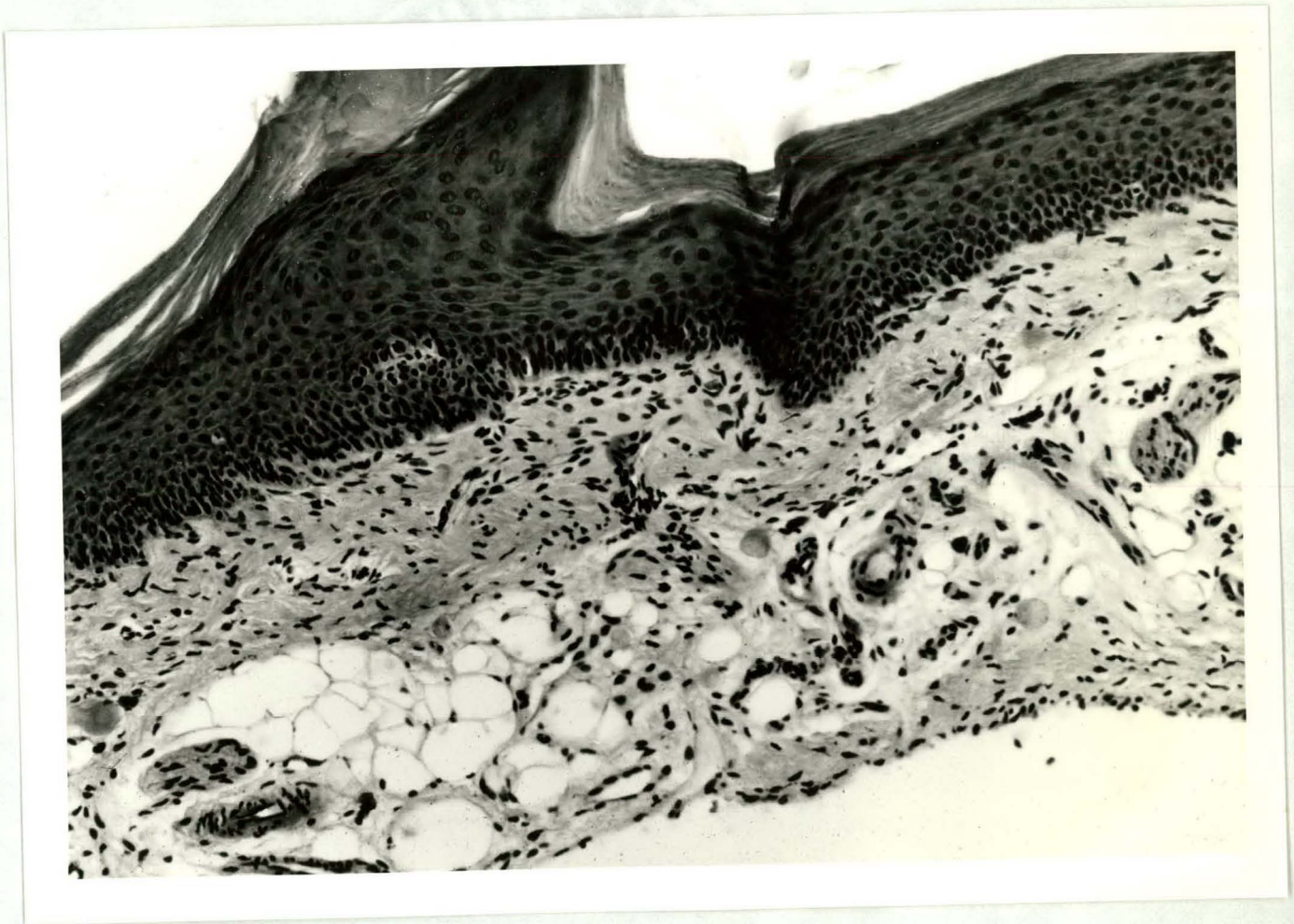


FIGURE 2

Cross section of rat palate after one hour of incubation in buffer containing enzymes (100 X).

PHOTOMICROGRAPH OF RAT PALATE: ONE HOUR DIGESTION

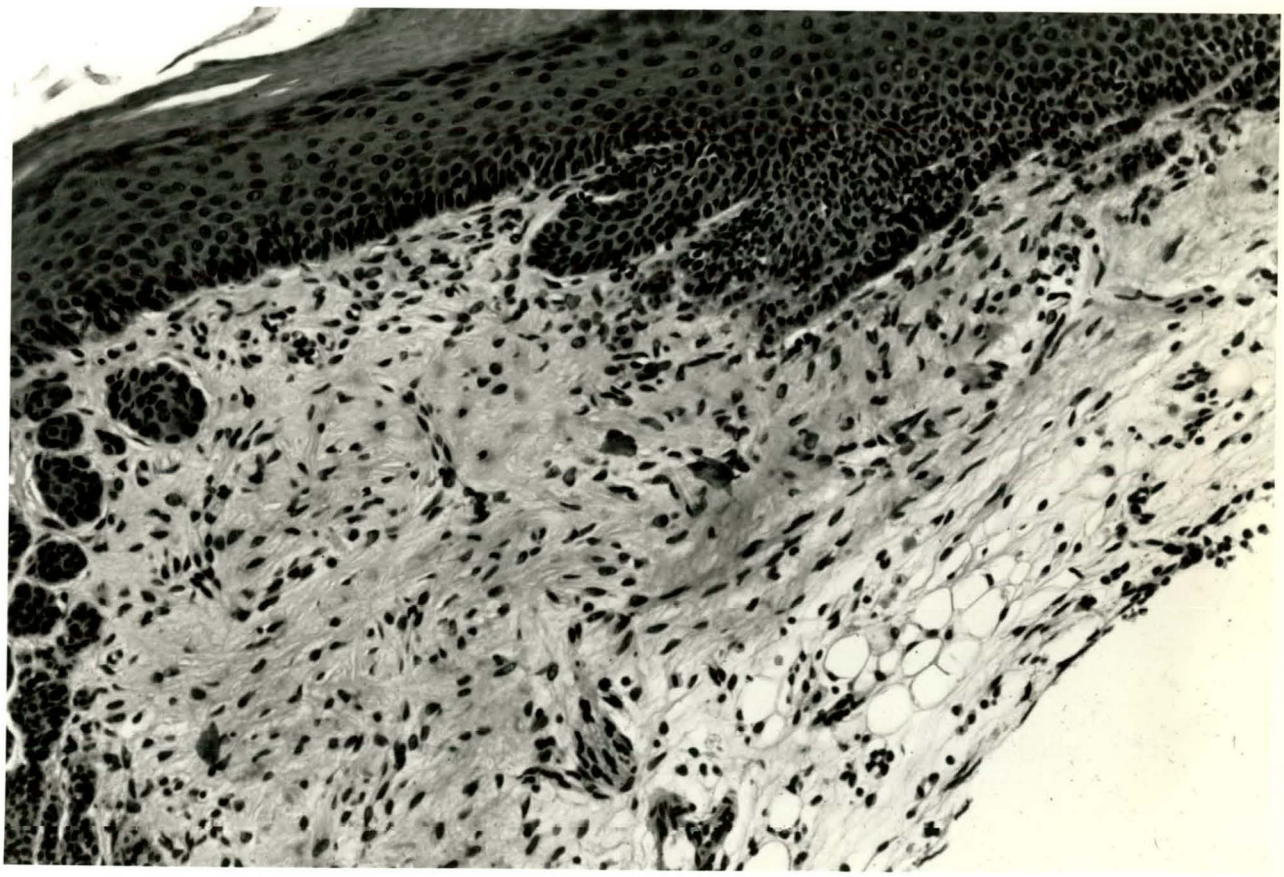


FIGURE 3

Cross section of rat palate after 1.5 hours of digestion (40 X).

PHOTOMICROGRAPH OF RAT PALATE: ONE AND

ONE HALF HOURS DIGESTION

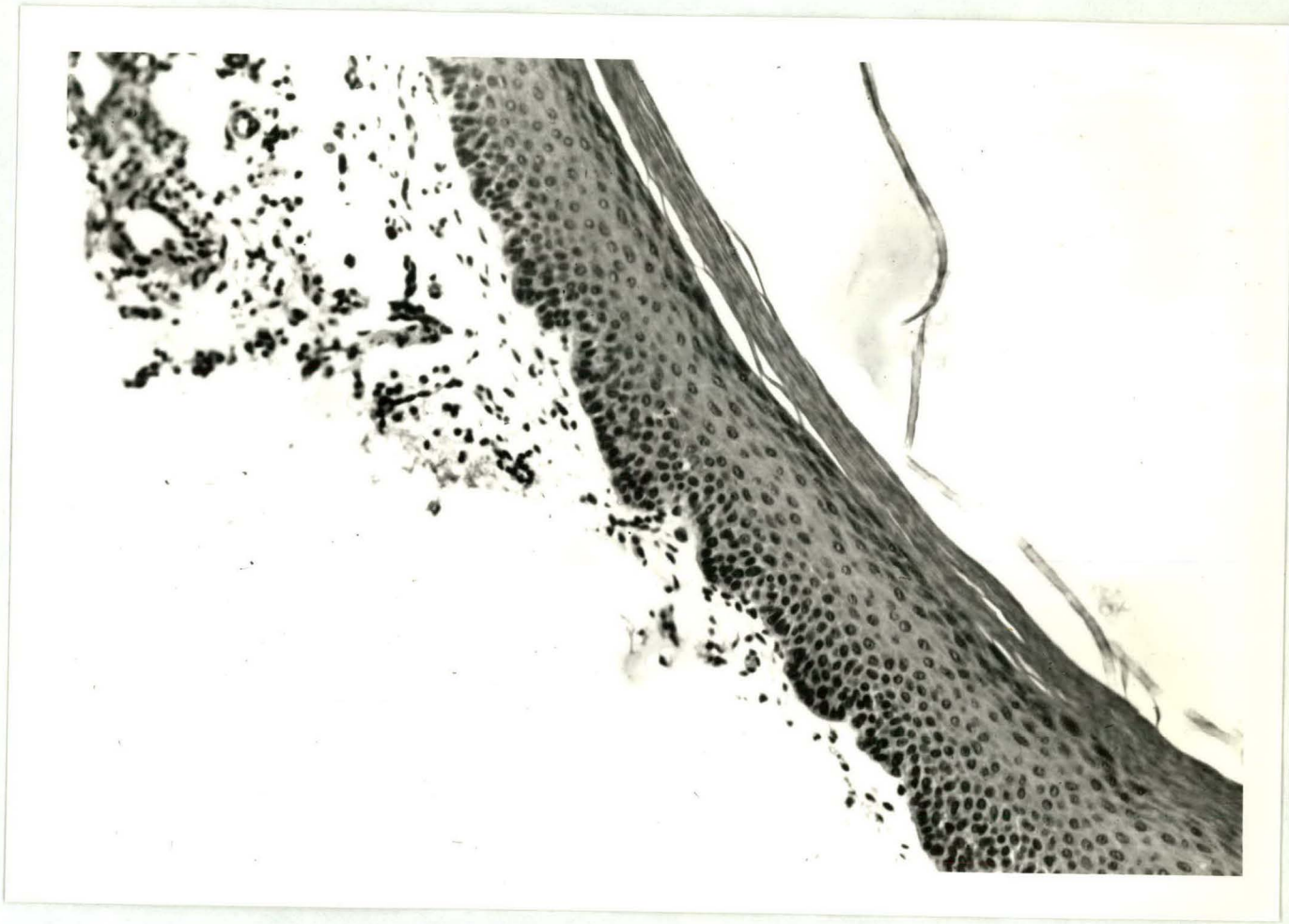


FIGURE 4

Cross section of rat palate after two hours of digestion (100 X).

