THE EFFECT OF TRIAMCINOLONE ACETONIDE ON COLLAGEN SYNTHESIS BY HUMAN AND MOUSE DERMAL FIBROBLASTS IN CELL CULTURE

by

ELAINE MEI LI TAN

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Department of Pharmaceutics & Biopharmaceutics

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date Jan 30, 1980

ABSTRACT

Glucocorticoids are known to affect metabolic activities of cells. The mechanism of glucocorticoid actions in adult human dermal and mouse L-929 fibroblasts have yet to be fully ascertained. This study endeavors to examine the effects of one glucocorticoid, triamcinolone acetonide, on cellular proliferation and collagen synthesis and to compare such effects in the human and mouse cell lines. Cellular proliferation and collagen synthesis are analyzed and quantitated by cell counts and selective digestion of the protein by bacterial collagenase, respectively. Further analysis of collagen synthesis is provided by polyacrylamide gel electrophoresis.

One-tenth triamcinolone acetonide per ml suppresses cellular proliferation of mouse L-929 fibroblasts. Proline incorporation into total and collagenase-sensitive protein is enhanced in the cell layer; that of medium is altered inconsistently. Polyacrylamide gel electrophoresis of proteins treated with pepsin show the abolition of total and collagenase-sensitive protein in the cell layer. Aberrations in hydroxylation and/or deformation in physical structure of protein may confer greater susceptibility to pepsin digestion. Cellular proliferation and proline incorporation into total and collagenase-sensitive protein of adult human dermal fibroblasts are affected inconsistently by the same dose of triamcinolone acetonide.

Except for the consistent suppression of cellular proliferation in the murine L-929 fibroblasts by triamcinolone acetonide, all observations pertaining to human dermal fibroblasts are incompatible with those obtained by other workers. Manipulation of culture conditions and glucocorticoid treatment dictate, to a large extent, the kind of responses observed. This could account for the wide variability and frequent contradictory findings reported in the literature.

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INTRODUCTION

Topical glucocorticoids effect alterations in the proliferation and collagen metabolism of fibroblasts *in vivo* and *in vitro* (Jablonska *et al.*, 1979; Ponec, 1977b). An attempt was made to predict and rank the intrinsic anti-inflammatory activities of these steroids by examining their effect on cellular proliferation. Indeed, Berliner and Ruhmann (1967) and Brotherton (1971) developed and claimed that their parallel line bioassay provided a correlation between the anti-inflammatory potencies and the inhibition of mouse L-929 fibroblast growth rate. This bioassay has since fallen in disrepute because it does not agree with clinical experience (Winter and Wilson, 1976).

Glucocorticoid effects on collagen metabolism and the association with dermal atrophy have not been fully ascertained. Dermal atrophy, thought to arise from alterations in collagen metabolism, is a common adverse phenomenon observed clinically after prolonged and intense glucocorticoid treatment (Snyder and Greenberg, 1977; Wilson, 1978).

This work attempts to examine the effects of a glucocorticoid, triamcinolone acetonide, which has been known to cause dermal atrophy (Snyder and Greenberg, 1977), on cellular proliferation and collagen synthesis. Mouse L-929 and adult human dermal fibroblasts are employed in this study, the former because glucocorticoid effects on their collagen metabolism have yet to be examined by other investigators and the latter for their obvious relevance to dermal atrophy.

LITERATURE REVIEW

A. ADVERSE CLINICAL ASPECTS OF GLUCOCORTICOID THERAPY

During the past quarter century many dermatological disorders have been treated with topical glucocorticoids.[1] Over the years, steroids of increasing anti-inflammatory potencies have been manufactured, dispensed, and used, frequently for prolonged periods. The consequence is the appearance, with greater frequency, of a wide range of diverse and adverse side-effects. Since the withdrawal of the glucocorticoid precipitates a rebound inflammatory reaction, therapy tends to continue, sometimes for several years. Occlusive dressings often exacerbate the adverse effects (Burry, 1973). For instance, daily applications of hydrocortisone 17-butyrate with occlusion produce dermal atrophy (Kirby and Munro, 1976). Other adverse effects include striae, telangiectasia, epidermal thinning, depigmentation especially in the black population, perioral dermatitis, purpura, rosacea-like dermatitis, ulceration, and erythema (Snyder and Greenberg, 1977). Dermal atrophy is thought to be produced by an aberration of collagen metabolism (Jablonska, 1979). Undesirable systemic response, such as Cushing's syndrome, does occur rarely. A single nonocclusive dose of clobetasone propionate applied to normal skin suppresses adrenal function completely within nine hours (Ortega et al., 1975).

Although atrophogenicity has been associated with fluorinated glucocorticoids (Saarni and Hopsu-Havu, 1976; Stevanovic, 1972), certain nonfluorinated glucocorticoids such as desonide and hydrocortisone 17-butyrate have also been implicated. Furthermore, betamethasone valerate, a potent fluorinated glucocorticoid, is less atrophogenic than either of the foregoing compounds (Kligman and Frosch, 1979). Therefore, the presence of a halogenated atom does not confer any greater atrophogenic activities (Winter and Wilson, 1976).

That the anti-inflammatory and the vasoconstrictive properties of topical glucocorticoids are parallel is debatable (Child, 1968; Zaun, 1966). Although anti-inflammatory potency is often associated with the intense side effects, the correlation is not consistent. Furthermore, the frequency and time of onset of these side effects do not correlate with the anti-inflammatory potency of the glucocorticoids (Kligman and Frosch, 1979). Of most importance is the observation that there is no consistent correlation of therapeutically beneficial biological responses with the degree of atrophogenicity. An excellent example is given by triamcinolone acetonide which produces an anti-inflammatory response of medium degree in the human skin but causes atrophy comparable in severity to that of the most atrophogenic glucocorticoid (Kligman and Frosch, 1979).

B. STRUCTURE OF VARIOUS TOPICAL GLUCOCORTICOIDS

The basic structure of the glucocorticoid is a cyclopentanoperhydrophenanthrene nucleus (Fig. 1). Certain radicals and functions appended to the basic nucleus produce hydrocortisone (Fig. 2). Such functions confer activity to the steroid. The molecule is rendered inactive with the elimination of these functions. Cortisone is inactive topically because the C-11 ketone is not converted to C-11 hydroxyl in skin (vide infra) (Fig. 3). Modifications of the hydrocortisone molecule to increase the anti-inflammatory potency include double bond at C1 to C2, fluorine atoms at C6 and C9 (alpha position), hydroxyl at C16 (alpha position), acetonide group of the hydroxyls at C16 and C17, and esterification of either or both hydroxyls in the C17 side chain (Fig. 4) (Lee and Rapp, 1975). Certain ring substituents, such as 11-hydroxyl groups, are determinants in the binding capacity of the glucocorticoid molecule to the receptors. Radicals such as the 21-hydroxyl groups have been known to

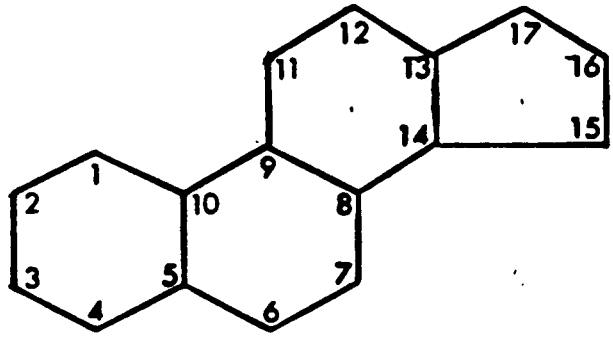


FIG. 1: CYCLOPENTANOPERHYDROPHENANTHRENE

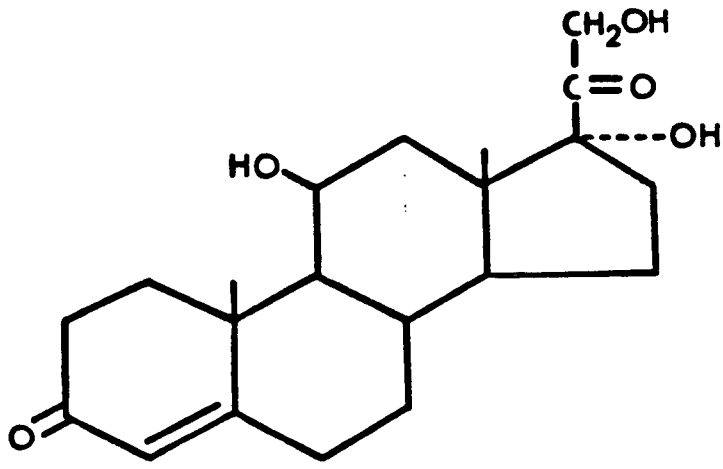


FIG.2: HYDROCORTISONE

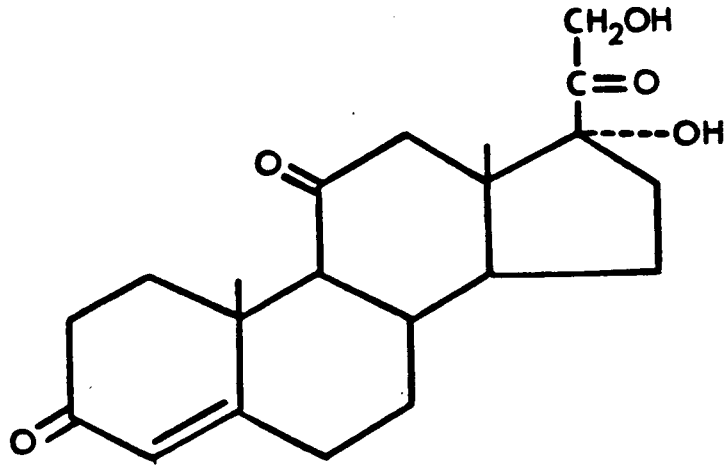


FIG. 3: CORTISONE

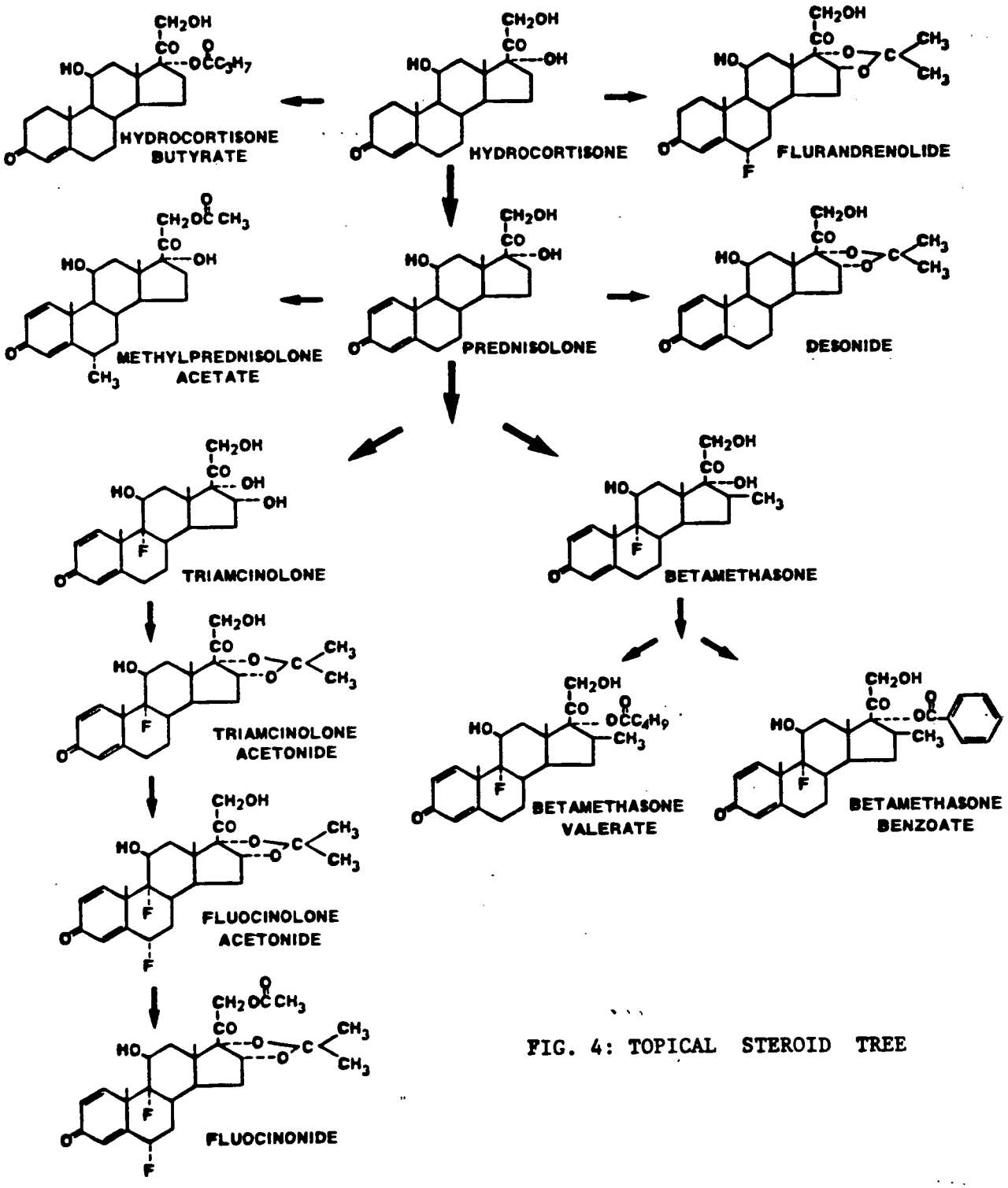


FIG. 4: TOPICAL STEROID TREE

affect the allosteric equilibrium of the receptors (Baxter and Forsham, 1972). Of the glucocorticoids seen in Figure 4, hydrocortisone is considered the least potent and fluocinonide the most potent anti-inflammatory glucocorticoids. Ranking of other topical glucocorticoids based on anti-inflammatory activity is difficult. Activity is not merely a function of chemical structure but includes the concentration and solubility of the steroid in the vehicle, the type of vehicle, and the partition coefficient of the steroid between the vehicles and the receptor phase which, in skin, would be the stratum corneum (Lee and Rapp, 1975).

C. INTERACTION OF TOPICAL GLUCOCORTICOIDS WITH CELLULAR MECHANISMS

Glucocorticoids penetrate the plasmalemma of the target cells and associate with specific, high-affinity, cytoplasmic receptor proteins evoking conformational changes in the receptor molecules (Buller and O'Malley, 1976). Glucocorticoid-sensitive cells possess cytoplasmic receptor sites; unresponsive ones contain few or none (Baxter and Forsham, 1972). Both human dermal and murine L-929 fibroblasts possess glucocorticoid receptors (Groyer et al., 1979).

Upon activation, the glucocorticoid-receptor complexes move to nuclear acceptor sites. A diminution of binding receptor sites and glucocorticoid-receptor complexes in the cytoplasm occurs concomitantly. Glucocorticoid resistance has been attributed to insufficient and inefficient translocation of cytoplasmic glucocorticoid-receptor complexes to the nucleus. Several thousand sites per cell are involved in the high-affinity binding of the glucocorticoid-receptor complexes to the chromatin of nuclei (Buller and O'Malley, 1976).

The biological responses elicited by the glucocorticoids are influenced by multiple factors. At those concentrations which would saturate their specific receptors, certain glucocorticoids do elicit an optimal response; with others, the response is only submaximal. The latter could be explained by binding of the glucocorticoid to both active and inactive conformations of the receptor sites. The glucocorticoids showing maximal response bind only to the active form of the receptor, thus generating an allosteric shift in equilibrium which, in turn, binds additional steroid, thereby favouring a maximal response. Biologically unresponsive glucocorticoids bind only to the inactive form of the receptor. At sufficiently high concentrations, such steroids are capable of inhibiting competitively the binding of the more active glucocorticoids (Baxter and Forsham, 1972).

D. RELEVANCE OF COLLAGEN

Collagen is the most abundant protein in the body, comprising one-third or one-half of the total protein (Gay and Miller, 1978). Collagen, not elastic fibres, provides the greatest resistance to tearing and deformation (Pierard and Lapiere, 1976). Collagen participates in tissue development and remodelling during growth, differentiation, ageing, wound healing as well as in pathological disorders such as fibrosis, cirrhosis and vascular diseases amongst others and, in a more specific manner, in cell attachment, chemotaxis and binding of antigen-antibody complexes (Gay and Miller, 1978). Although this protein is capable of restoring tensile strength to a weakened area, over-production hinders tissue mobility by generation of excessive scar tissue. The amount and type of collagen synthesized, conserved or destroyed

are related to the extent and form of insult to the body; genetic make-up may also be involved (Cohen et al., 1977).

Collagen is the principal structural element of the dermis and bone, comprising 34% of the proteins in the former and 57% in the latter . The metabolic turnover is very low in the adult (Klein, 1976).

E. BIOSYNTHESIS OF COLLAGEN

I. Transcription and Translation

The intracellular biosynthesis of collagen begins with the ribosomes of the rough endoplasmic reticulum. Little is known about the transcription of the monocistronic mRNA which is estimated to contain 4,500 nucleotides (Fessler and Fessler, 1979) and has a molecular weight greater than 1.5×10^6 (Klein, 1976; Fessler and Fessler, 1979). Unlike other proteins, hydroxyproline and hydroxylysine residues are not incorporated directly into the nascent pro α chains. Glycine, comprising 33% of the amino acids, occupies every third position of the tripeptide, glycine-X-Y, the basic amino acid sequence of 95% of the α chain. Proline or lysine is usually positioned in the X-position; the proline in the Y-position is usually hydroxylated (Klein, 1976).

The molecular weight of each pro α chain is 1.5×10^5. Each chain is composed of a collagenous portion with noncollagenous peptide extensions known as pro-peptides or registration peptides at the carboxy- and amino-terminal ends. These extensions possess few glycine, proline and hydroxyproline residues, some cysteine and tryptophan residues and an abundance of acidic amino acids (Martin et al., 1975). The carboxy-terminal propetide is composed of 200 to 300 amino acids and has a molecular weight of 3.5×10^4. The smaller amino-terminal propeptide has a molecular weight of 2.0×10^4 and at

least 100 amino acids (Klein, 1976; Uitto and Lichtenstein, 1976). The pro α chains, which are 40-50% larger than the α chains (Kivirikko and Risteli, 1976), are extruded across the membrane of the endoplasmic reticulum into the cisternae (Grant and Prockop, 1972; Bailey and Robins, 1976; Prockop et al., 1979).

II. Hydroxylation

Collagen requires several post ribosomal modifications of the primary structure. Proline and lysine residues of the completed polypeptide chains are hydroxylated by the enzymes prolyl and lysyl hydroxylase, respectively, located in the membrane and cisternae of the endoplasmic reticulum. Molecular oxygen, ferrous iron, a reducing agent, preferably ascorbate, and α-keto-glutarate (Kivirikko and Risteli, 1976) are required. One atom of oxygen is incorporated into hydroxyproline or hydroxylysine. Another atom of oxygen is incorporated into succinate, a byproduct of the decarboxylation of α-keto-glutarate (Kivirikko and Risteli, 1976; Lee and Rapp, 1975). The ferrous iron chelator, α,α'-dipyridyl, inhibits hydroxylation (Kivirikko and Risteli, 1976).

Jackson (1970) believes that most of hydroxylation occurs following the release of the pro α chains from the ribosomes. However, Grant and Prockop (1972) put forth the view that hydroxylation occurs within the membrane of the endoplasmic reticulum while the nascent chains are still lengthening. The current view is that both hydroxylation and glycosylation are initiated on the ribosomes during the generation of polypeptide chains and continue after release of the complete polypeptide chains into the cisternae of the endoplasmic reticulum (Oikarinen et al., 1976). These enzyme-mediated processes terminate upon formation of the triple helix (Kivirikko and Risteli, 1976).

The presence of hydroxyproline in the procollagen ensures the integrity of the triple helix (Rao and Adams, 1979) by conferring thermal stability to the molecule (Burjanadze, 1979). Hydroxylysine permits crosslinking of the molecules after extrusion into the extracellular matrix. Non-hydroxylated collagen, susceptible to thermal denaturation and proteolysis, gives rise to the weak tissues characteristic of scurvy (Klein, 1976).

