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A Comparative *In Vitro* Study of Growth and Metabolic Parameters of Fibroblasts from Human and Control and Streptozotocin Diabetic Rat Gingivae

by

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For my parents, Sobharam and Monica Singh.

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ABSTRACT

One of the most common diseases of man is periodontal disease. Diabetes mellitus, like periodontal disease, is also widespread. Diabetics are reported to be more susceptible to periodontal destruction and to experience impaired rates of oral healing. In addition, diabetics with periodontal disease appear to suffer from an increased severity of the disease and display inflammatory responses typical of severe periodontitis. Furthermore, both diabetes mellitus and periodontal disease involve alterations in collagen and connective tissue metabolism. Based on these clinical observations, this thesis describes an *in vitro* study of cellular metabolic responses, e.g. DNA, RNA, non-collagenous and collagenous protein production and of membrane transport capacity of human and rat (control and streptozotocin diabetic) gingival fibroblasts. The effects of varying insulin, ascorbate and serum concentrations were determined on fibroblasts isolated from the attached gingivae of rats and humans, grown and maintained in alpha MEM + 10% FCS. The effects of senescence on these cells are also reported. Since commercial insulin preparations contain bound zinc, rat gingival fibroblasts only (control and streptozotocin diabetic) were also grown in the presence of zinc chloride to assess the effects of the metal on *in vitro* fibroblast growth. The *in vitro* effects of streptozotocin on control rat gingival fibroblast growth and metabolism were also assessed. In addition, testosterone was also assessed as a gingival fibroblast growth factor in a preliminary experiment. With

the exception of the senescence studies, all experiments were done on cells in the 5th or 6th passages. It was found that:

1). Replicative rates of diabetic rat gingival fibroblasts were lower than those of control rat gingival fibroblasts. This finding was consistent in all experiments involving these two cell groups. Replicative rates and metabolic parameters of cells from diabetic rats treated in vivo with insulin prior to gingival excision and fibroblast isolation were similar to those of control rat cells.

2). Senescence induced changes in replication and metabolism of human and control and diabetic rat gingival fibroblasts do occur in vitro. Maximal replication of rat and maximal metabolism of both human and rat cells occurred in early passages (4-6) and declined with increasing passage number. Maximal replicative rates of human cells were distributed over a wider range of passages (2-8). Very old (24th passage) cells showed increases in DNA, RNA and protein content. The responses of both human and rat cells to insulin were variable. Diabetic cells were most sensitive to aging as indicated by their lifespan, which was less than half that of control rat cells.

3). Serum inhibited the stimulatory effect of insulin on control and diabetic rat gingival fibroblast metabolism. Maximal RNA and protein synthesis and the membrane transport of ^{3}H-2-deoxyglucose and ^{14}C-alpha-amino isobutyric acid all occurred at the lowest serum and the highest insulin concentrations studied (5% serum and 10^{-6} M insulin),

but declined as serum levels increased.

4). Human gingival fibroblasts responded differently to the addition of control and diabetic rat serum to the growth medium at levels of 5, 10 or 15%. Whereas, the control serum stimulated cellular replication in direct proportion to serum concentration, the diabetic serum was toxic to cell growth. Diabetic serum, however, produced slightly higher levels of cellular RNA and significantly more protein than cells grown in control serum.

5). Ascorbate uptake by control and diabetic rat gingival fibroblasts was not significantly different. Insulin treatment (10^{-6} M), however, significantly increased ascorbate uptake by the control cells, but was without effect on the ascorbate content of diabetic rat cells.

6). The DNA, protein and ascorbate content of human gingival fibroblasts were directly proportional to the ascorbate concentration of the growth medium. RNA levels were unaffected by changes in ascorbate concentration. At each ascorbate level assessed, insulin (10^{-6} M) further increased significantly all cellular parameters in direct proportion to the ascorbate concentration.

7). Control and diabetic rat gingival fibroblasts responded differently to cell density and its interaction with medium ascorbate and insulin. In general, cellular growth and metabolism were more sensitive to cell density changes than to the types of treatment. Maximal control and diabetic rat cell DNA and RNA levels occurred at the lowest and sec-

ond lowest densities (7.5×10^4 and 1.5×10^5 cells/dish) used respectively and declined as cell density was increased. Non-collagenous protein levels of the diabetic rat cells were directly proportional to cell density while levels in the control rat cells were either directly proportional to or unaffected by cell density. Control and diabetic cellular hydroxyproline accumulation was inversely proportional to cell density. Medium hydroxyproline levels were sensitive to both density and the type of treatment. The ascorbate content of control and diabetic rat cells was stimulated by both insulin and ascorbate.

8). Streptozotocin added in vitro to the growth medium of control rat gingival fibroblasts increased hydroxyproline accumulation in direct proportion to medium streptozotocin concentration. Cellular DNA, RNA and non-collagenous protein levels were unchanged.

9). Insulin bound zinc inhibited the stimulatory effect of insulin on the cellular growth rates of control and diabetic rat gingival fibroblasts, but was without similar effect on fibroblast metabolism. Two insulin preparations containing 0.2 and 0.5% bound zinc respectively were used at concentrations of 0, 10^{-8} and 10^{-6} M. Maximal control and diabetic rat cell replication occurred at 10^{-6} M containing 0.2% bound zinc. Maximal RNA and protein levels of control and diabetic cells occurred at 10^{-8} M insulin (0.2% bound zinc) and 10^{-8} M insulin (0.5% bound zinc) respectively. Early in the growth period (day 4), medium hydrox-

yproline levels were maximal at 10^{-8} M insulin (0.5% bound zinc) and more pronounced for diabetic cells. Thereafter, hydroxyproline levels were either inhibited or unchanged by insulin.

10). Zinc chloride in the growth medium was inhibitory to replication of control and diabetic rat gingival fibroblasts but without effect on RNA and non-collagenous protein synthesis. Medium hydroxyproline levels for both cell types were stimulated by both zinc and insulin although insulin produced the greater effect. Zinc chloride stimulation of hydroxyproline levels was more pronounced in the diabetic than control rat cells.

11). Testosterone in levels of 50, 100 and 150 ng/ml was without effect on the replication of control and the metabolism of control and diabetic rat gingival fibroblasts. DNA content of diabetic cells was equally increased by all concentrations of testosterone. Insulin alone increased DNA accumulation in both control and diabetic cells. Insulin and testosterone combined produced no further changes in any cellular parameters of both cell types.

The data indicate that healthy human and control rat gingival fibroblasts behave differently from diabetic rat cells. They describe and clarify the effects of insulin, ascorbate and serum concentrations on fibroblast growth and metabolism and support the use of gingival fibroblasts in tissue culture as a suitable model system for the study of connective tissue metabolism in both health and disease.

CHAPTER I

INTRODUCTION

INTRODUCTION

1-1 THE PERIODONTIUM-NORMAL TISSUE COMPOSITION

The periodontium is made up of the gingival epithelium and both soft and calcified connective tissue. The calcified connective tissue consists of the alveolar bone which houses the roots of the teeth and the cementum which covers the root surfaces and the periodontal ligaments (Schluger et al., 1977). The soft connective tissues of the periodontium are composed of collagens, non-collagenous glycoproteins, glycosaminoglycans and small quantities of elastin. Chemical, biochemical, histologic, morphologic and electron microscopic studies of the connective tissues around the teeth have contributed greatly to our knowledge of normal connective tissue formation and morphology as well as to the understanding of wound healing and pathologic tissue alterations. These tissues have served as a general model for connective tissues. The periodontium is affected by inflammatory, degenerative and proliferative diseases which produce changes in the quality and quantity of connective tissue constituents (Narayanan and Page, 1983). Gingival abnormalities are visible to the naked eye and methods to detect the presence of these diseases and their severity have been developed.

Connective tissue consists of a variety of cells and an organic matrix which is comprised of the secretory products of these cells. The main cell type of the gingival connective tissue and the periodontal ligament is the fibroblast which makes up

about 5.6% of the total connective tissue volume (Schroeder et al., 1973). It is the main collagen producing cell in the gingiva and is responsible for the maintenance of the connective tissue matrix (Narayanan and Page, 1983). Fibroblast functions are regulated by a variety of environmental ligands, some found in blood platelets and plasma and others produced and secreted by infiltrating leukocytes (Narayanan and Page, 1983). Fibroblasts can be easily grown in vitro as they remain diploid and grow and synthesize in a highly reproducible manner for more than 20 transfers (Narayanan and Page, 1983). In general, gingival fibroblasts behave like those derived from skin (Narayanan and Page, 1976), and show morphologic features similar to those in vivo (Engel et al., 1978). Gingival fibroblasts in vitro produce types I and III collagen and trace amounts of type V in approximately the same ratios as those found in normal gingival tissue (Narayanan et al., 1978, 1982). However, there appears to be functionally distinct subpopulations of human gingival fibroblasts (Engel et al., 1980; Hassell and Stanek, 1983), since some fibroblasts produce both types I and III collagen while others produce only type I (Engel et al., 1980).

Finally the similarities in growth and cellular metabolism between fibroblasts in vitro and those in situ have facilitated the in vitro study of biosynthetic properties and regulatory mechanisms (Kivirikko and Savolainen, 1981).

1-2a TISSUE DISTRIBUTION OF COLLAGEN AND ITS FUNCTIONS

Collagen is by far the most prevalent vertebrate protein. It represents about half of the total body protein in completely developed adult organisms. There are at least 6 known types of collagen in the body. The relative amounts of each type of collagen are tissue specific and it appears that almost all tissues contain several collagens, although in different proportions. Skin is mainly composed of type I collagen (Minor, 1980; Nimni, 1980). Type I trimer is found in skin, cartilage, aged chondrocytes and fibroblasts grown *in vitro* (Minor, 1980). Type II collagen is identified in cartilaginous structures (Minor, 1980; Bornstein and Sage, 1980; Nimni, 1980). The proportions of collagen types in a particular tissue change with time. For example, Type III collagen constitutes greater than 60% of foetal skin collagen while it represents less than 20% of adult skin collagen (Epstein, 1974). Consequently, type III has been falsely labelled "foetal collagen". This label is misleading since type III collagen is found in significant amounts in blood vessels and skin (Miller *et al.*, 1971) and in healing wounds regardless of subject age (Miller, 1976). Type IV is a basement membrane collagen (Nimni, 1980; Bornstein and Sage, 1980). Although type V collagen is assumed to be an interstitial collagen, it may be a part of the collagen of basement membranes in placenta, lung and muscle tissue (Burgeson *et al.*, 1976; Madri and Furthmayr, 1979).

Although various collagens in solid or fibrous form are primarily supporting structures in connective tissue, these proteins

also serve in a non-structural capacity which is ultimately derived from structural interactions. For example, collagen in conjunction with fibronectin and other matrix proteins is involved in the attachment of cells to a substratum, and in platelet aggregation (Bornstein and Sage, 1980). Collagenous surfaces, especially types I and III which are extensively co-distributed with fibronectin on fibroblast surfaces, are a major substratum for *in vivo* cell adhesion (Vaheri *et al.*, 1978). This interaction of the proteins is essential to the formation of an "external protein meshwork" at the cell surface that mediates the interaction of cells with the pericellular matrix (Bornstein *et al.*, 1978).

1-2b PHYSICAL AND CHEMICAL FEATURES OF THE COLLAGEN MOLECULE

The collagen molecule is a highly symmetric, rigid rod-like structure, approximately 3000 A long and 15 A wide (Gay and Miller, 1978; Nimni, 1980). It is composed of three individual polypeptide chains folded in a rod-like triple helical molecule. Each individual polypeptide chain, called an alpha chain, may be thought of as having three distinct domains. The NH_2 terminal non-helical region represents about 2% of the length of the molecule while the central portion and COOH terminal non-helical region account for the 95% and 3% respectively. The NH_2 and COOH terminal non-helical extensions are functionally important since they contain sites where cross links originate (Gay and Miller, 1978). Each alpha chain is coiled into a left-handed helix with about three amino acids per turn over a distance of 9 A (Gay and

Miller, 1978; Prockop et al., 1979). The three helices are further coiled around each other to form a right-handed helix with a repeat distance of about 100 A (Gay and Miller, 1978; Prockop et al., 1979). This helix is a rigid structure akin to a long thin segment of rope. The alpha chains contain about 1000 amino acid residues and approximately every third amino acid in each chain is glycine (Prockop et al., 1979). Thus, the molecular formula of an alpha chain may be represented as (X-Y-Gly) where X and Y are amino acids other than glycine (Minor, 1980). The triple helical conformation of collagen is also dependent on the presence of proline and hydroxyproline in the alpha chains (Prockop et al., 1979). In mammalian collagen, about 100 X positions are proline and about 100 Y positions are hydroxyproline (Prockop et al., 1979). Both of these amino acids are cyclic and rigid, thus limiting rotation of the polypeptide backbone. Therefore, they contribute to the stability of the collagen triple helix (Prockop et al., 1979; Minor, 1980). Specifically, the hydroxyl group of hydroxyproline is crucial to the stability of the triple helix (Prockop et al., 1979). Hydroxyproline is found almost exclusively in collagen (Prockop et al., 1979; Minor, 1980). The only other known vertebrate proteins containing this amino acid are elastin (Grant and Prockop, 1972) in the C1q subcomponent of the complement system (Porter and Reid, 1978), and acetylcholinesterase (Rosenberry and Richardson, 1977).

1-2c THE BIOSYNTHESIS OF COLLAGEN

Collagen is synthesized as a larger precursor protein called procollagen. The constituent chains of the procollagen molecule, the pro alpha chains are considerably larger than the alpha chains of the collagen molecule due to the presence of linear extension sequences at both the NH_2 and COOH termini of the chains. The intracellular assembly of procollagen involves a series of post-translational modifications. 1). Hydroxylation of selected prolyl and lysyl residues. 2). Glycosylation of some hydroxylysl residues with either galactose alone or galactose and glucose in disaccharide forms. 3). The addition of mannose and other sugars to the peptide extensions of procollagen (Clark and Kefalides, 1976; Olsen et al., 1977). 4). The synthesis of interchain disulphide bonds. Hydroxylation of about 100 prolyl residues in each of the three pro alpha chains of procollagen is necessary for the folding of the chains into the triple helical conformation characteristic of collagen. The triple helical conformation of collagen is stable at 37°C (Prockop et al., 1976).

Following the secretion of newly synthesized procollagen molecules into the extracellular spaces, the conversion of procollagen to collagen occurs. This process involves the cleavage of peptide bonds in all three pro alpha chains at both the NH_2 and COOH terminal regions of the procollagen molecule (Minor, 1980). Removal of the procollagen extension peptides results in the loss of about one-third of the molecular weight of the procollagen molecule, and the formation of collagen molecules. These collagen

molecules spontaneously aggregate into fibrils which are not microscopically distinguishable from mature fibrils found in tissues. The newly formed fibrils, however, lack the necessary tensile strength derived from the formation of covalent bonds (Prockop et al., 1979). Two major types of crosslinks are formed: intramolecular links which join alpha chains of the same molecule and intermolecular links which join collagen molecules (Prockop et al., 1979; Nimni, 1980). The intermolecular crosslinks are physiologically the most important ones (Nimni, 1980). Collagen fibres in the extracellular matrix of connective tissue vary in diameter and distribution (Minor, 1980; Nimni, 1980). These differences in appearance may reflect some basic structural characteristics, as well as the extra fibrillar matrix composition (glycoproteins, proteoglycans, etc.) (Minor, 1980; Nimni, 1980).

Collagen molecules produced by cleavage of procollagen, spontaneously aggregate into fibrils (Prockop et al., 1979). Specific lysine and hydroxylysine residues are oxidatively deaminated by the enzyme lysyloxidase to produce peptide bound aldehydes (Prockop et al., 1979; Nimni, 1980; Minor, 1980). The formation of intramolecular crosslinks between alpha chains of the same molecule results from an aldol condensation of two of the aldehydes (Prockop et al., 1979; Nimni, 1980). Intermolecular crosslinks are formed between peptide bound aldehydes of one collagen molecule and NH_2 groups of lysine and hydroxylysine residues on another collagen molecule (Nimni, 1980).

1-3 THE SIGNIFICANCE OF ASCORBIC ACID IN COLLAGEN METABOLISM

The involvement of ascorbate in connective tissue synthesis and metabolism is well documented. Ascorbic acid is essential for normal collagen formation (Chatterjee, 1978), by virtue of the fact that it is an essential cofactor in the hydroxylation of proline and lysine residues in collagen (Levene et al., 1972; Levene and Bates, 1975; Murad et al., 1981). The importance of proline hydroxylation is evident since it serves to stabilize the collagen triple helix (Ramachandran and Ramakrishnan, 1976) and its absence results in structurally unstable collagen (Berg and Prockop, 1973; Jimenez et al., 1973) which is not secreted from cells at a normal rate (Prockop et al., 1976). The hydroxylation of lysine to hydroxylysine is necessary both for the formation of intermolecular crosslinks in collagen, in addition to providing the molecular sites necessary for glycosylation. The importance of ascorbic acid in the crosslinking of collagen via hydroxylysine residues has been demonstrated by Levene et al. (1972) who found that ascorbic acid deficiency resulted in a decreased number of intermolecular crosslinks as determined by an increase in the salt solubility of the collagen. The present state of knowledge suggests that an increased salt solubility of collagen reflects a decrease in the number of crosslinks in the collagen structure. Bates et al. (1972) suggested that lysine hydroxylation was not as sensitive to ascorbic acid deprivation as was proline hydroxylation.

Mann and Newton (1975) reported that glucose inhibited the

uptake of ascorbate due to competition for the transport system. Glucose appears to have a higher affinity for the insulin receptor-site complex than ascorbate. The potential for impaired cellular ascorbate uptake in hyperglycemic states, not only produces an intracellular ascorbate deficiency, but may also contribute to the cellular pathology of diabetes mellitus. Verlangieri and Sestito (1981) reported that increasing insulin concentrations resulted in an increased uptake of radioactive ascorbate into foetal bovine heart endothelial cells. Maximum ascorbate uptake occurred at 4×10^{-4} U/ml and began levelling off thereafter. Further increases in insulin concentrations to as high as 4×10^{-3} U/ml produced no significant increase in ascorbate uptake. These results suggest a saturation of the insulin transport mechanism at or above 4×10^{-4} U/ml insulin under the experimental conditions of Verlangieri and Sestito (1981). Bigley et al. (1983), studying human neutrophils and fibroblasts and competition for transport mechanisms, confirmed that the transport of ascorbate was mediated by glucose transport mechanisms. Zebrowski and Bhatnagar (1979) have reported reduced serum and tissue levels of ascorbate due to enhanced urinary excretion in diabetic rats. Tissue and serum levels of ascorbate were restored and diabetic levels returned to the normal range in these animals following insulin therapy. Thus, it appears that in diabetes, ascorbate transport may be affected not only in the connective tissue matrix, but also in the kidney.

More recently, Liotti et al. (1983) reported that ascorbate in low doses (10 μg/ml) stimulated replication of human skin

fibroblasts in culture. Higher doses (30-150 µg/ml) produced the opposite effect. Ascorbate levels are elevated in tissues of high metabolic activity (Switzer and Summer, 1972). The metabolic turnover of gingiva is one of the highest of all connective tissues (Page and Ammons, 1974; Sodek et al., 1977). Consequently, gingival ascorbate levels may be expected to be significant compared to the content in other collagen producing tissues. The sensitivity of gingival collagen metabolism in the monkey to the ascorbate status of the animal has been reported by Ostergaard and Loe (1975). These workers found that collagen formed in the absence of ascorbate was collagenase degradable and low in hydroxyproline content.

The most widely used method for measuring collagen synthesis has been to measure the formation of hydroxyproline, which is found almost exclusively in collagen. However, it is apparent that this method would be inaccurate in situations where any of the required cofactors of proline hydroxylase or the actual enzyme, was rate limiting for the hydroxylation reaction. One example of a situation where this limitation might occur is during the growth of cultured fibroblasts which lack the ability to synthesize ascorbate (Priest and Bublitz, 1967). Ascorbate synthesis occurs mainly in the liver and kidney of ascorbate producing animals (Chatterjee et al., 1961), although some animals lack this ability. These are the bulbul bird, the Indian fruit bat, the guinea pig, man and the monkey (Chatterjee et al., 1961; Chatterjee, 1978). Cultured fibroblasts represent a convenient system for the study of the regulation of collagen synthesis. For

example, Chen and Postlethwait (1970), studying guinea pig tissue on scorbutic and ascorbate supplemented treatments, suggested that collagen was the only protein effected by the addition of ascorbate to the growth medium. This conclusion was based on the observation that ascorbate effected only the appearance of hydroxyproline and not the total proline incorporated into collagen. In a later study of human periodontal tissue, Aurer-Kozelj et al. (1982) reported an increase in the biosynthetic activity of collagen-producing fibroblasts, possibly due to ascorbate treatment. Based on electron microscopy data, these workers identified a greater number of excreted single collagen bundles in the vicinity of the fibroblasts. Ascorbate is thought to act on collagen at the site of proline hydroxylation. Blank and Peterkofsky (1975), although agreeing with the accepted site of ascorbate action, suggest that in chick embryo fibroblasts, ascorbate did not increase the activity of prolyl hydroxylase. Kao et al. (1977) reported that the addition of ascorbate to the incubation medium of cultured chick embryo tendon cells increased the synthesis of procollagen hydroxyproline without activation of prolyl hydroxylase. Therefore, it appears that the influence of ascorbate on hydroxyproline synthesis is ascribed to a cofactor effect on the hydroxylation reaction.

There appears to be some controversy surrounding the use of ascorbate <u>in vitro</u> to study collagen biosynthesis and metabolism. Peterkofsky (1972) has reported that ascorbate added to the culture medium, was extremely unstable. When monitored at a concentration of 0.25 mM in MEM and incubated at 37°C under 5% CO_2 and

95% air, only 2% of total ascorbate remained after 24 hours. In the presence of serum, the destruction of ascorbate was somewhat delayed and after 24 hours, 11-12% of the vitamin remained. Peterkofsky (1972) has suggested that the rapid disappearance of ascorbate in the growth medium may lead to anomalous results in studies where hydroxyproline is measured as an index of collagen production. Indeed, to compensate for this instability of ascorbate, several workers (Peterkofsky, 1972; Rowe et al., 1977-2; Faris et al., 1978; Liotti et al., 1983) have prepared ascorbate solutions immediately before use at daily medium change.

The physiological range of ascorbate in human serum is 20-60 μM (Rowe et al., 1977-2). To compensate for the loss of ascorbate in the growth medium, one might supplement the medium with super physiological levels of ascorbate. However, it would appear that the stimulatory effect of ascorbate is lost when physiological levels are exceeded. For example, Peterkofsky and Prather (1977) reported that 0.28 mM ascorbate in the growth medium inhibited the growth of chick embryo fibroblasts seeded at low density (2×10^5 cells/dish). Specifically, peroxides produced from the intracellular degradation of ascorbate caused the detachment and breaking up of cells. Levene and Bates (1975) reported that 0.28 mM ascorbate inhibited the plating efficiency of 3T6 fibroblasts seeded at low density (2.5×10^4 cells/dish). However, if ascorbate was added after 96 hours of growth, a stimulation in cellular proliferation of about 10% was observed. The optimal level of ascorbate appeared to be $0.5\text{-}2.8 \times 10^{-4}$ M. Later, Rowe et al. (1977-2) found that the same concentrations of ascorbate (0.28 mM) used by

Peterkofsky and Prather (1977) and Levene and Bates (1975) in the medium of cells seeded at low density (7.5×10^4 cells/dish), resulted in a low final cell density and abnormal cell morphology. The above studies suggest a relationship between ascorbate toxicity and cell density. However, none of these studies assessed cells at densities above 2×10^5 cells/dish. In addition, fibroblasts derived from tissue that may have high ascorbate requirements, e.g. gingiva, may respond favourably to levels of ascorbate that are toxic to fibroblasts derived from tissue with lower ascorbate requirements.

The toxic effect of ascorbate at super physiological levels has led some workers to study the potential use of ascorbate analogues that do not produce toxic end products. Peterkofsky and Prather (1977) reported that compounds containing the ene-diol reducing group, e.g. isoascorbate and dihydromaleate, produced the same effects on cells as ascorbate. Maleate was non-toxic. Glutathione and cysteine were almost as toxic as ascorbate, while dithiotreitol was much less toxic. Later, Nolan et al. (1978) suggested that reductants other than ascorbate, e.g. tetrahydropteridines, to some extent satisfied the requirement for a reductant in the prolyl hydroxylase reaction in vivo as well as in vitro.

It would seem that intracellular ascorbate levels are important to collagen formation since this process occurs within the cell. Yet, few studies have explored the relationship between extra and intracellular ascorbate levels. The fact that supplementing the growth medium with ascorbate does not produce signifi-

cant effects on collagen synthesis, suggests that intracellular levels are slowly lost. This may also explain the toxic effect of super physiological extracellular ascorbate levels on cells. Moreover, the relationship between ascorbate toxicity and cell density needs to be further explored. The interaction of insulin with ascorbate resulting in increased ascorbate uptake, suggests that more studies of membrane transport of the vitamin as it is affected by insulin are necessary.

1-4 EFFECTS OF PERIODONTAL DISEASE AND DIABETES ON COLLAGEN METABOLISM IN THE PERIODONTIUM

The connective tissues in the periodontium are composed of collagens, non-collagenous glycoproteins, glycosaminoglycans and small amounts of elastin (Narayanan and Page, 1983). Collagen is the most abundant of these macromolecular constituents and in all likelihood is the most significant in light of its contribution to the unique architecture and function of these tissues. Moreover, the collagen fibers contained are structurally organized in a consistent fashion from one mammalian species to another (Page and Schroeder, 1982).

Collagens represent 60% of the total tissue protein in the gingiva (Page, 1972). Of the extractable collagens, Types I and III account for 99% of the total and Types IV and V for about 1% (Narayanan et al., 1978, 1981, 1983; Hammouda et al., 1980, Dabbous et al., 1981). Although the rates of collagen synthesis and degradation appear to decrease greatly with age in connective

tissues, this does not seem to be the case in the periodontium where collagen is rapidly degraded and replaced (Narayanan and Page, 1983).

The gingival collagens differ in several respects from those of human skin origin. Most notably the amino acid 3-hydroxyproline present in skin collagens was not detected in gingival collagens (Narayanan et al., 1981). Thus, gingival tissue either lacks the enzyme capable of hydroxylating proline in the 3 position or the prolyl residues normally susceptible to hydroxylation in this position are blocked (Narayanan and Page, 1983). In addition, gingival collagens incorporate significantly less proline and more lysine than those from skin. Consequently, they may be expected to be lower in hydroxyproline and higher in hydroxylysine content than skin.

Gingival tissue contains a variety of non-collagenous glycoproteins but to date, the study of these components has been largely neglected. In addition little is known about the proteoglycan component of gingiva. Using histochemical methods, Schultz-Haudt et al. (1964) demonstrated the presence of chondroitin sulphate and hyaluronic acid in the gingiva. More recently, biochemical methods (Embery et al., 1979; Wiebkin et al., 1979) have facilitated the detection of these substances. It has been suggested that gingival acid mucopolysaccharides exert a protective effect on collagen and are important to the maintenance of collagen integrity (Cianco and Mather, 1971). These workers reported that lower levels of chondroitin sulphate were found in diseased human gingiva compared to normal tissue. Specifically,

it was found that healthy tissue contained 0.4% dry weight of gingiva, while periodontally involved tissue contained only 0.28%. Goggins and Billups (1972) reported that fibroblasts from both skin and gingiva have similar capabilities for the *in vitro* synthesis of acid mucopolysaccharides. These workers reported that hyaluronic acid was the major mucopolysaccharide synthesized along with lesser amounts of chondroitin sulphate. More recently, Bartold et al. (1982) reported that the extracellular proteoglycans of human gingiva were smaller and their stoichiometry less complex than those of gingival epithelium. Consequently, the gingival conective tissue may be more susceptible to endogenous enzymic degradation and toxins produced by components of the immune system than their counterparts in the gingival epithelium.

The above experimental data suggest that the molecular structures of gingival acid mucopolysaccharides predisposes them to degradation. Moreover, this depletion of acid mucopolysaccharides in periodontal disease removes the protective barrier guarding collagen and thus promotes collagen breakdown. Consequently, the importance of these substances to the integrity of the oral connective tissue cannot be ignored.

The periodontium is the tissue site of several diseases of which periodontal disease is the most common. In addition, several systemic diseases also have manifestations here (Narayanan and Page, 1983). Chronic periodontitis is a progressive, destructive disease caused by the apical extension of bacteria along the interface between the gingival tissue and the root surface (Page and Schroeder, 1982). Pocket formation occurs around the necks of

teeth, along with extensive destruction of the gingival connective tissue, periodontal ligament, alveolar bone and sometimes the roots of teeth (Narayanan and Page, 1983). Although the cellular population of the lesion is predominatly plasma cells and B lymphocytes, there are also a few macrophages and variable numbers of neutrophils present. Tissue destruction is characterized by acute inflammation and increasing numbers of neutrophils. Although there is a net decrease in the collagen content of diseased tissues, fibrosis and scarring of the gingiva sometimes occur (Avery and Simpson, 1973; Page et al., 1975). In the advanced disease state, destruction of the periodontal ligament and alveolar bone housing the tooth roots results in the loosening and/or exfoliation of the teeth, which may consequently require extraction.

Most studies directed at determining the connective tissue changes observed in periodontitis have focused on the collagens, since they are the major connective tissue components and the methodology required for their study is well developed. In view of the ease of access to the tissue, work has been done predominantly on gingival tissue (Narayanan and Page, 1983). The development of periodontitis is paralleled by increased rates of collagen production and degradation, while the total amount of collagen decreases (Narayanan and Page, 1983). For example, in vivo, the amount of Type V collagen increases about seven-fold with the concurrent appearance of an entirely new collagen, the Type I trimer, which may account for up to 2% of the total collagen present. Fibroblasts derived from inflamed tissue and cultured in vitro, also continue to synthesize the Type I trimer which may represent

up to 14% of the total collagen produced in some cultures. The proportion of Type III collagen is also increased (Narayanan and Page, 1983).

Among the mechanisms that may participate in connective tissue alterations is that of enzymic degradation of the connective tissue matrix components. Resident fibroblasts appear to be actively involved in the normal turnover and remodelling of the gingiva. These cells produce small amounts of collagenase (Birkedal-Hansen et al., 1976) and are capable of phagocytosis and digestion of collagen fibers present in the matrix (Ten Cate and Deporter, 1975; Soames and Davies, 1977). In addition, fibroblasts are capable of intracellular digestion of nascent chains prior to secretion. This process occurs in normal cells and may provide a mechanism by which defective polypeptide chains and molecules are destroyed (Bienkowski et al., 1978-1,2). In fact, Berg et al. (1980) reported that rapidly dividing fibroblasts degraded about 30% of their newly synthesized collagen while confluent cultures degraded about 10%. This mechanism may also be important in diseased tissues as it is significantly enhanced by prostaglandin E1 (Baum et al., 1980). Both normal and inflamed gingiva contain biologically significant amounts of prostaglandins (Loning et al., 1980).

Collagenase is the major enzyme involved in the degradation of the connective tissue matrix. The various collagen types demonstrate great differences in their susceptibility to collagenase, and the collagenases differ in their substrate specificities (Narayanan and Page, 1983).

The major portion of the collagenase in diseased gingiva originates from infiltrating leukocytes, especially macrophages and neutrophils (Schroeder et al., 1973). Macrophages are especially significant to matrix degradation since they can produce large amounts of enzymes including collagenase, elastase and lysosomal acid hydrolases (Page et al., 1973, 1974, 1978). The pathologic changes caused by periodontitis are typical of those observed in most other chronic inflammatory diseases. Therefore, the underlying mechanisms of these changes are probably similar in all connective tissues.

Diabetes mellitus, like periodontal disease, is wide-spread. In diabetes a number of physiological processes seem to be affected. For example, there appears to be an acceleration of the ageing process (Goldstein et al., 1978). Of central importance to senescence is the concept that the finite lifespan of cells in culture is the expression of senescence at the cellular level (Hayflick et al., 1975). In vitro studies of fibroblasts derived from diabetic rat gingiva have also demonstrated overall decreases in replicative and metabolic rates when compared to fibroblasts obtained from healthy experimental animals (Harrison, 1982). This evidence of accelerated senescence in diabetic tissues has formed the basis for the hypothesis to explain the diabetes associated endocrine, connective tissue and vascular pathology (McMillan, 1975; Vracko, 1974; Levin et al., 1976; Grgic et al., 1976).

The effects of diabetes on connective tissue, especially collagen, are well studied. In diabetic rats, there is a net loss of collagen mass in the skin (Schnier et al., 1979, 1982; Sato et

al., 1980-1,2). The acceleration of ageing with diabetes has been implicated in the onset of osteoarthritis in man, a phenomenon related to ageing (Caterson et al. (1980). These workers reported that the rate of osteoarthritis in streptozotocin diabetic rats was indeed accelerated by diabetes. Specifically, histological examination of diabetic articular cartilage showed morphological signs of cartilage degradation. Sepharose elutions indicated a smaller proportion of macromolecular aggregate structures. These changes are consistent with those occurring in osteoarthritis. The collagen of a diabetic resembles that of an older individual not only in tendon collagen, but in vessel walls and basement membranes (Spiro, 1973), both of which may precipitate vascular disease and disabilities characteristic of ageing. Moreover, the loss of structural and functional integrity of blood vessels and circulation may impair the normal homeostatic mechanisms and interfere with the responses of the system to the demands of local and distal tissues (Ellenberg, 1978).

The relationship between diabetes mellitus and periodontal disease has been extensively studied. Both diseases involve alterations in collagen and connective tissue metabolism. Diabetics are reported to be more susceptible to periodontal destruction and impaired rates of oral healing (Scopp, 1977; Cianciola et al., 1982; Munroe, 1983). Moreover, diabetics with periodontal disease suffer from an increased severity of the disease and display inflammatory responses characteristic of severe periodontitis (Scopp, 1977; Cianciola et al., 1982). The acceleration of gingival collagen breakdown is caused, at least in part,

by a diabetes-induced increase in proteolysis, arising from the increased activity of collagenase (Golub et al., 1978-2). The enhancement of collagenase activity along with elevated levels of other enzymes, e.g. elastase and acid hydrolases, produces periodontal destruction. This destruction activates the release of toxins by components of the inflammatory system. The interaction between enhanced enzyme activity and elevated levels of toxins probably results in periodontal destruction in diabetics at a rate more rapid than that normally seen in periodontal disease.

Diabetes is thought to produce a number of changes in the structure of the collagen molecule. Interstitial collagens isolated from diabetic rat tissue show decreased solubility of reconstituted fibrils, an increased intrinsic viscosity and reduced susceptibility to collagenase (Golub et al., 1978-1).

The involvement of the oral microflora in periodontal disease is well known, particularly the involvement of the black pigmented bacteroides (B. melaninogenicus) in periodontal destruction (Newman, 1979; Reed et al., 1980; Zambon et al., 1981). Grossi et al. (1983) have reported that in insulin-dependent diabetes mellitus, early periodontal loss was accompanied by the emergence of a microflora predominated by Capnocytophaga and B. melaninogenicus. Thus, the susceptibility of diabetics to periodontal disease may also be related to the presence of these microflora precipitated by the diabetic condition.

Collectively, the above information suggests that both diabetes and periodontal disease can have significant effects on connective tissue metabolism and the resultant structural integrity.

Moreover, the additive effect of both diseases can result in a more pronounced effect on the connective tissue matrix. The data suggests that the aberrations in connective tissue metabolism caused by diabetes may predispose diabetics to periodontal disease. However, the involvement of the oral microflora as an initiating factor in periodontal disease cannot be ignored, especially when the oral cavities of diabetics have been shown to house particularly virulent strains. In essence, the data have been derived largely from *in vitro* studies and attempt to emphasize the metabolic differences between healthy individuals and their counterparts with either periodontal disease or diabetes mellitus.

1-5 INSULIN: INVOLVEMENT IN FIBROBLAST METABOLISM

Insulin receptors are present in a large number of mammalian tissues. Among cells known to possess insulin receptors are erythrocytes, adipocytes, hepatocytes, cardiac muscle fibroblasts, placenta and brain cells (Rechler and Podskalny, 1976; Kaplan, 1980; Van Obberghen and Roth, 1981; Heidenrich et al., 1983). Properties of the insulin receptor in the various tissues are similar and include specificity and high affinity for insulin, rapid and reversible binding, saturability and a similar pH for optimal binding (Kuehn and Blundell, 1980; Beck-Nielsen, 1980; Van Obberghen and Roth, 1981; Heidenrich et al., 1983). The receptor serves to specifically recognize and bind insulin from among all other substances to which the cell is exposed. This specificity

manifests itself in an association constant or Ka value of 0.6 x 10^{-9} M (Caro and Amatruda, 1980). Moreover, since there are many more receptors than are needed for maximal physiological responses in the cells studied thus far, the binding of insulin to only approximately 5 percent of the high affinity receptors on adipocytes is sufficient to produce maximal activation of glucose transport (Kono and Barham, 1971).

Insulin receptors are found not only on the extracellular plasma membranes of cells, but also intracellularly on other membranous structures including the endoplasmic reticulum, golgi and mitochondria (Massague et al., 1980). However, the source, fate and function of these intracellular receptors are obscure (Van Obberghen and Roth, 1981).

Both insulin and its receptor are required for activation of the target cell (Van Obberghen and Roth, 1981). This includes not only all events which occur at the cell surface, but also the activation of intracellular enzymes such as glycogen synthase and pyruvate dehydrogenase. (Van Obberghen and Roth, 1981). The insulin-receptor interaction serves not only the function of cell-hormone recognition and activation, but acts also to regulate the affinity of the receptor for insulin. Exposure to high concentrations of insulin causes a sharp decrease in receptor affinity. This phenomenon is referred to as "down regulation" of insulin receptors (De Meyts et al., 1973; Gavin et al., 1974). In addition, by binding insulin, the cell surface receptor acts as a reservoir for the plasma hormone, binding hormone when the insulin levels rise and releasing the hormone back into the plasma as the

plasma level of the hormone decreases (Zeleznik and Roth, 1978).

The insulin receptor is considered to be an integral membrane glycoprotein since it can only be removed from the membrane with preserved structural integrity by detergents (Van Obberghen and Roth, 1981). The total amount of cell surface insulin receptors is less than 1 percent of the protein of the plasma membrane. The structure is similar to that of the immunoglobulin G molecule (Czech et al., 1981). Like other cellular components, insulin receptors are in a state of continual synthesis and degradation (Beck-Nielsen, 1980; Van Obberghen and Roth, 1981). Although there is little information concerning insulin receptor turnover, Kosmakos and Roth (1980) using insulin binding techniques, proposed a half life of 20-24 hours for IM-9 lymphocyte insulin receptors in the presence of cycloheximide and insulin. Both the rate of loss and the net loss of receptors were due to the insulin concentration. The insulin-induced receptor loss was mediated by insulin binding to the receptor. Insulins varying 200-fold in biopotency produced receptor loss in direct proportion to the ability of each insulin to occupy the receptor. Reed and Lane (1980) studied 3T3-L1 preadipocytes by density labelling, determined a half life of 6.7 hours for receptor degradation. Specifically, old "heavy" and new "light" receptors were solubilized with detergent and resolved by centrifugation. Insulin receptor synthesis and degradation in these cells showed an increase in insulin binding capacity during differentiation. The data indicated that an increase in insulin binding capacity was a result of new receptor synthesis. Consequently, the relative

amounts and positions of newly synthesized and old receptors in the gradient was determined by the capacity to bind ^{125}I-labelled insulin (Reed and Lane, 1980).

A change in receptor concentration related to cell differentiation has been documented. In the process of erythroid cell maturation, the number of receptor sites per cell was observed to decrease from 20,000 sites per cell in young cells to 8,000 sites per cell in old cells (Thomopoulos, 1980). Furthermore, Kaplan (1980) has determined from insulin binding studies that normal newborn humans possess about 50% more insulin binding sites per monocyte than in adults. The presence of insulin is crucial in regulating receptor concentration. Although Kosmakos and Roth (1980) have shown that the rate of onset of receptor loss is a direct function of insulin concentration, insulin concentration does not decrease the receptor number by more than 60-85 percent (Van Obberghen and Roth, 1981). Removal of insulin from the medium results in a rapid increase of the insulin receptor concentration (Van Obberghen and Roth, 1981).

Both insulin and its receptor are sensitive to temperature. Although, at 37°C and high levels of insulin (10^{-10} M), rapid steady states of binding occur, higher levels of binding are obtained at lower temperatures (4-20°C), due to decreased degradation of both receptor and hormone (Kuehn and Blundell, 1980, Beck-Nielsen, 1980). Insulin binding has also been reported to be affected by pH. Marshall et al. (1983) have reported that a lowering of pH from 7.8 to 7.0 reduced the insulin binding affinity from 3.3% to 1.0% by increasing the rate of insulin dissociation.

This effect was paralleled by a decrease in the rate of receptor mediated insulin degradation.

The physiological level of insulin is 10^{-11} - 10^{-10} M (Fujimoto and Williams, 1974). Insulin exerts its effects following interaction with specific receptors located in target cell surface membranes (Saunders, 1982; Jones, 1982; Jeffrey, 1982; Podlecki, 1983). It has been proposed that a clustering of hormone-receptor complexes results in cell membrane disruption which facilitates the transport of small molecules, e.g. glucose into the cell (Jeffrey, 1982). The insulin receptor complex is then internalized (Desbuquois and Postel-Vinay, 1980; Jones, 1982). Processes requiring insulin, e.g. stimulation of DNA and protein synthesis and regulation of receptor synthesis are carried out and the hormone is eventually degraded (Jeffrey, 1982).

Membrane transport of many metabolites required by cells both in vivo and in culture is facilitated by insulin. Insulin at levels as low as 10^{-10} M (Howard et al., 1979; Ishibashi et al., 1981) stimulated the uptake of 2-deoxyglucose (Vaheri et al., 1973) with maximal effects observed at 10^{-7} - 10^{-6} M insulin (Ishibashi et al., 1981). The stimulatory effect of insulin on alpha aminoisobutyric acid (AIB) transport has also been reported (Elsas et al., 1971; Manchester, 1972; Goldfine et al., 1972; Goldfine and Sherline, 1972; Risser and Gelehrter, 1973; Martin and Pohl, 1974). Goldfine and Sherline (1972) have shown that insulin at concentrations as low as 0.9 nM is capable of stimulating AIB uptake with a maximal effect seen at 40 nM insulin. Insulin has also been shown to increase DNA, RNA and protein

synthesis in cultured cells (Rubin, 1976; Fujimoto and Williams, 1974; Manchester, 1972).

An additive effect of insulin and serum resulting in increased cell numbers and DNA synthesis in vitro has been documented (Griffiths, 1972; Gospodarowicz and Moran, 1975). At low serum concentrations which arrest cell division due to limited availability of growth nutrients, insulin has been shown to potentiate the responses of cells to growth factors already present in the medium and delayed the degeneration of fibroblasts maintained at low serum concentrations (0.2 and 0.4% serum) (Gospodarowicz and Moran, 1975). The physiological level of insulin is 10^{-11} - 10^{-10} M and this level of insulin has been shown to stimulate glucose uptake and RNA and protein synthesis in fibroblasts maintained in serum free media (Fujimoto and Williams, 1974). However, it appears that cells grown in serum supplemented media require much higher levels of insulin to produce cellular uptake of alpha aminoisobutyric acid (Minemura et al., 1970; Hollenberg and Cuatrecasas, 1975), stimulation of RNA, protein synthesis and sugar uptake (Vaheri et al., 1973) and DNA synthesis and cellular replication (Jimenez de Asua, 1973; Vaheri et al., 1973). Hollenberg and Cuatrecasas (1975) have suggested that serum in the growth medium of cells maintained in culture interferes with or else inhibits the sensitivity of cells to insulin. Thus, it appears that in the presence of serum, higher levels of insulin are required to initiate a cellular response.

The binding of insulin to cells obtained from diabetics has received considerable attention. Some workers (Flier et al.,

1975; Olefsky and Reaven, 1977) report a decrease in insulin binding, while others report an increase (Kobayashi and Olefsky, 1979; Hansen et al., 1983). Olefsky and Reaven (1977) reported that the decreased insulin binding in diabetic monocytes was secondary to a reduction in the numbers of receptor sites per cell; 15,000 sites per normal monocyte versus 8,500 sites per diabetic monocyte. Flier et al. (1975) reported that a serum factor (most probably an antibody) alters the insulin receptor and impairs the subsequent binding of insulin. Kobayashi and Olefsky (1979) and Hansen et al. (1983) reported increased insulin binding by adipocytes of rat with insulin deficient streptozotocin diabetes due to enhanced receptor capacity and affinity. However, a lack of cellular response to insulin was manifested in decreased rates of glucose uptake and metabolism. This was thought to be due to cellular metabolic differences rather than insulin binding (Kobayashi and Olefsky, 1979; Hansen et al., 1983). Therefore, although there appears to be controversy surrounding insulin binding in diabetics, it is generally believed that cells from diabetics are less metabolically responsive to insulin than healthy cells.

Structural abnormalities in the insulin molecule and gene in diabetics have been reported. Tager et al. (1979) described a structurally abnormal insulin which precipitated diabetes. It was suggested that the abnormal insulin was a mixture of normal and an abnormal variant of insulin which contained leucine for phenyl alanine substitution at position 24 or 25 on the insulin B chain. The biological activity of this patient's insulin was only 11-12 percent of normal. Permutt et al. (1981) analyzed the structure

of the insulin gene in genomic DNA of humans with and without diabetes. Insertions of DNA between 1,500 and 3,400 base pairs have been detected near the transcription initiation site in 65% of Type II diabetics (non insulin dependent) and 25-30 percent of non-diabetics. Limitations of these insertions to this potential promoter region of the insulin gene suggests that they may alter gene expression in Type II diabetes. These insertions of DNA may prove to be useful genetic markers for diabetes.

In culture, fibroblasts from human diabetics resemble those of normal human cell cultures of more advanced age as measured by comparative growth rates. The slower growth rate resulted in diminished final cell densities (Rowe et al., 1977-1; Rosenbloom and Rosenbloom, 1978; Goldstein et al., 1978; Goldstein, 1979). Abnormal collagen synthesis has also been found in skin cultures from human diabetics (Rowe et al., 1977-1; Kohn and Hensse, 1977; Belleman et al., 1977; Tenni et al., 1980). Kohn and Hensse (1977), reported that cultures of fibroblasts from human diabetics produced a non-helical procollagen peptide which contains abnormal reducible crosslinks. The collagen was also devoid of normal non-reducible crosslinks and was of very high molecular weight. Similarly, Golub et al. (1978-1), described a collagen isolated from the tail tendon of streptozotocin diabetic rats that was of higher molecular weight than that found in the tail tendon of control rats. Although overall protein production is enhanced by insulin, concentrations of the hormone as high as 0.1 U/ml have been shown to be without effect on collagen secretion in human fibroblasts (Villee and Powers, 1977). The cellular metabolism of

fibroblasts in injured tissue of untreated diabetic mice is impaired (Weringer and Arquilla, 1981). Eight hour old wounds from diabetic hamsters contained 49% fibroblasts of control. Sixteen hours after injury the collagen content of diabetic wounds was still decreased (54% of control) (Weringer and Arquilla, 1981). Diabetic mice treated with insulin, however, exhibited many stimulatory effects of the hormone as evidenced by an increase in fibroblasts, capillaries, polymorphonucleocytes (PMNs) and collagen in the wounds 8 hours following insulin administration (Weringer et al., 1982). The observed growth and proliferation was attributed to insulin action on the cells involved. The insulin requirement in wounded tissue is further substantiated by the report of Javed et al., (1983), who suggested that diabetics with moderate or advanced periodontal disease required higher insulin doses than diabetics with mild periodontal disease.

The above studies suggest that diabetes mellitus is a complicated disease which causes a breakdown in cellular integrity. Not only is total body insulin reduced, but so are its mitogenic effects. For example, reduced cellular sensitivity to insulin due to serum inhibitors, reduced receptor binding or structural abnormalities in the insulin molecule itself, may reduce the stimulatory effects of the hormone. Although some studies report that the diminished effects of insulin are due to complications distal to the receptor, the exact mechanisms involved are vague. Clearly, more work needs to be focused on the mechanisms of insulin action.

1-6 THE RAT AS A MODEL FOR THE STUDY OF DIABETES MELLITUS

Spontaneous diabetes is a common occurrence in many animal species (Mordes and Rossini, 1981). In addition, animals can be rendered diabetic by a wide variety of experimental procedures.

Diabetic animals may be regarded as models of the disease in man (Mordes and Rossini, 1981). The use of diabetic animals offers many advantages. The genetics of humans are difficult to study and impossible to control while rodent colonies allow many generations to be accurately studied in a relatively short period of time (Mordes and Rossini, 1981). The occurrence of diabetes in animal colonies provides a powerful tool for the study of preventive therapies for the disease. Diabetes research in humans is impaired by the obvious ethical considerations. Although induction of the disease in man is strictly prohibited, it provides one of the most rewarding lines of animal study.

Extrapolation from animals to man is risky, especially in the case of diabetes. Despite the universal presence of hyperglycemia, none of the diabetic syndromes in animals correspond exactly to any of the forms of diabetes in man (Mordes and Rossini, 1981). Although spontaneous diabetes occurs commonly in animals, the disease is well characterized in only a few species. Small laboratory rodents because of their large numbers and low cost have provided an enormous amount of data.

The use of chemical agents to induce diabetes facilitates the detailed study of the biochemical, hormonal and morphologic events occurring during and after the induction of the diabetic state.

Several classes of agents produce such effects (Fischer and Rickert, 1975; Dulin and Soret, 1977). Of importance to this study is the class of agents described as cell specific toxins. These substances destroy the insulin producing beta cells of the pancreas resulting in the primary insulin deficient state (Mordes and Rossini, 1981). The two most studied agents in this class are alloxan and streptozotocin. Since our findings are reported from the study of streptozotocin diabetes, it is this chemical that will be highlighted.

Streptozotocin is a beta cytotoxin which in diabetogenic doses is free of non-specific toxic effects. Numerous studies indicate the toxicity of streptozotocin to be specific for the beta cell (Fischer and Rickert, 1975; Dulin and Soret, 1977). In vitro studies have shown it to have no effect on fibroblast protein synthesis (Wilson et al., 1983). This lack of effect may be due to lack of binding of streptozotocin by the cell membrane. Indeed the alpha monomer of the glucosamine moiety of streptozotocin has been shown to render the compound more cytotoxic than the beta anomer, suggesting the regulation of toxicity via specific recognition by the beta cell (Mordes and Rossini, 1981). It has been suggested that the glucose component of streptozotocin enhances its uptake into the beta cell where the cytoxicity of the nitrosurea moiety can be concentrated. Removal of the glucose moiety reduces the specific toxicity for beta cells. Once in the beta cell, streptozotocin is thought to lower levels of nicotine adenine dinucleotide (NAD) by both decreasing its synthesis and increasing its breakdown. NAD protects animals from the cytotox-

icity of streptozotocin (Wilson et al., 1983). The symptoms of insulin deficient diabetes mellitus induced by streptozotocin are similar to those seen in the natural disease state. Zebrowski and Bhatnagar (1979) induced diabetes in male Sprague-Dawley rats by an intravenous injection of streptozotocin. All animals designated as diabetic showed hyperglycemia, glycosuria, polydypsia, polyuria, hyperphagia and loss of body weight with time, criteria recognized as being characteristic of the diabetic state (Howland and Zebrowski, 1974), and all of which were responsive to insulin therapy.

Recent advances in the study of diabetes in animals have contributed to the understanding of its counterpart in man. Specifically, experimental animal models have been effectively used to study the etiologies, complications, treatments and prevention of diabetes.

1-7 REGULATION OF FIBROBLAST FUNCTION

Almost all cells in culture require serum for proliferation. The optimal concentration for promoting growth in cultured cells is 10-20 percent serum (Gospodarowicz and Moran, 1976). Most studies use 10 percent serum. The presence of serum in the culture medium influences collagen metabolism. It results in the increase of the total amount of collagen production and as well as in the ratio of Type I to Type III collagens (Narayanan and Page, 1977). Serum enhances, therefore, the production of more Type I than Type III collagen. DNA levels in cells grown *in vitro* are

directly proportional to the serum concentration (Holley and Kierman, 1968; Griffiths, 1972). Holley and Kierman (1968) proposed that serum contributes a factor or factors required by cultured cells for division. It is of interest that factors essential to tissue growth and repair, e.g. the somatomedins, have been reported to be in low levels in streptozotocin induced diabetes mellitus. In addition, inhibitors of somatomedin bioactivity have been demonstrated in the serum of untreated diabetic rats (Phillips et al., 1979-2).

Several investigations have produced in vitro evidence suggesting that cellular proliferation is impaired in cells grown in serum from diabetics of various species. This impairment of growth has been attributed to the elevated amount of very low density lipoprotein (VLDL) present in diabetic serum (Arbogast et al., 1982; Chi et al., 1982) and somatomedin inhibitory factors (Phillips et al., 1979-1,2). Specifically, Arbogast et al. (1982) reported that porcine aortic cells grown in 17 percent diabetic serum were severely contracted and 70 percent Trypan blue positive. By the fourth day in passage, only small islands of Trypan blue positive cells remained. The toxicity of diabetic serum was restricted to the VLDL fraction of diabetic serum since it was shown to inhibit the uptake of ^{3}H-thymidine in normal rat spleen cells (Chi et al., 1982). Moreover, removal of this VLDL fraction allowed the infranant from diabetic serum to support the growth of cultured cells as well as did normal serum (Chi et al., 1982). Other studies have suggested the presence of somatomedin inhibitors in diabetic serum (Phillips et al., 1979-1,2). It was

shown that the decreased mitogenic activity in diabetic serum was not only due to somatomedin inhibitors, but also to overall lowered levels of somatomedins (Phillips et al., 1979-2). In an attempt to determine whether circulating growth factors other than somatomedins were deficient in diabetic serum, Murphy and Lazarus (1983) grew human skin fibroblasts in medium containing diabetic and normal rat serum. Normal rat serum resulted in a dose dependent increase in the number of skin fibroblasts while diabetic serum resulted in considerably less cellular proliferation and DNA synthesis. The removal of small molecules such as ketones from the diabetic serum eliminated this source of inhibitors. Therefore, Murphy and Lazarus (1983) suggested that the defect in cellular proliferation in diabetes mellitus was due to the depletion of circulating growth factors other than insulin and somatomedins, since the fibroblasts survived in somatomedin depleted media. While the nature of the deficient growth factors in diabetic serum was unknown, these authors suggested that the deficiency of these growth factors may in concert with the presence of somatomedin inhibitors described by Phillips et al. (1979-2) cause disturbances in cell growth.

Despite the literature reports of the detrimental effects of diabetic serum on cells in culture, several studies have reported that diabetic serum does not inhibit cellular growth in vitro. Diabetic serum with the same concentration of cholesterol, phospholipid and triglyceride as normal serum, contains a factor or factors stimulatory to fibroblasts in vitro (Ledet et al., 1976; Ledet, 1976, 1977). This factor was not glucose, insulin or lipid

and could not be identified. However, Ledet (1977) suggested that the stimulation of growth caused by diabetic serum was due to increased serum growth hormone production.