PHOTOMICROGRAPH OF RAT PALATE: TWO HOURS DIGESTION

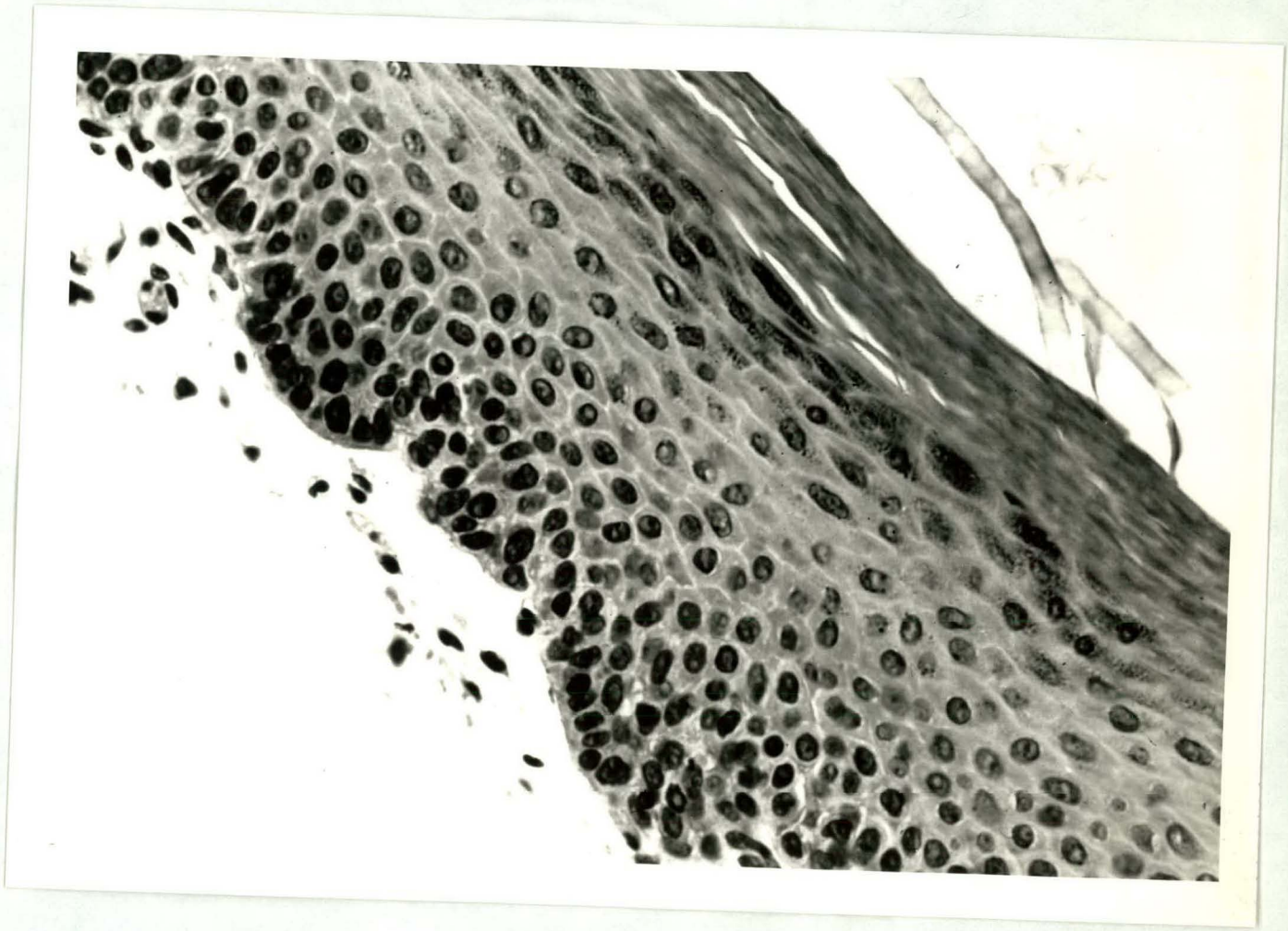


FIGURE 5

Cross section of rat palate after four hours of digestion (100 X).

PHOTOMICROGRAPH OF RAT PALATE: FOUR HOURS DIGESTION

protein measurements yielded 170 $\pm$ 10 µg protein per 10^6 connective tissue cells and 235 $\pm$ 15 µg protein per 10^6 epithelial cells.

3. Cell Viability

Routinely 90-95% of both epithelial and connective tissue cell populations excluded trypan blue dye. The ability of the cells to actively accumulate proline was also used as a criterion of their viability. Proline uptake per 10^6 cells was calculated for cells incubated with 0.2 mM ^{14}C-proline for various time points. In Figure 6 epithelial cells show a slightly higher uptake of free proline over connective tissue cells. However, due to the difference in ICF space measurements the epithelial cells have a lower distribution ratio than do the connective tissue cells (Fig. 7). Both epithelial and connective tissue cells for all time points do show distribution ratio's well over 1.0 and thus are actively accumulating proline.

Control data for total incorporation of ^{14}C-proline (Fig. 8) shows that during the four hour incubation period both the epithelial cell population and connective tissue cell population incorporate proline into protein at approximately the same rate after 30 minutes.

Data for hydroxylation of protein bound proline (i.e., hydroxyproline), (Fig. 9), shows that epithelial cells hy-

FIGURE 6

Uptake of proline by epithelial and connective tissue cells. Each point represents the mean with its upper and lower limits n = 10.

PROLINE UPTAKE BY ISOLATED CELLS FROM RAT PALATE

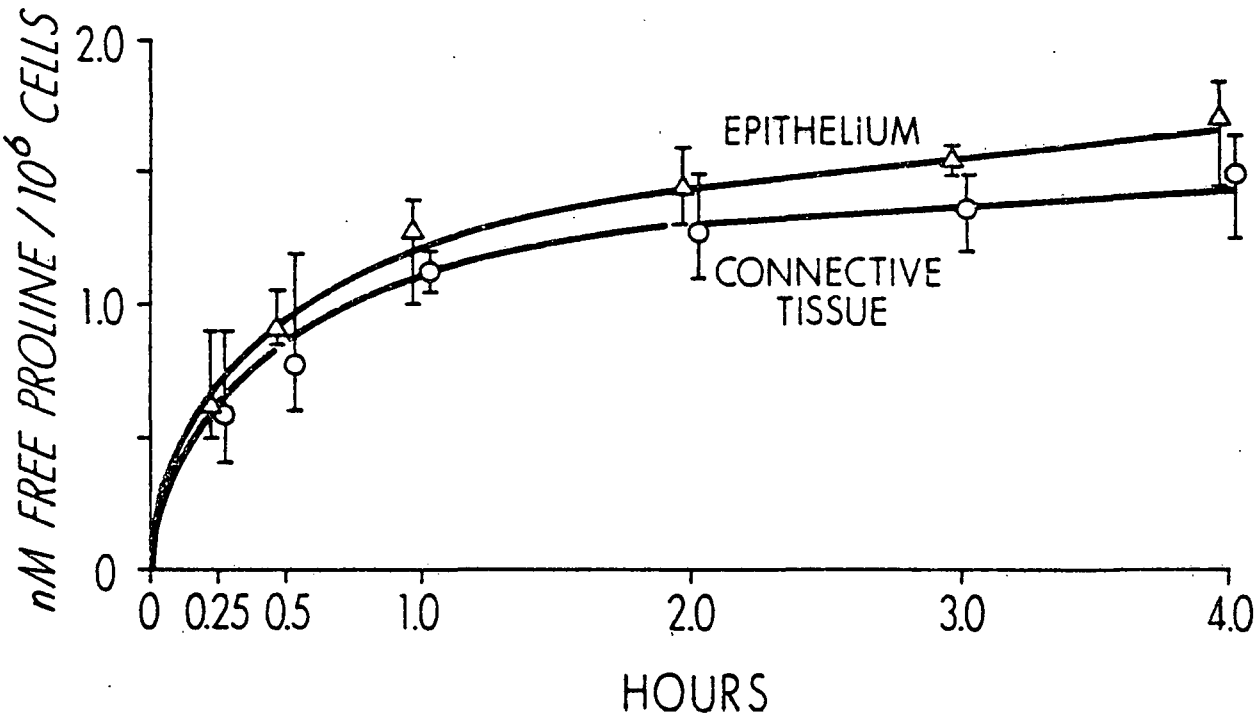


FIGURE 7

Distribution ratio = $\frac{\text{nM free proline}/\mu\text{l cell } H_2O}{\text{extraction proline concentration}}$ over a 4 hr. incubation of epithelial and connective tissue cells. Each point represents the mean with upper and lower limits determined from upper and lower limits of ICF measurements. n = 10.