III. Glycosylation

After hydroxylation, several hydroxylysine residues are glycosylated to form galactosylhydroxylysine and glucosylgalactosylhydroxylysine. The sugar molecules are attached to the hydroxylysines. Glycosylation is mediated by membrane-bound uridine diphosphate-galactosyl and uridine diphosphate-glucosyl transferases (Kivirikko and Risteli, 1976). Both enzymes require manganese to catalyze the transfer of the sugars from the corresponding uridine diphosphate glycoside to the hydroxyl groups of the hydroxylysine residues. The presence of the sugars is thought to determine the size and geometry of the collagen fibrils. Indeed, an inverse relationship has been suggested between the amount of sugars and the diameter of the fibrils (Grant, 1975). Glucosyl-galactosylhydroxylysine residues, more abundant than galactosylhydroxylysine in human skin (Krane, 1975a), increase with age from 30 to 75 years (Klein, 1976).

IV. Processing of Procollagen Molecules

Once released from the ribosomes, the pro α chains are converted to procollagen molecules. Interchain covalent disulphide bonding of the carboxy-terminal registration peptides permits chain association, proper

alignment of the pro α chains and the formation of the stable triple helix. The triple helix consists of three pro α chains that have wound around each other to form a supercoil (Hashimoto, 1978). The almost simultaneous formation of the interchain bonds and the triple helix occur before the procollagen molecules migrate from the cisternae of the endoplasmic reticulum to the Golgi apparatus. The registration peptides prevent intracellular fibre formation and facilitate the transport of the procollagen molecules in vesicles from the Golgi apparatus to the plasmalemma (Kivirikko and Risteli, 1976).

Microtubules are thought to participate in the movement of procollagen molecules to the plasmalemma prior to extrusion into the extracellular region. Agents such as colchicine and vinblastine, which disaggregate microtubules, delay the secretion of the protein (Bornstein and Ehrlich, 1973).

As extrusion occurs, the amino-terminal registration peptide is enzymatically cleaved en bloc by the amino-terminal procollagen endopeptidase to form p_c-collagen. The remaining registration peptide is cleaved separately by the carboxy-terminal endopeptidase to produce tropocollagen. Both enzymes require calcium for their activity. Peptide chains of molecular weight 11,500 or 17,500 are released by amino-terminal peptidase while cleavage at the carboxy-terminus generates trimeric, disulphide-linked fragments of molecular weight 75,000. Tropocollagen molecules with a molecular weight of 285,000 are each composed of three coiled chains, each having a molecular weight of 95,000 daltons and a composition of about 1,050 amino acids in a linear sequence (Gay and Miller, 1978).

V. Formation of Cross-links

Intermolecular covalent cross-links between adjacent tropocollagen monomers and the packing arrangement of tropocollagen in the fibril confer high tensile strength.

The initial step in the formation of cross-links requires oxidative deamination of the $-NH_2$ group of a specific lysine or hydroxylysine residue to produce the corresponding aldehydes, allysine or hydroxyallysine (Miller, 1976). The hydroxylysine-derived cross-links are more stable than those derived from lysine due to the formation of a stable keto form (Kivirikko and Risteli, 1976). Aldehyde synthesis is mediated by lysyl amine oxidase which requires molecular oxygen, pyridoxal phosphate and copper but is inhibited by copper chelators, pyridoxal phosphate trapping reagents and lathyrogens such as beta-aminopropionitrile (Kivikikko and Risteli, 1976); penicillamine, a copper chelator inhibits primarily by binding to the aldehyde groups (Klein, 1976).

Intramolecular covalent cross-links are formed by aldol condensation of two aldehyde groups near the amino-termini of two neighbouring α chains. Intermolecular cross-links are derived from the condensation of aldehydes with the $-NH_2$ group of lysine or hydroxylysine on neighbouring collagen molecules. Dehydrohydroxylysinonorleucine and dehydrodihydroxylysinonorleucine form the major intermolecular cross-links in part as galactosyl and glucosy-galactosyl derivatives. Dehydrohydroxylysinonorleucine arises from the condensation of lysine and hydroxylysine-derived aldehyde or of hydroxylysine and lysine-derived aldehyde; dehydrodihydroxylysinonorleucine from hydroxy-lysine and hydroxylysine-derived aldehyde. Lysinonorleucine cross-links also exist but are less stable (Kivirikko and Risteli, 1976).

F. PHYSICAL STRUCTURE OF COLLAGEN

The fundamental unit of collagen is tropocollagen, a rod-shaped molecule, measuring about 280 nm in length and 1.0 to 1.5 nm in diameter (Hashimoto, 1978). Each molecule is comprised of three α chains; each chain winds upon itself to form a left-handed coil with a pitch of 0.89 nm. A right-handed superhelix of pitch 8.7 nm is generated with the twining of the three α chains (Hashimoto, 1978; Viidik, 1979).

The aggregation of native tropocollagen chains proceeds in a "quarter-stagger" manner. Rather than being aligned so that the amino and carboxy termini match between tropocollagen molecules, each molecule is displaced, by about one quarter of its length (Hashimoto, 1978; Viidik, 1979).

G. DEGRADATION OF COLLAGEN

Collagen degradation occurs by enzymatic digestion with or without phagocytosis of the protein. In vivo, collagen is degraded by collagenase from neutrophilic granulocytes, fibroblasts or macrophages, or by cathepsin B from lysosomes of fibroblasts, chondrocytes or macrophages.

Fibroblasts from human skin are capable of synthesizing collagenase (Birkedale-Hansen et al., 1976; Bauer, 1977). This enzyme requiring calcium, with optimal activity at neutral or slightly alkaline pH, cleaves native tropocollagen three-quarters of the distance from its amino terminal end. The products are denatured at physiological temperatures and are degraded further by non-specific proteases (Birkedale-Hansen et al., 1976).

Collagenase can be found in several forms, a proenzyme or zymogen, a free active form, or as a complex with an inhibitor such as alpha 2-macroglobulin. The latent form of the enzyme is more prevalent in tissues where rate of

synthesis is greater than that of degradation, for instance, in inflammation or remodelling (Krane, 1975b).

Often collagen is phagocytosed by fibroblasts and other cells (Mitchell et al., 1978) prior to lysis by the lysosomal proteases. However, phagocytosis may be a process independent of collagenolysis (Svoboda et al., 1979).

More recently, another degradation process has been discovered. Within minutes of its synthesis, about 20 to 40% of intracellular collagen has been shown to undergo degradation (Bienkowski et al., 1978a). Such rapid destruction and the absence of collagenase activity argue against enzymatic or phagocytotic mechanisms. The newly synthesized collagen may not be intact α or pro α chains. Bienkowski et al. (1978b) demonstrated that the structure of the collagen is a factor in intracellular destruction as follows: Azetidine-2-carboxylic acid, a lower homologue of proline, was incorporated into collagen, the formation of the normal helical conformation being prevented by the azetidine ring. Intracellular degradation was elevated two-fold. Hence, these workers concluded that intracellular degradation regulates not only the amount of protein secreted extracellularly but the structural form of that protein (Bienkowski, 1978b).

H. TYPES OF COLLAGEN

Four types of collagen, I, II, III and IV have become well established (Kivirikko and Risteli, 1976). Several new types, A, B, C, D, E (Miller, 1979) have been discovered recently; little is known about types C, D and E. At least ten different genes are implicated in the synthesis of collagen. Each type is different in amino acid content and sequence, the degree of hydroxylation and the quantity of carbohydrate.

Type I, $[\alpha 1(I)]_2\alpha 2$, is composed of two α 1 chains and one α 2 chain which differs in its amino acid composition from that of α1. Although ubiquitous, it predominates in adult human skin, bone and tendon (Uitto and Lichtenstein, 1976). A trimer of type I, $[\alpha 1(I)]_3$, has been found recently and is associated with inflammed tissues, lathyritic chick embryo tendons and calvaria, and possibly normal human skin (Uitto, 1979). Type II, $[\alpha 1(II)]_3$, consists of three identical α chains genetically distinct from α1(I) and α2 and is found in hyaline cartilage (Uitto and Lichtenstein, 1976) and more recently, vitreous body (Schmut et al., 1979). Like type II, type III, $[\alpha 1(III)]_3$, is also composed of three identical α chains with high contents of hydroxyproline, glycine and cysteine. It is associated with extensible tissues. Although type III accounts for less than 20% of the total collagen in adult human skin, more than half of the total collagen in human foetal skin is type III (Uitto and Lichtenstein, 1976). The remainder is type I. Type IV $[\alpha 1(IV)]_3$, is also composed of three similar α chains and with A, B, C, D and E is exclusive to the basement membranes (Miller, 1979).

Types I and III have few hydroxylysine and carbohydrate residues while types II and IV possess a large quantity of both. Types I and II, unlike types III and IV, do not have cysteine residues to form interchain disulphide bonds (Veis, 1975).

Type I exists as densely packed rope-like fibrils in a highly ordered array. The fibrils form readily and provide high tensile strength (Hance and Crystal, 1975). Type III is composed of fine, pliable, randomly dispersed fibrils associated closely and loosely with other components of connective tissue (Perez-Tamayo, 1979); some is present as reticulin.

I. EXOGENOUS FACTORS AFFECTING CELLULAR PROLIFERATION AND COLLAGEN METABOLISM

I. Serum

Serum is known to contain poorly characterized factors essential for cellular movement, viability and proliferation. The mouse cell line, L-929, appears to be an exception to most cell types in that the generation times of these transformed fibroblasts are unaltered by exposure to 10% (v/v) foetal calf serum. However, these fibroblasts synthesize 20 to 30% more protein in the presence of 10% foetal calf serum than in its absence (Nolan et al., 1978). Studies using normal gingival fibroblasts (Narayanan and Page, 1977) showed that the presence of serum can effect a four and seven-fold increase in the incorporation of lysine and proline respectively, thus increasing protein and collagen synthesis. The incorporation of proline into protein is maximal at 20% (v/v) foetal calf serum; that of lysine, at 5% (v/v). The hydroxylation of collagen has been found to be enhanced by serum in fibroblasts of normal tissues (Bashey and Jimenez, 1977).

Serum also affects the type of collagen synthesized. Using human gingival fibroblasts, synthesis of type I collagen increases three-fold in 20% (v/v) foetal calf serum. In 10% (v/v) serum, type III production is increased but only by 1.5 fold. Further increases in serum concentration reverse the trend so that the ratio of type III to type I decreased. These observations were made in comparison to serum-free cultures (Narayanan and Page, 1977).

Collagen degradation is inhibited by the presence of serum. Mammalian collagenases are inhibited completely upon addition of 10% (v/v) foetal calf serum (Bienkowski et al., 1978a). Serum contains α1-antitrypsin and α2-macroglobulin, which bind to and inactivate collagenases (Birkedale-Hansen et al., 1976). Although human diploid dermal and lung fibroblasts have been found to

ingest extracellularly formed α_2-macroglobulin-protease complexes, no significance is attached to such endocytosis. Possibly, the proteases are liberated from the complex for re-excretion by the cells (Leuven et al., 1978).

To summarize, serum-mediated modifications of protein metabolism vary with the origin and type of cell but such effects have not been elucidated completely in human dermal and mouse L-929 fibroblasts.

II. Ascorbate

Ascorbate functions as a cofactor of lysyl hydroxylase and as cofactor and activator of prolyl hydroxylase (Peterkofsky and Udenfriend, 1965). In addition, ascorbate stimulates collagen secretion from the cells.

The role of ascorbate as an activating agent of prolyl hydroxylase in mouse L-929 fibroblasts was examined by Stassen et al (1973). Ascorbate, added to cultures in late logarithmic phase of growth, evoked a five-fold increase in the activity of prolyl hydroxylase; no dependence on RNA and protein synthesis was noted. The possibility that the stimulation of enzyme activity in the presence of ascorbate stemmed from the activation of a pre-formed precursor received support from immunological experiments in which a constant amount of prolyl hydroxylase precursor protein was found. Dithiothreitol converted quantitatively the active enzyme to smaller inactive molecules. Treatment with ascorbate restored the activity and proportionately decreased the concentrations of precursors. Therefore, it was suggested that, in the presence of ascorbate, the enzymatically-inactive subunits of prolyl hydroxylase (Stassen et al., 1973) aggregate to form an active tetramer (Chen-Kiang et al., 1977).

Later, Berg et al. (1976), using a different assay for prolyl hydroxylation, showed that ascorbate was incapable of activating prolyl hydroxylase in mouse L-929 fibroblasts. However, the lack of ascorbate did not appear to prevent completely the hydroxylation of proline (Nolan et al., 1978) nor affect cellular proliferation and rate of collagen synthesis (Peterkofsky, 1972). The absence of gulonolactone oxidase in L-929 cells confirmed the inability of these cells to synthesize ascorbate (Nolan et al., 1978). Although certain embryonic tissues, such as early chick blastoderm, embryonic mesenchyme and developing cartilage, do synthesize ascorbate endogenously (Meier and Solursh, 1978), cells derived from most adult tissues lose their capacity to produce ascorbate; adult chicken kidney and liver are exceptions.

The addition of 1.78 μg of ascorbate to 10^6 cells had the effect of hydroxylating collagen to 67%; this was reduced to 19% if only 50 ng ascorbate were added. Hence, although the absence of ascorbate did not inhibit hydroxylation completely, its presence did enhance the process (Nolan et al., 1978).

III. Glutamine

Glutamine is present in high concentration in most tissue culture media, the rationale being it is the sole precursor of purine bases. Hence, it promotes cell growth. Judged by incorporation of tritiated proline into hydroxyproline, the presence of 2 to 4 mM glutamine, the concentration usually found in tissue culture media, reduced collagen production by 80%. In contrast, decreasing the concentration of glutamine to 40 μM increased collagen synthesis by 40% (Ronnemaa et al., 1977). Corroborative data (Lehtinen et al., 1978) showed increases of collagen synthesis of 72.3% in cells and 20.8% in the

medium with 10 μM glutamine; 2 mM glutamine suppressed collagen synthesis by 62% in cells and 60% in the medium.

In high concentration, glutamine may compete with the uptake of proline into the cells or dilute the proline pool by metabolizing to glutamic acid to glutamic semialdehyde to proline. However, in low concentrations, glutamine provides nitrogen and ammonia, thus enhancing the intracellular production of amino acids and consequently, protein synthesis (Ronnemaa et al., 1977).

J. COLLAGEN METABOLISM OF CELLS IN VITRO

I. Mouse L-929 Fibroblasts

Cultured mouse L-929 cells, a well-established line of chemically transformed embryonic fibroblasts, synthesize and secrete collagen (Gribble et al., 1969). Indeed, collagen forms a higher proportion of the total protein than that found in human dermal fibroblasts (Kerwar et al., 1973). Matsubayashi et al., (1977) found that collagen accounted for 16% of protein synthesized during a 48 hour incubation period. During the logarithmic growth stage, 25 to 30% of the protein in the medium is representative of collagen. The activity of prolyl hydroxylase is low in the early logarithmic phase of growth (Kivirikko and Risteli, 1976). Since immunological methods have demonstrated the presence of the enzyme, it, most likely, is present in an inactive form. Hydroxyproline, indicative of increased enzymatic activity, does not appear until the late logarithmic phase when mitotic activity is low (Gribble et al., 1969). The collagen of the medium and the cell layer is hydroxylated to about 20% of maximum in the early logarithmic phase, increasing to 60% in the stationary phase. Activation of prolyl hydroxylase occurs through subunit aggregation (McGee and Udenfriend, 1972). Following hydroxylation, the

affinity of the collagen for prolyl hydroxylase diminishes with the subsequent release of the enzyme (Kuttan et al., 1975).

According to Kerwar et al. (1973), the predominant forms of the collagen in the medium are pro α1 and pro α2 chains; the low activity of procollagen peptidase precludes the presence of all but a few α chains. Only procollagen chains are found in the cellular collagen; procollagen peptidase activity is undetectable in the cellular compartment (Kerwar et al., 1973). In contrast, peptidase activity is noted in the study by Matsubayashi et al. (1977) who found tropocollagen molecules comprising α1 and α2 chains as well as two species of pepsin-sensitive collagen precursors. Unlike normal human dermal fibroblasts, mouse L-929 fibroblasts synthesize only one type of collagen, type I. Type III collagen is undetectable (Matsubayashi et al., 1977).

II. Human Dermal Fibroblasts

The human dermal fibroblast is thin, fusiform or oval-shaped with long cytoplasmic extensions. A small amount of cytoplasm and a large nucleus with prominent nucleoli constitute the main body of the cell. Prominent dilated rough endoplasmic reticula are present in the active collagen-synthesizing fibroblasts (Hashimoto, 1978).

The conversion of collagen precursors in culture requires many hours (Taubman et al., 1976; Goldberg, 1977). Consequently, most of the molecules in the extracellular medium are procollagen which dissociate upon reduction with 2-mercaptoethanol to form free single α1 and α2 chains (Lichtenstein et al., 1975; Goldberg et al., 1972). Associated with the smaller volume of the cell layer are the intermediate form of collagen, the p-collagen chains, and a small quantity of the native tropocollagen fibrils (Goldberg, 1977) which are

derived mainly from the conversion of the procollagen in the extracellular space of the cell layers (Taubman et al., 1976).

Collagen represents 5-10% of the total protein in the human dermal fibroblasts (Goldberg et al., 1964; Matsubayashi et al., 1977). Following a 24 hour incubation period with tritiated proline, 60 to 80% of the labelled collagen is present in the extracellular medium (Goldberg et al., 1972).

Types I and III collagens are synthesized by human dermal fibroblasts, the former predominating in normal healthy adult cells (Lichtenstein et al., 1975). Each cell is capable of synthesizing both types of collagen simultaneously (Gay et al., 1976). The conversion of type I procollagen to native tropocollagen molecules differs from that of type III collagenous proteins. In fibroblast cultures of murine and human dermal fibroblasts, native tropocollagen is generated from type I procollagen but such conversion is not observed with type III procollagen in the same cultures. These observations suggest that different enzyme systems are responsible for the processing of the two types of collagen. The enzymes converting type III procollagen appear to be lacking or less efficient than those for procollagen type I, leading to the disproportionate accumulation of type III procollagen in the medium (Goldberg, 1977).

K. GLUCOCORTICOID EFFECTS ON CELLULAR PROLIFERATION AND COLLAGEN METABOLISM

I. In Vitro Studies

(a) Cell Culture

Examination of the effects of glucocorticoids on collagen metabolism in vitro has not been confined to human dermal fibroblasts; at least as many studies have been directed towards fibroblasts from other tissues and

species. For instance, chick embryo tendon fibroblasts are popular, primarily because of their high metabolic activity and the fact that collagen represents 70 to 80% of the total protein (Saarni, 1977).

(i) chick embryo tendon fibroblasts

By measuring the incorporation of proline into hydroxyproline in these cells, Saarni et al. (1976), compared the effects on collagen synthesis of several glucocorticoids, including hydrocortisone butyrate. Kligman and Frosch (1979) believed the latter to be atrophogenic; Saarni et al. (1976) disagreed. Of the glucocorticoids studied, only hydrocortisone succinate at 10^{-4}M had no discernible effect on collagen synthesis (Saarni et al., 1976). Manipulation of the dosage of this glucocorticoid produced interesting and contrary responses. A slight increase in collagen synthesis was elicited at 10^{-5}M but increasing the concentration to 10^{-3}M produced a profound inhibition of 90% (Saarni, 1977). These observations clearly demonstrate the biphasic effect of glucocorticoids whereby enhancement of collagen synthesis is produced at low concentrations and inhibition is registered with elevated quantities of glucocorticoids. The free alcohol form of hydrocortisone was capable of suppressing hydroxyproline formation by 46% and 20% at 10^{-4}M and 10^{-5}M, respectively (Saarni, 1977). Similar results had been obtained earlier with the phosphate and butyrate derivatives of hydrocortisone as well as prednisolone (Saarni et al., 1976). Collagen synthesis was reduced 24 and 35% at 10^{-4}M hydrocortisone butyrate or prednisolone and 56% at 10^{-3}M hydrocortisone phosphate. Lower concentrations had no effect. With betamethasone-17-valerate, the depression was 90% at 10^{-4}M and 20% at 10^{-5}M. Dexamethasone, its phosphate derivative, and the alcohol and phosphate derivatives of betamethasone-17-valerate elicited no alterations in

collagen production perhaps due to the lack of conversion to some active forms of the steroid. With all dosages of glucocorticoids that were effective, neither an accumulation of unhydroxylated collagen in the cells nor an inhibition of the secretion of collagen was reported. The observed parallel decrease in quantity of collagen and total protein suggested a general decline of protein synthesis. These studies demonstrated the different potencies of various derivatives of hydrocortisone in the depression of collagen synthesis. Both monosodium phosphate and sodium succinate derivatives exhibited milder potencies than the free alcohol and butyrate forms of hydrocortisone and prednisolone. The most potent was betamethasone-17-valerate. Interestingly enough, betamethasone-17-valerate generates atrophy in greater severity than hydrocortisone, hydrocortisone butyrate or prednisolone. In clinical experience, hydrocortisone butyrate and betamethasone-17-valerate are comparable in anti-inflammatory potencies. No correlation exists between atrophogenic property and anti-inflammatory potency (Saarni, 1977).