The key factor in the Ledet study (1977) may be the normolipemic status of diabetic sera, since Arbogast et al. (1982) and Chi et al. (1982) attributed toxicity of the diabetic serum to increased VLDL levels. Thus, the stimulation caused by diabetic serum (Ledet et al., 1976; Ledet, 1976, 1977) may be due to the decreased toxicity of diabetic serum caused as a result of a more normal lipid level.

The effect(s) of normolipemic diabetic serum on collagen production in culture has also been assessed (Ledet and Vuust, 1980). These workers reported that diabetic serum enhanced the production of procollagen Type I and fibronectin by arterial myomedial cells. Moreover, the levels of these substances were higher than those in cells grown in normal serum. Although the factor(s) causing enhanced collagen production were not identified, growth hormone was proposed as a possible factor. In addition, these workers proposed that a more pronounced effect on collagen production might be seen using serum from diabetics in a more poorly controlled metabolic state.

The ageing of tissue and the resulting altered rates of metabolism observed in vitro have provided great insights into this universal phenomenon seen in man. Human fibroblasts (Hayflick, 1965), chick embryo cells (Hay and Strehler, 1967), human foetal lung cells (Maceira-Coelho and Ponten, 1969) as well as other cell types have been found to have a limited ability to

proliferate *in vitro*. In cultures the lifespan of normal human cells is inversely proportional to the donor's age (Martin et al., 1970).

Orgel (1973) proposed that fibroblast senescence resulted from an 'error catastrophe' in protein synthesis which causes a breakdown in vital cellular pathways that eventually leads to cell death. Of central importance to this hypothesis is the proposal that random errors in the synthesis of proteins concerned with DNA replication and transcription and translation of RNA, should increase the rate of intracellular accumulation of other faulty proteins.

Other theories which explain changes in cellular parameters have been proposed. Schneider and Fowlkes (1976) suggested the decrease in cellular DNA in senescent human fibroblasts to be due to several factors including an increased frequency of chromosomal deletions, a decreased amplification of specific DNA genes or a loss of cellular DNA. However, Lima and Macieira-Coelho (1972) reported that almost 100% of chick fibroblasts grown over 30 sub-cultures enter into DNA synthesis during their whole lifespan. Growth decline could be due mainly to an increase in cell loss associated with higher sensitivity to cell crowding, thus implying the involvement of the cell membrane. Further support for cell membrane involvement in senescence comes from the work of Schneider and Fowlkes (1976) and Mitsui and Schneider (1976). These workers, on the basis of their studies with cultured human fibroblasts, suggested that cell membrane alterations could be the primary event leading to a loss in replicative activity.

The literature data which describes RNA synthesis and its relationship to senescence are conflicting. Bowman et al. (1976) found a decrease in RNA synthesis in human fibroblasts as senescence progressed. On the other hand, Schneider and Shorr (1975) demonstrated an increase in cellular RNA levels with ageing in cells. Schneider and Shorr (1975) suggested the increased redundancy of DNA genes in senescent cells caused an increase in intracellular levels of RNA, mRNA and protein. This explanation is in accordance with the 'error catastrophe' hypothesis of Orgel (1973).

Senescent cells are increased in size and this may represent an attempt to maintain constant intracellular macromolecular concentrations in the face of increased intracellular volume. Goldberg (1971) proposed that the increased content in senescent cells was due to the cessation of protein synthesis with age which resulted in the accumulation of precursors such as aminoacyl tRNA.

Several studies have documented the accumulation of intracellular protein in senescent cells (Orgel, 1973; Fry and Weisman-Shomer, 1976; Kaftory et al., 1978). Capecchi et al. (1974) found mammalian cells to have a very sensitive mechanism for degrading proteins. However, Kaftory et al. (1978) proposed that there was a decrease in protein breakdown due to senescence rather than retarded proliferation. Collagen synthesis is greatly diminished in aged fibroblasts (Salcedo and Franzblau, 1981; Hildebran et al, 1983). These studies clearly show that cells do not produce as much collagen relative to total protein in the culture medium as they age. However, they do continue to accumulate

TCA precipitable hydroxyproline in the culture medium throughout the life of the culture. As expected, total protein synthesis decreases. These results suggest that age or proliferative capacity of cells, or both in culture may be closely related to the collagen production of these fibroblasts. The question as to how collagen accumulates in cultures of aged fibroblasts at a time when collagen synthesis is depressed may be explained by the reduction in protein breakdown in senescent cells (Kaftory et al., 1978). However, these mechanisms must be carefully explored to understand clearly the relationship between proliferative capacity and the deposition of this important connective tissue protein.

The rate of cell division in culture can depend greatly on the density of the cell population. Several reports have documented the influence of cell density on collagen production and metabolic activities of fibroblasts. It has been reported that a 'density dependent' regulation or 'contact inhibition' of growth occurs in several tissues in culture (Todaro et al., 1965; Castor, 1968; Vaheri et al., 1973). This regulation of growth is believed to be due to limitations of nutritional requirements in the culture medium (Holley, 1975). In accordance with these beliefs, Speicher et al. (1981) and Manner (1971) reported a decrease in DNA synthesis with an increase in cell density. However, Armato et al. (1978) found that up to a nineteen-fold increase in the degree of cell crowding of rat hepatocytes did not alter rates of DNA synthesis. These workers explain that a greater metabolic flexibility of hepatocytes might free them from the limitations of growth nutrients which result in decreased growth.

Protein synthesis appears to be very sensitive to cell density and is reported to decrease with increased cell density (Peterkofsky and Prather, 1977; Abe et al., 1979; McCoy et al., 1982). However, Speicher et al. (1981) found no effect of cell density on protein synthesis.

Collagen is produced more rapidly when cells are rapidly dividing than when they are quiescent (Manner, 1971; Tolstoshev et al., 1981; Aumailley et al., 1982). Several explanations have been offered by these various workers. The production of procollagen by cultured cells is constant, whether the cells are growing rapidly or are in the stationary phase (Tolstoshev et al., 1981). However, since significantly more newly synthesized collagen is degraded intracellularly in the log than in the confluent phase, overall procollagen synthesis is greater when the cells are rapidly dividing (Tolstoshev et al., 1981). Aumailley et al. (1982) reported that collagen synthesis per cell was higher in the exponential than in the quiescent state. In non-growing cultures, total protein synthesis was more decreased than collagen. As a consequence, the relative rate of collagen synthesis as measured by hydroxyproline content was less in the exponential than the stationary phase.

Cell density also affected the expression of the main interstitial collagen types, I and III. In rapidly dividing cultures, small amounts of Type III procollagen and collagen were synthesized relative to Type I collagen (Aumailley et al., 1982). This amount increased in the stationary phase of the culture and approached values similar to those found in skin <u>in vivo</u>. Cell

density and consequently, extracellular matrix formation, was also found to influence some steps in the processing of collagen alpha chains (Aumailley et al., 1982). Specifically, procollagens were converted into collagens at a higher rate in the stationary than in the exponential phase of the culture. The results suggest that cell density affects not only the types of collagen produced, but also the rates of production.

Studies on the role of metallic ions in various biological systems has aided in the understanding of several different cellular processes. Among these is zinc. This element is known to be necessary as a coenzyme for many different enzymes of diverse biological function. Studies with cultured cells have shown zinc to be stimulatory to DNA, RNA and protein synthesis (Rubin, 1973; Rubin and Koide, 1975; Epstein, 1982).

The interaction of zinc with insulin has been documented. Insulin used in most media is stabilized with zinc and ionic zinc is said to have insulin-like properties (May and Contoreggi, 1982). The physiological implication of this property is significant since insulin is stored in and probably secreted from the pancreas complexed with Zn^{2+} (Howell et al., 1969). The insulin-like effect of zinc upon lipogenesis <u>in vitro</u> has been described by Coulston and Dandona (1980) and these workers reported that zinc incubated with insulin had an additive effect on rat adipocytes.

However, the presence of zinc in pancreatic islets (Havu et al., 1977) has brought attention to the possible importance of this trace metal in the biosynthesis, storage and release of insu-

lin. Ghafghazi et al. (1980) have found that $ZnCl_2$ in doses of 0.15-0.05 mM inhibited in a dose dependent manner the stimulation by D-glucose, L-leucine and potassium of insulin secretion from isolated rat islets.

More recently, Chaproniere-Rickenberg and Webber (1983) have shown that growth inhibition caused by zinc limits the concentration of zinc stabilized insulin which can be used in tissue culture media. In other words, the presence of insulin bound zinc requires the use of higher levels of insulin to compensate for this inhibitory effect. Studies of the effects of the different commercially available insulin preparations, each with a different bound zinc level on cells in vitro, are clearly required.

The influence of sex steroid hormones on human gingiva has been investigated. Androgens and estrogens induce proliferation in gingival epithelium and other connective tissue (Shklar et al., 1967; Litwack et al, 1970; Rubright et al., 1971; El Attar et al., 1982). In fact human gingiva may function as a target organ for androgens (Southren et al., 1978) due to the presence of specific sex steroid receptors (Vittek et al., 1979-1). The studies suggest that sex steroids significantly influence the health of periodontal tissue. Testosterone has been shown to inhibit prostaglandin formation by gingiva, and thus could possess potent anti-inflammatory activity (El Attar et al., 1982). Since prostaglandins possess potent inflammatory effects, the inhibitory effect of testosterone could act to modify the inflammatory response in some human connective tissue disease states in males. The implication of this finding to periodontal disease clearly

emphasizes the need for more studies of the involvement of testosterone with both healthy and diseased gingiva.

The mechanism of testosterone action has been investigated. Keenan et al. (1975) reported that skin fibriblasts were able to convert testosterone to its active form dihydrotestosterone (DHT), bind DHT to a specific receptor protein and transport this complex into their nuclei. The presence of DHT receptors in gingiva has been reported (Southren et al., 1978). Vittek et al., (1979-2) suggested that pathologic periodontal changes may be causally related to alterations in circulating hormones, i.e. androgens, and their local tissue metabolism. In accordance with this suggestion, El Attar (1974) reported that enhanced formation of testosterone from androstenedione in inflamed gingiva might contribute to the accentuation of gingival inflammation. Systemic administration of DPH (diphenylhydantoin) (Vittek et al., 1979-1), has been shown to increase cellular mitotic activity (Vittek et al., 1979-1) and increase fibroblast metabolic activity, i.e. collagen, mucopolysaccharide and glycoprotein formation in connective tissue (Hassell et al., 1976). This enhancement of cellular activity appeared to be due to the increased stimulation of DPH on DHT activity. Several studies have reported that testosterone is more actively metabolized in sex skins than non-genital skins due to the presence of more androgen receptors in genital skins (Mulay et al., 1972; Keenan et al., 1975; Sultan et al., 1980). Therefore, while gingival tissues contain receptors for testosterone, these tissues may not be the most sensitive target organs to the stimulatory effect of the hormone.

In summary, the fibroblast content and matrix composition of normal gingival tissue from individuals with periodontitis and diseases affecting the periodontium are determined by cells, factors and systems acting in synchronicity. Enzymic breakdown of the matrix is important, as are the fibroblasts themselves in the modulation of the quantity and type of protein synthesized. Other constituents produced by cytotoxic reactions and selective growth pressures affecting fibroblast populations are also of importance. The pathologic changes observed in the periodontium in periodontal disease are typical of those observed in most other chronic inflammatory diseases, and the mechanisms underlying these alterations are most likely similar in all connective tissues.

CHAPTER II

EXPERIMENTAL OBJECTIVES

EXPERIMENTAL OBJECTIVES

The successful *in vitro* use of gingival fibroblasts from control and diabetic rats to study the effects of diabetes on oral connective tissues has been previously reported from this laboratory (Harrison, 1982).

Fibroblasts which are the principal producers of collagen in connective tissues also possess insulin receptors. Consequently, the effects of diabetes on collagen metabolism must be exerted at the cellular level. Tissue from diabetics demonstrates accelerated ageing (Goldstein et al., 1978) and may approach a scorbutic state (Mann, 1974). Although ascorbate in super physiological levels is inhibitory to the proliferation of skin fibroblasts *in vitro* (Peterkofsky and Prather, 1977), tissue with high metabolic turnover rates appear to have higher ascorbate requirements. The metabolic turnover rates of gingiva are among the highest in the body (Page and Ammons, 1974; Sodek et al., 1977). Therefore, these tissues may be expected to require greater ascorbate levels than tissues with lower turnover rates. Serum from diabetics is reported to impair cellular proliferation *in vitro* due to elevated levels of very low density lipoprotein (Arbogast et al., 1982; Chi et al., 1982) and somatomedin inhibitory factors (Phillips et al., 1979-1,2). Moreover, Hollenberg and Cuatrecasas (1975) have suggested that serum in the growth medium of cultured cells interferes with or else inhibits their sensitivity or response to insulin. Commercially available insulin preparations are stabilized with zinc (May and Contoreggi, 1982) and this trace metal is

thought to be involved in the biosynthesis, storage and release of insulin (Havu et al., 1977). More recently, Chapronière-Rickenberg and Webber (1983) have reported an inhibition of fibroblast growth caused by the presence of bound zinc in zinc stabilized insulin. Gingiva is reported to be a target for testosterone (Southren et al., 1978), which is mitogenic to cellular proliferation (El Attar et al., 1982) and is thought to modify the inflammatory response in connective tissue disease states via prostaglandin inhibition (El Attar et al., 1982).

In an attempt to transfer some information obtained from the rat model system to the human system, human gingival fibroblasts were studied and compared with rat data in several experiments presented in this thesis. These human data are, however, restricted to the study of gingival fibroblasts from healthy adults. The objectives of this thesis were, therefore, to:

1 (a). Isolate and grow in culture gingival fibroblasts from healthy human adults and control and streptozotocin diabetic rats. Rat and human fibroblasts were then treated with various physiological agents, e.g. insulin, serum, ascorbate and testosterone. Cellular metabolic responses, e.g. cellular DNA, RNA, non-collagenous and collagenous protein synthesis were determined by comparison of their respective sensitivity to the foregoing physiological agents. The effects of cell age and cell density on cellular metabolic responses were also determined as were membrane transport of ascorbate and certain metabolites.

(b). Assess in vivo insulin treatment of diabetic rats on the response of their gingival fibroblasts to in vitro insulin treatment, by measurement of cellular metabolic processes, e.g. cellular DNA, RNA, non-collagenous protein levels as compared to the metabolism of cells from control and untreated diabetic rats.

(c). Investigate and compare several metabolic responses, e.g. cellular DNA, RNA, non-collagenous and collagenous protein levels of human and control and diabetic rat gingival fibroblasts to age induced changes in cellular insulin sensitivity. Human cells only were assessed for age altered uptake of ^{3}H-2-deoxyglucose.

(d). Determine the effects of medium serum levels and their interactions with insulin on cellular replicative rates, RNA, protein synthesis, membrane transport of ^{3}H-2-deoxyglucose and ^{14}C-alpha-aminoisobutyric acid by control and diabetic rat gingival fibroblasts.

(e). Quantitate cellular metabolic responses of human gingival fibroblasts to varying medium concentrations of control and diabetic rat serum.

(f). Assess the uptake of ascorbate by control and diabetic rat gingival fibroblasts as affected by medium ascorbate both in the presence and absence of medium insulin.

(g). Measure the effects of cell density and its interaction with insulin and ascorbate on cellular metabolism, e.g. cellular DNA, RNA, non-collagenous and collagenous pro-

tein synthesis by control and diabetic rat gingival fibroblasts, and determine intracellular ascorbate levels due to the interaction of cell density with ascorbate and insulin.

(h). Study the metabolic processes, e.g. DNA, RNA, non-collagenous and collagenous protein levels and membrane transport of ascorbate in human gingival fibroblasts as affected by different concentrations of ascorbate both in the presence and absence of medium insulin.

(i). Investigate and compare several metabolic parameters, e.g. cellular DNA, RNA, non-collagenous and collagenous protein levels of control and diabetic rat gingival fibroblasts due to the addition to the growth medium of (i) different levels of testosterone both in the presence and absence of insulin, (ii) two insulin preparations with different levels of bound zinc, and (iii) zinc stabilized insulin, zinc chloride and the two combined.

(j). Determine the effect of <u>in vitro</u> streptozotocin treatment on cellular metabolic responses, e.g. DNA, RNA, non-collagenous and collagenous protein production by control rat gingival cells.

NOTE: The term "diabetic cells" as it appears in this thesis refers only to the source of tissue from which the fibroblasts were isolated, i.e. streptozotocin diabetic

rats. This term does not infer any distinct physiological differences between the two cell types, i.e. control and diabetic.

CHAPTER III

MATERIALS AND METHODS

MATERIALS AND METHODS

3-1 EXPERIMENTAL ANIMAL TREATMENT AND MAINTENANCE

(a) Induction of Streptozotocin Diabetes

Diabetes was induced in two month old male Sprague-Dawley rats by an intravenuos injection of streptozotocin as previously described by Zebrowski and Bhatnagar (1979). The administered dose was 55 mg/Kg body weight. The streptozotocin was purchased from Sigma Chemical Co., St. Louis, USA, No. S-0130).

(b) Assessment of the Diabetic State

All streptozotocin treated animals designated as diabetic showed hyperglycemia, glycosuria, polydypsia, polyuria and hyperphagia. A loss of body weight with time was also observed. These criteria are recognized as being characteristic of the diabetic state (Howland and Zebrowski, 1974). Twenty-four hours post streptozotocin treatment, all treated animals showed urine glucose concentrations of at least 2% as measured with Eli Lilly Testape. These criteria persisted throughout the experimental period for each of the animals used. Rat gingival tissue was removed for tissue culture no sooner than one month following the induction of diabetes.

(c) Housing of Experimental Animals

Experimental animals used were outbred, male Sprague-Dawley rats of the Faculty of Dentistry breeding colony at the University of Manitoba. Animals were housed in individual cages and were provided ad libitum with both food (Wayne Laboratories, Allied Mills Inc., Chicago, Ill. Blox F6), and distilled water. Environmental conditions were kept as constant as possible, i.e. constant temperature 72 $\pm$ 2°F and light; 10 hours of light and 14 hours of darkness.

3-2 TISSUE CULTURE SYSTEM

(a) Fibroblast Isolation

(i) Rat gingival fibroblasts

Gingival fibroblasts were isolated and cultured using essentially the technique of Marmary et al. (1976). Animals were lightly anaesthetized with ether and killed by severing the abdominal aorta. The gingival tissue used was removed from the attached gingiva around the maxillary incisors of the rats. The piece of excised tissue (20-60 mg fresh weight) was submerged in sterile alpha MEM (Minimum Essential Medium) with 2% Penicillin Streptomycin for 1 hour at room temperature as an initial sterii-zation step. The tissue was then removed, minced with scissors and/or scalpel and digested with 12 mg collagenase (Peck et al., 1964) in 4 ml fresh alpha MEM. The flask and its contents were

incubated in a shaking water bath at 37°C for 90 minutes. At time intervals of 30 minutes the suspension of tissue and medium was manually mixed 30 times by drawing the suspension into a sterile Pasteur pipette. Following the incubation period, the suspension of medium and digested tissue was separated from the undigested tissue with a Pasteur pipette and centrifuged at room temperature at 1600 RPM for 5 minutes. After decanting the supernatant, the pellet was twice washed, carefully resuspended and recentrifuged.

After the second washing, a 25 μl sample of cell suspension was counted using a haemocytometer. Tissue culture dishes (60 mm) were then seeded with 5×10^5 cells/dish in 5 ml alpha MEM with 10% Foetal Calf Serum (FCS, Flow Laboratories, Va., USA) and 2% Penicillin Streptomycin.

(ii) Human Gingival Fibroblasts

Human gingiva was obtained from medically healthy patients undergoing required dental treatment in either the Oral Diagnosis or Graduate Periodontics Clinics at the Faculty of Dentistry, University of Manitoba. The tissue collection procedures were approved by the Faculty of Dentistry Human Ethics Committee.

Human gingival fibroblasts were isolated from the excised tissue and initiated in culture using the explant method. After surgical removal, the isolated tissue was submerged in 4 ml alpha MEM with 2% penicillin streptomycin and maintained at room temperature for 1 hour as an initial sterilization step. The tissue was then removed and chopped into pieces approximately 1 mm^3 with

scissors and/or scalpel. Nine pieces of tissue were placed onto a 60 mm tissue culture dish to form 3 grids of 3 fragments each. A sterile glass coverslip with silicone grease on each corner was then placed on top of each triangular tissue grid to stabilize the tissue fragments against the surface of the dish. Approximately 3 grids were placed on each culture dish with 5 ml alpha MEM containing 10% FCS and 2% penicillin streptomycin being added. After about a fortnight, fibroblasts growing out of the explanted tissue produced confluent cultures. Following trypsinization, the cells were repeatedly subcultured (1:2 or 1:3 splits) until used.

(iii) Cell number

The cell number was determined using a haemocytometer. Twenty-five µl of cell suspension was mixed with 50 µl alpha MEM containing 2% penicillin streptomycin and 10% FCS, and 75 µl Trypan Blue cell stain. Ten µl of this mixture was placed in the haemocytometer and counted. The number of cells in the original suspension was determined using the mean of the 6 grids counted per sample in the haemocytometer.

(b) Medium and Growth Conditions

The medium used was alpha MEM (Gibco Canada, Burlington, Ontario, No. 410-1900). The medium as used contained 2% penicillin streptomycin (20,000 U penicillin and 20 mg streptomycin per 100 ml of medium. Ascorbate is present in the medium at a concentration of 50 µg/ml. FCS was added at 10% concentration unless otherwise stated. Growth medium was changed at least twice

weekly. The plated cells were maintained in a CO2 incubator at a temperature of 37°C and in a humidified atmosphere of 95% air and 5% CO2. Subculture splits were either 1:2 or 1:3 on confluent cultures. Unless otherwise stated, all experiments used 5th passage fibroblasts grown on 100 mm tissue culture dishes.

In most studies, fibroblasts were studied from day 1 of the experimental subculture while in some studies, in accordance with the experimental objectives, confluent cultures were used. Control rat cells in culture reached confluency at 7 days, while cells from the diabetic rat tissue were confluent at 8 days. Human fibroblasts achieved confluency at about 6 days. All cells were harvested and subcultured at 8 days to allow maximal cell growth and replication.

The above protocol was used to maintain gingival fibroblasts up to the subculture used experimentally. Thereafter, any variation in cell treatment or maintenance has been described in the individual experimental protocols.

3-3 STUDIES OF THE REGULATION OF HUMAN AND OF CONTROL AND DIABETIC RAT GINGIVAL FIBROBLAST METABOLISM

(a) Preliminary Studies on Control and Streptozotocin Diabetic Rat Gingival Fibroblast Metabolism: A Comparison of Biochemical Parameters

Thirty-one 2 month old male Sprague-Dawley rats were utilized in this study. The experimental protocol is shown in

Figure 1. Streptozotocin diabetes was induced as previously described (Methods). Twenty-four hours post-streptozotocin administration, all treated animals showed urine glucose concentrations of at least 2% as measured with Eli Lilly Testape. Sufficient insulin was given to 10 of the diabetic rats to maintain the urine glucose in a range of 0-1/10% (range of insulin doses = 0-25 IU*/day). On day 28 (Fig. 1) 4 control, 4 diabetic and 4 insulin treated diabetic animals were sacrificed, and the excised gingival tissue cultured in vitro as described (Methods). The remaining control rats were maintained without any further change in their treatment until day 56 when they were killed and their gingival tissue removed for tissue culture. The diabetic animals, however, were divided into two groups (Fig. 1), one of which remained diabetic and untreated, and the other, which from day 28-56 received daily doses of insulin adequate to maintain urine glucose in a range of 0-1/10%. The insulin treated diabetic animals (animals receiving insulin from day 1 of diabetes onset) were also divided into 2 groups. One group continued to receive daily doses of insulin while the other was taken off insulin therapy (Fig. 1). On day 56 all 5 remaining groups of animals were sacrificed, gingivae removed, the gingival fibroblasts isolated and cultured in vitro. Fibroblasts from these 5 groups were maintained in tissue culture until the 5th passage at which time each cell group was further subdivided into 2 treatment groups; one which received in vitro insulin (Connaught Labs. Ltd., Willowdale, Ontario) therapy

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*IU = International Units, 1 IU = 1/22 mg insulin.

at a concentration of 10^{-6} M and the other which received none. This division of animal and cell treatments yielded a total of 10 experimental groups (Fig. 1). The experimental period extended over 9 days. The growth medium was changed on days 3 and 6 and collected and saved for hydroxyproline determinations on days 3, 6 and 9. At these same times, cellular fractions were also collected for DNA, RNA, collagenous and non-collagenous protein determinations.

(b) The Effect of Senescence on the Sensitivity of Human Gingival Fibroblasts to Insulin Treatment

Human gingival fibroblasts 4, 8, 12 and 16 days old in the 5th, 8th, 12th and 18th passages were studied. From the start of each experimental passage, fibroblasts were grown in alpha MEM with 10% F.C.S.. Medium was changed on days 4, 8 and 12. Cellular DNA, RNA, non-collagenous and collagenous protein determinations were carried out on the fibroblasts at these times. Fibroblasts were grown in 60 mm culture dishes.

To assess the effect of senescence on the integrity of the cell membrane and its transport functions, the uptake of the non-metabolizable sugar metabolite, ^{3}H-2-deoxyglucose, was carried out both in the absence and presence of 10^{-9} M insulin (Sigma No. 15500). On each of days 4, 8, 12 and 16 of each experimental passage, the cells were washed twice with Hank's neutral salt solution and the original growth medium replaced with Hepes buffered medium (alpha MEM + 2% BSA-bovine serum albumin) containing insulin (Sigma No. 15500) at concentrations of 0 or 10^{-9} M. These

cells were then preincubated at 37°C for 4 hours to be conditioned to insulin before the isotope uptake was assessed. Following the preincubation period, 1 μCi of ^{3}H-2-deoxyglucose was added to each plate, and the plates preincubated in an air incubator at 37°C for 30 minutes. At the end of this incubation period, the medium was decanted, the cell layer washed 3 times with Hank's neutral salt solution, once with 0.2 N $HClO_4$ and the cell layer then scraped with 0.3 N NaOH. Aliquots of the cell layer were then counted in a Searle Mark III Liquid Scintillation Counter to determine the uptake of the ^{3}H labelled metabolite. The remaining cell fraction was analyzed for DNA, RNA, collagenous and non-collagenous protein.

(c) The Growth and Metabolic Responses of Control and Diabetic Rat Gingival Fibroblasts to Serum Modulation of Insulin Action

To assess the effect(s) of serum concentration on the responsiveness of control and diabetic rat gingival fibroblasts to insulin, the fibroblasts were maintained from the start of the 6th passage in alpha MEM with 5, 10, 15 or 20% FCS. Insulin (Sigma No. 15500) at levels of 0, 10^{-10}, 10^{-8} and 10^{-6} M was added at each serum concentration to give a total of 40 treatment groups (20 control and 20 diabetic). Insulin effects in the absence of serum were not assessed. The medium was changed on days 3, 6 and 9 and collected on days 6, 9 and 12 for hydroxyproline analyses. For each of the control and diabetic rat treatment groups, a total of 2 and 3 plates respectively for each of the serum and insulin

levels was used. Cellular DNA, RNA, non-collagenous and collagenous protein determinations were made on confluent 12 day old fibroblasts. At this time as well, the effect(s) of serum and insulin on membrane transport of simple non-metabolizable metabolites was also assessed by studying the uptake of the monosaccharide and the amino acid analogues ^{3}H-2-deoxyglucose and ^{14}C-alpha-aminoisobutyric acid respectively. In practice, confluent cultures were washed twice with Hank's neutral salt solution and fresh medium containing the respective serum-insulin concentration was then added to each plate. After the addition of 1 μCi each of ^{14}C-alpha-aminoisobutyric acid and ^{3}H-2-deoxyglucose to each plate, the cultures were incubated at 37°C for 30 minutes. At the end of the incubation period, the medium was decanted, the cell layer washed 3 times with Hank's neutral salt solution, once with 0.2 N $HClO_4$ and scraped with 0.3 N NaOH. Aliquots of the cell layer were then counted in a Searle Mark III liquid scintillation counter to determine the uptake of ^{3}H and ^{14}C labelled metabolites. The remaining cell fraction was analyzed as described for cellular constituents.

(d) The Growth and Metabolic Responses of Human Gingival Fibroblasts to Control and Diabetic Rat Serum.

Serum from diabetics is reported to be different from that of healthy individuals with regard to the concentrations and composition of growth factors, lipids and other serum constituents. To assess the effects of these serum differences on cellular proliferation and metabolism, human gingival fibroblasts were

grown in alpha MEM to which was added control or diabetic rat serum at concentrations of 5, 10 and 15%. With each type and concentration of rat serum, 9 plates were seeded to give a total of 6 treatment groups. Medium was changed on days 4 and 8 and collected on days 4, 8 and 12 for hydroxyproline determinations. On each of days 4, 8 and 12, 3 plates of fibroblasts per serum type were scraped and analyzed for cellular DNA, RNA, non-collagenous and collagenous protein. Cells at each serum type and level were photographed on days 1, 2, 5, 8 and 13.

(e) The Effect of Insulin on Ascorbate Uptake by Control and Diabetic Rat Gingival Fibroblasts

To determine the effect of insulin on the uptake of ascorbate by control and diabetic rat gingival fibroblasts, the cells were grown for 9 days in alpha MEM with 10% FCS. Insulin (Sigma No. 15500) was added at concentrations of 0 and 10^{-6} M to produce 4 treatment groups (2 control and 2 diabetic), and a total of 24 dishes (12 control and 12 diabetic). The medium was changed and discarded on days 3 and 6. On day 9, 3 plates per treatment group were scraped with 0.3 N NaOH for cellular DNA. The remaining 3 plates per treatment group were also terminated at this time and the fibroblasts scraped in the ice cold TCA for the determination of intracellular ascorbate content, using the method of Zannoni et al. (1974).

(f) The Effects of Ascorbate and its Interaction with Insulin on Human Gingival Fibroblasts

Human gingival fibroblasts were grown for 12 days in alpha MEM with 10% FCS to which was added ascorbate at 50, 100 and 200 µg/ml respectively. Since alpha MEM already contained 50 µg/ml ascorbate, L-ascorbate (Fisher, USA) was added at 0, 50 and 150 µg/ml to obtain the desired concentrations. At each concentration of ascorbate, insulin (Sigma No. 15500) at levels of 0 or 10^{-6} M was added to the medium to give a total of 6 treatment groups. A total of 108 dishes were used in this study. The medium was changed on days 4 and 8 and collected on days 4, 8 and 12. At the times of medium collection, 3 plates per treatment group were terminated and analyzed for cellular DNA, RNA, non-collagenous and collagenous protein. To determine the effects of insulin and cell age on the intracellular accumulation of ascorbate, 3 plates per treatment were also terminated on days 4, 8 and 12. The fibroblasts were removed by scraping in ice cold TCA and their ascorbate content determined by the method of Zannoni et al. (1974).

(g) The Relationship and Interaction of Cell Density, Insulin and Ascorbate on Control and Diabetic Rat Gingival Fibroblast Metabolism

To assess the impact of cell density on cellular metabolism and replication of control and diabetic rat gingival fibroblasts, cells in the 6th passage were seeded at densities of

7.5×10^4, 1.5×10^5, 6.0×10^5 and 1.0×10^6 cells/dish. In addition the effect of cell density on cellular responses to insulin and ascorbate was evaluated. Control and diabetic rat gingival fibroblasts at each cell density were grown from the start of the 6th passage in alpha MEM with 10% FCS or alpha MEM with 10% FCS to which was added 150 µg/ml L-ascorbate (Fisher), 10^{-6} M insulin (Sigma No. 15500) or both ascorbate and insulin at concentrations of 150 µg/ml and 10^{-6} M respectively. The protocol yielded a total of 32 treatment groups, 16 control and 16 diabetic, with 5 plates per group. The medium was changed on day 3 and collected for hydroxyproline analysis on day 3 and on the day at which the cells reached confluency. Fibroblasts seeded at densities of 6×10^5 and 1.0×10^6 cells/dish reached confluency at day 5, while fibroblasts at densities of 7.5×10^4 and 1.5×10^5 cells/dish were confluent at day 6. At these times of confluency 3 plates per treatment group were terminated for cellular DNA, RNA, collagenous and non-collagenous protein determinations. The remaining 2 plates per treatment group were scraped of fibroblasts in ice cold TCA for the determination of cellular ascorbate as described.

(h) The Effect of Cell Age on the Sensitivity to Insulin of Human and Rat (Control and Diabetic) Gingival Fibroblasts

Human and control and diabetic rat gingival fibroblasts were carried through a total of 24 consecutive passages respectively. Fibroblasts from diabetic rat gingiva did not sur-

vive beyond the 10th passage. Insulin effects were assessed and compared on human fibroblasts in passages 3, 6, 9, 12, 15, 20 and 24, on control rat gingival fibroblasts in passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and on diabetic rat fibroblasts in passages 3, 4, 6, and 9. Insulin (Connaught) was added to the growth medium at the beginning of each experimental passage at concentrations of 0 or 10^{-6} M. The medium was changed 3 times in 8 days at all passages (both experimental and non-experimental) and discarded. Cellular DNA, RNA, collagenous and non-collagenous protein determinations were made on 8 day old fibroblasts. Non-insulin treated fibroblasts grown in alpha MEM with 10% FCS were maintained at all cell passages to propagate the cell line.

(i) The In Vitro Effect of Streptozotocin on Control Rat Gingival Fibroblast Metabolism

To assess the effect(s) of the diabetogenic agent streptozotocin on gingival fibroblast metabolism, 63-60 mm dishes of control rat gingival fibroblasts were grown in alpha MEM with 10% FCS to which was added streptozotocin (Sigma No. S-0130) at concentrations of 0, 0.5, 1.0, 5.0, 10.0, 50.0 and 100.0 µg/ml. Nine plates at each streptozotocin level were monitored over a 9 day period. The medium was changed on days 3 and 6 and collected on days 3, 6 and 9 for hydroxyproline determinations. At each day of medium collection, 3 plates per treatment group were also terminated and the cell fractions collected for the assessment of cellular DNA, RNA, collagenous and non-collagenous protein levels.

(j) The Effects of Two Insulin Preparations with Different Concentrations of Bound Zinc on Control and Diabetic Rat Gingival Fibroblast Metabolism

Essentially all commercial insulin preparations contain some quantity of bound zinc for structural stability of the hormone. Control and diabetic rat gingival fibroblasts were maintained over a 12 day growth period in alpha MEM + 10% FCS with either of the 2 insulin preparations (Connaught with 0.2% bound Zn and Sigma No. 15500 with 0.5% bound Zn) added at concentrations of 0, 10^{-8} and 10^{-6} M. A total of 12 treatment groups, 6 control and 6 diabetic, each of 9 plates, were studied. Three plates per treatment group were analyzed on each of days 4, 8 and 12 for cellular DNA, RNA, collagenous and non-collagenous protein. Growth medium was changed on days 4 and 8 and collected on days 4, 8 and 12 for hydroxyproline determinations.

(k) The Effects of Zinc and Insulin on Control and Diabetic Rat Gingival Fibroblast Metabolism

To evaluate in vitro the effects of zinc on gingival fibroblast metabolism, control and diabetic fibroblasts were monitored over a 9 day growth period in alpha MEM + 10% FCS to which was added $ZnCl_2$ at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Insulin (Connaught) effects were assessed at hormone concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M in the growth medium. The effects of insulin-zinc interactions on fibroblast metabolism were also determined. Control and diabetic rat gingival fibroblasts

were grown in alpha MEM + 10% FCS with 10^{-8} M insulin to which was added $ZnCl_2$ at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. This treatment protocol resulted in a total of 24 treatment groups, 12 control and 12 diabetic with each group consisting of 3 plates. The medium was changed on days 3 and 6 and collected on days 3, 6 and 9 for hydroxyproline analyses. All plates in each treatment group were analyzed on day 9 only for cellular DNA, RNA, non-collagenous and collagenous protein.

(1) The Growth Rates and Metabolic Activity of Control and Diabetic Rat Gingival Fibroblasts to Varying Concentrations of Testosterone and Insulin

Control and diabetic rat gingival fibroblasts were monitored over a 12 day growth period in alpha MEM + 10% FCS to which was added testosterone (Sigma NOT-1500) at concentrations of 0, 50, 100 and 150 ng/ml. At each testosterone concentration, insulin (Connaught) was added at either 0 or 10^{-6} M, to provide a total of 144 plates consisting of 8 control and 8 diabetic treatment groups. Three plates per treatment group were terminated on day 6, 3 on day 9 and the remaining 3 on day 12. The fibroblast layers were scraped in 0.3 N NaOH and analyzed for cellular, DNA, RNA, collagenous and non-collagenous protein. The growth medium was changed on days 3, 6 and 9 and discarded.

3-4 ANALYTICAL PROCEDURES

a). Extraction of cellular DNA, RNA and protein was done according to the procedure of Patterson (1979).

b). Cellular DNA was determined by the method of Burton (1968), a modification of Giles and Myers (1965).

c). Cellular RNA was analyzed following the procedure of Schneider (1957).

d). Determination of non-collagenous protein was done according to the procedure of Lowry et al. (1951).

e). Cellular hydroxyproline analyses were done following the method of Stegemann (1958).

f). Medium hydroxyproline levels were done using the procedure of Bannister and Burns (1970). Samples were hydrolyzed in 6 N HCl for 24 hours at 110°C and then analyzed on a Technicon Auto Analyzer. Hydroxyproline is not present in alpha MEM. There is, however, a small amount of hydroxyproline present in FCS, but this amount is accounted for by using serum blanks in the analytical procedure. Since hydroxyproline is a unique amino acid found essentially only in collagen and is synthesized de novo by fibroblasts from peptide bound proline, hydroxyproline levels are, therefore, indicative of collagen production.

3-5 RADIOACTIVE DETERMINATIONS

All radioactive chemicals were purchased from the New England Nuclear Corp., Boston, Mass. All radioactive counting data were measured in a Searle Model 6880 Mark III Liquid Scintillation System with Scinti Verse I used as the fluor system. In practice 0.5-1.0 ml of the cell fraction to be counted was mixed with 15 ml Scinti Verse I in a 20 ml glass scintillation vial.

3-6 STATISTICAL ANALYSES

All data are reported at a 5% confidence level of statistical significance. The data were analyzed by use of the analysis of variance and t test and are presented as Mean $\pm$ SEM.

3-7 CHEMICALS

All chemicals were of reagent grade or of the highest purity commercially available.

CHAPTER IV

RESULTS

RESULTS

4-1 Preliminary Studies on Control and Streptozotocin Diabetic Rat Gingival Fibroblast Metabolism: A Comparison of Biochemical Parameters

The effects of *in vitro* insulin treatment on the metabolism of fibroblasts from control, diabetic and insulin treated diabetic rats are shown in Figures 2-6. These fibroblasts all showed an increase with time in cellular DNA levels over days 3, 6 and 9 (Fig. 2). Cells obtained from diabetic animals that received insulin therapy prior to fibroblast isolation produced levels of DNA similar to cells obtained from control rats. Moreover, when fibroblasts derived from insulin treated diabetic rats were further supplemented with insulin *in vitro*, their DNA content was similar to that of control cells supplemented *in vitro* with the same concentrations of insulin (Fig. 2). Diabetic cells produced the least amount of DNA. Supplementing these cells with insulin in culture did not produce significant increases in DNA levels. These data suggest that fibroblasts obtained from insulin treated diabetic animals were more responsive to insulin *in vitro* than were fibroblasts obtained from diabetic rats (Fig. 2).

All three cell preparations demonstrated a decrease in cellular RNA levels with time (Fig. 3). As was seen with cellular DNA, cellular RNA levels in diabetic rat cells were unaffected by insulin therapy *in vitro*. Control cells and those derived from insulin treated diabetic rats produced similar levels of RNA with the

exception of day 3 when higher levels of RNA were found in the fibroblasts obtained from the insulin treated diabetic rats (Fig. 3). RNA synthesis in cells isolated from insulin treated rats was not stimulated by insulin *in vitro*.

Non-collagenous protein levels (Fig. 4) in all cell groups increased with time in passage. Control rat fibroblast non-collagenous protein levels increased significantly from day 3 to 6 but showed little change thereafter. At no time and for no cell group did insulin cause a significant increase in cellular non-collagenous protein concentration. Protein synthesis initially lagged in the diabetic cells, but by day 9 approached the levels found in both the control and the insulin treated diabetic rats cells.

Medium hydroxyproline data are shown in Figure 5. Medium hydroxyproline accumulation by fibroblasts from control and insulin treated diabetic rats was not significantly stimulated by insulin (Fig. 5). Insulin treatment of diabetic rat fibroblasts significantly elevated hydroxyproline levels at days 3 and 6 (Fig. 5). Hydroxyproline levels in control and diabetic cells peaked at day 6 and in cells from insulin treated diabetic rats at day 9. Control cells secreted more total hydroxyproline into the medium than did cells of diabetic or insulin treated diabetic rats. Figure 6 shows that collagen accumulation on all 3 cell groups paralleled that of DNA.

Statistical analyses of DNA, RNA and protein data fibroblasts isolated on day 56 (Fig. 1), and all the treatment interactions are shown in Tables 1-4. Data are presented at a 5 percent level

of significance. With the exception of medium hydroxyproline, all other parameters were significantly effected by insulin treatment in vitro (Tables 1, 2, 3). DNA, non-collagenous protein and medium hydroxyproline levels were not significantly different due to the cell preparation. Cellular RNA levels were, however, significantly affected by the insulin status of the cells (Table 2).

4-2 The Effect of Senescence on the Sensitivity of Human Gingival Fibroblasts to Insulin Treatment

Figures 7-12 illustrate the effects of cell age and insulin on human gingival fibroblasts. The fibroblasts were used on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Maximum DNA levels were found at the 8th passage (Fig. 7). This observation was consistent with the occurrence of maximal cell density at passages 8 and 9 (Fig. 8). Cellular RNA, non-collagenous protein and cellular hydroxyproline levels were highest at the 5th passage, and lowest at the 8th (Figs. 9-11). The apparent inverse relationship in passages 5 and 8 between levels of DNA (Fig. 7) and comparative levels of RNA and protein suggest that when cells are rapidly dividing, other cellular metabolic pathways are slowed down.

The maximum uptake of 2-deoxyglucose observed at the 5th passage (Fig. 12) suggests that the transport of certain metabolites was also affected by cellular replicative rates. The sharp decline of 2-deoxyglucose uptake at passage 18 was more pronounced than that of cellular DNA, RNA and non-collagenous protein (Figs. 7,9,10), suggesting perhaps a greater sensitivity of membrane

transport to ageing. The effects of insulin on cellular replicative and metabolic parameters were at best variable (Figs. 7-12). Medium hydroxyproline data were not available for this experiment.

4-3 The Growth and Metabolic Responses of Control and Diabetic Rat Gingival Fibroblasts to Serum Modulation of Insulin Action

The results of serum modulation of insulin action on rat gingival fibroblasts are presented in Figures 13-18, respectively. Highest levels of cellular RNA were found at 5% FCS for both control and diabetic cells (Fig. 13). The only exception to this observation occurred in insulin "free" media, where the maximal level of RNA was seen at 10% FCS for control cells and 5% FCS for diabetic cells. For both cell groups studied, the highest level of RNA was seen at 10^{-6} M insulin. The magnitude of the serum effect was greatest for the control rat cells (Fig. 13).

The highest levels of non-collagenous protein also occurred at 5% FCS and 10^{-6} M insulin for both control and diabetic rat cells (Fig. 14). At each concentration of insulin, maximal levels of control cellular non-collagenous protein occurred at 5% FCS. In the absence of insulin, the highest levels of non-collagenous protein were observed at 10% FCS for control and 5% FCS for diabetic cells. Consistent with the cellular RNA data, the control cells demonstrated higher concentrations of non-collagenous protein than did the diabetic cells (Fig. 14). Non-collagenous protein, when expressed on a RNA basis, indicated that protein

synthesis was directly proportional to the serum concentration but was not significantly affected by insulin concentration (Fig. 15). On a RNA basis, diabetic cells produced slightly more or the same amount of protein as did the control fibroblasts (Fig. 15), while on a DNA basis, the control cells contained higher levels of protein. Cellular hydroxyproline levels were not detectable in this experiment. Similarly to the other parameters studied, medium hydroxyproline levels were highest at 5% FCS and 10^{-6} M insulin and decreased as serum levels were increased (Fig. 16). Medium from the control fibroblasts contained more hydroxyproline per cell than did the diabetic culture medium. In the absence of insulin, maximal levels of medium hydroxyproline occurred at 10% serum for control fibroblasts and 5% serum for diabetic rat fibroblasts (Fig. 16).

Uptake of ^{3}H-2-deoxyglucose by both the control and diabetic cells was maximal at 5% FCS and 10^{-6} M insulin (Fig. 17). At all insulin concentrations used, significantly greater uptake of the labelled metabolite occurred in the control cultures at 5% FCS. In insulin "free" media maximal uptake was seen at 10% FCS for control and 5% FCS for diabetic cells. Overall, control cells took up more deoxyglucose than did diabetic cells (Fig. 17).

Maximal uptake of ^{14}C-alpha-amino isobutyric acid in both control and diabetic cells also occurred at 5% FCS at all concentrations of insulin (Fig. 18). In insulin free media, maximal uptake of the amino acid metabolite occurred at 10% FCS for the control culture and at 5% FCS for the diabetic cells (Fig. 18). Control rat cells took up more total alpha-amino isobutyric acid

than did the diabetic cells.

4-4 The Growth and Metabolic Responses of Human Gingival Fibroblasts to Control and Diabetic Rat Serum

The effects of control and diabetic rat serum on human gingival fibroblast growth and metabolism are presented in Figures 19-27 and Table 5. Over the 12 days studied, the fibroblasts grown in control rat serum produced DNA in direct proportion to serum concentration (Fig. 20). Diabetic rat serum used at low concentrations did not promote fibroblast growth. Cells grown in 5% and 10% diabetic rat serum levels showed no detectable amounts of DNA (Fig. 20). The toxic effects of diabetic serum on cell growth as compared to control serum are shown in Figure 19a-e. Very low levels of DNA were detected in cultures grown in 15% diabetic serum only and these levels decreased with time in passage (Fig. 20). Although RNA levels did not appear to vary significantly with differences in control serum concentrations, they increased significantly over the 12 day growth period (Fig. 21). No detectable levels of RNA were found in the sparse cultures grown in 5% and 10% diabetic serum. These results are consistent with the DNA data for these cells (Fig. 20). However, on day 4 only, cells grown in 15% diabetic serum contained more RNA than those grown in 15% control serum. Similarly, cellular non-collagenous protein, cellular and medium hydroxyproline levels of cells grown in control rat serum and expressed on either a DNA or a RNA basis were not significantly affected by differences in

serum concentration of the growth medium (Figs. 22-27). However, non-collagenous protein levels increased significantly over the 12 day growth period, while hydroxyproline levels remained essentially unchanged. Cells grown in 15% diabetic serum showed non-collagenous protein and collagenous protein levels that were as much as 5-fold greater than levels obtained with 15% control serum (Figs. 22-27). Fibroblasts grown in diabetic serum accumulated significantly higher levels of hydroxyproline and non-collagenous protein when expressed on a RNA basis than cells grown in control serum (Figs. 23, 25, 27). Table 5 shows the effects of control and diabetic rat serum on hydroxyproline accumulation by human gingival cells. On a plate basis, cells grown in control serum accumulate about 8 times as much hydroxyproline as cells grown in diabetic serum. Hydroxyproline levels were directly proportional to control serum concentration, while 10% diabetic serum appeared to be most favorable to hydroxyproline accumulation. In the presence of control serum, about 85% of the total hydroxyproline detected was present in the growth medium. In the presence of diabetic serum, the distribution of hydroxyproline was more variable (Table 5). Cells grown in control serum produced only about 50% of their total collagen output by day 8. By comparison, cells grown in the diabetic rat serum had accumulated essentially their entire collagen output by day 8.

4-5 The Effect of Insulin on Ascorbate Uptake by Control and Diabetic Rat Gingival Fibroblasts

Ascorbate uptake by control and diabetic rat gingival fibroblasts is shown in Table 6. There was no difference in ascorbate uptake between the control and diabetic rat cells. Insulin produced a significant increase in ascorbate uptake by the control rat cells, but was without effect on the diabetic cells.

4-6 The Effects of Ascorbate and its Interaction with Insulin on Human Gingival Fibroblasts

The effects of insulin and ascorbate on human gingival fibroblast metabolism are shown in Figures 28-32 and Table 7. Cellular DNA levels were directly proportional to medium ascorbate concentrations (Fig. 28). At all levels of ascorbate, insulin treatment produced significant increases in DNA levels over cells treated with ascorbate alone (Fig. 28).

Cellular RNA levels were not significantly changed by ascorbate. Although they were elevated on days 8 and 12 by insulin treatment (Fig. 29), the RNA levels which were maximal on day 4, declined with time over the 12 day growth period. On day 4 non-collagenous protein levels were unchanged by varying ascorbate concentrations, while on day 8 they were directly proportional to ascorbate levels, and on day 12 elevated only at 200 μg/ml ascorbate (Fig. 30). Cellular hydroxyproline levels were directly proportional to ascorbate concentrations (Fig. 31). Insulin treat-

ment produced further significant increases in protein levels over cells treated with ascorbate alone (Figs. 30, 31). Medium hydroxyproline levels were unaffected by ascorbate concentration on day 4 and increased as ascorbate levels increased on days 8 and 12 respectively (Table 7). Insulin was equally stimulatory to ascorbate effects on medium hydroxyproline accumulation at all levels of ascorbate. Cellular ascorbate levels were significantly increased only at medium ascorbate levels of 200 μg/ml on day 8 (Fig. 32). Levels were further significantly increased by insulin treatment only at 200 ug/ml ascorbate on days 4 and 8 (Fig. 32).

4-7 The Relationship and Interaction of Cell Density, Insulin and Ascorbate on Control and Diabetic Rat Gingival Fibroblast Metabolism

The effects of cell density, medium ascorbate and insulin concentrations on rat gingival fibroblast metabolism are presented in Figures 33-37 and Table 8. Cell replication patterns between control and diabetic rat gingival fibroblasts varied with cell density and treatment (Fig. 33). Maximal replication of control rat fibroblasts at all treatments, except insulin, occurred at the lowest cell density (7.5×10^4 cells/dish). Maximal replication of the diabetic cells occurred at the second lowest cell density (1.5×10^5 cells/dish) at all treatments except the control treatment. At all cell densities and treatments, control cells consistently showed higher levels of DNA than did the diabetic cells (Fig. 33). Maximal RNA levels for control cells were at the sec-

ond lowest density (1.5×10^5 cells/dish) at all treatments and thereafter declined with increases in cell density. With the exception of the control treatment, diabetic cells at all other treatments accumulated most RNA at the lowest density (7.5×10^4 cells/dish) and thereafter, likewise control cell levels were inversely proportional to cell density (Fig. 34). The highest amount of RNA was produced by control cells seeded at 1.5×10^5/dish and treated with ascorbate. Non-collagenous protein levels in the control cells were unaffected by cell density, and in the diabetic cells, were directly proportional to cell density (Fig. 35). Diabetic cells contained significantly higher levels of non-collagenous protein than did control cells if treated with ascorbate or ascorbate with insulin, but not when treated with insulin alone. Cellular hydroxyproline levels were inversely proportional to cell density (Figure 36). Diabetic cells contained more cellular hydroxyproline than did control cells at all but the control treatment when seeded at the two lowest cell densities (Fig. 36). Cellular hydroxyproline levels of control cells seeded at the lowest density were markedly reduced following treatment of the cells with insulin and ascorbate either alone or together (Fig. 36). Otherwise, there was no effect of cell treatment on cellular hydroxyproline accumulation. Cellular ascorbate levels were markedly increased, especially in cells seeded at the higher cell densities with medium ascorbate levels of 200 µg/ml. Ascorbate uptake by the control cells was greatest at the lower cell densities seeded (Fig. 37) and increased by insulin when ascorbate was present at 200 µg/ml. Cell density and medium

ascorbate concentrations had similar effects on the cellular ascorbate content of the diabetic cultures. In addition, insulin had a stimulatory effect on the ascorbate content of the diabetic cells at medium ascorbate concentrations of 50 μg/ml, a finding not seen with the control cultures (Fig. 37). Accumulation of hydroxyproline in the medium of the control and diabetic fibroblast cultures was generally reduced following treatment with insulin, ascorbate or both combined (Table 8). With the exception of insulin treatment, medium hydroxyproline levels of control cells varied inversely with cell density (Table 8). By contrast, and with the exception of the highest cell density, medium hydroxyproline levels of the diabetic rat cultures increased with cell density (Table 8). The effects of insulin and ascorbate treatment on medium hydroxyproline in the experiment were most inconsistent.

4-8 The Effect of Cell Age on the Sensitivity to Insulin of Human and Rat (Control and Diabetic) Gingival Fibroblasts

A comparison of age and insulin effects on human and rat gingival fibroblast growth and metabolism are shown in Figures 38-45. Human gingival fibroblasts produced maximal levels of DNA at the 2nd and 6th passage (Fig. 38). The effects of insulin on cell replication were somewhat variable, since only 4 of the 7 passages studied showed an increase in DNA levels following insulin treatment (Fig. 38). Cellular RNA content of the cells was maximal in the 6th passage and generally decreased thereafter with passage number (Fig. 39). Insulin effects on RNA levels were

variable, but the cells did show an insulin sensitivity in the 12th and 24th passages. Non-collagenous protein levels increased with passage number and were stimulated by insulin in the 3rd and 12th passages (Fig. 40). Cellular hydroxyproline levels in non-insulin treated cells were maximal at the 6th passage and declined thereafter (Fig. 41) with cell age. Cellular hydroxyproline content subsequent to the 12th passage appeared to respond to insulin treatment, an observation not made with these cells in earlier passages.

Cellular DNA content of rat gingival fibroblasts was maximal in the 4th passage for diabetic and in the 4th and 24th passages (Fig. 42) for control rat cells. Fibroblast growth was not stimulated by the addition of insulin to the medium. Diabetic cells consistently produced less DNA than control and did not survive in culture past the 10th passage. All fibroblasts, both control and diabetic, showed decreased replicative rates with age. However, just before cell death, there was a marked increase in cellular replication as indicated by the increased DNA levels seen at the 9th passage for diabetic cells and the 24th for control cells (Fig. 42). Cellular RNA levels peaked in the 4th passage for both control and diabetic cells and showed a general decrease with increasing passage number for only the control cells (Fig. 43). As with cellular DNA, cellular RNA levels increased just prior to cell death. These data are seen in the 9th passage for diabetic and the 24th passage for the control rat cells. RNA levels in control and diabetic cells were generally quite similar over passages 3, 4, 6 and 9. The effects of insulin on RNA levels in this

study are not clear. Cellular non-collagenous protein levels were very similar for both cell groups and, in general, no response to insulin treatment was noted (Fig. 44). Cellular hydroxyproline levels were maximal at the 4th and 24th passages for control cells and the 4th and 9th for diabetic rat cells (Fig. 45). Medium hydroxyproline data were not available for this senescence study of human and rat gingival fibroblasts.