DISTRIBUTION RATIO OF ISOLATED RAT PALATAL CELLS

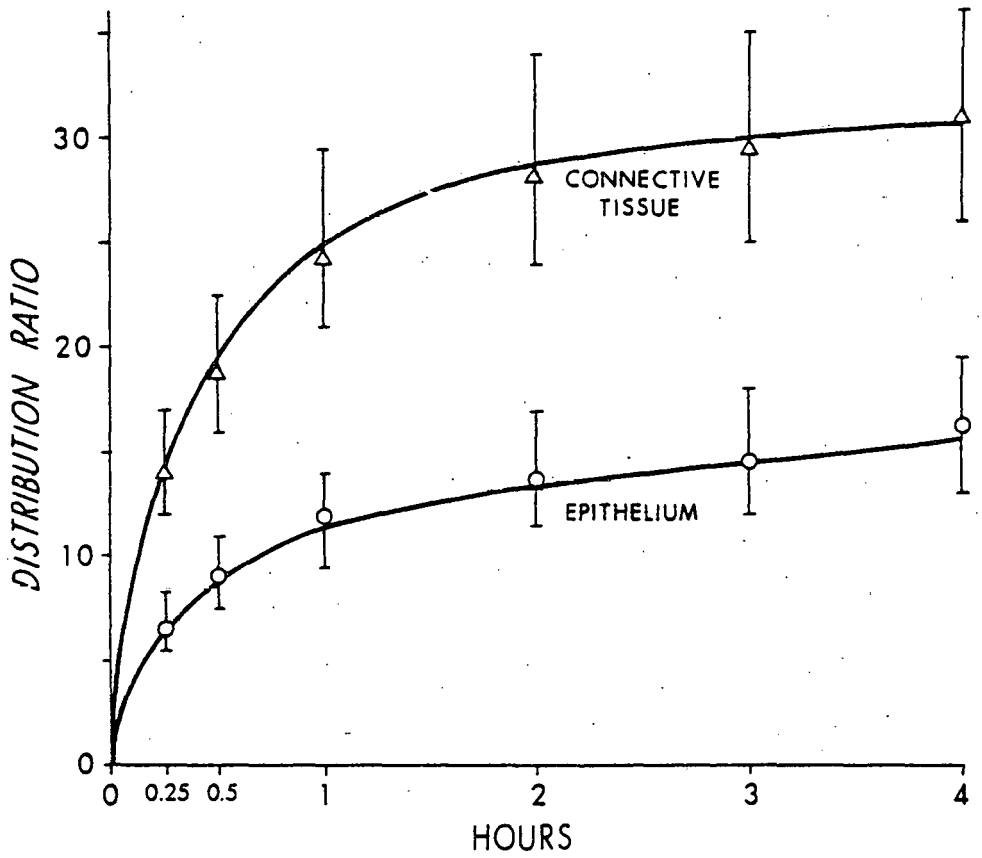
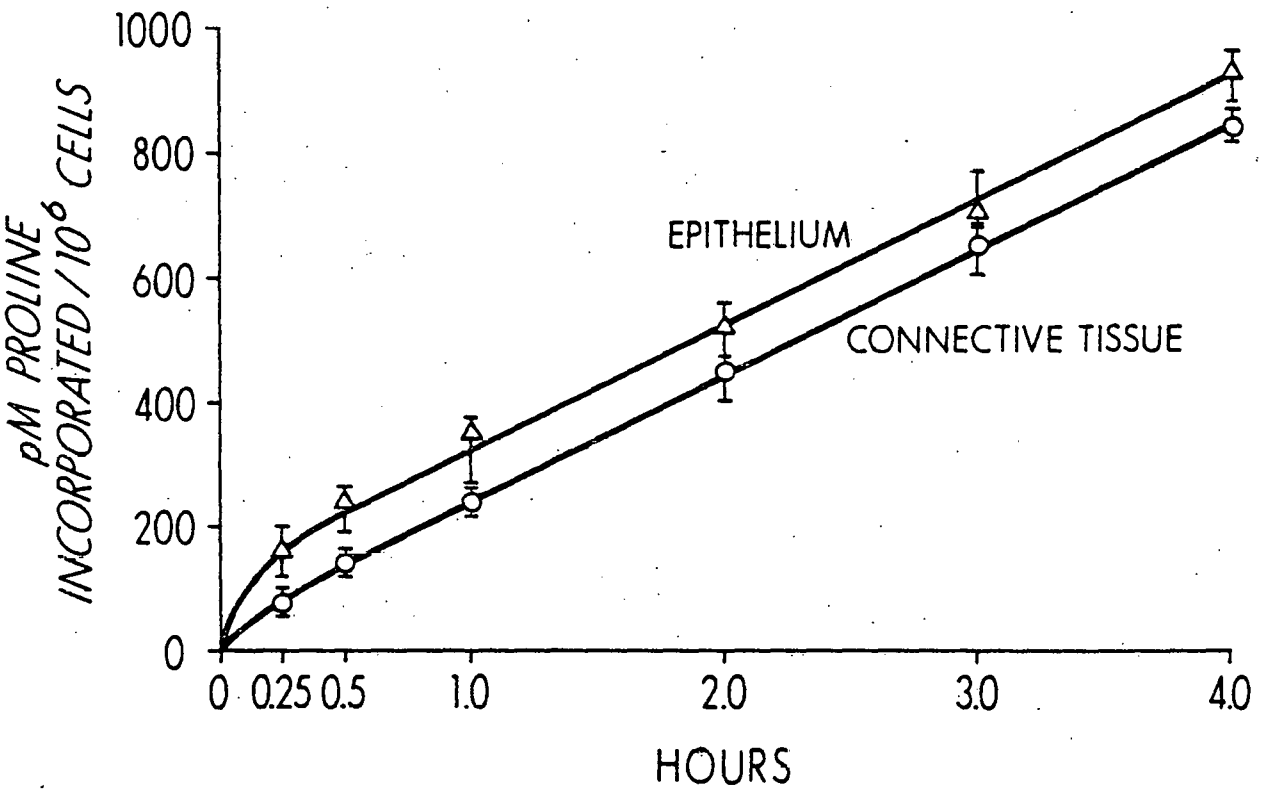


FIGURE 8

Incorporation of proline into protein by 10^6 epithelial and connective tissue cells. Points are the mean with upper and lower limits of 10 experiments.

INCORPORATION OF PROLINE INTO PROTEIN BY

ISOLATED RAT PALATAL CELLS



droxylate 20 pM/hr/10^6 cells more proline than do connective tissue cells.

Combining the control data for proline and hydroxyproline the per cent of protein made by the cell that is collagen, over the four hour incubation, is 26% for connective tissue cells and 43% for epithelial cells, assuming an imino acid content of 20 mole percent for collagen and an average proline content of 5 mole percent for non-collagen protein.

The results of the PTH experiments indicate that the PTH decreases protein synthesis in the connective tissue cell population as can be seen in Figure 9. Proline incorporation over the 4 hour incubation is decreased by 20 pM protein bound hydroxyproline per hour per 10^6 cells. Total incorporation of ^{14}C-proline at the end of the four hour incubation is decreased by 28%. As a further control and as could be expected no effect of PTH is seen on the epithelial cell population (Fig. 9) subjected to preincubation with PTH.

Combining the PTH data for proline and hydroxyproline the epithelial cells still produce 43% collagen and 57% non-collagen protein. Data for the connective tissue cell population shows that these cells are making protein 27% of which is collagen and 73% of which is non-collagen protein.

4. ^{14}C-D-Valine Metabolism

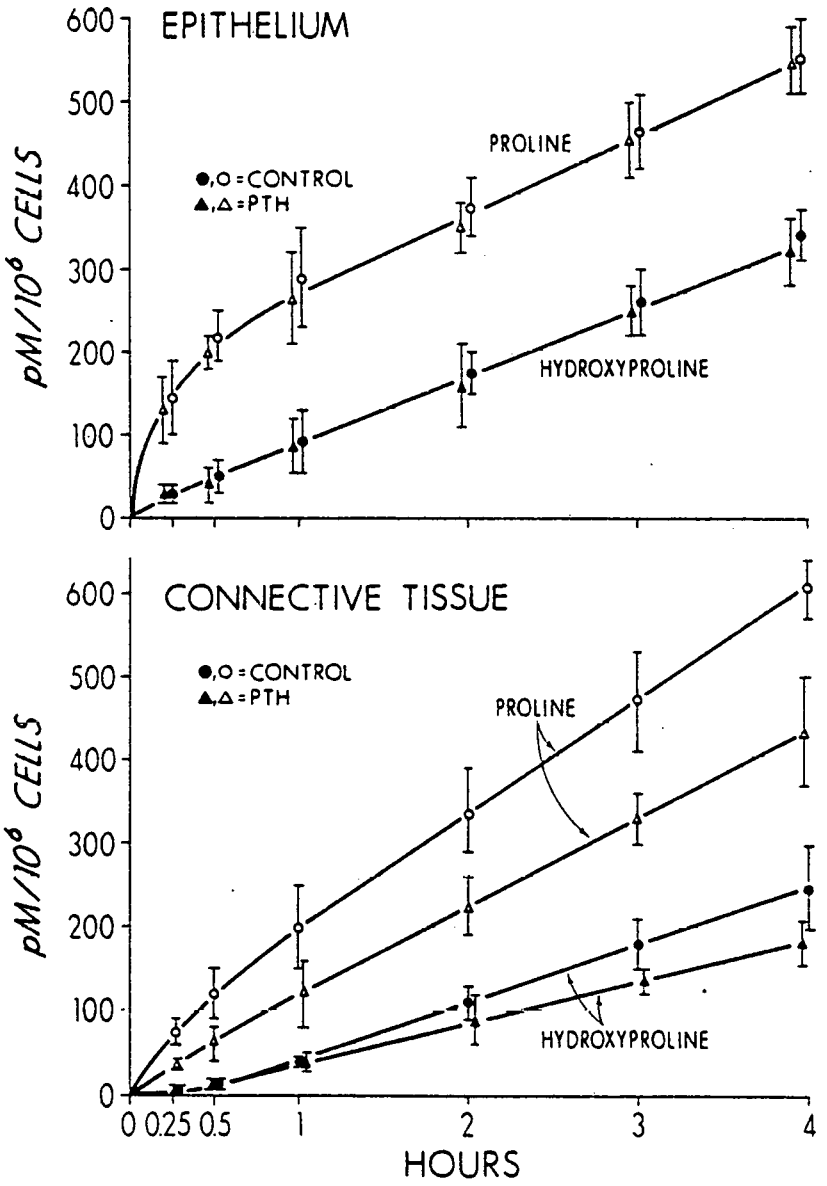
Gilbert and Migion[10] developed a nutrient media that provides for normal growth in culture of epithelial cells

FIGURE 9

Effect of PTH on connective tissue cells and epithelial cells as determined by pM proline incorporated per 10^6 cells and pM of hydroxylated protein bound proline per 10^6 cells. Results are from thin layer chromatography resolution of dansylated amino acids. Points represent the mean $\pm$ 1 SEM of 5 experiments.

COLLAGEN SYNTHESIS BY ISOLATED RAT PALATAL CELLS

AND EFFECT OF PTH



while selectively inhibiting fibroblast proliferation. In this medium D-valine is substituted for L-valine and only the epithelial cells having a D-amino oxidase can utilize the D-valine. D-amino acid oxidase catalyzes the oxidative deamination of D-valine to 2-ketoisovaleric acid. Although the cell does not possess a specific transport mechanism for D-amino acids, D-valine is free to enter by passive diffusion. D-valine, once inside the cell and deaminated to 2-ketoisovaleric acid can then be further metabolized.

Dansylation of ^{14}C-D-valine will give dansylated ^{14}C-D-valine (1-dimethylaminonaphthaline-5-sulfonyl-^{14}C-D-valine) which is soluble in toluene and will count in the absence of a solubilizer. 2-ketoisovaleric acid or any further metabolic products, lacking an amino group, will not react with dansyl chloride and hence are not soluble in toluene but can be solubilized by the addition of BBS-3[a]. Thus, the percent D-valine remaining in the buffer is equal to the counts per minute without BBS-3 divided by the counts per minute plus BBS-3, (Fig. 10). Figure 11 shows that connective tissue cells do not metabolize D-valine whereas epithelial cells show a continuous breakdown of D-valine through a six hour incubation.

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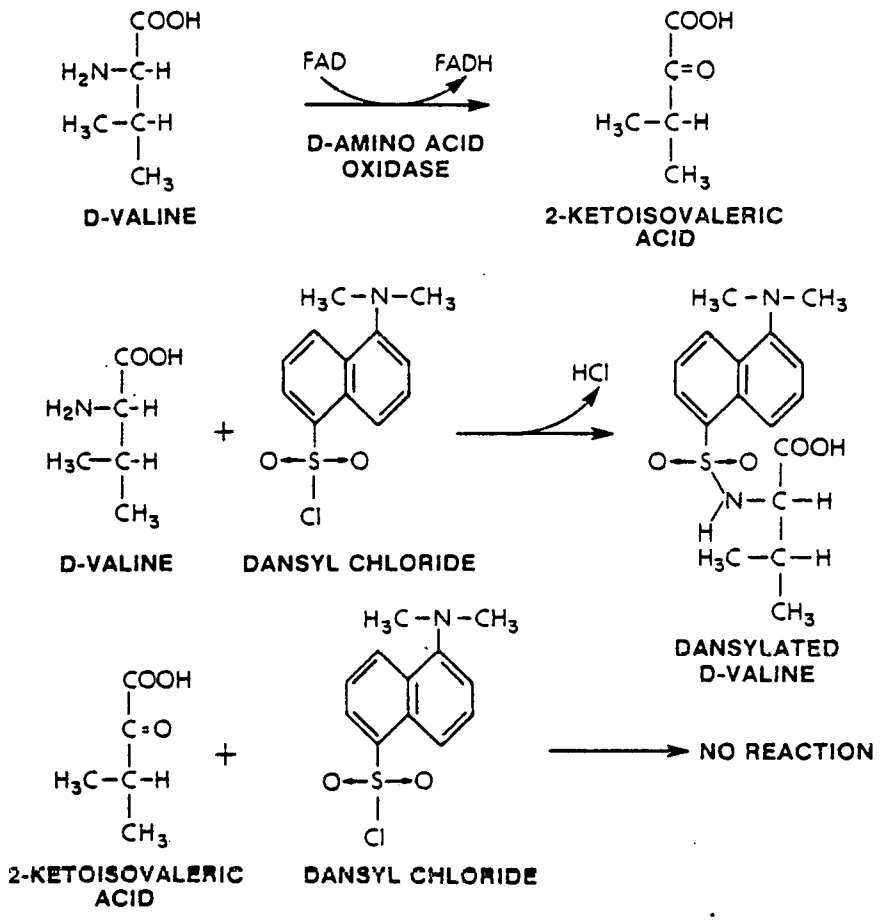
a. Beckman Instruments Inc., Fullerton, Ca.