Oikarinen (1977a), using the same cell type, also observed reduced synthesis of collagen in the presence of betamethasone-17-valerate. A specific alteration in collagen synthesis was suggested by depression of proline incorporation and by the marked reduction in hydroxyproline biosynthesis. The decline in collagen synthesis was not associated with any aberration in prolyl hydroxylase activity. However, if exposed to the glucocorticoid in vivo, prolyl hydroxylase activity decreased significantly suggesting either inactivation or inhibition in the synthesis of the enzyme (Oikarinen, 1977a). In a later in vitro study using the same cell type, Oikarinen (1977b) observed no alteration in the activities of prolyl hydroxylase and the other enzymes, lysyl hydroxylase, galactosyl and glucosyl transferases in the presence of

cortisol acetate despite a depression in collagen synthesis. Prolonged or repeated exposure to the glucocorticoid did result in reduction of the enzymatic activities, especially of the hydroxylases. Thus, it would appear from the foregoing data (Saarni, 1977; Oikarinen, 1977a) that certain glucocorticoids inhibit collagen synthesis by affecting the synthesis of the polypeptide chains directly and possibly by inhibiting the activities of those enzymes responsible for the post-translational changes in collagen.

(ii) foetal human dermal fibroblasts

Kirk and Mittwoch (1977) compared the effects of the glucocorticoid, cortisol, and its more potent synthetic fluorinated derivative, fluocinolone acetonide, on the cellular proliferation of human diploid dermal fibroblasts from aborted foetuses. Treatment with these glucocorticoids for 24 hours in doses ranging from a physiological level of 0.05 µg per ml to a pharmacological level of 150 µg per ml of growth medium produced higher mitotic indices (percentage of cells in mitotic phase) than untreated control cultures. The increase in the mitotic index could have arisen from an inhibition of a certain mitotic phase or could reflect a stimulation of proliferation. Cortisol, at concentrations up to 50 µg per ml produced no significant increase in the proliferative rate. However, at similar concentrations, fluocinolone acetonide did produce a slight but significant increase in the rate of proliferation. Furthermore, the cells were induced to enter earlier into the period of DNA synthesis which was increased substantially following glucocorticoid treatment (Kirk and Mittwoch, 1977).

Harvey et al. (1974) found that 10 µg prednisolone or cortisol per ml of medium inhibited the proliferation of human embryonic skin fibroblasts after a 9 day incubation period. When the glucocorticoid concentration was reduced to

0.1 µg cortisol or 0.01 µg prednisolone per ml of medium, the number of cells floating in the culture medium was decreased, possibly indicating enhanced cellular adhesion. The rate of tritiated thymidine incorporation was elevated by 0.01 µg of either glucocorticoid but decreased by 20% in the presence of 1.0 µg per ml and 50% at 10 µg per ml. The depression of thymidine incorporation preceded the reduction of cell numbers. The observed stimulation and suppression of thymidine incorporation at the lower and higher glucocorticoid concentrations, respectively, reflects the biphasic effect of glucocorticoids (Ueki et al., 1976). Collagen synthesis was enhanced by cortisol and prednisolone at concentrations ranging from 0.01 to 1.0 µg per ml medium; maximal stimulation occurred at 0.1 µg per ml of each steroid. These concentrations extend over the range of physiological and pharmacological doses (Harvey et al., 1974). The concentration of 0.1 µg per ml of cortisol is within the range of 0.04 to 0.2 µg per ml found in plasma (Asfeldt, 1971).

A later study of Doherty et al. (1976) also examined glucocorticoid effects on collagen synthesis by confluent human foetal skin fibroblasts of passage 5 to 10. At concentration of 10^{-7} M hydrocortisone sodium succinate did not inhibit the synthesis of collagen or non-collagen protein after a 5 hour incubation. But, at 10^{-5} M, collagen synthesis was stimulated markedly, as determined by incorporation of radioactive proline into hydroxyproline and total protein. Non-collagen protein synthesis was stimulated but not to a statistically significant degree. The stimulation of non-collagenous protein and collagen syntheses both in the medium and in the cell layer was enhanced by prolonging the incubation to 11 h in the presence of 10^{-5} M hydrocortisone sodium succinate (Doherty et al., 1976).

More recently, studies using normal human foreskin cells before the twentieth passage confirmed the concentration-dependent alteration of cellular proliferation. At concentrations between 0.001 µg per ml to 1.0 µg per ml of medium, cellular proliferation was enhanced by hydrocortisone, betamethasone valerate and clobetasol propionate. Significant depression of proliferation was evident with 10 µg of the foregoing glucocorticoids per ml medium (Priestley, 1978). Again, the biphasic effect of glucocorticoids is evident. These findings are in contrast to those of Berliner and Ruhmann (1967) and Brotherton (1971) who observed a progressive depression of cellular proliferation of mouse L-929 fibroblasts with increased glucocorticoid dosage. Clobetasol propionate at 10 µg per ml inhibited collagen synthesis by 42%, as measured by susceptibility of the collagen to digestion by protease-free collagenase. At 25 µg per ml of medium, collagen production was depressed more than 50% by betamethasone valerate and clobetasol propionate but total protein synthesis was reduced to a lesser extent, thus adding support to the view that glucocorticoids affect the metabolism of collagen specifically (Priestley, 1978).

A dose- and time-dependent effect of glucocorticoids on collagen synthesis was demonstrated by Saarni and Tammi (1978). Incubation of confluent cultures of human foetal dermal fibroblasts of passage 9 to 11 with 10 µM cortisol for 12 to 36 hours produced a significant increase in collagen synthesis. Stimulation was also reported with 1 µM cortisol at 12 hours, and with 10 nM cortisol at 96 hours. However, dosages in excess of 10 nM were inhibitory when incubated for 96 hours. The synthesis was followed by measurement of the incorporation of proline into hydroxyproline. Saarni and Tammi (1978) claimed that collagen synthesis was altered more than that of other proteins, suggesting

that cortisol, like betamethasone valerate and clobetasol propionate (Priestley, 1978) affects collagen synthesis specifically. Thymidine uptake was unaffected over the first 12 hours but dropped significantly after 36 hours with 10 μM cortisol. At 10^{-1} μM, DNA synthesis was inhibited after 96 hours. Saarni and Tammi (1978) suggest that cortisol affects the earlier part of G_1 phase of the cell cycle supporting an earlier report that showed the retardation of the cells to the G_1 phase. In contrast, Kirk and Mittwoch (1977) found that cortisol and fluocinolone acetonide shorten the G_1 phase, thereby promoting an early entry into the S phase. Thus, the effect of glucocorticoids on the cell cycle remains unclear in light of such contradictory findings.

The previous studies have demonstrated the dose- and time-dependent alterations of cellular proliferation and collagen synthesis by glucocorticoids in cultures of human foetal fibroblasts. Different techniques have been employed to determine collagen synthesis and cellular proliferation, producing varying results. The great number of variable parameters, for instance, dosage, duration of glucocorticoid treatment and stage of cell growth, unfortunately, hinder the elucidation of glucocorticoid actions *in vitro*.

(iii) adult human dermal fibroblasts

Human foetal cells have been used widely, as has been evident from the papers discussed so far. Less data is available on cells isolated from adult man. Ponec *et al.* (1977a) subjected logarithmic cultures of dermal fibroblasts from a young adult and of passage 17 to 21 to glucocorticoids at concentrations compatible with those found in skin after topical treatment with clinically employed dosages. At 5 μg per ml of medium for 5 days, triamcinolone acetonide, betamethasone-17-valerate or hydrocortisone 17-

butyrate caused 30 to 50% inhibition of cellular proliferation. Hydrocortisone at 5 µg per ml had no discernible effect but the same concentration of clobetasol-17-propionate suppressed cellular division almost completely. Either 16 µg hydrocortisone or 1 µg clobetasol-17-propionate per ml produced 30 to 50% inhibition.

Different results were generated in confluent cultures of adult human dermal fibroblasts. Neither depression of cellular proliferation nor increase in cell turnover was observed. Instead, with triamcinolone acetonide, hydrocortisone 17-butyrate and betamethasone-17-valerate, the cell density at confluency was 20% greater than that of control cultures (Ponec et al., 1977b). These observations lend support to those of Cunningham et al. (1974) who also found that triamcinolone acetonide, dexamethasone, cortisol and corticosterone increased cell density of confluent cultures of 3T3 fibroblasts. The synthesis of collagen was followed by measurement of the formation of hydroxyproline. Total protein synthesis was affected to a lesser extent than that of collagen following a 6 day incubation with the glucocorticoids. The depression, though slight at 5 µg hydrocortisone per ml of medium, was pronounced at 16 µg/ml. However, only 5 µg triamcinolone acetonide, betamethasone-17-valerate or hydrocortisone 17-butyrate per ml was necessary to cause a 30 to 50% reduction in hydroxyproline content. Clobetasol-17-propionate was even more active, 5 µg per ml limited total protein synthesis markedly; 1 µg per ml depressed hydroxyproline synthesis by 60%. The degree of inhibition of hydroxyproline synthesis was unrelated to fluorination, cellular proliferation, cellular turnover or protein synthesis (Ponec et al., 1977a,b).

(iv) mouse L-929 fibroblasts

Mouse L-929 fibroblasts are also susceptible to glucocorticoids. Unlike primary human skin fibroblasts, the proliferation of these cells was inhibited by glucocorticoids at concentrations 10^3 to 10^4 times lower than those encountered in the topical application of glucocorticoids. The inhibition of cellular proliferation of the L-929 cells extended over a range of concentrations, 10^4 to 10^5 times broader than that of the human fibroblasts. The effective minimal concentrations were 0.1 pg clobetasol-17-propionate per ml, 1 pg triamcinolone acetonide per ml, 10 pg betamethasone-17-valerate or hydrocortisone 17-butyrate per ml, 100 pg hydrocortisone-21-acetate per ml and 1 ng hydrocortisone per ml. Inhibition occurred even at 10 ug per ml, the highest concentration studied. Thus, only alterations in degree, not kind, of response were produced by the glucocorticoids in this established mouse line (Ponec et al., 1977a).

The inhibitory effects of hydrocortisone and triamcinolone acetonide on cellular proliferation of the mouse L-929 fibroblast were confirmed by Runikis et al., 1978. The effective range was 10 ng to to 1 ug hydrocortisone per ml; for triamcinolone acetonide and desonide, 100 pg to 1 ug/ml. Desonide was the most, hydrocortisone the least, potent inhibitor of cell proliferation. Dermal diploid fibroblasts from adult humans, in contrast to the mouse cells, were stimulated consistently by comparable concentrations of the foregoing glucocorticoids (Runikis et al., 1978). Although the latter results are in contrast to those of Ponec et al. (1977a), it must be noted that the glucocorticoid concentrations used by Ponec were much higher, ranging from 1 to 16 ug per ml. Indeed, the data from both studies are not necessarily conflicting but

may merely reflect the variable dose-response effect of glucocorticoids on cellular proliferation.

(b) Organ culture

Organ cultures, although not as widely employed as cell culture systems, have also been used to investigate the effects of glucocorticoids on collagen metabolism. Although concentrations of 10 µg hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate or flupredny1iden-21-acetate per ml had no discernible effect on collagen synthesis in human skin slices, increasingly severe inhibition of hydroxyhydroxyproline formation was observed from 30 to 300 µg of the glucocorticoid per ml. Betamethasone-17-valerate exhibited the most pronounced inhibition, hydrocortisone acetate the least, at all concentrations used. The amount of proline incorporation into total protein paralleled that of hydroxyproline, an index of collagen synthesis (Uitto et al., 1972).

A study by Koob et al. (1974) showed that human skin in organ culture was capable of synthesizing collagenases. Contrary to Houck's (1968) findings of increased collagen degradation following glucocorticoid treatment, Koob et al. (1974) found that 10 nM dexamethasone and 100 nM hydrocortisone were incapable of eliciting increased collagenolysis. However, collagenase activity was reduced by 80 to 90%, the degree of inhibition depending upon the steroid concentration, the lower limits being 1 pM dexamethasone and 10 nM hydrocortisone. Protein synthesis remained unaffected overall. The inhibition of collagenase expression was specific; this was corroborated by a similar decrease in the level of dialyzable hydroxyproline-containing peptides. From these data, Koob et al. (1974) concluded that in human skin explants glucocorticoids prevent endogenous collagen degradation.

The effects of glucocorticoids on collagen metabolism have also been examined in organ cultures of tissues other than skin or of humans. Based on a collagenase assay, treatment of chick embryo calvariae with large doses of triamcinolone acetonide had no effect on collagen synthesis (Cohen et al., 1977). However, collagen formation was depressed in tibiae exposed to cortisol. High doses of cortisol reduced the levels of hydroxyproline, glycosylated and total hydroxylysine as well as the uptake of proline and lysine residues. Also, high doses of hydrocortisone acetate, hydrocortisone phosphoric acid complex or hydrocortisone sodium succinate inhibited prolyl hydroxylation, resulting in reduction of collagen secretion into the medium (Blumenkrantz and Asboe-Hansen, 1976). Thus, the lower level of collagen found in the medium of organ cultures could be the consequence of two processes: (1) a defect in peptide synthesis and (2) a suppression of the post-translational modifications of collagen.

(II) In Vivo Studies

Conflicting, varying results and occasional misinterpretations have prevented the elucidation of glucocorticoid effects in in vitro systems. Unfortunately, the in vivo studies shed little light on the action of glucocorticoids on collagen metabolism. Admittedly, biochemical data from human subjects is sparse. Nonetheless, the histopathological findings of several studies of human subjects do present some common features.

Treatment of normal human skin with fluocinolone acetonide induced early onset of skin atropy. Significant decreases in the diameter of collagen fibrils, thinning, disorganization and fragmentation of collagen bundles and reduced numbers of fibroblasts were observed following fluocinolone acetonide

and flumethasone pivalate treatment. Characteristics of active protein synthesis, dilated rough endoplasmic reticulum, elaborate golgi apparatus and numerous mitochondria were not present. A prominent feature was the endocytosis of collagen fibrils. In close vicinity were dense lysosomal bodies, an indication of the formation of phagolysosomes (Jablonska et al., 1979; Groniowska et al., 1976). That dermal atrophy could be a consequence of collagen degradation is further supported by the biochemical findings of Cohen et al. (1977) who observed regression of human keloids following intralesional injections with triamcinolone. Regression was not due to decreased collagen synthesis. Indeed, collagen synthetic rate was comparable with that of untreated bilateral earlobe keloids. Although collagenolysis was not investigated, Cohen et al. (1977) hypothesized that triamcinolone enhanced collagen degradation.

More studies have been performed on rodents. Dexamethasone was found to inhibit collagen synthesis in skins of two strains of mice. One strain had a higher collagen synthetic rate. Following glucocorticoid treatment, collagen synthesis in this strain was depressed to a greater extent than that of the other strain. The former strain also encountered greater loss of non-collagenous proteins after dexamethasone administration. Hence, the proportion of collagen was altered. Two important conclusions emerged from this study. Strain differences determined the effect of glucocorticoids on dermal collagen. Both synthesis and degradation of proteins were affected by dexamethasone (Robey, 1979).

Extensive studies of glucocorticoid modifications of collagen metabolism have been reported for organs other than skin as well as skin from other species. Prednisolone, a popular glucocorticoid, was examined for its effects

on the activities of the hydroxylases and glycosyltransferases in rat skin and liver. The inhibition of activities of prolyl and lysyl hydroxylases, galactosyl- and glucosyl-transferases was dose-dependent; all were depressed to a similar degree at each pharmacological dose level. Suppression of enzyme activities could possibly arise from a general reduction in enzyme protein synthesis. Indeed, prolyl hydroxylase activity diminished to the same degree as the amount of the enzyme. Quantitation of the other enzyme proteins has yet to be done. Any reduction in the activity of enzymes responsible for post-translational modifications of collagen can be manifested in a suppression of collagen synthesis (Risteli, 1977). These findings corroborated the earlier work on rat liver by Cutroneo et al. (1971). Prolyl hydroxylase was inhibited in granuloma tissue by increasing concentrations of the glucocorticoids, methylprednisolone, triamcinolone diacetate, hydrocortisone acetate; triamcinolone diacetate had the most inhibitory effect. Dexamethasone also caused a 50 to 70% inhibition of collagen synthesis in mouse granulomas; total protein synthesis was depressed by only 25%. Kruse et al. (1978) reported a concomitant reduction of fibroblast number. The degree of depression of prolyl hydroxylase activity and of total protein synthesis was comparable. Hence, in the latter study neither hydroxylation of prolyl residues nor collagen synthesis was specifically affected by dexamethasone. Unlike Kruse et al. (1978), a more marked depression of the enzymatic activity relative to protein synthesis was observed by Cutroneo et al. (1971), indicating a specific effect of glucocorticoids on collagen synthesis.

Unlike the glucocorticoids discussed above, betamethasone disodium phosphate had no discernible effect on the activity of prolyl hydroxylase. The incorporation of labelled proline into hydroxyproline was inhibited to a

greater extent than that into non-collagen protein, indicating a specific effect of betamethasone on collagen synthesis (Nakagawa et al., 1971).

A significant specific dose- and time-dependent suppression of collagen polypeptide synthesis was observed in skin of newborn rats injected intraperitoneally with triamcinolone diacetate (Cutroneo and Counts, 1975; Newman and Cutroneo, 1978). Although similar reductions in the activities of prolyl and lysyl hydroxylases and a concomitant decline in hydroxyproline formation were observed, no synthesis or accumulation of underhydroxylated collagen occurred. Hydroxyproline formation paralleled the synthesis of the nascent collagen chains. DNA synthesis was suppressed only after multiple intraperitoneal injections of triamcinolone diacetate. In a later study (Slaga et al., 1978), DNA synthesis was shown to be stimulated 48 hours after topical application of fluocinolone acetonide to newborn mice. These contrasting observations were due not only to the glucocorticoids used but also to differences in the animal species, route of glucocorticoid administration, dosage and duration of treatment and animal species. Data corroborating those of Cutroneo and Counts (1975) and Newman and Cutroneo (1978) were obtained using modified techniques (Counts et al., 1979). Rats were injected intraperitoneally with 15 mg triamcinolone diacetate per kg body weight, and fibroblasts were derived from the dermis for analysis for collagen. These cells were incubated for 0, 1.0, 3.0 and 6.5 hours prior to the estimations of prolyl hydroxylase activity, total proline incorporated using a collagenase assay, hydroxyproline formation and lastly, the ratio of hydroxyproline to proline in the intracellular collagen peptides. Suppression was observed in the total incorporation of proline, the hydroxyproline formation and in the content of cellular collagen.

Kruse et al. (1978) also grew fibroblasts from granuloma tissue of mice injected intramuscularly with 0.35 mg dexamethasone per kg body weight. The results obtained contradicted those of Counts et al. (1979), for the rate of cellular growth and syntheses of total protein and collagen were unaffected. The differences in type, dosage and route of glucocorticoid administration and the animal species, most likely, account for the discrepancy. Only with the addition of 0.3 μM hydrocortisone to the culture medium of these fibroblasts were total protein and collagen syntheses suppressed. The activity of prolyl hydroxylase was also reduced. Again, the degree of inhibition varied with dose and duration of incubation with the steroid (Kruse et al., 1978).

A different in vivo system from the foregoing experiments was employed by Uitto and Mustakallio (1971) to test the effects of hydrocortisone acetate, fluocinolone acetonide, fluclorolone acetonide, betamethasone-17-valerate and fluprednyliden-21-acetate on collagen biosynthesis determined as the amount of hydroxyproline formed. All the glucocorticoids inhibited the formation of hydroxyproline in chick embryo tibiae when applied onto chorioallantoic membranes of chick embryos. The degree of suppression was dependent on dose and duration of treatment but was comparable with all the glucocorticoids. A distinctive observation that evolved from this study was the requirement of higher concentrations to inhibit collagen formation in vitro than in vivo (Uitto and Mustakallio, 1971).

SUMMARY

The mechanism of glucocorticoid interaction with collagen metabolism and its relevance to dermal atrophy are still obscure, largely because of the many variables involved in the experimental models, the culture systems, the assays, and the glucocorticoid treatment. Confusion is heightened by lack of information about glucocorticoid interactions along the entire metabolic pathway of collagen synthesis and degradation. Most studies have been devoted to an analysis of one or two aspects of collagen synthesis after glucocorticoid treatment. Often no association exists between biochemical, ultrastructural and clinical data, thereby making interpretation of data difficult, if not impossible.

