THE ROLE OF ASCORBIC ACID IN THE PERIODONTIUM

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Declaration

This report is submitted in partial fulfillment of The M.D.S. Degree of The University of Adelaide. This report contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this report contains no material previously published, except where due reference has been made in the text.

NAULCHAVEE HONGPRASONG

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Summary

Periodontal disease is a chronic condition that over a long period of time has the potential to destroy sufficient of the supporting bone to loosen and eventually become non-functional. There is a wide range of responses between individuals to the parasite bacteria that trigger the host defence system and which is itself responsible for the local damage. There has been an increasing awareness that host response is important in the quality of the disease experienced. This research has been directed toward the clarification of the role of one of these factors (vitamin C). This factor appears to have a role in the formation of collagen, a vital gingival structure and it plays a key role in host defence as a consequence of its requirement by neutrophils for effective chemotaxis and phagocytosis.

This report examines the possible role of vitamin C in the aetiology of periodontal disease. The investigation revealed a poor correlation between ascorbate levels of human gingivae and plasma but there was a good correlation in the animal model.

Gingival ascorbate concentration was found to be ten-fold higher than the plasma ascorbate level, suggesting that a high concentration of the vitamin occurs in the gingivae. Chronic marginal vitamin C deficiency may play a significant part in periodontal disease by affecting the host defense mechanism and the collagen structural components, including the microvascular system. The value of vitamin C in the treatment of periodontal disease may be found to improve host response mechanism via neutrophil function, promotion of repair and healing via collagen synthesis, and improved microvascular integrity in conditions where the gingival microcirculation has been impaired by environmental factors.

Chapter 1

Introduction

Periodontal disease has been recognised as the most widespread chronic disease of modern man. It is widely agreed that the disease is a product of a disturbance of the host-parasite relationship, and that bacterial plaque and its products are the major aetiological factors causing the disease (Egelberg, 1965; Loe et al, 1965; Socransky, 1970; Kelstrup and Theilade, 1974). Endotoxins derived from disrupted bacteria in the gingival sulcus are thought to gain entry to the body via the junctional epithelium to sensitise the host and then evoke an immune response on subsequent challenge (Wilde et al, 1977).

Antibody-bacterial antigen reactions induce the components of the complement system to attract polymorphonuclear leucocytes (PMN's) to the region where they phagocytose foreign particles in both the tissue and the sulcus (Attstrom, 1971; Listgarten and Ellegaard, 1973). Endotoxins may activate the clotting, kinin and complement systems resulting in the establishment of chronic inflammation (Mergenhagen, 1970). They also stimulate the release of lysosomal products from neutrophils and macrophages (Taichman et al, 1977; Baehni et al, 1979 and Norman et al, 1979) and promote bone resorption in vitro (Hausmann et al, 1970).

Other factors that can influence the host-parasite relationship and which may influence the course and severity of the disease are endocrines, systemic diseases, therapeutic agents and drugs, genes, and nutrition. Severe vitamin C deficiency is known

to induce scurvy, characterised in the gingivae by haemorrhagic swelling. The changes may be caused by the direct effect of lowered vitamin C or by bacterial derivatives operating in a less resistant environment.

The role of vitamin C in gingival metabolism is complicated but certainly it acts as a reducing agent in fibroblast metabolism promoting the hydroxylation of proline and lysine in collagen synthesis (Peterkofsky, 1972a) and collagen turnover in gingivae and periodontal ligaments (Page and Ammons, 1974; Rippin, 1976,1978). Furthermore, vitamin C also affects the chemotactic and migratory activity of leukocytes (De Chatelet et al, 1972; Goetzl et al, 1974; Alvares et al, 1981) which may represent an important mechanism for the host-parasite relationship. In addition, gingival blood supply in its crestal aspects is end-arterial without collateral circulation. It has been shown that stress, and habits such as smoking, greatly reduce gingival circulation (Clarke et al, 1981). This may reduce the availability of vitamin C to the region in an individual with normal plasma levels. The host defence could be compromised by reduced local vitamin C levels with less than optimal phagocytic activity, and lowered collagen turnover and repair.

Although acute ascorbic acid deficiency is a rare occurrence in developed countries, subclinical deficiency is not uncommon (Burr et al, 1974; Lee, 1978; Vir and Love, 1979; Ginter, 1979). Recently, Alvares and his associates studied the effect of subclinical ascorbate deficiency on periodontal health in Macaca monkeys. They found that the periodontium was vulnerable to chronic inflammation and that there was an impairment of polymorphonuclear leukocyte chemotaxis and phagocytosis (Alvares et al, 1981).

An ultrastructural improvement of epithelium and connective

tissue was also demonstrated in patients with progressive periodontitis following the administration of high doses of ascorbic acid (Aurer-Kozelj et al, 1982).

The concentration of ascorbate in various tissues varies in accordance with the vitamin C intake (Keith and Pelletier, 1974). The distribution of ascorbic acid between various cellular components of blood, eg. monocytes and polymorphs, relates to the plasma concentration (Evans et al, 1982). Ascorbic acid level of gingival tissue has been studied, and no correlation was found between ascorbate concentration in plasma and gingival tissue. When the plasma ascorbate increased significantly the increase in the gingivae was not significant (Glickman and Dines, 1963). However it appears that no studies have investigated the correlation of ascorbate levels in white cells, plasma and gingival tissue.

Accordingly, this study was designed to evaluate the ascorbate levels in gingival tissue, plasma and lymphocytes in chronic vitamin C deficient and normal guinea pigs. Control groups were also established for both gingival inflammation and subclinical vitamin C deficiency. Histological studies of the gingival tissues were also made in an attempt to correlate the ascorbate assays with gingival pathology. A human study was undertaken simultaneously to determine whether similar changes could be observed.

Chapter 2

Review of the literature

An early publication entitled "Treatise of Scurvy" showed that fresh vegetables, oranges and lemons could prevent and cure the disease (Lind, 1757, cited from Blockley and Baenziger, 1942). Since then little work has been done on the aetiology and pathology of scorbutic lesions until the 1970's.

In 1912 Holst and Frolish induced scurvy experimentally in guinea pigs (cited from Chatterjee, 1978). When the animals were kept on a diet deficient in vitamin C, changes occurred in the gingivae, periodontal membrane and alveolar bone consistent with those shown by Howe, Westin, Wohlbach, Hojor, Day, Fish, Harris, Boyle and Bessay in their work during 1920-1941 (Cited from Burrill, 1942).

2.1 Epidemiological studies

It was not clear whether the conditions observed in the guinea pigs also occurred in humans, and therefore epidemiological studies to determine the relationship between ascorbate status and periodontal disease were begun. In 1942, Blockley and Baenziger investigated patients attending the Northwestern University Dental School diagnostic clinic by recording their periodontal tissue conditions and plasma ascorbate levels. They found that in patients with normal gingivae plasma ascorbate varied from 0.38-1.6 mg% with a mean of 0.76 mg%, while patients with inflamed gingivae had levels which were 0.08-0.76 mg% with a mean of 0.31 mg%. The gingival condition improved after the administration of 200-500 mg

ascorbic acid daily. They concluded that there was a correlation between a lowered ascorbate content of the blood and periodontal disturbances. It was suggested that vitamin C might play a part as a primary factor in the aetiology of the disease because the deficient gingivae are prone to infection and complete repair is probably impossible when the disease is advanced.

Cloutier (1933) claimed that periodontal patients who were lacking in vitamin C could be cured by instituting a vitamin C rich diet. He withheld local treatment from his periodontal patients until he had observed the result of the diet correction.

Burrill (1942) confirmed this work by keeping records of the condition of the gingivae, periodontal tissues, and blood plasma ascorbate of 1396 patients. It was found that plasma ascorbate ranged from 0.04 mg% to 2.16 mg%, but he was unable to demonstrate the correlation between plasma ascorbate level and the periodontal condition. Burrill concluded that the vitamin C level tended to be lower in the presence of gingivitis compared with those free of disease.

Russel (1963) summarized studies of the periodontal status of 21,559 children and adults in nutritional field studies conducted in eight different countries: the United States (Alaska), Ethiopia, Ecuador, South Vietnam, Chile, Columbia, Thailand and Lebanon. The survey teams included specialists in nutrition, medicine, biochemistry, food technology, etc. Periodontal disease was examined using a periodontal index, including an assessment of present and active disease and the oral hygiene index. Vitamins C and A were measured from the plasma concentration, while thiamine and riboflavin were measured from the urine. It was found that the Alaskan diet was very low in vitamin A and C and in Ethiopia the diet was critically low in ascorbic acid intake. Ascorbic acid was high in the diets of Ecuador and Thailand but thiamine and

riboflavin were low. South Vietnam, Columbia and Lebanon were also low in thiamine and riboflavin while Chile was low in vitamin A. Russel could not find a correlation between the periodontal disease and plasma ascorbate, and he concluded that most of the variance in periodontal disease was associated with oral hygiene status and with age.

Russel et al, (1965) made both an epidemiological and nutritional study of periodontal disease in South Vietnam; no relationship between ascorbic acid intake and periodontal disease could be found. Correlation between plasma ascorbate and gingival indices was found in two small groups of U.S. Naval personnel stationed in Antarctica for a twelve month period (Perlitsch et al, 1961). A statistical association was found between serum vitamin C, oral hygiene and periodontal indices in the ten state nutritional survey of 38,000 subjects between 1968 and 1970 in the United States (Dept. of H.E.W 1968-1970). Shannon (1973) also demonstrated a statistically significant correlation between gingival scores and ascorbate levels in whole blood and urine.

2.2 Vitamin C and collagen formation

2.2.1 Histopathological studies

Histological studies of vitamin C deficient gingival tissues are numerous and many of them have concentrated on collagen formation relative to periodontal structure and functions (Glickman, 1948a,b; Waerhaug, 1958; Messer, 1972; and Aurer-Kozelj et al, 1982). Glickman (1948a) used twenty-five guinea pigs to study the effect of acute ascorbic acid deficiency. Sixteen of the guinea pigs were fed with a vitamin C-free diet, and the nine controls received the same diet supplemented with vitamin C, 1 mg/100 gm body weight. The histopathology of the mandible, maxilla

and knee joint were studied after thirty-five days. He found that in the periodontal membrane and alveolar bone there were destructive changes in the absence of gingival inflammation or pocket formation. The changes were generalized in pattern, although acute vitamin C deficiency caused oedema of gingival connective tissue, collagen degeneration and haemorrhage. The deficiency itself did not increase the incidence of gingivitis, but Glickman concluded that in vitamin C deficiency there is an increase in the frequency and severity of local infection which results from the deficiency state. In the same year Glickman demonstrated the effect of induced acute inflammation on the periodontium by applying 10% silver nitrate solution in the labial gingival sulcus of guinea pigs in both the deficient and control groups. After application of silver nitrate for four days the animals were sacrificed and studied histologically. Inflammation induced in the gingival sulcus resulted in pocket formation, localized necrosis and acute inflammation in the underlying tissues of both vitamin C deficient and control animals. In the deficient animal inflammatory cell infiltration was slight, with polymorphs diffusely scattered throughout the tissue. The vessels had collapsed and were barely discernible against the background of degenerated collagen (Glickman, 1948b).

Acute vitamin C deficiency resulted in an exaggerated inflammatory and destructive states in gingival tissue with no evidence of collagen repair in progress. Osteoporosis of alveolar bone had occurred and there was little ability of the bone to produce a satisfactory matrix. Glickman concluded that the factors that contributed to the exaggerated destruction of periodontal tissue included the inability to form a peripheral delimiting connective tissue barrier, a reduction in inflammatory cells, a diminished vascular response and the inhibition of fibroblast formation and differentiation of osteoblasts (Glickman, 1948 a,b).

Waerhaug (1958) studied the supporting structures of the teeth in long term vitamin C-deficient monkeys. He reported that two of the monkeys died after 42 and 75 days respectively without exhibiting typical clinical signs of scurvy. Signs of scurvy in two other monkeys appeared after 90 days. Teeth had increased mobility after 120 days and at the end of the experiment the teeth were loose and the gingivae were flabby and haemorrhagic. The odontoblasts of the dentine in the erupting teeth were dedifferentiated although some dentine formation occurred in the apical part. The pulp tissue showed some degeneration with few vessels while bone formation was reduced. The newly formed bone revealed thin trabeculae and small marrow spaces. Osteoclastic resorption was a common finding and in many areas extensive resorption of the alveolar wall had taken place. The collagen underlying connective tisue had broken down, and only scattered "islands" could be observed in the section. Hemorrhages were found in the periodontal membrane, the gingivae and the bone. The epithelium was not affected by the deficiency to the same extent as bone and connective tissue.

It was concluded that the clinical appearance of the periodontal structures in severe scurvy resembled that of periodontitis in man. The histological picture in scurvy is different as the damage in the periodontal membrane is generalised, whereas in periodontitis it is localised to areas below and adjacent to the deepened pocket. Tooth mobility in scurvy is caused by the generalized destruction of collagen of the periodontal membrane and the resorption of the inside of the alveolar wall, whereas in periodontitis it is facilitated by pocket deepening and localized destruction of the periodontal membrane.

Dreizen et al (1969) studied in marmosets the effect of vitamin C deficiency and showed that the fibres of the periodontal ligament were affected. In addition minor changes were observed in

the gingival fibres and alveolar bone, which resulted in a small increase of the periodontal space. Haemorrhage within the periodontal fibres occurred increasingly in the later stages of the scorbutic state and the marked disintegration of the periodontal fibres led to increased mobility of the teeth.

Messer (1972) studied dentine and bone formation in monkeys. He found that dentine formation decreased by approximately 30% in the vitamin C deficient animals, while bone formation ceased completely.

Kramer et al, (1979) studied collagen formation during wound healing and they demonstrated no normal collagen ingrowth into implanted sponges at 7 or 14 days in the scorbutic guinea pigs when compared to the supplemented animals. However, there was no histological difference in the amount of collagen formed between the group receiving the recommended daily (5 mg/day) and the supplemented group (200 mg/day).

Recently, the effect of ascorbic acid supplementation on periodontal tissue ultrastructure has been studied in subjects with progressive periodontitis. It was found that the plasma vitamin C level of 21 patients ranged from 0.18-0.30 mg%, corresponding to an intake of 30-40 mg of vitamin C daily. After supplementation with 70 mg of ascorbic acid daily for six weeks, plasma vitamin C levels were again measured and biopsies were done. An improvement in the organisation of the cytoplasmic joint bridging the intercellular spaces of proximal epithelial cells or the bundles of cytoplasmic tonofilaments and the connective tissue of the gingival lamina propria was found (Aurer-Kozelj et al, 1982).

2.2.2 Biochemical studies

There are many reports of investigations of collagen synthesis by biochemical means. Peterkofsky (1972a) studied the regulation of collagen secretion by ascorbic acid in 3T3 culture supplemented with ascorbate. The collagen synthesized and secreted in the scorbutic condition was extensively underhydroxylated. The result using chick embryo fibroblasts was the same, but total collagen synthesis was decreased in the absence of vitamin C. Peterkofsky suggested that the reduced secretion of collagen was probably caused by a deficiency of hydroxylation of proline and lysine during collagen turnover.

The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts was also studied by the use of an assay that measured ^{14}C-proline incorporation into the polypeptide chains of collagen (Peterkofsky, 1972b). The study failed to demonstrate the effect of vitamin C on growth and collagen synthesis rate in L-929 cells, suggesting that a cofactor which can substitute for ascorbate is synthesized in these cells.