FIGURE 10

Chemical rationale for the quantitative assay of the metabolism of labeled amino acids.

BIOCHEMISTRY OF D-VALINE ASSAY

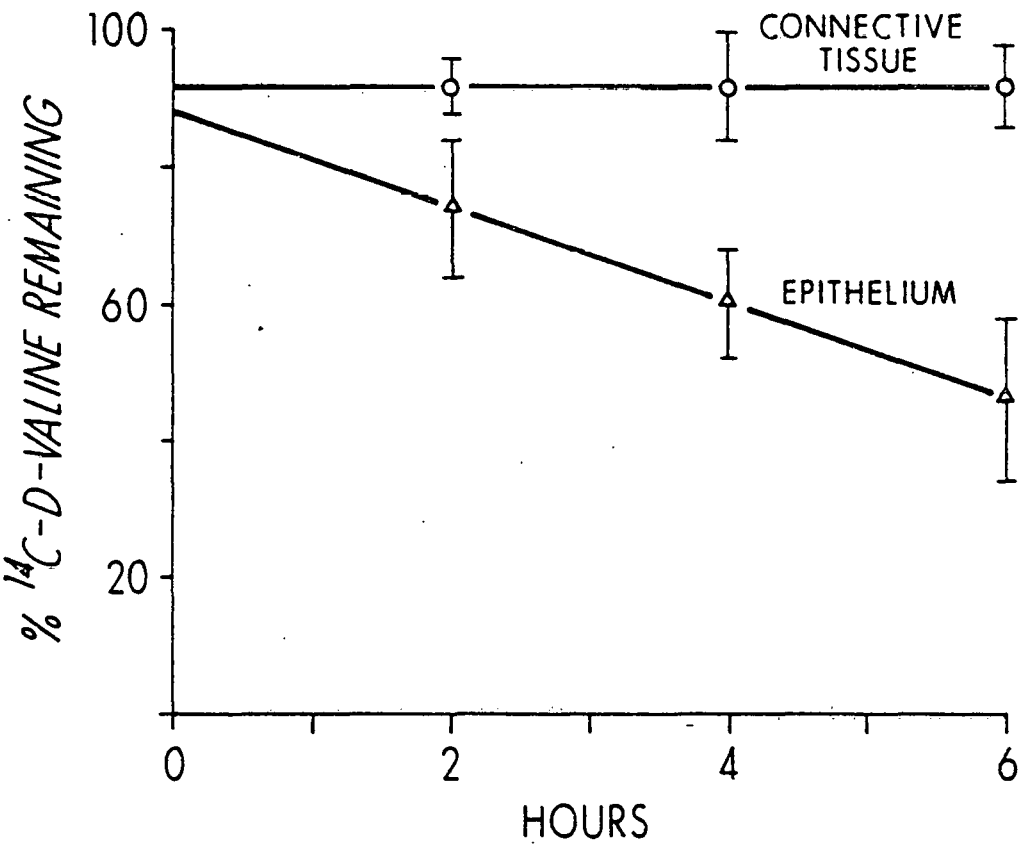


$$\% \text{ D-VALINE REMAINING} = \frac{\text{CPM} - (\text{BBS-3})}{\text{CPM} + (\text{BBS-3})} \times 100$$

FIGURE 11

Metabolism of D-valine by epithelial cells over a six hour incubation. % D-valine remaining in the media is an indication of metabolism of the D-amino acid by epithelial cells. Points are the mean $\pm$ 1 SEM of 4 experiments.

D-VALINE METABOLISM



DISCUSSION

The results presented in this paper indicate that by sequential digestion of keratinized oral tissue two morphologically and biochemically distinct populations of cells can be obtained.

In the studies with D-valine, we have developed a method for a quantitative assay of the metabolism of any labeled amino acid with the exception of the basic amino acids which have in addition to the alpha amino group, a nitrogen which will react with dansyl chloride.

Using this technique, we have demonstrated that rat oral epithelial cells contain a D-amino acid oxidase and thus provided additional evidence that we have two distinct populations of cells. This evidence strongly supports what was observed histologically, namely, a sequential digestion in which the connective tissue cells are dispersed during the first two hours and the epithelial cells during the last two hours.

Further biochemical evidence supports the observation that we have isolated distinct cell populations. The distribution ratios for both populations demonstrate that the isolated cells are able to accumulate proline against a concentration gradient. This requires that the plasma membrane is intact and is capable of active transport. The amount of free proline accumulated by the epithelial cells at steady state

(1.7 nM/10^6 cells) is slightly higher than that found for connective tissue cells (1.40 nM/10^6 cells). However, from ICF measurements the epithelial cells have nearly double the volume of connective tissue cells which are accumulating proline to a higher intracellular concentration. One might expect that the connective tissue cell population would incorporate more proline into protein than the epithelial cells. On histological ground this should also be expected in that the cells of the connective tissue are embedded in a matrix of collagen fibers. There is also a wealth of literature concerning collagen biosynthesis in the fibroblast[11]. From the results obtained this was not the case. Total proline incorporated into protein per 10^6 cells was found to be about the same in epithelial cells (860 pm) as in connective tissue cells (820 pm). The observation becomes more apparent if one examines the amount of hydroxylation of protein bound proline. Epithelial cells hydroxylate protein bound proline at a 30% higher rate than do connective tissue cells. The amounts of proline and hydroxyproline incorporated converted to percent collagen synthesized indicates that the epithelial cells, over a four hour incubation, have synthesized 60% more collagen than the connective tissue cells. From studies presently underway it appears that the basal cell layer of the epithelium is the most metabolically active. Grant et al. (12,13) have shown that the collagen component of basement membranes contain

higher quantities of hydroxyproline and hydroxylysine than other interstitial collagen. This leads one to speculate that most of this collagen is synthesized by the basal layer epithelial cells and is basement membrane collagen. Studies will be undertaken to define the type of collagen produced by each cell type.

Smith and Johnson (14) have shown that periosteal cells from rat skeletal tissue respond to PTH. Vaes (15) found that in a bone tissue culture system, bone cells respond to PTH whereas cultured fibroblasts show no effect to added hormone. Peck et al. (16,17) demonstrated that primary cultures of isolated bone cells are most responsive to PTH. The effect of hormone diminished with repeated subculturing. The results of the PTH experiments indicate that this hormone decreases protein synthesis in the connective tissue cell population. PTH decreases the rate of proline hydroxylation by 30% and decreases the rate of proline incorporated by 22%. Thus, what we are observing is equivalent to a 30% decrease in collagen synthesis. This apparent decrease in collagen synthesis could be due in part to a PTH induced inhibition of prolyl hydroxylase (18). The effect is not strictly limited to inhibiting collagen synthesis or prolyl hydroxylase since PTH causes a comparable decrease in synthesis of both collagen and non-collagen protein by connective tissue cells. Since it has previously been shown that PTH has no effect on fibroblasts the effect observed must be on cells

of osseous or preosseous origin, probably periosteal cells. From histological observations these are the cells that are liberated during the first hour of incubation with enzyme. It is of interest that the most metabolically active population of epithelial cells is the basal layer and apparently the most metabolically active segment of the connective tissue is the periosteal cell layer. As a further control to demonstrate PTH effect, epithelial cells subjected to preincubation with and without PTH showed no response to hormone with respect to incorporation of proline into protein and hydroxylation of protein bound proline.

Further experiments are planned to separate these two populations into subpopulations by the use of Ficoll gradients. Thus, the inhomogeneity of the connective tissue cell populations could be resolved and the epithelial cell population could be examined for connective tissue cell contaminates.

CONCLUSIONS

It was demonstrated that sequential isolation techniques yielded separate populations of cells from the epithelium and underlying connective tissue of rat palate. These two populations exhibit distinct biochemical differences. The correlation observed between the morphology and the biochemical evidence suggests that the technique of isolation and identification, described here for cells from the rat palate, can be applied to biochemical study of specific cell populations from normal and diseased oral tissues.

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CHAPTER II

COLLAGEN SYNTHESIS BY BASAL EPITHELIAL CELLS ISOLATED FROM RAT PALATE

INTRODUCTION

The epithelium of the periodontium contains four distinct cell subpopulations: basal cells, spinous cells, granular cells and cornified squamous cells. The region between the epithelium and the underlying stroma is traditionally described as a basement membrane. From morphological study of the periodontium it was originally believed that the basement membrane was a connective tissue derivative (Gersh and Catchpole, 1949). It was later postulated that this basement membrane was not a product of the connective tissue but rather of the basal epithelium (Schroder and Theilade, 1966). While conclusive evidence for the origin of periodontal basement membrane is still lacking, this structure has been shown to derive in some other tissues from the synthetic activity of epithelial cells (Kefalides, 1973).

Previous work in our laboratory demonstrated that a mixed epithelial cell population can synthesize collagen in vitro. The objective of this study was 1) to separate the basal epithelial cells from this mixed cell population; 2) to determine if these cells were capable of collagen synthesis; and 3) to establish whether the collagen synthesized showed any of the characteristics of basement membrane collagen.

The technique chosen for epithelial cell separation was density gradient centrifugation. This procedure has been effective in resolving distinct subpopulations from several

heterogeneous preparations of mammalian cells (Boone, 1968; Pretlow and Boone, 1969).

There are some marked differences in the amino acid composition of basement membrane collagen compared to other types of collagen (Kefalides, 1975; Kefalides, 1973). This information provided a basis for identification of the collagen synthesized by these epithelial cells as basement membrane collagen.

RESULTS

Cell Separation:

Figure 1 shows the results of sedimentation to equilibrium of a suspension of isolated connective tissue cells from rat palate. The mean density as measured from the midpoint of the most prominent fraction was 1.076 $\pm$ 0.001 gm/cc in 6 experiments. Cell recovery was 94 $\pm$ 5%. Equilibrium sedimentation of a suspension of isolated epithelial cells is illustrated in Figure 2. The mean density for these cells was 1.086 $\pm$ 0.001 gm/cc in 8 experiments; 92 $\pm$ 3% of the cells were recovered. In each set of experiments (Figures 1 & 2) the density gradients were consistently linear with an average r^2 value of 0.998.

The two populations of cells, connective tissue and epithelium, are thus distinct and each population homogenous with respect to density.

Velocity centrifugation of epithelial cells on a shallow density gradient is illustrated in Figure 3. This profile was remarkably reproducible in twenty experiments with the largest variation in fractions 19 and 20. As with the equilibrium gradients, cell recovery was routinely above 90%. Smears of cells from each gradient fraction, stained with Papanicolaou stain, revealed an increase in cell size from the top of the gradient, fraction 2, to the bottom, fraction 20 as seen in Figure 4. Thus, a distribution of the epithelial cells based on cell size was achieved but it was not possible

FIGURE 1

Percent recovered (left ordinate) refers to the percent of cells recovered in each fraction using the starting sample as 100% (40 x 10^6 cells). Density (right ordinate) is determined by using the index of refraction and comparing it against standard solutions of 40% ($^w/_w$) Ficoll. Each fraction is 4.0 ml. n = 6. For centrifugation conditions see Materials and Methods section of text.