In vivo experimental models have varied; they include chick embryos, rats, mice, monkeys and man. Within each model are such variables as age, sex, and species. The type and concentration of glucocorticoid, duration and frequency of glucocorticoid treatment, and the route of administration are but a few variables in in vivo experiments. The solvent or vehicle for the glucocorticoid, yet another variable, determines the ease and rapidity of its penetration.

Using culture systems, the investigator has at his disposal the use of cells or organs. Popular cell types have been fibroblasts derived from chick embryos, human foetuses and neonatal babies. The sites from which the cells have been derived are, largely, from tendon and tibiae in chick embryos, dermis and, of greater popularity, foreskin of humans. Surprisingly few studies centre on adult human individuals. Indeed, a survey to date has produced one study that employed fibroblasts derived from adult human models.

Human skin and liver have been the predominant tissues in organ cultures. Regardless of the choice of culture systems, the studies discussed thus far are fraught with a large number of variables. Where cellular aspects are concerned, the growth phase of fibroblasts and the passage number of the cell cultures are important considerations. The lack of optimal conditions for cellular growth can prevent the elucidation of biochemical effects of glucocorticoids on cellular metabolic activities. More variables are posed in the form of type, dosage, frequency and duration of glucocorticoid treatment of cells and organs, as well as the kind of solvent or vehicle which determines the ease and rapidity of penetrance into the fibroblasts or tissues.

Some workers (Kruse et al., 1978; Counts et al., 1979) have gone so far to combine in vivo and in vitro experiments. For instance, collagen metabolism of granuloma fibroblasts derived from dexamethasone-treated mice was examined in cell culture; in fact, some of these fibroblasts were later further exposed to hydrocortisone (Kruse et al., 1978). Such experiments can add only further confusion.

The most popular collagen assays have been the estimation of prolyl hydroxylase activity and the incorporation of proline into collagen estimated either with bacterial collagenase or by the amount of hydroxyproline formed. The latter procedure should not be regarded as an index of collagen synthesis, although it has been used as such by several investigators (Nakagawa et al., 1971; Ponec et al., 1977a,b; Saarni and Tammi, 1978), since the content of hydroxyproline reflects the hydroxylation process as well.

Information about the effects of glucocorticoids on other aspects of collagen metabolism is scant because few studies have been devoted to the interaction of glucocorticoids with cellular uptake of precursor amino acids,

specific activity and size of intracellular proline pool, intracellular collagen degradation, glycosylation, secretion of peptide chains, activity of the procollagen peptidase, the extent of cross-linking of the collagenous fibrils and finally, the types of collagen formed.

APPROACH

Despite the fact that glucocorticoids have been in use clinically for many years, virtually nothing is known about their action on certain metabolic activities in many types of cells. Glucocorticoids have been found to consistently inhibit the cellular proliferation of murine L-929 fibroblasts. However, no study to date has been performed on glucocorticoid effects on collagen metabolism in these cells which were of great interest because they had been used to predict anti-inflammatory potencies of glucocorticoids based on growth inhibition (Berliner and Ruhmann, 1967; Brotherton, 1971; Runikis et al., 1978). Since collagen metabolism in these cells is not unlike that of human dermal fibroblasts (Gribble et al., 1969), an attempt was made to examine collagen synthesis of mouse L-929 fibroblasts in the presence of a steroid. Cellular proliferation in glucocorticoid-treated fibroblasts of human origin are, reportedly, not altered consistently in kind and degree. Very few biochemical analyses have been performed on collagen synthesis in glucocorticoid-treated adult human dermal fibroblasts. Thus, this work was also undertaken to attempt to clarify the actions of a glucocorticoid on growth and collagen production in adult human dermal fibroblasts.

The cell culture system, as opposed to organ culture system, was chosen to study the effects of a glucocorticoid. The former system permits the potentially obscuring homeostatic mechanisms and humoral factors to be bypassed. Furthermore, only one cell type is exposed to a given concentration of glucocorticoid, thus permitting quantitative measurements to be made. Lastly, the cell culture system is amenable to modifications of the environment and so may

lead to the further understanding of the role of exogenous factors on cellular proliferation and collagen synthesis. To study the synthetic processes of collagen metabolism without interference from the degradative aspects, serum must be present. Aside from being essential for maintenance of cell growth, attachment and other metabolic activities, it has the added advantage of suppressing collagenase activity due to the presence of α2-macroglobulin and α1-antitrypsin which complex with the enzyme (Birkedale-Hansen et al., 1976).

The glucocorticoid of choice was triamcinolone acetonide which has been known to cause dermal atrophy in man (Kligman and Frosch, 1979) and animal models (Young et al., 1977). The degree of atrophy has been reported to be of great severity (Kligman and Frosch, 1979). As dermal atrophy has been postulated to arise from an aberration of collagen metabolism (Jablonska, 1979), this glucocorticoid was selected to test its actions on collagen biosynthesis in vitro. An added reason for the choice of this glucocorticoid is that triamcinolone acetonide does not bind to transcortin or corticosteroid-binding globulin in serum (Runikis et al., 1978; Ganong, 1975).

To obtain optimal collagen synthesis, confluent cultures were chosen because the activities of enzymes responsible for post-translational modifications are maximal during this phase of growth (Gribble et al., 1969). However, if triamcinolone acetonide was not effective during this growth phase, the logarithmic stage of growth would be selected for the administration of the glucocorticoid.

Unlike many of the studies mentioned (Ponec et al., 1977a,b; Saarni and Tammi, 1978) where incorporation of proline into hydroxyproline was used as an index of collagen synthesis, this present study employed a different means of quantitating collagen production, namely, the use of bacterial collagenase.

This enzyme selectively degrades collagen. The incorporation of proline into collagenase-sensitive proteins was examined in medium and cellular fractions, independently of the hydroxylation process. To seek confirmation of results obtained, radioactive proteins containing collagen were subjected to pepsin treatment and electrophoresed in polyacrylamide gels which were sliced and counted for radioactivity.

MATERIALS AND METHODS

A. MATERIALS

Triamcinolone acetonide, a gift from E.R. Squibb and Sons, Montreal, P.Q., was dissolved in propylene glycol at a concentration of 116 or 98 µg per ml and stored in the dark at 4°C. The concentration was confirmed during the course of experimentation by gas-liquid chromatography (Au, Runikis, Abbott, unpublished procedure).

G-(^{3}H)-L-proline of specific activity 677 Ci per mol in 2% (v/v) ethanol was purchased from Amersham-Searle Corp., Oakville, Ont.; U-(^{14}C)-L-proline of specific activity 225 Ci per mol in 0.01 N-HCl was purchased from New England Nuclear (Canada) Ltd., Lachine, P.Q.; acetyl-labelled collagen of specific activity 0.3 Ci per kg was a gift from M. Gisslow and B.C. McBride, Dept. of Microbiology (1975). Collagenous proteins were estimated using chromatographically-purified Clostridium histolyticum collagenase (Form III) (Advanced Biofactors Corp., Lynbrook, NY, USA). Twice-recrystallized pepsin containing approximately 2,500 units per mg was purchased from Worthington Biochemical Corporation, Freehold, NJ, USA.

All other chemicals were the purest grade commercially available.

B. CELL LINES

Stock L-929 mouse dermal fibroblasts were obtained from Flow Laboratories, Inglewood, CA, USA. Human dermal fibroblasts, derived from explants of a biopsy from a healthy male in his late twenties, were used in passages 5 to 8. Cells not required immediately were transferred to plastic vials containing medium plus 20% (v/v) foetal bovine serum and 10% (v/v) dimethyl sulphoxide and stored in liquid nitrogen.

C. CELL CULTURE

The cells were maintained in Dulbecco's Modified Eagle's Medium containing L-glutamine but no proline (Grand Island Biological Co., Berkeley, CA, USA), supplemented with 1.8 g $NaHCO_3$ per litre, 100 units of penicillin per ml, 100 µg of streptomycin per ml (Grand Island Biological Co., Berkeley, CA, USA) and 15% (v/v) foetal bovine serum (Flow Laboratories, Inglewood, CA, USA). Stock cells were maintained and grown to confluency at 37°C in 5% (v/v) CO_2-95% (v/v) air in 100 mm plastic culture dishes (Falcon Plastics, Fisher Scientific Co., Ltd., Montreal, P.Q.). The medium was changed twice a week. Confluent cells were treated for 10 min at 37°C with 0.25% (w/v) trypsin in Hank's buffered saline solution (Grand Island Biological Co., Berkeley, CA, USA), detached by gentle scraping with a policeman, then disaggregated by drawing up and expelling the suspension repeatedly with a pasteur pipette. Cells from several plates were pooled for each experiment.

Mouse (10^6) or human (1.5×10^6) cells were seeded into roller bottles of surface area 780 cm^2 containing 150 ml medium supplemented with 15% (v/v) foetal bovine serum and incubated at 37°C. Cellular morphology was examined daily; those bottles showing infection, cellular degeneration or abnormally retarded growth were discarded. The medium was changed once every three or four days.

To expose the cells to triamcinolone acetonide, the medium was replaced with fresh medium containing the supplements, 50 µg ascorbic acid (J.T. Baker Chemical Co., Phillipsberg, NJ, USA) per ml and triamcinolone acetonide in propylene glycol to a final concentration of 0.1 µg per ml; simultaneous preparations of control bottles were identical but contained no glucocorticoid.

Forty-eight hours following the initial administration of triamcinolone acetonide to the cells, the medium was replaced with 50 ml of medium containing the glucocorticoid or propylene glycol, ascorbic acid, serum, 10 µCi of ^{3}H- or ^{14}C-L-proline and 50 µg of the lathyrogen, beta-amino-propionitrile (Sigma Co., St. Louis, MO, USA), per ml to prevent the formation of cross-links in the collagen. The cells were incubated for a further twenty-four hours.

D. CELL HARVESTING, COUNTING AND SIZING

After incubation with radioactive-labelled proline, the medium was decanted, the cell layers washed twice with 10 ml serum-free medium and the washes pooled with the decanted medium. The cell layer was trypsinized, disaggregated and suspended in 100 ml serum-free medium. Within an hour of detachment, aliquots of suspended cells were counted with a haemacytometer and an impedance-triggered electronic counter. One hundred to four hundred cells were counted in each of four charges of the counting chamber of a haemacytometer; 1500 to 3000 cells were counted in each of five aliquots in an electronic counter (Celloscope Model 112 CLTH/RWP, Particle Data Inc., Cordis Laboratories, Miami, FL, USA).

The size distribution of both human and mouse fibroblasts was determined by a filar micrometer. Harvested cells were spotted on a haemacytometer and measured under high dry magnification using an eyepiece micrometer calibrated with a stage micrometer. A total of 125 to 200 cells were measured. Size distributions were also determined using an electronic counter in which the impedance was altered to provide a range of thresholds corresponding to different cell diameters.

E. ISOLATION OF RADIOACTIVE-LABELLED COLLAGEN AND TREATMENT WITH COLLAGENASE

All manipulations were carried out at 4°C. Collagen was isolated using a modification of the procedure of Uitto et al. (1976). 100 µg of cycloheximide (Sigma Co., St. Louis, MO, USA) per ml and 1 mM α,α'-dipyridyl (Fisher Scientific Co., Vanc., B.C.) were added to the pooled medium to inhibit protein synthesis and hydroxylation, respectively. Proteolysis was inhibited by the presence of 10 mM N-ethylmaleimide (Pierce Chemical Co., Rockford, IL, USA), 0.3 mM α-toluenesulphonylfluoride (Sigma Chemical Co., St. Louis, MO, USA) and 20 mM disodium ethylenediaminetetraacetate (Fisher Scientific Co., Vanc., B.C.). After removal of cellular debris by centrifugation at 9,000 g for 30 min, the collagenous proteins were precipitated by the addition of ammonium sulphate as a saturated solution to give a concentration of 20% (w/v) (Church et al, 1973). The precipitate was collected after 16 h by centrifugation at 18,000 g for 1 hour, washed twice with 20% (w/v) ammonium sulphate, dissolved in 2.0 ml 0.15 M NaCl containing 0.01 M $CaCl_2$ and 0.05 M Tris.HCl, pH 7.6, then dialyzed against the foregoing buffered saline. A 1.0 ml portion of the retentate was removed, weighed, and digested for 15 hours at 37°C with 100 µg of bacterial collagenase in the presence of 2.5 mM N-ethylmaleimide. The digestion was halted by the addition of 0.1 ml 0.25 M EDTA and the small collagenous peptides were eliminated by dialysis versus 0.15 M NaCl containing 0.05 M Tris.HCl, pH 7.6. Control samples in which the bacterial collagenase was absent were run simultaneously. The difference between the counts observed in the presence and absence of collagenase digestion was used as an index of the amount of radioactively-labelled collagen synthesized.

Treatment of the cellular fraction consisted of centrifugation at 9,000 g for 20 min to sediment the cells. The pellet from one bottle was suspended in

10 ml 0.5 M acetic acid and placed on a shaker overnight. The extract, collected by centrifugation, was dialyzed against 0.02 M disodium phosphate. The insoluble collagenous proteins were collected by centrifugation at 9,000 g for 30 min, dissolved in 2.0 ml 0.15 M NaCl containing 0.01 M $CaCl_2$ and 0.05 M Tris.HCl, pH 7.6, then dialyzed against the same buffer. Enzymatic digestion of 1.0 ml portions of the retentate was done as before.

F. PEPSINIZATION OF NATIVE COLLAGEN

Collagenous proteins isolated from both cells and medium, by the foregoing procedure, were dissolved in 0.5 M acetic acid. To ensure maximal cleavage of the telopeptides and complete digestion of the noncollagenous proteins, pepsin was added to each sample to a final concentration of 100 ug per ml (Burke et al., 1977). After digestion for 5 hours at 15°C, the reaction was halted by neutralization with 0.5 N NaOH. The telopeptide digests were then removed by dialysis against 0.4 M NaCl containing 0.1 M Tris.HCl, pH 7.6.

G. IDENTIFICATION AND QUANTITATION OF PEPSINIZED ^{14}C-LABELLED COLLAGEN IN POLYACRYLAMIDE GELS

The procedure used was a modification of that of Furthmayr and Timpl (1971), Byers et al. (1974) and Sykes et al. (1976). Reagent A: To 10 g acrylamide (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH, USA) and 0.26 g N,N'-methylenebisacrylamide (Eastman Kodak Co., Rochester, NY, USA) dissolved in 50 ml 0.2 M phosphate buffer, pH 7.2, were added 10 ml 2% (w/v) sodium dodecyl sulphate (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH, USA) and 0.1 ml N,N,N',N'-tetramethylethylenediamine (Eastman Kodak Co., Rochester, NY, USA) followed by dilution to 100 ml with distilled water. Reagent B: 150 mg ammonium persulphate (Fisher Scientific Co., Vanc., BC) were dissolved in 50 ml 0.1 M phosphate buffer, pH 7.2. Both

reagents A and B were stored at 4°C in the dark. When equal volumes of solutions A and B were mixed, polymerization occurred within two hours.

To the collagen extracts was added one-tenth volume of a tracking dye comprising of 50% (w/v) sucrose in 0.1% (w/v) bromophenol blue. The collagen was denatured for 30 minutes at 50°C before application of 50 µl to the gel. Reference type I and type III collagens derived from rat tail tendon and human placenta (prepared by Tan, E.M.L. and Tan, S.Y.L.) were treated similarly and included in each electrophoretic run. The gels were electrophoresed at 8 mA per gel in 0.1 M phosphate buffer, pH 7.2 containing 0.1% (w/v) sodium dodecyl sulphate. When the tracking dye had migrated to the end of each gel, the electrophoresis was terminated. The reference gels were removed and stained overnight in 0.2% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad Co., Richmond, CA, USA) in 50% (v/v) methanol/5% (v/v) acetic acid. The unstained radioactive gels were sliced into 2 mm slices. Each slice was solubilized with 0.2 ml 60% perchloric acid (Fisher Scientific Co., Vanc., BC) and 0.4 ml 30% (v/v) hydrogen peroxide (Fisher Scientific Co., Vanc., BC) for 3 to 5 hours at 60°C (Kobayashi and Harris, 1974) in separate 22 mm linear polyethylene scintillation vials (New England Nuclear (Canada) Ltd., Lachine, PQ),.

H. ASSAY OF RADIOACTIVITY OF COLLAGEN

For the initial experiment with L-929 cells, 1.0 ml of the non-dialyzable collagenase-resistant protein was placed in a plastic minivial (New England Nuclear (Canada) Ltd., Lachine, PQ). An equal volume of 0.15 M NaCl containing 0.05 M Tris.HCl, pH 7.6 and 4 ml of Aquasol-2 (New England Nuclear (Canada) Ltd., Lachine, PQ) were added and the mixture shaken. These samples were loaded in a Unilux II liquid scintillation spectrometer (Searle Analytic Inc., Des Plaines, IL, USA) and counted by a channels ratio method at 4°C.

For the experiment with the human cells, the collagenous proteins were counted in 22 mm linear polyethylene scintillation vials with 9.0 ml Aquasol-2 in a Mark III liquid scintillation system (Searle Analytic Inc., Des Plaines, IL, USA) using a channels ratio method.

The reference standard was 7-(^{14}C) benzoic acid (New England Nuclear (Canada) Ltd., Lachine, PQ) dissolved in a small excess of saturated NaOH, neutralized to pH 7.0 and diluted gravimetrically with distilled water.. A weighed aliquot of benzoic acid was adjusted to 0.15 M NaCl buffered to pH 7.6 with 0.05 M Tris.HCl. 9.0 ml Aquasol-2 was added to a 1.0 ml weighed aliquot of the benzoic acid. Volumes of 5 to 50 µl nitrobenzene were added to produce variable quenching. The absolute counts of the collagen samples were calculated from a plot of channels ratio versus counting efficiency.

For the gel electrophoretic experiment with the L-929 fibroblasts, the slices were solubilized at 60°C, cooled and 10 ml Aquasol-2 added to each vial with thorough mixing. These samples and a set of commercial ^{14}C-standards (New England Nuclear (Canada) Ltd., Lachine, PQ) were counted as before in a Mark III liquid scintillation system.

I. STATISTICAL ANALYSIS

Student's t-test was used to compare two groups of replicates with cells derived from a common pool. A two-way analysis of variance was used to analyze experiments where replicate pairs of bottles utilizing different pools of cells were involved. The latter technique permitted the separation of the effect of the glucocorticoid from that of batch-to-batch variability of the cells. In this analysis, the residual is used as the error and included both the variance of replicates and the batch-to-batch differences in response to the drug.

EXPERIMENTAL AND RESULTS

A. CELL SIZE AND DISTRIBUTION

Fibroblasts were initially counted in haemacytometers but were later counted in an electronic counter. The haemacytometer affords the advantage of visual elimination of cellular debris and nonviable cells but lends itself to greater variability in counting. Therefore, the electronic counter was substituted because the counts tended to be more reproducible and accurate. However, the electronic counter does not discriminate between viable and nonviable cells.

To use the electronic counter, one had to screen out electric noise to avoid false elevated counts. The lower and upper thresholds had to be set accordingly. To do this required information about cell sizes. The cells used for sizing were mouse L-929 and human dermal fibroblasts.