4-9 The In Vitro Effect of Streptozotocin on Rat Gingival Fibroblast Metabolism

The *in vitro* effects of streptozotocin on control rat gingival fibroblast metabolism are presented in Tables 9-13 and Figure 46. Although cellular DNA levels were not affected by varying streptozotocin concentration (Table 9), they increased normally with time over the 9 day growth period. RNA levels also were unaffected by streptozotocin at any of the concentrations studied (Table 10), but were highest at day 3 when DNA levels were very low and then diminished with time to day 9. Cellular RNA varied inversely with cellular DNA levels. Non-collagenous protein levels were also unaffected by streptozotocin concentration, but increased with time from day 3 to 6 at which time they plateaued (Table 11). Cellular hydroxyproline levels were unaffected by streptozotocin at concentrations under 100 µg/ml (Table 12). The higher concentrations of streptozotocin added *in vitro* appeared to affect the cellular content of hydroxyproline. On day 6, this level of streptozotocin had produced a significant

increase in cellular hydroxyproline content, while by day 9 it resulted in a marked reduction in hydroxyproline content, suggesting an effect of streptozotocin on either or both the cellular synthesis of collagen or of its transport into the extracellular medium (Table 12). Medium hydroxyproline levels on a DNA basis were unaffected by streptozotocin on day 3 (Table 13). However, on days 6 and 9, streptozotocin at all concentrations but 0.5 µg/ml on day 6 produced significant increases in medium hydroxyproline accumulation over cells grown in the absence of streptozotocin. Although the data in Table 13 do not clearly indicate an optimal concentration of streptozotocin for maximal hydroxyproline accumulation, the data in Figure 46 show that cummulative hydroxyproline levels (cellular + medium) on a plate basis were directly proportional to streptozotocin concentration. Collectively, these data show streptozotocin to be without effect on cellular replicative rates, RNA and non-collagenous protein accumulation. However, collagenous protein accumulation in the growth medium was significantly increased by its presence. Streptozotocin may act to enhance the extracellular transportation of procollagen.

4-10 The Effects of Two Insulin Preparations with Different Concentrations of Bound Zinc on Control and Diabetic Rat Gingival Fibroblast Metabolism

The effects of two insulin preparations, each containing a different level of bound zinc on the growth and metabolism of control and diabetic rat gingival cells, are presented in Figures

47-51 and Table 14. Maximal levels of DNA on all 3 days occurred at 10^{-6} M insulin with 0.2% bound zinc (Fig. 47). Diabetic culture produced less DNA than did the control cells at all treatments during the 12 day growth period (Fig. 47). Maximal amounts of RNA were produced by the control cells at 10^{-8} M insulin (0.2% bound zinc), and by the diabetic cells at 10^{-8} M insulin (0.5% bound zinc) (Fig. 48). On days 4 and 12, the control rat cells showed similar to or higher levels of RNA than did the diabetic cells, but on day 8, RNA levels in the control cultures were significantly increased (Fig. 48). Insulin effects on non-collagenous protein levels paralleled those seen with RNA. The control cells contained maximal levels of non-collagenous protein at 10^{-8} M insulin (0.2% bound zinc) while the highest levels in the diabetic cells were found at 10^{-8} M insulin (containing 0.5% bound zinc) (Fig. 49). When expressed on a RNA basis (Fig. 50), the greatest non-collagenous protein levels were found throughout the 12 day growth period in the control cultures. The highest levels of non-collagenous protein in control cells were produced in the absence of insulin, and in the diabetic cells at 10^{-8} M insulin (0.2% bound zinc) on days 4 and 8. Cellular hydroxyproline data are presented in Figure 51. Hydroxyproline levels of control cells were increased by both insulin preparations, although the increases were variable due to both insulin type and concentration. Maximal levels of cellular hydroxyproline in diabetic cells occurred at days 8 and 12 at 10^{-8} M insulin (containing 0.5% bound zinc). Medium hydroxyproline accumulation by control and diabetic rat cells is shown in Table 14. Medium

hydroxyproline accumulation by both cell types on day 4 was maximal at 10^{-8} M insulin (containing 0.5% bound zinc), although on days 8 and 12, hydroxyproline levels by both control and diabetic rat cells were greatly diminished in the presence of insulin.

4-11 The Effects of Zinc and Insulin on Control and Diabetic Rat Gingival Fibroblast Metabolism

The effects of zinc and insulin on control and diabetic and rat gingival fibroblast metabolism are presented in Figures 52-55 and Table 15. Control cells produced more DNA than did diabetic cells at all treatments (Fig. 52). Maximal DNA levels were found in cells grown in the the presence of insulin alone (Fig. 52). DNA levels were directly proportional to insulin concentration. In contrast, zinc was inhibitory to cell growth. The reduction in cellular DNA due to zinc paralleled medium zinc concentration. A similar effect was observed when the cells were treated simultaneously with both insulin and zinc. The stimulatory effect of insulin decreased as the concentration of zinc was increased (Fig. 52). These data collectively suggest that zinc is inhibitory to cellular growth.

RNA levels were not changed by different types of treatment, except when zinc and insulin combined in 10^{-8} M insulin with 10^{-10} and 10^{-8} M zinc respectively diminished levels of RNA in control and diabetic cells (Fig. 53). Varying the levels of zinc or insulin did not cause any significant differences in control cell RNA levels, although diabetic cell RNA levels were diminished at all

levels of insulin (Fig. 53). Cellular non-collagenous protein levels were consistently higher in the control cells irrespective of treatment (Fig. 54). Moreover, the treatments described were without apparent effect on non-collagenous protein levels in either the control or diabetic cultures. Cellular hydroxyproline data are shown in Figure 55. Insulin increased the cellular content of hydroxyproline by the control cells, but was without effect on the diabetic rat fibroblasts. Zinc at all concentrations used, markedly reduced the cellular levels of hydroxyproline in both cell groups when present alone in the medium or added with insulin (Fig. 55). Regardless of treatment, the control cultures consistently contained higher levels of hydroxyproline. The presence of either zinc or insulin alone in the medium resulted in a significant increase of hydroxyproline levels in both control and diabetic rat gingival fibroblasts, although maximal hydroxyproline levels occurred at insulin treatment (Table 15). Zinc was more stimulatory to the medium hydroxyproline content of diabetic than control cells.

4-12 The Growth Rates and Metabolic Activity of Control and Diabetic Rat Gingival Fibroblasts to Varying Concentrations of Testosterone and Insulin

The effects of testosterone, insulin and their interaction on control and diabetic rat gingival fibroblast metabolism are presented in Tables 16-19. Replicative rates of control fibroblasts were significantly higher than those isolated from the gin-

giva of diabetic rats (Table 16). There did not appear to be any significant effect of testosterone on DNA synthesis of control cells, although testosterone increased DNA levels in diabetic cells (Table 16). In the presence of testosterone there was no significant stimulation due to insulin treatment (Table 16). However, in the absence of testosterone both control and diabetic rat fibroblasts showed increased levels of DNA when grown in the presence of insulin (Table 16).

Both control and diabetic fibroblasts showed no significant differences in RNA levels due to testosterone treatment (Table 17).

Fibroblasts from the control rats produced more non-collagenous protein than did those isolated from the gingiva of diabetic rats (Table 18). Non-collagenous protein levels in both fibroblast groups increased significantly over the 12 day growth period (Table 18). Only testosterone concentrations of 100 and 150 ng/ml with 10^{-6} M insulin decreased control cell non-collagenous protein levels compared to cells at other treatments at day 6 (Table 18). Overall, non-collagenous protein levels were unchanged by insulin treatment (Table 18).

Cellular hydroxyproline levels in both control and diabetic fibroblasts were unchanged by testosterone at any of the concentrations studied, except at day 9 when insulin and 150 ng/ml testosterone produced increased levels of hydroxyproline (Table 18). In general, there was no significant change in hydroxyproline levels due to insulin treatment (Table 19). Hydroxyproline levels in control and diabetic fibroblasts increased with time from day 6 to

9 and then decreased from day 9 to 12 (Table 19). No data were available for medium hydroxyproline.

CHAPTER V

DISCUSSION

DISCUSSION

It is generally been thought that with the exception of varying rates of replication, all fibroblasts in a given connective tissue are similar and that all serially subcultured fibroblasts contain heritably identical phenotypes (Narayanan and Page, 1983). However, recent data suggest otherwise. For example, Engel et al. (1980) reported the presence of functionally distinct subpopulations of human gingival fibroblasts, some of which produced both types I and III collagen and others which produced type I only. In addition, Hassell and Stanek (1983) reported significant differences in proliferative rates, replicative life spans and protein and collagen synthesis of 6 mass cultures derived from a single biopsy of a healthy gingiva papilla tip.

Fibroblasts from gingiva which are constantly under the threat of invasion by plaque, may maintain some subpopulations in an active state. Any changes in the cellular environment, e.g. systemic drug therapy may affect additional subpopulations and subsequently change the composition of various resident subpopulations.

Cell selection of this kind could play an important role in the pathogenesis of diseases affecting the connective tissue. Moreover, the systemic use of streptozotocin in our studies to induce diabetes may in fact be triggering a different set of subpopulations and we, in using these cells in culture, may be studying cells that are phenotypically different from those obtained from control rats. This may in part explain the observed differ-

ences in cellular behaviour between these two cell types.

In diabetes mellitus normal cellular metabolism is disrupted and even with insulin therapy, the quality of metabolic control produced by the exogenous insulin is not the same. The stimulatory effect of insulin on cultured mammalian cells which results in increased cellular DNA (Rechler and Podskalny, 1974), RNA (Fujimoto and Williams, 1974) and protein synthesis (Jefferson, 1980) has been well documented. The data described in this thesis indicate that diabetic rat cells in tissue culture consistently showed lower replicative rates as measured by DNA/plate. These results are completely supported by the findings of Rowe et al. (1977-1) and Goldstein et al. (1979) who reported decreased replicative rates in cultures of skin isolated from human diabetics and of Harrison (1982), who reported decreased replicative rates in gingival fibroblasts of diabetic rats. Diabetic rat cells tended to produce less cellular RNA and protein as compared to the control cells or to cells isolated from insulin treated diabetic rats. While this lower metabolic rate of the diabetic cells in culture may be attributed to the diabetic state of the animals from which they were obtained, of equal importance may be the fact that lower replicative rates of diabetic cells as compared to the control cultures (fewer fibroblasts per plate) resulted in equal or lower RNA and protein levels per cell as compared to the control cells. Harrison (1982) found that whereas diabetic cell dishes contained only 86% of the number of cells found in control dishes at the end of the first subculture, this fraction was further reduced to approximately 48% by the end of the fourth subcul-

ture. All experiments reported in this thesis (except senescence studies) were performed on fibroblasts in the 5th or 6th subculture.

No reports to our knowledge have documented the metabolic study of fibroblasts obtained from insulin treated diabetic rats. Our data show that gingival fibroblasts isolated from these animals and maintained in tissue culture behaved metabolically very similarly to fibroblasts obtained from control rats. This similarity was observed in DNA, RNA and protein levels of the two cell preparations. Fibroblasts obtained from insulin treated diabetic rats sacrificed on day 28 did not seem to respond to insulin *in vitro*. However, fibroblasts from these animals which were sacrificed on day 56 of the experiment showed a definite stimulatory response to exogenous insulin which was reflected in an increase in cellular DNA, RNA and protein synthesis. The data further show that supplementing the growth medium of these cells with insulin generally produced no significant change in the levels of medium hydroxyproline for all cell types isolated from rats sacrificed on either day 28 or 56. The lack of stimulation of collagen secretion by insulin has been reported by Villee and Powers (1977), who showed that levels of insulin as high as 0.1 u/ml were without effect on collagen secretion by human skin fibroblasts. Cellular DNA, RNA and non-collagenous protein levels of cells of rats sacrificed on day 56 were insulin sensitive. These results suggest that different responses of the cellular parameters to exogenous insulin treatment may result as a consequence of the different type of animal treatment.

In the study of senescence on human gingival fibroblasts, the lowest DNA levels were detected at the 5th passage where maximal RNA and protein synthesis and the cellular uptake of 2-deoxyglucose occurred. Thus, it appeared that cells actively involved in intracellular metabolic processes demonstrate decreased replicative rates. Consistent with the occurrence of elevated cell density (Fig. 8), maximal DNA levels occurred in human gingival fibroblasts at the 8th passage and generally decreased thereafter. This is in accordance with several reports in the literature that document the peaking of DNA levels in early passages which decreased thereafter with an increase in age in vitro (Weissman-Shomer and Fry, 1975; Schneider and Fowlkes, 1976; Linn et al., 1976; Mitsui and Schneider, 1976). Schneider and Fowlkes (1976) have proposed that in human fibroblasts, a decrease in cellular DNA content was due to several factors including an increased frequency of chromosomal deletions, a decreased amplification of specific DNA genes, or a loss of cellular DNA. However, Lima et al. (1972) found that almost 100% of chick fibroblasts grown over 30 subcultures entered into DNA synthesis during their whole lifespan. Growth decline could be due mainly to an increase in cell death associated with a higher sensitivity to cell crowding, which implies involvement of the cell membrane. Data on the involvement of the cell membrane in senescence comes from the work of Schneider and Fowlkes (1976) and Mitsui and Schneider (1976), who have suggested that cell membrane alteration in human fibroblasts could be the primary event leading to a loss in replicative activity. The stimulatory effect of insulin on DNA synthesis has

been documented (Vaheri et al., 1973). Under our experimental conditions there was a general lack of response to insulin which was independent of cell age. Our cells were incubated in 10^{-9} M insulin for 4 hours. At this level of insulin we observed no significant stimulation of DNA synthesis. This is in agreement with the data of Hollenberg and Cuatrecasas (1973), who found that insulin at physiological levels (10^{-10} M, especially if used along with serum in the growth medium) had no observable effects on DNA synthesis *in vitro*.

Cellular RNA was also affected by cell age. The highest RNA levels occurred in cells in the 5th passage. Lowest RNA levels were seen at the 8th passage, the passage at which maximal DNA synthesis and cell density occurred. Schneider and Shorr (1975) have reported a decrease in RNA synthesis in logarithmically growing cultures. In addition, Bowman et al. (1976) found a decrease in RNA synthesis in human fibroblasts as senescence progressed. Our results are in agreement with both of these findings.

The highest levels of non-collagenous protein were also found at the 5th passage. Similarly, as was the case with RNA, the lowest levels of non-collagenous protein occurred at the 8th passage, suggesting that the rapid rates of cellular proliferation, which did not favour RNA synthesis also had similar effects on non-collagenous protein synthesis. Several studies have documented the accumulation of intracellular protein in senescent cells (Orgel, 1973; Fry and Weisman-Shomer, 1976; Kaftory et al., 1978). Capecchi et al. (1974) found mammalial cells to have a very sensitive mechanism for degrading proteins. Kaftory et al. (1978),

however, suggest that there is a decrease in protein breakdown due to senescence rather than retarded cell proliferation. Overall, a decrease in protein levels with age, could suggest either that defective proteins are being degraded (Capecchi, 1974), or that total non-collagenous protein synthesis is decreased. These data agree with the reports of Salcedo and Franzblau (1981) and of Hildebran et al. (1983), who documented a decrease in protein synthesis with increasing cell age in culture. Cellular hydroxyproline accumulation was very sensitive to senescence. There were no detectable levels at the 12th passage presumably due to either the degradation of faulty molecules (Capecchi et al., 1974), decreased synthesis, or increased rates of secretion of collagenous protein into the medium, the levels of which we did not determine. Salcedo and Franzblau (1981) and Hildebran et al. (1983) showed that rat muscle and human skin fibroblasts respectively did not produce as much total collagen relative to total protein as they aged. This finding is in complete agreement with our data which show a general decrease in cellular collagenous protein levels with age, and non-collagenous protein to represent a significantly larger fraction of the total cellular protein content.

The uptake of 2-deoxyglucose by human gingival fibroblasts was extremely sensitive to senescence and showed a general decrease with increasing passage number to the 18th passage at which point the lowest level of ^{3}H-2-deoxyglucose uptake occurred. Insulin at 10^{-9} M did not significantly stimulate the uptake of ^{3}H-2-deoxyglucose over that by cells which received no insulin.

This lack of insulin effect was similarly observed with cellular DNA, RNA and protein levels. In our study, cells had been incubated with insulin at 37°C for 4 hours. Marshall and Olefsky (1980), studying rat hepatocytes, reported that incubating the cells with insulin for 4 hours decreased the percentage of insulin bound to the cell receptors. Moreover, this decrease in insulin binding to receptors was directly proportional to the concentration of insulin used (1 - 100 ng/ml). In addition, these workers also showed that cells incubated for 4 hours with 100 ng/ml insulin internalized only 58% as much as ^{3}H-2-deoxyglucose as did control cells receiving no insulin. While our data did not show such a marked inhibition in ^{3}H-2-deoxyglucose uptake by the insulin treated cells, they did show that the insulin did not facilitate an increased uptake of ^{3}H-2-deoxyglucose. Eng et al. (1980) and Kaplan (1980) have reported a decrease in the number of insulin receptors on human erythrocytes and monocytes as these cells matured. Thus, although only 5% of adipocyte insulin receptors need to be occupied to produce maximal stimulation of glucose transport (Kono and Barham, 1971), the decrease in number of receptors with age, coupled with the insulin induced decrease of hormone binding, may together produce a decrease in ^{3}H-2-deoxyglucose internalization and explain the lack of insulin stimulation observed in our studies. The low ^{3}H-2-deoxyglucose uptake seen at the 8th passage may well be due at least in part to the rapid cellular proliferation which occurred at this time.

Insulin within the physiological range (10^{-9} M) and in the presence of serum was insufficient to stimulate human gingival

fibroblasts to divide and metabolize at higher rates than did cells grown and maintained in the absence of added insulin. Membrane transport of glucose appeared more sensitive to senescence than did the other intracellular parameters measured, e.g. DNA, RNA and protein.

The effects of serum on insulin stimulation of control and diabetic rat gingival fibroblasts showed that serum inhibited the stimulatory effect of insulin on these cells in tissue culture. The reports of others (Vaheri et al., 1973; Fujimoto and Williams, 1974) which show that insulin does stimulate RNA synthesis were confirmed in our studies. The highest levels of RNA occurred at the lowest serum concentration we used (5% FCS) in combination with the highest and non-physiological insulin concentration (10^{-6} M). Fujimoto and Williams (1972) found that the presence of serum obscured any potential effect of insulin on the conversion of glucose to CO_2. Consequently, in a later study (1974) these workers removed serum from their culture medium 24 hours prior to adding insulin and observed a clear effect of insulin at concentrations of 0.1 mU (approximately 10^{-10} M) on uridine incorporation into RNA. In addition, it seemed that the inhibitory effect of serum on insulin also affected protein synthesis, since these workers further reported that cells maintained without serum responded to physiological levels of insulin (10^{-10} M) with an increased rate of protein synthesis. These observations of Fujimoto and Williams (1974) are consistent with our results in that we found the highest levels of protein (both non-collagenous and collagenous) to occur in the presence of lowest serum concentration and at the

highest insulin concentration (10^{-6} M) studied. Membrane transport phenomena are also sensitive to the serum-insulin interaction. The stimulatory effect of insulin on alpha-amino isobutyric acid uptake has been well documented (Elsas et al., 1971; Goldfine and Gardner, 1972; Risser and Gelehrter, 1973; Martin and Pohl, 1974). Goldfine et al. (1972) for example, have shown that insulin at concentrations as low as 9.0×10^{-10} M stimulated alpha-amino isobutyric acid uptake with the maximum effect observed at 4.0×10^{-8} M insulin. These insulin effects were well within range of our data which demonstrated the uptake of alpha-amino isobutyric acid at insulin levels as low as 10^{-12} M. In our studies, the maximal uptake of ^{14}C-alpha-amino isobutyric acid occurred at the lowest serum concentration and highest insulin level. Risser and Gelehrter (1973) observed an insulin effect at about 7×10^{-7} M and in the absence of serum on alpha-amino isobutyric acid transport in rat hepatoma cells incubated for 30 minutes with a maximum effect at 2 hours. This period of incubation is within the range of our incubation time of 30 minutes. Hollenberg and Cuatrecasas (1975) have also reported insulin stimulation of alpha-amino isobutyric acid transport, but only at concentrations well above the physiological range. Their maximum effect was observed at 10^{-8} M insulin. Although we observe insulin stimulation of membrane transport at super physiological levels, our data also showed that the membrane transport of alpha-amino isobutyric acid was stimulated by physiological levels of insulin, especially when serum is either absent or present in low concentrations. Contrary to our findings, Minemura (1970) observed no effects on

alpha-amino isobutyric acid transport in rat adipocytes incubated with insulin at a concentration of 7 mU/ml (approximately 7 X 10^{-9} M) in serum free media. These workers concluded that insulin effects were exerted at some unidentified step in protein synthesis rather than on the transport of amino acid.

Insulin stimulates 2-deoxyglucose uptake by cultured cells (Vaheri et al., 1973 Howard et al., 1979; Ishibashi et al., 1981). Our results indicate that maximal uptake of 2-deoxyglucose occurred at 5% FCS and 10^{-6} M insulin. These results agree with those of Ishibashi et al. (1981) who also found that insulin in serum free media stimulated 2-deoxyglucose uptake in human skin fibroblasts at levels as low as 10^{-10} M. They found their maximum effect at 10^{-7} - 10^{-6} M insulin. Further support for our observations come from the work of Howard et al. (1979) who found that insulin at concentrations as low as 10^{-10} M in serum free media stimulated 2-deoxyglucose uptake by human diploid fibroblasts. In their study, half maximal uptake occurred at approximately 3 X 10^{-9} M insulin. Moreover, Howard et al. (1979) found as we also observed, that insulin action on 2-deoxyglucose uptake occurred within a short period of time (30 minutes). On the basis of their findings that insulin stimulated glucose uptake by cells maintained in serum free medium, Fujimoto and Williams (1974) suggest a decreased effect of insulin in the presence of serum. Our results confirm these observations and show, in addition, an inverse relationship between medium serum and insulin concentrations as measured by the insulin responsiveness of numerous cellular metabolic parameters. In addition, at all concentrations of

serum and insulin used in our studies, we found that control cells took up more ^{3}H-deoxyglucose than did diabetic cells. This finding is supported by the work of Kobayashi and Olefsky (1979), who reported that diabetic rat adipocytes took up less glucose than did control cells, apparently due to a decreased responsiveness to insulin, although these cells bound more total insulin than did the control cells.

Optimal concentration of serum for promoting growth in cultured cells is 10-20% FCS (Gospodarowicz and Moran, 1976). Most studies use 10% FCS. In accordance with this information, we found that in the absence of exogenous insulin, maximal DNA, RNA, protein levels and membrane transport occurred in control cells grown in 10% FCS and in diabetic cells at 5% FCS. In the presence of insulin, maximal cellular activity was seen at the lowest serum concentration (5%) and the highest insulin level (10^{-6} M). Narayanan and Page (1977) reported that serum lowers the hydroxyproline/proline and hydroxylysine/lysine ratios, indicating a decrease in collagen synthesis caused by serum. Collagen synthesis was inhibited above 20% serum. Fujimoto and Williams (1974) suggest that cells maintained in serum free medium were responsive to physiological levels of insulin while our data, and that of Hollenberg and Cuatrecasas (1975) both suggest that cells maintained in serum supplemented media require higher than physiological amounts of insulin to evoke cellular responses. We did not assess cells grown in serum free media. Thus all of the available data agree that serum significantly reduced the cellular response to insulin in culture.

The mechanisms of this interaction of serum and insulin warrant further investigation. It may well be that serum contains protein capable of binding insulin. This could be investigated by the use of ^{125}I insulin binding studies using both regular and heat inactivated serum. If serum contains small molecules that inhibit or destroy insulin, these may be dialyzed out of the serum and the remaining serum fraction used in a repeat of this study.

A few recent studies have investigated the growth sustaining effects of serum other than fetal calf on a variety of mammalian cells. For example, Murphy and Lazarus (1983) have described a stimulatory effect of control rat serum on the replicative rate of human skin fibroblasts. Their observations are consistent with the results of our study on the effects of varying concentrations of control and diabetic rat serum on human gingival fibroblasts, which demonstrate a dose dependent increase in replicative rates of human gingival fibroblasts grown in media with normal rat serum. Diabetic rat serum, however, was inhibitory to human cell growth in culture. The inhibition increased both with serum concentration and with time in passage. In general, fibroblasts in our study which were grown in diabetic serum, showed minimal replication, did not attach and in essence, showed characteristics of dying cells. These observations are in agreement with those described by Arbogast (1982), who found that porcine aortic cells grown in 17% diabetic rat serum were severely contracted, 70% Trypan Blue positive and by day 4, only small islands of cells remained. Our cells grown in 5 and 10% diabetic rat serum showed no detectable levels of DNA, while cells in 15% serum showed

barely detectable levels (Fig. 20). This may be due to the detachment or non-attachment of cells with arrested metabolic function and their subsequent loss by being washed away in the medium (Fig. 19a-e). The inhibitory effect of diabetic serum on cells in culture has been attributed to the toxicity of a very low density lipoprotein fraction in the sera (Arbogast et al., 1982; Chi et al., 1982) and a lack of somatomedins and somatomedin inhibitory factor(s) (Phillips et al., 1979-1,2). On the other hand, normolipemic diabetic serum has been shown to stimulate the growth of cells in vitro (Ledet, 1976; Ledet et al., 1976, 1977). The diabetic rat serum used in our study contained a much higher lipid concentration than did the control serum. Visually the lipids could be readily observed in the freshly drawn rat serum as well as floating in the prepared medium. Therefore, the high level of lipid may have contributed at least in part to the toxic effect we observed on cell replication. The widespread detrimental effect of diabetic serum on cell replication may in part be responsible for the enhanced inflammatory state and/or the reduced rate of wound healing observed in diabetics. Whether there was a lack of somatomedins or there were somatomedin inhibitors present in our diabetic serum is unknown to us since we did not assay for either the presence of these substances or their inhibitors.

Of great interest was the data which indicated that while diabetic serum was toxic to cellular growth, human cells grown in diabetic serum contained slightly more RNA and significantly more collagenous and non-collagenous protein than did the same cells grown in the same concentration of control rat serum. These

results are in agreement with those of Ledet and Vuust (1980), who found that factor(s) in normolipemic human insulin dependent diabetic serum enhanced the production of procollagen type I and fibronectin by rabbit aortic myomedial cell cultures. Their cells grown in 10% diabetic serum produced more procollagen type I and fibronectin than did cells grown in 10% normolipemic control serum. These workers further reported that although they were unsure of the specific source of stimulation of collagen production, a more pronounced effect might be seen using serum from animals in a more poorly controlled diabetic state. The diabetic serum used in our study was obtained from rats that had received no insulin therapy and were consequently in a poorly controlled diabetic state. These animals readily exhibited a variety of criteria well described for the diabetic state including urine glucose levels that were at least 2 g % (Howland and Zebrowski, 1974). We also found pronounced differences in collagen production between the cells grown in control and diabetic serum. For example, on a cell basis, cells grown in control serum produced as little as 10-15% the amount of collagen produced by cells grown in diabetic serum from poorly controlled diabetic rats. The study of Ledet et al. (1980) used normolipemic serum from patients with routine insulin therapy and some of which had glycosuria. In our study, diabetic serum contained considerably higher amounts of total lipid. Therefore, although Ledet et al. (1980) did not attribute their enhanced cellular cellular production of collagen to ketone bodies, hyperglycemia or insulin in the diabetic serum, we have no evidence not to rule out the possibility of the stimu-

latory effect originating in the lipid fraction and this in fact may have been the source of the factor(s) which enhanced the collagen production by diabetic serum. Cells grown in all the levels (5, 10, 15%) of the control serum contained about 14% intracellular collagen, while the remaining 86% was found in the medium. A significantly different cell medium distribution of collagen was found when the cells were grown in diabetic rat serum. With the exception of cells grown in 10% diabetic serum, these cells contained approximately 20-30% of collagen intracellularly, with the remaining 70-80% secreted into the medium (Table 5). Whether this pattern reflects a slow down of the collagen secretion system caused by the diabetic serum, is presently uncertain. However, the cells grown with normal rat serum produced approximately 50% of their total collagen production by day 12, approximately 35% by day 8 and approximately 15% by day 4, while cells grown in medium with diabetic serum produced 98-99% of collagen between days 0 and 8 and almost none between days 8 and 12. Thus it appears that the mechanisms of collagen synthesis are regulated differently for cells grown in control and diabetic rat serum.

Collectively, the results suggest that human gingival cells grown in medium containing normal rat serum behave differently from cells grown in medium with diabetic rat serum. The diabetic serum was inhibitory to DNA synthesis and cellular replication, but was stimulatory to RNA, non-collagenous and collagenous protein synthesis. While it was not possible to identify the serum factor(s) that caused the alteration in cellular behaviour, the available literature would suggest the elevated lipid levels in

diabetic serum to be a likely source.

Our study of the effects of ascorbate and of its interaction with insulin on human gingival fibroblasts indicates a significant stimulatory effect of ascorbate on DNA synthesis in direct proportion to ascorbate concentration (Fig. 28). This finding is in accordance with results of Rowe et al. (1977-2), Peterkofsky and Prather (1977) and more recently, Krystal et al. (1982). However, Krystal et al. (1982) showed that ascorbate was stimulatory to DNA synthesis of rabbit articular chondrocytes in organ and cell culture only at a limited range of concentrations (10-40 µg/ml), and killed the cells at concentrations of 160 µg/ml. We found human gingival cells to demonstrate maximal replicative rates at ascorbate levels of 200 µg/ml. These data may reflect a higher requirement of gingival tissue for ascorbate, compared to other skin fibroblasts. Ascorbate levels are known to be greater in tissue with high turnover rates. Since gingival tissue has one of the highest turnover rates of any tissue (Page and Ammons, 1974; Sodek et al., 1977), it, therefore, would not be unreasonable to expect a stimulatory response by ascorbate in gingival tissue. However, contrary to our findings, Levene and Bates (1970) and Tajima and Pinnell (1982) found no significant affect of ascorbate on cellular proliferation in mouse and human skin fibroblasts. Furthermore, Levene and Bates (1970) found that proliferation of ascorbate treated mouse fibroblasts decreased with time in culture, contrary to our findings. Our observations of no significant stimulation of RNA by ascorbate agrees with the findings of Tajima and Pinnell (1982), who found no significant differences in

RNA levels between cells treated with (100 μM) or without ascorbate.

Our results indicate that ascorbate was stimulatory to non-collagenous protein levels, but only at 200 μg/ml medium ascorbate (Fig. 30). Non-collagenous protein levels decreased with time over the 12 day culture period, an observation made also by Levene and Bates (1970). They found a decrease with age in culture in the protein levels of ascorbate (50 μg/ml) treated cells over a 14 day growth period, with this level of ascorbate producing no effect on non-collagenous protein synthesis. However, Tajima and Pinnell (1982) found no significant stimulation of non-collagenous protein synthesis in cultured human skin fibroblasts treated with (100 μM) or without ascorbate, while this level of ascorbate, being much higher than the range used in our study, produced a stimulatory effect on collagen synthesis as measured by procollagen in RNA.

Collagenous protein synthesis by human gingival fibroblasts was extremely sensitive to ascorbate. Whereas levels of cellular hydroxyproline were directly proportional to the concentration of ascorbate used, medium hydroxyproline accumulation was increased only at 100 and 200 μg/ml ascorbate on days 8 and 12 (Fig. 31, Table 7). This indicates increased collagen production at high ascorbate levels, resulting in greater secretion into the medium. Moreover, our findings as well as those of Tajima and Pinnell (1982), indicate that the stimulatory effect of ascorbate on collagenous protein was more pronounced than for non-collagenous protein. This is further supported by the work of Chen and

Postlethwait (1970), who in studying guinea pig tissue, suggested that collagen was the only protein effected by the addition of ascorbate to the growth medium. Unlike cellular RNA or non-collagenous protein, only cellular and medium hydroxyproline levels increased with time in culture. This concurred with the results of Levene and Bates (1970), who found that collagen synthesis in mouse fibroblasts continued at a steady or increased rate with ageing in culture over days 8, 10, 12 and 14.

Insulin has been shown to stimulate the uptake of ascorbate in cells grown in vitro (Mann and Newton, 1975; Verlangieri and Sestito 1981; Dowse et al., 1983). Verlangieri and Sestito (1981) reported that increasing insulin levels produced an increased uptake of ascorbate into foetal bovine endothelial cells. In this study, maximum ascorbate uptake occurred at 4×10^{-4} U/ml insulin and levelled off thereafter. Similarly, the uptake of ascorbate by human gingival fibroblasts under our experimental conditions was significantly elevated at a concentration of 200 µg/ml ascorbate in the growth medium, and was further increased significantly at only this level of ascorbate by insulin at levels of 10^{-6} M. The stimulatory effect of insulin on the metabolism of cells grown in tissue culture is well known. Rowe et al. (1977-2) claimed that insulin in concentrations greater than physiological levels (10^{-10} - 10^{-11} M) decreased the stimulatory effect of ascorbate. Our results are in direct conflict with these since insulin at 10^{-6} M potentiated the effect of ascorbate on cellular metabolic activity at all concentrations used (50, 100, 200 µg/ml). This relationship was seen for all cellular parameters measured; cellu-

lar DNA, RNA, non-collagenous protein, and hydroxyproline (collagen). Even cellular RNA and non-collagenous protein, which were not significantly responsive to ascorbate alone, showed significant increases in their levels when insulin was also included with ascorbate. These data suggest that insulin potentiated the stimulatory effect of ascorbate on human gingival fibroblast metabolism. Bigley et al. (1983), while studying human neutrophils and skin fibroblasts, confirmed that the transport of ascorbate was mediated by glucose transport mechanisms. This probably explains our observed stimulation of ascorbate effects by insulin. Ascorbate is known to be in high concentrations in tissues with high turnover rates. Gingiva has one of the highest turnover rates of any tissue in the body (Page and Ammons, 1974; Sodek et al., 1977). Therefore, the stimulatory response of gingival fibroblasts to ascorbate concentrations as high as 200 μg/ml may reflect a high requirement for ascorbate.

The relationship of cell density to the metabolic activity of cells in culture is currently poorly understood. Under the experimental conditions we have used, DNA levels in control and diabetic rat gingival cells were inversely proportional to cell density when grown under essentially all treatment conditions. This relationship did not hold for control cells grown in the presence of insulin, or for the diabetic cells treated with both insulin and ascorbate (Fig. 33). These results concur with those of Speicher et al. (1981) and Manner (1971) who have also described a decrease in DNA synthesis with an increase in cell density. With all of the other treatments employed, the diabetic fibroblasts

produced maximal DNA at the second lowest cell density seeded, while the control cells produced maximal DNA at their lowest cell density. This disparity reflects replicative differences between control and diabetic fibroblasts that are probably density dependent. Control fibroblasts treated with 10^{-6} M insulin and diabetic cells treated with 10^{-6} M insulin + 200 μg/ml ascorbate did not show significant differences in DNA levels with changes in cell density. This finding is in accordance with those of Armato et al. (1978) who reported that up to 19-fold increases in the degree of rat hepatocyte cell crowding did not alter the rates of DNA synthesis.

The stimulatory effect of ascorbate on DNA synthesis has been well documented (Peterkofsky and Prather, 1977; Rowe et al., 1977-2; Krystal et al., 1982). We observed no significant stimulation of DNA synthesis by ascorbate under the conditions we used. For all treatments used, maximal RNA levels in the control cells at all treatments were observed at the second lowest fibroblast density. The diabetic fibroblasts, with the exception of the control treatment, showed maximal RNA levels at the lowest cell density. The apparent inverse relationship between cell replication and RNA synthesis as reported by Schneider and Shorr (1975), is confirmed by our findings. For both the control and the diabetic rat fibroblasts, cellular RNA levels decreased with an increase in cell density. The range of ascorbate in normal serum is 0.02-0.06 mM (Rowe et al., 1977-2). Rowe et al. (1977-2) have reported that 0.28 mM ascorbate, when added to human skin fibroblasts at low densities (7.5×10^4 cells/dish), resulted in cell death.

Peterkofsky and Prather (1977) have also reported that 0.28 mM (2.8×10^{-4} M) ascorbate inhibited the growth of chick embryo (skin) and human embryonic and adult skin cells seeded at low densities (2×10^5 cells/dish) and that the inhibitory effect was due to the formation of peroxides. Our study which used ascorbate at lower levels 200 µg/ml (approximately 1.1×10^{-6} M) on control and diabetic gingival fibroblasts at low densities (7.5×10^4 and 1.5×10^5 cells/dish) reports that in general more DNA, RNA and cellular hydroxyproline were found in cells at low densities (7.5×10^4 and 1.5×10^5 cells/dish) than at higher densities. Gingiva is known to have a high turnover rate (Page and Ammons, 1974; Sodek et al., 1977). It is thought that tissues of high turnover rate have higher ascorbate requirements than tissues of lower turnover rates (Switzer and Summer, 1972). This might explain the higher rates of replication and of cellular metabolism of the gingival fibroblasts grown at low cell density in the presence of ascorbate. The lack of cellular inhibition by ascorbate may also have been due to the lower levels of peroxides produced from lower ascorbate concentrations than those used by Peterkofsky and Prather (1977) and Rowe et al. (1977-2).

Under these experimental conditions, diabetic gingival fibroblast collagenous protein levels at the two lowest cell densities and non-collagenous protein at all cell densities, were higher than those of their density matched control cells following treatment with either ascorbate alone or ascorbate with insulin. Since these differences did not occur when the fibroblasts were treated with insulin, ascorbate alone would, therefore, seem

responsible for this stimulation. Tissue from diabetic animals may approach a scorbutic state (Mann, 1974) due to inhibition of tissue ascorbate uptake by the hyperglycemic state (Mann and Newton, 1975). Diabetic rat serum and tissue ascorbate levels are reduced due to increased urinary excretion (Zebrowski and Bhatnagar, 1979). Therefore, it is not unreasonable that diabetic tissue cells in culture, removed from the hyperglycemic environment, would be more sensitive than the control cells to ascorbate stimulation. Diabetic cell non-collagenous protein levels increased with increased cell density. This is in conflict with the findings of Abe et al. (1979) and McCoy et al. (1982) in their studies of normal human skin fibroblasts, all of whom reported that protein (collagenous and non-collagenous) synthesis was inversely proportional to cell density. However, since our results showed cellular hydroxyproline levels to be inversely proportional to cell density, the existing disparity between our data relates only to non-collagenous protein synthesis and by diabetic rat gingival cells. The lack of a significant change in the non-collagenous protein levels of the control cells due to cell density is in accordance with the findings of Speicher et al. (1981) who also reported no effect of cell density on protein synthesis when cells were seeded at densities of 2.0 and 5.0 X 10^6 cells/dish. Although we did not study cellular metabolism at such high cell densities, our results are nonetheless in agreement. Cellular hydroxyproline levels in both the control and the diabetic rat gingival fibroblasts decreased with an increase in cell density. Control cells contained maximal levels of hydroxyproline

under control treatment conditions. All other treatments produced a reduction of cellular hydroxyproline levels when control cells were seeded at low densities. This may reflect a decrease in collagen secretion at these treatments. Medium hydroxyproline levels of control cells were generally inversely proportional to cell density. This indicates that cells producing large amounts of collagen secrete larger quantities than cells producing less collagen. This implies the involvement of a mechanism very sensitive to intracellular collagen levels. Diabetic cells with the exception of the highest density showed medium hydroxyproline levels that were directly proportional to cell density. This density related difference in medium hydroxyproline accumulation between control and diabetic cells may be due only to differences in sensitivity to cell crowding, which may affect the comparative rate at which the cells secrete procollagen across the membrane and into the medium. Insulin has been shown to stimulate the uptake of ascorbate in cells grown in vitro (Mann et al., 1975; Verlangieri and Sestito, 1981). These findings support our observations of increased ascorbate uptake by insulin when ascorbate is present at 200 µg/ml in the growth medium.

Ascorbate uptake by the control and diabetic cells was quite variable and when present at 200 µg/ml only, was stimulated by insulin treatment. Nonetheless, our data obtained at lower medium ascorbate levels are also consistent with the results reported in the above quoted literature.

The variability in ascorbate uptake by cells grown in medium with added ascorbate may in part be related to the chemical insta-

bility of ascorbate in the growth medium. Peterkofsky (1972) showed that ascorbate present in the growth medium at 0.25 mM, and incubated at 37°C under 5% CO_2 and 95% air, was almost all gone after 24 hours. Only 2% remained. If serum was present in the medium, the destruction of ascorbate was delayed, and 11-12% of the vitamin remained after 24 hours. Peterkofsky (1972) suggested that the destruction of ascorbate may lead to anomalous results in cell growth and collagen production. We prepared our ascorbate medium solutions just prior to each experiment. The medium used was always incubated overnight in an air incubator at 37°C before use on days 0, 3 and 6, to ensure the growth of fibroblasts in constant temperature conditions. This incubation may in fact have enhanced the breakdown of ascorbate as described by Peterkofsky (1972). Our fibroblasts were incubated under essentially the same conditions as those of Peterkofsky (1972). Under the conditions employed, control and diabetic fibroblast metabolism appeared to be more sensitive to density differences than to treatment differences. Optimal cellular activity for both groups of fibroblasts, with the exception of non-collagenous and medium collagenous protein, occurred at the two lowest cell densities studied (7.5×10^4 and 1.5×10^5 cells/dish).

In an attempt to clarify the ascorbate uptake data for the control and diabetic rat cells, a subsequent study was carried out to re-measure intracellular ascorbate levels in these cells. We found that diabetic and control cells contained similar levels of ascorbate when grown in medium with 50 µg/ml ascorbate. Harrison (1982) reported that on a plate basis, diabetic rat gingival cells

contained less ascorbate than control cells. This would seem correct, given that the reduced numbers of cells per dish of diabetic cells might result in lower levels of ascorbate than would control cultures. However, whether the diabetic content was more or less than the control cellular ascorbate on a cell basis, was not determined. In our study, insulin increased ascorbate uptake in control rat fibroblasts, but was without effect on diabetic cells when the medium ascorbate level was 50 μg/ml. This agrees with the findings of Harrison (1982) who reported that insulin pretreatment of control and diabetic rat cells increased the uptake of ^{14}C ascorbate by control rat gingival cells, but was without effect on diabetic cells when uptake was expressed as disintegrations per minute.

A comparative senescence study of gingival fibroblasts of human, and of control and diabetic rats showed that overall DNA synthesis in all of these types of fibroblasts was variably affected by insulin. Plisko and Gilchrest (1983) have reported that early passage fibroblasts (3-5) derived from newborn foreskin had greater proliferative responses to insulin (as measured by cell density) than did early passage fibroblasts derived from adult skin. The gingival tissue used in this study was isolated from adult human and adult male Sprague-Dawley rats and showed this relationship at early passages. Fibroblasts from the gingiva of control rats showed two peaks of maximum DNA levels, one at the 4th and the other at the 24th passage. Maximal DNA levels in diabetic rat cells also occurred as two peaks, but at the 4th and 9th passages. By comparison, the human cells showed their maximal

levels of DNA in the 2nd and 6th passages. Viable fibroblasts from diabetic rat gingiva could not be maintained in culture beyond the 10th passage. DNA levels in these cells decreased at the 6th passage and suggested a slowed replicative rate, but again increased by the 9th passage just before the cells died. DNA synthesis in the control rat fibroblasts was lowest at the 15th passage and gradually increased in the 24th passage to the highest level seen. Human cellular DNA levels generally decreased with increasing passage number. Decreased replicative rates observed with age have been reported by several workers (Weisman-Shomer and Fry, 1975; Schneider and Fowlkes, 1976; Linn et al., 1976; Mitsui and Schneider, 1976). Schneider and Fowlkes (1976) have suggested that a decrease in cellular DNA in senescent human fibroblasts was due to several factors including an increased frequency of chromosomal deletions, a decreased amplification of specific DNA genes or to a loss of cellular DNA. Overall, our human cell data agree with these results. However, the increased levels of DNA seen in rat cells at the 9th and 24th passages, may be attributed to an accumulation of DNA due to deficiencies in the cellular degradative mechanisms. Fry and Weisman-Shomer (1976), Linn et al. (1976) and Epstein (1974) have all shown that DNA polymerases are altered in senescent fibroblasts in vitro. Structural conformational changes in these crucial enzymes could cause the experimentally observed decreased rates of DNA replication and repair and the accumulation of somatic mutations in cultured cells (Epstein, 1974). Thus, although the rates of DNA synthesis and replication are decreased, the accumulation of faulty DNA molecules could be

detected in an assay for cellular DNA, such as the Burton Method (1968) used for DNA analysis in our studies.

Cellular RNA in human and rat cells decreased after early passages up to the 20th passage for human and rat control cells and to subculture 6 for diabetic rat cells. Thereafter, levels of RNA increased at the 9th passage for diabetic rat cells and the 24th passage for human and control rat cells. Bowman et al. (1976) have reported a decrease in RNA synthesis in human fibroblasts with an increase in cell age. Our data agrees with this finding. However, our observed increase in RNA in very old cells does not suggest a sudden increase in RNA synthesis, but rather an accumulation of cellular RNA. This conclusion is supported by the report of Schneider and Shorr (1975), who suggested that the increased redundancy of DNA genes in senescent cells cause an increase in the cellular levels of RNA, mRNA and protein. Furthermore, Goldberg (1971) proposed that the increased RNA content of senescent cells was due to the cessation of protein synthesis with age, which resulted in the accumulation of precursors such as aminoacyl tRNA. In our experiments we assayed only for total cellular RNA. Non-collagenous protein levels of the rat cells studied under our experimental conditions were generally constant throughout the life cycle of the fibroblasts. This relatively constant rate of protein synthesis throughout the life cycle of the cells has also been observed by Schneider and Shorr, 1975) in their study of ageing cultured human fibroblasts. Both control and diabetic rat cells showed similar rates of non-collagenous protein synthesis. Non-collagenous protein levels in

the human cells were slightly increased by passage number. This latter finding is in complete agreement with the Orgel "error catastrophe" hypothesis which proposes that random errors in protein synthesis should increase the intracellular accumulation of faulty proteins. Capecchi et al. (1974) found mammalian cells to have a very sensitive mechanism for degrading proteins. However, Kaftory et al. (1978) claim that a decrease in protein breakdown was due more to senescence than to a retarded cellular proliferation. Cellular hydroxyproline levels were maximal in the 4th and and 6th passages for control and diabetic rats and in the 6th passage for human cells. There did not appear to be any significant effect of insulin on collagen production.

Collagen levels of diabetic tissue fibroblasts were less than those of control fibroblasts. Salcedo and Franzblau (1981) and Hildebran et al. (1983) have reported a decrease in collagen synthesis with age. Our results concur with these reports. However, the increases just before cell death were probably due to the intracellular accumulation of hydroxyproline caused by defective degradative mechanisms (Orgel, 1973; Kaftory et al., 1978) and/or to a reduced secretion of collagen into the medium. A reduction in the cellular secretory mechanisms with age is not unreasonable since the earlier study of human cells reported a decrease in the membrane transport (uptake) of ^{3}H-2-deoxyglucose with age.

It would appear that rat and human gingival fibroblasts were not significantly stimulated by insulin (containing 0.2% bound zinc). Intracellular DNA levels, cellular RNA and collagenous

protein content of both human and rat cells decreased with age in passage, but was increased in very old rat cells only. The lifespan of the diabetic cell was less than half that of control cells and all of their cellular parameters were similar to those found in the older control cells, for example, cells in passages 12-24. Therefore, these data permit us to conclude that diabetic cells do in fact exhibit accelerated ageing as described by Goldstein et al., 1978. Moreover, senescence studies of both rat and human cells indicate that optimal cellular metabolism as measured by cellular DNA, RNA, protein synthesis and membrane transport occurred at the 5th or 6th passages in the early portion of the cell lifespan. This is especially reassuring since all of our studies were done on fibroblasts in the 5th or 6th passage. Our results suggest that the Orgel theory (1973) of cellular catastrophe is valid for rat cells. The fact that human cells did not by comparison accumulate DNA, RNA and collagenous protein in very old cells suggests perhaps that their cellular degradative mechanisms were still efficient.

Streptozotocin, added to the growth medium in a range of concentrations, did not appear to visually have any deleterious effects on control rat gingival fibroblasts in culture. The control rat gingival cells appeared to grow and divide as they normally did. The results indicate that streptozotocin, in concentrations as high as 100 μg/ml (3.77×10^{-4} M) had no significant effects on cellular replicative rates, RNA and non-collagenous protein production. These findings agree with those of Wilson et al. (1983) who found that streptozotocin at concentrations of 10^{-3}

to 10^{-2} M was not cytotoxic to pancreatic fibroblasts, but were toxic to the pancreatic beta cells. These workers also reported that the experimental levels of streptozotocin produced no significant changes in total cellular protein levels. Our results indicated, however, that streptozotocin was stimulatory to collagenous protein levels of the control rat cells. The decreasing intracellular levels, coupled with the corresponding increasing medium levels of hydroxyproline, expecially at the higher streptozotocin concentrations used, would suggest that the stimulatory effect of streptozotocin probably acts in part on the mechanism of procollagen secretion into the growth medium. The stimulation of *in vitro* collagenous protein production by streptozotocin occurred in levels as low as 1.0 μg/ml. Diabetes was induced in our rats by an intravenous dose of streptozotocin at 55 mg/kg. The effects of such elevated levels of streptozotocin on *in vivo* collagenous protein production may have profound effects on *in vitro* collagen studies, since the altered collagen production may not only be due to the metabolic effects of diabetes secondary to streptozotocin, but also to streptozotocin itself.

Chaproniere-Rickenberg and Webber (1983) recently reported that zinc levels in zinc stabilized insulin were inhibitory to the growth of cells *in vitro*. Two different commercial preparations of insulin, each with a different quantity of bound zinc had inadvertently been used in our studies. Moreover, the results of these studies showed less than equal sensitivity of the cells to these two insulin preparations. Therefore, the metabolic effects of these two insulin preparations with different levels of bound

zinc were assessed on control and diabetic rat cells. Under the described experimental conditions the only known sources of zinc were the insulin fractions. The alpha-MEM used was devoid of this metal. Foetal calf serum may well contain trace levels of zinc, although we did not analyze for zinc. Epstein (1982) using atomic adsorption spectrophotometry indicated the presence of zinc in foetal calf serum, which, when used at a 10% concentration, corresponded to a zinc level of 2×10^{-6} M. This level of zinc, which Epstein claimed was negligible, produced an inhibition of DNA synthesis by rat cells under our experimental conditions.

Control cell RNA and non-collagenous proteins were maximal at 10^{-8} M insulin (0.2% bound zinc), while maximal levels of these parameters in the diabetic cells occurred at 10^{-8} M insulin (0.5% bound zinc). Both cellular and medium hydroxyproline levels of the diabetic cells were maximal at 10^{-8} M insulin (0.5% bound zinc) as were the medium hydroxyproline levels of the control cells. The diminished hydroxyproline levels for both the control and the diabetic cells in the latter part of the growth period was possibly due to a lack of cellular response to insulin (Table 14). It is suggested that the lack of metabolic response to insulin may be due to the levels of insulin bound zinc. The levels seen in 10^{-6} M insulin (0.2% bound zinc) and 10^{-6} M insulin (0.5% bound zinc) were 2×10^{-9} M and 5.0×10^{-9} M zinc respectively. These levels of zinc diminished the anabolic effect of insulin on control and streptozotocin diabetic rat gingival fibroblast metabolism. Diabetic rat cells appeared to tolerate a higher level of zinc than did the control cells as their maximal metabolic rates

were at 10^{-8} M insulin (0.5% or 5 X 10^{-9} M bound zinc). This may be due to the fact that streptozotocin in producing the diabetic state by killing of pancreatic B cells which produce insulin, may in fact be reducing serum and tissue zinc levels, thus producing a higher tolerance for zinc in these cells. However, Levine et al. (1983) reported no differences in serum and tissue zinc concentrations between control and streptozotocin diabetic mice.

These results suggest that the cellular metabolic and replicative rates respond differently to insulin bound zinc. In both the control and diabetic rat cells, maximum DNA synthesis was seen at 10^{-6} M insulin with 0.2% bound zinc corresponding to 2 X 10^{-9} M zinc. This level of zinc may represent the optimal threshold of the metal for DNA synthesis in these cells as both control and diabetic cells grown in the other insulin preparation at 10^{-6} M (0.5% bound zinc or 5 X 10^{-9} M) were considerably less responsive. In fact, Chapronière-Rickenberg and Webber (1983) have found maximal replication in adult human prostatic epithelium to occur at an insulin concentration of 0.03-0.12 U/ml corresponding to a bound zinc concentration of 1.4 X 10^{-7} M zinc. Although we observed maximal replicative rates at higher insulin levels and lower zinc levels, our results are nonetheless in agreement in that the same metabolic effect was observed. These workers also showed that zinc free insulin produced significantly higher increases in growth. However, they (Chapronière-Rickenberg and Webber, 1983) did not assess effects of insulin and zinc free medium, or zinc alone in the absence of insulin. Therefore, whether their cells grown in medium with zinc stabilized insulin actually produced

less DNA than cells without insulin is unknown.

May and Contoreggi (1982), attempted to explain any inhibitory effect of zinc on insulin by suggesting that zinc inhibits certain membrane bound enzymes which are implicated in the mechanism of insulin action, e.g. adenylate cyclase, guanylate cyclase, Ca^{2+} ATPase and Na^{+}, K^{+} ATPase. Thus, while zinc in certain concentrations may be anabolic and stimulatory, its effects become inhibitory at greater than threshold levels.

An involvement of serum with zinc has also been suggested. Epstein (1982) in a study with human diploid fibroblasts proposed that although there are negligible amounts of zinc in serum, there could be some alteration in zinc toxicity by certain proteins, especially proteins capable of binding zinc. This author also showed that foetal calf serum used at the 10% level may exert some protective effect on normal human cells. Our cells were maintained in 10% foetal calf serum, but whether or not this serum level provided any measure of protection to the rat gingival system cannot be determined from our experiments.

In accordance with the work of Chapronière-Rickenberg and Webber (1983), we conclude that the inhibitory effect of zinc limits the anabolic ability and concentration of zinc stabilized insulin that can be used in culture media. Furthermore, we observed that the zinc affects both the control and the streptozotocin diabetic fibroblasts, although streptozotocin diabetic cells appear to have a higher zinc tolerance for the metal ion than do the control cells.

To further clarify whether zinc bound insulin alone and not

"free" zinc in the growth medium limited the stimulatory effect of insulin, control and diabetic rat cells were grown in varying levels of $ZnCl_2$, insulin (0.2% bound zinc) and the two factors combined. The results indicate that all experimental parameters measured, cellular DNA, RNA, non-collagenous and collagenous protein, were lower at all treatments in diabetic cells than control cells. Maximal cell replication and DNA synthesis were seen with insulin treatments alone. Cellular DNA levels increased with the insulin concentration in the growth medium. This finding agrees with our previous results involving varying levels of insulin with 0.2% bound zinc in the growth medium and its effect on DNA synthesis. Second highest levels of DNA were observed in cells grown in the presence of both insulin and $ZnCl_2$. The lowest DNA levels were seen in cells grown in medium containing $ZnCl_2$ alone and were inversely proportional to zinc concentration. These results, therefore, suggest that zinc is inhibitory to DNA synthesis in levels of 10^{-8} - 10^{-5} M. This finding has been confirmed by the work of Chaproniere-Rickenberg and Webber (1983) who grew human prostatic epithelial cells in varying concentrations of zinc chloride and found growth inhibition at concentrations above 3 X 10^{-8} M zinc chloride. Our observation of inhibition of growth at 10^{-8} M zinc shows at least a 3-fold greater sensitivity of the cells to zinc than was shown by the above workers. However, contrary to our results, Epstein (1982) reported that human diploid fibroblasts show no differences in cell numbers at day 9 when grown in medium without and with 1.75 X 10^{-4} M zinc, while zinc in higher levels was shown to decrease cell numbers. Rubin and Koide (1975)

have shown that Zn^{2+} increased the uptake of uridine and its incorporation into acid soluble material. However, zinc at levels of 8×10^{-5} M or higher were shown to inhibit DNA synthesis, an effect not inconsistent with our data. Cells grown in medium with $ZnCl_2$ and 10^{-8} M insulin contained significantly lower levels of DNA than cells grown in 10^{-8} M insulin alone which suggests that $ZnCl_2$ inhibits the mitogenic effect of insulin on DNA synthesis.