DENSITY DETERMINATION OF ISOLATED

CONNECTIVE TISSUE CELLS

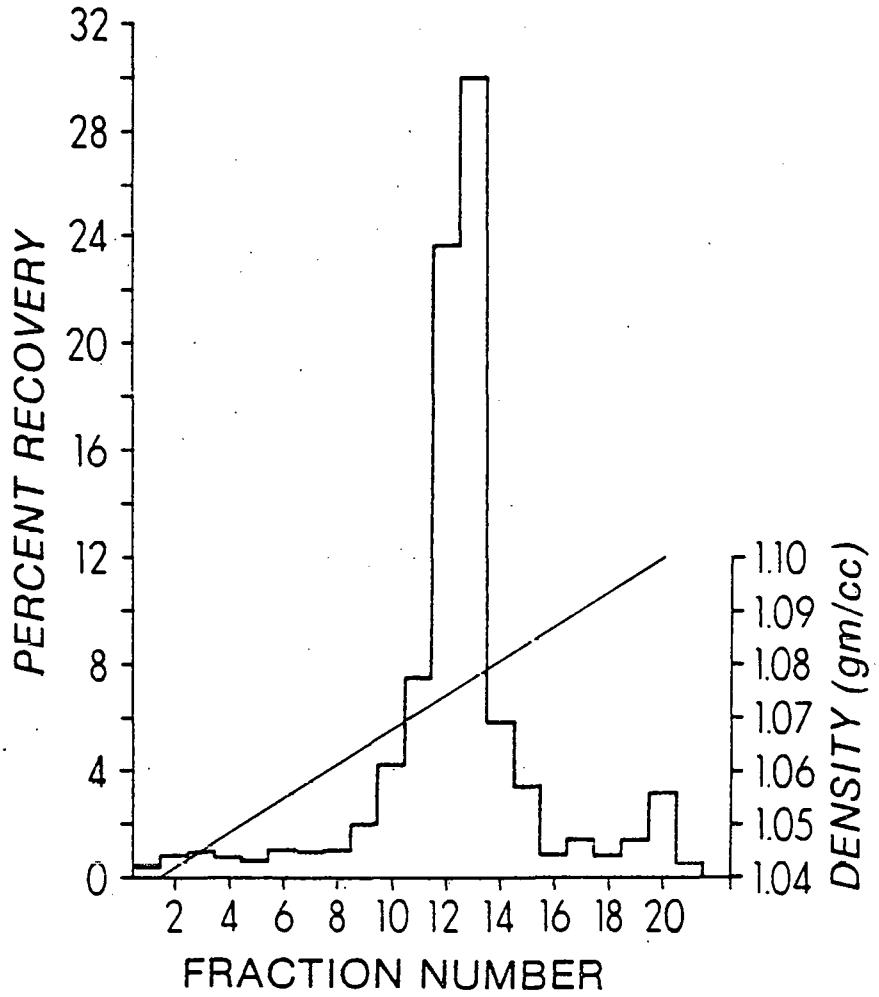


FIGURE 2

Percent recovered (left ordinate) refers to the percent of cells recovered in each fraction using the starting sample as 100% (40 x 10^6 cells). Density (right ordinate) is determined by using the index of refraction and comparing it against standard solutions of 40% ($^w/_w$) Ficoll. Each fraction is 4.0 ml. n = 6. For centrifugation conditions see Materials and Methods section of text.

DENSITY DETERMINATION OF ISOLATED

EPITHELIAL CELLS

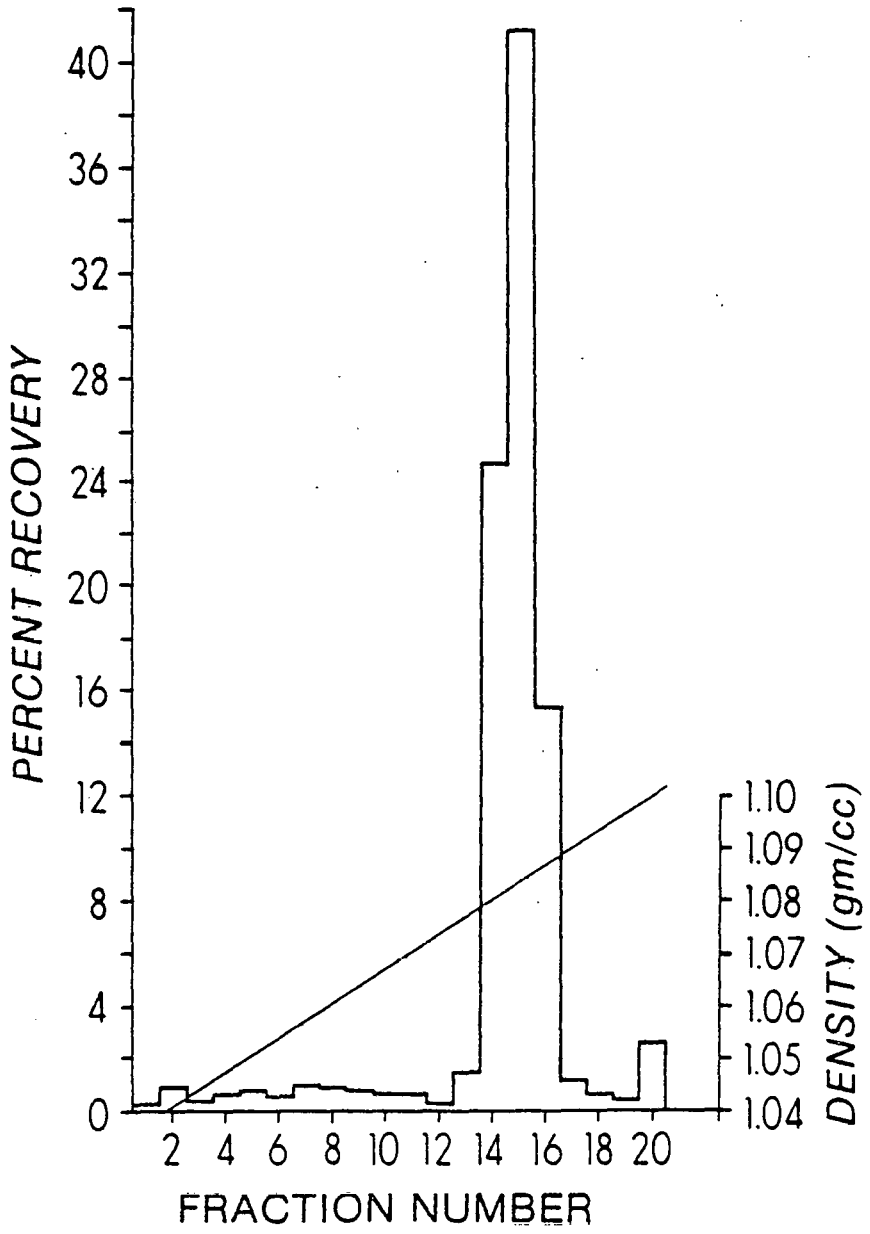


FIGURE 3

Number of cells x 10^6 is shown on the left ordinate. Each fraction is 4.0 ml. n = 20. For gradient construction and centrifugation conditions see Materials and Methods section of text.

GRADIENT PROFILE RESULTING FROM A VELOCITY CENTRIFUGATION OF ISOLATED EPITHELIAL CELLS

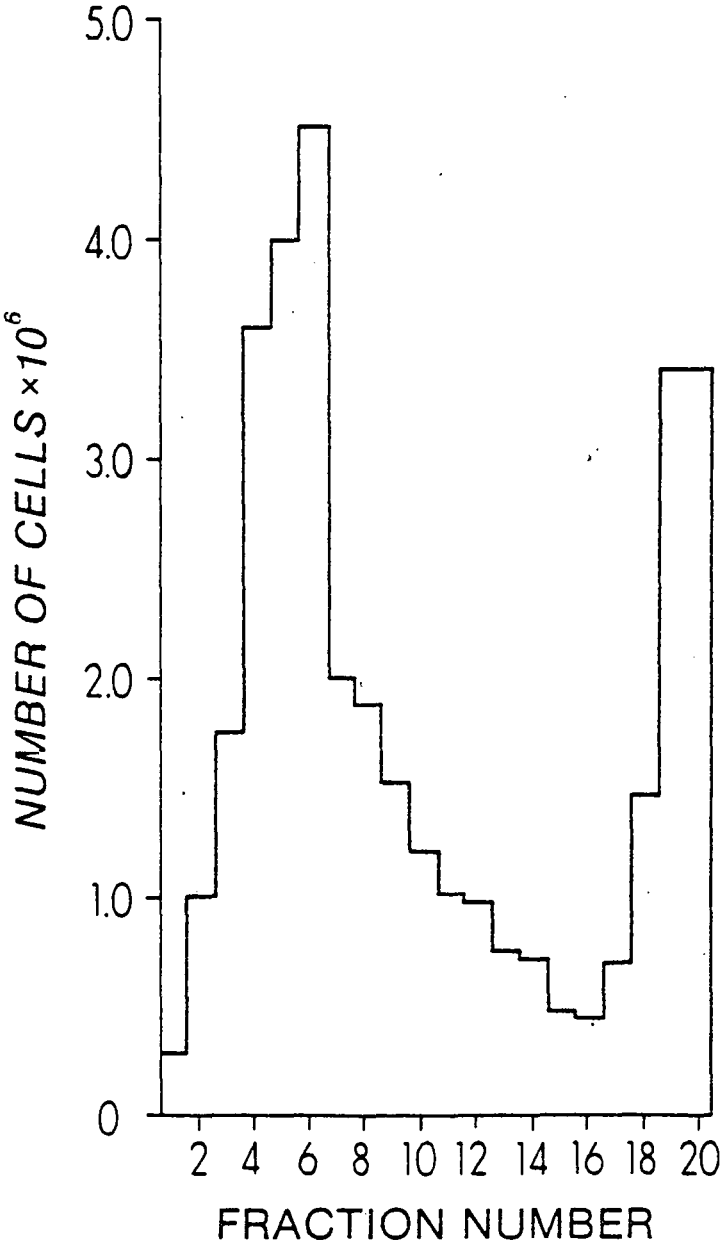
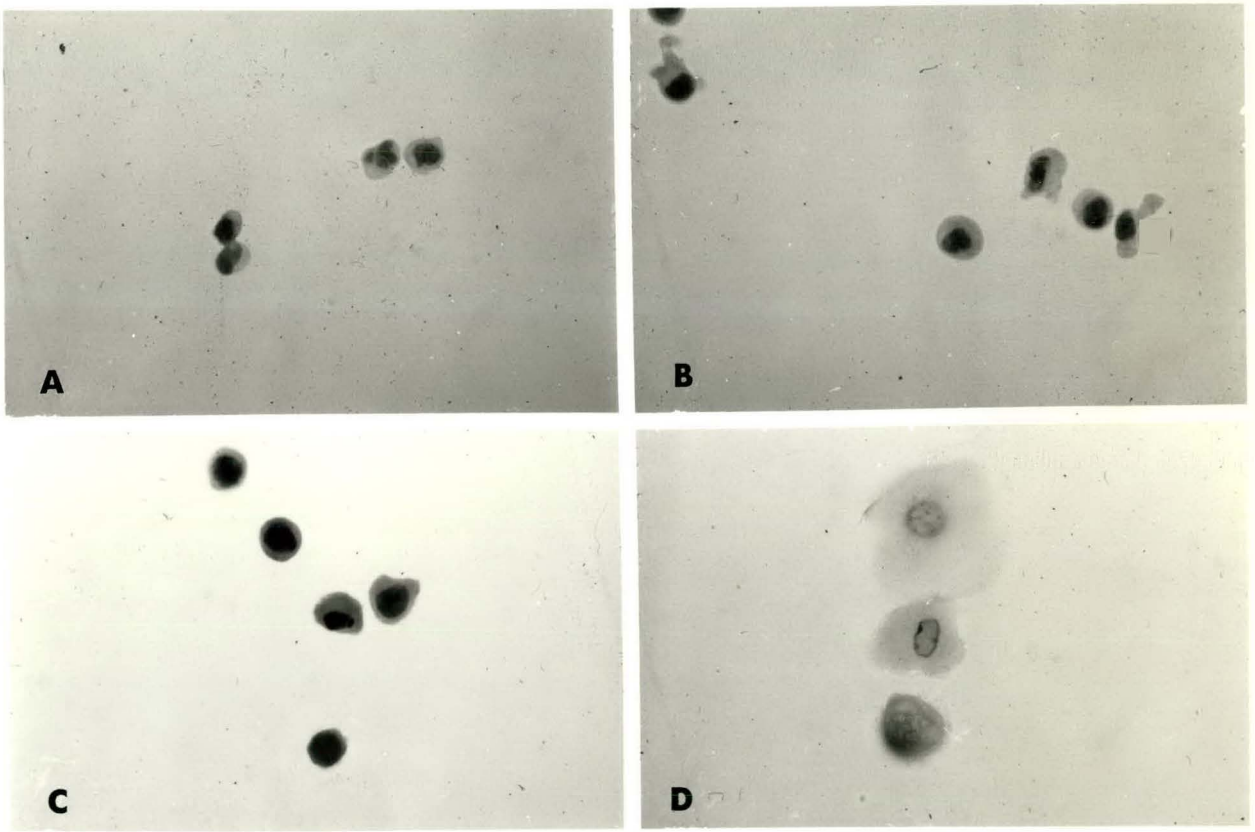