A plate of murine L-929 fibroblasts was trypsinized and disaggregated. An aliquot of the cell suspension was applied to the haemacytometer. An eyepiece micrometer was attached to a light microscope and calibrated with a stage micrometer. Under 10x magnification 120 mouse L-929 fibroblasts were examined. Their diameters ranged from 12 to 25 µm. Approximately 23% of the cells, representing the majority of the cell population, measured 16 µm as evident by the prominent peak in figure 5. 34% and 43% of the cells fell below and above this peak, respectively (Fig. 5). To confirm these findings, cell distribution according to diameter sizes was examined in a calibrated impedence-triggered electronic counter. A series of thresholds was set and the difference in cell number between thresholds generated a similar distribution curve of diameter sizes as that obtained with micrometer measurements. The cells examined in the electronic counter were derived from the same pool

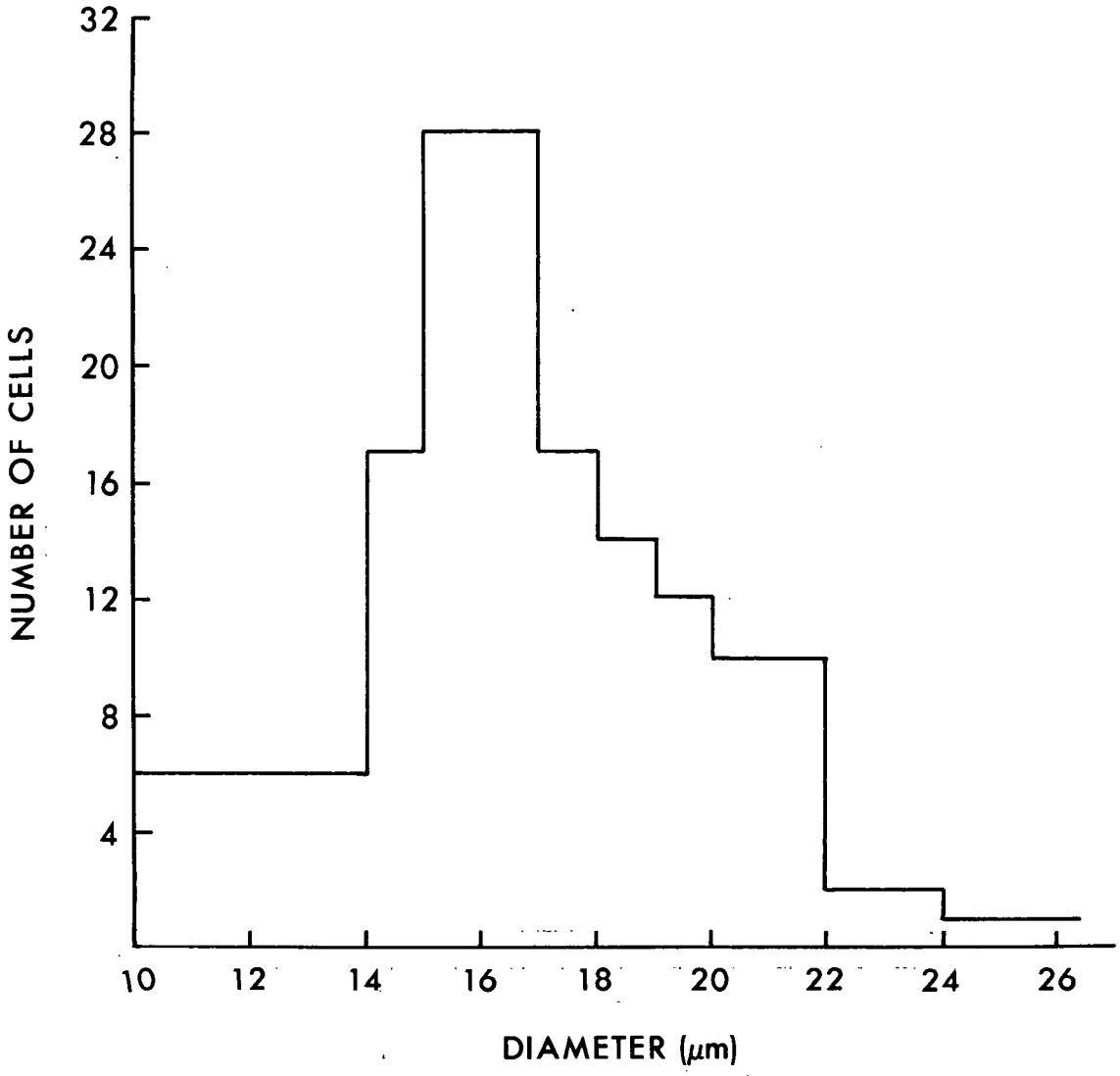


Figure 5. The distribution of diameters of mouse L-929 fibroblasts measured by micrometer. Cells were harvested, suspended in medium and the diameters measured in a wet mount under 10 x magnification using a micrometer eyepiece calibrated with a stage micrometer. 120 cells were measured.

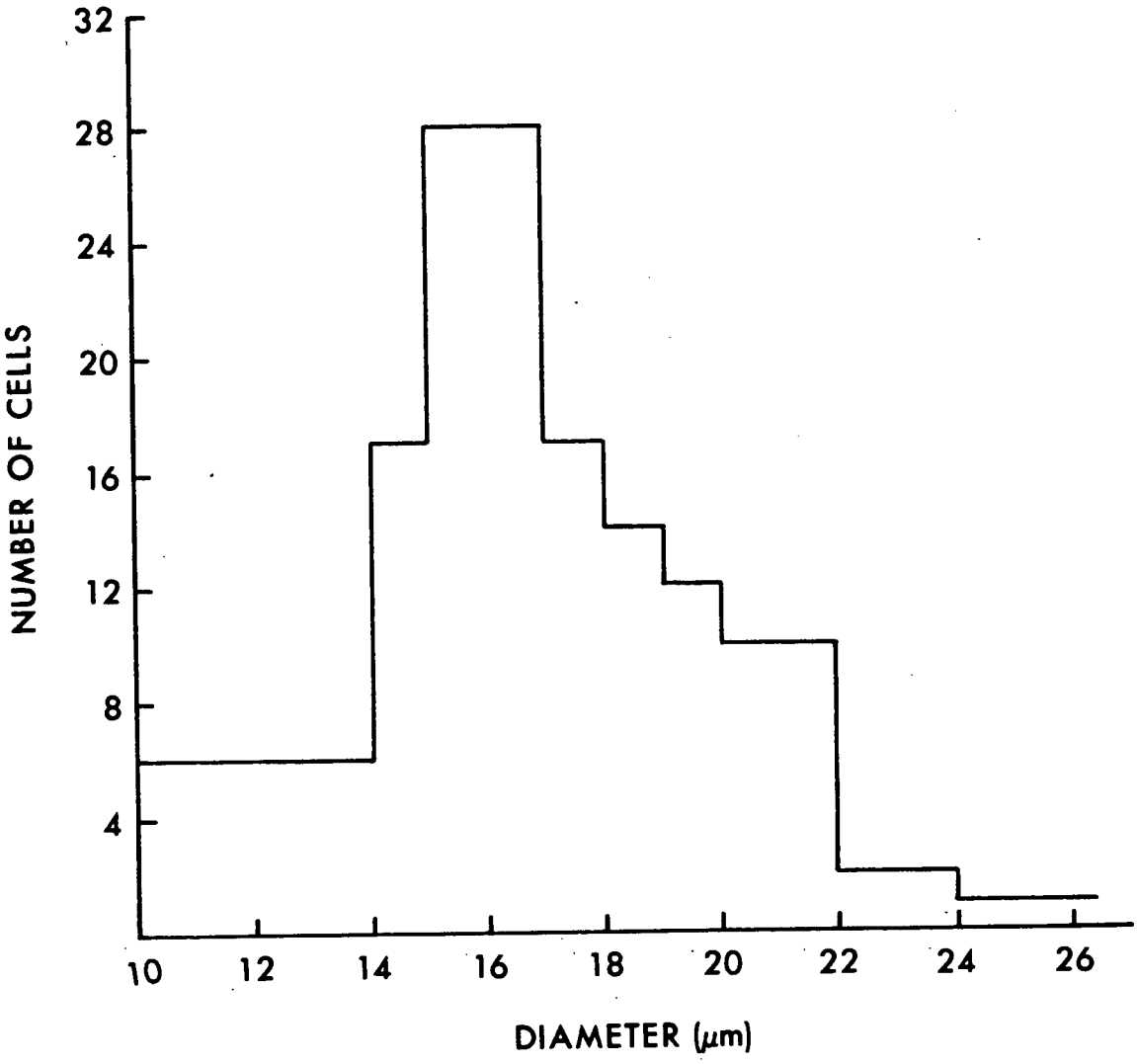


Figure 5. The distribution of diameters of mouse L-929 fibroblasts measured by micrometer. Cells were harvested, suspended in medium and the diameters measured in a wet mount under 10 x magnification using a micrometer eyepiece calibrated with a stage micrometer. 120 cells were measured.

of cells observed under light microscope at 10x magnification. Measurements by both means were performed within the hour of one another. To avoid error arising from cellular aggregation, counts were completed not later than an hour following isolation and disaggregation procedures.

Similar procedures were applied to adult human dermal fibroblasts. A plate of cells was trypsinized and disaggregated. An aliquot was applied to a haemacytometer. Under 10x magnification, micrometer readings indicated diameter sizes of 10 to 30 µm. Of 126 cells, 46% had diameter sizes of 18 to 20 µm. About 23% of the cells were smaller than 18 µm and 31% larger than 20 µm (Fig. 6). Cellular distribution according to diameter sizes was then examined within an hour of the micrometer readings in an electronic counter. The difference in cell numbers between thresholds was calculated and generated a similar distribution curve (Fig. 7). As was observed previously, a prominent peak representing the majority of cells was obtained at 18 to 20 µm. The distribution curve was fairly reproducible within two hours of isolation and disaggregation of cells. Two hours after isolation there was a slight decrease in cells measuring 25 to 30 µm. There was a concomitant increase in those measuring 12 to 15 µm. After 2 hours, cells began to aggregate giving rise to erroneous counts. Cell counts for subsequent experiments were performed within an hour of isolation and disaggregation.

Comparison of the range of diameter sizes of murine L-929 and human dermal fibroblasts by both micrometric and electronic means indicated that they were similar in size. Settings for the electronic counter were, therefore, identical. Particles less than 10 µm were eliminated in counting for both cell lines.

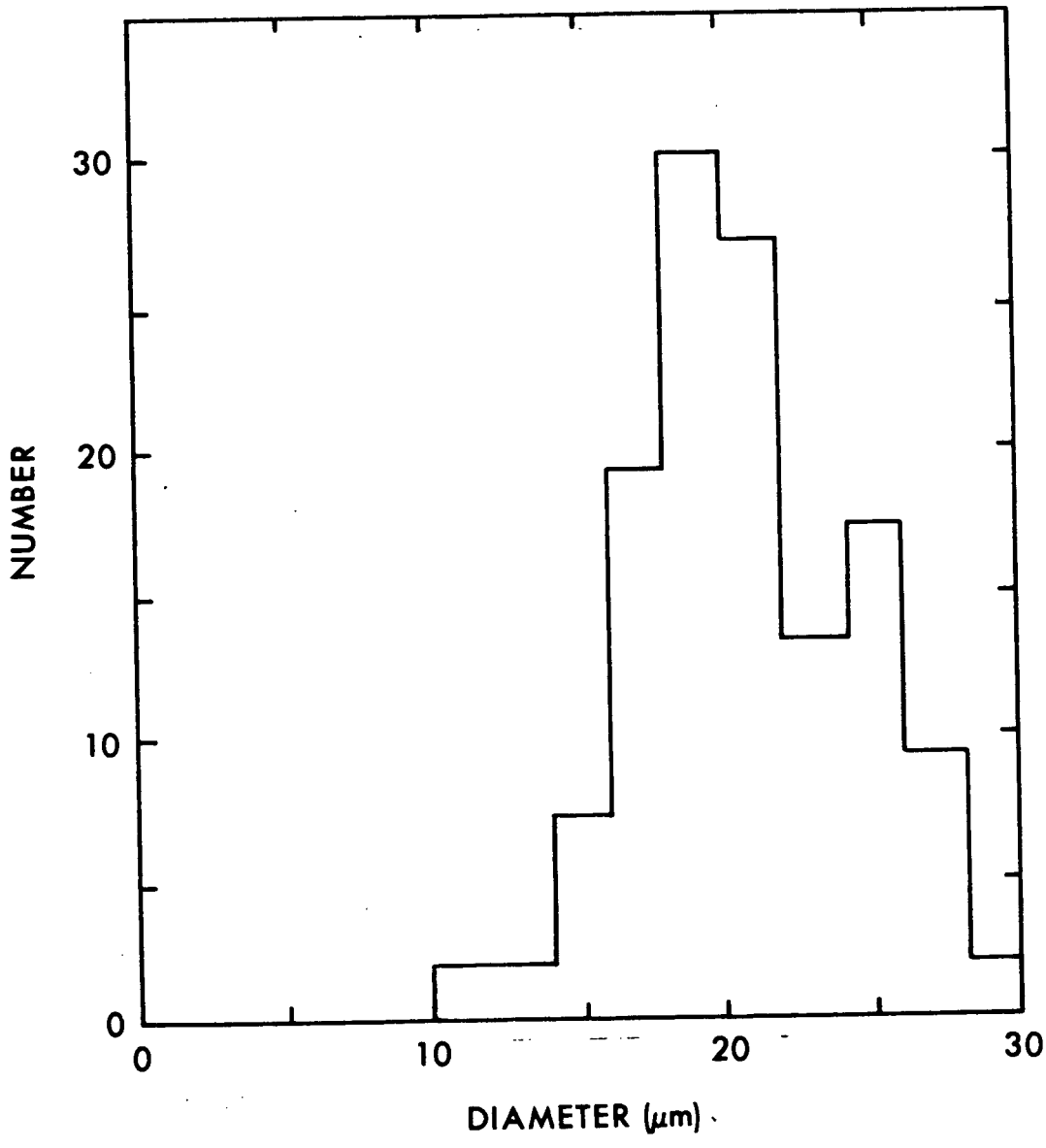


Figure 6. The distribution of human fibroblasts measured by micrometer. Cells were harvested, suspended in medium, and the diameters measured in a wet mount under 10 x magnification using a micrometer eyepiece calibrated with a stage micrometer; 126 cells were measured.

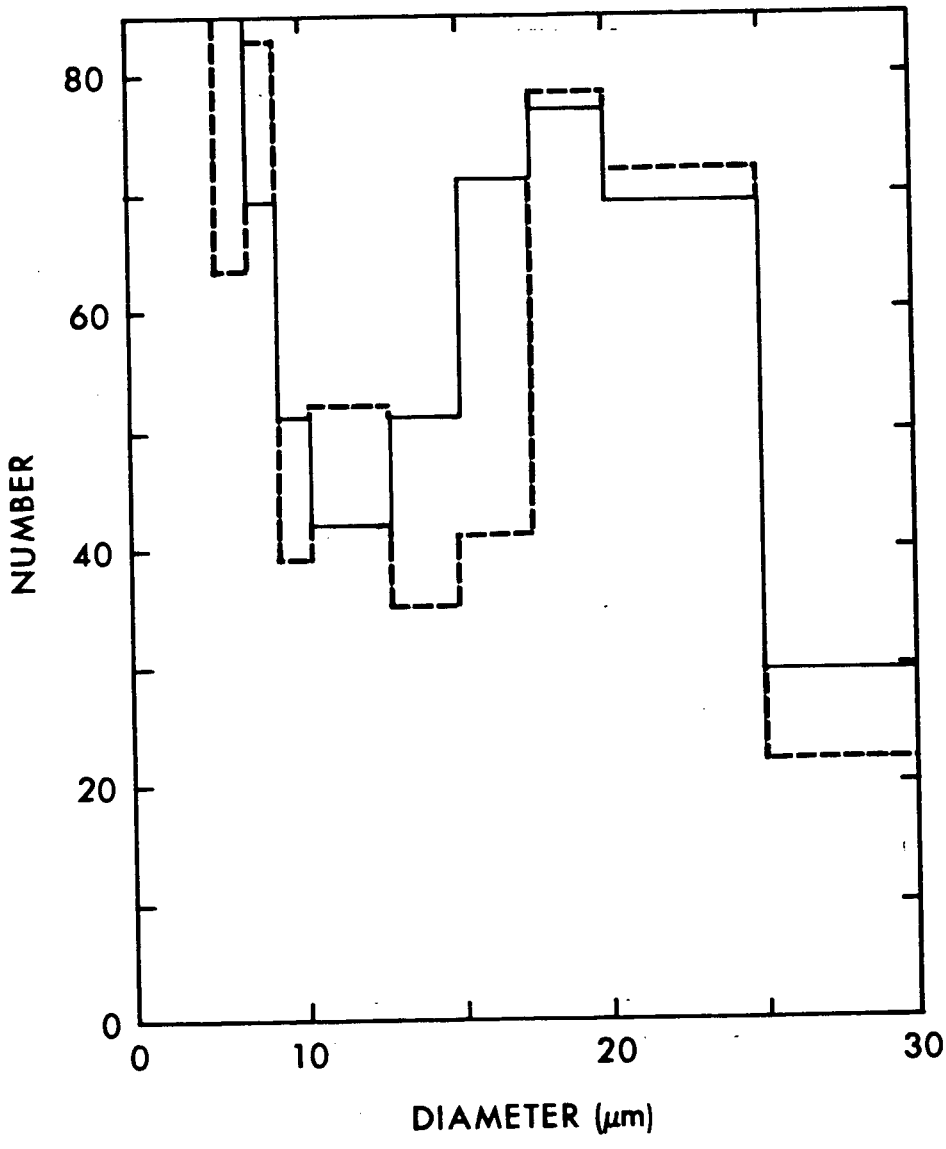


Figure 7. The distribution of diameters of human fibroblasts measured in an electronic particle counter. Cells were harvested, suspended in medium and the number of cells over a series of thresholds was measured in a calibrated particle counter. The number of cells between thresholds was calculated by difference. The solid line represents counts obtained immediately after isolation; the dotted line represents counts determined 2 hours after isolation.

B. GLUCOCORTICOID EFFECTS ON CELLULAR PROLIFERATION AND PROLINE INCORPORATION: COLLAGENASE ASSAY STUDIES

I. Mouse L-929 Fibroblasts

The study of mouse L-929 fibroblasts was initiated before that of the human dermal fibroblasts because of the greater ease in maintaining the former cells in culture. Also, analysis of collagen synthesis in glucocorticoid-treated murine fibroblasts had not been done by other investigators. Since collagen synthesis in cultured fibroblasts had been reported to be maximal at late logarithmic phase of growth (Gribble et al., 1969), mouse L-929 fibroblasts were seeded and grown to this stage at which time triamcinolone acetonide was administered to the cells for 72 hours.

In the first experiment, A, triplicate control and glucocorticoid-treated bottles were derived from a common pool of cells. Due to the inconvenience of seeding triplicate samples and the high incidence of contamination, later experiments, B, consisted of bottles in which each pair was seeded from a different pool of cells. The results of experiments A and B are in Table I, Parts 1 and 2.

At least four separate cell counts were obtained from each sample; counts in excess of 10^4 cells were corrected for coincidence. At no time did the standard deviation of counts exceed 1% of the total number of cells. The number of cells floating in each bottle in the medium was counted but deemed negligible.

In every experiment, triamcinolone acetonide suppressed cellular proliferation, thus confirming a previous study (Runikis et al., 1978). In experiment A, analysis by Students' t-test showed that suppression of cellular proliferation by triamcinolone acetonide was consistent and significant

Table I, Parts 1 and 2: The effect of triamcinolone acetonide on cellular proliferation and synthesis of collagenase-sensitive protein of confluent mouse L-929 fibroblasts.

Roller bottles containing 150 ml medium were seeded with 10^6 cells and maintained until the cells reached the late logarithmic phase of growth (4 to 5 days). Medium containing ascorbic acid and either 0.116 µg triamcinolone acetonide per ml in propylene glycol (present) or propylene glycol alone (absent) was added and the incubation continued 48 hours at which time the medium was replaced with 50 ml medium containing, in addition to the above, beta-aminopropionitrile and either G-(^{3}H)- or U-(^{14}C)-L-proline. After 24 hours, the medium was removed, the cell layer washed, and the cells harvested and counted. Collagen was isolated from both medium and cells; portions of each fraction were treated with collagenase and dialyzed. The digest and an undigested control were assayed for radioactivity.

In experiment A, triplicate control and glucocorticoid-treated samples were derived from a common pool of cells; in the subsequent experiments (B), each pair of bottles was derived from a different pool of cells.