Golub (1973) also studied the effect of ascorbic acid on the collagen turnover in bone in tissue culture media to label the newly synthesised ^{3}H-hydroxyproline. This study showed that collagen synthesis in tissue culture was markedly accelerated by increasing the ascorbic acid concentration (0.05-50.0 µg/ml) but the degradation of ^{3}H-hydroxyproline in collagen was inhibited. High ascorbic acid levels seemed to increase the crosslinking between molecules synthesised and labelled in tissue culture. Ascorbic acid deficiency in tissue culture appeared to inhibit collagen remodelling in bone by decreasing the accumulation of newly synthesised collagen and the breakdown of older preformed collagen.

Ostergaard and Loe (1975) used monkeys to study the collagen content of skin and gingival tissues in ascorbic acid deficiency by determining the amount of proline and hydroxyproline from tissue collagen extraction treated with bacterial collagenase. They found that the content of hydroxyproline in the scorbutic monkeys decreased throughout the experimental period and the rate of decrease from the gingivae was faster than that of the skin. The hydroxyproline reduction rate was probably dependent upon the collagen turnover rate in the tissue. The ratio of proline and hydroxyproline in the collagenase treated collagen extracts was higher in the deficient group than the controls. They concluded that ascorbic acid is a prerequisite for the maintenance of the collagen pool in the tissue. Lack of vitamin C results in lower hydroxyproline content in a collagenase degradable protein fraction.

Barnes (1975) offered the opinion that ascorbic acid had no effect on the preformed collagen, but the amount of soluble collagen was reduced in scorbutic animals because of the lack of synthesis of new collagen while normal loss from the pool was occurring.

Kramer et al, (1979) analysed implanted sponges in three groups of guinea pigs. The first group received no ascorbate and were examined after seven and 14 days. A daily allowance dose (5 mg/day) was given to the second group and a supplemented dose of (200 mg/day) was given to the third group. After 7 days the first group had a minimal level of hydroxyproline (0.54 µg) and at 14 days the hydroxyproline concentration was higher but it was still low (0.62 µg) relative to the control group. In the animals receiving 5 mg/day and 200 mg/day, significantly greater amounts of hydroxyproline were present than in scorbutic animals. Although the supplemented group showed significantly greater amounts of hydroxyproline than the daily allowance group at seven days, there was no significant difference at 14 days.

Kramer et al, (1979) concluded that the large supplement of ascorbic acid appears to have at least a transient effect on wound healing collagenation, thereby supporting the case for large dose ascorbate therapy during acute phases of healing.

It is known that ascorbic acid is involved in the hydroxylation reaction in collagen synthesis. A role was proposed for vitamin C that suggested the vitamin was required for the reduction of enzyme bound Fe^{++} that may become oxidised to Fe^{+++} in a side reaction after the conclusion of prolyl hydroxylation. Reduction of the ferric form to the ferrous state would be required before the hydroxylation cycle could proceed. Ascorbic acid would appear to maintain enzyme function in the hydroxylation of peptide bound proline (Anonymous, 1979).

2.3 Vitamin C and mucosal permeability

It has been shown that ascorbic acid deficiency reduces the mucosal barrier function and alters the synthesis of basement membrane collagen (Priest, 1970; Alfano et al, 1975). It was suggested that the basement membrane region of epithelium is the rate-limiting barrier to endotoxin penetration. The effect of ascorbic acid deficiency on oral mucosal permeability to the penetration of endotoxin using quantitative methodology in guinea pigs was evaluated. It was shown that ascorbic acid deficiency significantly impaired the synthesis of basement membrane collagen and increased the permeability of mucosal epithelium (Alfano et al, 1975a,b,c). However, they were unable to establish that alterations in mucosal permeability were caused by a direct effect of the nutrient deficiency on the epithelium or by modification in endotoxin-mediated processes. Therefore, Alfano studied the effect of acute ascorbic acid deficiency on the DNA content and permeability of guinea pigs' oral mucosal epithelium. He found

that there was a decrease in penetration which was paralleled by a decrease in the DNA content with time in the control group. The experimented animals had an altered epithelial DNA content which did not correlate with permeability. He concluded that DNA content and epithelial permeability was a reflection of the functional maturation of structures such as membrane-coating granules or the basal lamina (Alfano, 1978).

Mallek and Miller (1979) showed that supplementation of ascorbic acid (1000 mg/day) for four weeks in humans resulted in a significant increase in tissue and white blood cell ascorbate levels. The increased ascorbate levels correlated with a reduced penetration coefficient of tritiated dextran. They concluded that crevicular tissue was able to take up a large moiety of ascorbate and suggested that the sulcular epithelium required an optimal level of ascorbate for the maintenance of effective barrier function.

Alvares and Siegel (1981) recently demonstrated that the permeability of the gingival sulcular epithelium was increased two or three weeks prior to the development of the clinical scorbutic gingivitis in acute vitamin C deficient monkeys. However the role of ascorbate in the permeability of mucosa is not understood. It may be related to the effect of vitamin C on collagen formation, including a lack of formation of the triple helical structure followed by degradation of the unassociated alpha chains (Barnes, 1975), and an impairment of oral epithelial basement membrane synthesis (Alfano *et al,* 1975c). The increase in permeability of sulcular epithelium could allow endotoxins or antigenic substances from the gingival crevice to enter the underlying connective tissue and could contribute to the pathogenesis of scorbutic gingivitis.

2.4 Vitamin C and host response

2.4.1 Immunity

Vitamin C was proposed as an antiviral and antibacterial agent by Klenner (1951, 1953) and McCormick (1952) (cited from Leibovitz and Siegel, 1978), and this hypothesis recently has been re-emphasised (Pauling, 1970; Mc Call et al, 1971). Pauling noted in connection with vitamin C intake and the common cold that the morbidity of the patients who take one gramme of vitamin C daily was decreased by approximately 36% (Pauling, 1976).

The administration of megadoses of vitamin C have been shown to prevent the common cold and upper respiratary disease (Chalmers, 1975; Thomas and Holt, 1978). Baird et al, (1979) found that 80 mg daily of vitamin C reduced the incidence of respiratory symptoms by 14%-21% in 362 volunteers but the control group gave similar results. Siegel (1974) demonstrated that there was a significant increase in the circulation of interferon after induction with murine leukemia virus in mice fed with 250 mg% of vitamin C. He also found that adding L-ascorbic acid in the mouse cell cultures stimulated with polyinasinic acid.polycytidylic acid [Poly(rI).poly(rC)] enhanced the interferon synthesis (Siegel, 1975).

Siegel and Morton (1977) studied vitamin C and the immune responses in mice supplemented with L-ascorbate (250 mg%) in their drinking water ad libitum. Splenic T-cells activity was enhanced, including the release of a variety of lymphokines which were probably responsible for effecting cell-mediated immune responses.

Prinz et al, (1977) also demonstrated a small but

significant increase in serum IgA, IgM, and complement C3 in 25 healthy male university students fed 1gm of ascorbic acid per day for 75 days. Anderson et al, (1980) reported that there was a stimulation of lymphocytic transformation by Phyto-haemaglutinin (PHA) and concanavalin A (Con A) after the daily ingestion of 1-3gm of ascorbic acid in human volunteers.

Thomas and Holt (1978) concluded that ascorbate supplements appeared to increase antibody production against antigen. Banic (1982) concluded from his experiments that high and low doses of vitamin C increased slightly the effectiveness of immunisation of guinea pigs with a human cell culture rabies vaccine. However Beisel (1982) reviewed the literature and concluded that an ascorbic deficient state could not prevent the formation of antibodies or the agglutinin response but that supplementation might enhance those reactions.

2.4.2 Neutrophil function

Goetzl et al, (1974) reported that the incubation of human leucocytes with ascorbic acid resulted in enhanced chemotactic and random migratory responses of the cells. The neutrophil hexose monophosphate shunt activity was also increased when determined by the conversion extent of 1-^{14}C glucose to $^{14}CO_2$. This finding was confirmed by Sandler et al, (1975) who showed that ascorbic acid enhanced the chemotactic responsiveness of mononuclear cells. It was also demonstrated that ascorbic acid increased the cyclic guanosine monophosphate (GMP) content in the cells. Leukocyte chemotaxis was also enhanced by ascorbic acid but no significant increase in cyclic GMP was found.

Shilotri (1977a,b) also studied glycolytic, hexose monophosphate shunt and bactericidal activities of leukocytes in

ascorbic acid deficient guinea pigs. He found that the phagocytosis induced glycolytic, hexose monophosphate shunt activities and bactericidal activity of guinea pig's PMNs were impaired in ascorbic acid deficiency.

Leibovitz and Siegel (1978) reviewed the relationship between ascorbic acid, neutrophil function and the immune response. They studied ascorbic acid with regard to antimicrobial activity, interferon production and humoral and cellular immune response. Ascorbic acid was thought to play a role in neutrophil functions, such as phagocytosis and migration, including increased chemotaxis and stimulated hexose monophosphate shunt activity, etc.

Anderson (1982) studied the effects of ascorbate on both normal and abnormal leucocyte functions. It was found that the stimulation of neutrophil migration and lymphocyte transformation related to the inhibition of the auto-oxidative effect of the myeloperoxidase/hydrogen peroxide/halide system ($MPO/H_2O_2/Hal$ sys). Neutrophils and lymphocytes exposed to the $MPO/H_2O_2/Hal$ sys demonstrated an impaired responsiveness in their chemotactic and transformation responses, respectively. Ascorbate was found to protect neutrophils and lymphocytes during exposure to the $MPO/H_2O_2/Hal$ sys and also ascorbate improved the primary immunological function of these defence cells in patients with chronic granulomatous disease. White cell function was also found to be improved in patients suffering from bronchial asthma.

The effect of subclinical ascorbic acid deficiency on host defense in monkeys was studied. The PMN's phagocytic activity of the experimental group was decreased significantly, and there was increased susceptibility to periodontal disease (Alvares et al, 1980, 1981).

Patients with a family history of diabetes mellitus and

demonstrating the existence of periodontal disease were found to have a depressed neutrophil chemotaxis when compared to the other periodontal patients (McMullen et al, 1981). Aleo (1981) proposed a possible role of vitamin C deficiency in periodontal disease in diabetics. His hypothesis was based upon the following assumptions:

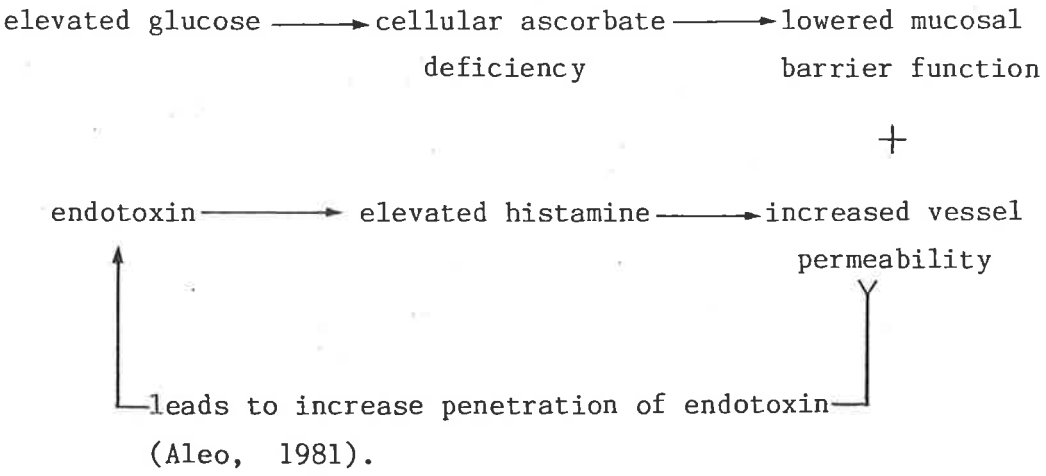
1. Transport of ascorbate across cell membranes may be impaired by glucose but facilitated by insulin.

2. Glucose utilization was significantly accelerated by sublethal concentrations of endotoxin.

3. Endotoxin - induced histamine sensitivity of tissue was enhanced by ascorbic acid deficiency.

4. Ascorbic acid deficiency altered mucosal barrier function.

The cycle was summarized as follows:



(Aleo, 1981).

Stankova et al, (1975) reported that neutrophils collected from scorbutic guinea pigs were able to produce H_2O_2 and kill bacteria as well as PMNs from the control. There was no significant impairment of phagocytosis of bacterial cells by macrophages of vitamin C deficient guinea pigs but the migration of macrophages was reduced significantly in vitro (Ganguly et al, 1976).

Shilotri and Bhat (1977) studied the effect of mega doses of vitamin C on the bacteriocidal activity of normal human leukocytes by measuring the conversion of glucose $-1-^{14}C$ to $^{14}CO_2$ in the hexose monphosphate shunt during resting and phagocytosing states. The number of viable bacteria was counted and it was found that supplemented doses did not affect the bactericidal activity of leukocytes. The phagocytic effect of supplemented doses was not different from the initial intake. Therefore, they concluded that there was no beneficial effect of using megadoses of vitamin C on bactericidal activity but there was a distinct decrease of the activity.

Neutrophils were shown to contain a relatively high concentration of ascorbic acid which reflected the vitamin status of humans more accurately than serum (Crandon et al, 1940, Butler and Cushman, 1940 and Srikantia et al, 1970). The concentration of ascorbic acid in neutrophils isolated from rabbits or guinea pigs is between ten and forty times that of serum levels (De Chatelet et al, 1974), and various organs also selectively concentrate vitamin C (Keith and Pelletier, 1974). High concentration of ascorbic acid in leukocytes may suggest that the vitamin plays a vital role in the activity of these cells. The role may be involved with the properties of the cell in body defences, i.e. antibody production and phagocytosis.

2.5 Vitamin C and host environment

2.5.1 Smoking

Ascorbic acid metabolism is compromised by the environmental factors of stress and smoking. Brook and Grimshaw (1968) showed that moderate smokers of both sexes had decreased levels of plasma ascorbic acid. Males appeared to experience the greatest reduction of vitamin C titres for all smoking intensities. These findings were confirmed by Pelletier (1968), who found that after taking saturating doses of vitamin C (two gm/day for five days) both smokers and non-smokers had similar white cell and plasma ascorbate levels. In addition it was found that in the elderly, plasma and leukocyte vitamin C levels were lower than in the young and in smokers of more than 15 cigaretts per day, the vitamin C content was lower than that in non-smokers (Burr et al, 1974).

It was also demonstrated that PMN leukocytes collected from smokers were less vital and less able to phagocytose particles when compared to those of non-smokers (Kenney et al, 1977). Further studies of the response of polymorphonuclear leukocytes to chemotactic stimulation for smokers and non-smokers failed to demonstrate a difference between the ability of the PMN from smokers and non-smokers to react to chemotactic agents. No difference was found between the ability of chemotactic agents from smokers and non-smokers to attract PMNs. However, significant differences in the ability of PMNs to migrate towards an attractant occurred from pair to pair. It was concluded that cigarette smoking has a lasting effect on the ability of circulating PMNs to react to chemotactic stimulation, or on the ability of cytotaxins or cytotaxigens to attract PMNs (Kraal and Kenney, 1979).

Recently Thyberg and Nilson (1982) noted that mouse macrophages cultivated in a medium containing nicotine (1nM - 1μM)

slightly decreased the ability of the cells to phagocytose latex beads. Moreover, it has been found that nicotine can affect blood circulation in gingiva. The effects of intra-arterial epinephrine and nicotine on gingival circulation were studied using the heat diffusion principle. It was found that nicotine caused a severe reduction in blood flow rates despite greatly increased pressure within the system (Clarke et al, 1981). The reduction in flow was shown to be due to a severe constriction of the afferent gingival vessels (Shephard and Clarke, 1984).