FIGURE 4

Photomicrographs (magnification equals 252 x) of separated epithelial cells stained with Papanicolaou stain. (A) Cells from fraction number 3, (B) Cells from fraction number 6, (C) Cells from fraction number 10 and (D) Cells from fraction number 20.

EPITHELIAL CELLS SEPARATED BY MEANS OF

VELOCITY CENTRIFUGATION



to identify the basal cells morphologically.

Pulse labeling with ^{3}H-thymidine for one hour prior to sacrifice provided the results shown in Figures 5 to 8. Autoradiographs of the tissue (Fig. 5) show that only the basal cells have incorporated label. Velocity centrifugation of prelabeled cells gave a distribution of label as shown in Figure 6, with predominant cell label appearing in fractions 9, 10, 11. Autoradiographs of cell smears from fractions 3, 6, 10 and 20 (Fig. 7) show a notable increase in cell size moving down the gradient and the highest percentage of cells with label in fraction 10. Some labeled cells also appear in fraction 20.

Samples of each fraction from a velocity centrifugation of labeled epithelial cells were then assayed for total DNA, and radioactivity measured. The results, expressed as CPM/µg DNA, are presented in Figure 8. Two peaks of specific activity are apparent, one at fraction number 1 and the second at fraction number 10. The peak of cells, at fraction numbers 19 and 20, show minimal radioactivity. It was concluded from these ^{3}H-thymidine incorporation experiments, that the basal cells distributed within fractions 9 thru 12 on the velocity gradients.

Collagen Synthesis by Basal Epithelial and Connective Tissue Cells:

Analysis of labeled amino acids incorporated into the

FIGURE 5

Only basal epithelial cells show labeling over the nucleus after sacrificing animal one hour post injection. (Magnification equals 100 x). Tissue section stained with H and E.

PHOTOMICROGRAPH OF ^{3}H-THYMIDINE LABELING OF

BASAL EPITHELIAL CELLS

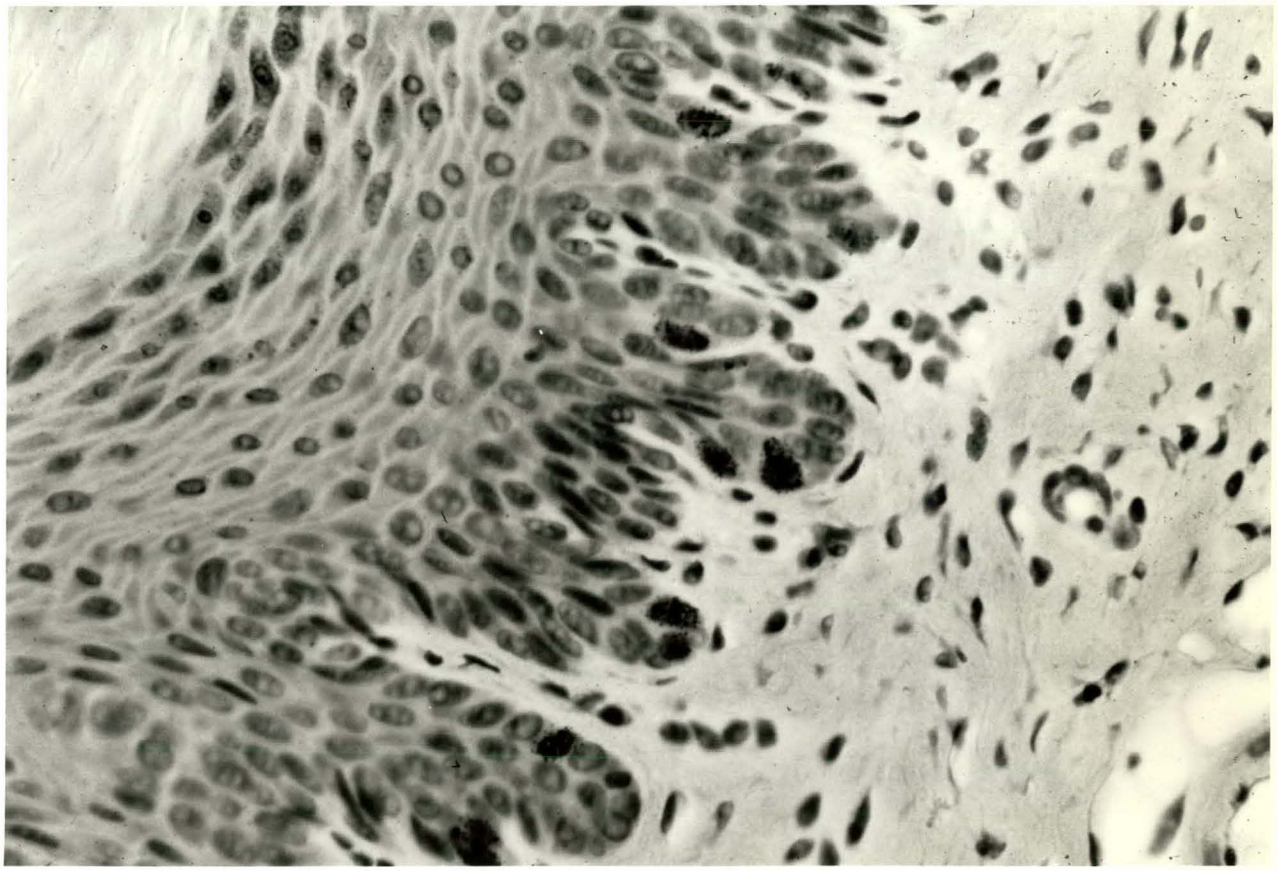


FIGURE 6

Number of cells x 10^6 is shown on the left ordinate. Percent labeling is shown on the right ordinate and is defined as the number of cells with more than 6 silver grains over the nucleus out of 1500 cells counted per fraction.

LOCATION OF BASAL EPITHELIAL CELLS

ON AN ISOKINETIC GRADIENT

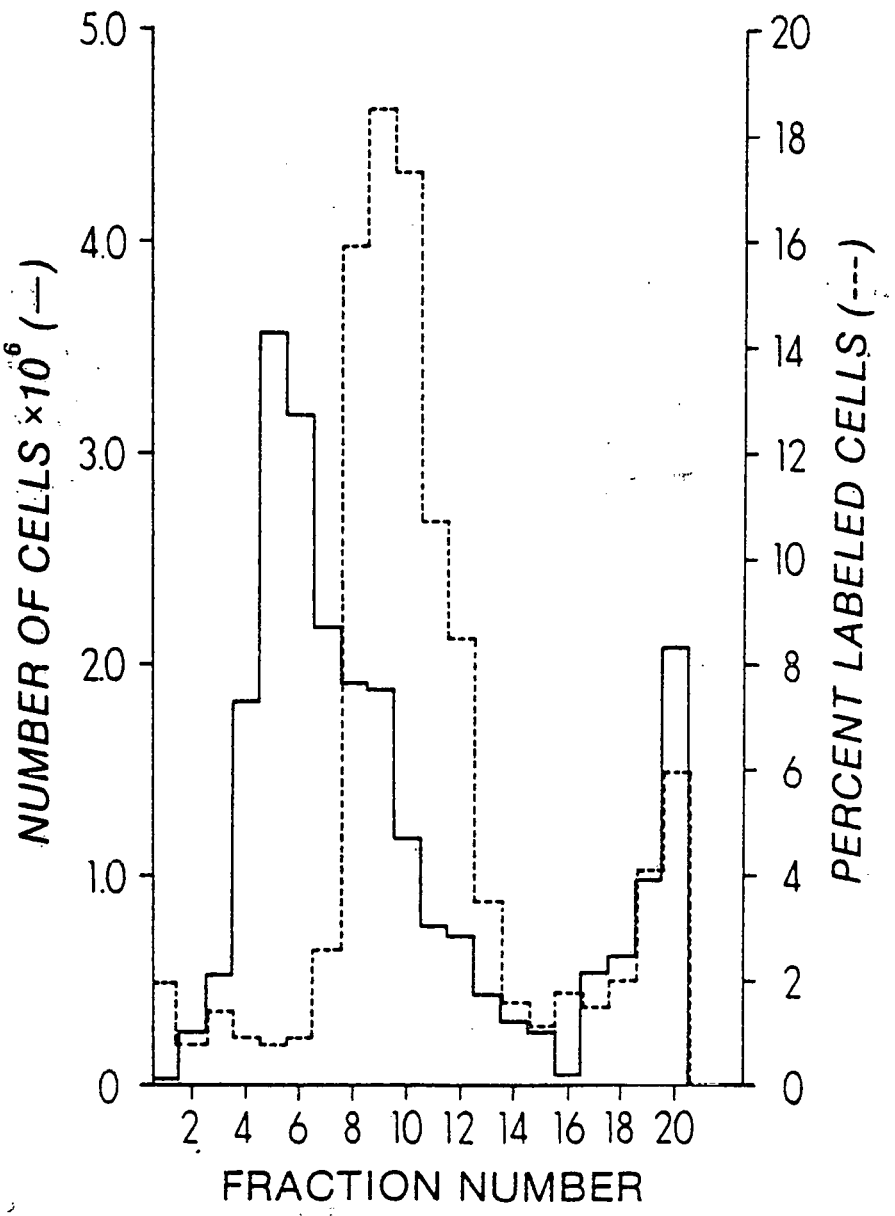


FIGURE 7

Autoradiographs of separated epithelial cells from: (A) Fraction number 3, (B) Fraction number 6, (C) Fraction number 10 and (D) Fraction number 20. The cells were stained with H & E, magnification is 252 X.