Cellular RNA and non-collagenous protein levels were not significantly affected by $ZnCl_2$, insulin or both combined, suggesting that cellular metabolic and replicative rates have different sensitivities to zinc. Non-collagenous protein and RNA levels of cells grown in varying concentrations of zinc with 10^{-8} M insulin were directly proportional to the zinc concentration. A possible explanation for this effect of zinc has been put forth by Arquilla et al. (1978) who found that zinc in concentrations of 6×10^{-6} to 6×10^{-4} M increased the binding and decreased the degradation of ^{125}I iodoinsulin, and this effect was directly proportional to zinc concentration. This increased binding of insulin might serve to increase the effect of insulin on cellular metabolism.

Both the medium and the cellular collagen content of cells were higher when grown in insulin than when grown in zinc alone or with zinc and insulin. Although the levels of cellular hydroxyproline caused by the latter two treatments were significantly decreased, the increased medium levels suggest that zinc alone enhanced the secretion of collagen, but it diminished the effect of added insulin since cells grown in 10^{-8} M insulin alone accumulated significantly higher levels of hydroxyproline than cells

grown in zinc with 10^{-8} M insulin. Tengrup et al. (1981) reported increased collagen accumulation in the granulation tissue of zinc treated rats. This accumulation was, however, not due to increased rates of collagen synthesis but rather to decreased rates of collagen breakdown. Our data agrees with the findings of Tengrup et al. (1981) regarding zinc induced accumulation of collagenous protein. However, since we did not assess collagenous protein turnover, we cannot say whether zinc stimulated the synthesis or decreased the breakdown of collagenous protein. Cells grown in insulin alone accumulated similar levels of hydroxyproline at all concentrations studied. $ZnCl_2$ was more stimulatory to medium hydroxyproline levels of diabetic than control cells, suggesting a greater sensitivity of diabetic cells to zinc stimulation of collagen secretion. This is not unreasonable since our previous study reported that diabetic cells were more tolerant of zinc than control cells.

In conclusion and in accordance with the findings of Chapronière-Rickenberg and Webber (1983), as well as with the preceding study in this thesis, it is proposed that not only is zinc inhibitory to the growth of control and streptozotocin diabetic gingival fibroblasts, but it also limits the effect of insulin both when it is bound as an integral part of the insulin molecule and when it is added as $ZnCl_2$ to the growth medium. Moreover, the similarity of growth responses of rat gingival and human prostatic epithelial cells (Chapronière-Rickenberg and Webber, 1983) to zinc suggests that its effects are universal. Zinc chloride, however, did not alter cellular RNA and non-collagenous protein levels, but

was stimulatory to medium hydroxyproline accumulation. It appears that these effects of zinc on hydroxyproline accumulation are mediated at the cell membrane where it increases the secretion of collagenous protein.

The identification of specific receptors for dihydrotestosterone in the gingiva suggests that this is a target organ for the androgen (Southren et al., 1978). Studies have focused on the metabolism of testosterone in the gingiva (Keenan, 1975; Vittek et al., 1979-2) and its effects on the inflammatory responses in connective tissue (El Attar et al., 1982). However, little attention has been directed to the cellular metabolic responses to testosterone i.e. replicative rates, RNA, non-collagenous and collagenous protein production. Our study utilized the gingiva of male control and diabetic Sprague-Dawley rats. Vittek et al. (1979-2) has reported that greater amounts of testosterone were metabolized by male gingiva in vitro. Testosterone has been shown to induce proliferation of gingival connective tissue (Shklar et al., 1967). These workers reported that rats receiving daily doses of testosterone (0.25 mg) for 30 days showed slight increases in normal connective tissue activity, while hypophysectomized rats (with reduced anabolic activity of connective tissue) showed a marked anabolic effect in their gingival connective tissues. Our results indicate that testosterone added to the growth medium did not produce any significant increase in DNA synthesis of control rat gingival cells. DNA production in fibroblasts from diabetic rats which showed decreased replicative rates and metabolism were stimulated significantly by testosterone. However, the effect was the

same at all of the concentrations of testosterone used. Our results do agree with those of Shklar et al. (1967) who reported that where the anabolic capacity of the tissues was not reduced, testosterone had only a mild effect, while in tissues with decreased anabolic activity, as exists in the diabetic fibroblasts, testosterone was stimulatory to cellular proliferation.

Our results showed no stimulatory effects of testosterone on cellular RNA, non-collagenous protein or cellular hydroxyproline levels in either of the cell types studied. Kochakian (1960) demonstrated a summation of protein anabolic effects of testosterone and growth hormone in rats. Since insulin is a known mitogen (Gospodarowicz and Moran, 1976), one might expect a similar summation of the stimulatory effects of insulin and testosterone on rat gingival fibroblast metabolism. However, only when grown in the presence of insulin alone did we observe a slight stimulation of cellular DNA levels in both control and diabetic cultures. It seems, therefore, that the two mitogens, when present together in the growth medium, may in some yet unknown manner compete with each other resulting in a general lack of stimulation to cellular metabolic and replicative rates.

Our findings indicate a similarity in behaviour between cultured streptozotocin diabetic rat gingival fibroblasts and those obtained from diabetic human skin (Goldstein et al., 1978; Rosenbloom and Rosenbloom, 1978). We also found that fibroblasts isolated from streptozotocin diabetic rats which were treated *in vivo* with insulin 24 hours post streptozotocin treatment (Fig. 1) behaved similarly to fibroblasts derived from healthy rats. On

the other hand, cells derived from untreated diabetic rats, but treated in vitro with insulin, did not show a similar quality of metabolic control. Clinically, these results suggest that early detection of diabetes coupled with insulin therapy, could possibly halt the irreversible effects of diabetes on these cells.

Of relevance to the diabetic patient is our observed stimulation of collagen accumulation by zinc. Thus, while zinc inhibits cellular growth, it may enhance the compromised integrity of diabetic connective tissue, especially in wound healing. Our observed stimulation by ascorbate of cell growth and collagen metabolism, similarly promotes the use of this vitamin in diabetic tissues, especially since these tissues are thought to approach a scorbutic state (Mann, 1974). In addition, the presence of ascorbate especially in gingiva may elevate the turnover rates of diabetic gingiva to more normal rates. Under our experimental conditions, insulin did not increase ascorbate uptake in diabetic rat cells. Mann and Newton (1975) and Bigley et al. (1983) attributed this phenomenon to hyperglycemia of the diabetic serum, which reduced the insulin stimulated uptake. Indeed, we found that diabetic serum was toxic to in vitro cell growth. This was possibly due to elevated lipid and glucose levels, which in a diabetic patient might explain the clinically observed vascular pathology. Our observation of accelerated ageing is not unusual since this phenomenon is known to occur in diabetics.

Our data suggests that fibroblasts isolated from control and streptozotocin diabetic rats may represent phenotypically different subpopulations. Elucidation of the mechanisms by which these

subpopulations are modulated could lead to the manipulation of connective tissue metabolism by endogenous and exogenous agents, and by doing so, improve the quality of connective tissue, especially in diseased states such as diabetes mellitus.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

These studies describe the successful growth and maintenance of healthy human control and streptozotocin diabetic rat gingival fibroblasts *in vitro*. In summary it has been shown that:

1). Human and rat gingival fibroblasts do represent an acceptable model for the study of oral connective tissue metabolism, both in healthy and diseased states.

2). Under identical growth conditions and handling, the diabetic cells consistently showed lower replicative rates as measured by DNA production than did the control rat cells.

3). The responses of human and rat cells to insulin and ascorbate were variable and depended on the experimental conditions.

4). Both human and rat gingival fibroblasts were sensitive to senescence induced changes in cellular replication and metabolism. In general, cellular responses to insulin were lacking. Diabetic cells were most sensitive to ageing as indicated by their lifespan, which was less than half that of control cells.

5). Replication and metabolism of human gingival fibroblasts were stimulated by control rat serum. Diabetic rat serum was, however, toxic to the cells.

6). Serum inhibited the stimulatory effect of insulin on

control and diabetic rat gingival fibroblast metabolism.

7). There was no significant difference in ascorbate uptake between control and diabetic rat gingival fibroblasts. Ascorbate uptake by control rat cells only was stimulated by insulin.

8). Ascorbate stimulated replication and metabolism of human gingival fibroblasts. The effects were directly proportional to the medium ascorbate concentration. Insulin produced a further stimulation of the ascorbate effects.

9). Control and diabetic rat gingival fibroblast metabolism was more sensitive to changes in cell density than to ascorbate or insulin treatment. These two groups of cells responded differently to changes in cell density.

10). Streptozotocin, when added to the growth medium, was without effect on DNA, RNA and non-collagenous protein synthesis of control rat gingival fibroblasts. Collagenous protein production, however, was stimulated in direct proportion to streptozotocin concentration.

11). Insulin bound zinc limited the stimulatory effect of insulin on the replication and metabolism of control and diabetic rat gingival fibroblasts. Cellular replicative and metabolic rates showed different sensitivites to zinc.

12). Zinc chloride was inhibitory to replication by control and diabetic rat gingival fibroblasts, but metabolic parameters

such as RNA and non-collagenous protein synthesis were not affected. Insulin in the absence of added zinc produced maximal stimulation of DNA synthesis. By comparison, zinc added with insulin to the growth medium resulted in no increase in DNA levels over cells treated with zinc alone. Collagenous protein production was equally stimulated by insulin, zinc and the two factors combined. Both cell types produced maximal levels of collagenous protein in response to insulin.

13). Testosterone in the growth medium was without effect on cellular DNA, RNA and protein synthesis by control rat gingival fibroblasts. DNA levels in diabetic cells were stimulated by testosterone. Testosterone and insulin added together to the medium did not produce any synergistic changes in cellular replicative and metabolic rates.

The data collectively suggest that the differences in cellular biochemical responses of healthy and streptozotocin diabetic rat cells may be due to differences in cellular response to their chemical environment and also are probably related to the health status of the organism from which the cells originated. The fact that both healthy and diseased tissue fibroblasts behave similarly in vivo and in vitro should facilitate more future studies on the relationship of the chemical composition of the cellular environment to cellular behaviour. Studies outlining the mechanism of insulin action in diabetic tissue fibroblasts are clearly required

in view of the altered metabolism seen in these cells and the importance of insulin to cellular metabolism.

CHAPTER VII

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FIGURE

with either 0 or 10^{-6} M insulin (containing 0.2% bound zinc). Hydroxyproline levels are presented on a DNA basis for a 9 day growth period.

6 Relationship of medium hydroxyproline levels to cellular replication by control, diabetic and insulin treated diabetic rats. Medium hydroxyproline and cellular DNA levels are presented on a plate basis for a 9 day growth period.

7 Relationship of insulin and cell age to cellular replication of human gingival fibroblasts. Fibroblasts were used on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin (containing 0.5% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-9} M.

8 Effect of cell age on cellular replication by human gingival fibroblasts. Growth is presented as cell density (cells/cm^2 of culture dish X 10^{-4}). Cell densities were determined at each of passages 1 through 24.

9 Relationship of insulin and cell age to cellular RNA concentration in human gingival fibroblasts. RNA expressed on a DNA basis was measured on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin containing 0.5% bound zinc was added to the growth medium at concentrations of 0 and 10^{-9} M.

10 Relationship of insulin and cell age to cellular non-

collagenous protein levels in human gingival fibroblasts. Protein expressed on a DNA basis was measured on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin (containing 0.5% bound zinc) was added at concentrations of 0 and 10^{-9} M.

11 Relationship of insulin and cell age to cellular collagenous protein concentration in human gingival fibroblasts. Collagenous protein was expressed as hydroxyproline per DNA and measured on days 4, 8, 12 and 16 of passages 5, 8 and 18. There were no detectable levels of cellular collagenous protein in the 12th passage. Insulin (containing 0.5% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-9} M.

12 Relationship of insulin and cell age to membrane transport of ^{3}H-2-deoxyglucose by human gingival fibroblasts. The uptake of ^{3}H-2-deoxyglucose was measured on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin (containing 0.5% bound zinc) was added at concentrations of 0 and 10^{-9} M.

13 Comparative effects of the interaction of medium serum and insulin on cellular RNA levels in control and diabetic rat gingival fibroblasts. RNA levels are presented on a DNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc)

was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

14 Comparative effects of the interaction of medium serum and insulin on the cellular non-collagenous protein concentrations of control and diabetic rat gingival fibroblasts. Non-collagenous protein is presented on a DNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

15 Comparative effects of the interaction of medium serum and insulin on the cellular non-collagenous protein concentrations of control and diabetic rat gingival fibroblasts. Non-collagenous protein is presented on a RNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

16 Comparative effects of the interaction of medium serum and insulin on the accumulation of medium collagenous protein by control and diabetic rat gingival fibroblasts. Collagenous protein, measured as medium hydroxyproline, is presented on a DNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing

0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

17 Comparative effects of the interaction of medium serum and insulin on membrane transport of ^{3}H-2-deoxyglucose by control and diabetic rat gingival fibroblasts. ^{3}H-2-deoxyglucose uptake was assessed on 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

18 Comparative effects of the interaction of medium serum and insulin on membrane transport of ^{14}C-alpha-aminoisobutyric acid (AIB) by control and diabetic rat gingival fibroblasts. ^{14}C-alpha-AIB uptake was assessed on 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 and 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

19a-e Comparative effects of substituting control or diabetic rat serum for FCS in the growth medium of human gingival fibroblasts at levels of (i) 5%, (ii) 10% and (iii) 15% rat serum. (a) Day 1, (b) Day 2, (c) Day 5, (d) Day 8, (e) Day 13.

20 Effects of replacing FCS in the growth medium with

control and diabetic rat sera on the cellular growth of human gingival fibroblasts. Cell growth was measured as DNA per plate on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 and 15% respectively.

21 Effects of replacing FCS in the growth medium with control and diabetic rat sera on cellular RNA levels of human gingival fibroblasts. RNA was measured on a DNA basis on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

22 Effects of replacing FCS in the growth medium with control and diabetic rat sera on cellular non-collagenous protein levels in human gingival fibroblasts. Protein, measured as Lowry detectable protein and expressed on a DNA basis, was monitored for a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

23 Effects of replacing FCS in the growth medium with control and diabetic rat sera on cellular non-collagenous protein levels in human gingival fibroblasts. Protein, measured as Lowry detectable protein and expressed on a RNA basis, was monitored for a 12 day growth period. Control and diabetic rat sera were added to the growth

medium at concentrations of 5, 10 or 15% respectively.

24 Effects of replacing FCS in the growth medium with control and diabetic rat sera on intracellular concentrations of collagenous protein in human gingival fibroblasts. Collagenous protein was measured as μg cellular hydroxyproline per 100 μg DNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

25 Effects of replacing FCS in the growth medium with control and diabetic rat sera on intracellular concentrations of cellular collagenous protein in human gingival fibroblasts. Collagenous protein was measured as μg cellular hydroxyproline per 100 μg RNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

26 Effects of replacing FCS in the growth medium with control and diabetic rat sera on the accumulation by human gingival fibroblasts of medium collagenous protein. Collagenous protein was measured as μg hydroxyproline per 100 μg DNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

27 Effects of replacing FCS in the growth medium with

control and diabetic rat sera on the accumulation by human gingival fibroblasts of medium collagenous protein. Collagenous protein was measured as μg hydroxyproline per 100 μg RNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

28 Growth response of human gingival fibroblasts to growth medium ascorbate concentrations and the interaction of ascorbate with insulin. Growth was measured as μg DNA per plate on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 μg/ml respectively. Insulin (containing 0.5% bound zinc) was added at each level of ascorbate in concentrations of 0 or 10^{-6} M.

29 Comparison of the effects of medium ascorbate concentration and its interaction with insulin on cellular RNA levels of human gingival fibroblasts. RNA was measured as μg RNA per μg DNA on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 μg/ml respectively. Insulin (containing 0.5% bound zinc) was added at each level of ascorbate in concentrations of 0 or 10^{-6} M.

30 Comparison of the effects of growth medium ascorbate concentration and its interaction with insulin on the intra-

cellular levels of non-collagenous protein in human gingival fibroblasts. Protein synthesis was measured as μg protein per μg DNA on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 μg/ml respectively. At each level of ascorbate, insulin (containing 0.5% bound zinc) was added at concentrations of 0 or 10^{-6} M.

31 The effects of medium ascorbate concentration and its interaction with insulin on the intracellular levels of collagenous protein in human gingival fibroblasts. Collagen production was measured as μg cellular hydroxyproline per 100 μg DNA on days 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 μg/ml respectively. At each level of ascorbate, insulin (containing 0.5% bound zinc) was added in concentrations of 0 or 10^{-6} M.

32 The effects of medium ascorbate and insulin concentrations on the intracellular accumulation of ascorbate in human gingival fibroblasts. Intracellular ascorbate levels were measured as μg ascorbate per μg DNA on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 μg/ml respectively. Insulin (containing 0.5% bound zinc) was added at each level of ascorbate in con-

centrations of 0 and 10^{-6} M.

33 Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on the growth of control and diabetic rat gingival fibroblasts. Cell growth, expressed as DNA per plate, was measured at time of confluency for each cell density (F3, F4 on day 5, and F1 and F2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 µg/ml to 200 µg/ml.

34 Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on cellular RNA content of control and diabetic rat gingival fibroblasts. Cellular RNA expressed on a DNA basis, was measured at time of confluency for each cell density (F3, F4 on day 5, and F1 and F2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 µg/ml to 200 µg/ml.

35 Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on cellular non-collagenous protein content of control and diabetic rat gingival fibroblasts. Cellular non-collagenous protein expressed on a DNA basis, was measured at time of con-

fluency for each cell density (F3, F4 on day 5, and F1 and F2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 μg/ml to 200 μg/ml.

36 Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on cellular collagenous protein content of control and diabetic rat gingival fibroblasts. Collagenous protein, measured as hydroxyproline and expressed on a DNA basis, was determined at time of confluency for each cell density (F3, F4 on day 5, and F1 and F2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 μg/ml to 200 μg/ml.

37 Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on the intracellular accumulation of ascorbate by control and diabetic rat gingival fibroblasts. Ascorbate levels expressed on a DNA basis, were measured at time of confluency for each cell density (F3, F4 on day 5, and F1 and F2 on day 6). In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 μg/ml to 200 μg/ml.

38 Comparative effects of cell age and insulin on the growth of human gingival fibroblasts. Cell growth, presented as DNA per plate, was determined on 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

39 Comparative effects of cell age and insulin on cellular RNA concentrations of human gingival fibroblasts. RNA levels of 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24 are presented on a DNA basis. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

40 Comparative effects of cell age and insulin on cellular non-collagenous protein concentrations of human gingival fibroblasts. Non-collagenous protein of 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24 are presented on a RNA basis. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

41 Comparative effects of cell age and insulin on cellular collagenous protein concentrations of human gingival fibroblasts. Collagenous protein measured as hydroxyproline is presented on a DNA basis for 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24. At these pas-

sages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

42 Comparative effects of age and insulin on the growth of control and diabetic rat gingival fibroblasts. Cell growth, expressed as μg DNA per plate, was measured on 9 day old cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 for the control rat cells and at passages 3, 4, 6 and 9 only for the diabetic rat cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

43 Comparative effects of age and insulin on cellular RNA levels of control and diabetic rat gingival fibroblasts. RNA expressed on a DNA basis, was determined on 9 day old control rat cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and 9 day old diabetic rat cells and at passages 3, 4, 6 and 9 only. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

44 Comparative effects of age and insulin on cellular non-collagenous protein levels of control and diabetic rat gingival fibroblasts. Non-collagenous protein expressed on a DNA basis, was determined on 9 day old control rat cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and 9 day old diabetic rat cells at passages 3, 4, 6 and 9

only. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

45 Comparative effects of age and insulin on cellular collagenous protein levels of control and diabetic rat gingival fibroblasts. Collagenous protein, measured as hydroxyproline and expressed on a DNA basis, was determined on 9 day old control rat cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and 9 day old diabetic rat cells at passages 3, 4, 6 and 9 only. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

46 Effects of streptozotocin added <u>in vitro</u> to the growth medium on total hydroxyproline (cellular + medium) production by control rat gingival fibroblasts. Total hydroxyproline expressed on a per plate basis was determined on days 3, 6 and 9 of a 9 day growth period. Streptozotocin was added to the growth medium at concentrations of 0, 1.0, 10.0 and 100.0 µg/ml.

47 Comparative cell growth of control and diabetic rat gingival fibroblasts in the presence of insulin preparations containing different amounts of bound zinc. Growth was measured as DNA per plate on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ1 and IZ2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6}

M.

48 Comparison of cellular RNA levels of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Cellular RNA was expressed on a DNA basis and measured on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ1 and IZ2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

49 Comparison of cellular non-collagenous protein concentrations of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Protein was expressed as non-collagenous protein on a DNA basis and measured on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ1 and IZ2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

50 Comparison of cellular non-collagenous protein levels of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Protein levels, expressed as non-collagenous protein on a RNA basis, were measured on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ1 and IZ2, containing 0.2 and 0.5% bound zinc respectively,

were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

51 Comparison of cellular collagenous protein content of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Collagenous protein is expressed as µg cellular hydroxyproline per 100 µg DNA on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ1 and IZ2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

52 Relationship of the effects of insulin and zinc concentrations alone and of their interaction on the growth of control and diabetic rat gingival fibroblasts. Growth was measured as DNA per plate on 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

53 Relationship of the effects of insulin and zinc concentrations alone and of their interaction on cellular RNA concentration of control and diabetic rat gingival fibroblasts. Cellular RNA is expressed on a DNA basis

for 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

54 Relationship of the effects of insulin and zinc concentrations alone and of their interaction on cellular non-collagenous protein levels of control and diabetic rat gingival fibroblasts. Cellular non-collagenous protein is expressed on a DNA basis for 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

55 Relationship of the effects of insulin and zinc concentrations alone and of their interaction on cellular collagenous protein concentration of control and diabetic rat gingival fibroblasts. Cellular collagenous protein is expressed on a DNA basis for 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were deter-

mined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

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and of insulin and ascorbate combined on the accumulation of medium hydroxyproline by control and diabetic rat gingival fibroblasts.

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10 In vitro effect of streptozotocin on RNA production by rat gingival fibroblasts.

11 In vitro effect of streptozotocin on non-collagenous protein produced by rat gingival fibroblasts.

12 In vitro effect of streptozotocin on cellular hydroxyproline production by rat gingival fibroblasts.

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14 In vitro effect of testosterone and testosterone-insulin interaction on rat gingival fibroblast growth.

15 In vitro effects of testosterone and testosterone-insulin interaction on RNA production by rat gingival fibroblasts.

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17 In vitro effects of testosterone and testosterone-insulin interaction on cellular hydroxyproline production by rat

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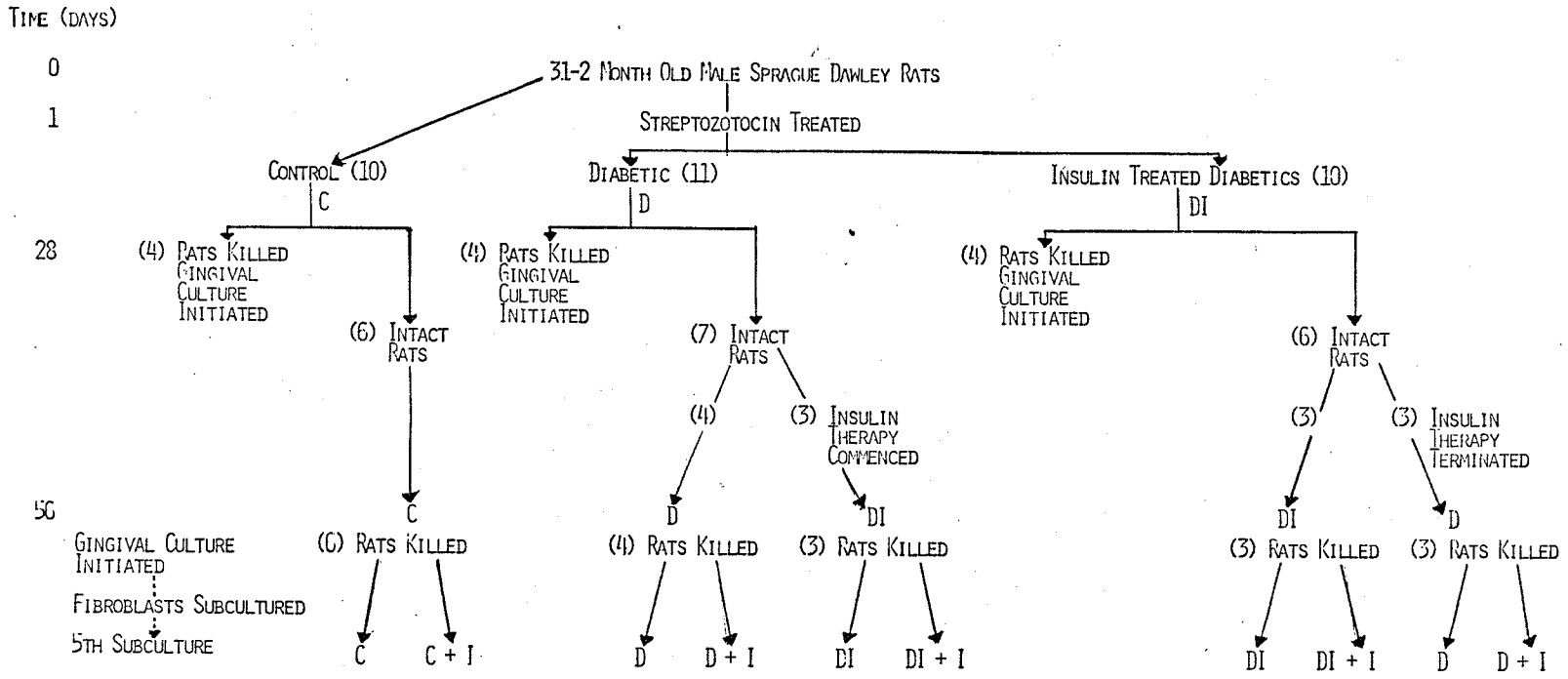
18 Comparative effects of 2 insulin preparations with different amounts of bound zinc on medium hydroxyproline accumulation by rat gingival fibroblasts.

19 Comparison of *in vitro* effects of insulin, zinc chloride and their interaction on medium hydroxyproline accumulation by 9 day old control and diabetic rat gingival fibroblasts.

CHAPTER IX

FIGURES AND TABLES

FIGURE 1: FLOW CHART OF ANIMAL TREATMENT



DAY 1 = 24 HOURS POST STREPTOZOTOCIN TREATMENT

INSULIN CONCENTRATION = 10^{-6}M

C - CONTROL, D - DIABETIC, DI - INSULIN TREATED DIABETIC, + - INSULIN ADDED IN VITRO

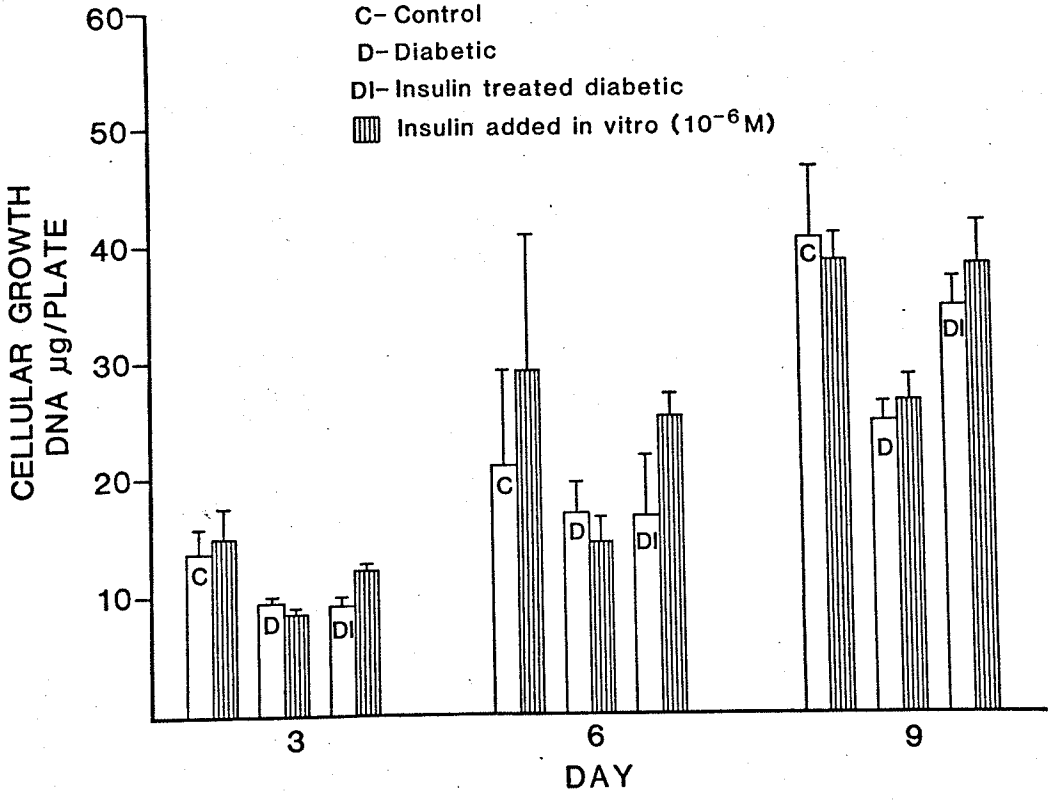


FIGURE 2: Comparative cellular growth of gingival fibroblasts from control, diabetic and insulin treated diabetic rats, maintained in medium supplemented with either 0 or 10^{-6} M insulin (containing 0.2% bound zinc). Growth was measured as DNA per plate and monitored for a 9 day period.

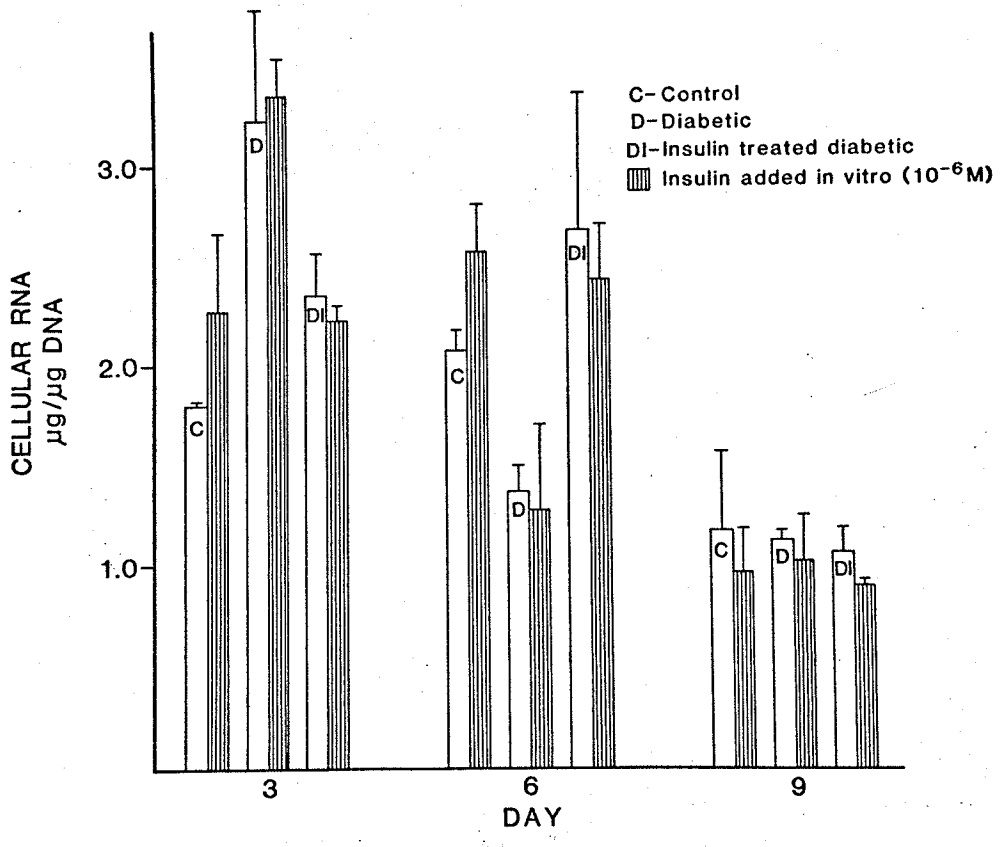


FIGURE 3: Comparison of cellular RNA levels in gingival fibroblasts from control, diabetic and insulin treated diabetic rats, maintained in medium supplemented with either 0 or 10^{-6} M insulin (containing 0.2% bound zinc). RNA was measured on a DNA basis for a 9 day growth period.

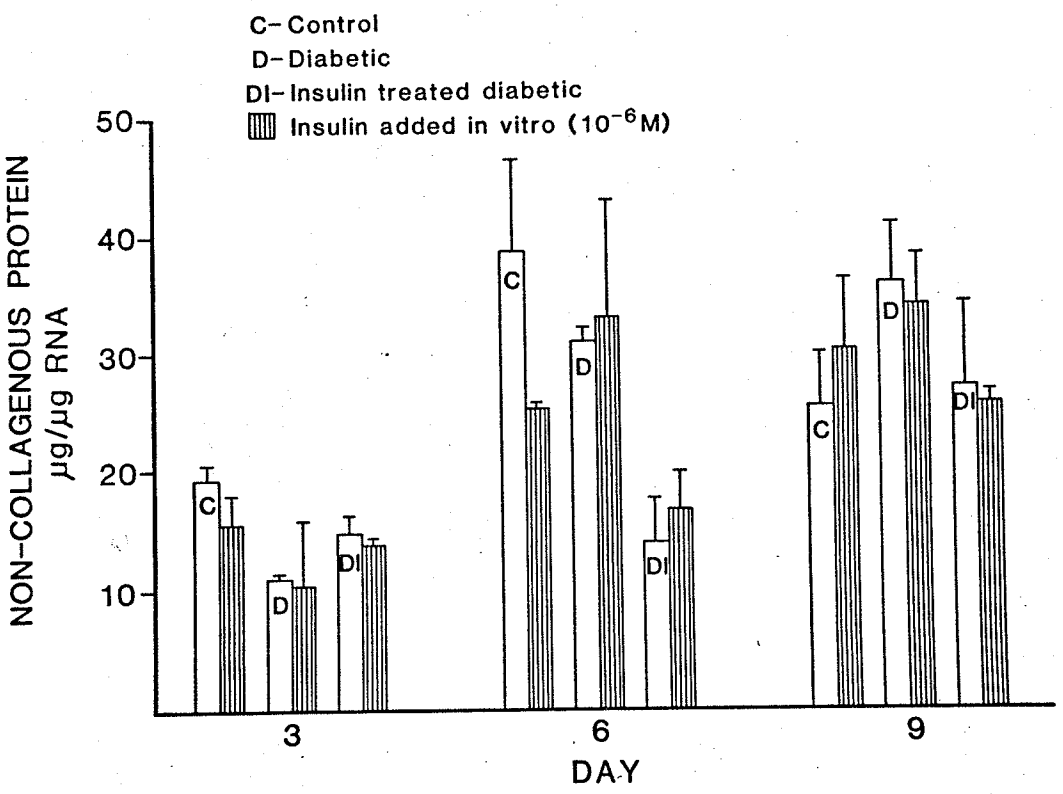


FIGURE 4: Comparison of non-collagenous protein levels in gingival fibroblasts from control, diabetic and insulin treated diabetic rats maintained in medium supplemented with either 0 or 10^{-6} M insulin (containing 0.2% bound zinc). Non-collagenous protein levels are presented on a RNA basis for a 9 day growth period.

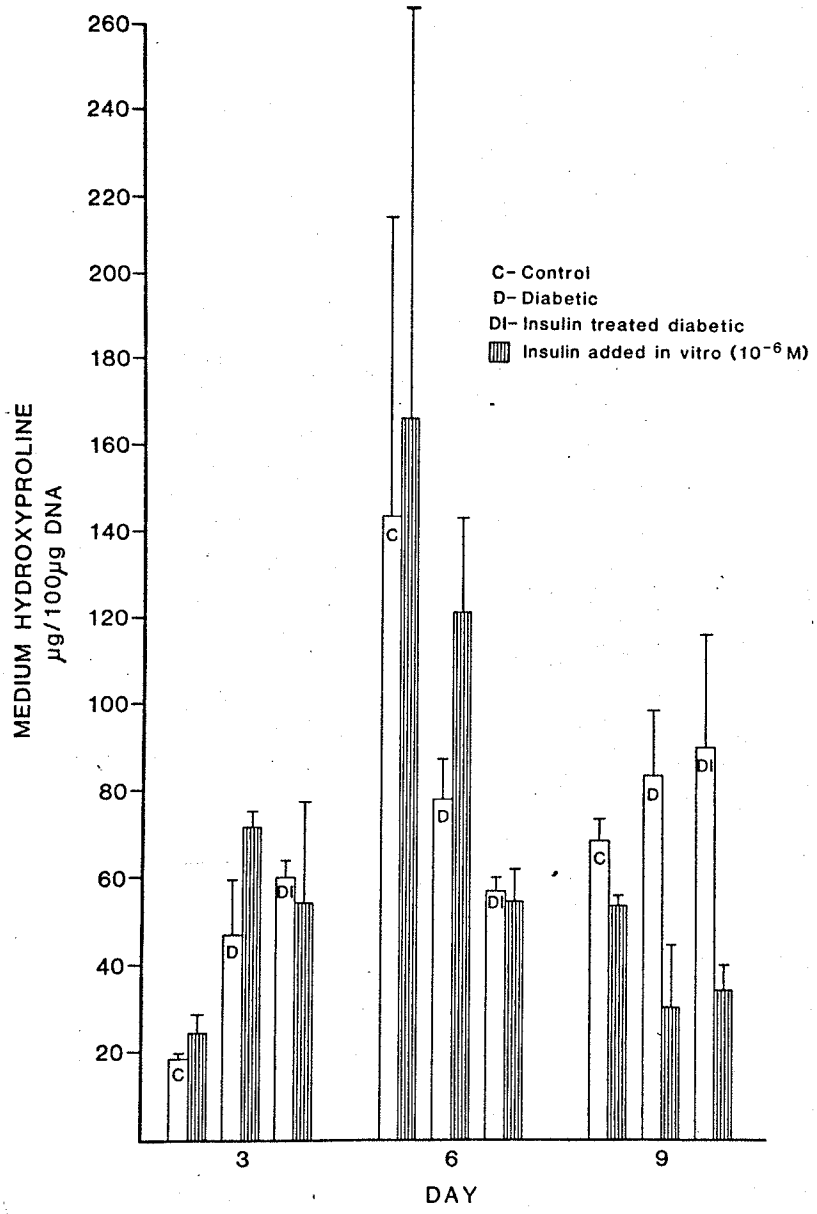


FIGURE 5: Comparison of medium hydroxyproline accumulation by gingival fibroblasts from control, diabetic and insulin treated diabetic rats, maintained in medium supplemented with either 0 or 10^{-6} M insulin (containing 0.2% bound zinc). Hydroxyproline levels are presented on a DNA basis for a 9 day growth period.

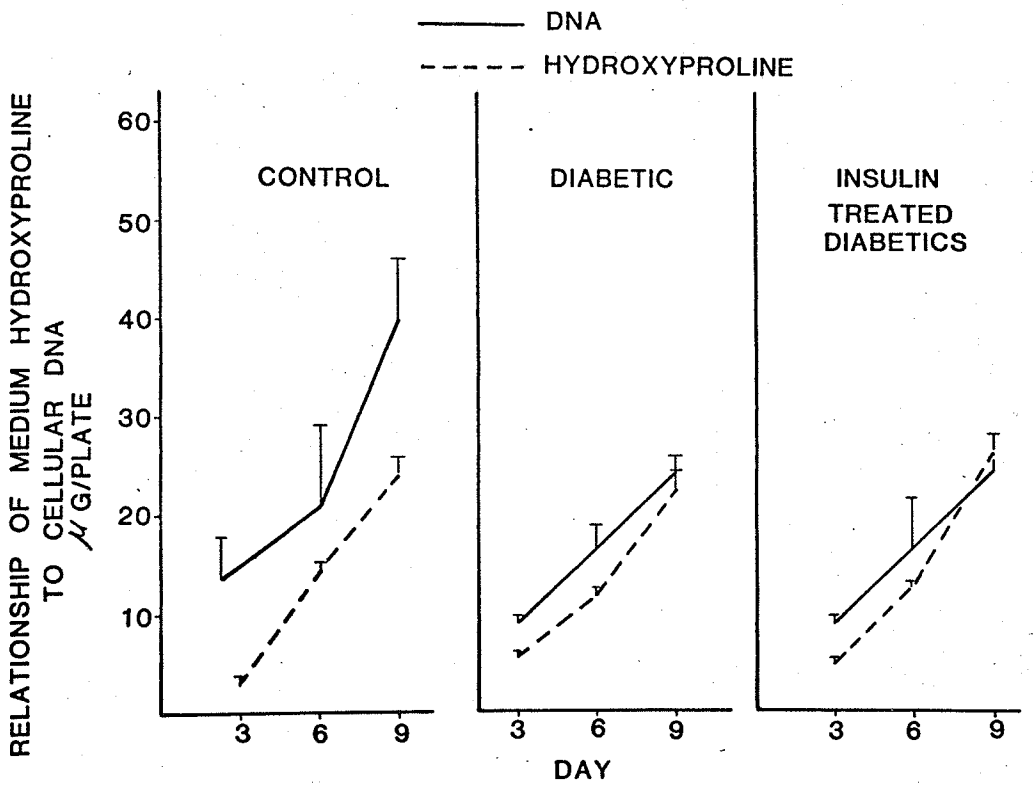


FIGURE 6: Relationship of medium hydroxyproline levels to cellular replication by control, diabetic and insulin treated diabetic rats. Medium hydroxyproline and cellular DNA levels are presented on a plate basis for a 9 day growth period.

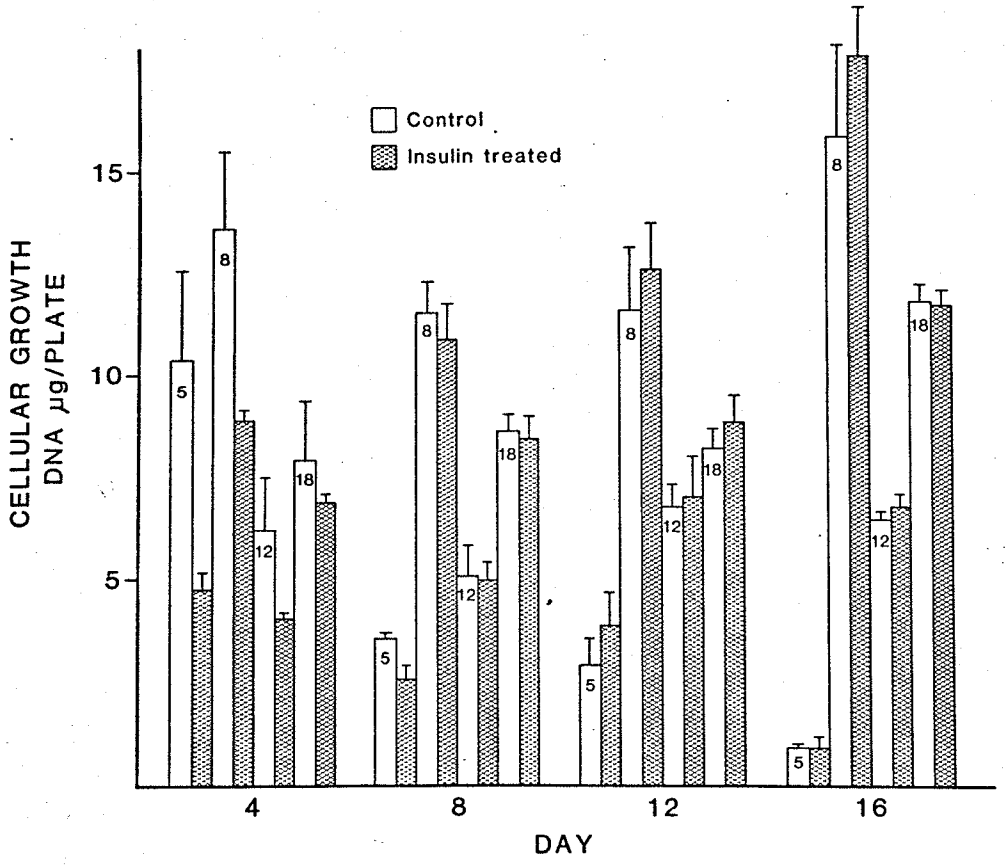


FIGURE 7: Relationship of insulin and cell age to cellular replication of human gingival fibroblasts. Fibroblasts were used on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin (containing 0.5% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-9} M.

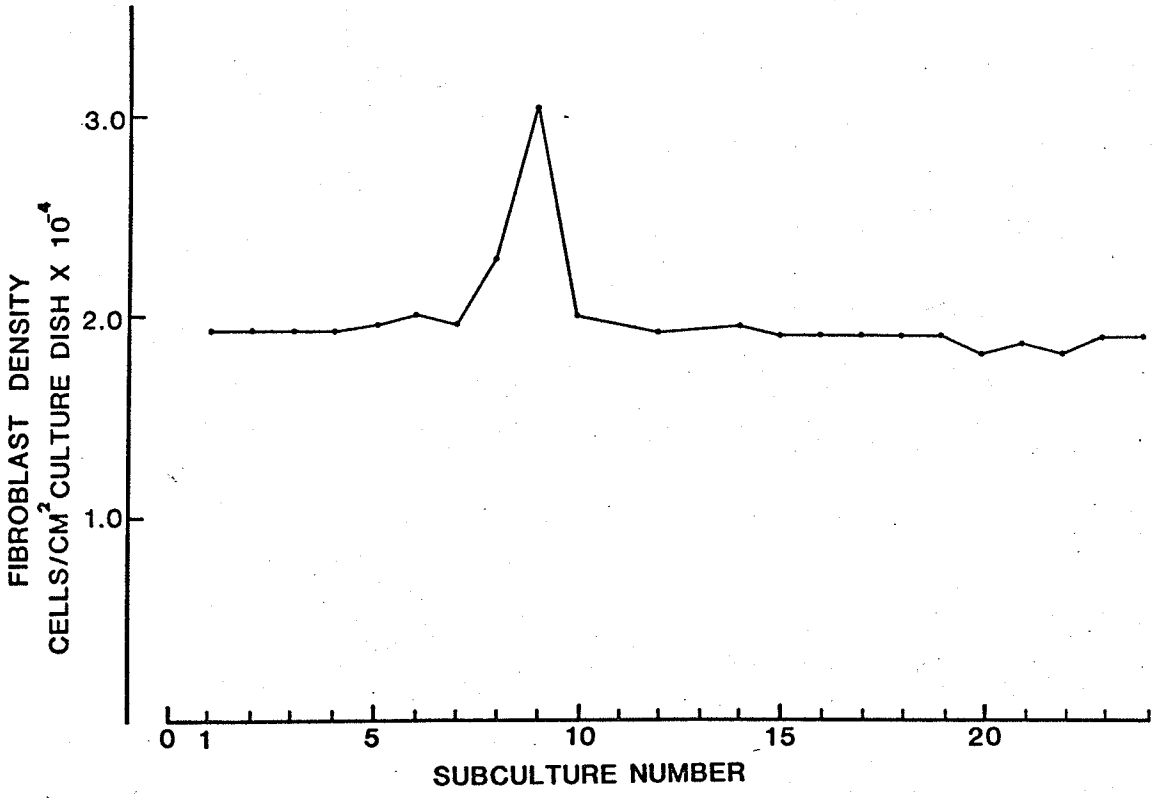


FIGURE 8: Effect of cell age on cellular replication by human gingival fibroblasts. Growth is presented as cell density (cells/cm^2 of culture dish x 10^{-4}). Cell densities were determined at each of passages 1 through 24.

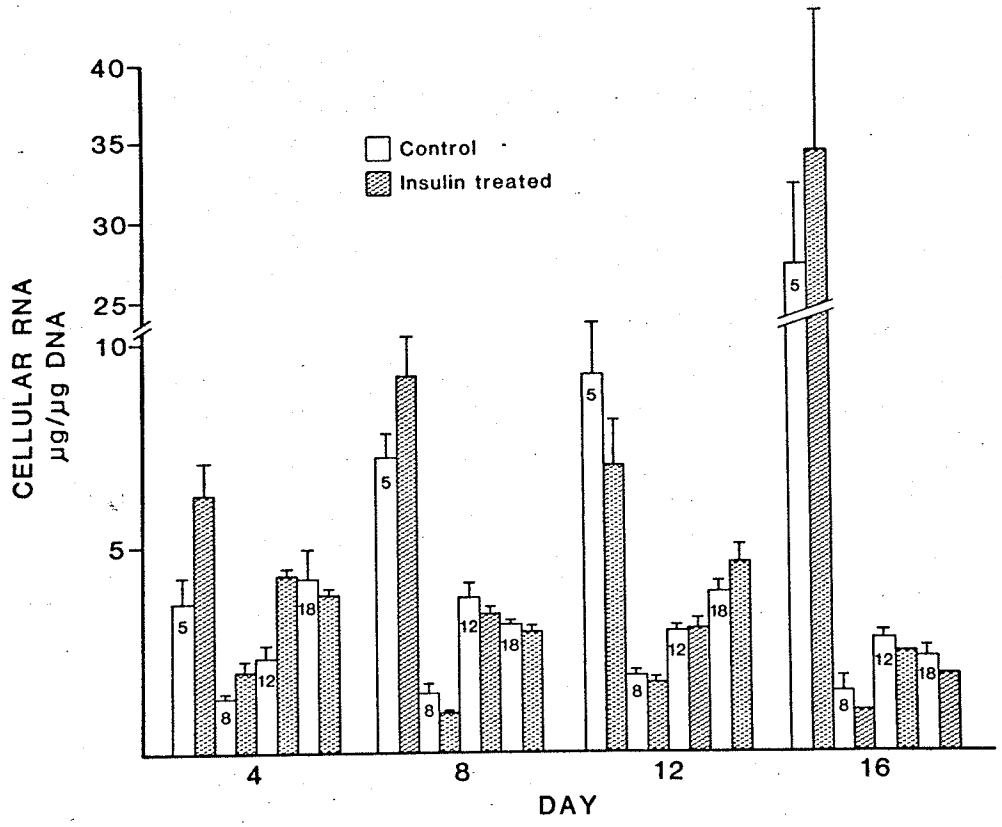


FIGURE 9: Relationship of insulin and cell age to cellular RNA concentration in human gingival fibroblasts. RNA expressed on a DNA basis was measured on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin containing 0.5% bound zinc was added to the growth medium at concentrations of 0 and 10^{-9} M.

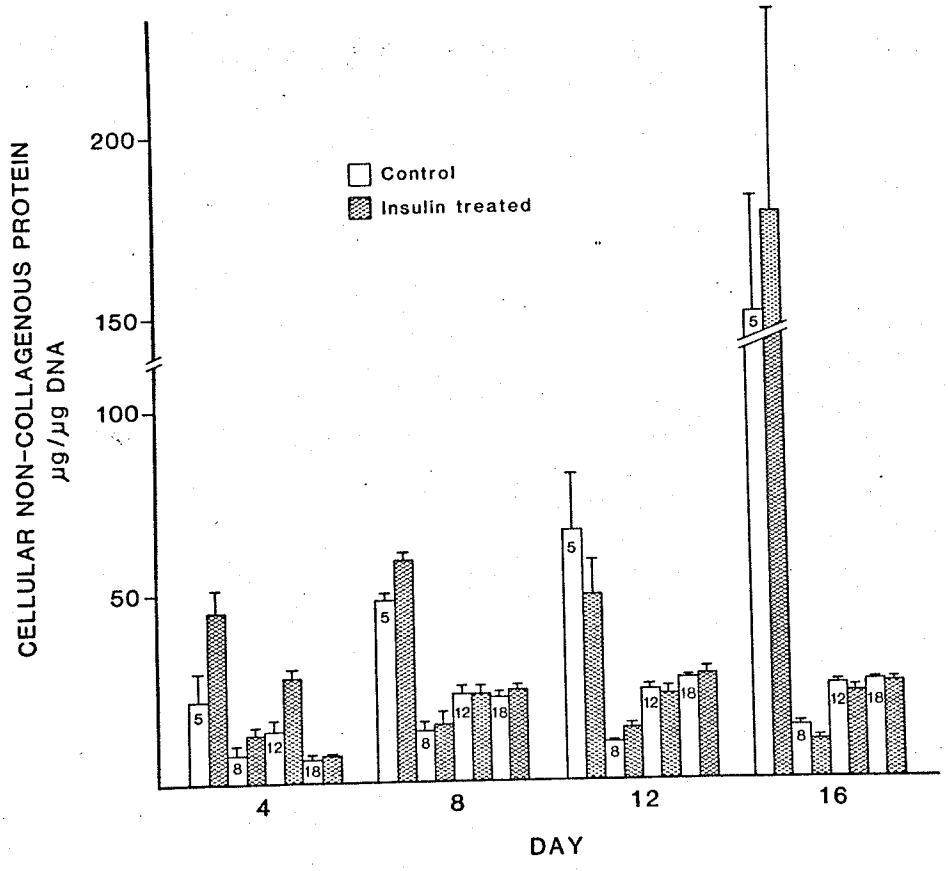


FIGURE 10: Relationship of insulin and cell age to cellular non-collagenous protein levels in human gingival fibroblasts. Protein expressed on a DNA basis was measured on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin (containing 0.5% bound zinc) was added at concentrations of 0 and 10^{-9} M.