2.5.2 Alcohol

Since the human cannot synthesise ascorbic acid, dietary ascorbate deficiency can occur, especially in selected groups such as alcoholics and the elderly taking inadequate care of themselves. Bonjour (1979) suggested that ascorbic acid deficiency in alcoholics was demonstrated by lowered leucocyte and plasma concentrations of ascorbate when compared with a control group. The reduction may be a simple correlation with intake levels but may indicate a high demand for the vitamin in that group. Krasner etal, (1974) researched the clearance of ethanol from plasma in healthy male volunteers before and after the administration of vitamin C and found that ascorbate accelerated the catabolism of ethanol. Fazio et al, (1981) found that plasma ascorbic acid concentration was significantly decreased in healthy subjects when ethanol was ingested with ascorbic acid.

2.5.3 Oral contraception

Oral contraceptives consist of female sex hormones known to be conditioning factors of gingivitis. It is possible that these hormones play a role in ascorbic acid metabolism since both plasma and leucocyte ascorbic acid levels are reduced in women taking 'the

pill' (Mcleroy and Schendel, 1973; Briggs and Briggs, 1972; Rivers and Devine, 1972). It may be advisable for users of these agents to supplement their dietary ascorbate to acheive optimal levels of plasma and leucocyte ascorbate (Weininger and King, 1982).

2.6 Vitamin C and histamine metabolism

Vitamin C breaks down histamine to aspartic acid and therefore functions as a detoxifying agent for histamine (Chatterjee et al, 1975a). In scorbutic guinea pigs ascorbic acid would not be available for histamine catabolism and tissue and plasma levels could be expected to increase. It was found that the restoration of ascorbate in guinea pigs rapidly reduced their histamine levels to within normal limits (Chatterjee et al, 1975b). Furthermore, in the human plasma values for ascorbate below 0.7 mg% were associated with a marked rise in histamine values (Clemetson, 1980). Sharma et al, (1981) failed to demonstrate a correlation between histamine and plasma ascorbate in the third trimester of pregnancy. It was thought that the patients probably had adequate ascorbate and the addition of further moities was of little value in modifying histamine levels. Ascorbate values above 1.2 mg% achieved a virtually constant level of histamine.

Histamine has a powerful vasodilatory effect on small blood vessels and high histamine levels lead to hyperaemia and increased capillary permeability. In the scorbutic condition there may be a correlation between high and persistent endogenous histamine levels and either an increased penetration of endotoxin or capillary degeneration (Chatterjee, 1978).

2.7 Vitamin C and drug metabolism

The effect of ascorbic acid on drug metabolism in scorbutic guinea pigs has been studied (Zannoni et al, 1972; Zannoni and Lynch, 1973; Zannoni and Sato, 1975). Plasma, leucocyte and urinary ascorbate concentrations were decreased when ascorbic acid and aspirin were given simultaneously (Basu, 1982).

It has been shown that vitamin C increased the activity of liver enzymes that enhanced drug detoxification, reduced the potential of drug sensitivity and improved efficiency of antibiotics (Ringsdorf et al, 1978, 1980).

In conclusion, gingival health could be influenced by ascorbate in many ways; in collagen formation, epithelial permeability, histamine metabolism, production of antibodies and interferon, neutrophil function and in the promotion of wound healing. The ascorbate content of the various tissues correlates with plasma or leucocyte levels, which in turn reflect the status of body ascorbate. However gingival ascorbate did not correlate with plasma values in patients with periodontal disease (Glickman and Dine, 1963). Possibly this paradox may be explained by the operation of local factors that restrict the free circulation of blood in the end-arterial condition resulting in vasoconstriction such as smoking and stress, or in sluggish flow such as in the presence of chronic inflammation.

2.8 Vitamin C deficiency models

The choice of a suitable laboratory animal is very limited since most vertebrates synthesise ascorbate from glucose in the liver or kidney; microsomal L-gulonolactone oxidase is the enzyme employed in the final stage of the conversion of glucose to ascorbic acid (Chatterjee et al, 1961). Only man, anthropoid apes,

monkeys, bats and guinea pigs are without the ability to synthesize ascorbate. The cost and availability of the monkey animal model leaves the guinea pig as the only real option for vitamin C experiments but it's ascorbate metabolism is different from that of the human.

2.8.1 Biochemistry of vitamin C in the guinea pig

The guinea pig is herbivorous, consuming large quantities of vegetation in it's native habitat of the Andean mountain ranges. The teeth are those of a herbivore and the eating patterns once established, are difficult to change. A high fibre diet is required but a purified pellet has been used successfully in young guinea pigs (Navia and Lopez, 1973).

The half-life of ascorbate in guinea pigs is short, about four days and so the animals quickly show signs of deficiency. The rate of ascorbic turnover in guinea pigs is rapid and tissue storage is limited, making a frequent and regular intake of ascorbate essential. Guinea pigs obtaining two mg of ascorbic acid daily or four mg on alternate days or six mg every third day appeared to remain healthy. However the supply of 14 mg of ascorbate on a weekly basis was inadequate and failed to preserve adequate health of the animal (Zilva, 1941 -cited from Navia and Hunt, 1976).

The tissue concentration of ascorbate in most guinea pigs' organs is proportional to the dietary intake and the plasma concentration (Navia and Hunt, 1976). Retention and loss of ascorbate by the different tissues of guinea pigs fed scorbutigenic diet varied considerably. The adrenals, spleen, and aqueous humour lose ascorbate rapidly while the loss from the brain and lens is slower. After 14 days on an ascorbate-free diet the tissue levels of ascorbate were 25% of the initial concentration. Specific organ

tissues from the adrenal and spleen retained only 5% of their initial ascorbate (Hughes et al, 197la,b). Guinea pigs given a vitamin C-free diet usually displayed overt signs of deficiency within three weeks and died after approximately one month (Ginter, 1979). It was found that female guinea pigs adjust their ascorbate metabolism in the terminal phases of the scorbutic state (Odumosu and Wilson, 1971).

The ascorbate requirements of guinea pigs has been difficult to determine and a range from 0.4-25 mg per day is required, dependent upon the conditions. No urinary ascorbate excretion was detected when seven mg of ascorbic acid per kilogram of body weight was fed to guinea pigs, a level of ascorbate calculated to be ten times higher than the human requirement (Navia and Hunt, 1976).

2.8.2 Chronic marginal vitamin C deficiency model

In an attempt to more closely reproduce the human nutritional status, a model of marginal vitamin C deficiency was developed (Ginter et al, 1968). Guinea pigs were given a vitamin C free diet for two weeks to reduce the body pool of ascorbate without producing obvious signs of deficiency. A maintenance dose of 0.5 mg of ascorbate per day or for heavier animals (over 500 gm), a dose of 0.1mg/100 gm body weight was given. This concentration of ascorbate appeared to maintain the animals in acceptable health, as indicated by their food consumption and their weight curves compared with controls. Their tissue ascorbate levels were very low and were found to be close to the values of the scorbutic condition but no obvious deficiency condition arose even in experiments lasting for several months. The condition was probably comparable to subclinical hypovitaminosis C in man and was described as marginal vitamin C deficiency (Ginter, 1979).

Chapter 3

Materials and Methods

3.1 Animal experiment

Twenty-eight male guinea pigs, three months old and weighing between 300-400 gm, had their incisal teeth cleaned twice weekly with cotton buds soaked in 0.2% chlorhexidine solution for three weeks prior to the onset of the experiment. All of the animals were fed with autoclaved chow pellets*, distilled water ad libitum, and a supplement of five mg of L-ascorbic acid in one ml of 20% sucrose solution given orally thrice weekly. The supplement was freshly prepared each week and refrigerated. The weight and temperature measurements of the animals were taken once a week.

* Composition of guinea pig chow pellet:

lucerne	19.0%	wheat	20.0%
barley	25.5%	pollard	20.0%
soya bean meal	7.5%	meat meal	5.0%
limestone	0.5%	calcium phosphate	0.5%
salt	1.0%	vitamins (minerals)	1.0%

The pellets were manufactured by Milling Industries, Adelaide.

3.1.1 Experimental periodontal disease

Chronic inflammation was induced by the application of dental floss ligatures reinforced by composite resin. The ligatures were placed on the central incisors of either jaw and the opposite pair acted as controls. The ligatures were checked twice a week and replaced as necessary. All procedures were performed under general anaesthesia acheived by intramuscular injection [Ketamine hydrochloride (Ketalar^{R}) 50 mg/kg].

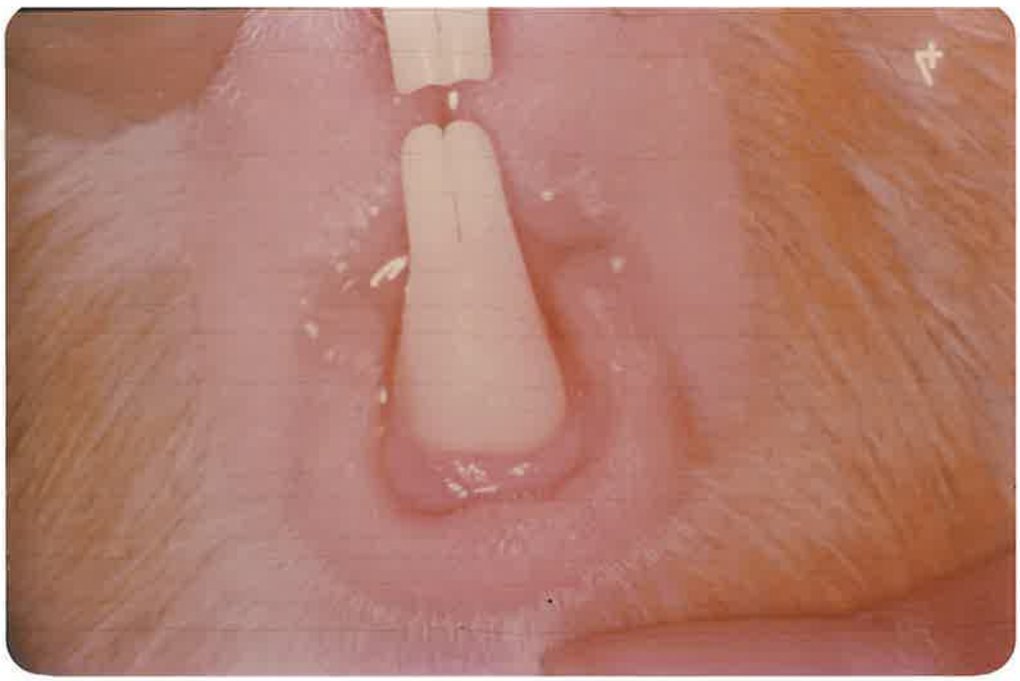


Fig. 1 Position of incisal ligature

3.1.2 Experimental protocol

The animals were separated into two groups after the induction of disease. The experimental group was fed with chow pellets and water with a marginally deficient dose of vitamin C added (1mg in 1ml of 20% sucrose three times / week). The control animals received the same diet except that they received the vitamin supplement described in 3.1. The animals were maintained on this regime for at least 12 weeks. Once a month during the experimental period, random selection of animals from both groups was made to obtain blood samples for ascorbate assay. The blood was obtained by cardiac puncture under an inhalation anaesthesia (2% halothane in oxygen).

After six weeks, three animals of the experimental group expired and were replaced by two controls. The marginally deficient dose of ascorbic acid solution was increased to 2mg/ml of 20% sucrose solution, 3 times/week for five weeks and then returned to the original level until sacrifice (six weeks) for all the animals in the test group. Two more animals died one week before the conclusion of the experiment. The control animals received increased ascorbate in the seventh week (10 mg/ml) and increased to 20mg/ml until the conclusion of the experiment.

At sacrifice, blood was collected from the abdominal aorta for plasma and lymphocyte preparation in all animals under halothane anaesthesia. Ascorbate assay was made from the gingivae of five pairs of animals and blocks of the incisal region were collected from the remaining animals for histopathological investigation.

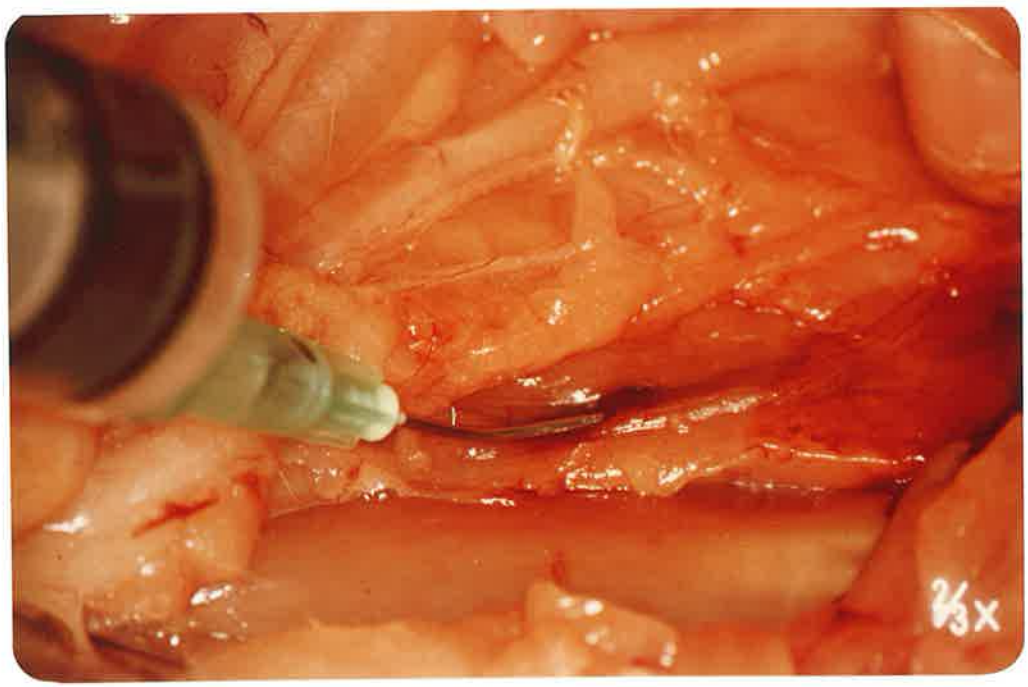


Fig. 2 Abdominal aorta blood collection

3.1.3. Vitamin C assay sample preparation

A. Plasma ascorbate preparation

Two ml of blood in an EDTA tube were centrifuged for 15 minutes at 3,000 rpm to separate plasma from red cells while avoiding the buffy layer. Half a ml of plasma was added to 1.5 ml of cold Trichloroacetic acid (TCA) 6% W/V solution. The tube was shaken until the mixture was mixed well and left at room temperature for 5 minutes. The solution was centrifuged for 10 minutes at 3,000 rpm. The supernatant was kept for plasma ascorbate measurement after being frozen at -20°C until the time of assay.

B. Lymphocyte ascorbate preparation

1. Separation of lymphocytes

Two ml of EDTA blood were diluted with 2 ml of buffered salt solution (Ringer's solution). The diluted blood was carefully layered on 3 ml of Ficoll-paque^{R}, lymphocyte separator solution in a 10 ml centrifuge tube. The tube was centrifuged at 400g for 40 minutes at 18° C in a refrigerated centrifuge (Ice-Centra-7R). The upper layer containing plasma was discarded. The lymphocytes in the middle layer were harvested into a centrifuge tube. Ringer's salt solution was added to make up 10 ml and the cells were suspended by gently drawing in and out of a Pasteur pipette. The lymphocytes were diluted in isotonic solution (Isoton II) to make a 1:200 dilution for lymphocyte count by adding 0.1 ml of lymphocyte solution to 19.9 ml of Isoton II. The lymphocytes in Ringers salt solution were centrifuged at 400 g at 18°C to obtain a cell pellet that was washed with Ringer's salt solution; the supernatent was discarded. The cell pellet was then ready for ascorbate extraction.