PHOTOMICROGRAPH OF ISOLATED ^{3}H-THYMIDINE LABELED EPITHELIAL CELLS AFTER VELOCITY CENTRIFUGATION

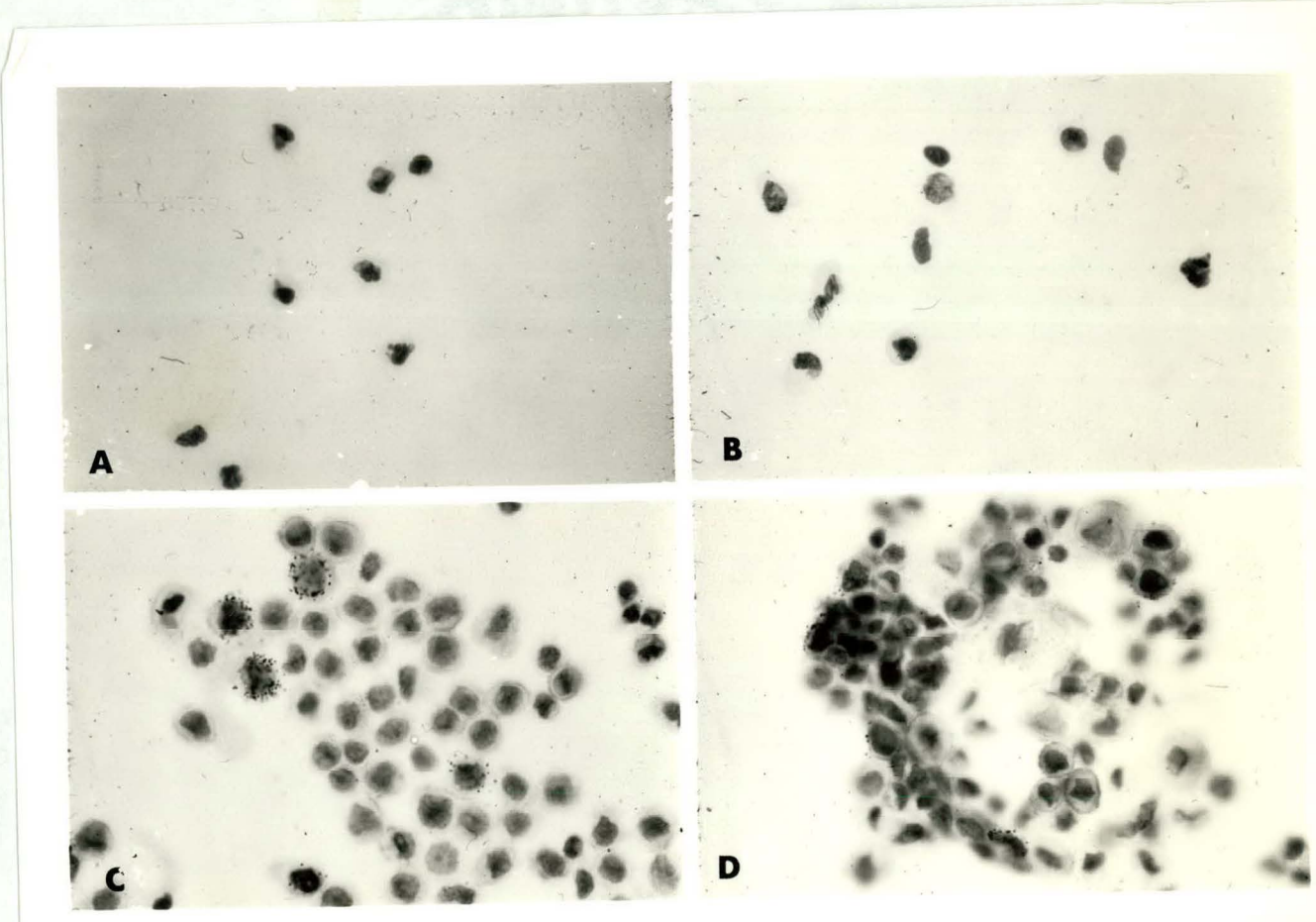
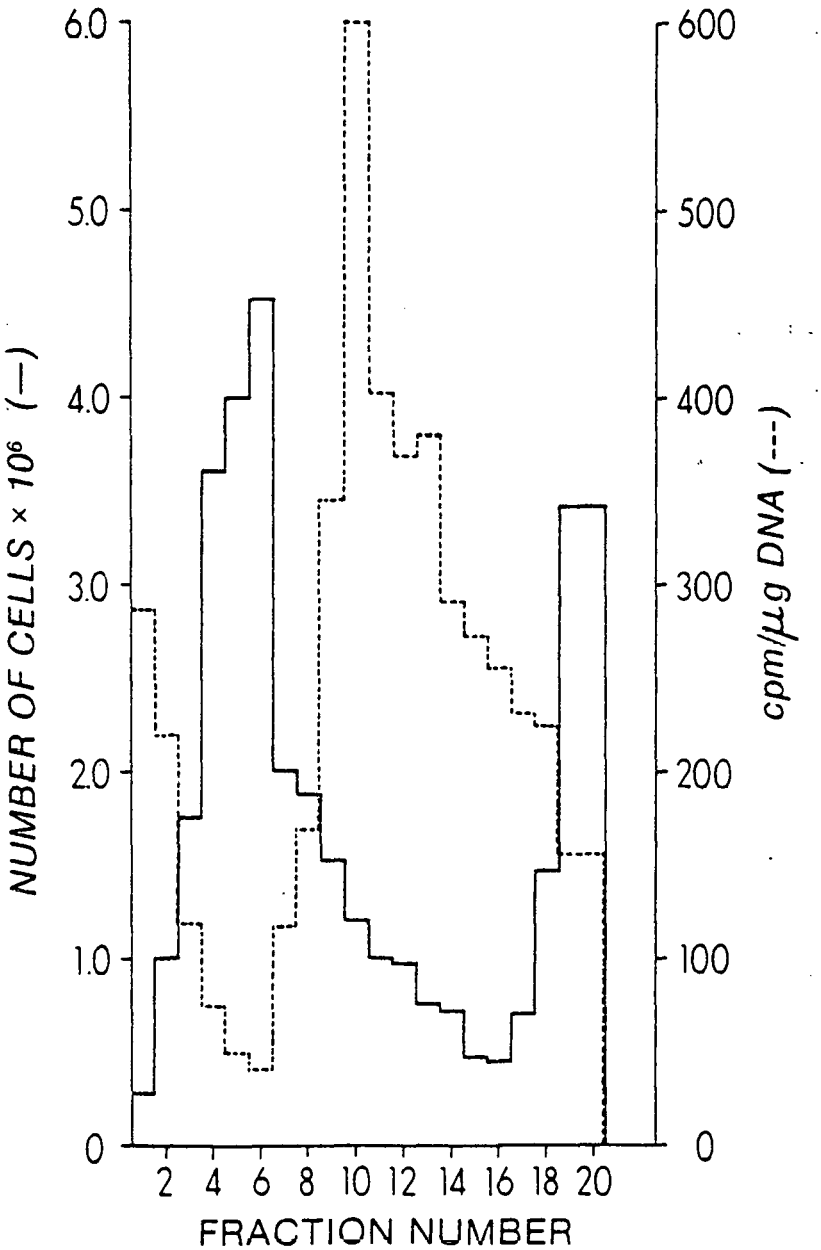


FIGURE 8

The left ordinate shows number of cells x 10^6. The right ordinate represents CPM of ^{3}H-thymidine per µg cellular DNA. All DNA determinations were run in duplicate.

DETERMINATION OF THE LOCATION OF ^{3}H-THYMIDINE LABELED BASAL EPITHELIAL CELLS IN AN ISOKINETIC GRADIENT



collagen synthesized by 1) a mixture of isolated connective tissue cells and 2) a highly enriched basal epithelial cell population from fractions 9, 10, 11 and 12 of a velocity centrifugation is presented in Table I. Epithelial cells synthesize a collagen with a proline:hydroxyproline ratio of 0.47 ± 0.04 and an alanine:leucine ratio of 0.69 ± 0.03. In comparison, connective tissue cells synthesized a collagen with a proline:hydroxyproline ratio of 1.48 ± 0.06 and an alanine:leucine ratio of 5.92 ± 0.16. Total incorporation of labeled amino acids in nmoles/10^6 cells is also shown in Table I. Epithelial cells incorporate 47% more proline into collagen than do an equivalent number of connective tissue cells. Mean proline incorporation values are 4.1 nM/10^6 epithelial cells and 2.79 nM/10^6 connective tissue cells. Furthermore, epithelial cells incorporate 18% more alanine into collagen then do connective tissue cells and show over a 9 fold increase in incorporation of leucine over connective cells. Mean incorporation values for alanine and leucine by epithelial cells are respectively 1.20 nm/10^6 cells and 1.75 nM/10^6 cells. The connective tissue cell population incorporated 1.02 nM alanine per 10^6 cells and 0.17 nM leucine per 10^6 cells.

TABLE I

INCORPORATION OF AMINO ACIDS INTO COLLAGEN BY BASAL EPITHELIAL AND CONNECTIVE TISSUE CELLS

		nMoles/10^6 cells				nMoles/10^6 cells		
	Experiment Number	Alanine	Leucine	Ala/Leu	Experiment Number	Proline	Hydroxy-proline	Pro/Hyp
Basal Epithelial Cells	1	1.20	1.82	0.66	4	1.24	2.75	0.45
	2	1.21	1.68	0.72	5	1.21	2.66	0.45
	3	1.20	1.75	0.69	6	1.37	3.06	0.45
					7	1.41	2.71	0.52
Connective Tissue Cells	1	1.12	0.19	6.03	4	1.65	1.12	1.48
	2	0.92	0.16	5.73	5	1.65	1.18	1.40
	3	1.02	0.17	6.00	6	1.67	1.11	1.51
					7	1.67	1.09	1.54

DISCUSSION

A well defined basement membrane lies between the epithelium and connective tissue of the palate. The origin of this structure, as well as other basement membranes is of particular interest because it is rich in collagen with distinct characteristics. Prior to any studies of basement membrane collagen biosynthesis it was assumed that this protein, like other collagens, was produced by connective tissue cells, i.e. cells derived from a mesenchymal line. Recent evidence that the collagen of other basement membranes is a secretory product of epithelial cells (Kefalides, 1973), and our previous work, showing that both epithelial and connective tissue cells isolated from rat palate synthesize collagen (Terranova & Brand, 1978) led us to investigate the source of the collagen of this basement membrane.

It appeared that the most direct approach to this question was to separate from a mixture of isolated epithelial cells, those cells which lie along this basement membrane (the basal cells) and study the synthesis of collagen by these cells. The density gradient technique for cell separation developed by Pretlow and Boone (1969) was found to be remarkably effective in preparing a basal cell population. The only significant modification introduced into their procedure was the addition of sodium metrizoate to the polysucrose (Ficoll) medium used for equilibrium sedimentation. When Ficoll alone was used, the high density solution was extremely viscous

and difficult to use. Addition of sodium metrizoate, a density medium widely used in the separation of blood cells, (Munthe-Kass & Seglen, 1974) provided iso-osmotic solutions of the desired densities and greatly reduced the viscosity.