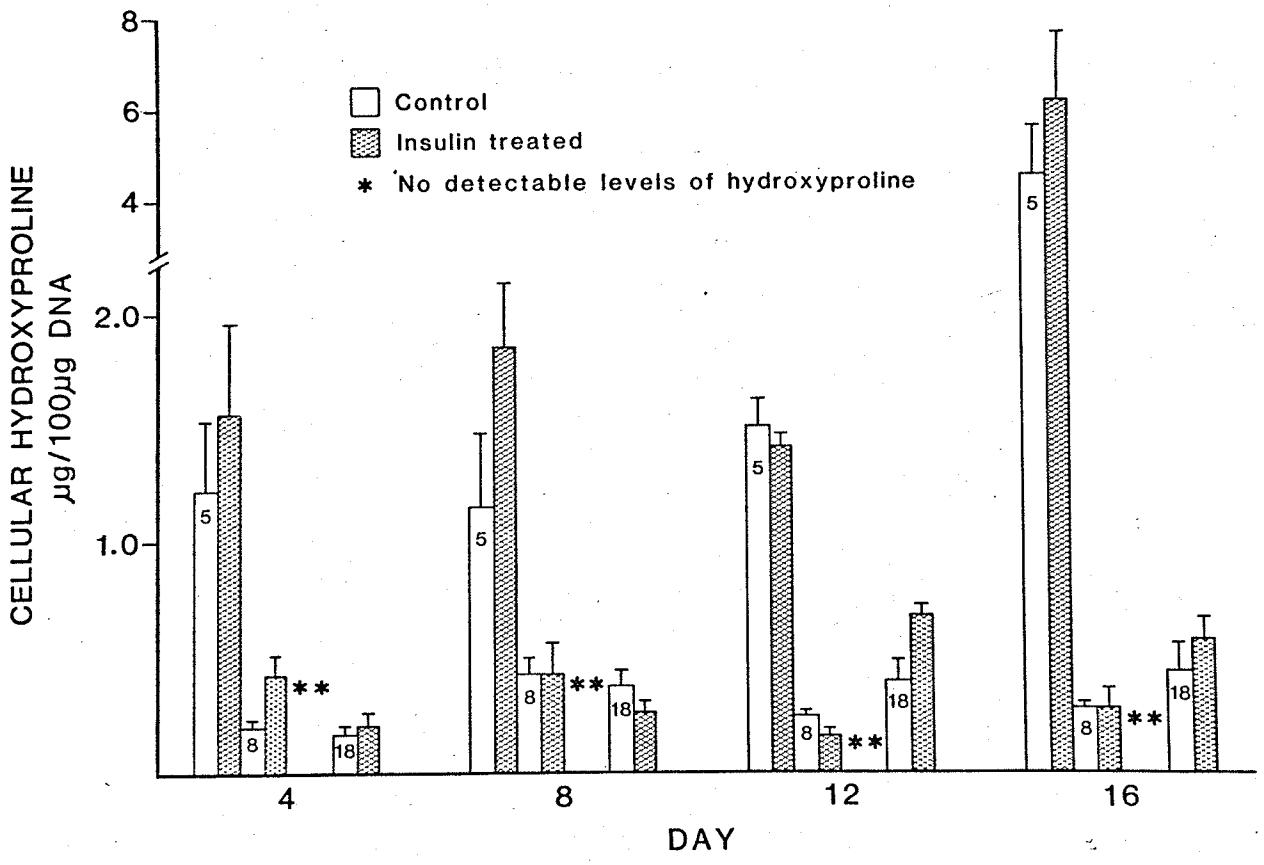


FIGURE 11: Relationship of insulin and cell age to cellular collagenous protein concentration in human gingival fibroblasts. Collagenous protein was expressed as hydroxyproline per DNA and measured on days 4, 8, 12 and 16 of passages 5, 8 and 18. There were no detectable levels of cellular collagenous protein in the 12th passage. Insulin (containing 0.5% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-9} M.

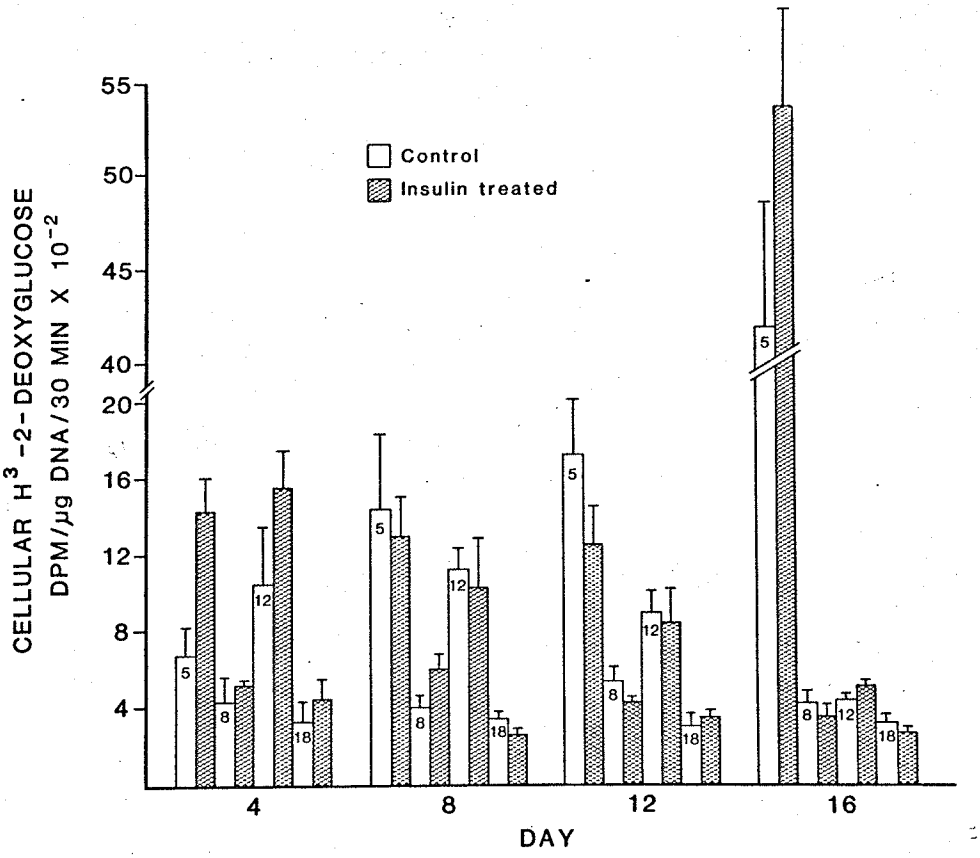


FIGURE 12: Relationship of insulin and cell age to membrane transport of ^{3}H-2-deoxyglucose by human gingival fibroblasts. The uptake of ^{3}H-2-deoxyglucose was measured on days 4, 8, 12 and 16 of passages 5, 8, 12 and 18. Insulin (containing 0.5% bound zinc) was added at concentrations of 0 and 10^{-9} M.

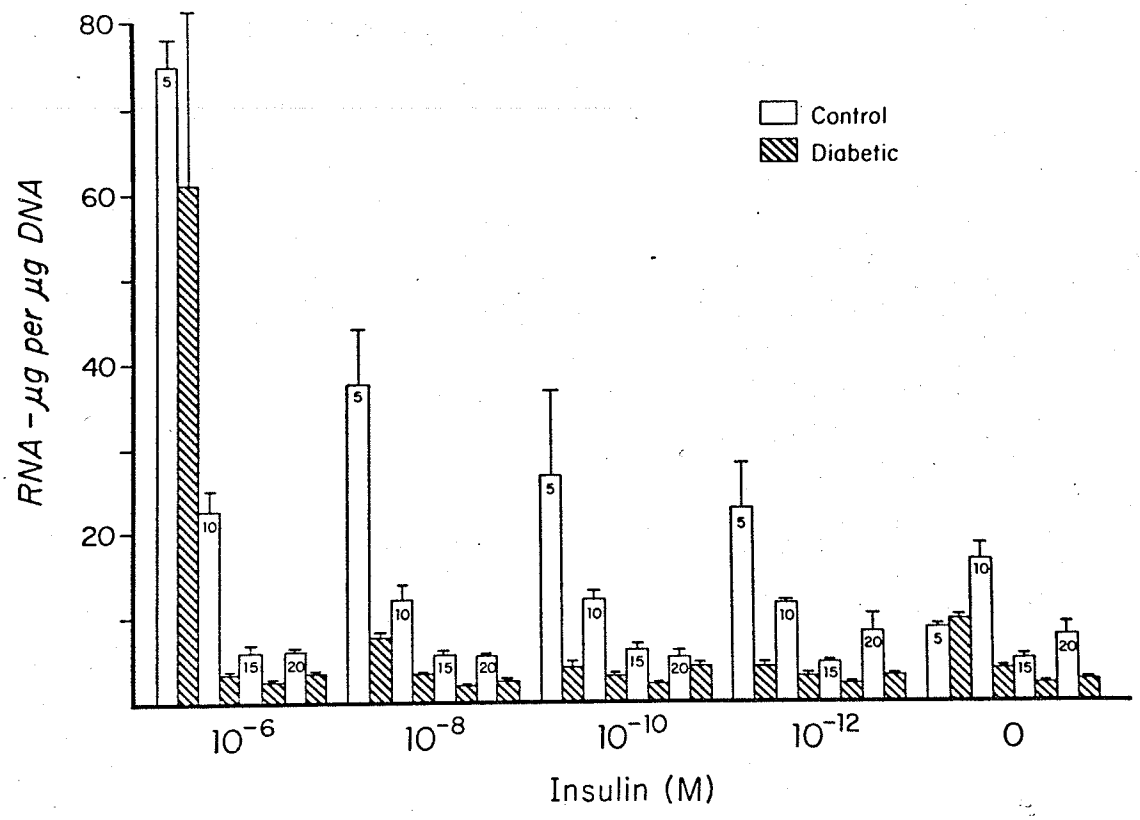


FIGURE 13: Comparative effects of the interaction of medium serum and insulin on cellular RNA levels in control and diabetic rat gingival fibroblasts. RNA levels are presented on a DNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level insulin (containing 0.5% bound zinc was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

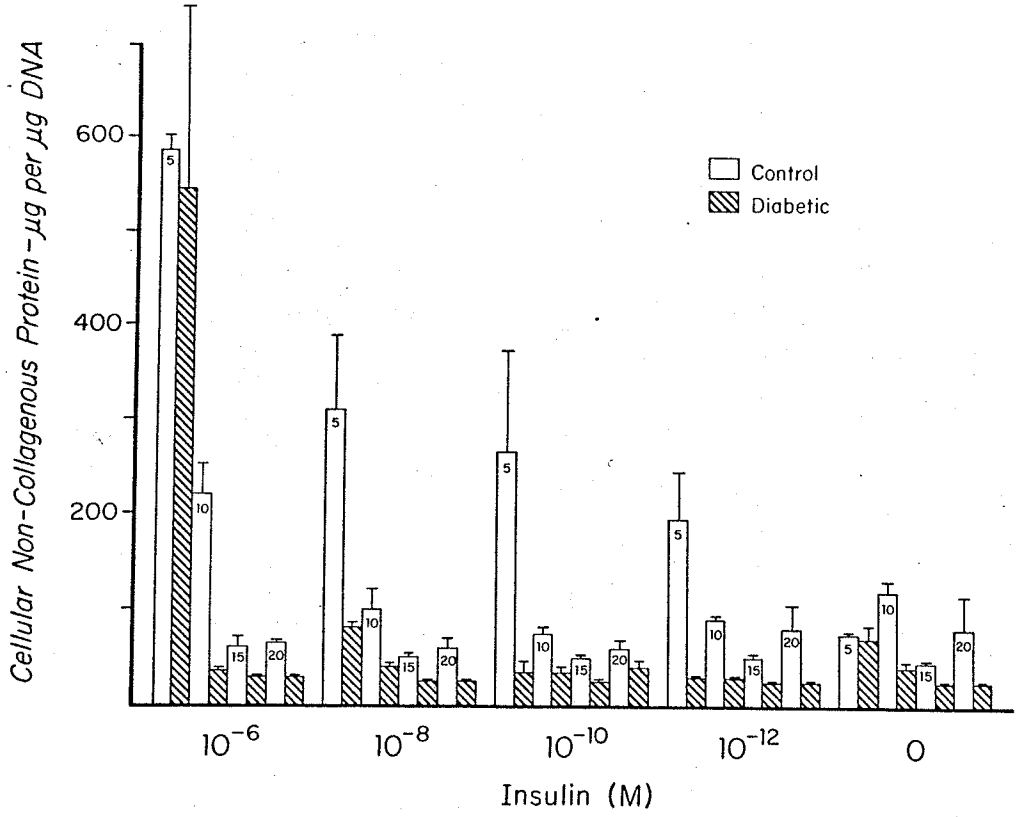


FIGURE 14: Comparative effects of the interaction of medium serum and insulin on the cellular non-collagenous protein concentrations of control and diabetic rat gingival fibroblasts. Non-collagenous protein is presented on a DNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

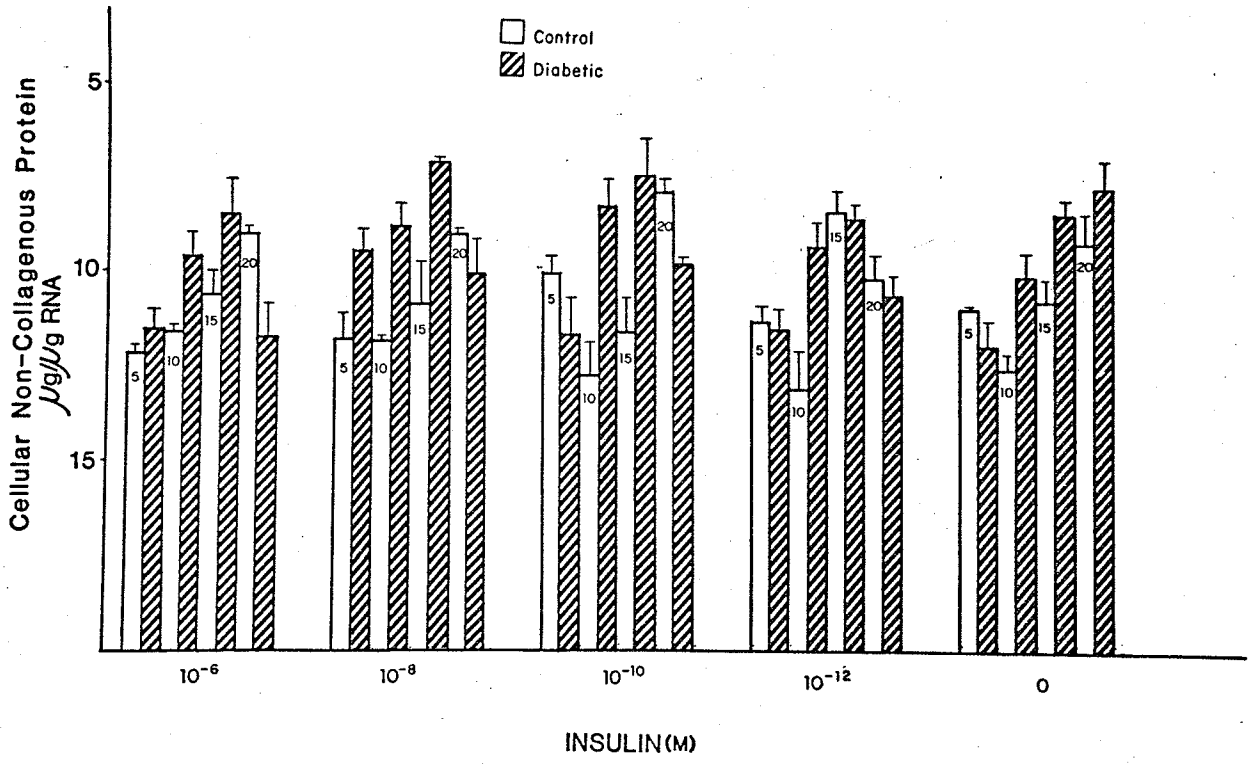


FIGURE 15: Comparative effects of the interaction of medium serum and insulin on the cellular non-collagenous protein concentrations of control and diabetic rat gingival fibroblasts. Non-collagenous protein is presented on a RNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

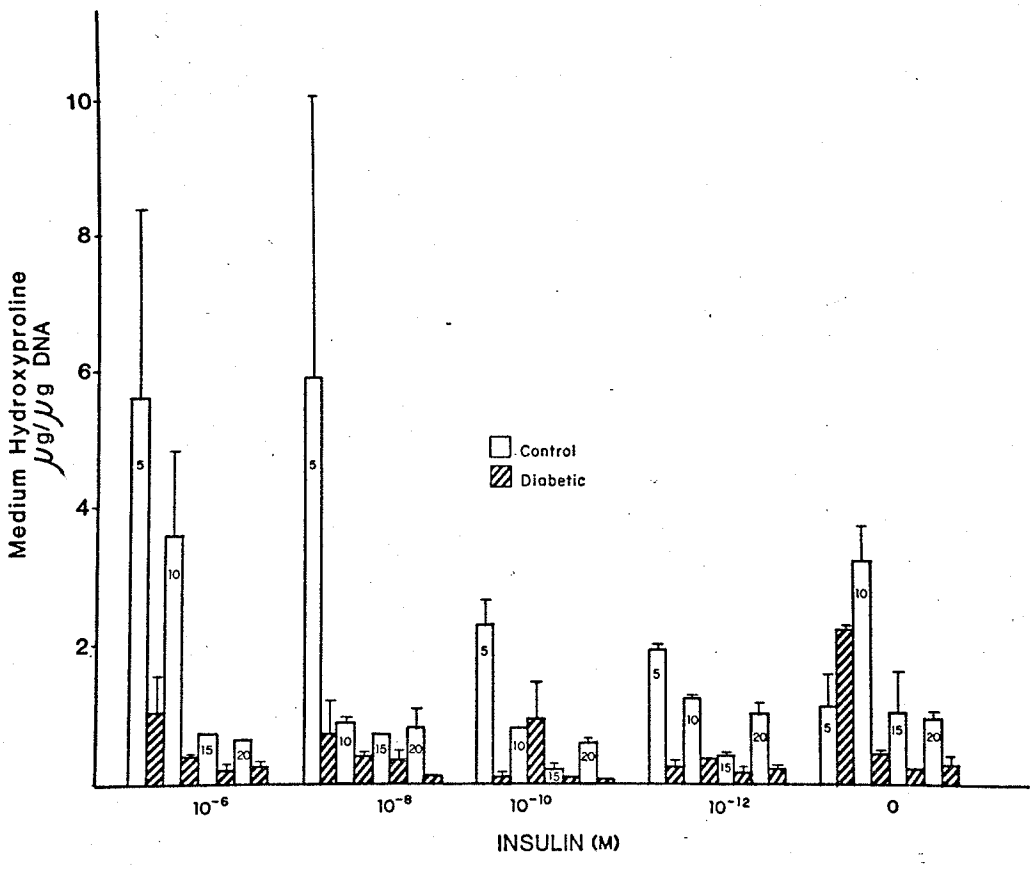


FIGURE 16: Comparative effects of the interaction of medium serum and insulin on the accumulation of medium collagenous protein by control and diabetic rat gingival fibroblasts. Collagenous protein, measured as medium hydroxyproline, is presented on a DNA basis for 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

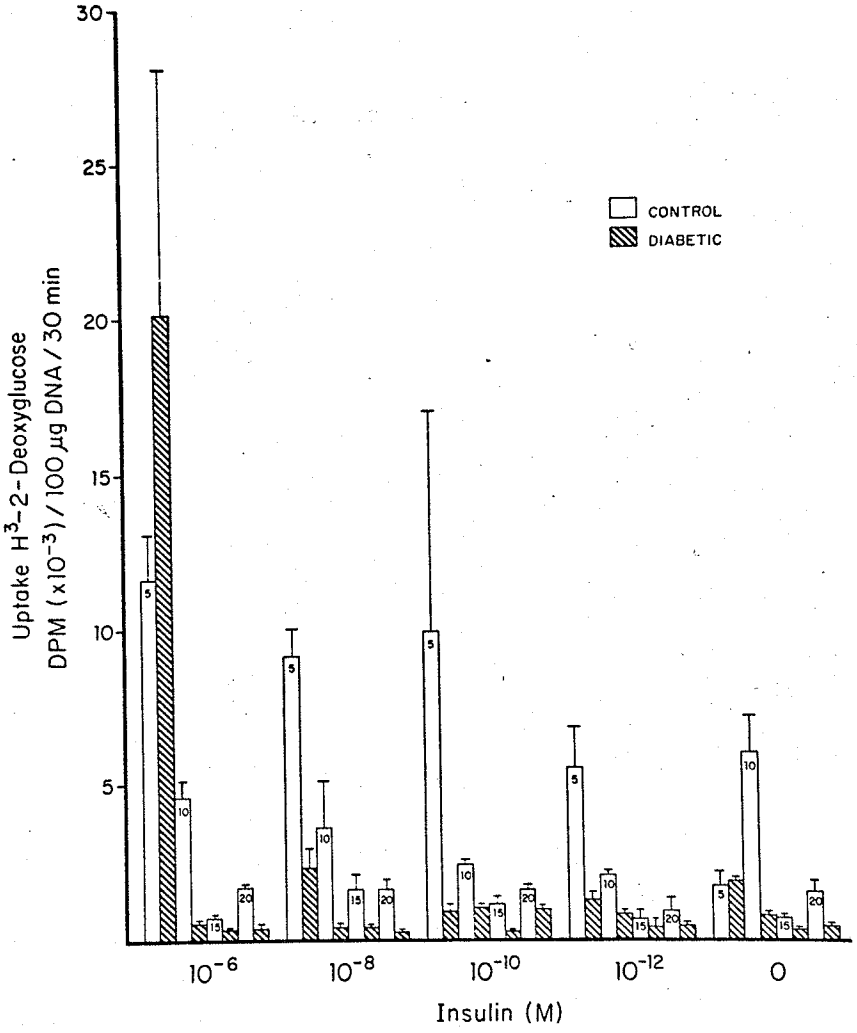


FIGURE 17: Comparative effects of the interaction of medium serum and insulin on membrane transport of ^{3}H-2-deoxyglucose by control and diabetic rat gingival fibroblasts. ^{3}H-2-deoxyglucose uptake was assessed on 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 or 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

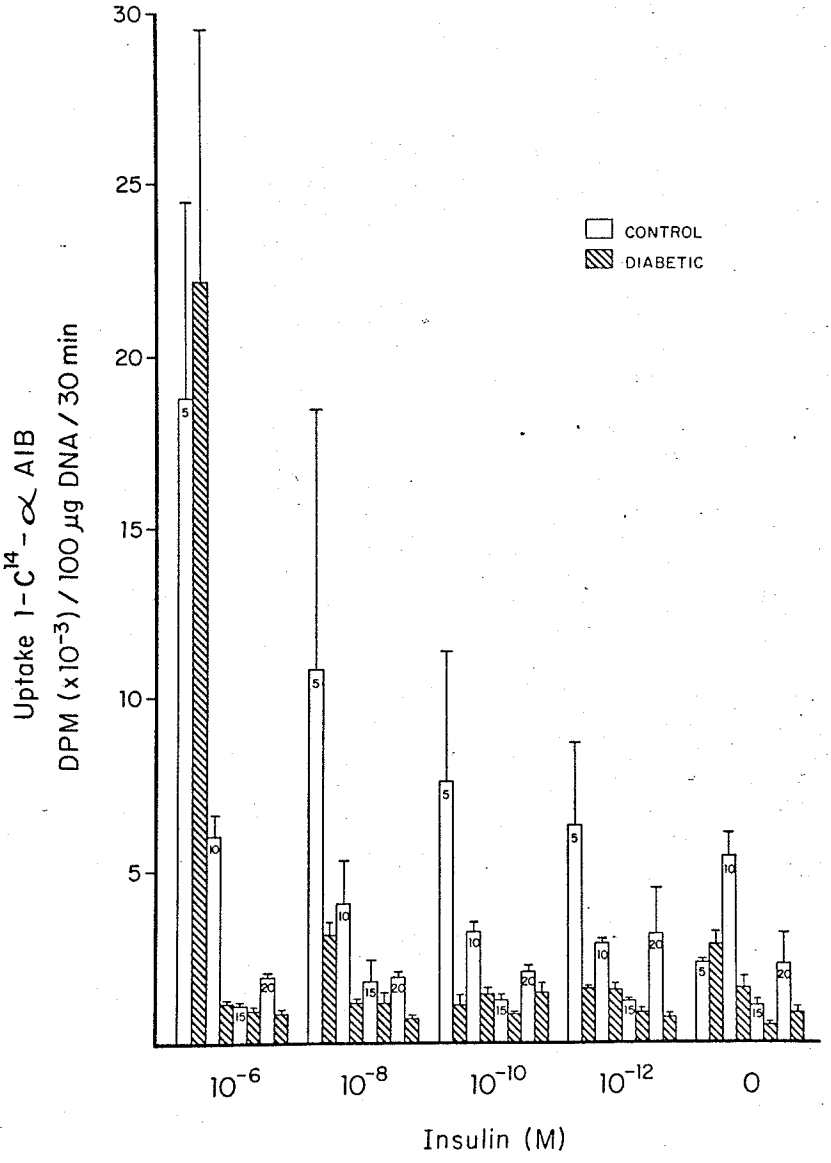


FIGURE 18: Comparative effects of the interaction of medium serum and insulin on membrane transport of ^{14}C-α-aminoisobutyric acid (AIB) by control and diabetic rat gingival fibroblasts. ^{14}C-α-AIB uptake was assessed on 12 day old cells. FCS was added to the growth medium at concentrations of 5, 10, 15 and 20%. At each serum level, insulin (containing 0.5% bound zinc) was added at 0, 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6} M respectively.

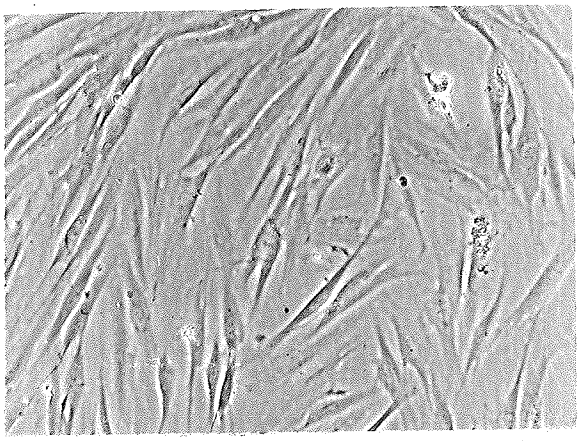
Figure 19 (a) - (e)

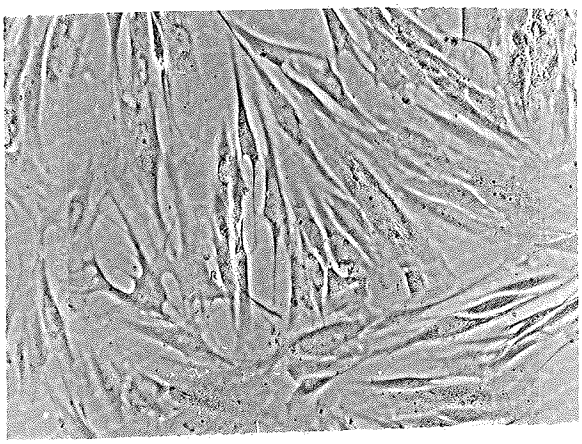
Comparative effects of substituting control or diabetic rat serum for FCS in the growth medium of human gingival fibroblasts at levels of (i) 5%, (ii) 10% and (iii) 15% rat serum.

(a) Day 1
(b) Day 2
(c) Day 5
(d) Day 8
(e) Day 13

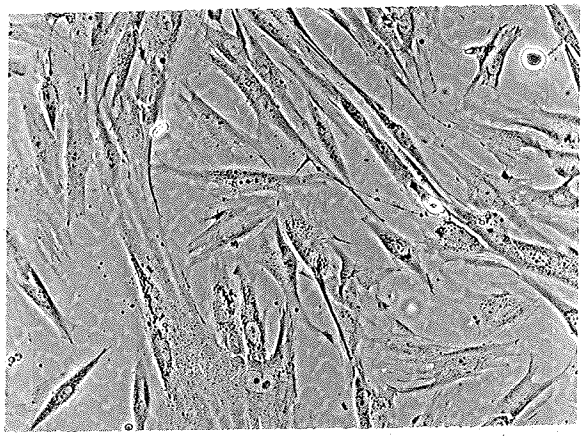
Figure 19 (a)

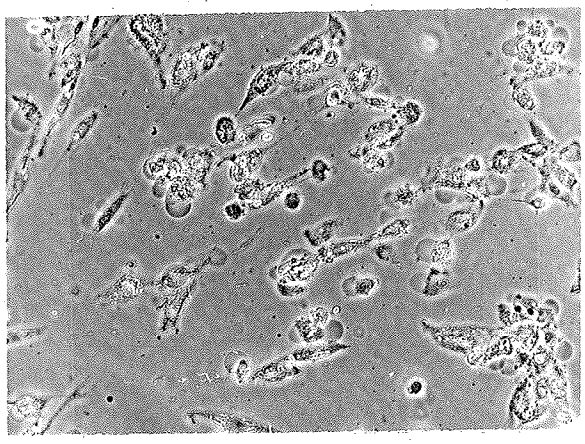
CONTROL (i) 5% DIABETIC



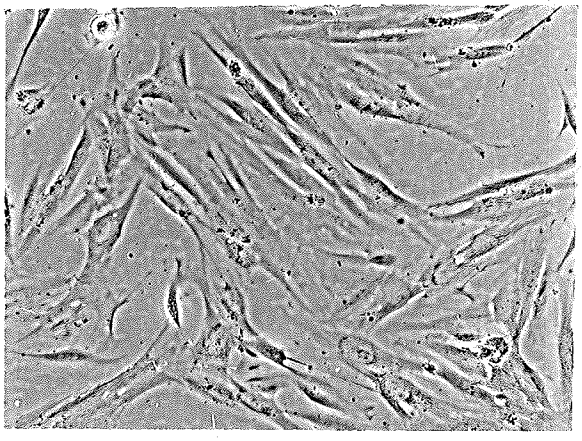


(ii) 10%





(iii) 15%



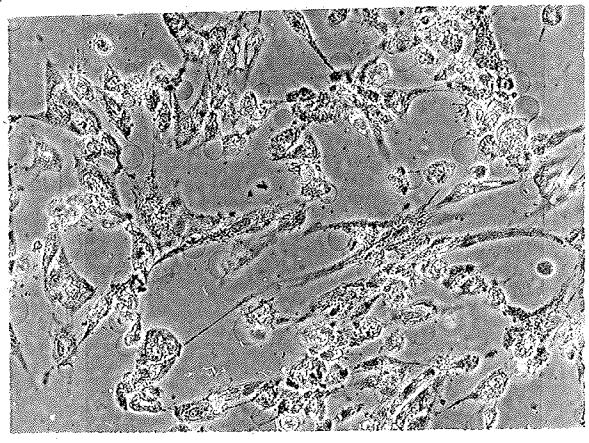
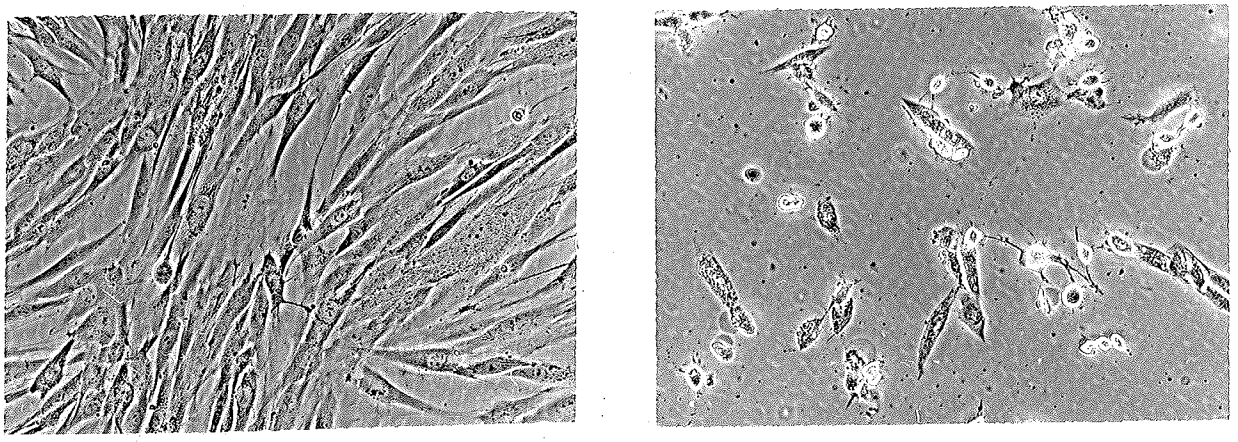
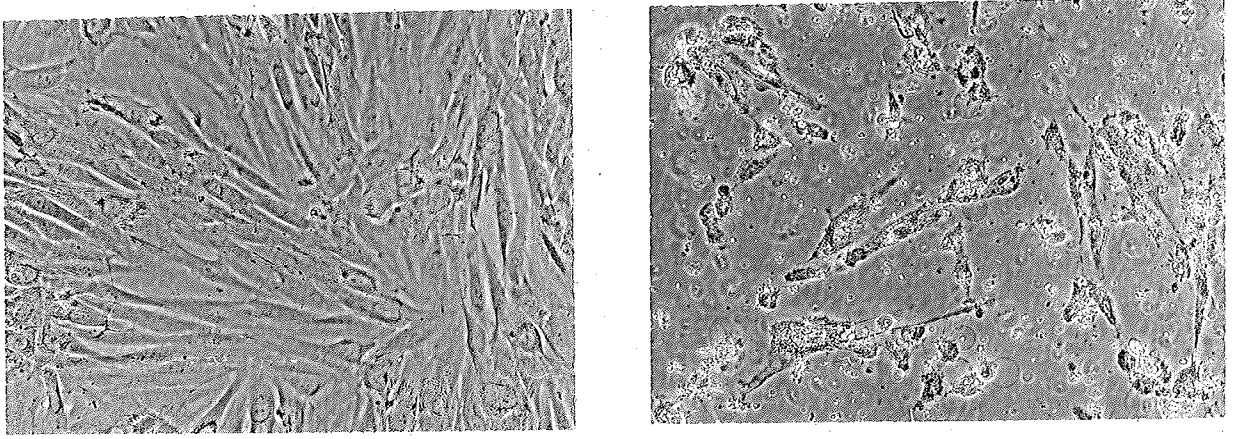


Figure 19(b)

CONTROL (i) 5% DIABETIC



(ii) 10%



(iii) 15%

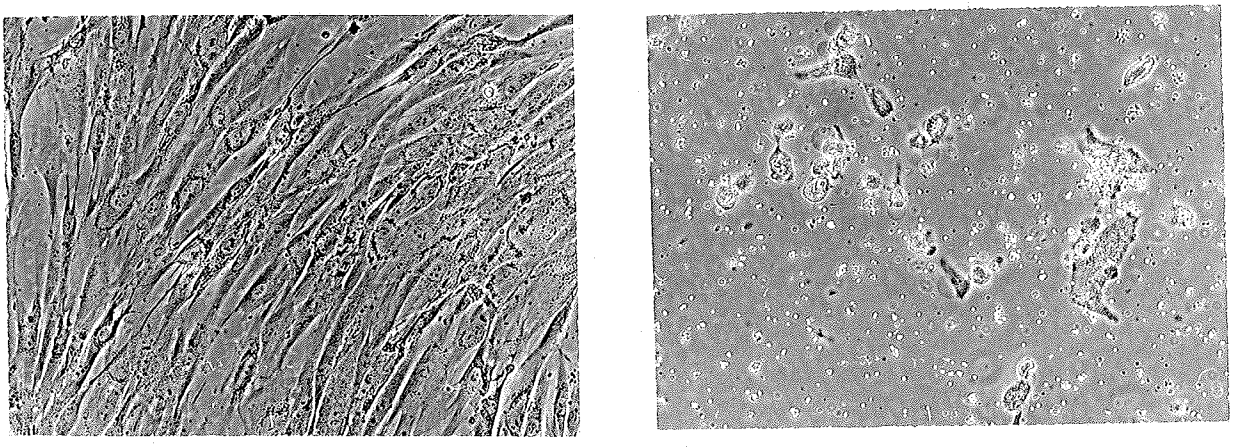
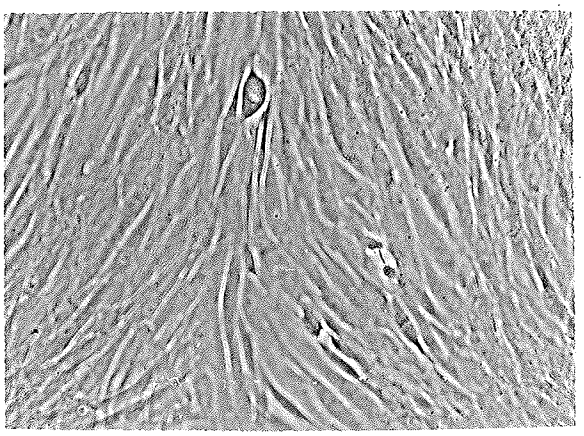
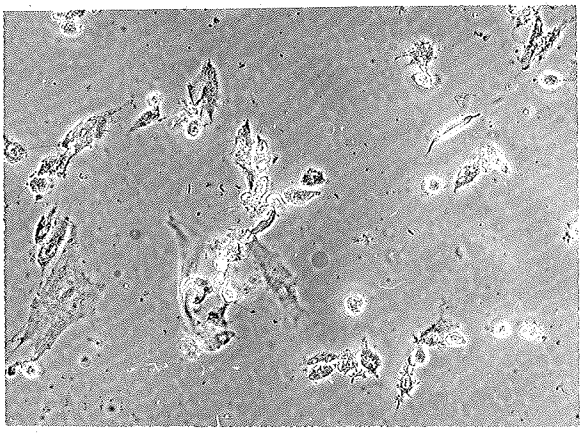


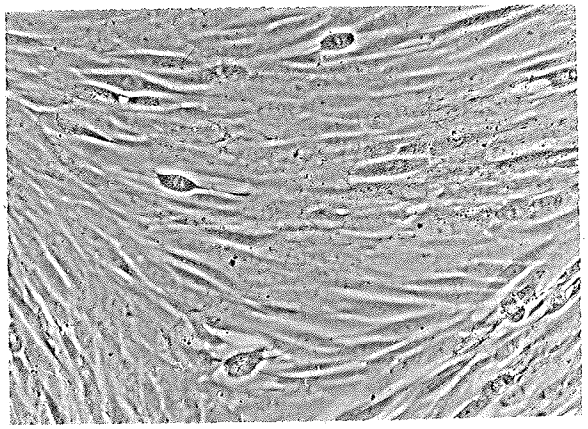
Figure 19 (c)

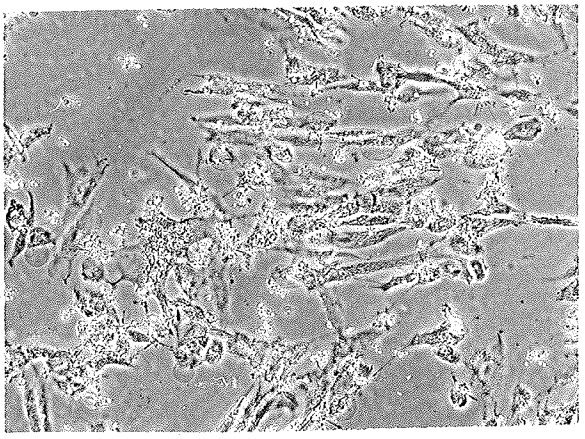
CONTROL (i) 5% DIABETIC



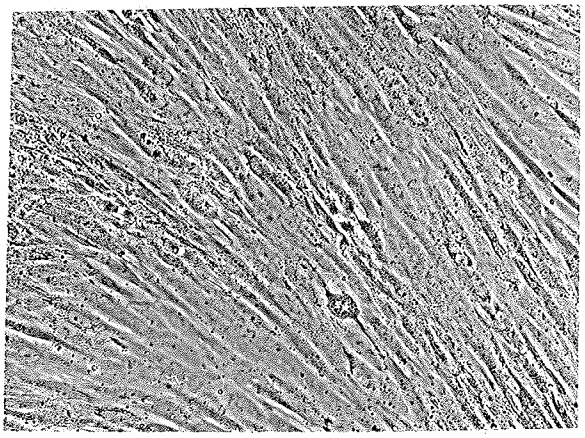


(ii) 10%





(iii) 15%



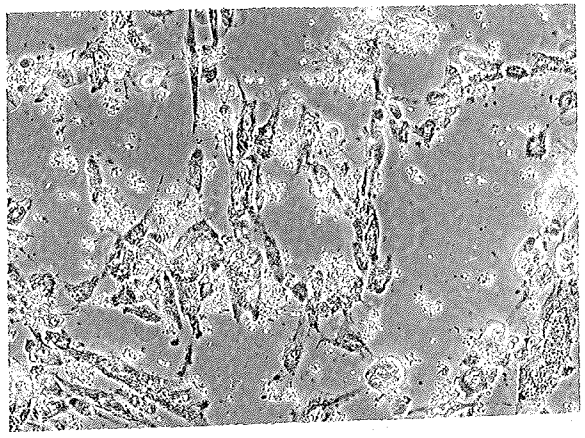
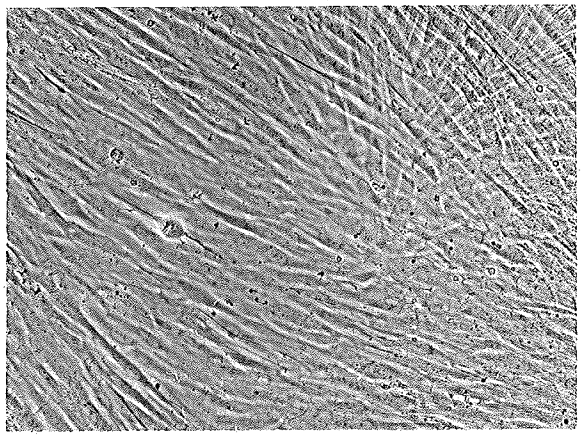
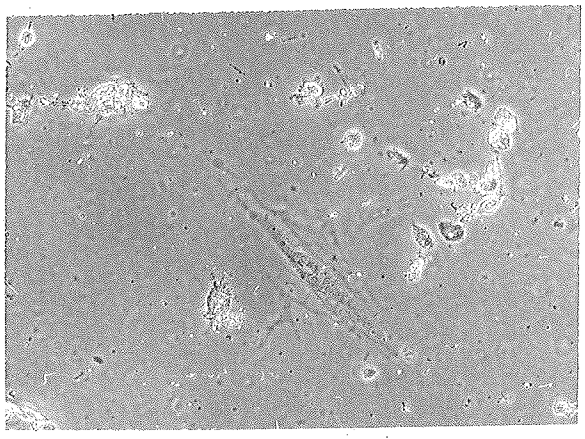


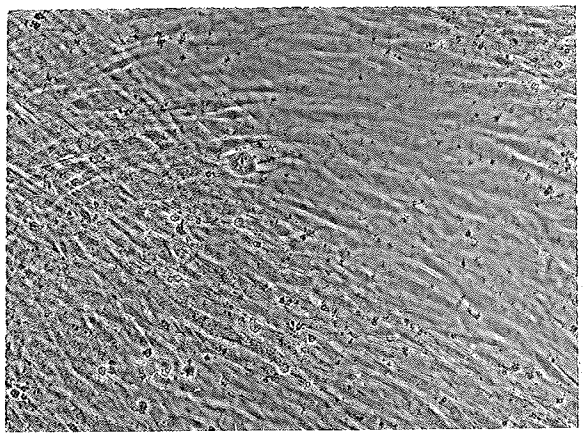
Figure 19 (d)

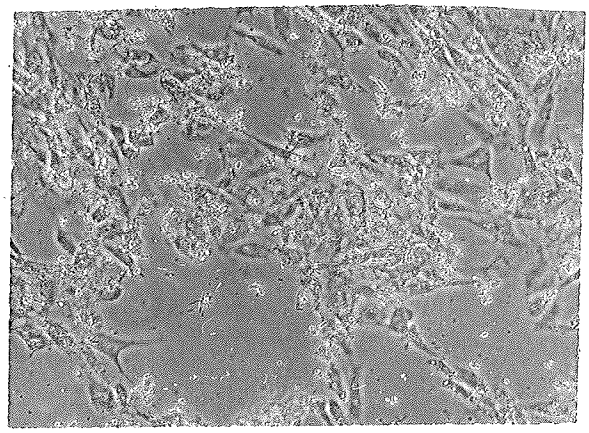
CONTROL (i) 5% DIABETIC



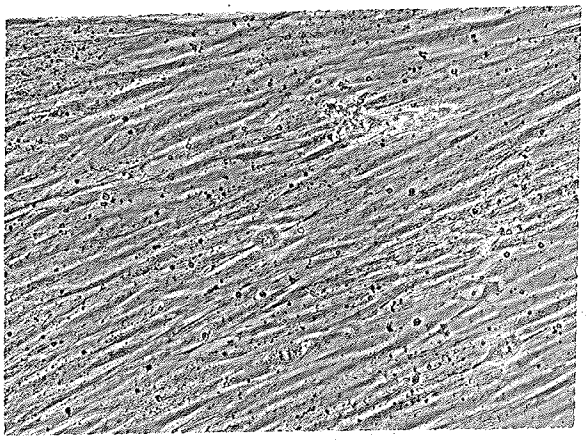


(ii) 10%





(iii) 15%



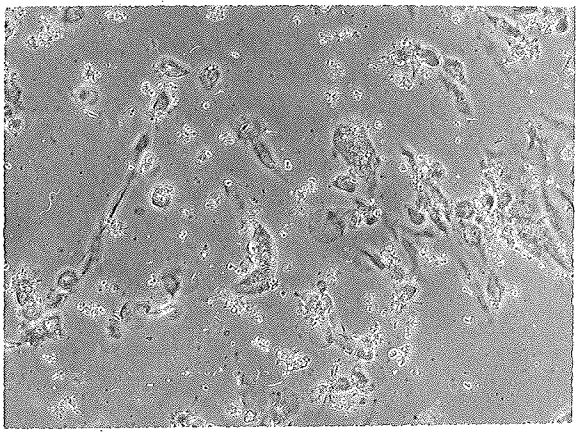
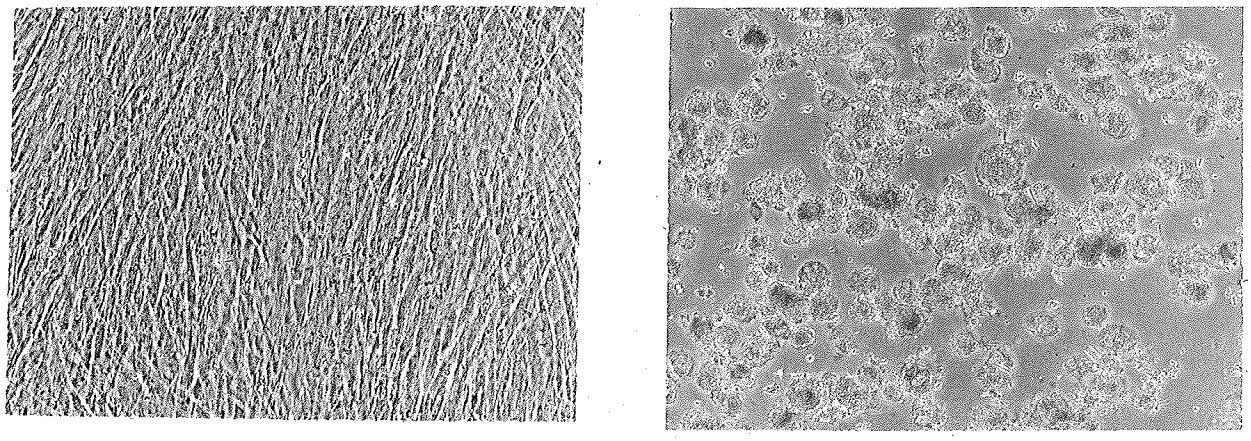
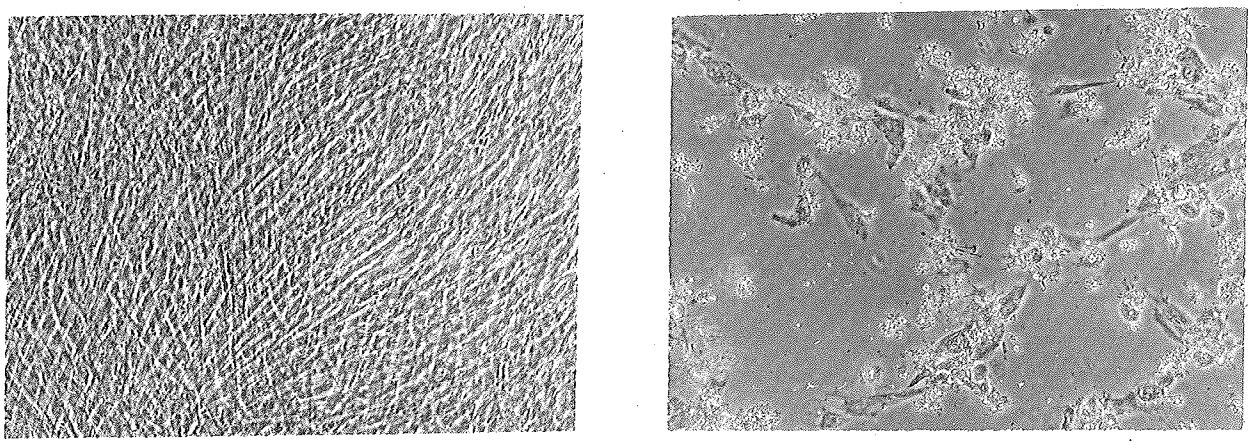


Figure 19 (e)

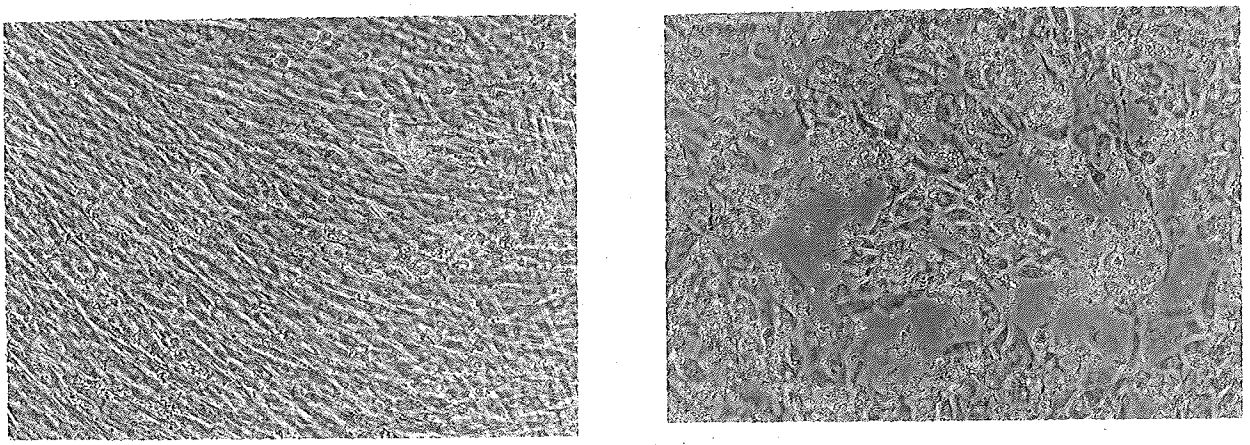
CONTROL (i) 5% DIABETIC



(ii) 10%



(iii) 15%



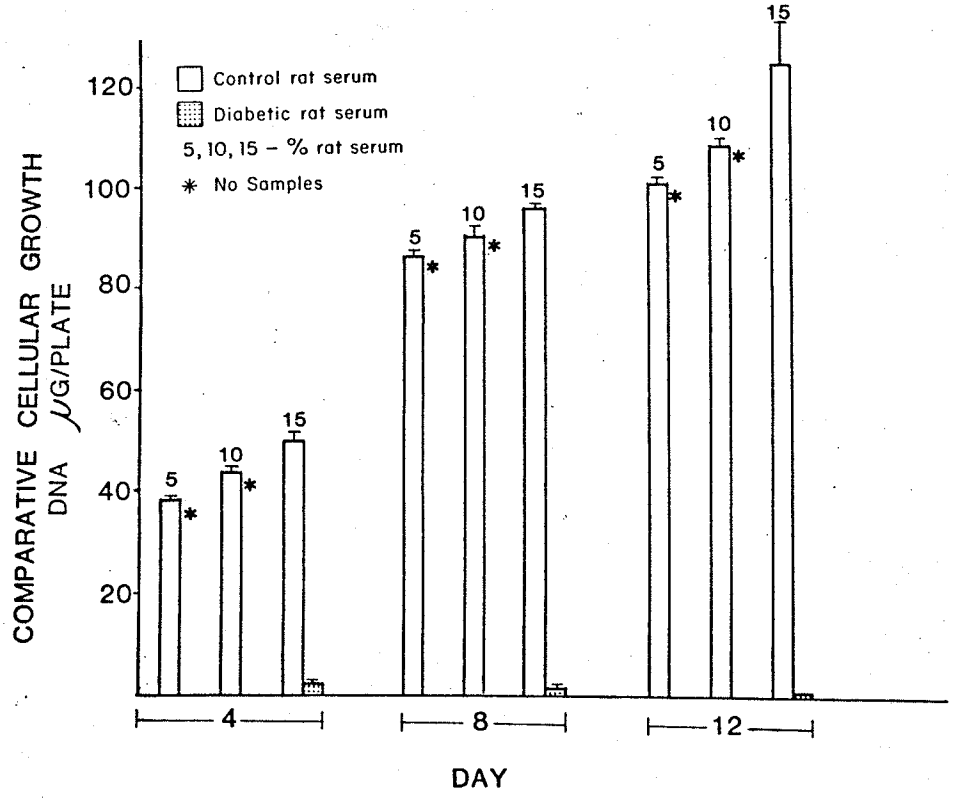


FIGURE 20: Effects of replacing FCS in the growth medium with control and diabetic rat sera on the cellular growth of human gingival fibroblasts. Cell growth was measured as DNA per plate on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 and 15% respectively.