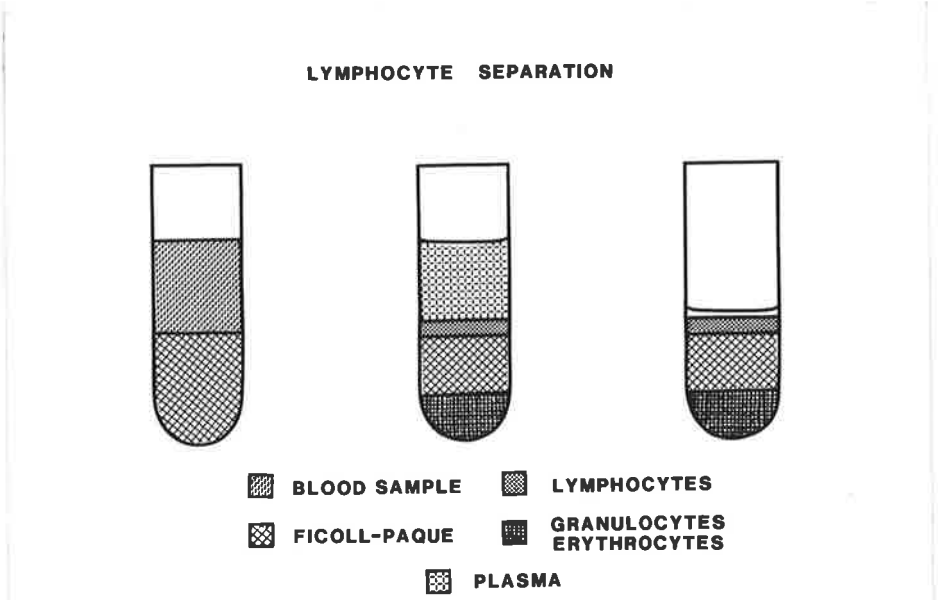


Fig. 3 Lymphocyte separation

2. Lymphocyte ascorbate preparation

The cell pellet was weighed and 2ml of 6% cold TCA were added to the pellet and transferred to a glass rod homogeniser. The homogenised lymphocyte solution was transfered to a centrifuge tube, while the homogeniser was washed by another 2 ml of 6% cold TCA solution, that subsequently was added to the centrifuge tube. The homogenised solution was centrifuged at 3000 rpm for 10 minutes at 18°C. The supernatant was frozen and kept at -20°C until the time of assay.

C. Gingival tissue ascorbate preparation.

After collection, the gingival tissue was washed with normal saline to remove blood. The tissue was dried with gauze and weighed and then cut into smaller pieces and homogenised with 2 ml of 6% cold TCA in a glass rod homogeniser. The homogenised solution was transferred to a centrifuge tube. The glass homogeniser was washed with another 2 ml of 6% TCA which was added to the tube. The mixture was then centrifuged at 3,000 rpm for 10 minutes. The supernatant was kept and frozen at -20°C for gingival vitamin C assay until the time of assay.

3.1.4 Vitamin C assay

The method of Roe and Kuether (1943) was used to measure plasma, lymphocyte and gingival tissue ascorbate concentrations. To develop the colour which was read at an absorbance of 520nm in the spectrophotometer (SP8-100 spectrophotometer, Rye Unicam); 2:4 dinitrophenylhydrazine (DNPH) was used as a working agent.

A. Reagents

1. TCA

Trichloroacetic acid 60 gm were dissolved in distilled water and made up to one liter (6%TCA). The 4% solution was derived by taking 66.7 ml of 6% TCA and making the volume up to 100 ml with distilled water. The TCA solutions were kept at 4°C.

2. 2:4 DNPH working agent

2:4 DPNH	2gm
Sulphuric acid (9N)	100ml
Thiourea	4gm

Two gm of 2:4 DNPH were dissolved in 100 ml of 9N H_2SO_4 (three parts of distilled water to one part of the concentrated acid). Four gm of thiourea were added to the solution with occasional shaking until dissolved. The mixture was filtered and kept at 4°C and prepared monthly.

3. Bromine solution

Bromine 5 ml was diluted to approximately 250 ml with distilled water. This solution was kept at room temperature.

4. Sulphuric acid 85%

B. Standard curve preparation.

Ten mg of L-ascorbic acid was dissolved in 10 ml of 4% cold TCA and 0.5 ml of the solution was diluted to 50 ml with 4% cold TCA to make a 10µg/ml standard ascorbic acid solution. This solution was oxidised by drop addition of bromine solution until the solution turned faint yellow. Excess bromine was removed by bubbling

nitrogen through the solution until it became clear. Different concentrations of vitamin C in 4% cold TCA were made up in 5 tubes. The first tube contained 2 ml of standard solution and was kept as a blank. The remaining four tubes contained 0.25, 0.5, 1.0 and 1.5 ml of standard solution respectively and were each diluted to 2ml with 4% cold TCA. At that time, 0.5 ml of 2:4 DNPH solution was added to all tubes except the blank. The tubes were incubated in the waterbath for 3 hours at 37° C and then 85% H_2SO_4 (2.5 ml) was slowly added to the standard solutions which were placed in an ice bath to avoid excessive heat production; 2:4 DNPH solution (0.5 ml) was added to the blank. The standard tubes were mixed thoroughly and left for 30 minutes at room temperature before reading the absorbance at 520 nm in the spectrophotometer. A standard curve was obtained for each assay plotting the concentration of vitamin C against absorbance (Fig. 4).

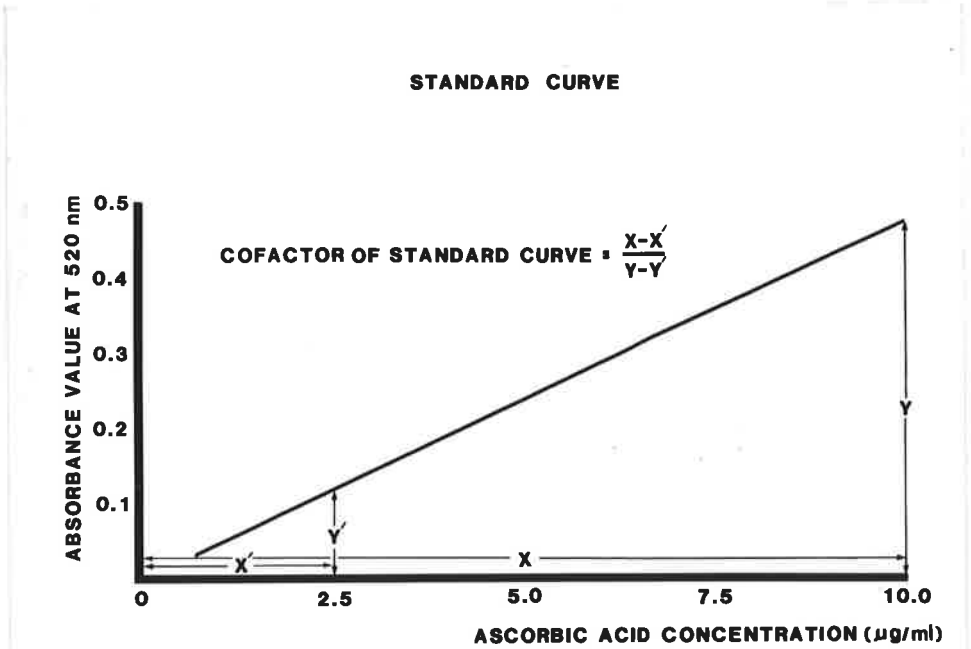


Fig. 4 Standard curve

C. Vitamin C assay preparation.

The thawed samples were oxidised with bromine solution and then gassed with nitrogen to remove the excess bromine until the solution became clear in colour. 0.5 ml of sample was placed in each of three tubes and 0.125 ml of 2:4 DNPH was added to two of the tubes. The third tube was kept as a blank. The samples were then incubated in a water-bath at 37°C for three hours. 0.625 ml of 85% Sulphuric acid (H_2 SO_4) was added to the sample tubes which were placed in an ice bath to avoid excessive heat generation. 0.125 ml of 2:4 DNPH solution was then added to the blank tube. The samples were mixed thoroughly and left at room temperature for half an hour before reading the absorbance in the spectrophotometer at 520 nm.

D. Calculation

$$\text{Cofactor of standard curve (cofac)} = \frac{\text{Difference between concentration}}{\text{Difference between the absorbance value}^*}$$

$$\text{Plasma ascorbate (mg \%)} = \frac{\text{cofac} \times \text{absorbance value}^* \times 4}{10}$$

$$\text{Lymphocyte ascorbate (mg/100gm lymphocyte pellet)} = \frac{\text{cofac} \times \text{absorbance value}^* \times 4 \times 100}{\text{weight of cell pellet}}$$

$$\text{Gingival ascorbate (mg/100 gm tissue)} = \frac{\text{cofac} \times \text{absorbance value}^* \times 4 \times 100}{\text{weight of gingival tissue}}$$

* absorbance value = read absorbance - blank absorbance

3.1.5 Histological preparation

A. Specimen processing

The block dissections of incisal areas were collected and fixed in 10% buffered formalin solution for one week. The specimens were washed in running water for one hour before putting in the formic formate decalcifying solution (sodium formate 68 gm in formic acid 340 ml to make 2 litres with distilled water). The solution was changed everyday until complete decalcification had occurred as determined by radiograph. Decalcified specimens were neutralized in the 5% sodium sulphate for approximately 12 hours and then washed in running water for one hour before embedding. The tissues were dehydrated in gradually increasing concentrations of alcohol and were finally placed in a solution of 50% alcohol 50% methyl salicylate for one hour each. The specimens were placed in methyl salicylate plus 0.5% celloidin for three days, followed by a period of four days in a similar solution with the concentration of celloidin raised to 1%. The specimens were then infiltrated with paraffin wax and mounted with the labio-lingual plane uppermost in the block.

B. Sectioning

Labio-lingual sections of 6 μm were cut using a rotary microtome until the middle of the first central incisor was reached, then the specimen was reblocked and cut in the mesio-distal plane.

C. Staining

Sequential sections were stained with haematoxylin and eosin (H and E). Other sections were stained with Herovici polychrome method (1963) that distinguishes between young and

mature collagen fibres. Representative sections were selected for photographic purposes.

D. Histopathological assessment

The tissue was assessed for inflammatory cell infiltration of the connective tissue adjacent to the junctional epithelium. The degree of induced periodontal disease within the tissue was also assessed. Inflammation was scored as follows:

0:- Little or no inflammatory cell infiltration

1:- Slight inflammatory cell infiltration

2:- Moderate inflammatory cell infiltration

3:- Heavy inflammatory cell infiltration

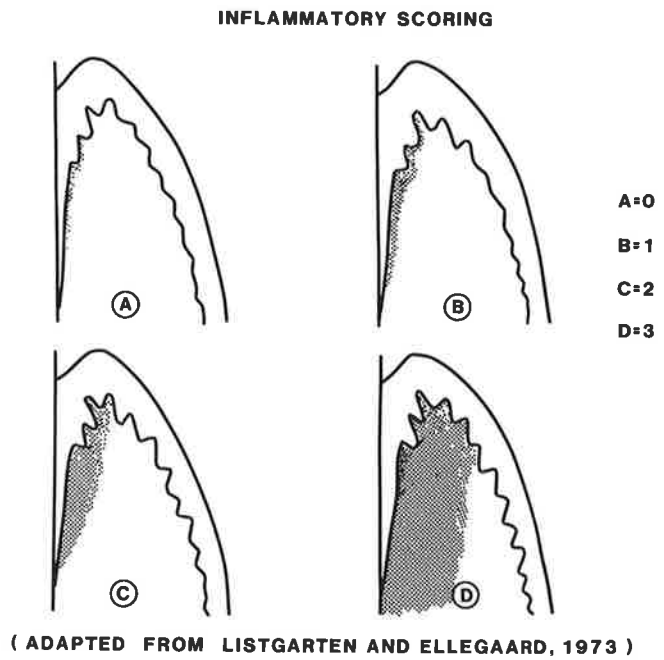


Fig. 5 Inflammatory scoring

3.2 Clinical experiment

Whenever possible gingival tissue was collected from patients during routine surgical periodontal procedures. The tissue was washed with water and wrapped in gauze soaked in normal saline. The tissues were homogenised within half an hour of collection in readiness for ascorbate measurement. Blood samples were collected from as many of the patients as possible for ascorbate assay. The samples were prepared as described above and stored at -20°C until the time of assay.

Dietary analysis and medications taken were recorded from the patients for two week-days and one weekend day. Cigarette and alcohol consumption were estimated and the vitamin C intake was estimated from the dietary records using the table of composition of Australian food (Thomas and Corden, 1970) and compared with tissue and plasma ascorbate levels.

3.3 Statistical analysis

A student t-test was used to assess the difference in the plasma, lymphocyte and gingival ascorbate levels in the animal experimental and control groups. The difference between non-inflamed and induced-inflamed gingival ascorbate levels and the degree of inflammation were tested by the same method. The simple correlation and regression analyses between plasma, lymphocyte and gingival ascorbate levels were also tested.

Significance of correlation and the slope of the fitted line were tested for statistically significant departure from zero by a two-tailed t test (Snedecor and Cochran, 1969).

Chapter 4

Results

4.1 Animal experiment

4.1.1 Physical health

Body weight

All animals were fed as described in Chapter 3, prior to the experiment; measurement of body weight was carried out weekly. Weights gradually increased during the pre-experimental period from 377.5 ± 37.2gm to 641.2 ± 42.2gm in the control group and from 396.6 ± 40.0gm to 652.8 ± 62.8gm in the experimental group. No significant difference was found in the weights of the groups before the experimental period began. After the ligatures were placed both groups continued to gain weight for one week and had stable weights for a further two weeks. At that point the experimental group began to lose weight but the controls continued to gain weight. After six weeks the weights were 738.4 ± 55.8gm and 646.6 ± 130.6gm in the control and experimental groups respectively. The control animals acheived satisfactory growth on the autoclaved pellet and vitamin C supplementation while three of the vitamin C deficient animals were terminally ill at that point and 2mg of ascorbic acid was given three times a week to the surviving animals of the experimental group. After 17 weeks the body weight of the deficient group was significantly lower than the control group ($p<0.001$). The results are shown (Fig. 6, Table 1).

Five guinea pigs of the experimental group died during the 17 weeks, while three of the controls died as a result of cardiac puncture. Three animals from the experimental group had paralysed rear legs for the final month of the experimental period. The paralysis may have resulted from Ketalar injections damaging the sciatic nerve or from the effect of severe vitamin C deficiency (Veen-Baigent 1975). No similar changes were found in the control group.

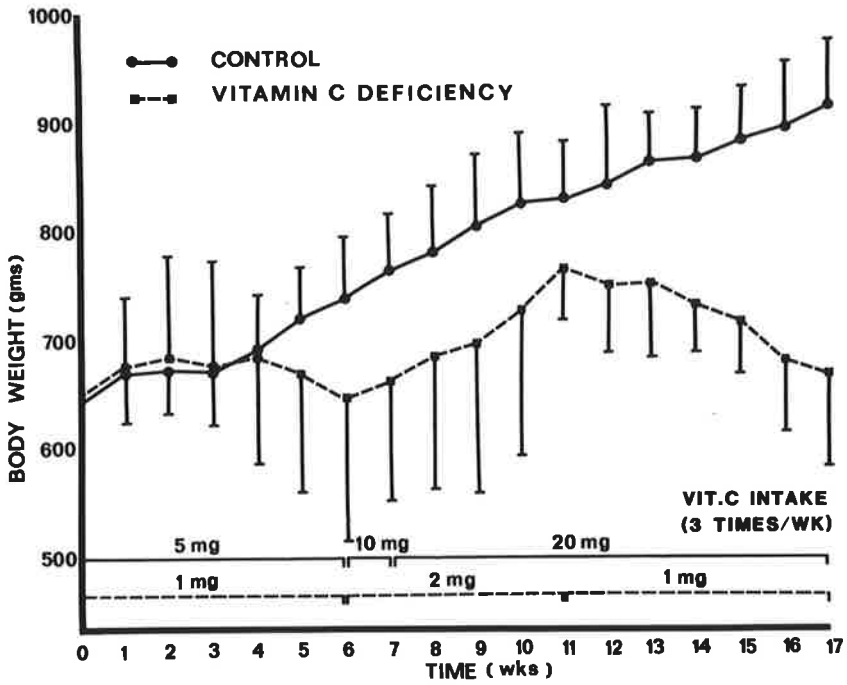


Fig. 6

Weekly mean body weights of 8-11 control and 7-11 vitamin C deficient guinea pigs with standard deviation bars.