Table I, Part 1: Medium fraction

Experiment	Glucocorticoid	Cells per bottle ($x10^{-6}$)	DPM per bottle	DPM total protein per cell ($x10^{6}$)	Loss with collagenase (%)	DPM collagenase-sensitive protein per cell ($x10^{6}$)
A	Present	44.22	45350	1025.55	12***	
		37.97	59710	1572.14	26***	-
		36.23	55168	1522.30	18***	
	Absent	174.94	214698	1227.27	23***	
		185.57	269976	1454.77	48***	-
		185.17	156168	843.33	7	
	P*	0.001				
B	Present	41.62	36196	896.68	75	704.44
	Absent	52.08	107840	2070.66	88	1780.77
	Present	18.79	42372	2255.03	75	1691.27
	Absent	64.56	146725	2272.69	88	1999.97
	Present	15.03	44304	2947.70	90	2652.93
	Absent	60.80	99569	1637.65	88	1441.13
	Present	16.12	33202	2060.00	80	1648.00
	Absent	69.68	127774	1833.73	79	1448.65
	P**	0.01				

*Probability that the drug had no effect, by Student's t-test
**Probability that the drug had no effect, by two-way analysis of variance
***Collagenase digestion at 4°C

Table I, Part 2: Cellular fraction

Experiment	Glucocorticoid	Cells per bottle ($x10^{-6}$)	DPM per bottle	DPM total protein per cell ($x10^{6}$)	Loss with collagenase (%)	DPM collagenase-sensitive protein per cell ($x10^{6}$)
A	Present	44.22	11060	250.11	67	167.57
		37.97	11482	287.27	63	180.98
		36.23	8700	240.13	64	153.68
	Absent	174.94	21350	122.04	69	84.21
		185.57	16928	91.22	68	62.03
		185.17	19614	105.92	70	74.14
	P*	0.001		0.01 P 0.001		0.01 P 0.001
B	Present	41.62	5078	122.01	-	59.71
	Absent	52.08	4022	77.23	16	18.92
	Present	18.79	5610	298.56	20	59.71
	Absent	64.56	6430	99.60	19	18.92
	Present	15.03	12668	842.85	7	59.00
	Absent	60.80	1360	22.37	31	6.93
	Present	16.12	218	13.52	48	6.49
	Absent	69.68	420	6.03	35	2.11
	P**	0.01		0.18		0.85
A and B	P**			0.08		0.004

*Probability that the drug had no effect, by Student's t-test
**Probability that the drug had no effect, by two-way analysis of variance

($P = 0.001$). The cells used in experiment B were derived from different pools; hence a two-way analysis of variance was done. Again, cellular proliferation was suppressed markedly and significantly ($P = 0.01$) (see Table I, Parts 1 and 2).

The incorporation of proline into total protein and collagenase-sensitive protein was studied to assess the effect of 0.1 µg triamcinolone acetonide per ml (0.23 µM) medium on biological activities other than proliferation. In all experiments, triamcinolone acetonide was administered to late logarithmic growing cultures for 72 hours; radioactive-labelled proline was present during the last 24 hours of incubation. Tritium-labelled proline was employed initially but because of some loss of radioactivity in the form of water molecules during hydroxylation, ^{14}C-proline was substituted. Fractions containing collagen in medium and cells were identified by susceptibility to protease-free Clostridium histolyticum collagenase (Peterkofsky and Diegelmann, 1971). In Table I, Part 1, proline incorporation into total protein per bottle of medium was consistently less with glucocorticoid treatment. Subsequent experiments (B) confirmed these results. However, the activity of total protein per cell was not consistently lower in the glucocorticoid-treated bottles either in experiment (A) or in subsequent experiments (B) ($P = 0.88$). Proline incorporation into the collagenase-susceptible protein of the first experiment A could not be measured due to the low fraction of radioactivity lost with collagenase at $4^{o}C$. 50 µg per ml of collagenase was used in the foregoing experiment (Table I, Part 1). The low losses suggested that the concentration or the temperature or both factors were not conducive for maximal enzymatic activity. Consequently, the experiments in Section C were undertaken to determine the conditions necessary for efficient collagenase

digestion. These experiments established an increase in activity at 37°C (see Tables IV and V). The concentration of collagenase used in subsequent experiments was 100 µg per ml.

Glucocorticoid effect on the collagenase-susceptible protein per cell of the medium was inconsistent in the last four experiments (B) (see Table I, Part 1). Collagenase eliminated 79% to 88% and 75% to 90% of the radioactive counts in the control and glucocorticoid-treated bottles, respectively; the samples were therefore relatively free of radioactive-labelled noncollagenous proteins.

Examination of the cell layers in experiments B showed that the amounts of radioactivity per bottle were altered inconsistently in control and experimental groups. However, a consistent increase in radioactivity per cell was observed in total ($P = 0.18$) and collagenase-susceptible protein ($P = 0.85$) of triamcinolone acetonide-treated bottles; the few samples analyzed did not allow this observation to be significant statistically. In the experiment A, the glucocorticoid treatment enhanced proline incorporation into total and collagenase-sensitive protein per cell significantly (0.01 P 0.001). The data for experiments A and B were confirmed by pairing replicates of experiment A arbitrarily. Although the elevated incorporation of proline into total protein per cell was not statistically significant ($P = 0.08$), that of collagenase-susceptible protein per cell was highly significant ($P = 0.004$) (see Table I, Part 2). Thus, the observation of a consistent positive effect of triamcinolone acetonide on proline incorporation into collagenase-susceptible protein was more significant when arbitrarily-paired bottles of experiment A were combined statistically with those of experiments B.

II. Adult Human Dermal Fibroblasts

(a) short-term incubation with triamcinolone acetonide

The effects of 0.1 µg triamcinolone acetonide per ml on cellular proliferation and collagen synthesis in a cell line derived from adult human were examined under identical conditions to those for the mouse L-929 fibroblasts. Under these conditions, triamcinolone acetonide had an effect on cellular proliferation and collagen synthesis in the cell layers of the mouse fibroblasts. Therefore, it was of interest to determine if human dermal fibroblasts responded similarly to the glucocorticoid.

Roller bottles were seeded with 1.5×10^6 cells per bottle, 1.5 times more cells per bottle than that required for the previous study. Unlike the mouse fibroblasts, human fibroblasts attained confluency an average of 24 hours later. The cells were grown to late logarithmic phase of growth at which time triamcinolone acetonide was administered. Triamcinolone acetonide, administered for 72 hours during late logarithmic phase of growth, increased cell numbers by 11 to 27% in four of the five experiments, confirming the findings of Runikis et al. (1978); the fifth decreased by 17% (Table II, Parts A and B). Therefore, triamcinolone acetonide had no consistent effect on cellular proliferation ($P = 0.15$). The effect of triamcinolone acetonide on cellular proliferation was tested using a two-way analysis of variance. Since, in every experiment, the paired bottles were derived from a separate pool of cells, the differences between batches of cells was also tested. However, this statistical analysis pools replicate variability with batch-to-batch differences in the response to glucocorticoid. Since the number of cells in both the control and glucocorticoid-treated bottles varied between 27 and 95 million, the differences between experiments was relatively large ($P = 0.0009$).

Table II, Parts A and B: The effect of triamcinolone acetonide on cellular proliferation and on the incorporation of proline into collagenase-sensitive protein by adult human dermal fibroblasts.

Roller bottles containing 150 ml medium were seeded with 1.5×10^6 cells and maintained until the fibroblasts had reached the late logarithmic phase of growth (5 to 6 days) when medium containing ascorbic acid and either 0.116 µg triamcinolone acetonide per ml in propylene glycol (present) or propylene glycol alone (absent) was added; the incubation was continued for 48 h. The medium was replaced with 50 ml medium containing, in addition to the above, beta-aminopropionitrile and U-(^{14}C)-L-proline. After 24 hours, the medium was decanted, the cell layer harvested and an aliquot counted for cell number. Fractions containing collagen were isolated from medium and cells; one portion of each fraction was treated with bacterial collagenase and dialyzed. The digest and undigested portions were assayed for radioactivity.

Each experiment consisted of paired bottles derived from a different pool of cells.

Table II, Part A: Medium fraction

Glucocorticoid	Cells per bottle ($x10^{-6}$)	DPM per bottle	DPM total protein per cell ($x10^6$)	Loss with collagenase (%)	DPM collagenase-sensitive protein per cell ($x10^6$)
Present	47.47	10664	224.65	53	119.06
Absent	42.77	23092	539.91	60	323.95
Present	28.57	7822	273.79	63	172.49
Absent	34.58	6926	200.29	56	112.16
Present	106.01	51126	482.29	72	347.25
Absent	94.81	75784	799.28	67	535.52
Present	78.88	60236	763.67	53	404.75
Absent	63.95	49782	778.40	70	544.88
Present	34.73	41454	1193.47	56	668.34
Absent	27.42	8138	296.82	48	142.47
P*	0.15		0.79		0.93

*Probability that the drug had no effect, by two-way analysis of variance.

Table II, Part B: Cellular fraction

Glucocorticoid	Cells per bottle ($x10^{-6}$)	DPM per bottle	DPM total protein per cell ($x10^{6}$)	Loss with collagenase (%)	DPM collagenase-sensitive protein per cell ($x10^{6}$)
Present	47.47	452	9.52	17	1.62
Absent	42.77	304	7.11	33	2.35
Present	28.57	658	23.03	59	13.59
Absent	34.58	1384	40.02	85	34.02
Present	106.01	6106	57.60	69	39.74
Absent	94.81	12388	130.65	81	105.83
Present	78.88	746	21.48	75	16.11
Absent	63.95	3588	56.10	70	39.27
Present	34.73	-	-	-	-
Absent	27.42	-	-	-	-
P*	0.15		0.09		0.08

*Probability that the drug had no effect, by two-way analysis of variance.

In this experiment, five bottles of confluent cultures of human dermal fibroblasts were treated with triamcinolone acetonide for 72 hours. U-(^{14}C)-L-proline was added during the 24 hour period prior to harvesting. No consistent increase or decrease was noted in the dpm per bottle of the medium. Nor was there a consistent difference in dpm per cell between the glucocorticoid-treated and control samples before or after digestion by collagenase ($P = 0.79$ and $P = 0.93$, respectively). Three out of the five bottles registered an increase in the amount of U-(^{14}C)-L-proline incorporated into total protein per cell. Incorporation was decreased in the other two. Interestingly enough, the incorporation into collagenase-susceptible protein was similarly affected. Where an increase in incorporation was registered in total protein of the medium, a similar elevation was also obtained with the collagenase-susceptible fraction. Loss of activity following bacterial collagenase treatment ranged from 48 to 72%. No differences were found between experiments within the control and glucocorticoid-treated samples ($P = 0.54$) (see Table II, Part A).

With glucocorticoid treatment three out of four bottles showed an inhibition, the last showed a stimulation ($P = 0.09$) of incorporation of proline into total protein of the cell layer per bottle. A similar trend was also observed when calculations were based on a per cell basis. The proline incorporation of both control and experimental groups differed from experiment to experiment ($P = 0.005$). Triamcinolone acetonide caused a consistent depression in the incorporation of proline into collagenase-sensitive protein per cell in all four bottles ($P = 0.08$). More experiments may have made this observation statistically significant. Bacterial collagenase digestion reduced the radioactivity from 33% to 85% in the control and 17% to 75% in the glucocorticoid-treated bottles (see Table II, Part B).

(b) Long-term incubation with triamcinolone acetonide

The foregoing experiments were conducted with confluent fibroblasts because the enzymes responsible for the post-translational modifications of collagen and the amount of collagen synthesized were maximal at that stage of growth (Gribble et al., 1969). Since human fibroblasts have been known to respond to glucocorticoids only during the logarithmic phase of growth (Kruse et al., 1978) later experiments using paired bottles of cells, one treated with triamcinolone acetonide and the other a control, were conducted with the addition of the glucocorticoid at this growth phase to determine if the cell numbers would be altered consistently and the proline incorporation suppressed more markedly. Saarni and Tammi (1978) reported a minimum incubation requirement of 90 hours to suppress proline into hydroxyproline and proteins in foetal human fibroblasts; hence, in the following experiments, the fibroblasts were exposed to triamcinolone acetonide 24 hours after seeding for a prolonged period of 180 hours.

Triamcinolone acetonide had no consistent effect on cell numbers in four bottles ($P = 0.44$). The cell numbers, among the control bottles, ranged from 7 to 23 million; those of the glucocorticoid-treated bottles, 6.5 to 25 million. There was a significant variation from experiment to experiment which was independent of the glucocorticoid ($P = 0.03$).

Examination of proline incorporation into the medium showed that the incorporation of radioactive-labelled proline into total protein per bottle was increased in one of the four bottles treated with triamcinolone acetonide. The other three registered a decline. Calculated on a per cell basis, no consistent effect was observed either on proline incorporation into total protein ($P = 0.25$) or on that of collagenase-susceptible protein ($P = 0.40$)

(Table III, Part A). Two of the four bottles showed an increase in total protein and collagenase-digestible protein per cell. Where an increase in total protein per cell was obtained, so was there a similar increase in the collagenase-digestible protein per cell. Following collagenase digestion 49% to 75% and 47% to 67% of the counts were removed from the control and glucocorticoid-treated samples, respectively. Between experiments, no difference in proline incorporation into total protein ($P = 0.12$) and collagenase-sensitive protein ($P = 0.21$) were present.

Unlike the initial experiments with short-term incubation of the glucocorticoid, no consistent trend was observed in the cell layer, either in proline incorporation per cell into total ($P = 0.71$) or into collagenase-susceptible protein ($P = 0.31$). When subjected to collagenase digestion, 72% to 90% of the counts were dialyzable in the control, but only 60% to 72% were in the glucocorticoid-treated samples, indicating lesser purity of collagen in the latter (Table III, Part B). Although, no statistical analysis could be done on possible variation of glucocorticoid effect between experiments, some glucocorticoid-independent variation was noted in the amount of radioactive proline incorporated into total ($P = 0.005$) and collagenase-digestible proteins ($P = 0.09$).

C. DETERMINATION OF CONDITIONS FOR EFFICIENT COLLAGENASE DIGESTION

In the initial experiment with murine L-929 fibroblasts, collagenase digestion was conducted at a concentration of 50 µg per ml of collagen solution at $4^{\circ}C$. A small proportion of radioactivity was lost from the medium fraction containing collagen (Table I, Part A). Hence, the temperature

Table III, Parts A and B: The effect of prolonged exposure to triamcinolone acetonide on cellular proliferation and the synthesis of collagen of human dermal fibroblasts.

Roller bottles containing 150 ml medium were seeded with 1.5×10^6 cells and maintained for 24 hours, when replacement medium was added containing either 0.098 µg triamcinolone acetonide per ml in propylene glycol (present) or propylene glycol alone (absent) and the incubation continued for 96 hours. The replacement medium contained 100 µg ascorbic acid per ml in addition to the above. After a further 84 hours, replacement medium was added containing 50 µg ascorbic acid per ml and, in addition to the above, beta-aminopropionitrile and U-(^{14}C)-L-proline. After 24 h, the medium was removed, the cells washed and harvested and those over 10 µm diameter counted electronically. Fractions containing collagen were isolated from medium and cells; one portion of each fraction was exposed to collagenase and dialyzed. The digest and undigested control were assayed for radioactivity.

Each experiment consisted of paired bottles derived from a different pool of cells.

Table III, Part A: Medium fraction

Glucocorticoid	Cells per bottle ($x10^{-6}$)	DPM per bottle	DPM total protein per cell ($x10^{6}$)	Loss with collagenase (%)	DPM collagenase-sensitive protein per cell ($x10^{6}$)
Present	6.51	446942	68,663.40	67	46004.48
Absent	16.39	677940	41,365.11	49	20268.90
Present	8.72	480544	55,123.18	47	25907.89
Absent	7.21	114290	15,859.21	60	9436.23
Present	25.75	149620	5,811.36	52	3021.91
Absent	23.42	352136	15,034.79	75	11276.09
Present	11.00	192234	17,475.82	48	8388.39
Absent	13.22	273972	20,721.29	72	14919.33
P*	0.44		0.25		0.40

*Probability that the drug had no effect, by two-way analysis of variance

Table III, Part B: Cellular fraction

Glucocorticoid	Cells per bottle ($x10^{-6}$)	DPM per bottle	DPM total protein per cell ($x10^{6}$)	Loss with collagenase (%)	DPM collagenase-sensitive protein per cell ($x10^{6}$)
Present	6.51	7380	1133.78	72	816.32
Absent	16.39	12242	746.96	72	537.81
Present	8.72	12130	1391.43	70	967.04
Absent	7.21	11924	1654.61	90	1489.15
Present	25.75	26606	1033.40	60	620.04
Absent	23.42	37486	1600.5	79	1264.40
Present	11.00	5418	492.55	62	305.38
Absent	13.22	4558	344.7	86	296.44
P*	0.44		0.71		0.31

*Probability that the drug had no effect, by two-way analysis of variance

and concentration of enzyme were tested to determine conditions required for increased collagenase digestion.

The activity of bacterial collagenase was tested by two methods: (1) formation of dialyzable hydroxyproline (Woessner, 1961) from purified placental collagen as the substrate, (2) formation of dialyzable radioactivity from acetylated collagen. In the first method, triplicate samples were employed. Zero, 50, 250 and 1250 µg of bacterial collagenase per ml were tested at $4^{o}C$ and $37^{o}C$. In the absence of the enzyme, the amount of dialyzable hydroxyproline was negligible. Therefore, no other proteases appear to be present to digest collagen. At 50 µg of enzyme per ml at $4^{o}C$, no digestion of collagen was apparent, indicating that the concentration or temperature or both were insufficient for degradation of collagen. Increasing the temperature from $4^{o}C$ to $37^{o}C$ resulted in a 75 to 80% loss of collagen. Increasing the enzyme concentration from 50 to 250 µg per ml did not have any effect at $4^{o}C$. But at $37^{o}C$, the loss of collagen by enzymatic digestion ranged from 75 to 83%. Similar observations were made with 1250 µg enzyme per ml at $4^{o}C$ and $37^{o}C$. Concentrations exceeding 50 µg per ml did not enhance the degradation of collagen at $37^{o}C$. Temperature manipulation had a profound effect on the amount of collagen digested (Table IV). Several investigators use bacterial collagenase at a concentration of 100 µg per ml (Peterkofsky et al., 1971; Priestley, 1978). Hence a second method was invoked to confirm the foregoing findings. Using acetylated collagen as the substrate, 100 µg bacterial collagenase per ml at $4^{o}C$ removed negligible radioactivity; at $37^{o}C$, 85% to 87% of the radioactivity was eliminated, thus confirming the increased activity of bacterial collagenase at this temperature and concentration (see Table V).

Table IV: The activity of bacterial collagenase at different concentrations and temperatures.

0.130 to 0.180 ug of purified placental collagen, a mixture of Types I and III, per ml were exposed to 0, 50, 250, or 1250 µg bacterial collagenase (Clostridium histolyticum) per ml at 4 or 37°C for 24 hours. The reaction was terminated with EDTA and the degradation products eliminated by dialysis against 0.15 M NaCl containing 0.05 M Tris HCl, pH 7.6. The retentate was analyzed for hydroxyproline.

Table IV. The activity of bacterial collagenase at different concentrations and temperatures.

	4°C		37°C	
Enzyme ug/ml	Expt. A ug/ml collagen	Expt. B ug/ml collagen	Expt. A ug/ml collagen	Expt. B ug/ml collagen
0	0.176 0.158 0.156	0.137 0.138 0.134	0.151 0.166 0.156	0.138 0.143 0.138
50	0.166 0.159 0.153	0.140 0.141 0.133	0.039 0.038 0.040	0.033 0.035 0.035
250	0.166 0.166 0.166	0.140 0.137 0.138	0.028 0.029 0.031	0.034 0.033 0.033
1250	-	0.138 0.137	-	0.026 0.028 0.028

Table V: Concentration and temperature-dependent degradation of acetylated collagen by bacterial collagenase.

Similar amounts of acetylated collagen was exposed to 100 µg bacterial collagenase per ml at 4^{o} or 37^{o}C. The reaction was terminated by addition of EDTA. The degraded products were removed by dialysis versus 0.15 M-NaCl containing 0.05 M Tris HCl, pH 7.6. The retentates were assayed for their radioactivity.

Table V. Concentration and temperature-dependent degradation of acetylated collagen by bacterial collagenase.

	Temperature	
	4°C cpm/ml	37°C cpm/ml
Control	20992	20938
0 µg/ml enzyme	22059	22201
100 µg/ml enzyme	20899	2892
	21496	3119
		2779

D. POLYACRYLAMIDE GEL ELECTROPHORETIC STUDIES

I. Rationale

Triamcinolone acetonide affected the incorporation of proline into collagenase-sensitive protein in the cell layer of mouse L-929 fibroblasts. A consistent, statistically significant increase in proline incorporation was obtained. To obtain confirmation of the identity of this protein, polyacrylamide gel electrophoresis was employed and an attempt was made to quantitate the amount of radioactivity of the peaks or bands. Reference collagens consisting of purified unlabelled type I collagens from human placenta and rat-tail tendon as well as collagen labelled by methylation were subjected to electrophoresis with the experimental samples to permit identification of the protein in the latter samples.

II. Methodological Tests for Effects of Staining and Destaining Polyacrylamide Gels

The recoveries of acetylated collagen from stained and destained gels were compared with those of unstained gels (see Table VI). The recovery of the radioactivity added to the gels was 80% to 83% in the triplicate samples of unstained gels, whereas only 8% to 18% of the activity was recovered after staining with Coomassie Blue and destaining in 7% (v/v) acetic acid (Table VI). Low recoveries were also obtained by Kerwar *et al.* (1972) who recovered 55% of the added counts from gels stained with Amido-Schwartz stain. Possible reasons for low recoveries are loss of radioactive material by elution during staining and destaining procedures and quenching due to the Coomassie Blue stain of the collagen bands. Thus, subsequent gels were not stained before slicing and digestion of gel slices.