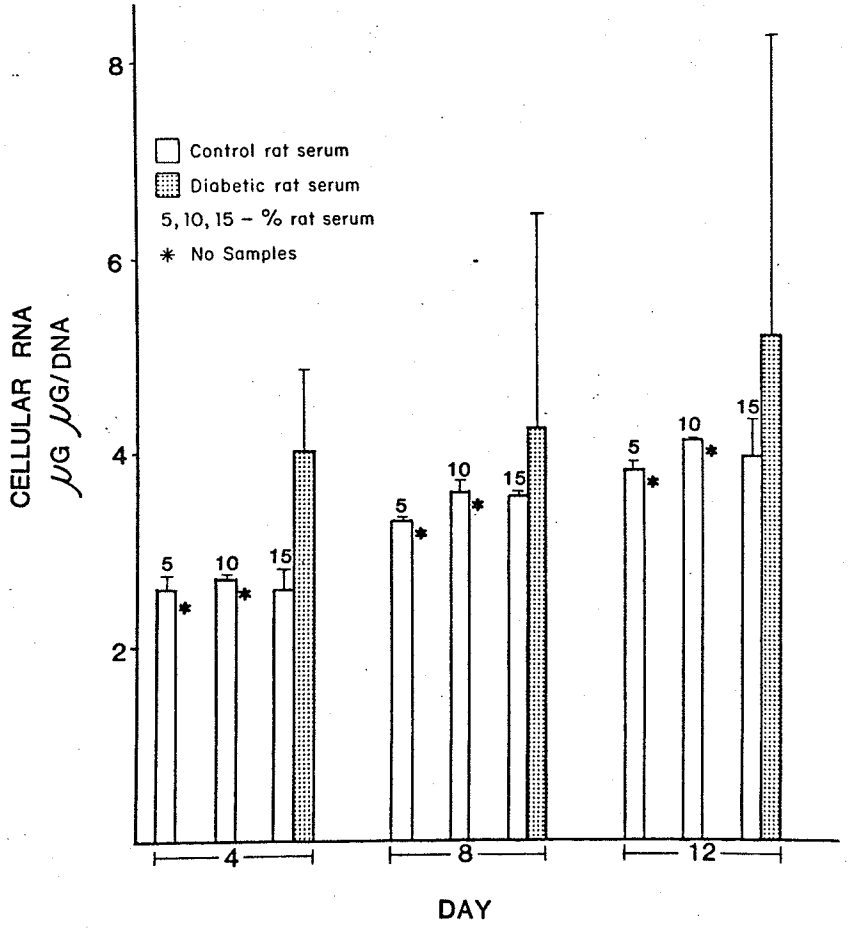


FIGURE 21: Effects of replacing FCS in the growth medium with control and diabetic rat sera on cellular RNA levels of human gingival fibroblasts. RNA was measured on a DNA basis on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

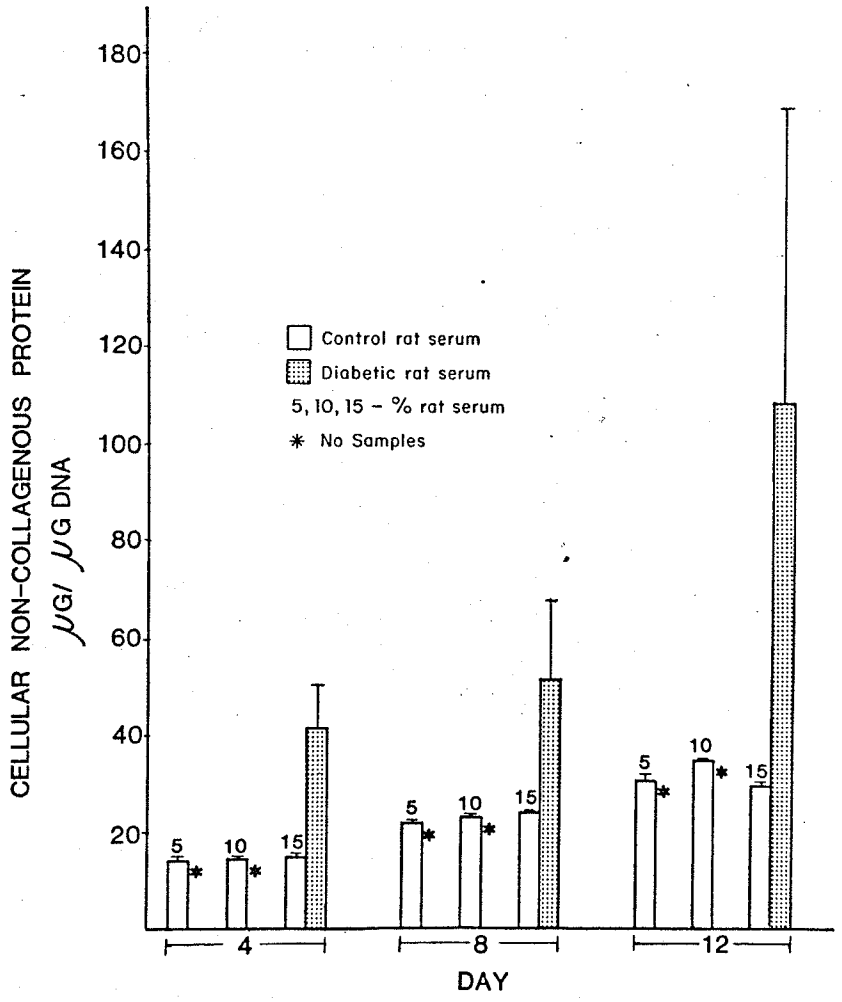


FIGURE 22: Effects of replacing FCS in the growth medium with control and diabetic rat sera on cellular non-collagenous protein levels in human gingival fibroblasts. Protein, measured as Lowry detectable protein and expressed on a DNA basis, was monitored for a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

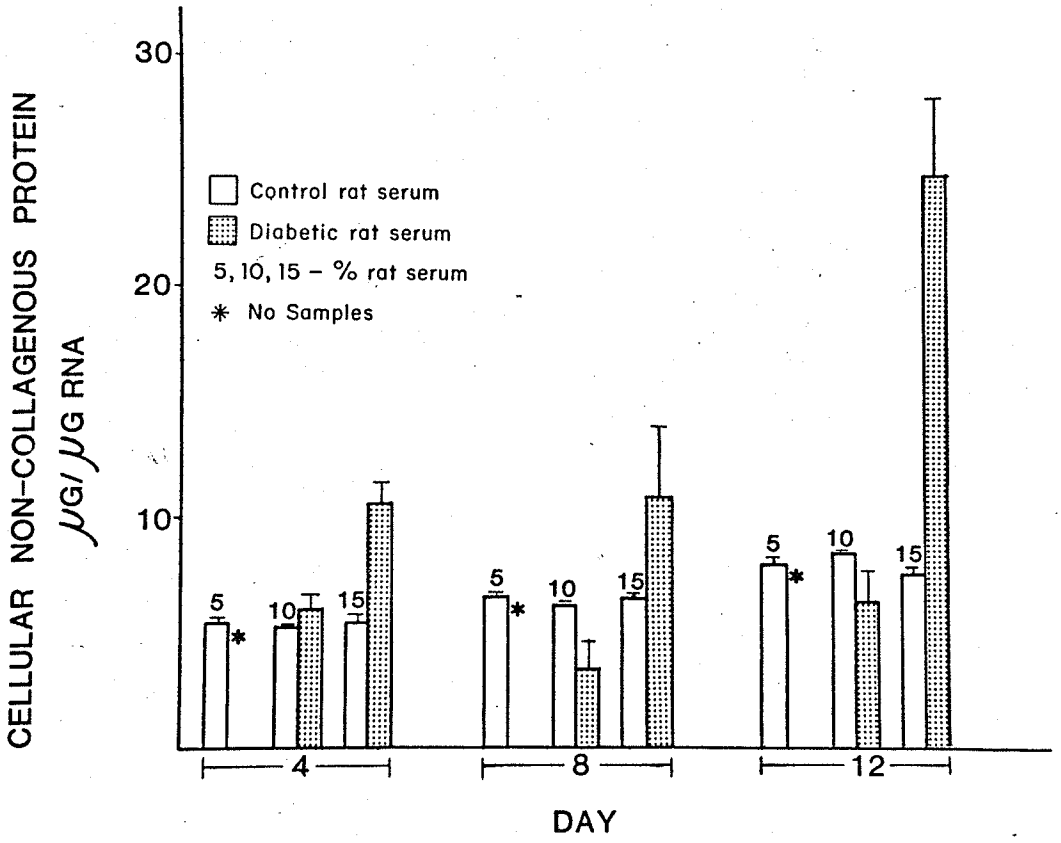


FIGURE 23: Effects of replacing FCS in the growth medium with control and diabetic rat sera on cellular non-collagenous protein levels in human gingival fibroblasts. Protein, measured as Lowry detectable protein and expressed on a RNA basis was monitored for a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

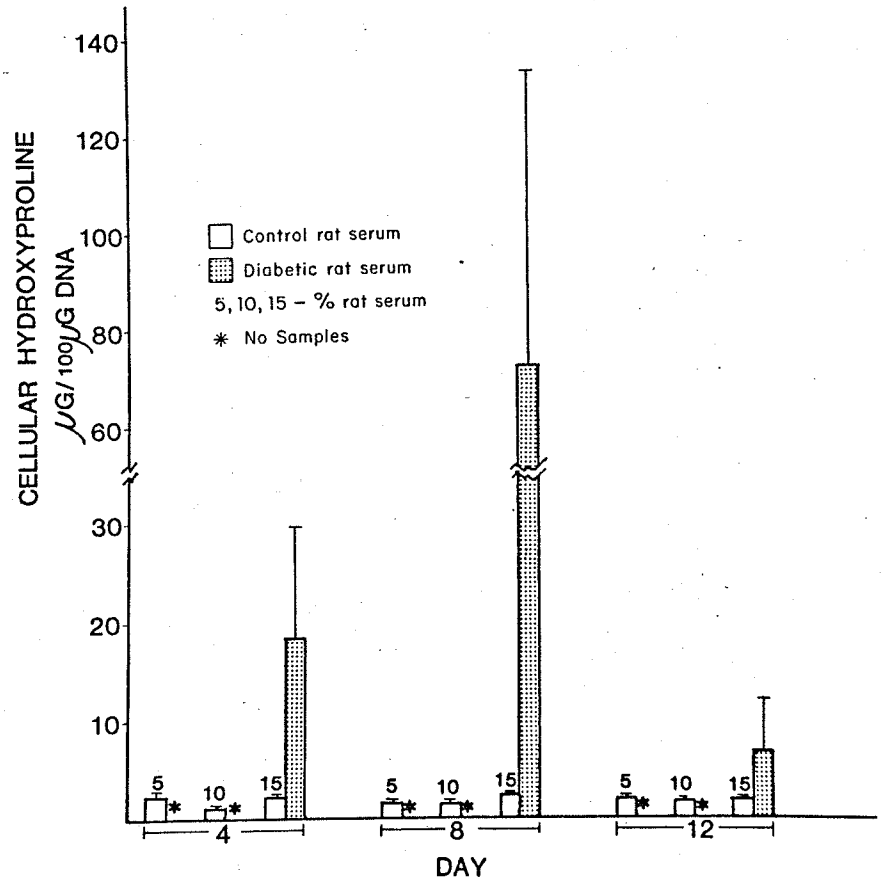


FIGURE 24: Effects of replacing FCS in the growth medium with control and diabetic rat sera on intracellular concentrations of collagenous protein in human gingival fibroblasts. Collagenous protein was measured as μg cellular hydroxyproline per 100 μg DNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

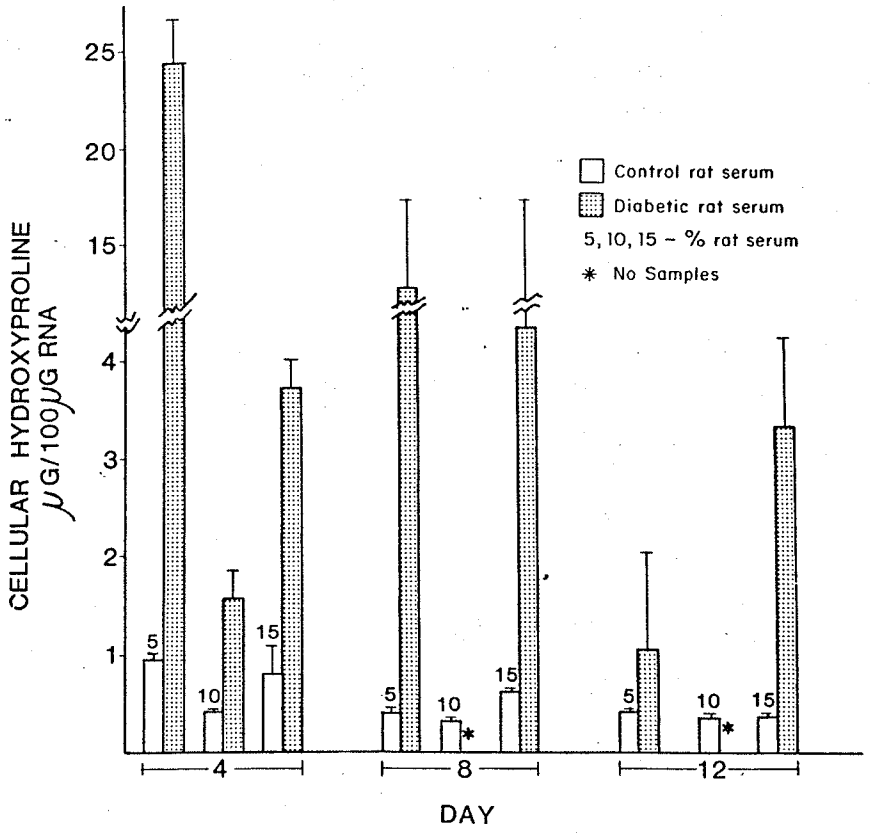


FIGURE 25: Effects of replacing FCS in the growth medium with control and diabetic rat sera on intracellular concentrations of cellular collagenous protein in human gingival fibroblasts. Collagenous protein was measured as μg cellular hydroxyproline per 100 μg RNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

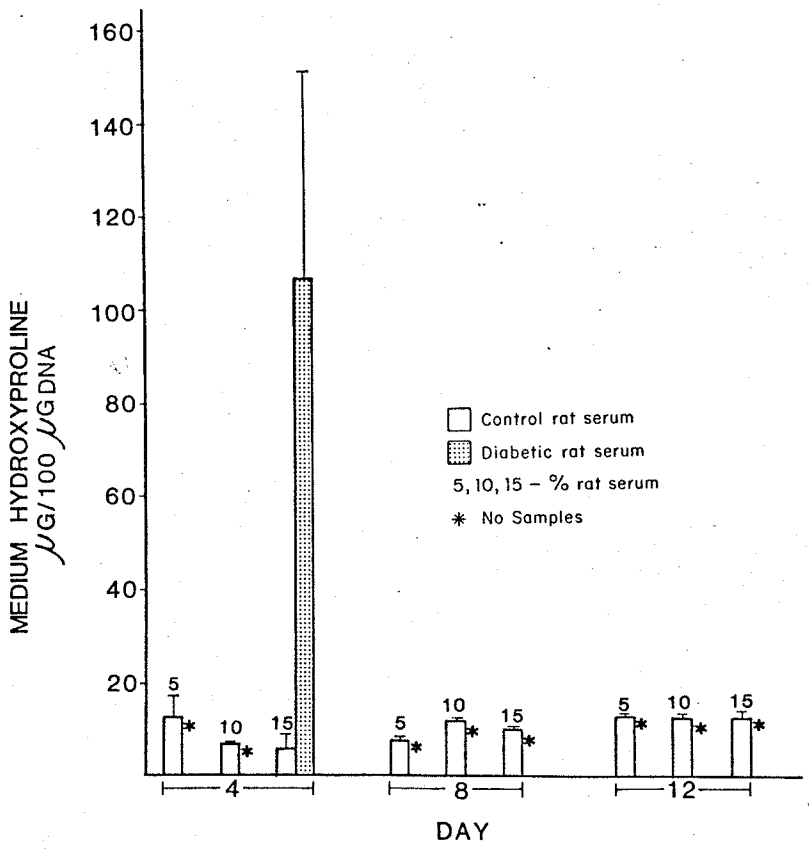


FIGURE 26: Effects of replacing FCS in the growth medium with control and diabetic rat sera on the accumulation by human gingival fibroblasts of medium collagenous protein. Collagenous protein was measured as µg hydroxyproline per 100 µg DNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

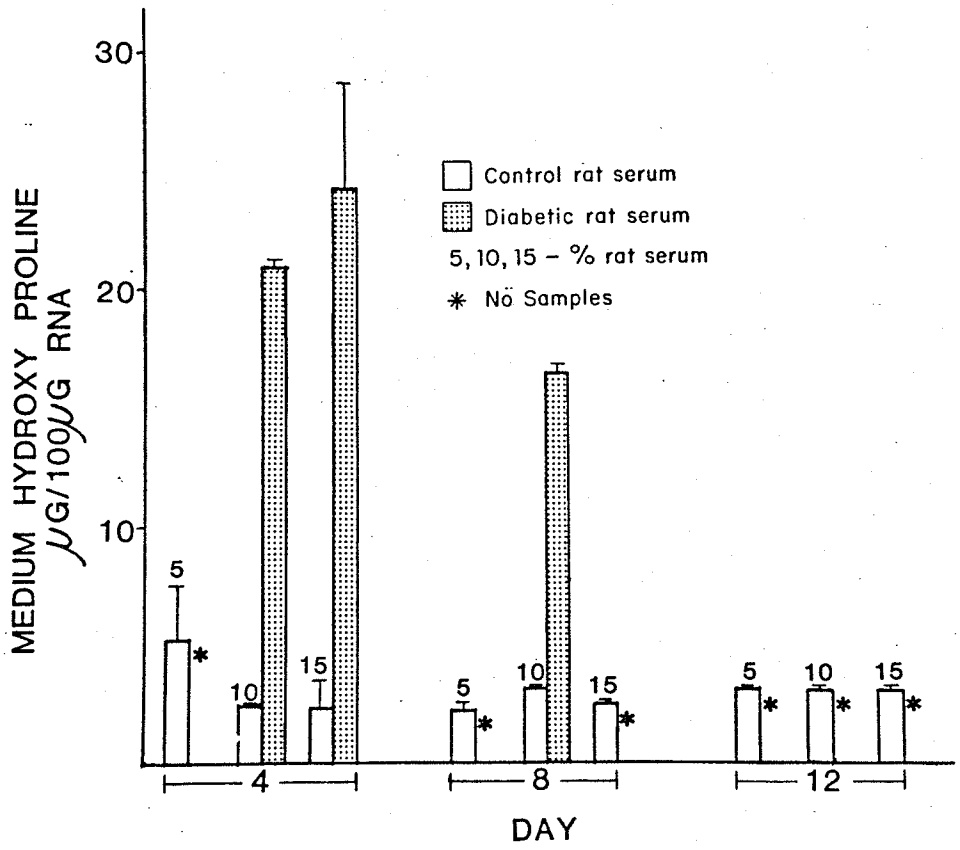


FIGURE 27: Effects of replacing FCS in the growth medium with control and diabetic rat sera on the accumulation by human gingival fibroblasts of medium collagenous protein. Collagenous protein was measured as µg hydroxyproline per 100 µg RNA on days 4, 8 and 12 of a 12 day growth period. Control and diabetic rat sera were added to the growth medium at concentrations of 5, 10 or 15% respectively.

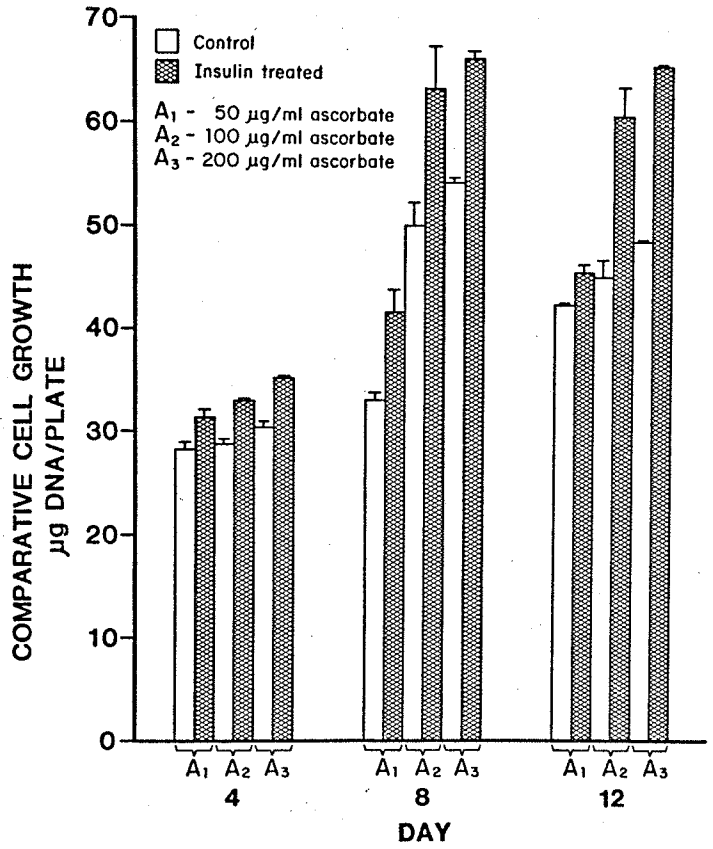


FIGURE 28: Growth response of human gingival fibroblasts to growth medium ascorbate concentrations and the interaction of ascorbate with insulin. Growth was measured as µg DNA per plate on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 µg/ml respectively. Insulin (containing 0.5% zinc bound) was added at each level of ascorbate in concentrations of 0 or 10^{-6} M.

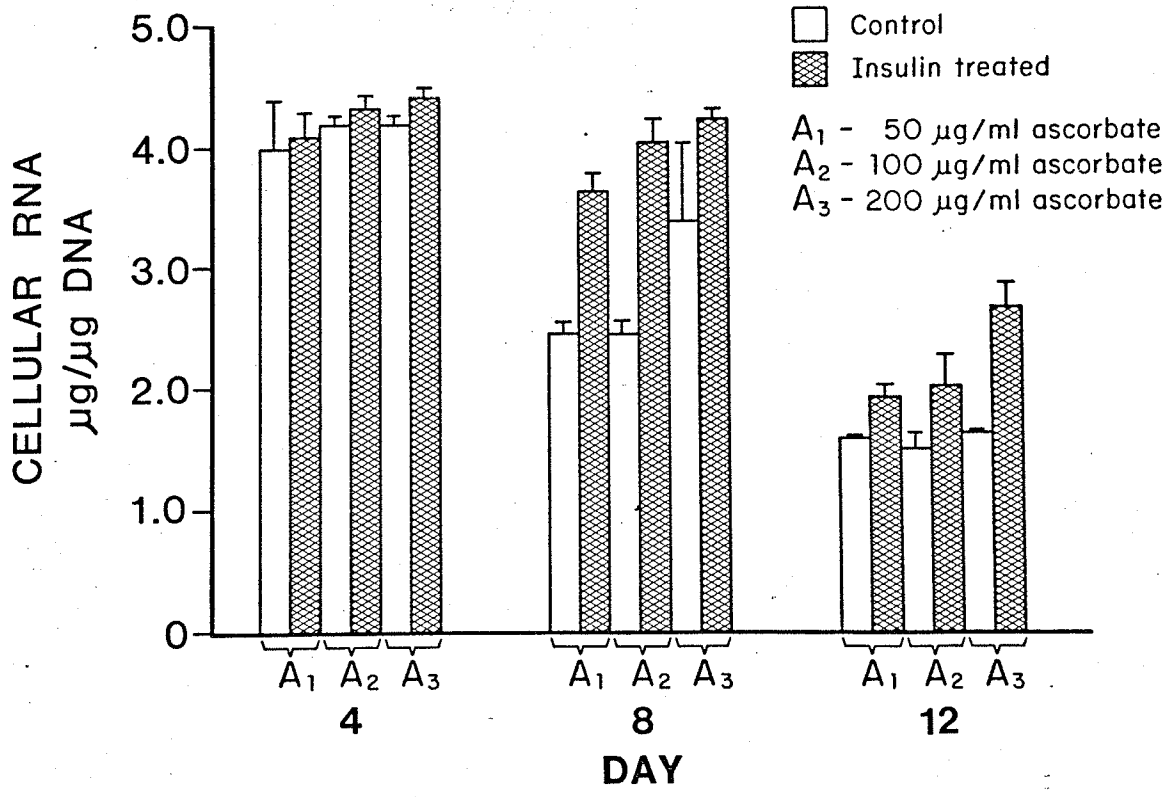


FIGURE 29: Comparison of the effects of medium ascorbate concentration and its interaction with insulin on cellular RNA levels of human gingival fibroblasts. RNA was measured as µg RNA per µg DNA on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 µg/ml respectively. Insulin (containing 0.5% bound zinc) was added at each level of ascorbate in concentrations of 0 or 10^{-6} M.

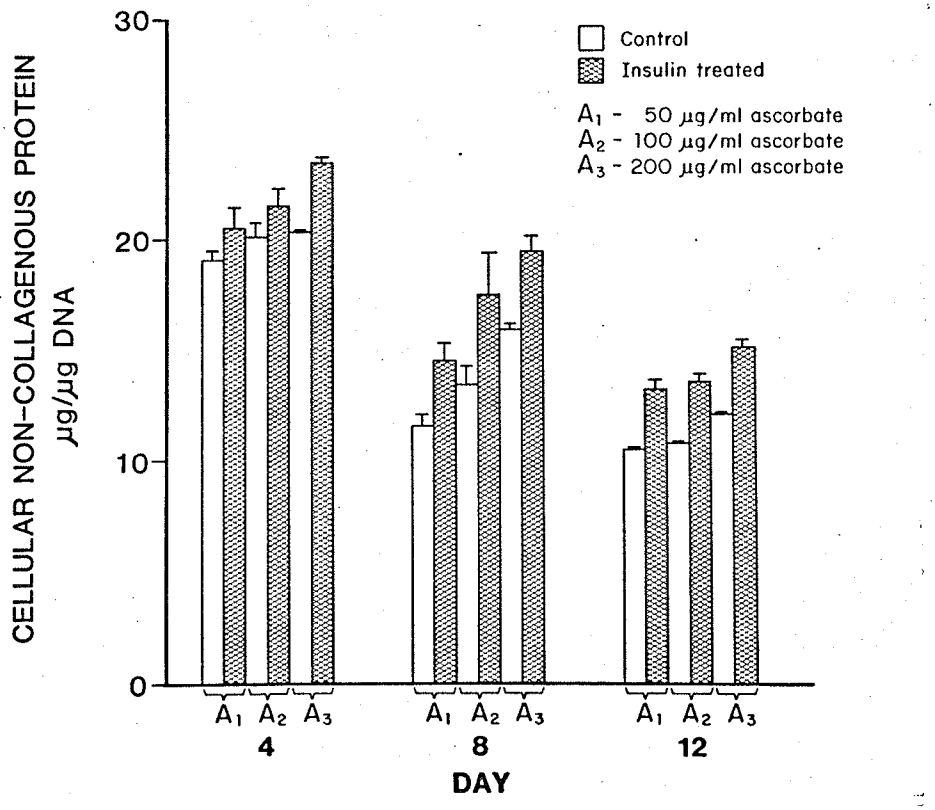


FIGURE 30: Comparison of the effects of growth medium ascorbate concentration and its interaction with insulin on the intracellular levels of non-collagenous protein in human gingival fibroblasts. Protein synthesis was measured as µg protein per µg DNA on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 or 200 µg/ml respectively. At each level of ascorbate, insulin (containing 0.5% bound zinc) was added at concentrations of 0 or 10^{-6} M.

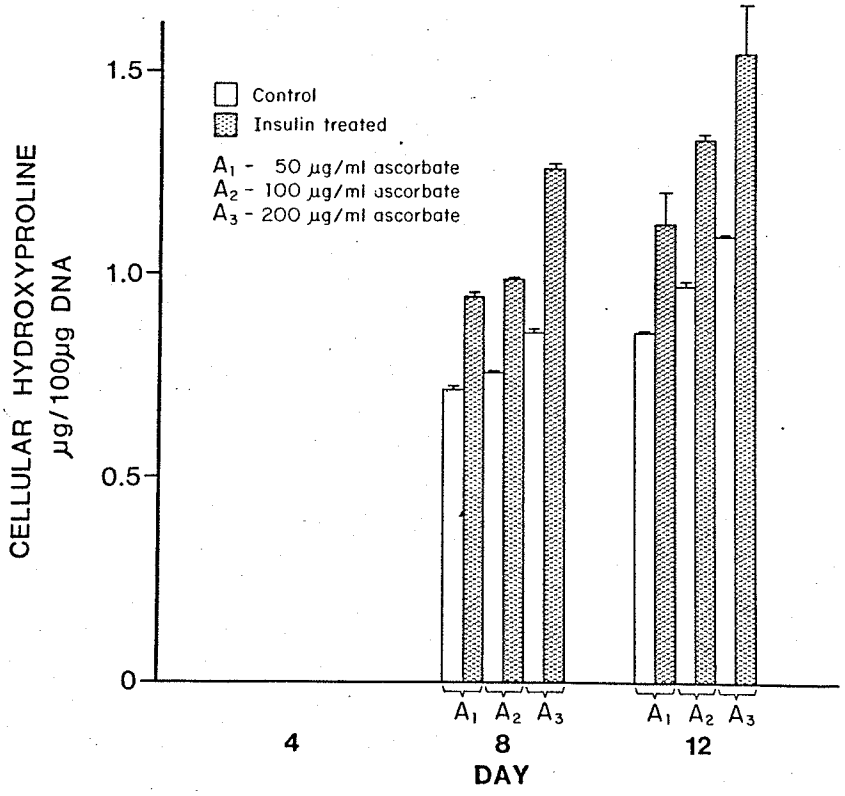


FIGURE 31: The effects of medium ascorbate concentration and its interaction with insulin on the intracellular levels of collagenous protein in human gingival fibroblasts. Collagen production was measured as µg cellular hydroxyproline per 100 µg DNA on days 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 and 200 µg/ml. At each level of ascorbate, insulin (containing 0.5% bound zinc) was added in concentrations of 0 or 10^{-6} M.

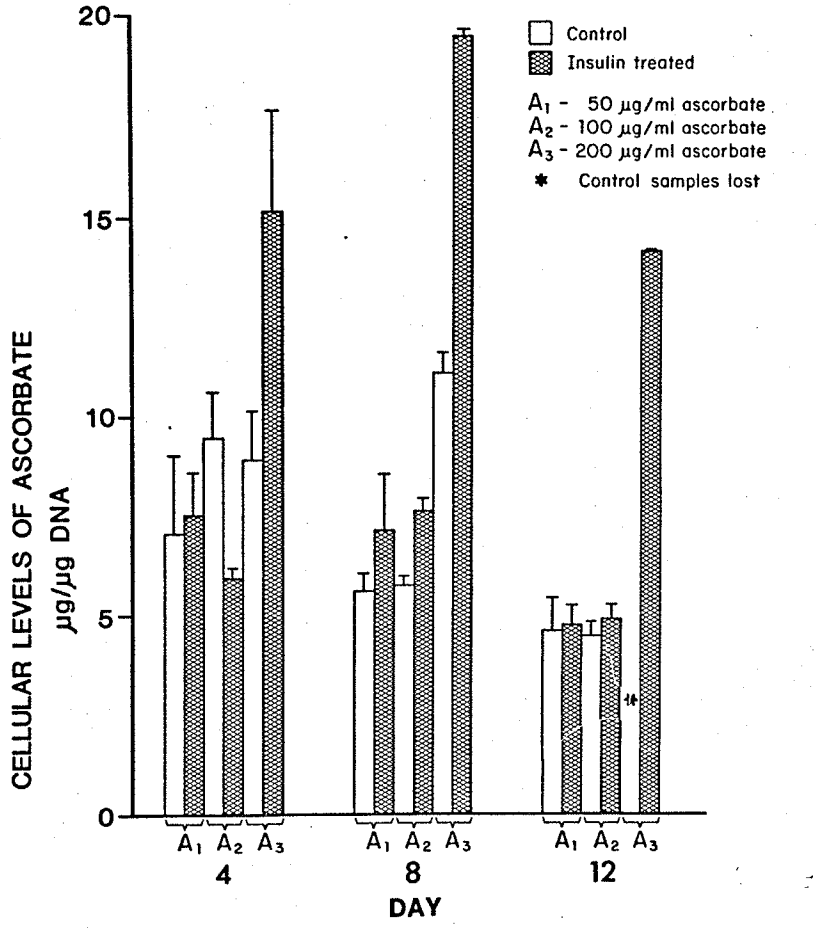


FIGURE 32: The effects of medium ascorbate and insulin concentrations on the intracellular accumulation of ascorbate in human gingival fibroblasts. Intracellular ascorbate levels were measured as µg ascorbate per µg DNA on days 4, 8 and 12 of a 12 day growth period. Ascorbate was added to the growth medium to yield final concentrations of 50, 100 and 200 µg/ml. Insulin (containing 0.5% bound zinc) was added at each level of ascorbate in concentrations of 0 and 10^{-6} M.

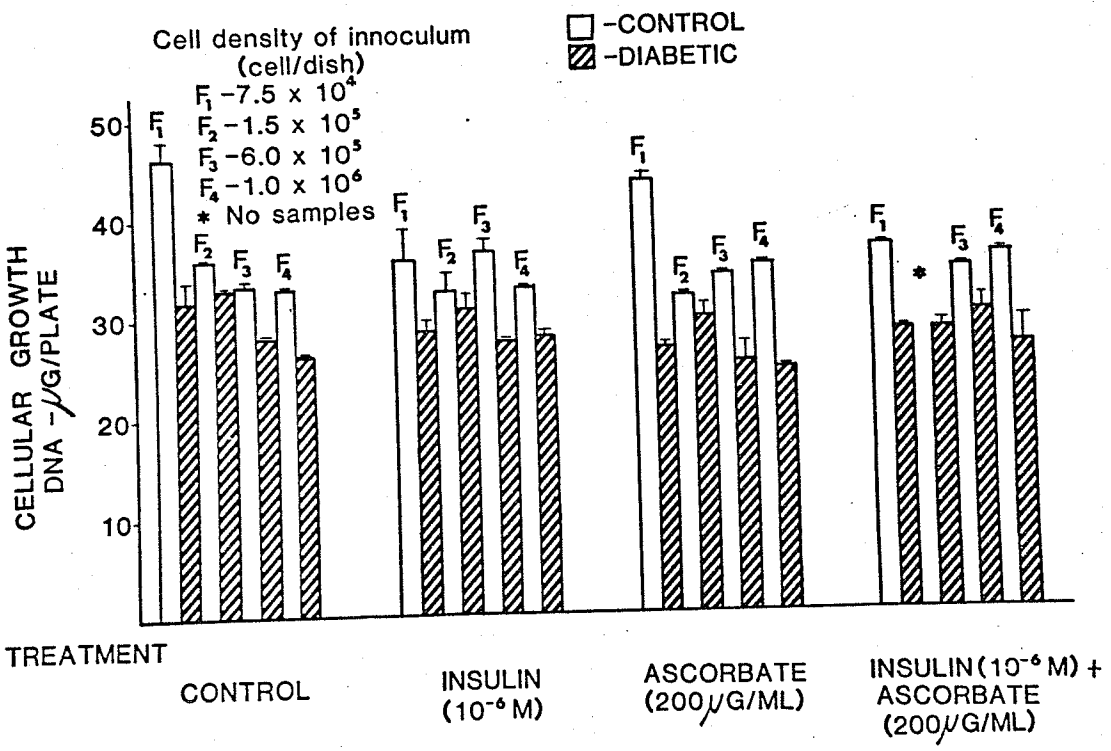


FIGURE 33: Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on the growth of control and diabetic rat gingival fibroblasts. Cell growth, expressed as DNA per plate, was measured at time of confluency for each cell density (F_3, F_4 on day 5, and F_1 and F_2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 μg/ml to 200 μg/ml.

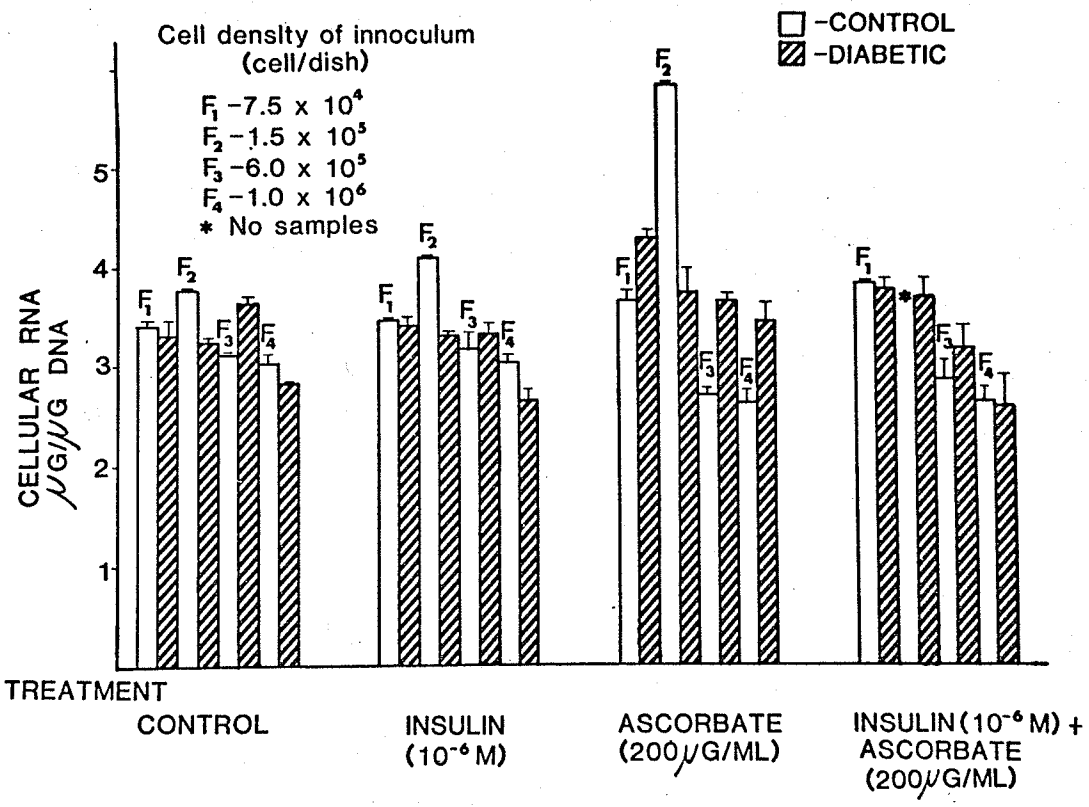


FIGURE 34: Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on cellular RNA content of control and diabetic rat gingival fibroblasts. Cellular RNA expressed on a DNA basis, was measured at time of confluency for each cell density (F_3, F_4 on day 5 and F_1 and F_2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 µg/ml to 200 µg/ml.

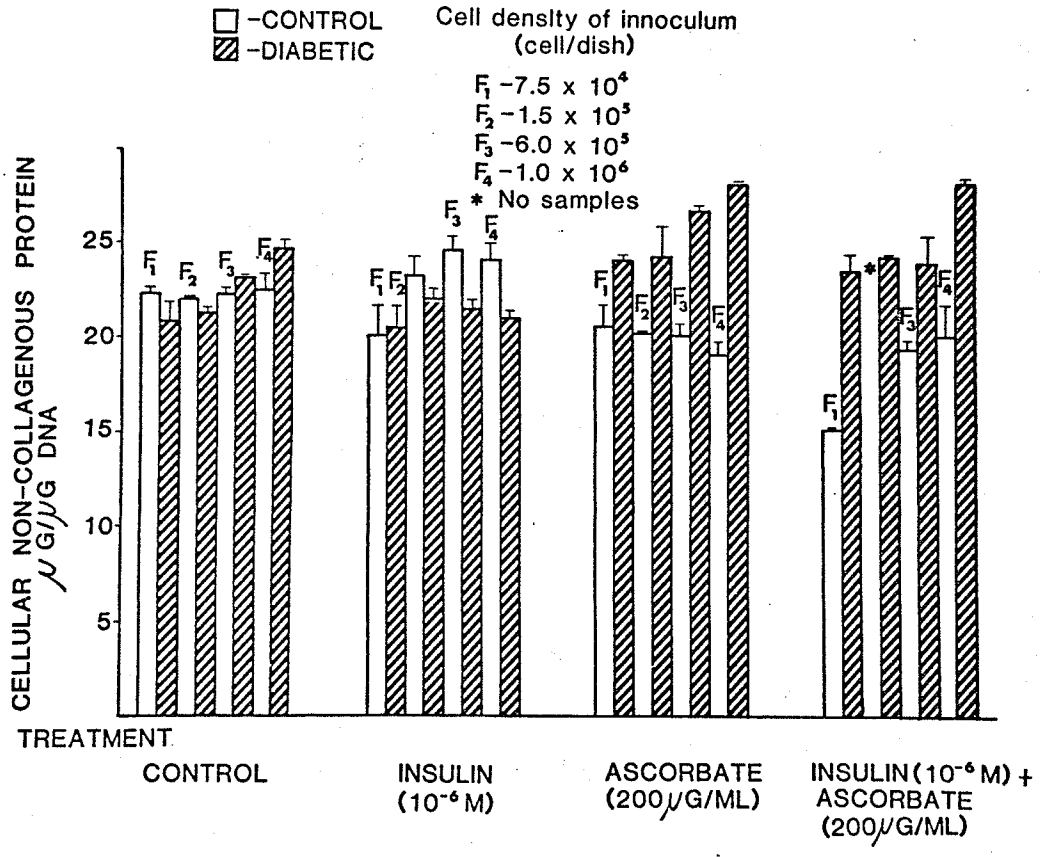


FIGURE 35: Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on cellular non-collagenous protein content of control and diabetic rat gingival fibroblasts. Cellular non-collagenous protein expressed on a DNA basis, was measured at time of confluency for each cell density (F_3, F_4 on day 5 and F_1 and F_2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 μg/ml to 200 μg/ml.

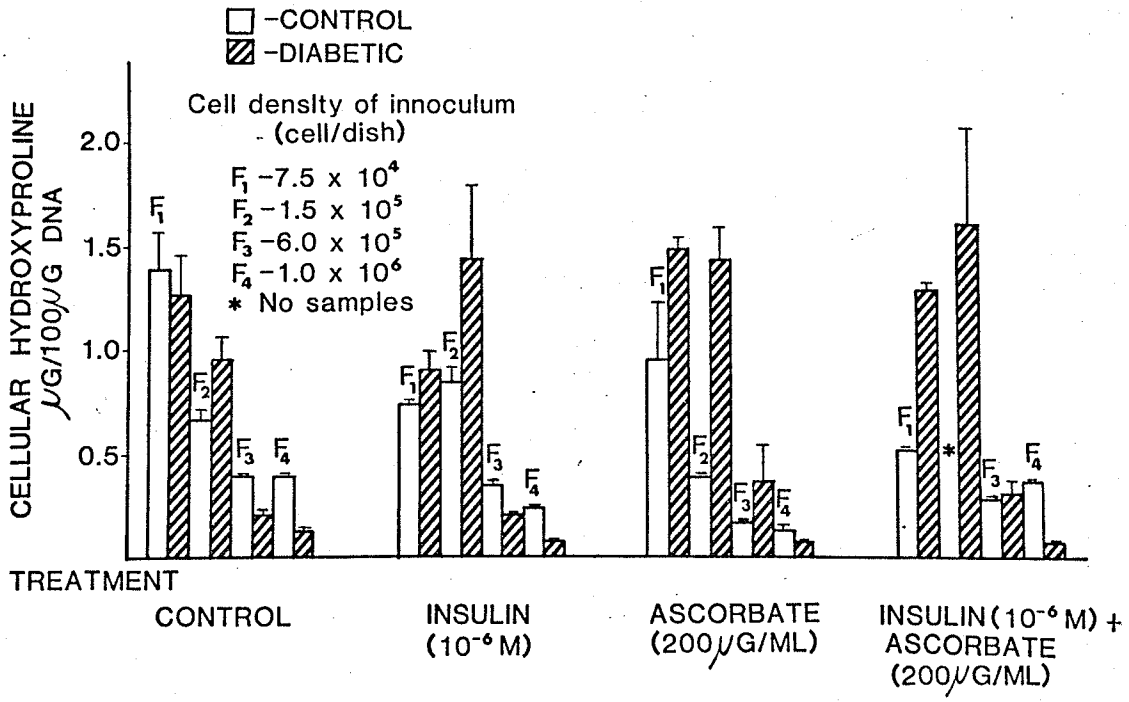


FIGURE 36: Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on cellular collagenous protein content of control and diabetic rat gingival fibroblasts. Collagenous protein, measured as hydroxyproline and expressed on a DNA basis, was determined at time of confluency for each cell density (F_3, F_4 on day 5 and F_1 and F_2 on day 6). Insulin was added to the medium at a concentration of either 0 or 10^{-6} M. In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 µg/ml to 200 µg/ml.

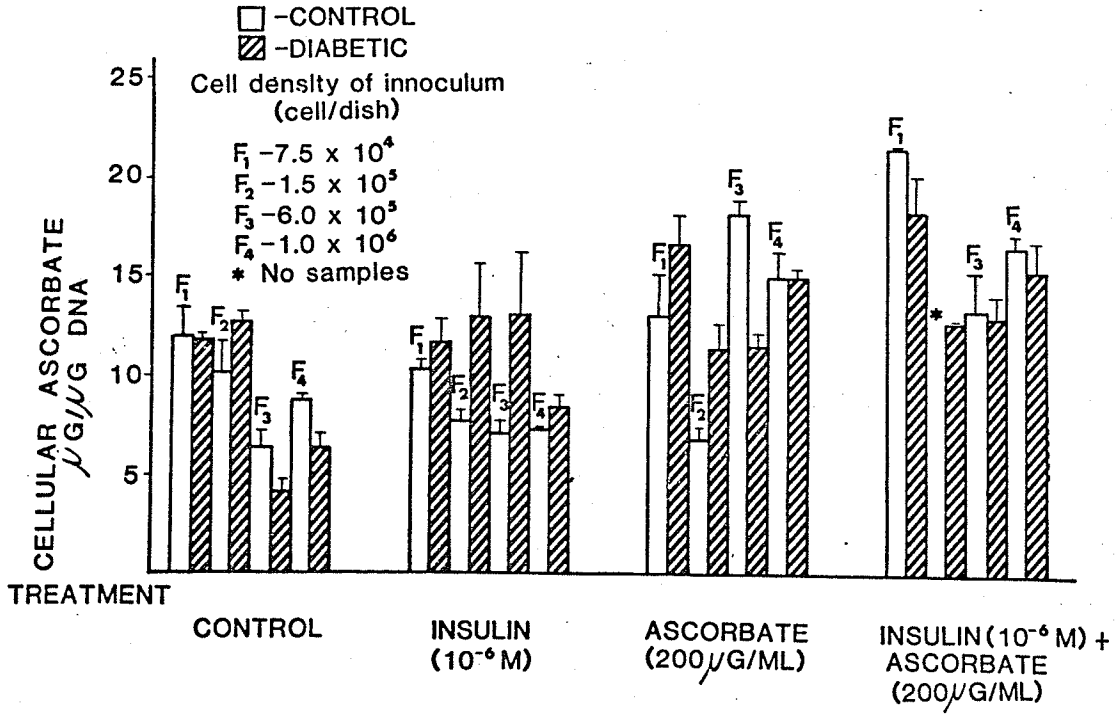


FIGURE 37: Relationship of cell density, medium insulin, ascorbate and of insulin and ascorbate combined on the intracellular accumulation of ascorbate by control and diabetic rat gingival fibroblasts. Ascorbate levels expressed on a DNA basis, were measured at time of confluency for each cell density (F_3, F_4 on day 5 and F_1 and F_2 on day 6). In the ascorbate treatment groups, ascorbate alone was added to raise the standard medium concentration from 50 µg/ml to 200 µg/ml.

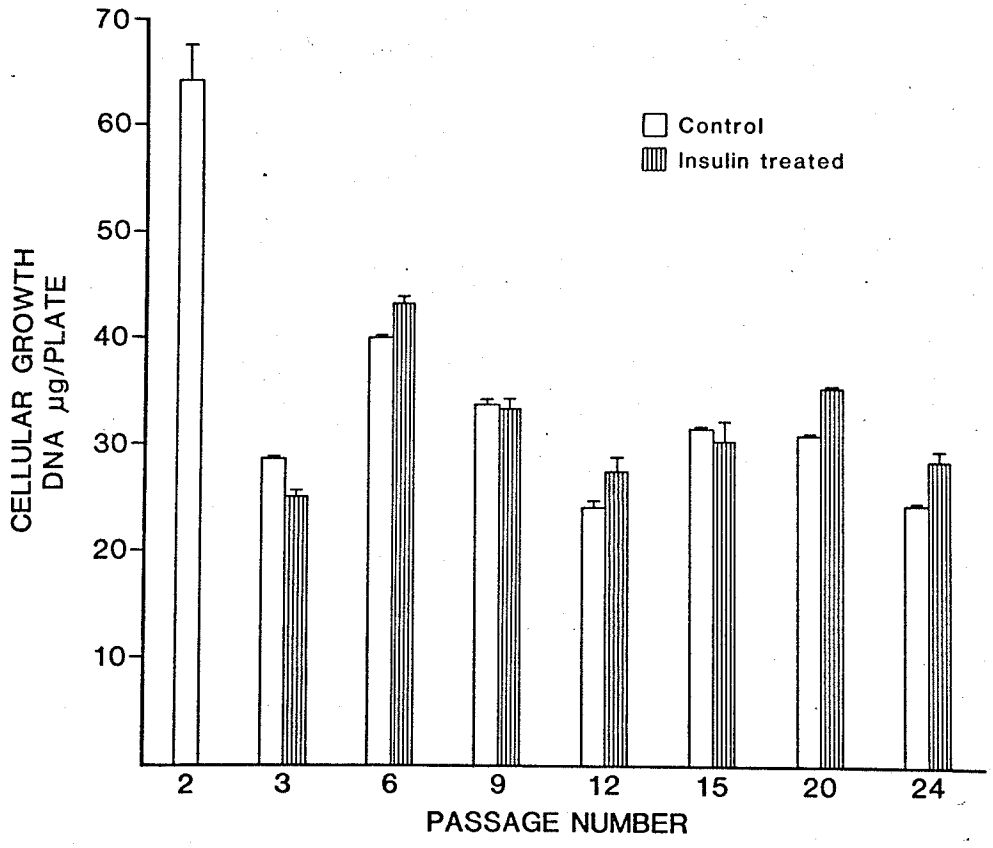


FIGURE 38: Comparative effects of cell age and insulin on the growth of human gingival fibroblasts. Cell growth, presented as DNA per plate, was determined on 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

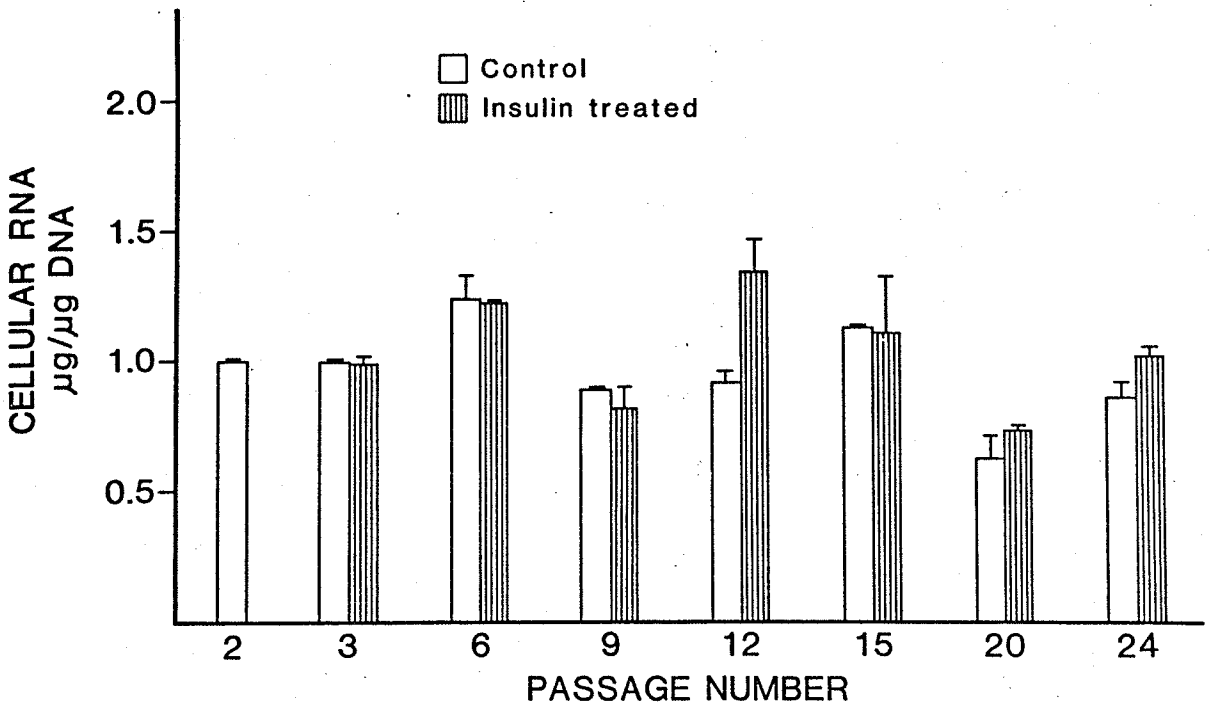


FIGURE 39: Comparative effects of cell age and insulin on cellular RNA concentrations of human gingival fibroblasts. RNA levels of 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24 are presented on a DNA basis. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentratins of 0 and 10^{-6} M.

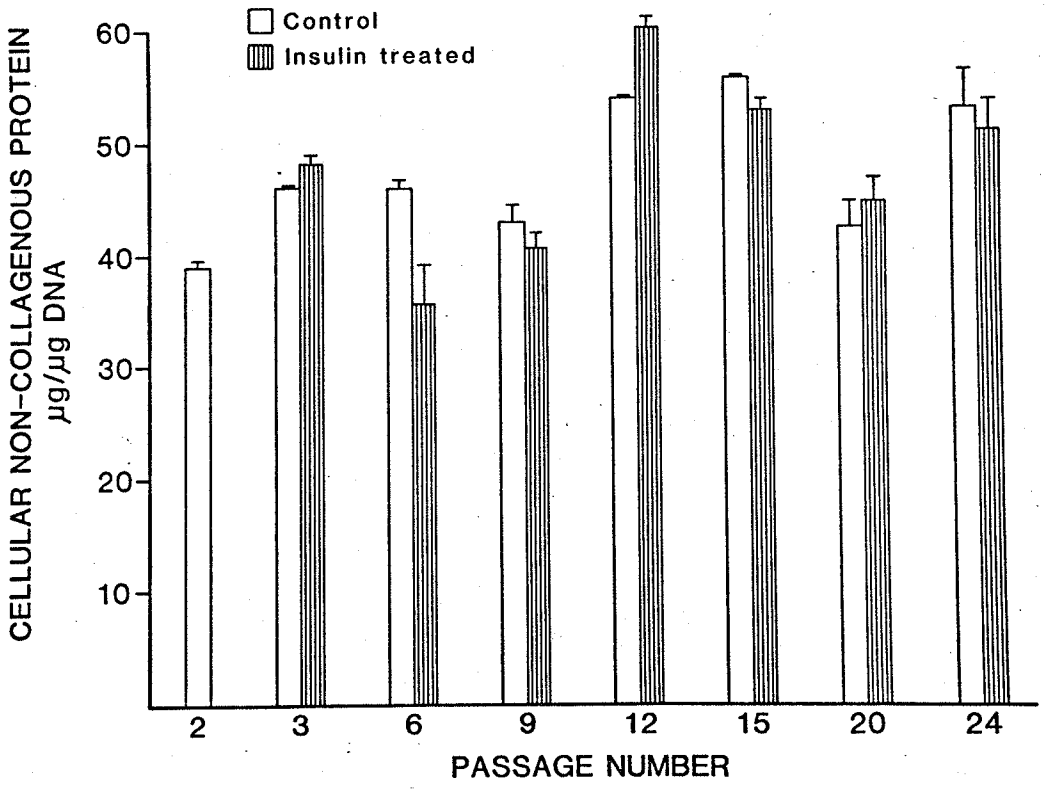


FIGURE 40: Comparative effects of cell age and insulin on cellular non-collagenous protein concentrations of human gingival fibroblasts. Non-collagenous protein of 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24 are presented on a RNA basis. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

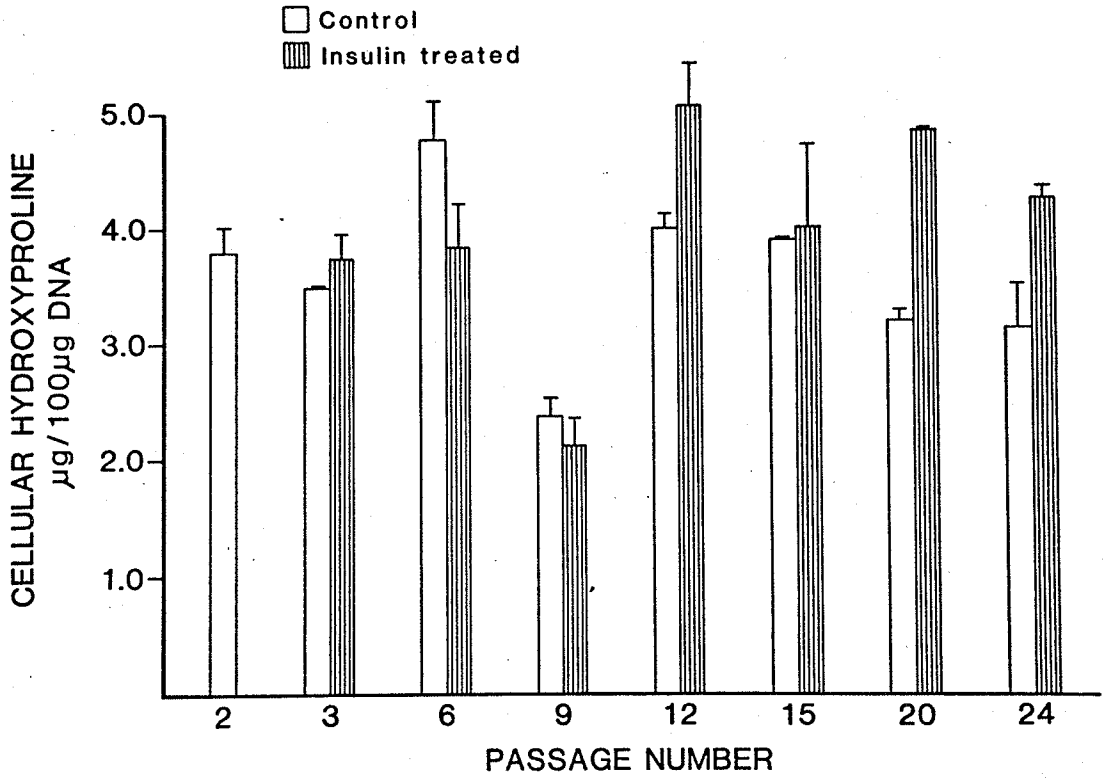


FIGURE 41: Comparative effects of cell age and insulin on cellular collagenous protein concentrations of human gingival fibroblasts. Collagenous protein measured as hydroxyproline is presented on a DNA basis for 9 day old cells at passages 2, 3, 6, 9, 12, 15, 20 and 24. At these passages only, insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 and 10^{-6} M.