Table 1

Mean body weights of guinea pigs (gm) during the vitamin C deficient regime.

Week	Control		Vit C deficiency	
	n	mean ± S.D.	n	mean ± S.D.
0	11	641.2 ± 42.2	11	652.8 ± 62.8 n.s
4	10	693.1 ± 52.3	11	686.7 ± 96.1 n.s
8	9	780.8 ± 60.6	9	685.6 ± 124.4 n.s
12	8	844.4 ± 71.5	7	751.0 ± 62.4*
17	8	916.0 ± 59.5	7	668.4 ± 84.3**

n = number of animals

n.s. = no significant difference

* significantly lower than control at $p<0.05$

** significantly lower than control at $p<0.01$

Temperature measurement

The temperature (°C) measurement was made sublingually once a week. There was no significant difference between the groups before the experiment began, however the temperature of the vitamin C deficient animals was slightly but significantly lower than that of the control group. The data are summarised in Fig. 7 and Table 2.

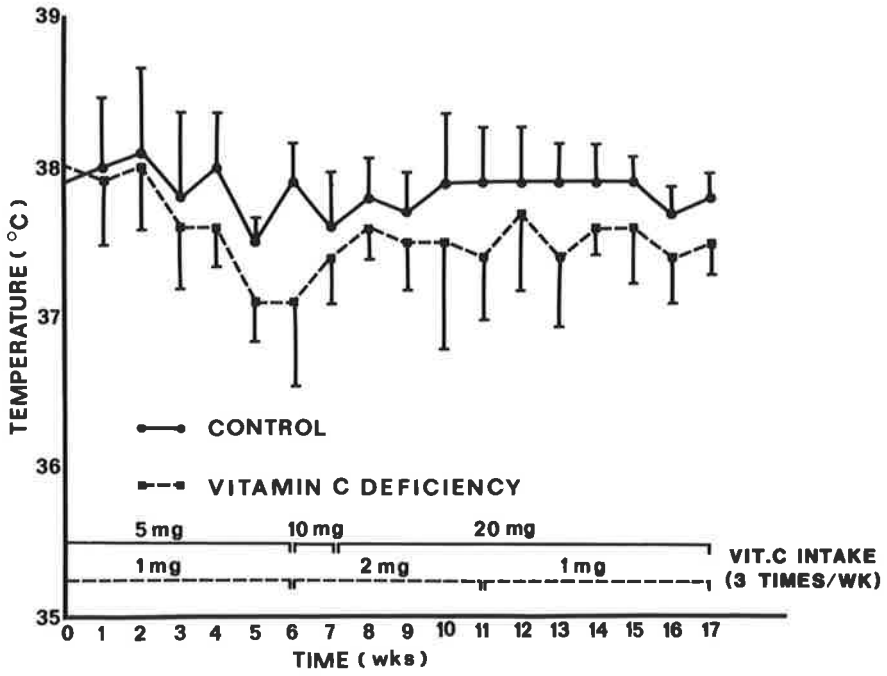


Fig. 7

Weekly mean body temperature (°C) of 8-11 control and 7-11 vitamin C deficient guinea pigs with standard deviation bars.

Table 2

Mean body temperatures (°C) of guinea pigs

Time	Control		Vit. C deficiency	
	n	mean ± S.D.	n	mean ± S.D.
Pre-experimental period	11	37.9 ± 0.1	11	38.0 ± 0.2
Experimental period	8-11[a]	37.8 ± 0.1	7-11[a]	37.5 ± 0.3**

n = number of animals

a = 3 control and 4 experimental animals died during the observation period

** significantly lower than the control group, $p<0.01$

4.1.2 Oral health

Oral hygiene was practised in the pre-experimental period and at the onset of the study the gingival tissues were considered to be healthy. One week after the placement of ligatures there were signs of inflammation in both groups but it was more severe in the ascorbate deficient animals. The obvious clinical difference between the groups was the susceptability of the experimental group to gingival haemorrhage. Also, the mobility of the teeth of the ascorbate deficient group increased, but if ligatures were lost these changes were rapidly reversed. The ligatures were checked twice a week to ensure that the local gingival irritation was consistently maintained. It was not possible to probe any pockets in animals of either group.

4.1.3 Vitamin C assay

A. Plasma ascorbate

The plasma ascorbate was measured at weeks 4, 8, 12 and 17 (Table 3). No significant difference was found at week 4, but significant reductions of ascorbate values were observed at the longer time periods.

Table 3

Mean plasma ascorbate concentration (mg/100 ml)

Time (weeks)	Control		Vit C deficiency	
	n	mean ± S.D.	n	mean ± S.D.
4	3	0.15 ± 0.04	3	0.10 ± 0.01n.s.
8	4	0.22 ± 0.03	3	0.14 ± 0.01**
12	5	0.29 ± 0.04	5	0.14 ± 0.02**
17	8	0.30 ± 0.09	7	0.15 ± 0.03**

n = number of animals

n.s. = not significantly different from control

** significantly lower than control at $p<0.01$

B. Lymphocyte ascorbate

The lymphocyte ascorbate concentration is shown (Table 4). The number of lymphocytes varied inversely with the total lymphocyte ascorbate content. The number of lymphocytes found in the experimental animals was significantly higher ($p<0.01$), while the ascorbate concentration was significantly lower ($p<0.01$) than in the controls.

Table 4

Mean lymphocyte ascorbate concentration and lymphocyte count at week 17

	Control		Vit C deficiency	
	n	mean ± S.D.	n	mean ± S.D.
LAA (mg%)	8	2.86 ± 1.38	7	1.08 ± 0.58**
LAA ($ug/10^8$cells)	7	13.71 ± 4.16	6	2.92 ± 1.47**
Lymphocyte count ($x10^3$cells/mm^3)	7	6.99 ± 3.02	6	10.9 ± 4.43**

n= number of animals

** significantly different at $p<0.01$.

C. Gingival ascorbate

The gingival ascorbate was measured in both non-inflamed and chronically inflamed tissue and the results are shown (Table 5). The gingival ascorbate in scorbutic animals was significantly lower than in controls in both inflamed (irritated by ligatures) and non-inflamed tissue, however no significant difference was found in either group between the inflamed and non-inflamed tissue. The higher levels of ascorbate in the inflamed tissue probably reflect the presence of a large number of inflammatory cells that also carry high levels of ascorbate.

Table 5

Mean gingival ascorbate concentration (mg/100gm of tissue)

	Control		Vit C deficient	
	n	mean ± S.D.	n	mean ± S.D.
Non-inflamed	5	5.12 ± 0.79	5	1.83 ± 0.37**
Inflamed	5	4.86 ± 0.94	5	2.27 ± 0.52**

n = number of animals

** significantly lower than control at $p<0.01$.

D. Relationship of plasma, lymphocyte and gingival ascorbate concentrations

The lymphocyte ascorbate was compared with plasma ascorbate of 15 guinea pigs. Good correlations were found ($r = 0.57$) at $p<0.05$. Correlations between plasma ascorbate content and gingival tissue in both inflamed and non-inflamed tissue were also found (Table 6). Lymphocyte ascorbate content was also related to the concentration of ascorbate in inflamed and non-inflamed tissue (Table 7). The regression analyses between plasma, lymphocyte and gingival ascorbate concentration are shown (Fig. 8 , 9)

Table 6

Correlation between lymphocyte, non-inflamed, inflamed gingivae and plasma ascorbate content.

	Plasma ascorbate content		
	n	correlation(r)	significance
Lymphocyte ascorbate	15	0.57	$p<0.05$
Non-inflamed gingival ascorbate	10	0.87	$p<0.01$
Inflamed gingival ascorbate	10	0.74	$p<0.05$

n = number of animals

Table 7

Correlation between inflamed, non-inflamed gingivae and lymphocyte ascorbate contents

		Lymphocyte ascorbate content	
	n	correlation(r)	significance
Non-inflamed gingival ascorbate	10	0.64	$p<0.05$
Inflamed gingival ascorbate	10	0.62	$p<0.05$

n = number of animals

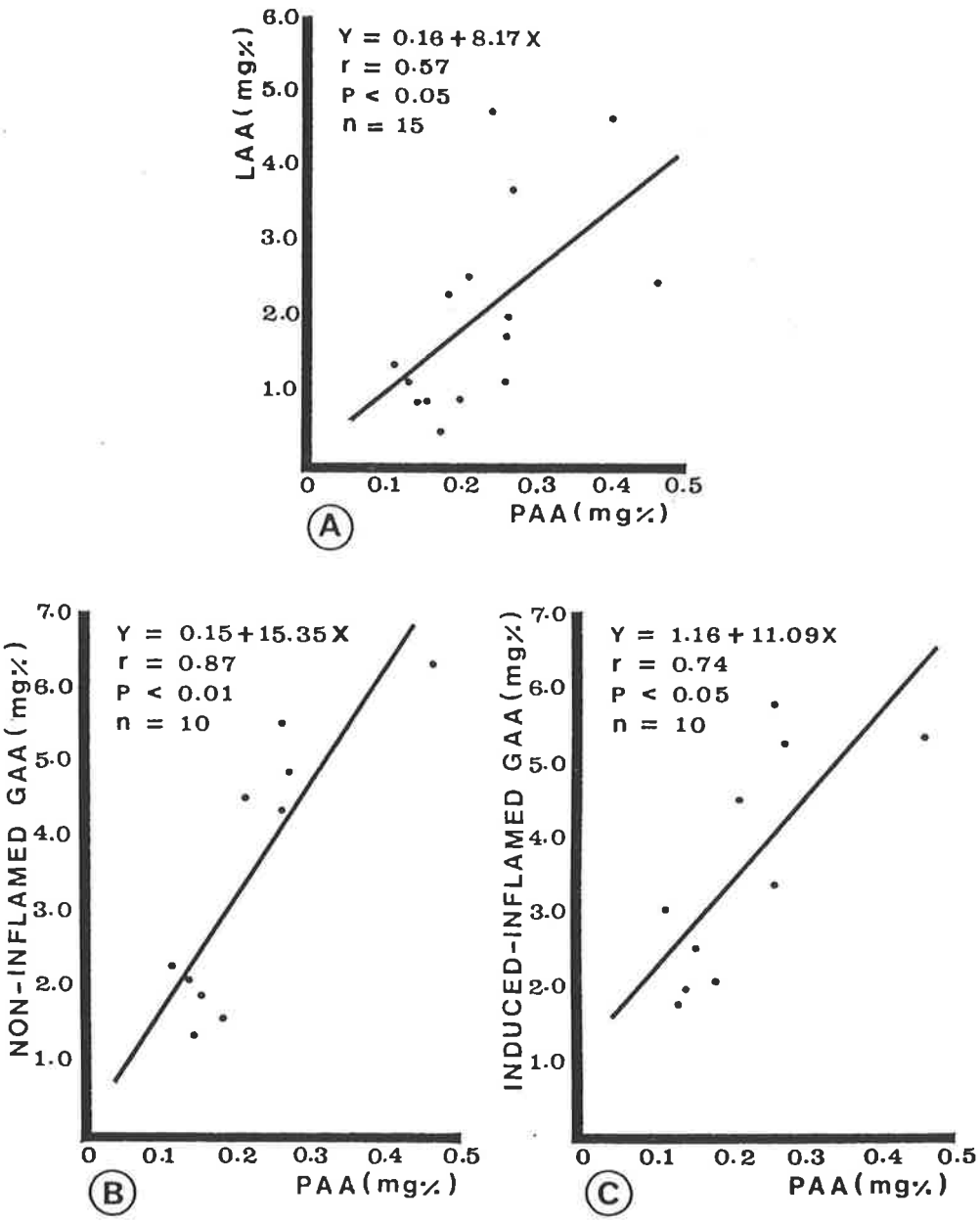


Fig. 8 The regression lines demonstrate the relationship between:

A. Plasma and lymphocyte ascorbate concentration.
B. Plasma and non-inflamed gingival ascorbate concentration.
C. Plasma and induced-inflamed gingival ascorbate concentration.

PAA = Plasma ascorbic acid concentration.
LAA = Lymphocyte ascorbic acid concentration.
GAA = Gingival ascorbic acid concentration.

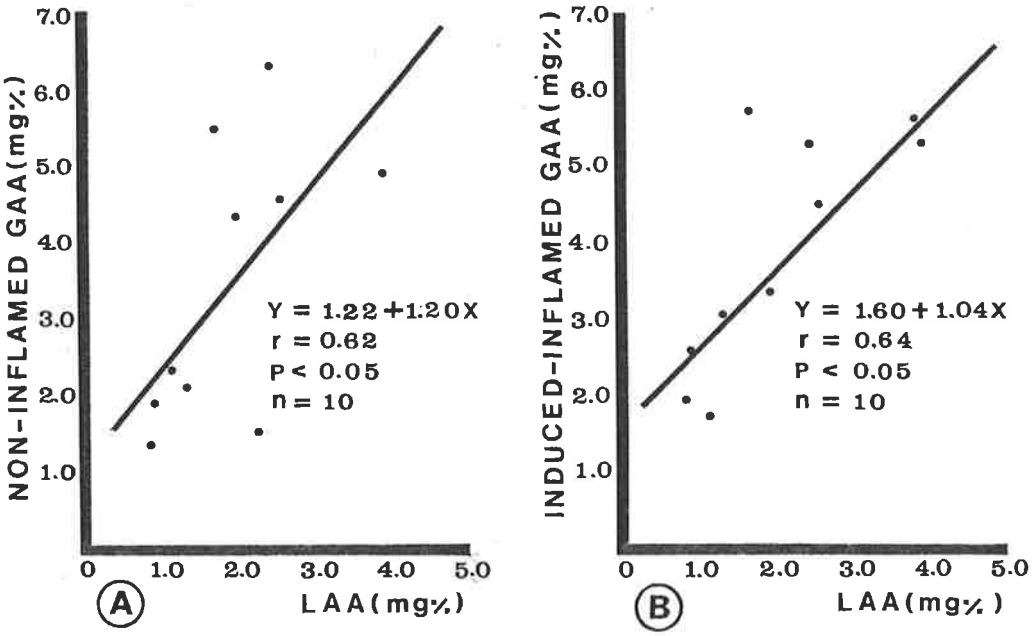


Fig. 9 The regression lines demonstrate the relationship between:

A. Lymphocyte and non-inflamed gingival ascorbate concentration.
B. Lymphocyte and induced-inflamed gingival ascorbate concentration.

4.1.4 Histological investigation

1. Degree of inflammation

The inflammatory scores were the mean values of scores taken from the labiolingual and mesiodistal sections. The results are shown (Table 8).

Table 8

Mean inflammatory scores of non-inflamed and inflamed gingival tissues

	Inflammatory score			
	Vit C deficiency		control	
	n	x ± S.D.	n	x ± S.D.
non-inflamed	10	1.45 ± 0.80	12	1.08 ± 0.79
inflamed	10	2.6 ± 0.57	12	2.5 ± 0.43

n= number of blocks

The degree of inflammation of the scorbutic animals in both series were not significantly higher than controls.