Equilibrium sedimentation of the epithelial cells showed that while there are four major cell subpopulations (basal cells, spinous cells, granular cells and cornified squamous cells) these cell types are all of near uniform density. In eight experiments, 85-94% of the cells recovered (Fig. 2, fractions 14,15,16) had a density of 1.086 $\pm$ 0.009 g/cc. (mean $\pm$ 1 S.D.). Similar results were obtained with the cells from the connective tissue. Of the cells recovered, 79-91% (Fig. 1, fractions 11,12,13) had a density of 1.076 $\pm$ 0.008 g/cc.

Separation of the subpopulations of a mixture of cells of uniform density will depend then only on cell size. Velocity sedimentation of the epithelial cells on a shallow linear density gradient gave the distribution illustrated in Figure 3. The problem which remained was to establish a method of accurately identifying the position of the basal cells in this gradient. The morphology of the cells did not offer reliable indicators for identification with the light microscope. However, the subpopulation of specific interest, the basal cells, contains the only proliferating cells in the epithelium. So the tissue was prelabeled in vivo with ^{3}H-thymidine. As seen in autoradiographs of the intact epi-

thelium (Fig. 4) the label was restricted primarily to the cells of the basal layer. Velocity sedimentation of prelabeled epithelial cells (Figs. 6 & 8) revealed that the basal cells were predominantly in fractions 8 thru 12 with a significant number of labeled cells in fractions 19 and 20 at the bottom of the gradients. The cells moving to the high density end of the gradient were found to be in aggregates or clumps. Aggregates of cells will sediment as single particles with a large relative size and thus a rapid sedimentation velocity. The clumps are not likely to result from incomplete digestion of the tissue since the cells are filtered through 35 μ pore nylon mesh during the isolation procedure. They may aggregate when centrifuged in the washing steps prior to application on the gradient. While loss of labeled cells due to clumping did not exceed 12%, attempts were made to disperse these cells. Reincubation with various combinations of the enzymes used in tissue digestion or reduction in the total number or concentration of cells applied to the gradient did not eliminate the problem.

The homogeneity of the cell population taken from fractions 9 thru 12 could not be determined conclusively although this pool of cells had the uniform appearance under the microscope that was observed for fraction 10 (Fig. 7). A count of the labeled cells in this pool, taken from the data in Figure 6, and in the radioautograph of a section

from the basal epithelium (Fig. 4) revealed that in each, approximately 15% of the cells contained ^{3}H-thymidine label. So it was evident that the cells used for subsequent study were a population which was highly enriched in basal epithelial cells.

In choosing criteria for identifying the collagen synthesized by the isolated cells in vitro, several possibilities were considered. The numerous marked differences in amino acid composition of basement membrane versus interstitial collagens provide a sound basis for distinguishing the two. The most prominent features of basement membrane collagen are the large fractions of proline and lysine which are hydroxylated, the high content of 3-hydroxyproline, leucine and isoleucine and the low content of alanine (Kefalides, 1973). Since the total amount of collagen synthesized by these cells in a 24 hour incubation is small, a reasonable recovery during extraction and purification required the addition of carrier collagen. However, the addition of carrier eliminates the possibility of total or even partial amino acid analysis of the product. It appeared the maximum information with the clearest interpretation would be obtained from a study of labeled amino acid incorporation, expressing the results as ratios. The incorporation of labeled proline into collagen proline and hydroxyproline was an obvious choice. The combination of alanine and leucine provided the largest difference in ratio for any

pair of amino acids. Mean values for the results in Table I are listed in Table II for comparison with data for other tissue collagens. The incorporation ratios for collagen synthesized by the connective tissue cells agree well with literature values for tendon, bone and skin. The pattern of incorporation of label into collagen by basal epithelial cells shows the same marked differences observed for basement membranes. We believe this is strong evidence that these epithelial cells synthesize a basement membrane collagen.

This investigation defines one basal epithelial cell contribution to the basement membrane, synthesis of the collagen component.

The techniques described for the isolation and purification of basal epithelial cells from rat palate appears to have considerable significance in several areas of investigation relating to periodontal disease. Specifically, epithelial-connective tissue interactions could be investigated in the normal and diseased states. Further characterization of basal epithelial cells could be achieved by sub-culturing and one could then investigate the influence of the basement membrane on epithelial cell differentiation. The same technique could be used to obtain PTH responsive periosteal cells. Once these cells are isolated one could examine the fate of periosteal cells during active bone resorption.

TABLE II

COMPARISON OF AMINO ACID RATIOS OF VARIOUS COLLAGENS

COLLAGEN SOURCE	Pro/Hyp	Ala/Leu	References
Interstitial Collagens			
1. Canine Tendon	1.38	5.81	Kefalides, 1975
2. Chick Bone	1.16	4.84	Miller et al., 1967
3. Rat Skin	1.30	4.64	Bornstein et al., 1966
Basement Membrane Collagens			
1. Human Glomerulus	0.43	0.61	Kefalides, 1971
2. Sheep Anterior Lens Capsule	0.51	0.74	Kefalides, 1971
3. Canine Descement's Membrane	0.62	0.62	Kefalides & Denduchis, 1961
Rat Palate Collagens			
1. Connective Tissue Cells	1.48	5.92	Data from Table I
2. Basal Epithelial Cells	0.47	0.69	Data from Table I

In recent years, there has been extensive investigation of the interactions between dental plaque and the periodontal unit. To our knowledge, the present investigation is the first reported methodology for studying individual isolated subpopulation of cells from oral tissue. We feel that by using this methodology the biochemical aberrations which develop in specific cell types in the periodontal unit due to the metabolic by-products of dental plaque, can be investigated with the purpose of further defining this disease process.

EXPERIMENTAL PROCEDURES

Cell Isolation:

The cell isolation technique is a modification of the method described by Terranova and Brand (1978). Palatal tissue, freshly excised from young adult female rats, was split into two segments under a dissecting microscope. One part was connective tissue only; the second segment was the epithelium with some adherent connective tissue. These tissues were incubated separately at 37^{o}C in a Hepes buffered, isotonic salt solution, pH 7.4. Changes in the digestion medium used previously included substitution of 100 µg/ml 3 x crystallized trypsin for elastase, reduction in the concentration of both crude collagenase and hyaluronidase to 2 mg/ml and addition of medium BGJ (Amino acids and vitamins only). Tissue concentration in the digestion medium was reduced to 20 mg/ml.

Cells dispersed during the first hour incubation of the epithelium were discarded and the digestion resumed for a second hour. The connective tissue was incubated for two hours without interruption. Cells from each segment of tissue were harvested as previously described (Dziak and Brand, 1974) and cell counts were made with a haemocytometer.

Density Gradient Centrifugation:

For equilibrium sedimentation an iso-osmotic, linear density gradient from 1.04 to 1.10 gm/cc was constructed

over a cushion of density 1.13 gm/cc in a 100 ml polycarbonate centrifuge tube. The gradients were prepared with a Pharmacia gradient maker from solutions of 4% sodium metrizoate and 21.4 to 5.0% Ficoll. Solutions were made up in pH 7.4 buffer containing 25 mM Hepes, 10mM $NaHCO_3$, 68mM NaCl, 1mM $MgSO_4$, 1mM K_2HPO_4, 1mM $CaCl_2$ and 1 mg/ml bovine serum albumin Fraction V. Gradients were cooled in an ice bath to 4^oC and 4.0 ml of a cell suspension containing 40 x 10^6 cells was carefully layered on. Gradient tubes were centrifuged in an International Model CRU 5000 refrigerated centrifuge at 870 x g for 105 minutes. Care was taken to accelerate and deaccelerate slowly to avoid disrupting the gradients.

Gradients were divided into 4.0 ml fractions by pumping a 40% Ficoll solution to the bottom of the tube and pushing the contents out thru a tapping cap similar to that described by Pretlow & Boone (1969). Cell counts for each fraction were made with a haemocytometer. The density of each fraction and thus the linearity of the density gradient was determined by measuring the index of refraction compared to a 40% ($^w/_w$) Ficoll standard solution.

For velocity sedimentation, shallow gradients of 10.96 to 2.72% Ficoll were prepared without sodium metrizoate. Samples of 40 x 10^6 cells were centrifuged for 30 minutes at 97 x g. Preparation and fractionation of these gradients was carried out by the same procedure described above for equilibrium sedimentation.

Preparation of Labeled Basal Epithelial Cells:

One hour prior to sacrifice each female Sprague-Dawley rat (210-250 gm) received 1 μCi/gm body weight ^{3}H-thymidine (47 Ci/mM Amersham Searle) intraperitoneally. One palate from each group of twenty animals was placed immediately in 10% neutral buffered formalin and subsequently dehydrated, embedded in parafin, sectioned (6 microns thick), mounted, coated with NTB2, exposed for two months and the sections were then developed.

The remaining palates were incubated as described above with 1.0 mM cold thymidine added to the incubation medium. The resulting cell suspensions were then subjected to velocity centrifugation on a shallow linear density gradient. Each fraction was centrifuged at 4°C for 10 minutes at 1000 x g. The resulting cell pellets were washed twice with Hepes buffered salt solution, recentrifuged and subsequently fixed in 10% neutral buffered formalin for 8 hours. Fractions were then centrifuged at 1000 x g for 10 minutes, the supernatant discarded, and 0.5 ml of 100% ethanol added. Five histological smears of each fraction were made, coated with NTB2, exposed for 2 months and developed. Three hundred cells per slide were counted.

In subsequent experiments the initial labeling, preparation and velocity centrifugation remained the same. However, after each fraction was counted for number of cells and washed twice, one half of the cell suspension was assayed for cellular

DNA by the method of Puzas and Goodman, (1978). DNA from the remaining half of the cell suspension was extracted using the same methodology and then counted in a liquid scintillation counter using 1.0 ml Beckman BBS-3 plus 9.0 ml toluene-omnifluor as a counting cocktail.

In further experiments all fractions from a velocity centrifugation of unlabeled epithelial cells, were centrifuged at 1000 x g for ten minutes, washed and the resulting cell pellet fixed and dehydrated as above. Cell smears of each fraction were made and stained with Papanicolaou stain.

Incorporation of Labeled Amino Acids Into Protein:

Isolated connective tissue cells and separated basal epithelial cells were treated identically. Ten million cells were incubated in 20.0 ml of a Hepes buffered isotonic salt solution pH 7.4 supplemented with an amino acid mixture. The ^{3}H and ^{14}C-labeled amino acids were added at 0.2 millimolar. All other amino acids were at the concentration in adult rat serum (Scharff and Wool, 1965). This incubation medium also contained 50 µg ascorbate, 50 µg β-amino propionitrile and 2.0 mg Type I collagen to be used as a co-precipitate. The culture vials were then flushed with 95% O_2 - 5% CO_2 and incubated at 37°C for 24 hours. After incubation the cell suspension was homogenized in a Vertis 45 homogenizer and transferred to dialysis tubing, molecular weight cut-off of 14,000. The homogenate was extensively dialyzed at 4°C against

0.1 M acetic acid for 48 hours. The dialyzate was centrifuged at 4°C at 10,000 x g for 30 minutes. The supernatant was then slowly brought up to 5% NaCl (approximately 30 minutes at 4°C) and recentrifuged. After carefully removing the supernatant the precipiate was dissolved in 0.1M acetic acid and dialyzed at 4°C for 48 hours against 1.0M NaCl, 0.05 M Tris, pH 7.4. This dialyzate was filtered through celite; the filtrate was dialyzed extensively against 0.02 M NaH_2PO_4 pH 7.5, and then centrifuged at 4°C at 10,000 x g for 30 minutes. To the pellet, 1.0 ml 6 N Hcl was added and this mixture hydrolyzed for 16 hours at 110°C. The samples were then dried under vacuum, dansylated and resolved via thin layer chromatography as described by Terrranova and Brand (1978).

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