Table VI. Recovery of radioactive collagen from polyacrylamide gels.

Known amounts of acetylated collagen were applied to duplicate polyacrylamide gels. Upon completion of the electrophoretic run, one gel of each pair was stained with 0.2% (w/v) Coomassie Blue R-250 and destained in 7% (v/v) acetic acid. All gels were cut into slices 2mm thick, each slice was digested with 0.2ml 60% (w/v) perchloric acid and 0.4ml 30% (v/v) hydrogen peroxide for 3 to 5h at 60^{o}C in separate scintillation vials. 9.0 ml Aquasol-2 was added to each vial. Samples were then assayed for radioactivity.

Table VI. Recovery of radioactive collagen from stained and unstained polyacrylamide gels.

Radioactivity (d.p.m.)		
Applied	Recovered	Recovery (%)
Stained		
96491.16	8023.20	8.3
12413.72	2263.40	18.2
14281.27	1688.70	11.8
Unstained		
96401.16	76511.10	79.6
89785.21	74515.70	82.9
12633.40	9636.70	76.3

III. Recovery of Radioactive Proteins Following Pepsin Treatment of Proteins Containing Collagen.

Following pepsin treatment of procollagen, telopeptides were removed by dialysis against 0.4 M NaCl containing 0.1 M Tris. HCl, pH 7.6. The dialysis solution was replaced with 0.1 M acetic acid and thereafter the proteins were lyophilized. An unsuccessful attempt was made to dissolve the lyophilized product in 0.5 M acetic acid before application to polyacrylamide gels. There was incomplete solubilization of the material in 0.5 M acetic acid. Modifications in the procedure were instigated to ensure complete solubilization. The efficacy of the procedures adopted was assessed by monitoring the radioactivity at each step (see Fig. 8).

Triplicate control and glucocorticoid-treated samples were treated as follows: proteins containing collagen isolated from the medium were treated with pepsin; the digest was neutralized and dialyzed. The cloudy retentate was centrifuged and the supernate A removed. To the precipitate C was added 2 ml of 0.5 M acetic acid. The resultant cloudy suspension was centrifuged, the supernate B decanted and the precipitate D was dissolved in 100 µl of 0.01 M phosphate buffer, pH 7.2, containing 0.2 (w/v) sodium dodecyl sulphate and 2 M urea, and heated at 60^{o} for 30 minutes. The precipitate dissolved. Both supernatant fractions (A and B) and the dissolved precipitate were pooled and lyophilized; the product was dissolved in 100 µl of the foregoing phosphate budfer and applied to polyacrylamide gels (see Fig. 8 and Table VII).

Cellular proteins containing collagen were also treated by this procedure. Cells from a common pool were seeded in six roller bottles of which three were control and three were treated with triamcinolone acetonide. The cells from each group were pooled (see Table VII).

Figure 8. Treatment of collagen-rich fractions of cellular layers for polyacrylamide gel electrophoresis. Procedure 1.

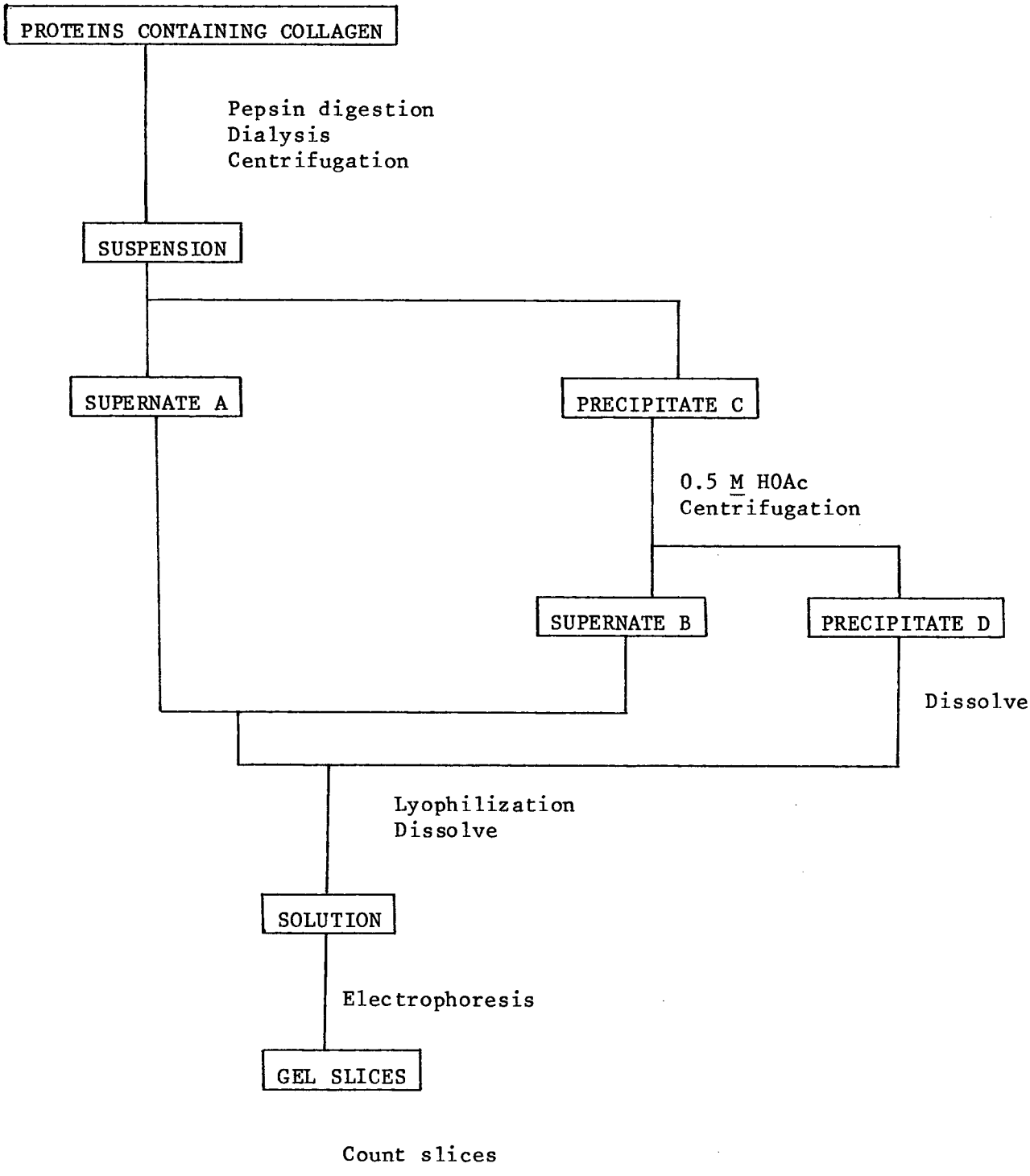


Table VII. Recovery of the radioactivity of proteins containing collagen from polyacrylamide gels.

Proteins containing collagen were treated with pepsin. The digest was neutralized, dialyzed and centrifuged. Supernate A was removed and 2 ml of 0.5 M acetic acid was added to precipitate C. The latter solution was centrifuged, supernate B decanted and the remaining precipitate D dissolved in 100 µl of 0.01 M phosphate buffer, pH 7.2, containing 0.2% (w/v) sodium dodecyl sulphate and 2 M urea. The precipitate was dissolved by heating for 30 minutes at 60°C. Both supernate A and B and the dissolved precipitate were pooled, lyophilized and dissolved in the foregoing buffer before application to polyacrylamide gels.

Table VII. Recovery of the radioactivity of proteins containing collagen from polyacrylamide gels.

Step	Medium %		Cell Layer %	
	Control	Triamcinolone Acetonide	Control	Triamcinolone Acetonide
(Supernate B + Precipitate D)/Precipitate C	89.0 117.6 92.9	99.4 95.1 92.2	92.4	107.4
Solution/(supernate A + supernate B + precipitate D)	81.4 86.3 100.2	90.4 84.3 80.9	98.3	83.9
Gel slices/solution	97.8 90.5 80.1	89.9 80.8 80.0	90.2	86.1
Gel slices/(supernate A + precipitate C)	91.0 89.0 88.8	81.2 82.9 78.8	81.7	89.9

Isolation of the collagenous proteins from the foregoing medium and cellular fractions was modified slightly in an unsuccessful attempt to minimize the excessive manipulative procedures. Again, cells from a common pool were used to seed eight roller bottles of which four were controls and four were treated with triamcinolone acetonide. The cells from each group were then pooled for the isolation of collagenous proteins (see Fig. 9 and Table VIII). As before, the protein fractions were treated with pepsin, and the digest was neutralized and dialyzed against 0.4 M NaCl containing the 0.1 M Tris. HCl, pH 7.6. The retentates were lyophilized. The products were suspended in 0.5 M acetic acid and the suspensions centrifuged to precipitate the insoluble proteins (Residue D). The supernate A was set aside and the residue D re-extracted with 0.5 M acetic acid and centrifuged. The residue was set aside, the supernate was pooled with supernate A. Saturated $(NH_4)_2SO_4$ was added to the pooled supernate B to a final concentration of 30% (w/v) and allowed to stand overnight at $4^{o}C$ to precipitate collagen. Assay of the supernate C showed negligible radioactivity. The precipitate and the residue D were dissolved in phosphate buffer as described previously. Recovery of the radioactivity was followed at each step (Table VIII). A major disadvantage of the foregoing procedures was the excessive manipulation involved. Hence, a simplified procedure was devised.

Fractions containing collagen from the medium and cellular layer were treated with pepsin. The enzymatic reaction was terminated by neutralization with 0.5 N NaOH and dialysis versus 0.4 M NaCl containing 0.1 M Tris. HCl, pH 7.6. The retentate was lyophilized, dissolved in 0.01 M phosphate buffer, pH 7.2, containing 0.2% (w/v) sodium dodecyl sulphate and 2 M urea and heated at $60^{o}C$ for 30 min. The sample was then applied to the gel (Fig. 10).

Figure 9. Treatment of collagen-rich fractions of medium and pooled cellular fractions for application to polyacrylamide gel electrophoresis. Procedure 2.

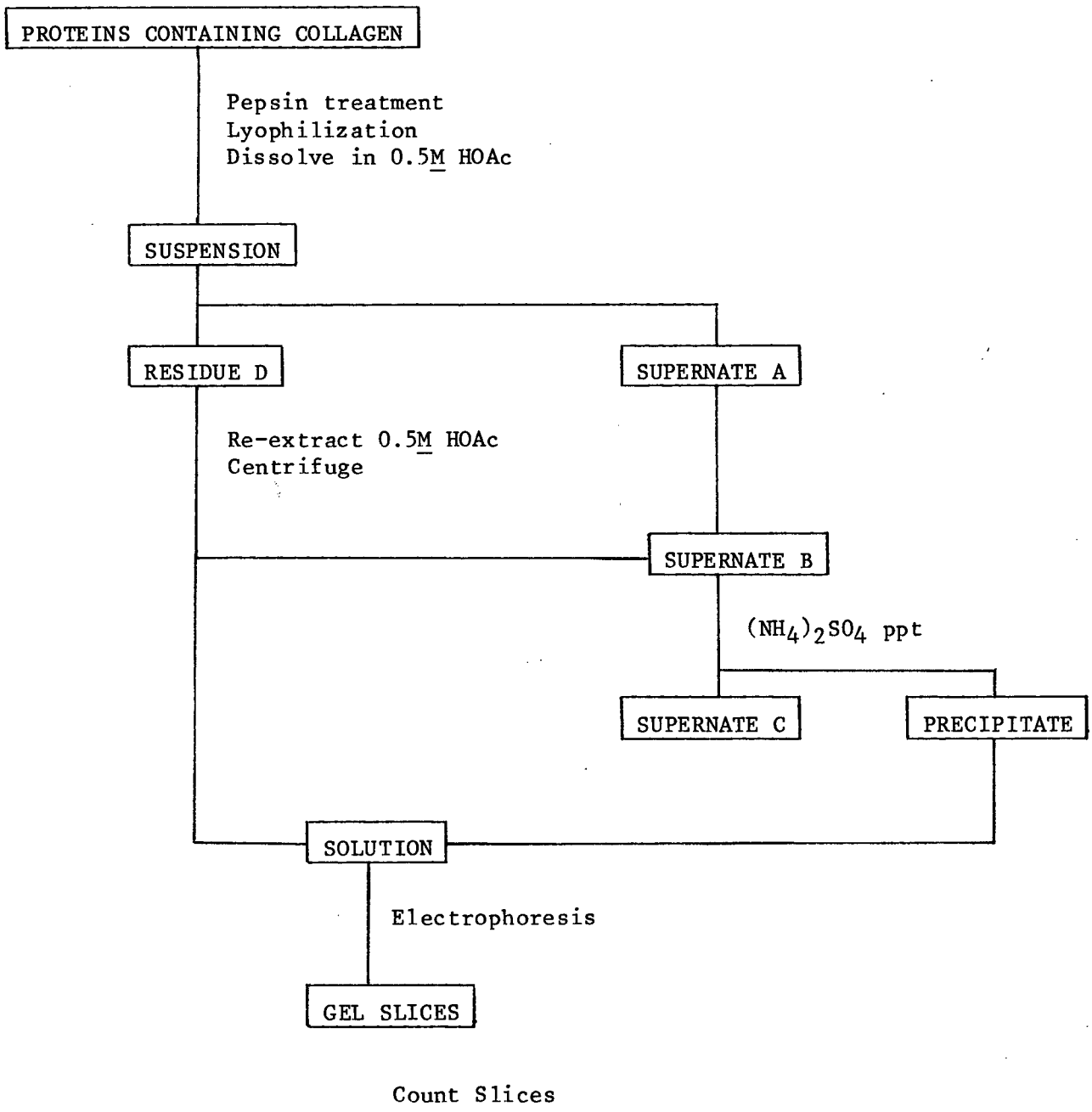
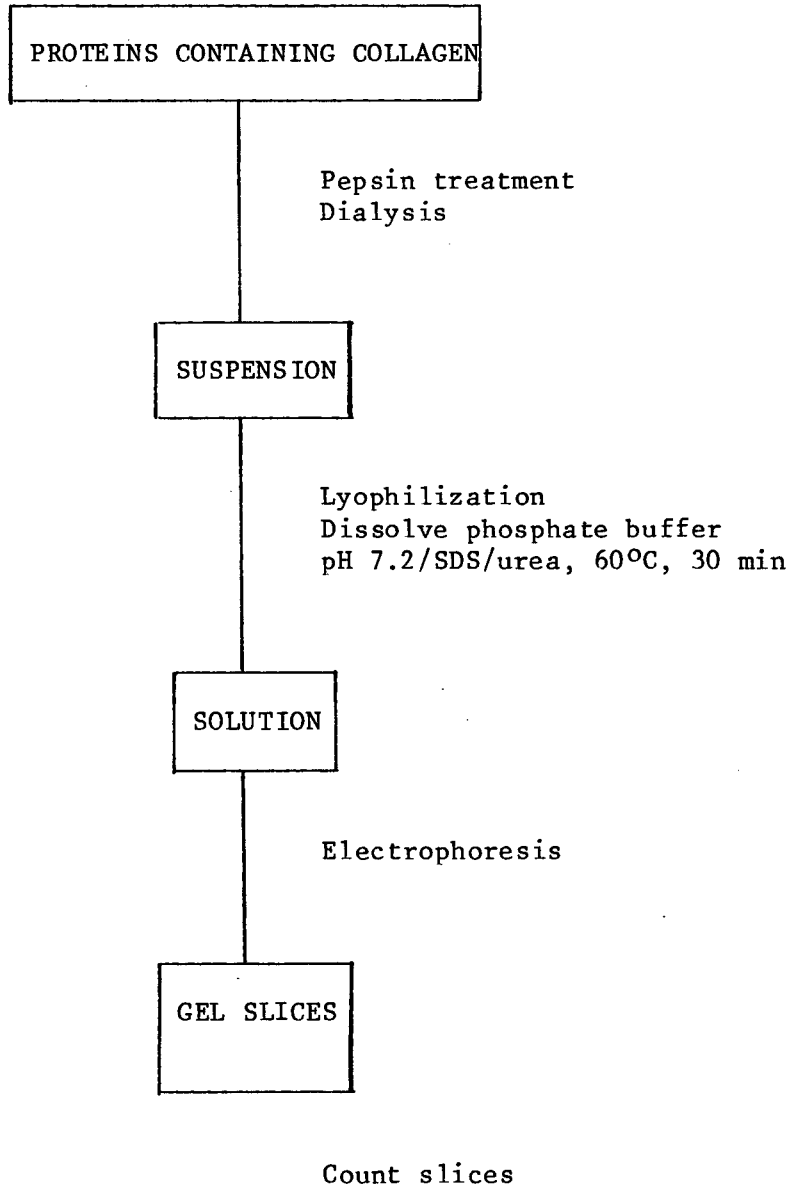


Table VIII. Recovery of radioactivity of pooled cellular proteins from polyacrylamide gel electrophoresis.

Proteins containing collagen were treated with pepsin. The digest was neutralized, dialyzed and lyophilized. The lyophilized product was suspended in 0.5 M acetic acid and the suspension centrifuged to precipitate the insoluble proteins (Residue D). Supernate A was decanted, residue D re-extracted with 0.5 M acetic acid and centrifuged. This supernate was pooled with supernate A. Saturated $(NH_4)_2SO_4$ was added to this pooled supernate B to precipitate collagen which was combined with residue D and dissolved in phosphate buffer to be applied to polyacrylamide gels.

Comparison of Steps	Recoveries (%) from cell layer	
	Control %	Glucocorticoid %
suspension/pepsin-treated collagen fraction	57.3	39.9
(precipitate + residue D)/suspension	94.6	81.1
gel counts/(precipitate + residue D)	81.6	82.3

Figure 10. Treatment of proteins containing collagen in medium and cellular layer for polyacrylamide gel electrophoresis. Procedure 3.



Recoveries of added radioactive counts did not fall below 80% (Table IX). In all samples, solubilization of the products derived from pepsin-treatment was more complete in the phosphate buffer, pH 7.2, containing 0.2% (w/v) sodium dodecyl sulphate and 2 M urea when heated at 60°C for 30 min, than in 0.5 M acetic acid.

IV. Glucocorticoid Effects on Proline Incorporation Into Collagenous Proteins of Medium and Cellular Layer Fractions of Mouse L-929 Fibroblasts: Polyacrylamide Gel Electrophoretic Studies

(a) comparison of peaks of medium fractions of control and triamcinolone acetonide-treated samples

A total of nine control and ten glucocorticoid-treated samples were examined. A representative from each of the control, glucocorticoid-treated and reference samples can be seen in figure 11. Three principal peaks appeared consistently in each experimental sample. The reference collagen (——) consisted of three peaks corresponding to γ, β, and α molecules. These radioactive peaks coincided with those observed visually in stained polyacrylamide gels containing unlabelled reference collagen (Plate 1). The γ molecules represent three α chains in association with one another, the β represent the association of two α chains and lastly, the α are single collagen chains. The γ bands or peaks have the slowest mobility on the polyacrylamide gels because of their high molecular weight. β bands have intermediate mobility and the α bands exhibit the most rapid mobility due to the low molecular weight of single α chains. Peaks obtained from control (---) and triamcinolone acetonide-treated samples corresponded to the γ and α peaks of the reference collagen. The intermediate peak of the control and glucocorticoid-treated

Table IX. Recovery of radioactivity of proteins containing collagen from polyacrylamide gels.

Collagenous proteins from the medium and cellular layer were treated with pepsin, neutralized and dialyzed against 0.4 M NaCl containing 0.1 M Tris. HCl, pH 7.6. The retentate was lyophilized and dissolved in 0.01 M phosphate buffer, pH 7.2, containing 0.2% (w/v) sodium dodecyl sulphate and 2 M urea by heating at 60°C for 30 min. The sample was applied to polyacrylamide gels.