The degree of inflammation was significantly higher in all ligature irritated tissues than in non-irritated tissues ($p<0.01$)

2. Histopathological studies

A. Gingivae

The appearance of normal gingivae (Fig. 10 A,B) showed keratin covered masticatory epithelium, crevicular and junctional epithelia and underlying connective tissue lightly infiltrated with inflammatory cells. The non-irritated regions of both control and experimental groups appeared to be similar with light infiltration of chronic inflammatory cells present(Fig. 11A) except for the col region of the interdental papillae where the infiltration became moderate. No significant difference between the groups could be found.

Where ligatures had been placed heavy infiltration was observed in the tissues of both the control and experimental groups (Fig. 10C, 11B). However no significant difference was found between either group.

B. Gingival fibres

Controls

The gingival fibres of the normal tissue demonstrated the usual arrangements of fibres between tooth, cementum and alveolar bone (Fig. 12A). The controls with ligatures demonstrated the presence of mild inflammatory changes within the connective tissue adjacent to the crevice (Fig. 12B).

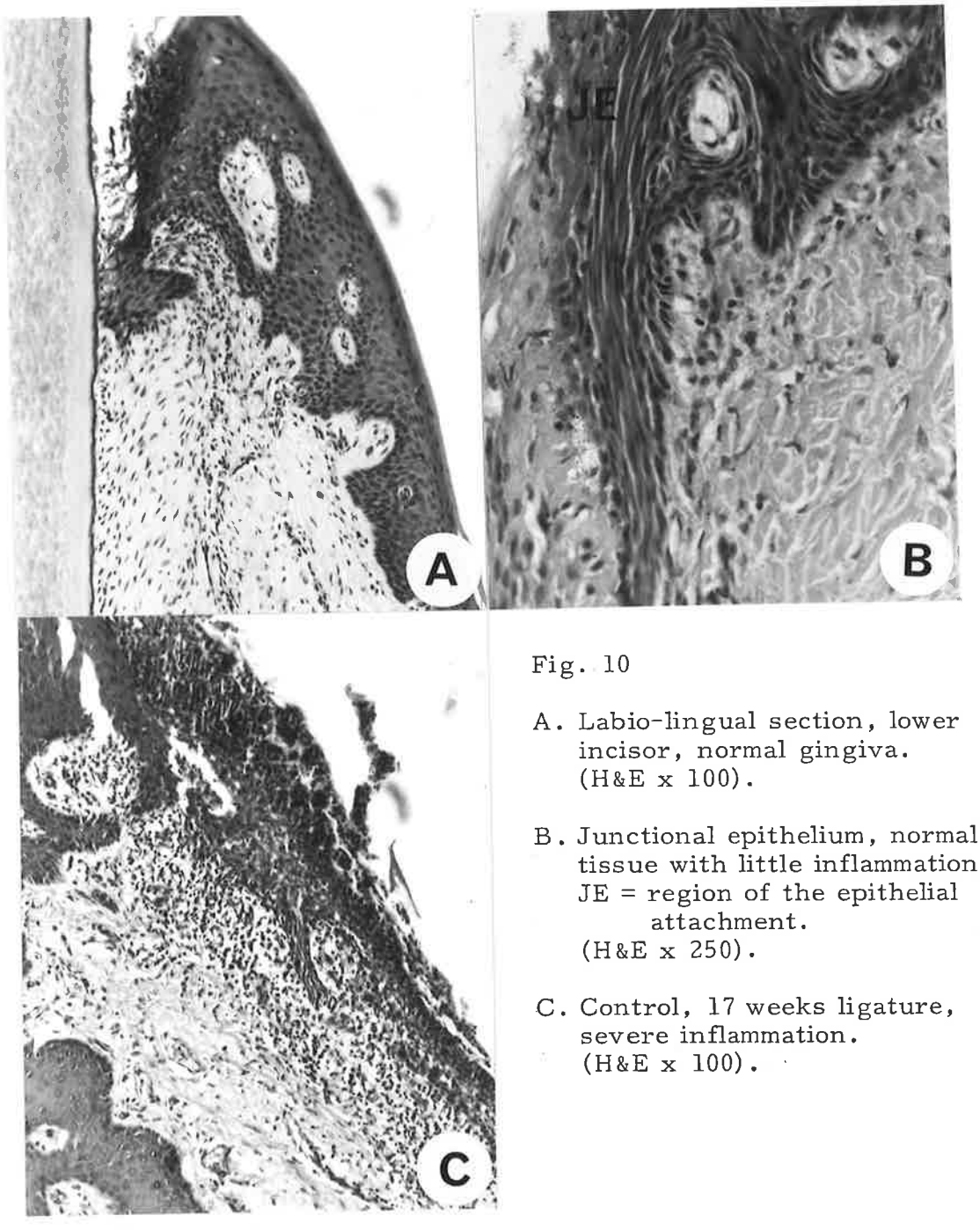


Fig. 10

A. Labio-lingual section, lower incisor, normal gingiva. (H&E x 100).

B. Junctional epithelium, normal tissue with little inflammation. JE = region of the epithelial attachment. (H&E x 250).

C. Control, 17 weeks ligature, severe inflammation. (H&E x 100).

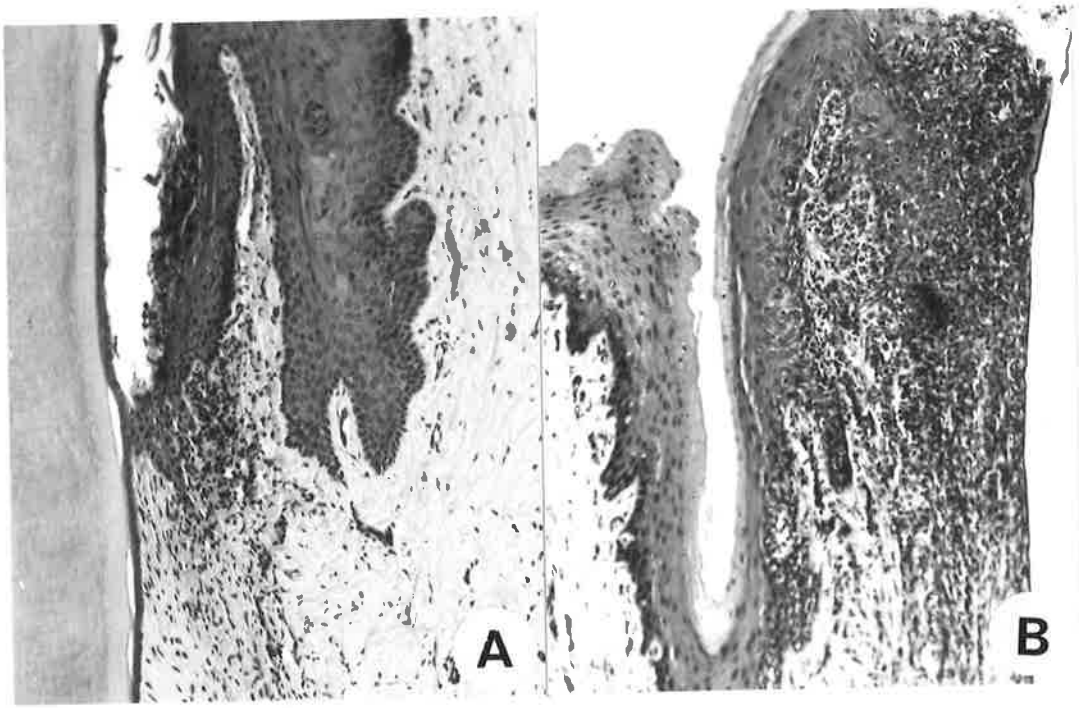


Fig. 11 Labio-lingual section, lower incisor, vitamin C deficient gingiva.

A. No ligature, slight inflammation. (H&E x 100).

B. 17 weeks ligature, heavy inflammatory cell infiltration. (H&E x 100).

Experimental group

The gingival fibres in the experimental group showed more disintegration than in the controls (Fig. 12C,D). The young collagen fibres were readily demonstrated in most specimens and the degree of fibre damage varied with the intensity of the inflammation.

C. Periodontal fibres

Controls

The periodontal fibres adjacent to the tooth were dense and ran parallel or oblique to the root surface. In the alveolar bone region the fibres were less dense and ran at 90° to the bone surface (Fig. 13A,B). In the control animals where ligatures were placed, the tissue appeared to be little changed from normal; some widening of the periodontal spaces due to lessened density of the fibres was possible. In some sections minor destruction of the bundles was noted (Fig. 13C).

Experimental group

Where no ligatures had been placed the results were variable; some samples appeared to be near-normal while others showed inflammatory changes with moderate destruction of the periodontal bundles (Fig. 14A). Ligatures were responsible for greatly intensifying the damage, the periodontal fibres adjacent to the bone were destroyed and those in the tooth region were very severely reduced in density (Fig. 14B). Young collagen fibres were demonstrated using a polychrome stain (Herovici 1963) and areas of haemorrhage were noted in some sections.

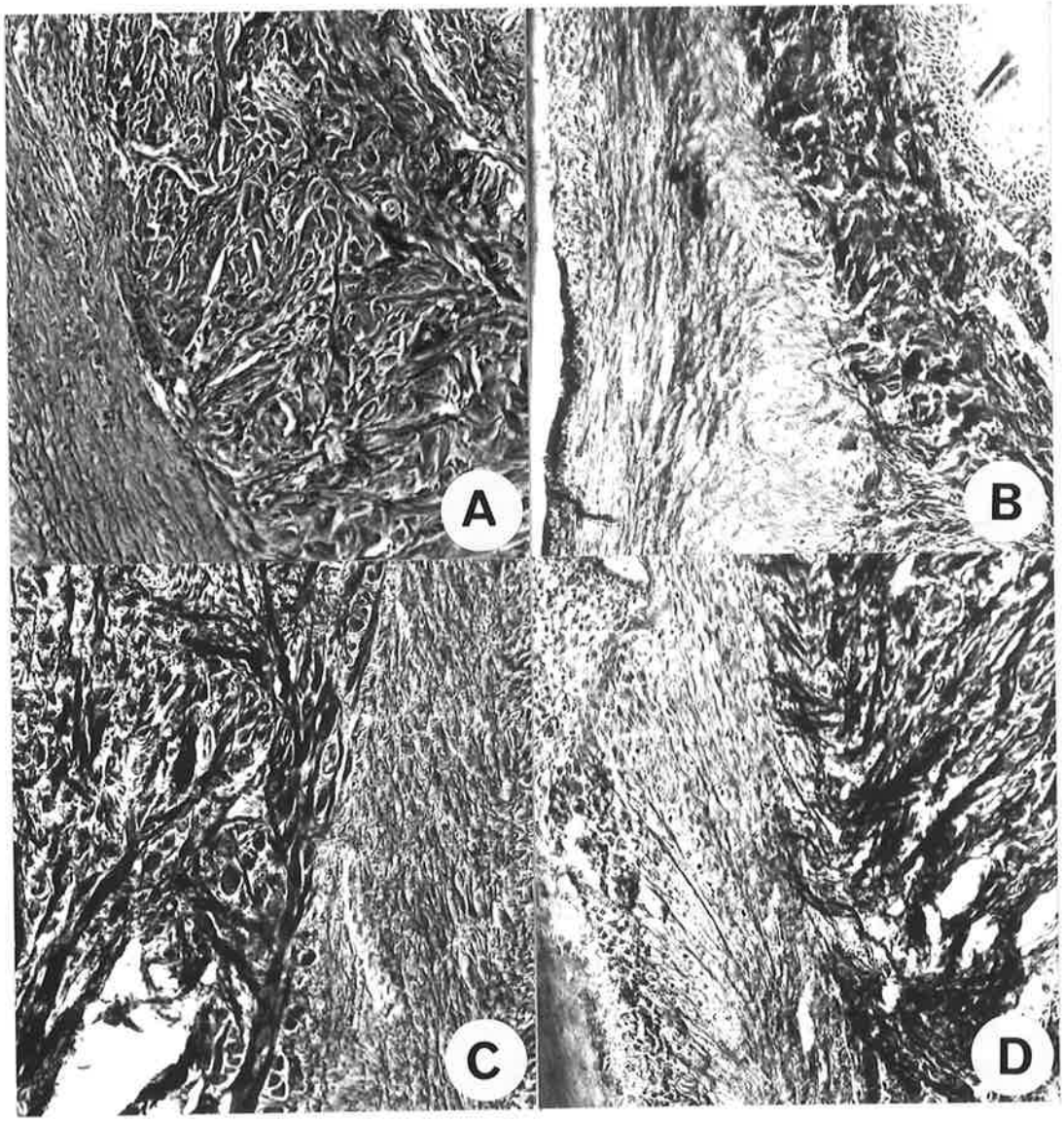


Fig. 12

A. Control, gingival fibres, no ligature. (Polychrome stain x 100).

B. Control, 17 weeks ligature, less dense and thinner fibres than normal fibres. (Polychrome stain x 100).

C. Fibres from vitamin C deficient animal, no ligature, some destruction of fibres. (Polychrome stain x 100).

D. Fibres from vitamin C deficient animal, 17 weeks ligature, disintegration of collagen fibres. (Polychrome stain x 100).