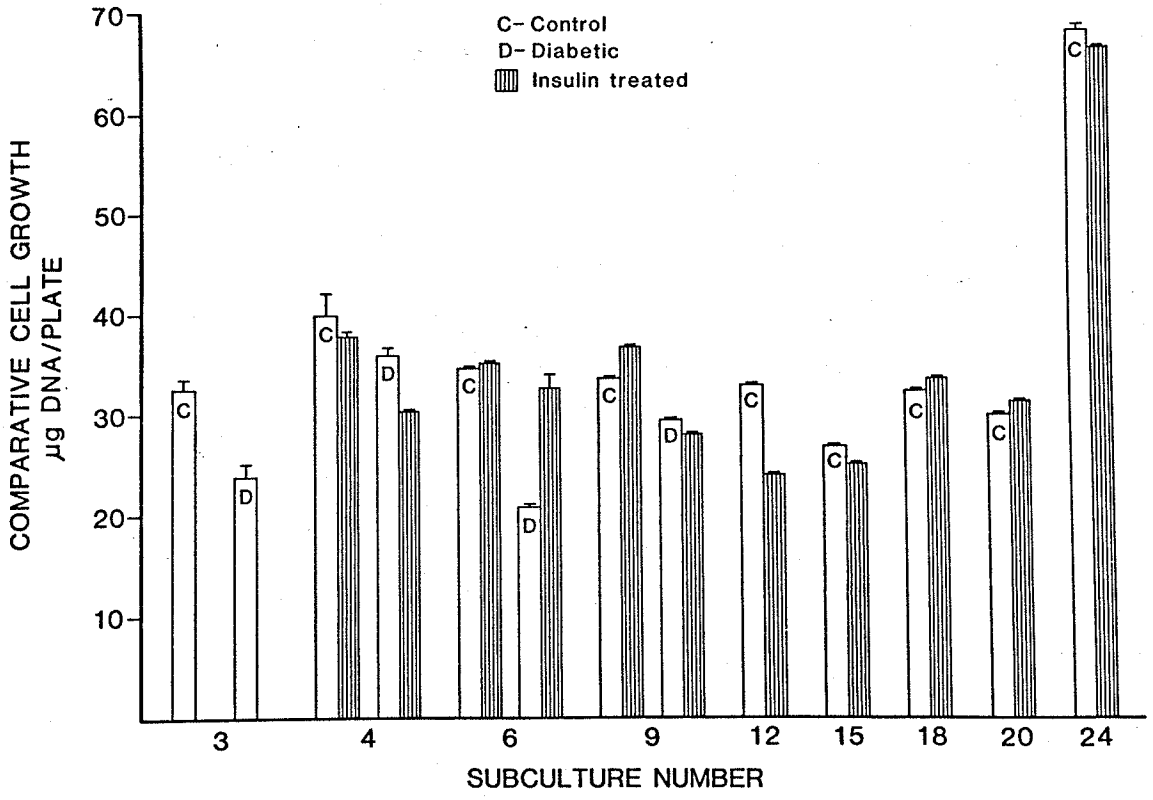


FIGURE 42: Comparative effects of age and insulin on the growth of control and diabetic rat gingival fibroblasts. Cell growth, expressed as µg DNA per plate, was measured on 9 day old cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 for the control rat cells and at passages 3, 4, 6 and 9 only for the diabetic rat cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

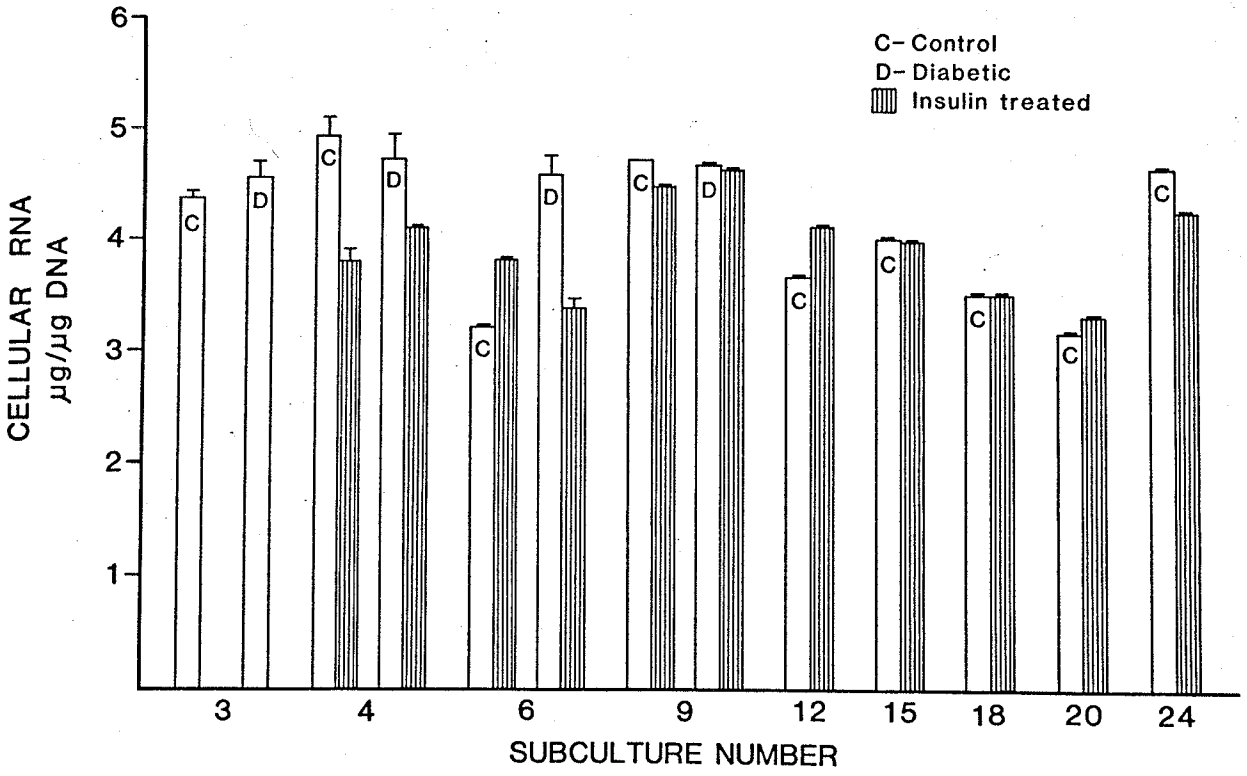


FIGURE 43: Comparative effects of age and insulin on cellular RNA levels of control and diabetic rat gingival fibroblasts. RNA expressed on a DNA basis, was determined on 9 day old control rat cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and 9 day old diabetic rat cells at passages 3, 4, 6 and 9 only. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

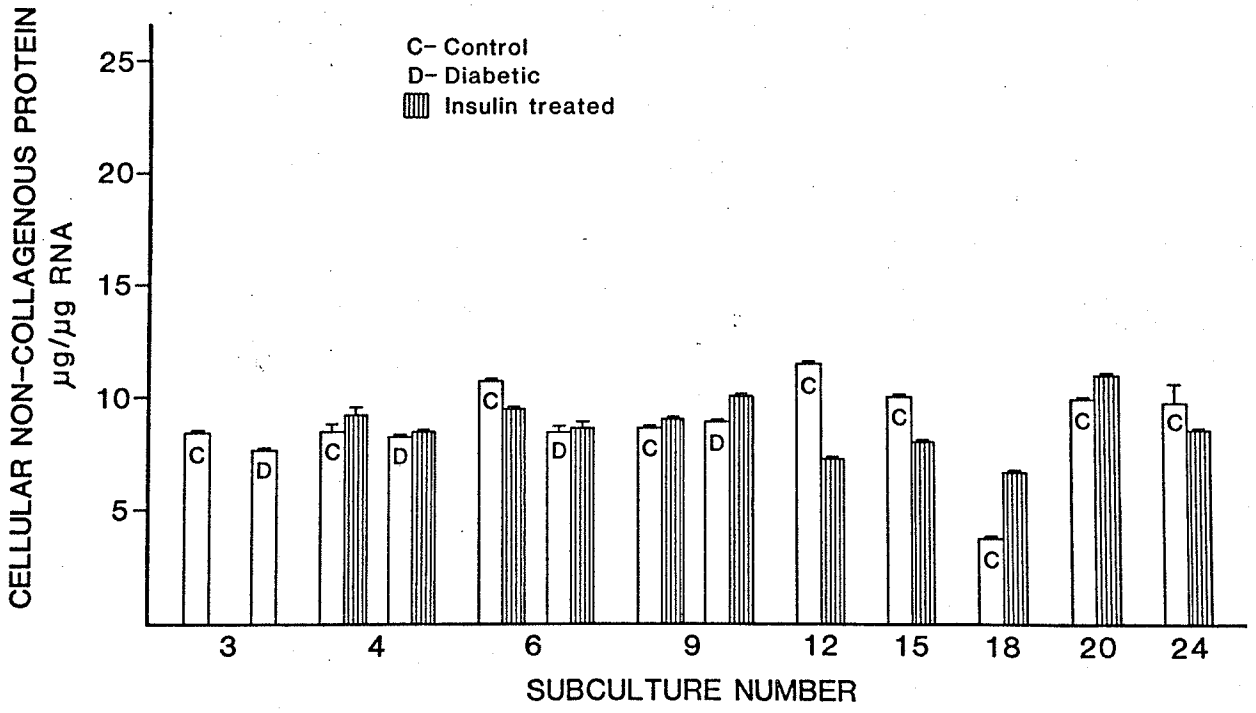


FIGURE 44: Comparative effects of age and insulin on cellular non-collagenous protein levels of control and diabetic rat gingival fibroblasts. Non-collagenous protein expressed on a DNA basis, was determined on 9 day old control rat cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and 9 day old diabetic rat cells at passages 3, 4, 6 and 9 only. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

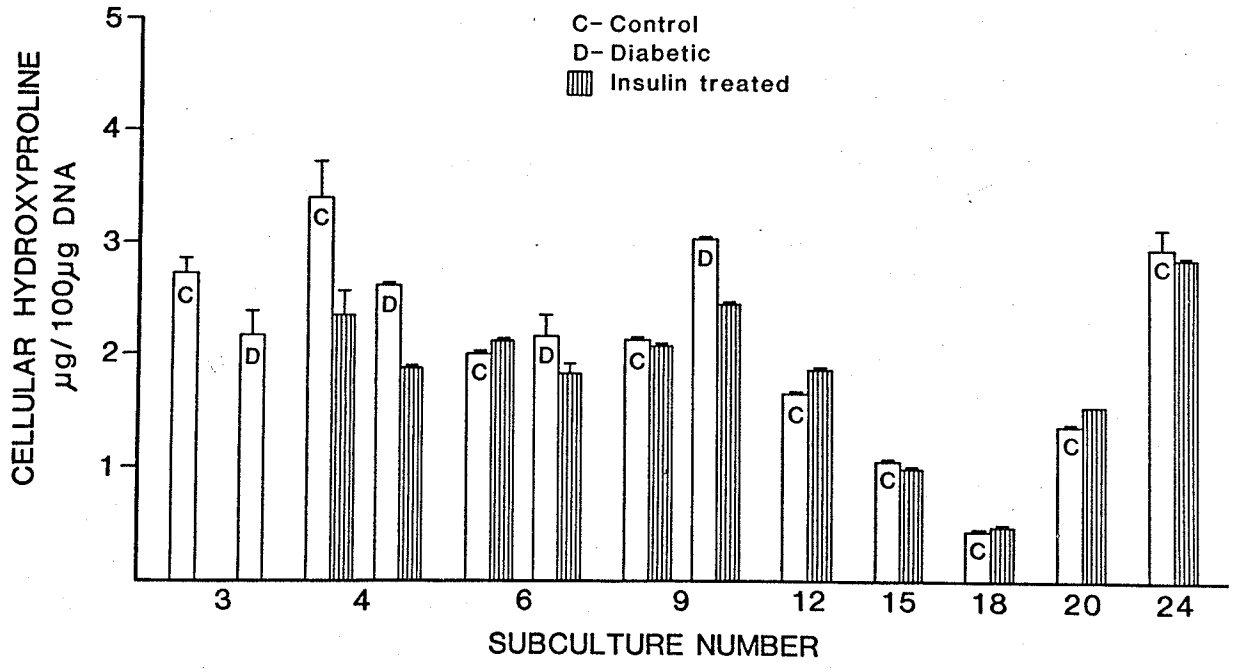


FIGURE 45: Comparative effects of age and insulin on cellular collagenous protein levels of control and diabetic rat gingival fibroblasts. Collagenous protein, measured as hydroxyproline and expressed on a DNA basis, was determined on 9 day old control rat cells at passages 3, 4, 6, 9, 12, 15, 18, 20 and 24 and 9 day old diabetic rat cells at passages 3, 4, 6 and 9 only. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0 or 10^{-6} M.

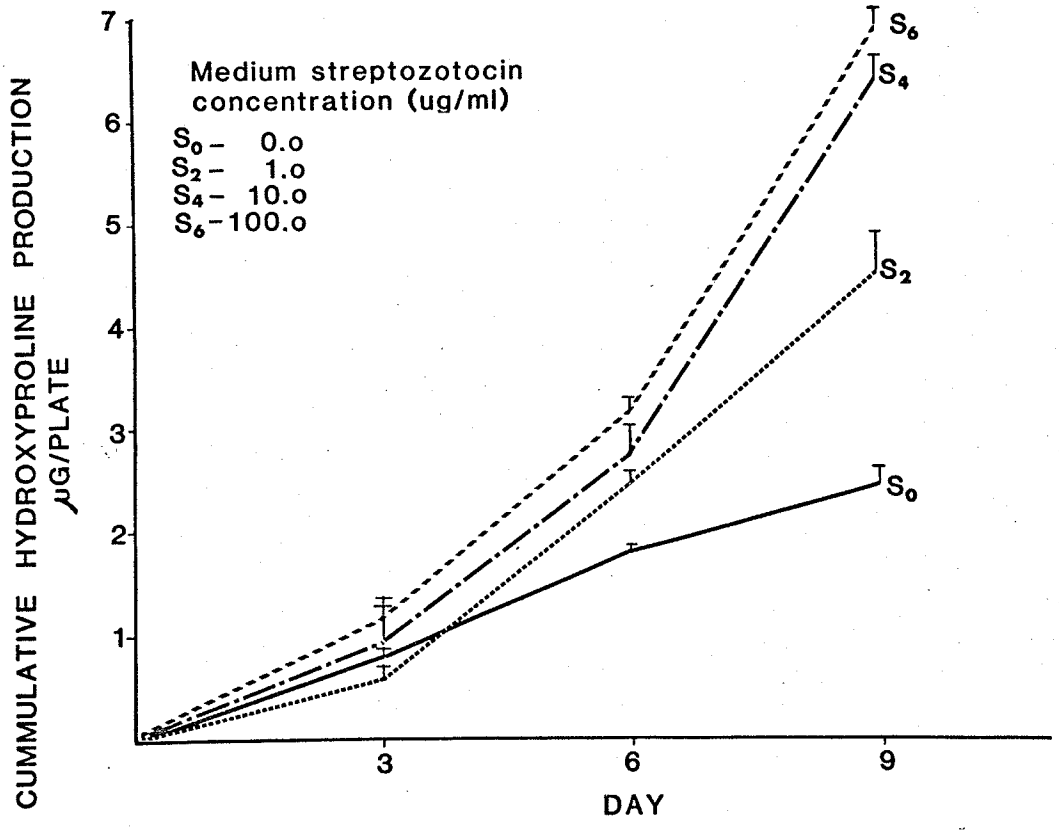


FIGURE 46: Effects of streptozotocin added in vitro to the growth medium on total hydroxyproline (cellular + medium) production by control rat gingival fibroblasts. Total hydroxyproline expressed on a per plate basis was determined on days 3, 6 and 9 of a 9 day growth period. Streptozotocin was added to the growth medium at concentrations of 0, 1.0, 10.0 and 100.0 ug/ml.

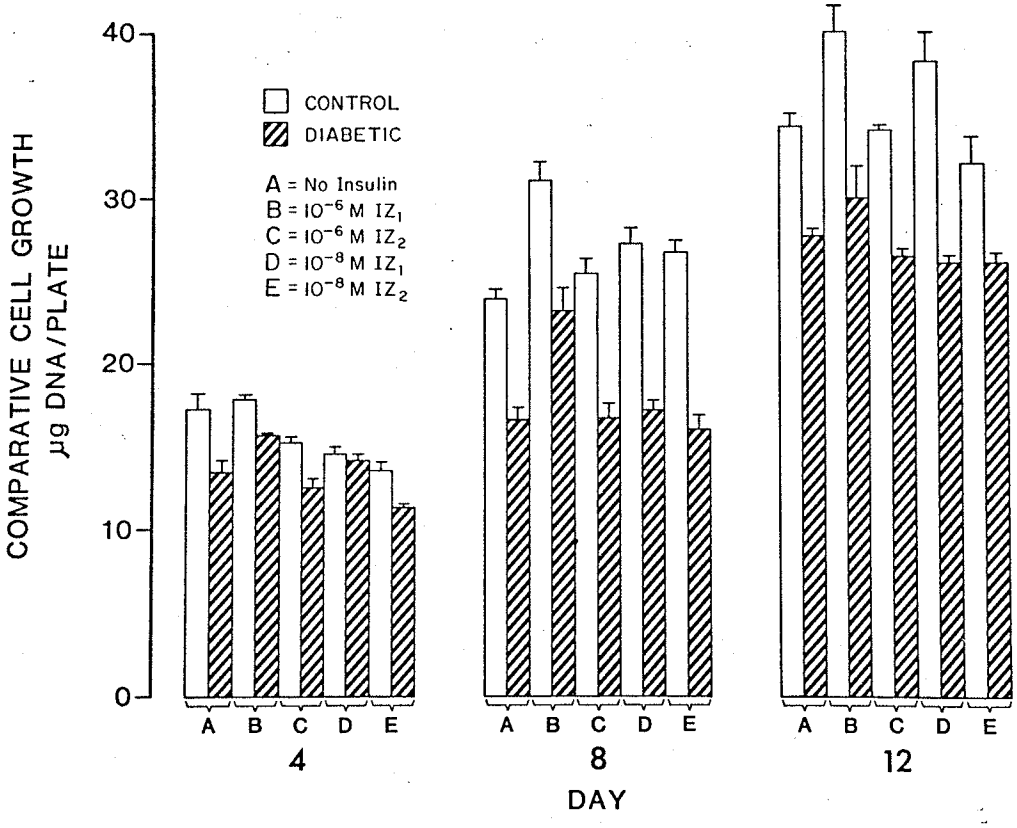


FIGURE 47: Comparative cell growth of control and diabetic rat gingival fibroblasts in the presence of insulin preparations containing different amounts of bound zinc. Growth was measured as DNA per plate on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ_1 and IZ_2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

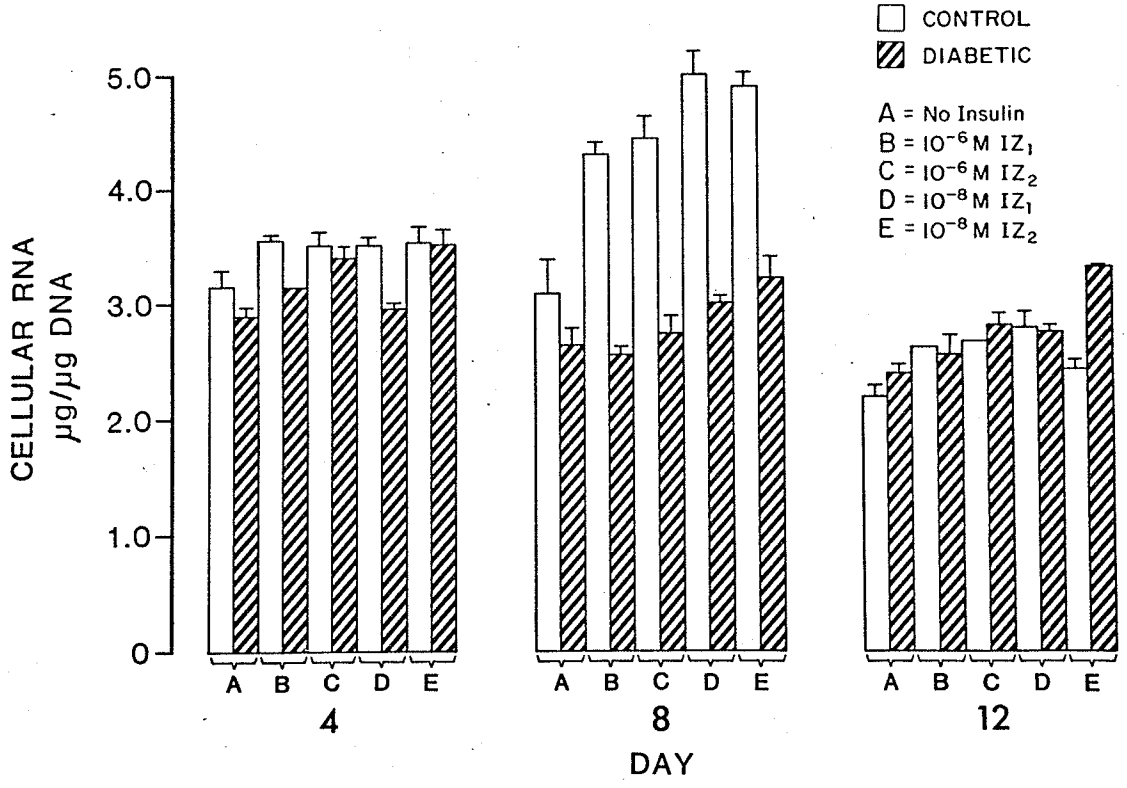


FIGURE 48: Comparison of cellular RNA levels of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Cellular RNA was expressed on a DNA basis and measured on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ_1 and IZ_2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

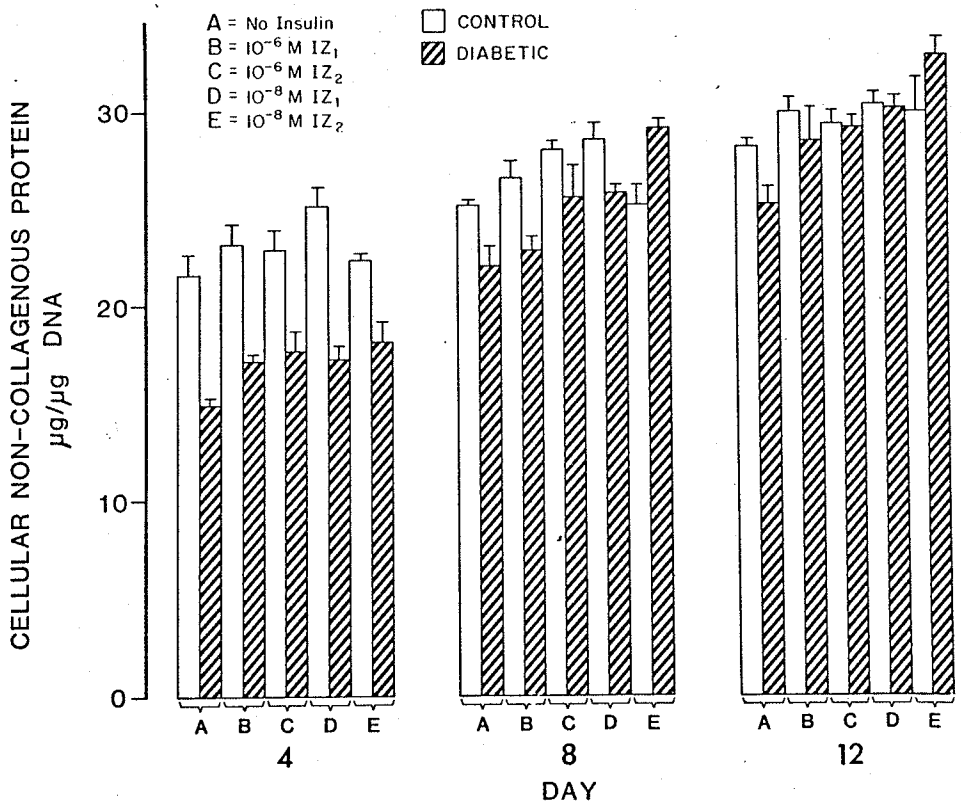


FIGURE 49: Comparison of cellular non-collagenous protein concentration of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Protein was expressed as non-collagenous protein on a DNA basis and measured on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ_1 and IZ_2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} and 10^{-6} M.

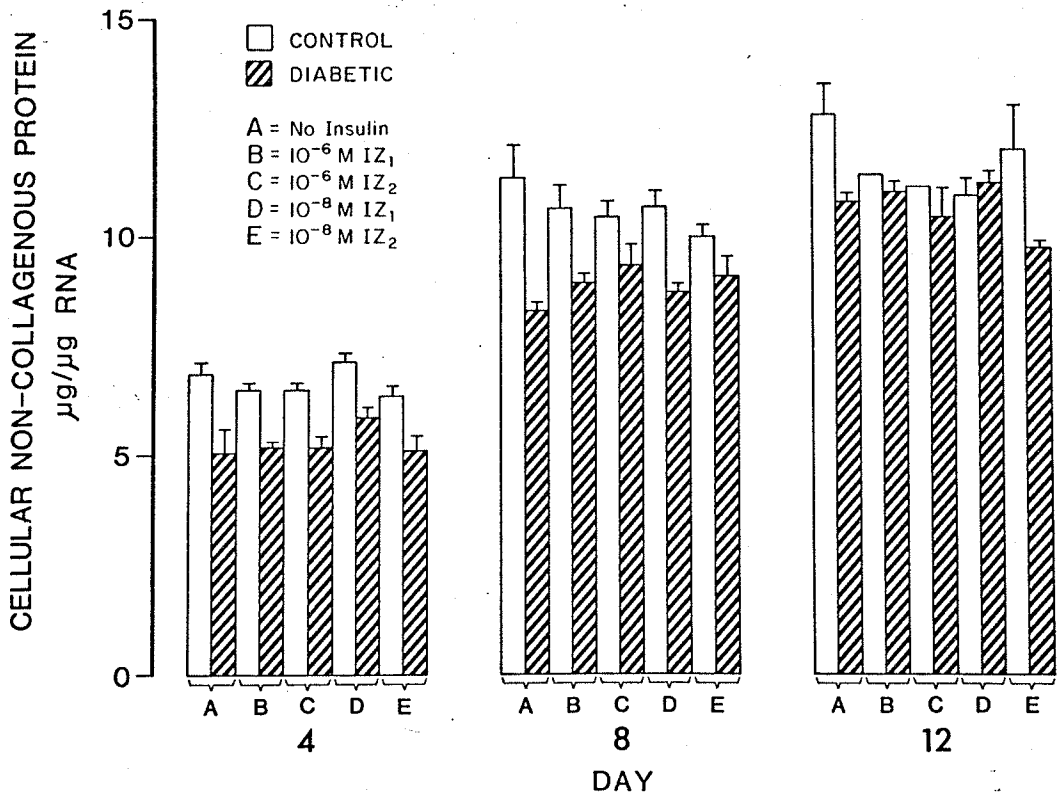


FIGURE 50: Comparison of cellular non-collagenous protein levels of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Protein levels, expressed as non-collagenous protein on a RNA basis, were measured on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ_1 and IZ_2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

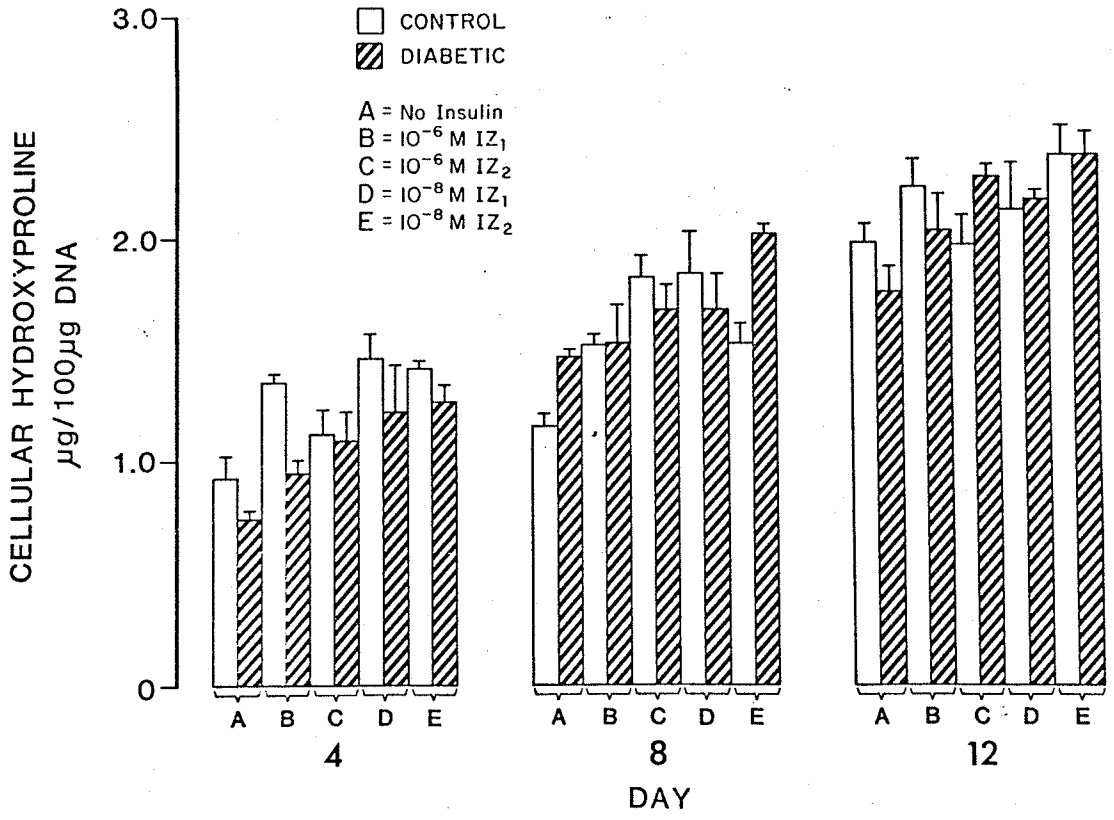


FIGURE 51: Comparison of cellular collagenous protein content of control and diabetic rat gingival fibroblasts treated with insulin preparations containing different amounts of bound zinc. Collagenous protein is expressed as µg cellular hydroxyproline per 100 µg DNA on days 4, 8 and 12 of a 12 day growth period. Insulin preparations IZ_1 and IZ_2, containing 0.2 and 0.5% bound zinc respectively, were added to the growth medium at concentrations of 0, 10^{-8} or 10^{-6} M.

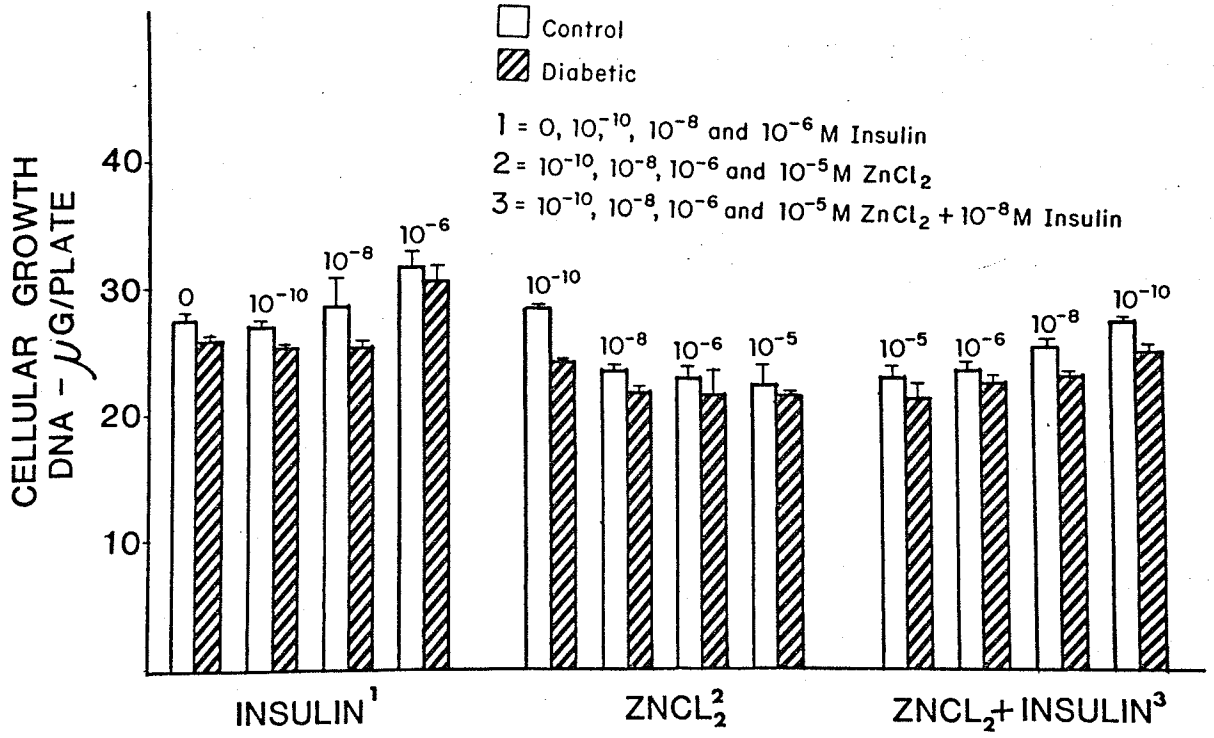


FIGURE 52: Relationship of the effects of insulin and zinc concentrations alone and of their interaction on the growth of control and diabetic rat gingival fibroblasts. Growth was measured as DNA per plate on 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

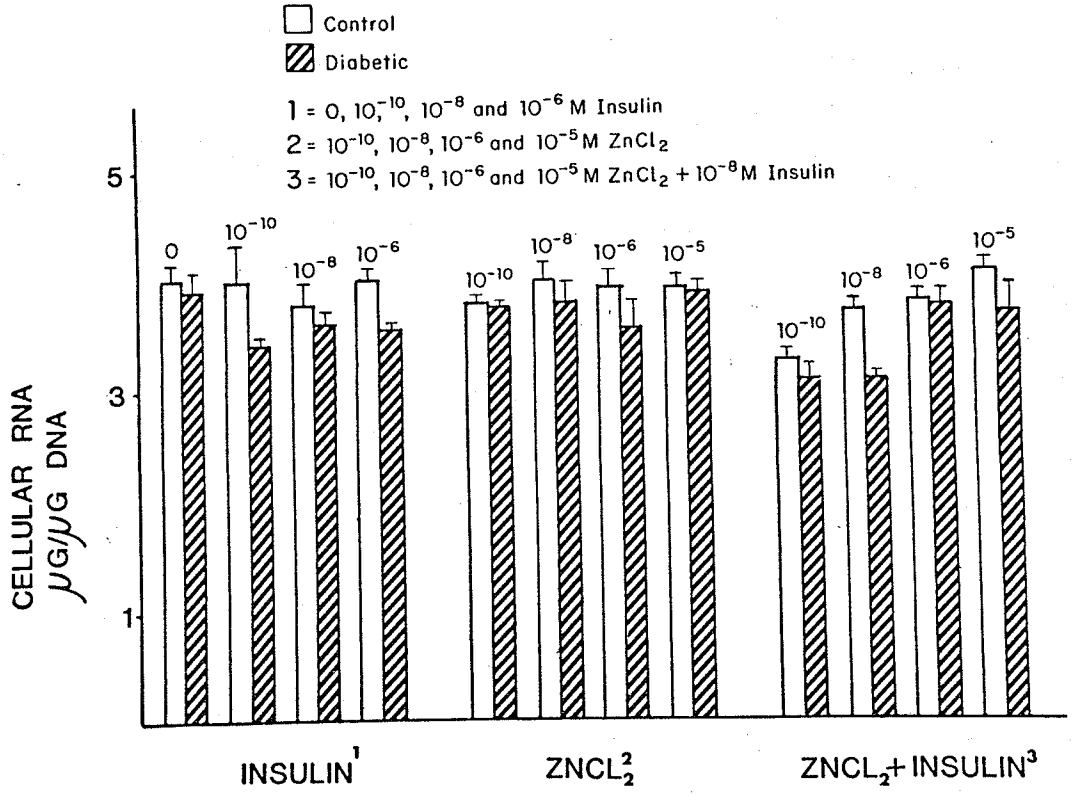


FIGURE 53: Relationship of the effects of insulin and zinc concentrations alone and of their interaction on cellular RNA concentration of control and diabetic rat gingival fibroblasts. Cellular RNA is expressed on a DNA basis for 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

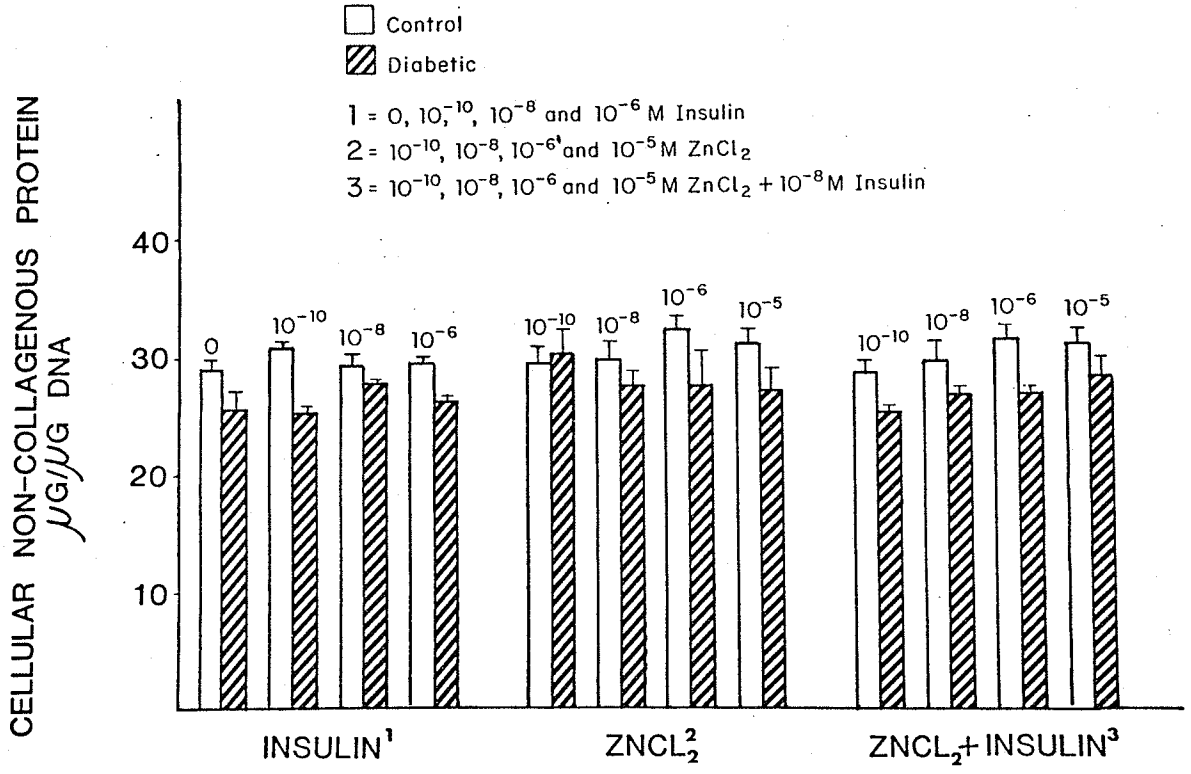


FIGURE 54: Relationship of the effects of insulin and zinc concentrations alone and their interaction on cellular non-collagenous protein levels of control and diabetic rat gingival fibroblasts. Cellular non-collagenous protein is expressed on a DNA basis for 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

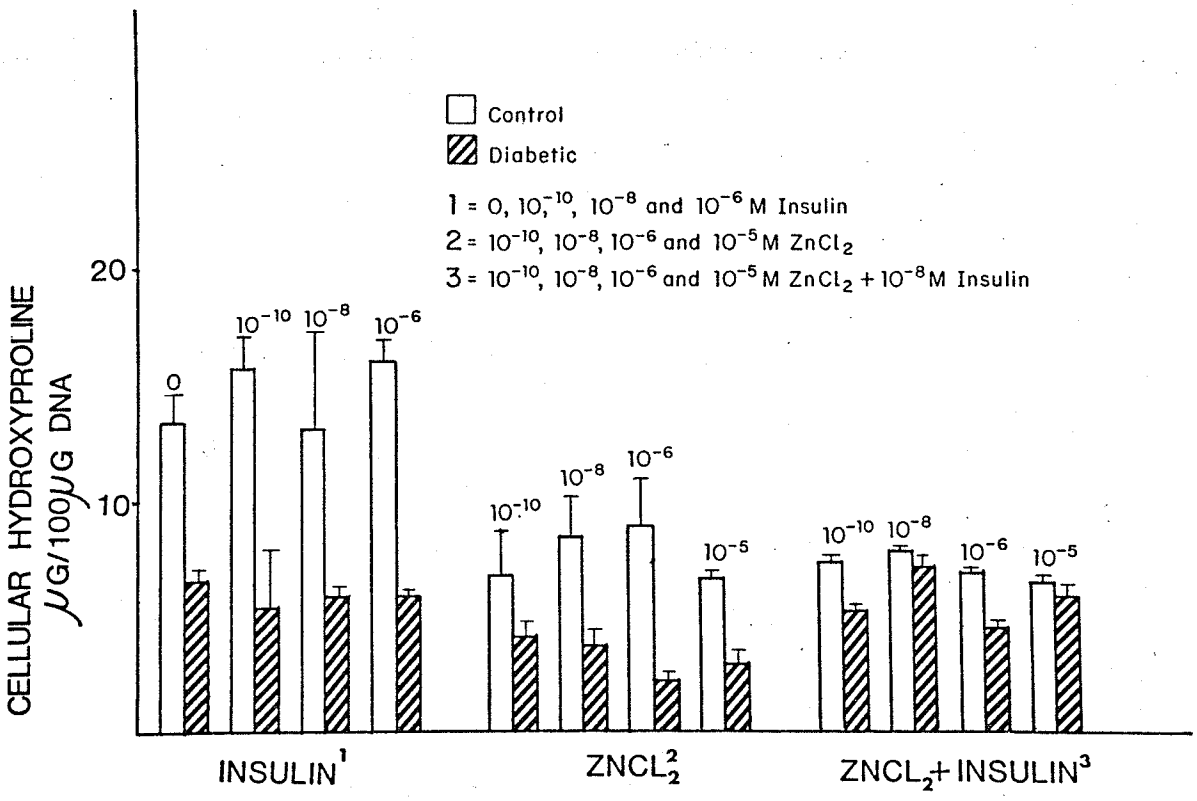


FIGURE 55: Relationship of the effects of insulin and zinc concentrations alone and of their interaction on cellular collagenous protein concentration of control and diabetic rat gingival fibroblasts. Cellular collagenous protein is expressed on a DNA basis for 9 day old cells. Insulin (containing 0.2% bound zinc) was added to the growth medium at concentrations of 0, 10^{-10}, 10^{-8} and 10^{-6} M. Zinc was added as zinc chloride at levels of 10^{-10}, 10^{-8}, 10^{-6} and 10^{-5} M. Zinc-insulin interactions were determined at the foregoing zinc levels in the presence of 10^{-8} M insulin only.

TABLE 1: COMPARISON OF DATA. ANALYSIS OF VARIANCE. ANIMALS SACRIFICED ON DAY 56. CELLULAR DNA (µg / PLATE).

ANALYSIS OF VARIANCE	F VALUE
A - DAYS 3, 6, 9	0.77
B - CELL TYPE, CONTROL, DIABETIC, INSULIN TREATED DIABETIC	2.88
AB - INTERACTION	1.00
C - INSULIN	53.58 †
AC - INTERACTION	0.60
BC - INTERACTION	1.31
ABC - INTERACTION	0.35

† $P < .05$

TABLE 2: COMPARISON OF DATA. ANALYSIS OF VARIANCE. ANIMALS SACRIFICED ON DAY 56. CELLULAR RNA (µg / µg DNA)

ANALYSIS OF VARIANCE	F VALUE
A - DAYS 3, 6, 9	0.03
B - CELL TYPE, CONTROL, DIABETIC, INSULIN TREATED DIABETIC	53.82 †
AB - INTERACTION	1.48
C - INSULIN	237.0 †
AC - INTERACTION	2.07
BC - INTERACTION	1.70
ABC - INTERACTION	0.80

† $P < .05$

TABLE 3: COMPARISON OF DATA. ANALYSIS OF VARIANCE. ANIMALS SACRIFICED ON DAY 56. CELLULAR NON-COLLAGENOUS PROTEIN (µg/ µg DNA)

ANALYSIS OF VARIANCE	F VALUE
A - DAYS 3, 6, 9	0.06
B - CELL TYPE, CONTROL, DIABETIC, INSULIN TREATED DIABETIC	0.50
AB - INTERACTION	0.76
C - INSULIN	15.88 †
AC - INTERACTION	1.00
BC - INTERACTION	2.64
ABC - INTERACTION	0.65

† $P < .05$

TABLE 4: COMPARISON OF DATA-ANALYSIS OF VARIANCE. ANIMALS SACRIFICED ON DAY 56. MEDIUM HYDROXYPROLINE (µg / 100 µg DNA).

ANALYSIS OF VARIANCE	F VALUE
A - DAYS 3, 6, 9	0.25
B - CELL TYPE, CONTROL, DIABETIC, INSULIN TREATED DIABETIC	2.53
AB - INTERACTION	5.69
C - INSULIN	3.24
AC - INTERACTION	0.39
BC - INTERACTION	0.26
ABC - INTERACTION	0.38

TABLE 5: EFFECT OF CONTROL AND DIABETIC RAT SERUM ON HYDROXYPROLINE ACCUMULATION BY HUMAN GINGIVAL FIBROBLASTS.

PERCENT SERUM	TOTAL (μg) HYDROXYPROLINE PRODUCED OVER 12 DAYS IN PASSAGE		DISTRIBUTION OF HYDROXYPROLINE IN CELLS AND MEDIUM				% HYDROXYPROLINE ACCUMULATION IN CONTROL RAT SERUM AT DAYS			% HYDROXYPROLINE ACCUMULATION IN DIABETIC RAT SERUM AT DAYS		
	CONTROL SERUM	DIABETIC SERUM	CONTROL SERUM % MED.	% CELL	DIABETIC SERUM % MED.	% CELL	4	8	12	4	8	12
5	75.973	8.784	88.48	13.52	68.31	31.39	15.28	30.11	54.61	81.09	17.47	1.43
10	87.986	16.835	86.6	13.40	98.01	1.99	11.93	38.51	49.55	28.72	71.28	0
15	94.846	12.486	85.41	14.60	79.29	20.71	12.66	35.73	51.62	49.77	49.01	1.22

TABLE 6: EFFECT OF INSULIN TREATMENT ON THE INTRACELLULAR ACCUMULATION OF [1]ASCORBATE BY CONTROL AND DIABETIC RAT GINGIVAL FIBROBLASTS.

FIBROBLAST SOURCE AND TREATMENT	ASCORBATE µg / µg DNA
CONTROL (5)[2]	0.868 ± 0.280
CONTROL + INSULIN (2)	1.460 ± 0.127
DIABETIC (5)	0.954 ± 0.313
DIABETIC + INSULIN (5)	0.764 ± 0.145

1. ASCORBATE LEVELS WERE DETERMINED ON 9 DAY OLD FIBROBLASTS. FIBROBLASTS WERE GROWN IN α MEM WHICH CONTAINED 50 µg / ML ASCORBATE. MEDIUM WAS CHANGED ON DAYS 3 AND 6.
2. ( ) NUMBER OF PLATES PER GROUP ANALYZED.
3. ANALYSIS OF VARIANCE — F VALUE

A - TREATMENT - DIABETES	2.37
B - INSULIN	5.46 †
AB - INTERACTION	8.95 †

† $P < .05$

TABLE 7: THE EFFECTS OF MEDIUM ASCORBATE AND OF ASCORBATE-INSULIN INTERACTION ON THE ACCUMULATION OF MEDIUM HYDROXYPROLINE BY HUMAN GINGIVAL FIBROBLASTS.

MEDIUM INSULIN CONCENTRATION (M)	DAY	MEDIUM HYDROXYPROLINE µg / 100 µg DNA		
		MEDIUM ASCORBATE µg / ML		
		50	100	200
0	4	$20.21 \pm 2.75^{A,a}$	$20.56 \pm 0.28^{C,a}$	$25.76 \pm 2.95^{G,a}$
	8	$22.47 \pm 0.86^{A,b}$	$22.07 \pm 0.15^{D,b}$	$30.87 \pm 3.60^{H,c}$
	12	$22.82 \pm 1.17^{A,d}$	$24.44 \pm 0.05^{E,d}$	$33.96 \pm 0.71^{H,e}$
10^{-6}	4	$27.67 \pm 2.68^{B,f}$	$20.23 \pm 0.08^{C,g}$	$31.58 \pm 1.63^{H,f}$
	8	$28.00 \pm 2.92^{B,h}$	$32.89 \pm 1.89^{F,h}$	$37.60 \pm 1.72^{H,i}$
	12	$26.80 \pm 1.95^{B,j}$	$30.27 \pm 3.01^{F,j}$	$39.09 \pm 9.90^{H,j}$

MEAN ± SEM, STATISTICAL ANALYSIS BY T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO TIME AND INSULIN CONCENTRATION, WHILE DIFFERENCES DUE TO ASCORBATE CONCENTRATION ARE INDICATED BY LOWER CASE SUPERSCRIPTS.

TABLE 8: RELATIONSHIP OF CELL DENSITY, MEDIUM INSULIN, ASCORBATE AND OF INSULIN AND ASCORBATE COMBINED ON THE ACCUMULATION OF MEDIUM HYDROXYPROLINE BY CONTROL AND DIABETIC RAT GINGIVAL FIBROBLASTS.

CELL TYPE	CELL DENSITY CELLS/DISH	MEDIUM HYDROXYPROLINE µg / 100 µg DNA			
		TREATMENT			
		CONTROL	10^{-6}M INSULIN	200 µg/ML ASCORBATE	200 µg/ML ASCORBATE +10^{-6}M INSULIN
CONTROL	7.5×10^4	$96.04 \pm 3.16^{A,a}$	$35.75 \pm 1.18^{F,b}$	$56.63 \pm 2.27^{I,c}$	$30.36 \pm 0.61^{L,d}$
	1.5×10^5	$65.40 \pm 2.20^{B,e}$	$38.66 \pm 4.52^{F,f}$	$32.92 \pm 0.78^{J,f}$	*
	5.0×10^5	$79.72 \pm 4.72^{C,g}$	$64.70 \pm 3.05^{G,h}$	$45.08 \pm 2.84^{K,i}$	$24.83 \pm 3.60^{L,j}$
	1.0×10^6	$71.87 \pm 7.33^{C,k}$	$72.81 \pm 4.15^{H,k}$	$26.33 \pm 8.68^{J,l}$	$28.94 \pm 6.54^{L,l}$
DIABETIC	7.5×10^4	$55.48 \pm 0.17^{D,m}$	$38.05 \pm 2.64^{F,n}$	$56.25 \pm 1.34^{I,m}$	$40.11 \pm 4.46^{M,n}$
	1.5×10^5	$70.66 \pm 1.96^{C,o}$	$52.28 \pm 8.43^{G,p}$	$58.23 \pm 5.60^{I,p}$	$62.93 \pm 1.60^{N,p}$
	5.0×10^5	$89.32 \pm 2.71^{A,q}$	$47.60 \pm 1.12^{G,r}$	$54.59 \pm 1.93^{I,s}$	$58.00 \pm 7.63^{N,s}$
	1.0×10^6	$39.95 \pm 0.01^{E,t}$	$43.92 \pm 13.84^{G,t}$	$64.49 \pm 1.14^{I,u}$	$40.28 \pm 3.48^{M,t}$

MEAN ± SEM, STATISTICAL ANALYSIS BY T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO CELL DENSITY AND CELL TYPE WHILE DIFFERENCES DUE TO TREATMENT ARE INDICATED BY LOWER CASE SUPERSCRIPTS.

* NO SAMPLES WERE AVAILABLE FOR CONTROL CELLS SEEDED AT 1.5×10^5 CELLS PER DISH AND TREATED WITH ASCORBATE AND INSULIN COMBINED.