Step	Medium % Control	Medium % Glucocorticoid	Cellular Layer % Control	Cellular Layer % Glucocorticoid
Solution/suspension	89.9	90.0	89.9	90.1
	-	-	97.5	-
Gel counts/solution	85.0	91.1	95.6	86.6
	-	-	92.8	89.9
Gel counts/suspension	81.9	82.0	85.0	78.0
	84.3	94.8	90.5	81.9

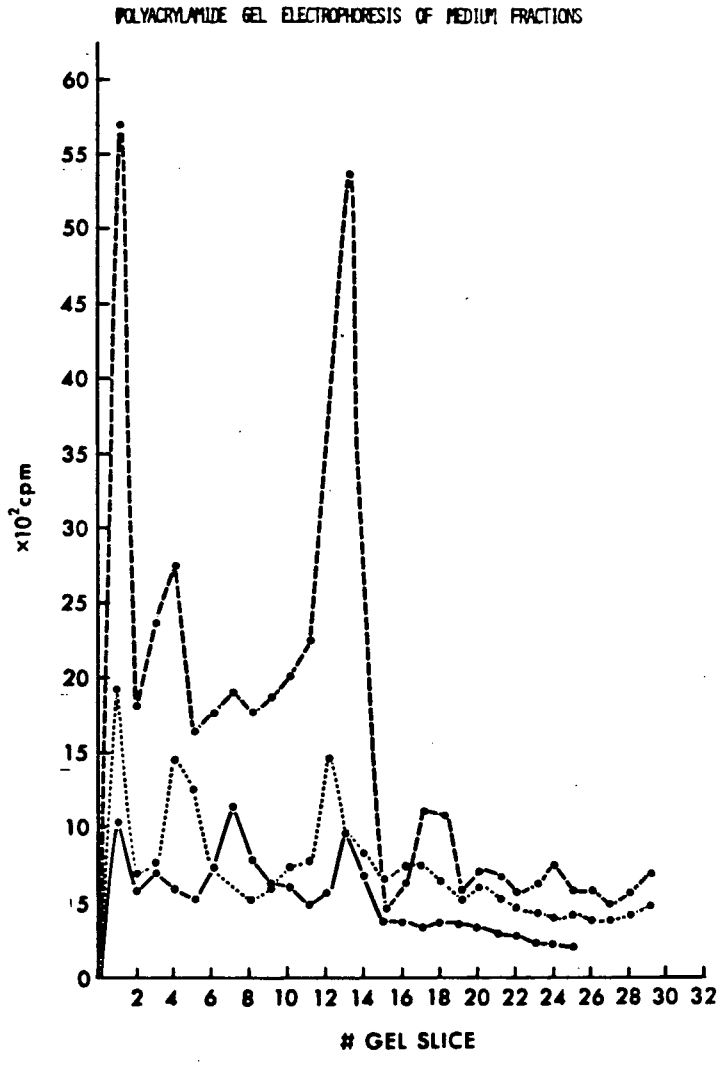


Figure 11. Polyacrylamide gel electrophoresis of proteins containing collagen of the medium fractions

Pepsinized products in phosphate buffer containing sodium dodecyl sulphate and urea were applied to polyacrylamide gels and subjected to electrophoresis. The gels were slices, digested and assayed for radioactivity. Typical purified reference collagen (——), control (---), and triamcinolone acetonide-treated (...) samples are shown.

samples was less mobile than the β peak of the reference sample. Nevertheless, the peaks of the experimental samples suggest the presence of collagen. Smaller extraneous peaks of mobility greater than the α peaks were also observed; none corresponded to any known collagen bands. Most likely, the peaks were due to pepsin-resistant non-collagenous proteins or, less likely, to degradation products of collagen (Fig. 11).

(b) comparison of peaks of cellular fractions of control and triamcinolone acetonide-treated samples

The three principal peaks observed previously (Fig. 11) were less or not distinctive in the cellular fractions of four control and four triamcinolone acetonide-treated samples. Indeed, only one control sample had a distinctive peak corresponding to the α band of the reference methylated collagen (——). None of the glucocorticoid-treated samples had such a peak. All samples had a peak corresponding to the γ band of the reference collagen. The presence of numerous peaks of mobility greater than the α molecules were indicative of pepsin-resistant proteins in the control samples (---). Such proteins were, apparently, absent in the triamcinolone acetonide-treated samples (....). Indeed, other than the peak corresponding to the γ band, no other peaks were observed in those pepsin-treated samples exposed to the glucocorticoid (Fig. 12).

(c) comparison of peaks of medium and cellular fractions

The three peaks resembling the γ, β, and α molecules of collagen were definitely more prominent in the medium suggesting that there are more collagenous proteins in the medium than in the cellular layer. There appeared to be more pepsin-resistant proteins in the cellular layer than in the medium as indicated by the numerous peaks associated with the control cellular sample (Fig. 11 and 12).

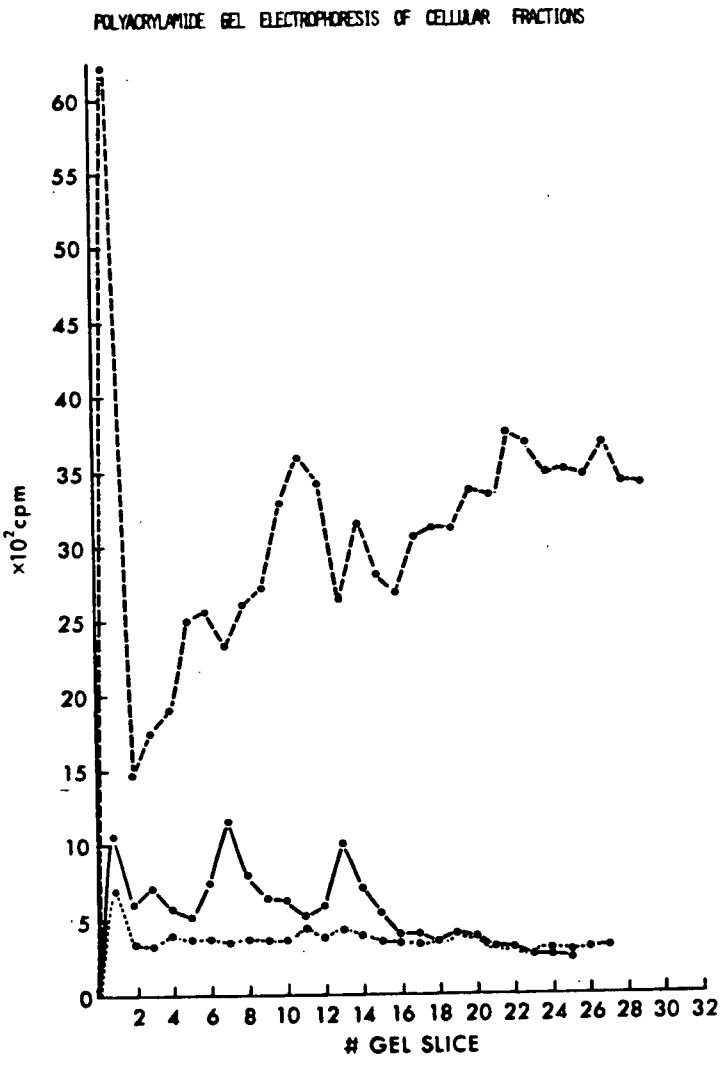


Figure 12. Polyacrylamide gel electrophoresis of proteins containing collagen of the cellular fractions

Pepsinized products in phosphate buffer containing sodium dodecyl sulphate and urea were applied to polyacrylamide gels and subjected to electrophoresis. The gels were sliced, digested and assayed for radioactivity. Typical purified reference collagen (——), control (---) and triamcinolone acetonide-treated (...) samples are shown.

DISCUSSION

In an effort to relate dermal atrophy to a loss of collagen, many studies have been performed on glucocorticoid effects on collagen metabolism in a variety of cell lines, including human dermal fibroblasts but not mouse L-929 fibroblasts, depression of collagen synthesis. The results obtained thus far have been contradictory, in part, because of the great number of variable factors, such as duration, frequency, type and dosage of glucocorticoid treatment, that bear investigation. Other variables include type of cell line, the site from which the tissue was biopsied, age and sex of donor, the passage number and stage of growth of fibroblasts. Often, the results have been misinterpreted, causing greater confusion. For instance, the measurement of hydroxyproline has often been used as an index of collagen synthesis (Saarni and Tammi, 1978; Ponec et al, 1977b). However, hydroxylation is but one of several steps in the biosynthesis of collagen and, indeed, hydroxyproline measurements reflect simultaneously the incorporation and hydroxylation of proline residues in the peptide chains.

Triamcinolone acetonide, a potent anti-inflammatory glucocorticoid, at a low pharmacological dosage of 0.1 µg per ml, has been found to affect cellular proliferation in adult human dermal and mouse L-929 fibroblasts (Runikis et al., 1978). This glucocorticoid has also been observed clinically to cause dermal atrophy (Snyder and Greenberg, 1977, 1974).

The objectives of this study were to determine the effect of 0.1 µg per ml of triamcinolone acetonide on the collagen synthesis of mouse L-929 fibroblasts. These cells respond to glucocorticoids. Their cellular proliferation is depressed by a variety of glucocorticoids at different

concentrations. The degree of inhibition was correlated with anti-inflammatory potencies in a parallel-line bioassay developed by Berliner and Ruhmann (1967) and Brotherton (1971). Since collagen synthesis in these cells treated with glucocorticoids had not been examined previously, it was of interest to us to perform this study. Secondly, due to the obvious relevance to the clinical findings of dermal atrophy, human dermal fibroblasts were included in the study. The cells employed were derived from an adult individual. Except for one study (Ponec et al., 1977b), all other human studies have used fibroblasts derived either from neonatal or foetal humans. Measurement and identification of collagen synthesis were accomplished by bacterial collagenase assays and polyacrylamide gel electrophoresis in our study.

Only the cellular layers of mouse L-929 fibroblasts were affected by the addition of 0.1 µg triamcinolone acetonide per ml of medium. Cell numbers decreased and the incorporation of proline into collagenase-sensitive protein per cell increased. The former observation supports that of Runikis et al. (1978). The accumulation of radioactivity within the cells might be indicative of either underhydroxylated or abnormally structured collagen; either would arrest triple helix formation. Hence, the secretion of collagen may be inhibited. Indeed, such has been observed in scurvy (Klein, 1976). Underhydroxylation of collagen in the presence of glucocorticoids was observed in chick embryo tibiae treated with high doses of hydrocortisone, hydrocortisone acetate, hydrocortisone phosphoric acid complex or hydrocortisone sodium succinate (Blumenkrantz and Asboe-Hansen, 1976). The observation, suggesting inhibition of the enzyme, prolyl hydroxylase, was supported by Counts et al. (1979). They observed decreases in cellular prolyl hydroxylase activity and in the amount of hydroxyproline in dermal fibroblasts in cell

culture derived from triamcinolone diacetate-treated rats. Earlier work (Cutroneo and Counts, 1975; Newman and Cutroneo, 1978) had shown that the decrease in prolyl hydroxylase activity was achieved only after multiple glucocorticoid treatments. Prolyl hydroxylase activity had been shown to be inhibited in rats treated with triamcinolone, hydrocortisone or methylprednisolone (Cutroneo et al., 1971), thus confirming the effect of glucocorticoids on this enzyme. Kruse et al. (1978) found that, although prolyl hydroxylase activity was reduced in sponge granulomas from dexamethasone-treated mice and in hydrocortisone-treated fibroblast cultures, protein synthesis was inhibited to the same degree. Hence, there was no effect on prolyl hydroxylation per se. Similar results were obtained from carrageenan granuloma of betamethasone disodium phosphate-treated rats (Nakagawa et al., 1971). Thus, the effect of glucocorticoids on prolyl hydroxylation remains unclear and may be dependent on the type and concentration of the glucocorticoid, period of incubation with the glucocorticoid, and the type and origin of the cells.

Accumulation of collagen need not arise only from underhydroxylation but could be a consequence of an aberration in the molecular structure which would arrest the triple helix formation. Azetidine-2-carboxylic acid, when incorporated into collagen, prevents the formation of the normal helical conformation. Secretion of collagen is depressed. Concomitant accumulation of collagen occurs (Bienkowski, 1978b).

The proteins of the cell layers of the mouse L-929 cells were further characterized in polyacrylamide gel electrophoresis because of the consistent increase in the radioactivity within the cells following glucocorticoid treatment. Also, the consistent low losses of radioactivity in the control and

experimental groups following collagenase digestion suggested the presence of large amounts of collagenase-resistant proteins which were also characterized in the gels.

Radioactive peaks of pepsin-treated proteins were compared with those of reference methylated collagen and the bands of purified collagen visualized in stained gels. Three principal peaks or bands of different mobilities were observed. The fastest migrating band or peak represented the single α chains which had the lowest molecular weight of the three peaks, the intermediate bands represented the β chains and the slowest migrating were the γ chains which have the largest molecular weight. These peaks were prominent in the medium from control and triamcinolone acetonide-treated samples. The collagen was relatively free of other proteins, supporting the findings of the previous experiment; high loss of radioactive counts was obtained after collagenase digestion. No differences were apparent in the control and glucocorticoid-treated samples.

Although, in the cell layer fractions of control samples, peaks corresponding to the β and α bands were present they were less distinct than those in the medium samples. Such peaks were completely absent in glucocorticoid-treated samples. Peaks corresponding to the γ bands appeared in all samples. Other peaks, indicative of pepsin-resistant proteins, were prominent in the control but absent in the triamcinolone acetonide-treated fractions. The presence of large amounts of non-collagenous pepsin-resistant proteins in the cell layers confirmed the data in the previous experiments; low removal of radioactivity was encountered after bacterial collagenase in both control and glucocorticoid treated samples of the cell layers.

Interpretations of the data obtained from the collagenase assay and electrophoretic studies are as follows: If the collagen which accumulated in the cell layer after glucocorticoid treatment was abnormally structured, its aberration might have allowed the pepsin to destroy it completely. The predominant effect of pepsin on normally structured collagen is the cleavage of the telopeptides at both ends of the amino and carboxy termini (Miller, 1972). Since the effect of glucocorticoids on collagen metabolism has not been examined in polyacrylamide gels before, comparisons with other work cannot be made.

Common to all control cell layer samples were numerous peaks, indicative of non-collagenous protein, that were absent in the glucocorticoid-treated samples after pepsin treatment. These observations further suggest that the intracellular proteins of triamcinolone acetonide-treated samples are more susceptible to pepsin digestion. Whether this is due to aberration in molecular structure is yet unconfirmed.

The possibility that the peak corresponding to the γ molecule may have represented most or all of the collagen present was considered; exposure of these molecules to bacterial collagenase would have identified the protein. However, the sample applied to the gel could have included other large macromolecules incapable of penetrating the gel and, therefore, would have been included in the γ peak.

Other types of experiments would have to be done to further characterize the collagen. For instance, the contents of hydroxyproline and proline within the cells should be examined. Unfortunately, many workers use hydroxyproline alone as an index of collagen synthesis. But the synthesis of collagen includes biochemical processes other than hydroxylation, for instance, amino

acid uptake into the cell, the size of the amino acid pool, incorporation of amino acids into the collagen molecule, post-translational modifications such as glycosylation, processing of the molecules into procollagen, and extrusion of collagen into the extracellular milieu. Any of these might be affected by the drug with effects apparent by one method and not by another.

Glucocorticoids have been reported to enhance cellular proliferation in logarithmically growing cultures of human dermal fibroblasts (Runikis et al., 1978; Kirk and Mittwoch, 1977) and inhibit that in confluent cultures (Ponec et al., 1977a,b; Priestley, 1978). One must question the relevance of employing human foetal fibroblasts (Priestley, 1978; Kirk and Mittwoch, 1977) whose metabolic activities differ from those of adult human fibroblasts. The use of human fibroblasts of late passages (15 to 21st) (Ponec et al., 1977a,b) to assess the adverse effects of glucocorticoids on collagen synthesis must also be questioned. Cellular metabolic activities generally decrease with the number of passages.

Using adult human dermal fibroblasts at confluency, our preliminary experiments have shown that triamcinolone acetonide does not have a statistically significant effect on cellular proliferation and incorporation of proline into collagenase-sensitive protein. The incorporation of radioactive proline into cellular collagenase-sensitive protein was suppressed after glucocorticoid treatment, though not significantly. Additional experiments might have clarified the significance of this observation. The incubation period with triamcinolone acetonide may have been too short to show a consistent effect on cellular proliferation. Hence, the glucocorticoid was added for a prolonged period; again no significant effects on cellular proliferation and proline incorporation were observed, possibly, because this

particular cell strain was not very responsive to triamcinolone acetonide. Verification of triamcinolone acetonide effects can be made only if several strains of adult human dermal fibroblasts have been examined.

The lack of a consistent effect of triamcinolone acetonide on proline incorporation into collagenase-sensitive protein in adult human dermal fibroblasts may have been due to the dosage, 0.1 µg per ml of medium. This dosage is exceedingly low when compared to those used by other investigators in their *in vitro* studies. For instance, proline incorporation into collagenase-sensitive protein was suppressed by 10 µg clobetasol propionate or betamethasone valerate per ml in human foetal fibroblasts; the concentration was 100 times greater than that we selected. Priestley (1978) claimed that although the concentrations employed *in vitro* do not necessarily reflect that found in the skin, the foregoing concentration was related to clinical use because about 2.2 µg hydrocortisone per ml was found in the dermis and was increased almost ninety-fold with the removal of stratum corneum before topical application of 1% glucocorticoid; indeed the concentration of the glucocorticoid in the dermis was expected to be increased further in diseased skin. Hence, 10 µg per ml was not unrealistic. Most other workers have also employed concentrations exceeding that used in our experiments (Ponec *et al.*, 1977a,b). Proline incorporation into hydroxyproline was also depressed by 1 or 5 µg hydrocortisone, hydrocortisone-17-butyrate, triamcinolone acetonide, betamethasone-17-valerate or clobetasol-17-propionate (Ponec *et al.*, 1977a,b). Hence, 0.1 µg triamcinolone acetonide per ml was probably not sufficiently high to induce changes in proline incorporation into collagen.

A survey of studies reported to date does not permit generalization of the effects of glucocorticoid on fibroblasts in tissue culture since these effects

were many, varied and often inconsistent. Too many variables, for instance, the type, passage number, origin, and growth phase of the cell, the age and sex of the donor, the type and concentration of glucocorticoid, the length of the incubation period, the frequency of glucocorticoid treatment and system used - in vivo, in vitro, organ or cell cultures - require systematic evaluation to elucidate the adverse effects of glucocorticoids. Recent personal communication with Booth and Uitto (1979) has failed to shed any light on the glucocorticoid effects on collagen synthesis in adult human dermal fibroblasts in cell culture. Indeed, there were no consistent effects despite numerous experiments which were designed and employed to examine many of the foregoing variables encountered in cell culture systems. One cannot discount the possibility that glucocorticoids may not affect collagen synthesis but may perhaps alter the degradative aspects of collagen metabolism. Therefore, all biochemical steps in collagen metabolism and other cellular metabolic activities as well as the overall physiological responses to glucocorticoids have to be examined separately and in concert if the adverse effects of glucocorticoids are to be understood completely.

SUMMARY AND CONCLUSION

Triamcinolone acetonide, at a concentration of 0.1 µg per ml had the following effects on mouse L-929 fibroblasts and a strain of adult human dermal fibroblasts:

1. Cellular proliferation of confluent mouse L-929 fibroblasts was consistently depressed.
2. Incorporation of proline into total protein and collagenase-sensitive material in the medium remained unaltered.
3. However, the incorporation of proline into cellular protein and collagenase-sensitive material was enhanced consistently and significantly ($0.01 > P > 0.001$ and $P = 0.004$ respectively).
4. Characterization of the collagenase-sensitive material was accomplished by polyacrylamide gel electrophoresis. Such material of the medium fraction had peaks whose mobilities coincided with those of purified collagen, therefore identifying the collagenase-sensitive protein as collagen. Collagenase-sensitive cellular material treated with pepsin displayed only one peak in polyacrylamide gels. Indeed, other proteins were not apparent in gels following pepsin treatment.
5. At the logarithmic and confluent phases of growth, cellular proliferation of adult human dermal fibroblasts exposed to the glucocorticoid was not altered consistently.
6. Incorporation of proline into collagenase-sensitive material in the medium and cellular fractions of human fibroblasts was affected inconsistently.
7. To conclude, 0.1 µg per ml triamcinolone acetonide does affect the metabolic activities of mouse L-929 fibroblasts. Both cellular

proliferation and collagen synthesis in the cell layer are altered by this concentration. Total protein and collagenase-sensitive protein appear to be degraded by pepsin according to polyacrylamide gels, indicating perhaps that the collagenase-sensitive protein may be underhydroxylated or abnormal in structure.

8. The line of adult human dermal fibroblasts used in this study does not appear to respond to the single dose of triamcinolone acetonide. This could be due to the low concentration of the glucocorticoid or perhaps this particular line is not responsive to glucocorticoids.

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