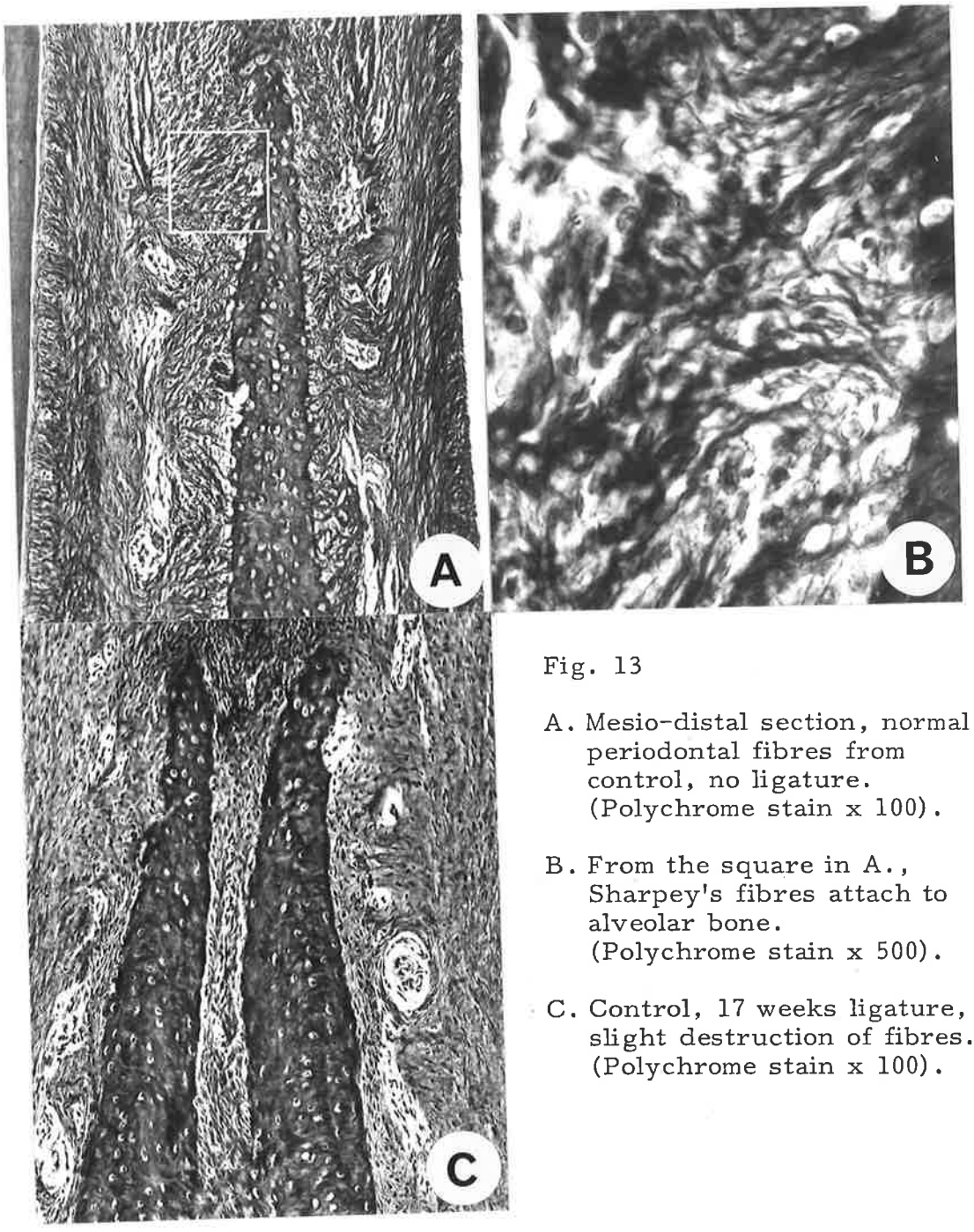


Fig. 13

A. Mesio-distal section, normal periodontal fibres from control, no ligature. (Polychrome stain x 100).

B. From the square in A., Sharpey's fibres attach to alveolar bone. (Polychrome stain x 500).

C. Control, 17 weeks ligature, slight destruction of fibres. (Polychrome stain x 100).

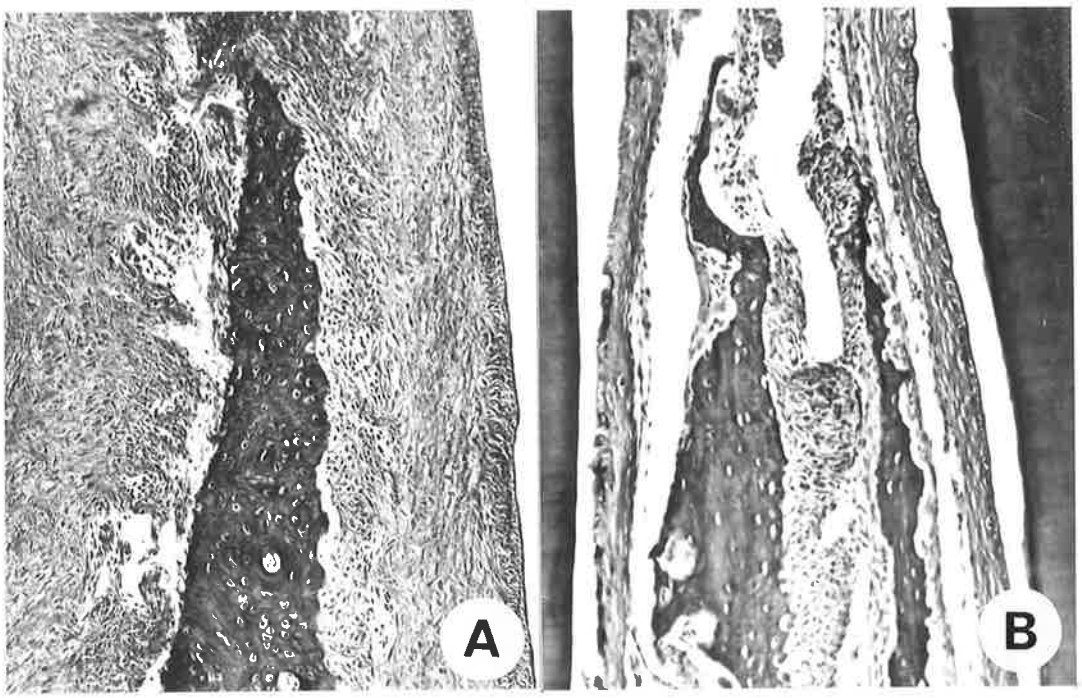


Fig. 14 Periodontal fibres of vitamin C deficient animal.

A. No ligature, destruction of Sharpey's fibres. (Polychrome stain x 100).

B. 17 weeks ligature, severe disintegration of most collagen fibres. (Polychrome stain x 100).

D. Alveolar bone

Controls

The normal alveolar bone showed both resorption and remodelling and there was no evidence of alveolar bone changes in half of the control group even where ligatures had been placed (Fig. 15A). Half of the specimens from the control group with ligature irritation demonstrated slight alveolar crest resorption (Fig. 15B).

Experimental group

Ligature-free specimens were similar to the controls but trabeculation was possibly a little thinner and more obvious resorption was seen (Fig. 16A). In regions adjacent to ligatures there were significant areas of resorption of the crestal bone (Fig. 16B,C). The resorption of bone also appeared to be correlated with the intensity of inflammation.

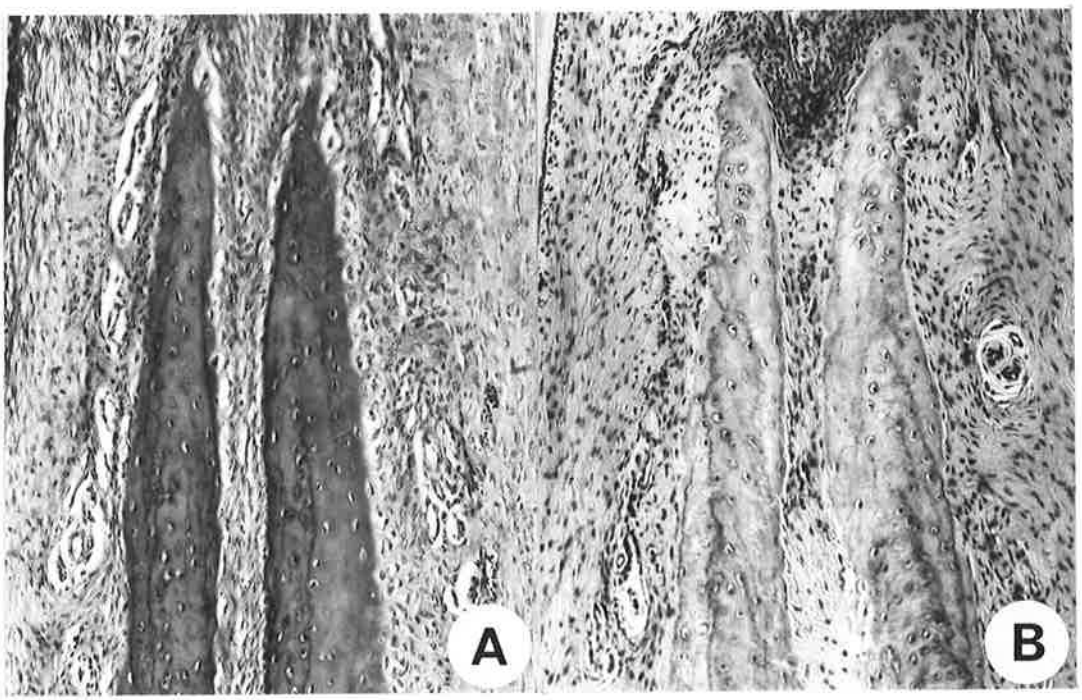


Fig. 15 Alveolar bone of control animal.

A. No ligature, normal bone remodelling. (H&E x 100).

B. 17 weeks ligature, slight bone resorption. (H&E x 100).

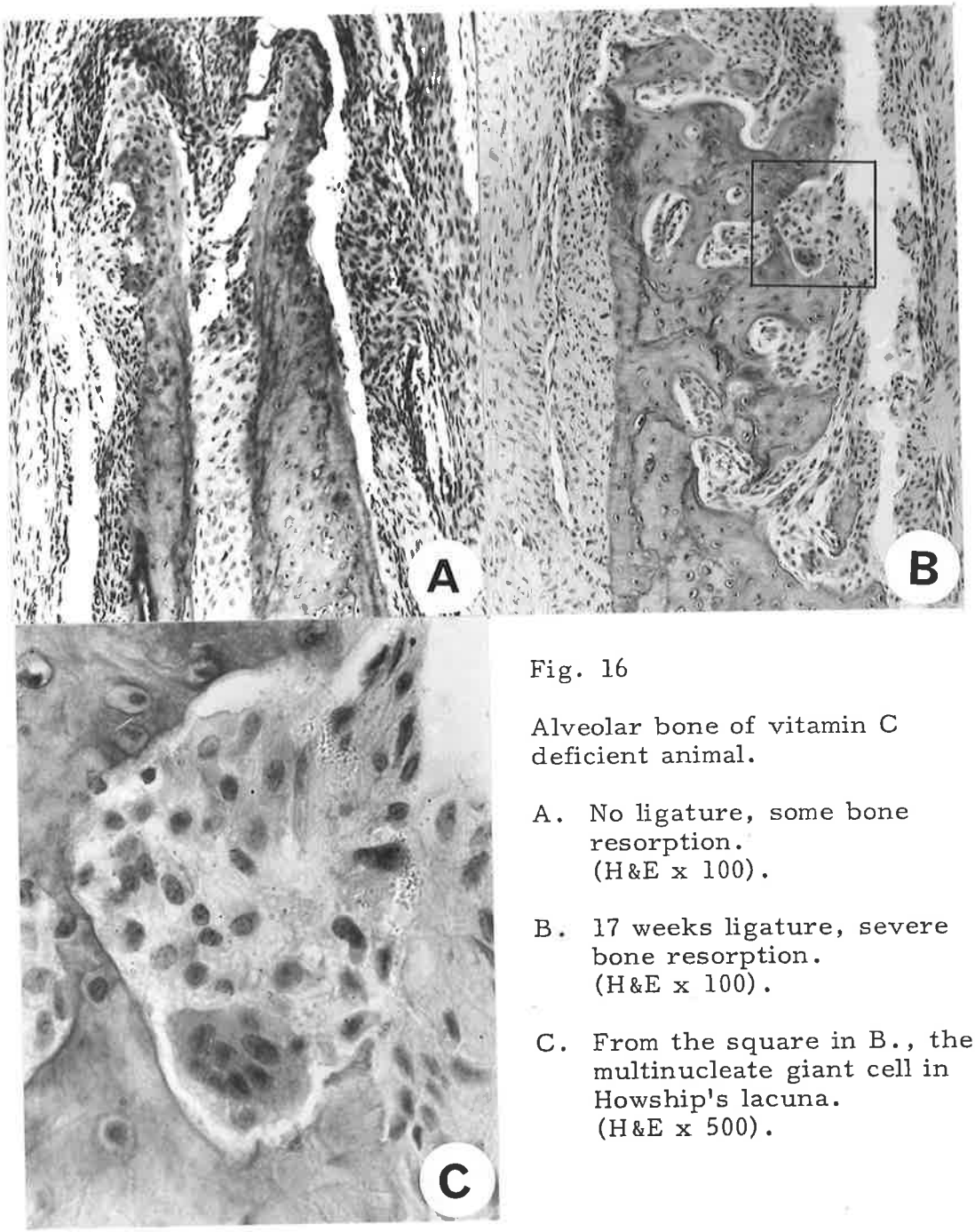


Fig. 16

Alveolar bone of vitamin C deficient animal.

A. No ligature, some bone resorption. (H&E x 100).

B. 17 weeks ligature, severe bone resorption. (H&E x 100).

C. From the square in B., the multinucleate giant cell in Howship's lacuna. (H&E x 500).

4.2 Human investigation

Thirty-seven patients, 19 females and 18 males (average age 50) with periodontal disease provided 65 gingival biopsies, 27 blood samples and 25 dietary intake records.

4.2.1 Plasma ascorbate concentration

The amount of ascorbate intake was calculated from the dietary records and was found to correlate with the plasma levels which varied from 0.37 - 2.6mg%. The mean level was 1.36 ± 0.56mg%, (1.53 ± 0.47mg% female, 1.15± 0.66mg% males), two females and five males (25.9%) had plasma ascorbate levels of less than 1mg% which should be classified as subclinical ascorbate deficiency (Ginter 1979). The data are summarised in Table 9.

Table 9

Mean plasma ascorbate concentrations

	Plasma ascorbate concentration(mg%)		
	Female	Male	Total
No. of patients	15	12	27
Mean ± S.D.	1.53 ± 0.47	1.15 ± 0.66	1.36 ± 0.56

No significant difference was found between any of the groups.

4.2.2 Gingival ascorbate concentration

The gingival biopsies were obtained from patients undergoing routine periodontal surgical procedures. Gingival ascorbate data are shown (Table 10).

Table 10

Mean gingival ascorbate concentration
65 biopsies from 37 patients

	gingival ascorbate concentration (mg%)		
	anterior	posterior	total
biopsy number	29	36	65
mean ± S.D.	10.90 ± 4.27	9.85 ± 3.16	10.32 ± 3.70

No significant difference was found between the groups.

4.2.3 Relationship between plasma and gingival ascorbate concentration.

Twenty-seven pairs of plasma and mean gingival ascorbate concentration were compared. The correlation was not strong ($r=0.34$) and t testing showed that there was a poor relationship. Although no significant relationship could be found, the gingival ascorbate concentration tended to increase when plasma ascorbate content increased. Linear regression showed a poorly fitted line when calculated from the least square (slope = 2.07, intercept = 7.48) that may imply that gingival ascorbate concentration was not dependent upon the plasma ascorbate levels. The data are summarised (Table 11 and Fig. 17).

Table 11

Relationship between gingival and plasma ascorbate concentration

	ascorbate content (mg%)	
	plasma (x)	gingival tissue*(y)
No. of patients	27	27
Mean	1.36	10.30
Sum of squares deviation from mean	8.01	297.15
Sum of products	16.6	
correlation coefficiency (r)	0.34 n.s.	

*mean value of gingival ascorbate content in each patient was used to match plasma ascorbate content.

n.s. = relationship between plasma and gingival ascorbate was not significant.

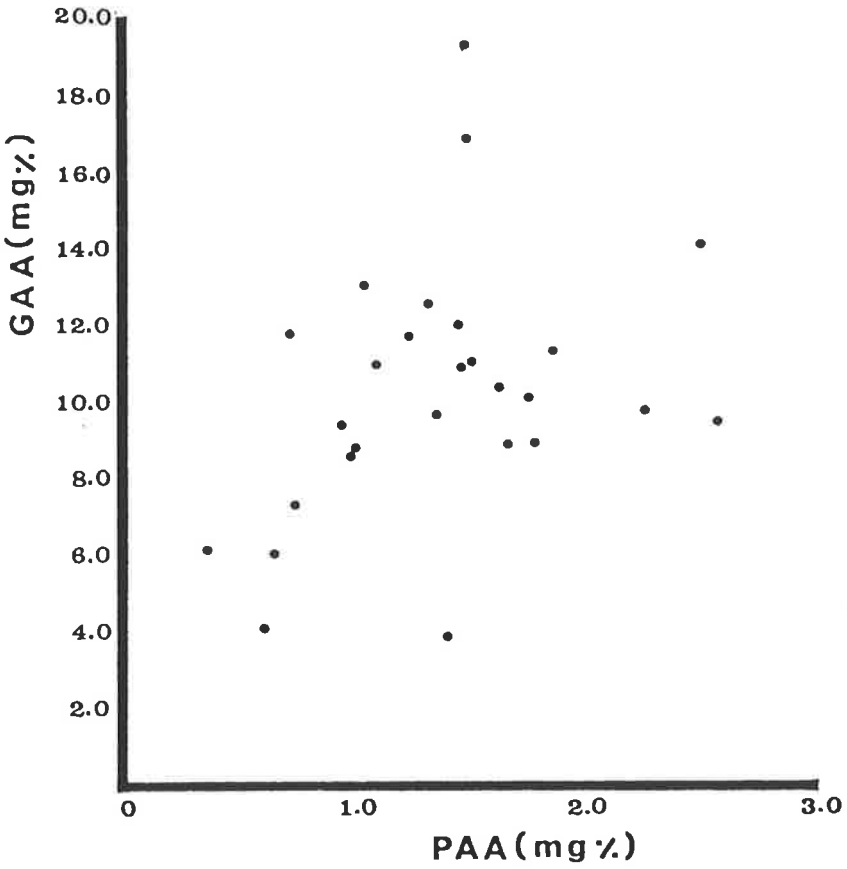


Fig.17 The relationship between plasma and gingival ascorbate concentration in 27 periodontal patients.

n.s. = The correlation between them was not significant.
(n=27, r=0.34, slope=2.07, intercept=7.48)

Chapter 5

DISCUSSION

As male guinea pigs are more stable than females in their expression of vitamin C deficiency under the influence of scorbutic diets (Odumosu and Wilson, 1971), they were chosen as the model for this study. In guinea pigs the vitamin C turnover is rapid, and the animals display distinct signs of deficiency within 3 weeks, and die with typical signs of scurvy within 4 weeks (Ginter, 1979). The guinea pigs in the present study survived for 120 days (17 weeks) without signs of scurvy except for some weight loss and slightly lowered body temperatures. Therefore, they could be described as exhibiting chronic vitamin C deficiency, which served our purposes admirably for the long term study.