TABLE 9: IN VITRO EFFECT OF STREPTOZOTOCIN ON RAT GINGIVAL FIBROBLAST GROWTH

STREPTOZOTOCIN CONCENTRATION µg / ML	DNA µg /PLATE DAY 3 †	DAY 6	DAY 9
0.0	0.25 ± 0.01	3.59 ± 0.13	3.79 ± 0.18
0.5	0.25 ± 0.01	3.58 ± 0.16	3.75 ± 0.33
1.0	0.25 ± 0.03	3.67 ± 0.21	3.83 ± 0.04
5.0	0.25 ± 0.02	3.70 ± 0.11	3.87 ± 0.09
10.0	0.25 ± 0.01	3.63 ± 0.09	3.91 ± 0.14
50.0	0.25 ± 0.01	3.58 ± 0.08	3.83 ± 0.19
100.0	0.25 ± 0.02	3.58 ± 0.08	3.83 ± 0.08

MEAN ± SEM, STATISTICAL ANALYSIS OF DATA BY ANALYSIS OF VARIANCE.

† DAY 3 < DAY 6 ≤ DAY 9, $p < .05$

TABLE 19: IN VITRO EFFECT OF STREPTOZOTOCIN ON RNA PRODUCTION BY RAT GINGIVAL FIBROBLASTS

STREPTOZOTOCIN CONCENTRATION µg / ML	RNA µg / µg DNA		
	DAY 3 †	DAY 6	DAY 9
0.0	50.23 ± 6.05	6.85 ± 0.20	4.52 ± 0.26
0.5	49.47 ± 1.24	6.62 ± 0.20	4.84 ± 0.67
1.0	50.6 ± 2.45	6.96 ± 0.14	4.96 ± 0.09
5.0	50.39 ± 1.13	6.96 ± 0.10	4.44 ± 0.24
10.0	48.48 ± 0.64	6.87 ± 0.41	4.70 ± 0.33
50.0	51.30 ± 1.34	6.89 ± 0.38	4.84 ± 0.28
100.0	51.91 ± 0.89	6.64 ± 0.45	4.68 ± 0.50

MEAN ± SEM, STATISTICAL ANALYSIS OF DATA BY ANALYSIS OF VARIANCE.
† DAY 3 > DAY 6 > DAY 9, $p < .05$

TABLE 11: IN VITRO EFFECT OF STREPTOZOTOCIN ON NON-COLLAGENOUS PROTEIN PRODUCTION BY RAT GINGIVAL FIBROBLASTS

STREPTOZOTOCIN CONCENTRATION µg / ML	NON-COLLAGENOUS PROTEIN µg / µg DNA		
	DAY 3 +	DAY 6	DAY 9
0.0	16.13 ± 1.01	30.98 ± 1.81	30.72 ± 3.06
0.5	16.96 ± 2.65	31.23 ± 0.73	32.07 ± 1.44
1.0	15.96 ± 2.00	31.51 ± 0.78	30.47 ± 3.34
5.0	16.96 ± 1.00	29.45 ± 1.06	32.27 ± 1.31
10.0	16.96 ± 1.00	30.33 ± 2.14	30.37 ± 0.88
50.0	15.96 ± 1.00	32.11 ± 0.80	31.02 ± 3.28
100.0	17.05 ± 0.91	32.10 ± 1.25	32.30 ± 1.77

MEAN ± SEM, STATISTICAL ANALYSIS OF DATA BY ANALYSIS OF VARIANCE.
+ DAY 3 < DAY 6 = DAY 9, $P < .05$

TABLE 12: IN VITRO EFFECT OF STREPTOZOTOCIN ON CELLULAR HYDROXYPROLINE PRODUCTION BY RAT GINGIVAL FIBROBLASTS.

STREPTOZOTOCIN CONCENTRATION µg / ML	CELLULAR HYDROXYPROLINE µg / 100 µg DNA		
	DAY 3	DAY 6	DAY 9
0.0	$19.07 \pm 0.87^{A,a}$	$0.80 \pm 0.10^{B,b,c}$	$1.36 \pm 0.17^{D,b,d}$
0.5	$16.00 \pm 3.63^{A,a}$	$0.83 \pm 0.06^{B,b,c}$	$1.10 \pm 0.33^{D,b,c}$
1.0	$18.00 \pm 2.77^{A,a}$	$0.77 \pm 0.04^{B,b,c}$	$0.97 \pm 0.11^{D,b,c}$
5.0	$16.53 \pm 3.33^{A,a}$	$0.84 \pm 0.24^{B,b,c}$	$0.98 \pm 0.27^{D,b,c}$
10.0	$19.73 \pm 1.73^{A,a}$	$0.82 \pm 0.17^{B,b,c}$	$0.78 \pm 0.18^{D,b,c}$
50.0	$18.13 \pm 2.89^{A,a}$	$1.09 \pm 0.15^{B,b,c}$	$0.77 \pm 0.22^{D,b,c}$
100.0	$19.73 \pm 0.87^{A,a}$	$1.14 \pm 0.08^{C,b,c}$	$0.64 \pm 0.13^{E,b,c}$

MEAN $\pm$ SEM, STATISTICAL ANALYSIS BY ANALYSIS OF VARIANCE AND T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO STREPTOZOTOCIN CONCENTRATION EFFECTS, WHILE DIFFERENCES DUE TO TIME ARE SHOWN BY LOWER CASE SUPERSCRIPTS.

TABLE 13: IN VITRO EFFECT OF STREPTOZOTOCIN ON MEDIUM HYDROXYPROLINE ACCUMULATION BY RAT GINGIVAL FIBROBLASTS.

STREPTOZOTOCIN CONCENTRATION μg / ML	MEDIUM HYDROXYPROLINE μg / μg DNA		
	DAY 3	DAY 6	DAY 9
0.0	$3.0 \pm 0.01^{A,a}$	$0.30 \pm 0.01^{B,b}$	$0.15 \pm 0.08^{D,c}$
0.5	$2.67 \pm 0.33^{A,d}$	$0.30 \pm 0.03^{B,e}$	$0.77 \pm 0.05^{E,f}$
1.0	$2.33 \pm 0.33^{A,g}$	$0.50 \pm 0.03^{C,h}$	$0.96 \pm 0.01^{F,i}$
5.0	$4.67 \pm 0.67^{A,j}$	$0.50 \pm 0.06^{C,k}$	$0.75 \pm 0.05^{E,l}$
10.0	$3.33 \pm 2.33^{A,m}$	$0.51 \pm 0.02^{C,n}$	$0.51 \pm 0.19^{E,n}$
50.0	$5.0 \pm 1.00^{A,o}$	$0.70 \pm 0.19^{C,p}$	$0.81 \pm 0.11^{E,p}$
100.0	$4.33 \pm 0.88^{A,q}$	$0.56 \pm 0.04^{C,r}$	$0.98 \pm 0.04^{F,s}$

MEAN = SEM, STATISTICAL ANALYSIS BY ANALYSIS OF VARIANCE AND TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO STREPTOZOTOCIN CONCENTRATION EFFECTS, WHILE DIFFERENCES DUE TO TIME ARE SHOWN BY LOWER CASE SUPERSCRIPTS.

TABLE 14: COMPARATIVE EFFECTS OF 2 INSULIN PREPARATIONS WITH DIFFERENT AMOUNTS OF BOUND ZINC ON MEDIUM HYDROXYPROLINE ACCUMULATION BY RAT GINGIVAL FIBROBLASTS.

CELL TYPE	DAY	INSULIN TYPE[1]	MEDIUM HYDROXYPROLINE µg / 100 µg DNA		
			INSULIN (M)		
			0	10^{-8}	10^{-6}
CONTROL	4	IZ_1	$21.70 \pm 2.03^{A,a}$	$24.55 \pm 6.77^{E,a}$	$44.80 \pm 7.36^{J,b}$
		IZ_2		$58.70 \pm 0.05^{F,c}$	$12.45 \pm 0.02^{K,d}$
	8	IZ_1	$55.10 \pm 5.40^{B,e}$	$48.65 \pm 6.68^{G,e}$	$59.99 \pm 7.03^{J,e}$
		IZ_2		$32.89 \pm 4.33^{E,f}$	$30.94 \pm 2.62^{L,f}$
	12	IZ_1	$52.28 \pm 5.96^{B,g}$	$45.68 \pm 2.25^{G,g}$	$22.54 \pm 4.30^{L,h}$
		IZ_2		$14.06 \pm 0.66^{H,i}$	$28.50 \pm 2.38^{L,j}$
DIABETIC	4	IZ_1	$14.65 \pm 2.94^{C,k}$	$19.50 \pm 5.12^{E,k}$	$25.60 \pm 0.77^{L,l}$
		IZ_2		$81.73 \pm 15.9^{I,m}$	$13.92 \pm 1.31^{K,k}$
	8	IZ_1	$90.11 \pm 11.96^{D,o}$	$47.82 \pm 3.67^{G,p}$	$38.46 \pm 8.79^{J,p}$
		IZ_2		$49.19 \pm 12.36^{G,q}$	$57.11 \pm 6.26^{J,q}$
	12	IZ_1	$48.04 \pm 3.14^{B,r}$	$30.10 \pm 6.77^{E,s}$	$18.29 \pm 1.22^{L,t}$
		IZ_2		$35.14 \pm 0.72^{E,u}$	$20.0 \pm 7.69^{L,v}$

MEAN ± SEM, STATISTICAL ANALYSIS BY T TEST.

[1] INSULIN PREPARATIONS IZ_1 AND IZ_2 CONTAIN 0.2 AND 0.5% BOUND ZINC RESPECTIVELY.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO TIME, CELL TYPE AND INSULIN TYPE, WHILE DIFFERENCES DUE TO INSULIN CONCENTRATION ARE SHOWN BY LOWER CASE SUPERSCRIPTS.

TABLE 15: COMPARISON OF IN VITRO EFFECTS OF INSULIN, ZINC CHLORIDE AND THEIR INTERACTION ON MEDIUM HYDROXYPROLINE ACCUMULATION BY 9 DAY OLD CONTROL AND DIABETIC PAT GINGIVAL FIBROBLASTS.

CELL TYPE	INSULIN (M)	HYDROXYPROLINE (µg / 100 µg DNA)				
		ZINC CHLORIDE (M)				
		0	10^{-10}	10^{-8}	10^{-6}	10^{-5}
CONTROL	0	$13.67 \pm 3.25^{A,a}$	$15.96 \pm 6.28^{D,a}$	$25.47 \pm 4.0^{G,b}$	$33.03 \pm 4.24^{H,b}$	$41.59 \pm 3.49^{J,c}$
	10^{-10}	57.46 ± 6.10^{B}				
	10^{-8}	$37.14 \pm 5.12^{C,d}$	$28.12 \pm 3.09^{E,e}$	$20.28 \pm 3.44^{G,f}$	$47.43 \pm 7.68^{J,d}$	$11.69 \pm 3.41^{K,g}$
	10^{-6}	58.01 ± 6.58^{B}				
DIABETIC	0	$12.63 \pm 4.04^{A,h}$	$36.10 \pm 2.83^{F,i}$	$30.86 \pm 2.85^{G,j}$	$33.83 \pm 9.4^{H,j}$	$46.03 \pm 5.66^{J,k}$
	10^{-10}	51.41 ± 8.38^{B}				
	10^{-8}	$59.52 \pm 5.28^{B,l}$	$16.41 \pm 1.76^{D,m}$	$29.11 \pm 5.49^{G,h}$	$33.70 \pm 2.53^{H,n}$	$12.64 \pm 7.26^{K,m}$
	10^{-6}	34.40 ± 9.37^{C}				

MEAN ± SEM, STATISTICAL ANALYSIS BY T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO CELL TYPE AND INSULIN TREATMENT, WHILE DIFFERENCES DUE TO ZINC CHLORIDE CONCENTRATION ARE SHOWN BY LOWER CASE SUPERSCRIPTS.

TABLE 16: IN VITRO EFFECT OF TESTOSTERONE AND TESTOSTERONE-INSULIN INTERACTION ON RAT GINGIVAL FIBROBLAST GROWTH.

CELL TYPE	MEDIUM INSULIN CONCENTRATION	DAY	DNA µg / PLATE			
			MEDIUM TESTOSTERONE ng / ML			
			0	50	100	150
CONTROL	0	6	$49.98 \pm 1.13^{A,a}$	$53.55 \pm 0.45^{E,b}$	$50.78 \pm 0.54^{I,a}$	$50.53 \pm 0.85^{L,a}$
		9	$59.03 \pm 0.74^{B,c}$	$60.80 \pm 0.20^{F,c}$	$60.70 \pm 0.53^{J,c}$	$60.15 \pm 2.00^{M,c}$
		12	$56.33 \pm 1.80^{B,d}$	$56.08 \pm 0.85^{E,d}$	$50.42 \pm 2.25^{I,d}$	$47.98 \pm 0.99^{L,e}$
	10^{-6}M	6	$56.52 \pm 0.99^{B,f}$	$53.17 \pm 0.53^{E,f}$	$52.16 \pm 0.60^{I,f}$	$52.90 \pm 1.14^{L,f}$
		9	$61.06 \pm 2.97^{B,g}$	$60.80 \pm 0.40^{F,g}$	$61.13 \pm 0.49^{J,g}$	$60.80 \pm 0.52^{M,g}$
		12	$57.08 \pm 0.30^{B,h}$	$57.08 \pm 0.50^{E,h}$	$51.00 \pm 1.75^{I,i}$	$52.42 \pm 2.25^{L,i}$
DIABETIC	0	6	$30.78 \pm 0.78^{C,j}$	$43.92 \pm 1.90^{G,k}$	$42.78 \pm 1.90^{K,k}$	$40.63 \pm 2.11^{N,k}$
		9	$43.98 \pm 0.13^{D,l}$	$48.02 \pm 0.47^{H,m}$	$49.43 \pm 0.48^{I,m}$	$43.20 \pm 2.00^{N,l}$
		12	$40.83 \pm 1.42^{D,n}$	$44.67 \pm 1.69^{G,n}$	$43.58 \pm 0.46^{K,n}$	$41.92 \pm 0.65^{N,n}$
	10^{-6}M	6	$48.27 \pm 1.56^{A,o}$	$44.45 \pm 1.40^{G,p}$	$43.23 \pm 0.40^{K,p}$	$44.87 \pm 2.29^{N,p}$
		9	$53.42 \pm 0.93^{B,q}$	$49.75 \pm 0.68^{H,r}$	$50.50 \pm 0.29^{I,r}$	$51.82 \pm 0.82^{L,r}$
		12	$47.33 \pm 0.50^{A,s}$	$45.92 \pm 1.01^{G,s}$	$45.83 \pm 0.17^{K,s}$	$44.92 \pm 0.67^{N,s}$

MEAN ± SEM, STATISTICAL ANALYSIS BY ANALYSIS OF VARIANCE AND T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO TIME, INSULIN CONCENTRATION AND CELL TYPE, WHILE DIFFERENCES DUE TO TESTOSTERONE CONCENTRATION ARE INDICATED BY LOWER CASE SUPERSCRIPTS.

TABLE 17: IN VITRO EFFECTS OF TESTOSTERONE AND TESTOSTERONE-INSULIN INTERACTION ON RNA PRODUCTION BY RAT GINGIVAL FIBROBLASTS.

CELL TYPE	MEDIUM INSULIN CONCENTRATION	DAY	RNA µg / µg DNA			
			MEDIUM TESTOSTERONE ng / ML			
			0	50	100	150
CONTROL	0	6	$4.21 \pm 0.03^{A,a}$	$4.07 \pm 0.12^{D,a}$	$3.77 \pm 0.18^{H,a}$	$4.03 \pm 0.15^{K,a}$
		9	$3.75 \pm 0.09^{B,b}$	$3.51 \pm 0.04^{E,b}$	$4.02 \pm 0.23^{H,b}$	$3.65 \pm 0.10^{L,b}$
		12	$3.66 \pm 0.07^{B,c}$	$3.43 \pm 0.05^{E,c}$	$3.58 \pm 0.18^{H,c}$	$3.45 \pm 0.14^{L,c}$
	10^{-6}M	6	$4.21 \pm 0.10^{A,d}$	$4.13 \pm 0.09^{D,d}$	$3.73 \pm 0.09^{H,e}$	$4.38 \pm 0.12^{K,d}$
		9	$4.12 \pm 0.38^{A,f}$	$4.31 \pm 0.17^{D,f}$	$4.38 \pm 0.06^{I,f}$	$4.10 \pm 0.20^{K,f}$
		12	$3.65 \pm 0.09^{B,g}$	$3.02 \pm 0.09^{F,h}$	$3.72 \pm 0.14^{H,g}$	$3.77 \pm 0.02^{L,g}$
DIABETIC	0	6	$3.32 \pm 0.07^{C,i}$	$3.42 \pm 0.15^{E,i}$	$3.42 \pm 0.09^{H,i}$	$3.74 \pm 0.16^{L,i}$
		9	$3.90 \pm 0.22^{A,j}$	$3.74 \pm 0.08^{G,j}$	$3.62 \pm 0.06^{H,j}$	$3.77 \pm 0.16^{L,j}$
		12	$3.60 \pm 1.26^{B,k}$	$3.22 \pm 0.09^{F,k}$	$3.24 \pm 0.02^{J,k}$	$3.15 \pm 0.03^{M,k}$
	10^{-6}M	6	$3.31 \pm 0.15^{C,l}$	$3.30 \pm 0.16^{E,l}$	$3.64 \pm 0.02^{H,l}$	$3.80 \pm 0.21^{L,l}$
		9	$4.23 \pm 0.05^{A,m}$	$3.98 \pm 0.07^{D,n}$	$4.15 \pm 0.25^{I,m}$	$3.92 \pm 0.00^{L,n}$
		12	$3.47 \pm 0.55^{B,o}$	$3.44 \pm 0.09^{E,o}$	$3.62 \pm 0.07^{H,o}$	$3.57 \pm 0.07^{L,o}$

MEAN ± SEM, STATISTICAL ANALYSIS BY ANALYSIS OF VARIANCE AND T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO TIME, INSULIN CONCENTRATION AND CELL TYPE, WHILE DIFFERENCES DUE TO TESTOSTERONE CONCENTRATION ARE INDICATED BY LOWER CASE SUPERSCRIPTS.

TABLE 18: IN VITRO EFFECTS OF TESTOSTERONE AND OF TESTOSTERONE-INSULIN INTERACTION ON NON-COLLAGENOUS PROTEIN PRODUCTION BY RAT GINGIVAL FIBROBLASTS.

CELL TYPE	MEDIUM INSULIN CONCENTRATION	DAY	NON-COLLAGENOUS PROTEIN µg / µg DNA			
			MEDIUM TESTOSTERONE ng / ML			
			0	50	100	150
CONTROL	0	6	$7.31 \pm 0.20^{A,a}$	$7.13 \pm 0.28^{J,a}$	$8.16 \pm 0.83^{O,a}$	$7.27 \pm 0.42^{S,a}$
		9	$26.42 \pm 0.56^{B,b}$	$26.31 \pm 0.18^{K,b}$	$23.29 \pm 1.14^{P,b}$	$23.42 \pm 1.17^{T,b}$
		12	$35.92 \pm 2.90^{C,c}$	$30.38 \pm 1.80^{L,c}$	$34.41 \pm 0.89^{Q,c}$	$28.76 \pm 2.31^{T,c}$
	10^{-6}M	6	$7.76 \pm 0.14^{D,d}$	$7.58 \pm 0.38^{J,d}$	$6.47 \pm 0.32^{O,e}$	$6.31 \pm 0.21^{S,e}$
		9	$28.03 \pm 0.44^{E,f}$	$26.44 \pm 1.00^{K,g}$	$25.9 \pm 0.17^{P,g}$	$25.72 \pm 1.07^{T,g}$
		12	$33.53 \pm 1.58^{C,h}$	$31.24 \pm 0.71^{L,h}$	$33.96 \pm 0.87^{Q,h}$	$27.83 \pm 0.30^{T,i}$
DIABETIC	0	6	$8.90 \pm 0.34^{F,j}$	$9.95 \pm 0.73^{M,j}$	$9.24 \pm 0.71^{O,j}$	$8.64 \pm 1.36^{S,j}$
		9	$22.83 \pm 0.41^{G,k}$	$19.80 \pm 1.34^{N,k}$	$21.59 \pm 0.39^{P,k}$	$22.63 \pm 1.22^{T,k}$
		12	$29.87 \pm 1.94^{E,l}$	$28.62 \pm 1.01^{L,l}$	$28.68 \pm 2.18^{R,l}$	$27.2 \pm 0.13^{T,l}$
	10^{-6}M	6	$10.73 \pm 0.88^{H,m}$	$9.51 \pm 0.28^{M,m}$	$9.80 \pm 0.41^{O,m}$	$9.01 \pm 0.53^{S,m}$
		9	$20.74 \pm 0.49^{I,n}$	$19.66 \pm 0.63^{N,n}$	$22.86 \pm 0.91^{P,n}$	$22.23 \pm 0.38^{T,n}$
		12	$30.62 \pm 0.83^{E,o}$	$26.36 \pm 1.11^{K,o}$	$26.78 \pm 1.35^{R,o}$	$27.70 \pm 1.40^{T,o}$

MEAN $\pm$ SEM, STATISTICAL ANALYSIS BY ANALYSIS OF VARIANCE AND T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO TIME, INSULIN CONCENTRATION AND CELL TYPE, WHILE DIFFERENCES DUE TO TESTOSTERONE CONCENTRATION ARE INDICATED BY LOWER CASE SUPERSCRIPTS.

TABLE 19: IN VITRO EFFECTS OF TESTOSTERONE AND TESTOSTERONE-INSULIN INTERACTION ON CELLULAR HYDROXYPROLINE PRODUCTION BY RAT GINGIVAL FIBROBLASTS.

CELL TYPE	MEDIUM INSULIN CONCENTRATION	DAY	CELLULAR HYDROXYPROLINE µg / 100 µg DNA			
			MEDIUM TESTOSTERONE ng / ML			
			0	50	100	150
CONTROL	0	6	$3.08 \pm 0.92^{A,a}$	$1.86 \pm 0.90^{C,a}$	$1.31 \pm 0.58^{F,a}$	$1.26 \pm 0.04^{I,a}$
		9	$2.34 \pm 0.55^{A,b}$	$2.46 \pm 0.15^{C,b}$	$2.30 \pm 0.33^{F,b}$	$2.84 \pm 0.26^{J,b}$
		12	$2.69 \pm 0.84^{A,c}$	$2.11 \pm 0.40^{C,c}$	$1.66 \pm 0.24^{F,c}$	$1.08 \pm 0.18^{I,c}$
	10^{-6}M	6	$1.62 \pm 0.69^{A,d}$	$2.02 \pm 0.12^{C,d}$	$1.94 \pm 0.53^{F,d}$	$1.74 \pm 0.04^{K,d}$
		9	$3.17 \pm 0.18^{A,e}$	$2.43 \pm 0.27^{C,e}$	$2.68 \pm 0.63^{F,e}$	$3.88 \pm 0.21^{L,f}$
		12	$1.17 \pm 0.08^{A,g}$	$1.32 \pm 0.23^{C,h}$	$1.46 \pm 0.01^{F,h}$	$1.71 \pm 0.09^{K,i}$
DIABETIC	0	6	$0.50 \pm 0.18^{B,j}$	$0.26 \pm 0.02^{D,j}$	$0.31 \pm 0.02^{G,j}$	$0.32 \pm 0.06^{M,j}$
		9	$2.51 \pm 0.95^{A,k}$	$3.32 \pm 0.57^{E,k}$	$3.65 \pm 0.24^{H,k}$	$5.64 \pm 0.78^{N,l}$
		12	$1.34 \pm 0.37^{A,m}$	$1.33 \pm 0.15^{C,m}$	$1.63 \pm 0.30^{F,m}$	$1.24 \pm 0.20^{I,m}$
	10^{-6}M	6	$0.49 \pm 0.08^{B,n}$	$0.35 \pm 0.05^{D,n}$	$0.45 \pm 0.10^{G,n}$	$0.29 \pm 0.06^{M,n}$
		9	$4.26 \pm 0.30^{A,o}$	$2.59 \pm 0.52^{E,p}$	$4.07 \pm 0.38^{H,o}$	$7.23 \pm 0.35^{N,q}$
		12	$2.14 \pm 0.54^{A,r}$	$1.34 \pm 0.28^{C,r}$	$1.82 \pm 0.14^{F,r}$	$1.13 \pm 0.15^{I,r}$

MEAN ± SEM, STATISTICAL ANALYSIS BY ANALYSIS OF VARIANCE AND T TEST.

UPPER CASE SUPERSCRIPTS DENOTE DIFFERENCES DUE TO TIME, INSULIN CONCENTRATION AND CELL TYPE, WHILE DIFFERENCES DUE TO TESTOSTERONE CONCENTRATION ARE INDICATED BY LOWER CASE SUPERSCRIPTS.

CHAPTER X

REFERENCES

REFERENCES

Abe, S., Steinmann, B.U., Wahl, L.M. and Martin, G.R. 1979. Nature 279, 442-444.

Arbogast, B.W., Lee, G.M. and Raymond, T.L. 1982. Diabetes 31, 593-599.

Armato, U., Andreis, P.G., Draghi, E. and Mengato, L. 1978. In Vitro 14, 479-484.

Arquilla, E.R., Packer, S., Tarmas, W. and Miyamoto, S. 1978. Endocrinology 103, 1440-1449.

Aumailley, M., Krieg, T., Razaka, G., Muller, P.K. and Bricaud, H. 1982. Biochem. J. 206, 505-510.

Aurer-Kozelj, J., Kralj-Klobucar, N., Buzina, R. and Bacic, M. 1982. Internat. J. Vit. Nutr. Res. 52, 333-341.

Avery, B.E. and Simpson, D.M. 1973. J. Periodontol. 44, 675-686.

Bannister, D.W. and Burns, A.B. 1970. Analyst 95, 596-600.

Bartold, P.M., Wiebkin, O.W. and Thonard, J.C. 1982. Arch. Oral Biol. 27, 1-7.

Bates, C.I., Prynne, C. and Levene, C.J. 1972. Biochem. Biophys. Acta 278, 610-616.

Baum, B.J., Moss, J., Breul, S.D., Berg, R.A. and Crystal, R.G. 1980. J. Biol. Chem. 255, 2843-2847.

Beck-Nielsen, H. 1980. Dan. Med. Bull. 27, 173-184.

Belleman, P., Fry, J.R., Bridges, J.W., Schlote, W. and Mecke, D. 1977. Diabetologia 13, 621-628.

Berg, R.A. and Prockop, D.J. 1973. Biochem. Biophys. Res. Comm. 52, 115-120.

Berg, R.A., Schwartz, M.L. and Crystal, R.G. 1980. Proc. Nat. Acad. Sci. USA 77, 4746-4750.

Bienkowski, R.S., Baum, B.J. and Crystal, R.G. 1978-1. Nature 276, 413-416.

Bienkowski, R.S., Cowan, M.J., McDonald, J.A. and Crystal, R.G. 1978-2. J. Biol. Chem. 253, 4356-4363.

Bigley, R., Wirth, M., Layman, D., Riddle, M. and Stankova, L. 1983. Diabetes 32, 545-548.

Birkedal-Hansen, H., Butler, W.T. and Taylor, R.E. 1976. Calcif. Tiss. Res. 23, 39-44.

Blanck, T.J. and Peterkofsky, B. 1975. Arch. Biochem. Biophys. 171, 259-267.

Bornstein, P., Duksin, D., Balian, G., Davidson, J.M. and Crouch, E. 1978. Ann. NY Acad. Sci. 312, 93-105.

Bornstein, P. and Sage, H. 1980. Ann. Rev. Biochem. 49, 957-1003.

Bowman, P.D., Meek, R.L. and Daniel, C.W. 1976. Exp. Cell Res. 101, 434-437.

Burgeson, R.E., Eladic, F.A., Kaitila, H. and Hollister, D.W. 1976. Proc. Nat. Acad. Sci. USA 73, 2579-2583.

Burton, K. 1968. IN: Methods in Enzymology, vol. 12, (Grossman, L. and Kivie, M., eds), Academic Press, New York, pp. 163-166.

Capecchi, M.R., Capecchi, N.E., Hughes, S.H. and Wahl, G.M. 1974. Proc. Nat. Acad. Sci. USA 71, 4732-4736.

Caro, J.F. and Amatruda, J.M. 1980. J. Biol. Chem. 255, 10052-10055.

Castor, L.N. 1968. J. Cell Physiol. 72, 161-172.

Caterson, B., Baker, J.R., Christner, J.E., Pollok, B.A. and Rostand, K.S. 1980. Alabama J. Med. Sci. 17, 292-299.

Chaproniere-RicKenberg, D.M. and Webber, M.M. 1983. In Vitro 19, 373-375.

Chatterjee, I.B., Kar, N.C., Ghosh, N.C. and Guha, B.C. 1961. Ann. N.Y. Acad. Sci. 92, 36-56.

Chatterjee, I.B. 1978. Wld Rev. Nutr. Diet. 30, 69-87.

Chen, R.W. and Postlethwait, R.W. 1970. Exp. Med. Surg. 28, 294-298.

Chi, D.S., Berry, D.L., Dillon, K.A. and Arbogast, B.W. 1982. Diabetes 31, 1098-1104.

Ciancio, S.G. and Mather, M.L. 1971. J. Periodont. Res. 6, 188-193.

Cianciola, L.J., Park, B.H., Bruck, E., Mosovich, L. and Genco, R.J. 1982. JADA 104, 653-660.

Clark, C.C. and Kefalides, N.A. 1976. Proc. Nat. Acad. Sci. USA 73, 34-38.

Cohen, M.P. and Klein, C.V. 1979. J. Exp. Med. 149, 623-631.

Coulston, L. and Dandona, P. 1980. Diabetes 29, 665-667.

Czech, M.P., Massague, J. and Pilch, P.F. 1981. TIBS 6, 222-225.

Dabbous, M.K., Hammouda, O. and Brinkley, S.B. 1981. Mol. Cell Biochem. 34, 87-93.

Desbuquois, B., Postel-Vinay, M.C. 1980. IN: Insulin, Chemistry and Structure and Function of Insulin and Related Hormones (Brandenburg, D. and Wollmer, A., eds), de Gruyler, Berlin, pp. 285-292.

De Meyts, P., Roth, J., Neville, D.M., Garn, J.R. and Lesniak, M.A. 1973. Biochem. Biophys. Res. Comm. 55, 154-161.

Dowse, C.M., Zebrowski, E.J., Gaschuetz, H.H. and Brunka, J.R. 1983. J. Dent. Res. 62, 228, Abst. No. 527.

Dulin, W.E. and Soret, M.G. 1977. Chemically and hormonally induced diabetes. IN: The Diabetic Pancreas. (Volk, B.W. and Wellman, K.F., eds), N.Y. Plenum Press, pp. 425-465.

El Attar, T.M. 1974. Arch. Oral Biol. 19, 1185-1190.

El Attar, T.M., Lin, H.S. and Tira, D.E. 1982. Prostaglandins. Leukotrienes and Med. 9, 25-34.

Ellenberg, M. 1978. N.Y. State J. Med. 78, 2214-2218.

Elsas, L.J., MacDonell, R.C. and Rosenberg, L.E. 1971. J. Biol. Chem. 246, 6452-6459.

Embery, G., Oliver, W.M. and Stanbury, J.B. 1979. J. Periodont. Res. 14, 512-519.

Eng, J., Lee, L. and Yalow, R.S. 1980. Diabetes 29, 164-166.

Engel, L.D., Schroeder, H.E. and Page, R.C. 1978. Infect. Immun. 19, 287-297.

Engel, L.D., Schroeder, H.E., Gay, R. and Clagett, J. 1980. Arch. Oral Biol. 25, 283-296.

Epstein, E.J. Jr. 1974. J. Biol. Chem. 249, 3225-3331.

Epstein, J. 1982. J. Cell. Physiol. 110, 17-22.

Faris, B., Snider, R., Levine, A., Moscaritolo, R., Salcedo, L. and Franzblau, C. 1978. In Vitro 14, 1022-1027.

Fischer, L.J. and Rickert, D.E. 1975. Pancreatic islet-cell toxicity. CRC Crit. Rev. Toxicol. pp. 231-263.

Flier, J.S., Kahn, C.R., Roth, J. and Bar, R.S. 1975. Science 190, 63-65.

Fry, M. and Weisman-Shomer, P. 1976. Biochemistry 15, 4319-4328.

Fujimoto, W.Y. and Williams, R.H. 1972. Diabetes 21, 360.

Fujimoto, W.Y. and Williams, R.H. 1974. Diabetes 23, 443-448.

Gavin, J.R., Roth, J., Neville, D.M., DeMeyts, P. and Buell, D.N. 1974. Proc. Nat. Acad. Sci. USA 71, 84-88.

Gay, S. and Miller, E.J. 1978. IN: Collagen in the Physiology and Pathology of Connective Tissue, N.Y., pp. 1-24.

Ghafghazi, T., McDaniel, M.L. and Lacy, P.E. 1981. Diabetes 30, 341-345.

Giles, K.W. and Myers, A. 1965. Nature 206, 93.

Goggins, J.F. and Billups, L.C. 1972. J. Dent. Res. 51, 303-307.

Goldberg, A.L. 1971. Proc. Nat. Acad. Sci. USA 68, 362-366.

Goldfine, I.D., Gardner, J.D. and Neville, D.M. 1972. J. Biol. Chem. 247, 6919-6926.

Goldfine, I.D. and Sherline, P. 1972. J. Biol. Chem. 247, 6927-6931.

Goldstein, S., Littlefield, J.W. and Soeldner, J.S. 1969. Proc. Nat. Acad. Sci. USA 64, 155-160.

Goldstein, S., Moerman, E.J., Soeldner, J.S., Gleason, R.E. and Barnett, D.M. 1978. Science 199, 781-782.

Goldstein, S. 1979. J. Invest. Dermatol. 73, 19-23.

Golub, L.M., Greenwald, R.A., Zebrowski, E.J. and Ramamurthy, N.S. 1978-1. Biochem. Biophys. Acta 534, 73-81.

Golub, L.M., Schneir, M. and Ramamurthy, N.S. 1978-2. J. Dent. Res. 57, 520-525.

Gospodarowicz, D. and Moran, J.S. 1975. Exp. Cell. Res. 90, 279-284.

Gospodarowicz, D. and Moran, J.S. 1976. Ann. Rev. Biochem. 45, 531-558.

Grant, M.E. and Prockop, D.J. 1972. N. Engl. J. Med. 286, 194-199, 242-249, 291-300.

Grgic, A., Rosenbloom, A.L., Weber, F.T., Giordano, B., Malone, J.I. and Shuster, J.S. 1976. J. Pediatr. 88, 584-588.

Griffiths, J.B. 1972. Exp. Cell. Res. 75, 47-56.

Grossi, S., Christersson, L.A., Park, B., Nakamura, M., Mashimo, P.A. and Genco, R.J. 1983. J. Dent. Res. 62, 178, Abst. No. 81.

Hammouda, O., Brinkley, B. and Dabbous, M.K. 1980. Fed. Proc. 39, 1789, Abst. No. 965.

Hansen, F.M., Nilson, P., Sonne, O., Hustvedt, B.E., Nilsson-Ehle, P., Nielsen, J.H. and Lovo, A. 1983. Diabetologia 24, 131-135.

Harrison, T.W. 1982. MSc. Thesis. University of Manitoba.

Hassell, T.M., Page, R.C., Narayanan, A.S. and Cooper, C.G. 1976. Proc. Nat. Acad. Sci. USA 73, 2909-2912.

Hassell, T.M. and Stanek, E.J. 1983. Arch. Oral Biol. 28, 617-625.

Havu, N., Lundgren, G. and Falkner, S. 1977. Acta Endocrinol. 86, 570-577.

Hay, R.J. and Strehler, B.L. 1967. Exp. Geront. 2, 123-135.

Hayflick, L. 1965. Exp. Cell. Res. 37, 614-636.

Hayflick, L. 1975. Fed. Proc. 34, 9-13.

Hayflick, L. 1979. Fed. Proc. 38, 1847-1850.

Heidenrich, K.A., Zahniser, N.R., Berhanu, P., Brandenburg, D. and Olefsky, J.M. 1983. J. Biol. Chem. 258, 8527-8530.

Hildebran, J.N., Absher, M. and Low, R.B. 1983. In Vitro 19, 307-314.

Hollenberg, M.D. and Cuatrecasas, P. 1973. Proc. Nat. Acad. Sci. USA 70, 2964-2968.

Hollenberg, M.D. and Cuatrecasas, P. 1975. J. Biol. Chem. 250, 3845-3853.

Holley, R.W. and Kiernan, J.A. 1968. Proc. Nat. Acad. Sci. USA 60, 300-304.

Holley, S.L. 1975. Nature 258, 487-490.

Howard, B.V., Mott, D.M., Fields, R.M. and Bennett, P.H. 1979. J. Cell. Physiol. 101, 129-138.

Howell, S.L., Young, D.A. and Lacy, P.E. 1969. J. Cell. Biol. 41, 167-176.

Howland, B.E. and Zebrowski, E.J. 1974. Hormone Metab. Res. 6, 121-124.

Ishibashi, F., Hidaka, H. and Howard, B.V. 1981. J. Clin. Endocrinol. Metab. 54, 34-39.

Javed, T., Collins, C., Burton, C. and Miller, R. 1983. J. Dent. Res. 62, 179, Abst. No. 82.

Jefferson, J.J. 1980. Diabetes. 29, 487-496.

Jeffrey, P.D. 1982. Diabetologia 23, 381-385.

Jimenez, S., Marsch, M. and Rosenbloom, J. 1973. Biochem. Biophys. Res. Commun. 52, 106-114.

Jimenez de Asua, L., Surian, E.S., Flawia, M.M. and Torres, H.N. 1973. Proc. Nat. Acad. Sci. USA 70, 1388-1392.

Jones, R.H. 1982. Diabetologia 23, 391-392.

Kaftory, A., Hershko, A. and Fry, M. 1978. J. Cell. Physiol. 94, 147-160.

Kao, W.W., Berg, R.A. and Prockop, D.J. 1977. J. Biol. Chem. 252, 8391-8397.

Kaplan, S.A. 1980. Pediatr. Res. 15, 1156-1162.

Keenan, B.S., Meyer, W.J., Hadjian, A.J. and Migeon, C.J. 1975. Steroids 25, 535-552.

Kivirikko, K.I. and Savolainen, E.R. 1981. Focus on Research, Med. Biol. 59, 1-6.

Kobayashi, M. and Olefsky, J.M. 1979. Diabetes 28, 87-95.

Kochakian, C.D. 1960. Proc. Soc. Exp. Biol. Med. 103, 196-197.

Kohn, R.R. and Hensse, S. 1977. Biochem. Biophys. Res. Comm. 76, 765-771.

Kono, T. and Barham, F.W. 1971. J. Biol. Chem. 246, 6210-6216.

Kosmakos, F.C. and Roth, J. 1980. J. Biol. Chem. 255, 9860-9869.

Kuehn, L. and Blundell, T. 1980. IN: Cellular Receptors for Hormones and Neurotransmitters. (Ed.) Schulster and Levitski, Wiley: NY, pp. 185-195.

Krystal, R.G., Morris, G.M. and Sokoloff, L. 1982. Arthritis Rheum. 25, 318-325.

Ledet, T., Fischer Dzoga, K. and Wissler, R.W. 1976. Diabetes 25, 207-215.

Ledet, T. 1976. Acta. Path. Microbiol. Scand. Sect. A 84, 508-516.

Ledet, T. 1977. Diabetes 26, 798-803.

Ledet, T. and Vuust, J. 1980. Diabetes 29, 964-970.

Levene, C.I. and Bates, C.J. 1970. J. Cell. Sci. 7, 671-682.

Levene, C.I. Shoshan, S. and Bates, C.J. 1972. Biochem. Biophys. Acta 257, 384-388.

Levene, C.I. and Bates, C.B. 1975. Ann. N.Y. Acad. Sci. 258, 288-306.

Levin, M.E., Boisseau, V.C. and Anoli, L.V. 1976. N. Engl. J. Med. 294, 241-245.

Levine, A.S., McClain, C.J., Handwerger, B.S., Brown, D.M. and Morley, J.E. 1983. Am. J. Clin. Nutr. 37, 382-386.

Lima, L. and Macieira-Coelho, A. 1972. Exp. Cell Res. 70, 279-284.

Linn, S., Kairis, M. and Holliday, R. 1976. Proc. Nat. Acad. Sci. USA 73, 2818-2822.

Liotti, F.S., Bruschelli, G. and Mariucci, G. 1983. IRCS Medical Science 11, 502-503.

Litwack, D., Kennedy, J.E. and Zander, H.A. 1970. J. Periodont. Res. 5, 263-268.

Loning, T., Albers, M., Lisboa, B., Burkhardt, A. and Caselitz, J.

1980. J. Periodont. Res. 5, 525-535.

Lowry, O.H., Rosebrough, N.J., Farr, A.C. and Randall, R.J. 1951. J. Biol. Chem. 193, 265-275.

Macieira-Coelho, A. and Ponten, J. 1969. J. Cell. Biol. 43, 374-377.

Madri, T.A. and Furthmayr, H. 1979. Am. J. Pathol. 94, 323-332.

Manchester, K.L. 1972. Diabetes 21, Suppl. 2, 447-452.

Mann, G.V. 1974. Perspect. Biol. Med. 17, 210-217.

Mann, G.V. and Newton, P. 1975. Ann. N.Y. Acad. Sci. 258, 243-251.

Manner, G. 1971. Exp. Cell. Res. 65, 49-60.

Marmary, Y., Brunette, D.M. and Heersche, J.N.M. 1976. Arch. Oral Biol. 21, 709-716.

Marshall, S. and Olefsky, J.M. 1980. J. Clin. Invest. 66, 763-772.

Marshall, S., Podlecki, D.A. and Olefsky, J.M. 1983. Endocrinology 113, 37-42.

Martin, G.M., Sprague, C.A. and Epstein, C.J. 1970. Lab. Invest. 23, 86-92.

Martin, M.S. and Pohl, S.L. 1974. J. Biol. Chem. 254, 9976-9978.

Massague, J., Pilch, P.F. and Czech, M.P. 1980. Proc. Nat. Acad. Sci. USA 77, 7137-7141.

May, J.M. and Contoreggi, C.S. 1982. J. Biol. Chem. 257, 4362-4368.

McCoy, B.J., Galdun, J. and Cohen, I.K. 1982. In Vitro 18, 79-86.

McMillan, D.E. 1975. Diabetes 24, 944-957.

Miller, E.J., Epstein, E.H. and Piez, K.A. 1971. Biochem. Biophys. Res. Comm. 42, 1024-1029.

Miller, E.J. 1976. Mol. Cell. Biochem. 13, 165-188.

Minemura, T., Lacy, W.W. and Crofford, O.B. 1970. J. Biol. Chem. 245, 3872-3881.

Minor, R.R. 1980. Am. J. Pathol. 98, 227-277.

Mitsui, Y. and Schneider, E.L. 1976. Mech. Ageing Dev. 5, 45-56.

Mordes, J.P. and Rossini, A.A. 1981. Am. J. Med. 70, 353-360.

Mulay, S., Finkelberg, R., Pinsky, L. and Solomon, S. 1972. J. Clin. Endocrinol. 34, 133-143.

Munroe, C.O. 1983. Dental Clinics of North America 27, 329-340.

Murad, S., Grove, D., Lindberg, K.A., Reynolds, G., Sivarajah, A. and Pinnell, S.R. 1981. Proc. Nat. Acad. Sci. USA 78, 2879-2882.

Murphy, L.J. and Lazarus, L. 1983. Endocrinology 112, 1026-1034.

Narayanan, A.S. and Page, R.C. 1976. J. Biol. Chem. 251, 5464-5471.

Narayanan, A.S. and Page, R.C. 1977. FEBS Lett. 80, 221-224.

Narayanan, A.S., Page, R.C. and Kuzan, F. 1978. Lab. Invest. 39, 61-65.

Narayanan, A.S., Engel, L. and Page, R.C. 1981. J. Dent. Res. 60, 337, Abst. No. 107.

Narayanan, A.S., Page, R.C. and Meyers, D.F. 1982. J. Dent. Res. 61, 315.

Narayanan, A.S. and Page, R.C. 1983. Coll. Relat. Res. 3, 33-64.

Narayanan, A.S., Engel, L.D. and Page, R.C. 1983. Coll. Relat. Res. 3, 323-334.

Newman, M.G. 1979. Rev. Infect. Dis. 1, 313-323.

Nimni, M.E. 1980. Biorheology 17, 51-82.

Nolan, J.C., Cardinale, G.J. and Udenfriend, S. 1978. Biochem. Biophys. Acta. 513, 116-122.

Olefsky, J.M. and Reaven, G.M. 1977. Diabetes 26, 680-688.

Olsen, B.R., Guzman, N.A., Engel, J., Condit, C. and Aase, S. 1977. Biochemistry 16, 3030-3036.

Orgel, L.E. 1973. Nature 243, 441-445.

Ostergaard, E. and Loe, H. 1975. J. Periodont. Res. 10, 103-114.

Page, R.C. 1972. Developmental Aspects of Oral Biology. (Eds.) Slavkin, H. and Bavetta, L. Academic Press, N.Y., pp. 291-308.

Page, R.C., Davies, P. and Allison, A.C. 1973. Arch. Oral Biol. 18, 1481-1495.

Page, R.C., Davies, P. and Allison, A.C. 1974. J. Reticuloendothel. Soc. 15, 413-438.

Page, R.C. and Ammons, W.F. 1974. Arch. Oral Biol. 19, 651-658.

Page, R.C., Simpson, D.M. and Ammons, W.F. 1975. J. Periodontol. 46, 144-155.

Page, R.C., Davies, P. and Allison, A.C. 1978. Int. Rev. Cytol. 52, 119-157.

Page, R.C. and Schroeder, H.E. 1982. Periodontitis in Man and Other Animals. S. Karger Publishing Co., Basel, Switzerland, pp. 1-45, 239-251.

Patterson, M.K., Jr. 1979. IN: Methods in Enzymology, vol. 58, Cell Culture (Jakoby, W.B. and Pastan, I.H., Eds.), Academic Press, New York, pp. 147-148.

Peck, W.A., Birge, S.J., Jr. and Fedak, S.A. 1964. Science 146, 1476-1477.

Permutt, A., Chirgwin, J., Giddings, S., Kakita, K. and Rotwein, P. 1981. Clin. Biochem. 14, 230-236.

Peterkofsky, B. 1972. Arch. Biochem. Biophys. 152, 318-328.

Peterkofsky, B. and Prather, W. 1977. J. Cell. Physiol. 90, 61-70.

Phillips, L.S., Belosky, D.C. and Reichard, L.A. 1979-1. Endocrinology 104, 1513-1518.

Phillips, L.S., Belosky, D.C., Young, H.S. and Reichard, L.A. 1979-2. Endocrinology 104, 1519-1524.

Plisko, A. and Gilchrest, B.A. 1983. J. Gerontol. 38, 513-518.

Podlecki, D.A., Frank, B.H., Kao, M., Horikoshi, H., Freidenberg, G., Marshall, S., Ciaraldi, T. and Olefsky, J.M. 1983. Diabetes 32, 697-704.

Porter, R.R. and Reid, K.B. 1978. Nature 275, 699-704.

Priest, R.E. and Bublitz, C. 1967. Lab. Invest. 17, 371-379.

Prockop, D.J., Berg, R.A., Kivirikko, K.I. and Uitto, J. 1976. Biochemistry of Collagen, (Eds.) Ramachandran, G.N. and Reddi, A., Plenum Publishing Co., N.Y., pp. 163-273.

Prockop, D.J., Kivirikko, K., Tuderman, L. and Guzman, N.A. 1979. N. Engl. J. Med. 301, 13-23.

Ramachandran, G.N. and Ramakrishnan, C. 1976. Biochemistry of Collagen, (Eds.) Ramachandran, G.N. and Reddi, A., Plenum Publishing Co., N.Y., pp. 45-84.

Rechler, M.M. and Podskalny, J.M. 1976. Diabetes 25, 250-255.

Reed, B.C. and Lane, D.M. 1980. Proc. Nat. Acad. Sci. USA 77, 285-289.

Reed, M.J., Slots, J., Mouton, C. and Genco, R.J. 1980. Infect. Immun. 29, 564-574.

Risser, W.L. and Gelehrter, T.D. 1973. J. Biol. Chem. 248, 1248-1254.

Rosenberry, T.L. and Richardson, J.M. 1977. Biochemistry 16, 3550-3558.

Rosenbloom, A.L. and Rosenbloom, E.K. 1978. Diabetes 27, 338-341.

Rowe, D.W., Starman, B.J., Fujimoto, W.Y. and Williams, R.H. 1977-1. Diabetes 26, 284-290.

Rowe, D.W., Starman, B.J., Fujimoto, W.Y. and Williams, R.H. 1977-2. In Vitro 13, 824-830.

Rubin, M.H. 1973. J. Cell. Physiol. 82, 231-238.

Rubin, M.H. 1976. J. Cell. Physiol. 91, 249-259.

Rubin, M.H. and Koide, T. 1975. J. Cell. Physiol. 86, 47-58.

Rubright, C.W., Higa, L.H. and Yannone, M.E. 1971. J. Periodont. Res. 6, 55-64.

Salcedo, L.L. and Franzblau, C. 1981. In Vitro 17, 114-120.

Sato, T., Saito, T., Kokubun, M., Ito, M., Inoue, M., Saito, K. and Yoshinaga, K. 1980-1. Tohoku J. Exp. Med. 131, 97-98.

Sato, T., Oguma, S., Saito, T. and Yoshinaga, K. 1980-2. Tohoku J. Exp. Med. 132, 207-212.

Saunders, D.J. 1982. Diabetologia 23, 386-390.

Schluger, S., Yuodelis, R. and Page, R.C. 1977. Periodontal Disease, Lea and Febiger, Philadelphia.

Schneider, W.C. 1957. IN: Methods in Enzymology, vol. 3, (Coloeick, S.P. and Kaplan, N.O., Eds.), Academic Press, New York, pp. 680-684.

Schneider, E.L. and Fowlkes, B.J. 1976. Exp. Cell. Res. 98, 298-302.

Schneider, E.L. and Shorr, S.S. 1975. Cell. 6, 179-184.

Schneir, M., Bowersox, J., Ramamurthy, N.S., Yavelow, J., Murray, J., Edlin-Folz, E. and Golub, L.M. 1979. Biochim. Biophys. Acta. 583, 95-102.

Schneir, M., Ramamurthy, N.S. and Golub, L.M. 1982. Diabetes 31, 426-431.

Scopp, I.W. 1977. N.Y. J. Dent. 47, 9-11.

Schroeder, H.E., Munzel-Pedrazzoli, S. and Page, R.C. 1973. Arch. Oral Biol. 18, 899-923.

Schultz-Haudt, S.D., From, S.H. and Nordbo, M. 1964. Arch. Oral Biol. 9, 17-25.

Shklar, G., Chauncey, H.H. and Shapiro, S. 1967. J. Periodontol. 38, 25-32.

Soames, J.V. and Davies, R.M. 1977. J. Periodont. Res. 12, 378-386.

Sodek, J., Brunette, D.M., Feng, J., Heersche, J.N.M., Limeback, H.F., Melcher, A.H. and Ng, B. 1977. Arch. Oral Biol. 22, 647-653.

Southren, L.A., Rappaport, S.C., Gordon, G.G. and Vittek, J. 1978. J. Clin. Endocrinol. 47, 1378-1382.

Speicher, D.W., Peace, J.N. and McCarl, R.L. 1981. In Vitro 17, 863-870.

Spiro, R.G. 1973. N. Engl. J. Med. 288, 1337-1342.

Stegemann, H. 1958. Physiol. Z. Chem. 311, 41-45.

Sultan, C., Migeon, B.R., Rothwell, S.W., Maes, M., Zerhouni, N. and Migeon, C.J. 1980. J. Pediatr. Res. 13, 67-69.

Sultan, C., Terraza, A., Devillier, C., Michel, B., Descomps, B.

and Jean, R. 1982. Br. J. Dermatol. 107, Suppl. 23, 40-46.

Switzer, B.R. and Summer, G.K. 1972. J. Nutr. 102, 721-728.

Tager, H., Given, B., Baldwin, D., Mako, M., Markese, J., Rubenstein, A., Olefsky, J.M., Kobayashi, M., Kolterman, O. and Poucher, R. 1979. Nature 281, 122-125.

Tajima, S. and Pinnell, S.R. 1982. Biochem. Biophys. Res. Comm. 106, 632-637.

Ten Cate, A.R. and Deporter, D.A. 1975. Anat. Rec. 182, 1-14.

Tengrup, I., Ahonen, J. and Zederfeldt, B. 1981. Surg. Gynecol. Obstet. 152, 323-326.

Tenni, R., Tavella, D., Donnelly, P., DiFerrante, N., Hill, L., Leach, C. and Hatton, D. 1980. Biochem. Biophys. Res. Commun. 92, 1071-1075.

Terris, S. and Steiner, D.F. 1975. J. Biol. Chem. 250, 8389-8398.

Thomopoulos, P., Testa, U., Flamier, A. and Berthelier, M. 1980. Diabetes 29, 820-824.

Todaro, G.J., Lazar, G.K. and Green, H. 1965. J. Cell. Comp. Physiol. 66, 325-333.

Tolstoshev, P., Berg, R.A., Rennard, S.I., Bradley, K.H., Trapnell, B.C. and Crystal, R.G. 1981. J. Biol. Chem. 256, 3135-3140.

Toth, M. and Zakar, T. 1982. J. Steroid Biochem. 17, 653-660.

Vaheri, A., Ruoslahti, E., Hovi, T. and Nordling, S. 1973. J. Cell. Physiol. 81, 355-364.

Vaheri, A., Kurkinen, M., Lehto, V.P., Linder, E. and Timpl, R. 1978. Proc. Nat. Acad. Sci. USA 75, 4944-4948.

Van Obberghen, E. and Roth, J. 1981. Receptor Regulation (Ed.) Lefkowitz, R.; Chapman and Hall, N.Y., pp. 9-40.

Verlangieri, A.J.and Sestito, J. 1981. Life Sciences 29, 5-9.

Villee, D.B. and Powers, M.L. 1977. Nature 268, 156-158.

Vittek, J., Gordon, G.G., Rappaport, S.C. and Southren, A.L. 1979-1. J. Dent. Res. 58, 642-645.

Vittek, J., Rappaport, S.C., Gordon, G.G., Munnangi, P.R. and Southren, A.L. 1979-2. J. Periodontol. 50, 254-264.

Vracko, R. 1974. Diabetes 23, 94-104.

Weissman-Shomer, P. and Fry, M. 1975. Mech. Ageing Dev. 4, 159-166.

Weringer, E.J. and Arquilla, E.R. 1981. Diabetologia 21, 394-401.

Weringer, E.J., Kelso, J.M., Tamai, I.Y. and Arquilla, E.R. 1982. Acta Endocrinol. 99, 101-108.

Wiebkin, O.W., Bartold, P.M. and Thonard, J.C. 1979. Biochem. J. 183, 467-470.

Wilson, G.L., Mossman, B.T. and Craighead, J.E. 1983. In Vitro 19, 25-30.

Zambon, J.J., Reynolds, H.S. and Slots, J. 1981. Infect. Immun. 32, 198-203.

Zannoni, V., Lynch, M., Goldstein, S. and Sato, P. 1974. Biochem. Med. 11, 41-48.

Zebrowski, E.J. and Bhatnagar, P.K. 1979. Pharmacol. Res. Commun. 11, 95-103.

Zeleznik, A.J. and Roth, J. 1978. J. Clin. Invest. 61, 1363-1371.