The plasma ascorbate concentration of the deprived animal was higher than that reported for guinea pigs by earlier workers (Zannoni et al, 1974; Odumosu, 1982). Nevertheless, it was significantly lower than in the controls. The normal plasma ascorbate level of male guinea pigs is approximately 0.4 mg%, which was higher than that of the control group. This probably reflects the different dose of vitamin C intake and method of administration, as the plasma ascorbate level reflects closely the ascorbate intake (Veen-Baigent et al, 1975). However, circulating levels following oral administration of ascorbic acid could be influenced by other dietary factors and/or microbiological activity in the intestine.

In healthy guinea pigs, lymphocytes are the predominant white blood cell, the lymphocyte count ranging from 4.2 to 13.4 x $10^3/mm^3$ (Zeman and Wilber, 1965). The lymphocyte counts in the depleted and the control guinea pigs in this study were in the normal range for the guinea pigs, which confirmed previous reports

(Ginter, 1968; Fraser et al, 1980). However, the lymphocyte count in the deficient animals was higher than in the control group. This was probably due to the severity of the ligature-induced periodontal disease, which was more advanced in the deficient animals than in the control group. Furthermore, the ligature-induced periodontitis was reported to produce an increase of peripheral lymphocytes, as well as an increased lymphocytic infiltration of the affected area (Kornman et al, 1982). The lymphocyte ascorbate concentration of the deficient animals was depleted relative to the control animals. It varied in proportion to the ascorbic acid intake and plasma ascorbate level. The lymphocyte ascorbate varied inversely with the number of lymphocytes, as has been observed by other authors (Schorah et al, 1978; Vallance, 1979; Snook et al, 1983), which suggests that available ascorbic acid is shared by the number of cells, i.e. the higher the lymphocyte count the lower the ascorbate content. In chronic periodontal disease, the lymphocytes contained lower levels of ascorbate than normally found, leading to compromised physiological functions.

The average lymphocyte ascorbate value was lower than the leukocyte ascorbate values reported in acute vitamin C deficiency (Odumosu and Wilson, 1971; Odumosu, 1982). This was probably due to prolonged depletion of vitamin C, since leukocyte ascorbate drops gradually during vitamin C deficiency (Lowry et al, 1946; Loe and Wilson, 1971; Keith and Pelletier, 1974). However, at the same level of vitamin C intake the lymphocyte ascorbate content in the present study was higher than the leukocyte ascorbate concentration reported in guinea pigs by Keith and Pelletier (1974). This evidence could imply that guinea pig lymphocytes carry higher ascorbate than other white blood cells, which was similar to recent reports from human material (Evans et al, 1982; Castelli et al, 1982; Lee et al, 1982).

The gingival ascorbate concentration in the depleted guinea pig was significantly lower than in the control animals. The gingival ascorbate level correlated with both plasma and lymphocyte ascorbate values, and in this respect was similar to that seen in other organs (Keith and Pelletier, 1974; Hornig, 1975). The concentration of gingival ascorbate in the inflamed tissues was higher, but not significantly so, than in non-inflamed tissue in the deprived group, while there was no difference beteewn these tissues in the control group. It was expected that the ascorbate concentration would be higher in the inflamed gingivae than in normal gingivae because of the increased inflammatory cell infiltration. However the need for vitamin C in healing periodontal tissue could be increased and may explain why no differences were measurable.

The gingival ascorbate concentration was ten times that of plasma ascorbate, suggesting a high demand by the tissue which may make it more vulnerable to a deficiency of the vitamin. Periodontal tissues are highly collagenous and have a rapid turnover of collagen (Page and Ammons, 1974; Rippin, 1976 and 1978). Since vitamin C plays an important role in hydroxylation of proline and lysine in collagen synthesis (Barnes, 1975), a deficiency might be expected to lead to defects in collagen synthesis in the periodontal tissues. In addition, neutrophil function can be affected by vitamin C deficiency, leaving the first line of the host defence mechanism compromised (Anderson, 1982). Neutrophil migration and lymphocyte transformation by mitogens were impaired when they were exposed to the myeloperoxidase/hydrogen peroxide/halide system. This function can be improved by ascorbic acid as it inhibits the effect of the halide system and stimulates the activities of neutrophils and lymphocytes (Anderson, 1982).

In subclinical vitamin C deficient monkeys, an impairment of the chemotaxis and phagocytic activity of PMN were observed

(Alvares et al, 1981). In addition, the impairment of neutrophil function has also been reported in advanced periodontitis, Juvenile periodontitis, and periodontal patients with diabetes and other systemic diseases (Cianciola et al, 1977; Van Dyke et al, 1980 and 1982; Bissada et al, 1982). Van Dyke et al (1982) also demonstrated that the impairment of neutrophil function was caused by bacterial endotoxins, which were generally accepted to be an important etiological factor in periodontal disease.

Vitamin C deficiency, affects the blood vessel walls by impairing collagen and glycosaminoglycan metabolism, resulting in increased endothelial turnover and destruction of endothelial cells, which with the concurrent hypercholesterolemia and hypertriglyceridemia leads to atherosclerosis (Ginter, 1978). The deficiency of vitamin C also causes an increased tissue and plasma histamine level leading to vessel damage (Chatterjee, 1978). In addition, vitamin C deficiency results in delayed wound healing (Kramer et al, 1979; Ringsdorf and Cheraskin, 1982). A compromised gingival anatomy and end-arterial circulation in the periodontium by chronic vitamin C deficiency could predispose the host to chronic infection.

The results of the histological investigation in the guinea pigs were consistent with the biochemical results. They showed that in chronic vitamin C deficiency the periodontium was vulnerable to chronic inflammation and progressive periodontal disease, as has been observed also in monkeys with subclinical vitamin C deficiency (Alvares et al, 1981). In the deficient guinea pigs with ligatures, severe changes occurred when compared to the ones without ligatures. The inflammatory cell infiltration in the ligature-induced inflamed gingivae was significantly higher than in the non-inflamed tissues, but consistent with peripheral lymphocyte count in the deficient animals (Fig. 11b). It seems the degree of inflammation in the depleted animals with ligatures was

the highest for all groups, although it was not significantly different from contol animals with ligatures (Fig. 10 c). The inflammatory cell infliltration in the non-ligatured gingivae in the deficient animals was also higher than in the control group (Fig. 11a). The findings suggest that chronic vitamin C deficiency is a predisposing factor for gingival infection, and hence inflammation in the ligature-induced gingivae of the deficient group developed rapidly.

The destruction of collagen fibres was obvious (especially of Sharpey's fibres) in the deficient animals with ligatures (Fig. 12d, 14b). Evidence of collagen disintegration in the non-ligatured gingivae was less than in those with ligatures (Fig. 12c, 14a). The findings support the hypothesis that the severity of the disease was increased when local irritation was superimposed in vitamin C deficiency (Glickman, 1948b). In contrast, Sulkin and Sulkin (1975) reported that there was proliferation of collagen fibres in the prolonged marginal vitamin C deficient guinea pigs within most of the organs, especially the internal layer of blood vessels. In the present study the gingival tissue was subjected to a severe local irritation as well as to a vitamin C deficiency and that may account for the differences from those cited above. In the control group with ligatures, only minor changes in collagen fibres were seen.

Haemorrhage in the periodontium was rarely seen in the specimens from the deficient group, either with or without ligatures. This confirmed that the deprived animals in this study were not in a state of scurvy, as described by other authors (Waerhaug, 1958; Dreizen et al, 1969).

Alveolar bone resorption was seen in the experimental animals with ligatures (Fig. 16b), non-ligatured animals had minor changes (Fig.16a). Alveolar bone resorption in the deficient

animals without ligatures was similar to that of the control animals with ligatures (Fig. 15b), which differs from previous reports of acute vitamin C deficiency (Glickman, 1948a; Waerhaug, 1958). Dreizen et al (1969) reported the same result in scorbutic marmosets. Minor alterations were seen in the alveolar bone and gingival fibres, with the main destruction occurring in periodontal fibres. The progress of periodontal disease was invariably related to the presence of local irritants. It has also been reported that the vitamin C deficient volunteers showed more rapid alveolar crest resorption than the control group using radiographic asessment (Thomas et al, 1962).

Hunt and Paynter (1959) claimed that guinea pigs fed 5 mg of ascorbic acid daily showed no microscopic changes, while those given 0.4 mg daily for 75 days of feeding had mild changes in the pattern of alveolar bone deposition and remodelling. The findings of the present study are consistent with those of Hunt and Paynter. The minor resorption of alveolar bone in the control group with ligatures presumably resulted from ligature-induced periodontitis.

From these results in the animal experiment it is concluded that the low gingival ascorbate concentration associated with chronic vitamin C deficiency makes the periodontium more vulnerable to local irritants, leading to progressive inflammation and advanced periodontal disease.

The results from human study were different from the animal experiment since it was not possible to control environmental factors such as smoking, alcohol consumption, and stress from everyday life. The plasma ascorbate concentrations of the patients varied widely from 0.37 to 2.6 mg%, which was similar to previous reports (Burrill, 1942; Glickman and Dines, 1963). Patient plasma ascorbates reflected vitamin C intakes, as recorded by a dietary recall survey, but there was no plateau effect above 100 mg per day,

as has been suggested by Ginter (1979).

A lower plasma ascorbate concentration was observed in smokers, which confirmed the observations of others (Brook and Glimshaw, 1968; Pelletier, 1975). The clinical manifestation was consistent with the biochemical result and the tissue response improved with 250 mg of ascorbic acid supplementation daily, along with conservative periodontal treatment. A lower plasma ascorbate level was also evident in males than females in this study, although this difference was not statistically significant. A lower plasma ascorbate level is also commonly found in the aged (Burr et al, 1974; Vir and Love, 1979; Schorah et al, 1981), while periodontal disease is also commonly found in adults over 35 years of age. However, in the present study the 15% of patients with plasma concentrations lower than 0.7 mg% were all smokers.

The gingival ascorbate concentrations in the patients correlated poorly with the plasma ascorbate levels, which confirmed previously published findings by Glickman and Dines (1963). They found gingival ascorbate levels twice the plasma ascorbate levels in gingivitis patients. In the present study there was a ten-fold increase in gingival ascorbate over plasma concentration. This may reflect a difference between the patients studied, or differences in the methods used. Susceptibility of ascorbic acid to break down and loss prior to and during assay is a significant problem with some analytical procedures.

The gingival ascorbate concentrations in the patients in this study showed considerable variation due to location and type of biopsies, i.e. gingivectomy, flap operation. However, the gingival ascorbate concentrations from different locations were not significantly different. Although the gingival ascorbate content did not correlate well with the plasma ascorbate content, it tended to be high when the plasma ascorbate levels were high, except for

some patients with deep periodontal pockets. These patients with periodontal pocket depth < 9 mm had very low gingival ascorbate levels despite normal plasma ascorbate levels. It seems that the gingival ascorbate content decreased with the progress of the disease. This confirms the hypothesis that localized vitamin C deficiency develops in chronic inflammatory periodontal disease.

It is more difficult for smokers (Pelletier, 1975; Kallner et al, 1981), alcohol users (Bonjour, 1979; Fazio et al, 1981), oral contraceptive users (Weininger and King, 1982), or people taking drugs such as aspirin (Basu, 1982), and some antibiotics (Ringsdorf et al, 1980) to obtain an adequate vitamin C intake. Furthermore, the body ascorbate level could be affected by the microbiological degradation of ascorbate and incomplete absorption by the gastrointestinal tract of single large doses of vitamin C. Therefore, a daily recommended allowance of 30-40 mg of ascorbic acid seems insufficient under many circumstances. As shown in Table 12, it has been claimed that ascorbate tissue saturation occurs with intakes of approximately 75 to 150 mg daily with plasma levels of 1.4 mg% (Ginter, 1979), while similar intakes in the healthy elderly population maintained plasma ascorbate levels at 1.0 mg% (Garry et al, 1982).

The recommended dietary allowance of 45 mg of ascorbic acid daily for healthy people does not account for losses of nutrients during processing and preparations of foods, nor increased needs associated with modern life (Harper, 1975). Therefore, a person who has a plasma ascorbate above 0.3 mg% but less than 1.0 mg% could be subject to subclinical vitamin C deficiency. Recently Aurer-Kozelj et al (1982), in support of this view, demonstrated the improvement of the gingival connective tissue ultrastructure when a 70 mg supplement of ascorbic acid was given to periodontal patients to maintain plasma ascorbate levels at approximately 1.3 mg%.

The condition of subclinical vitamin C deficiency is relevant not only to periodontal disease, but also to other important degenerative diseases such as hypercholesterolemia leading to atherosclerosis, gallstone formation (Ginter, 1979), susceptibility to infectious diseases due to impairment of neutrophil function (Anderson, 1982), etc. Supplementation with vitamin C in such groups has led to an improvement in some of these conditions. Hence, the optimum requirement of ascorbic acid for maintaining people in good health should be reviewed.

TABLE 12

Plasma and dietary ascorbic acid correlations

Plasma ascorbate mg%	Ascorbic acid intake mg	Conditions/comments	Investigators
1.4	75 - 150	Maximum steady plasma ascorbate level (tissue saturation)	Ginter, 1979
1.3	70	Improvement of ultra-structural connective tissue	Aurer-Kozelj et al, 1982
1.0	75 - 150	Healthy elderly population	Garry et al, 1982
↑		Hypercholesterolemia, Atherosclerosis, Gall stone formation	Ginter, 1979
Subclinical vitamin C deficiency		Hyperhistaminemia	Chatterjee, 1978
↓		Periodontal disease	Alvares et al 1981
0.6	45	For healthy people without consideration of environmental factors	R.D.A. (USA) Harper, 1975
0.3	30	Prevention of scurvy	WHO/FAO
0.2	<10-20	First signs of scurvy	Sauberlich, 1975

From the data obtained in Table 12 and from data obtained from the patients in this study, it could be concluded that approximately 75 - 100 mg of ascorbic acid intake daily (approx. 1.0 mg% of plasma ascorbate concentration) is needed for good health. That is approximately double the current recommended dietary allowances. Snook et al (1983) supported this view by demonstrating the stability of leukocyte ascorbate concentration in healthy male subjects who were supplemented with approximately 70 mg daily. Recently, the RDA (1980) in America was revised and the recommended dietary allowance for vitamin C was increased to 60 mg for adults. In addition, Anderson (1975) recommended daily intakes of 100 or 200 mg of ascorbic acid except for brief periods during acute infection when larger doses could be beneficial.

Safety considerations with higher ascorbic acid dosages have been discussed (Barness, 1975; Beisel, 1982). Toxic effects associated with large intakes of vitamin C are probably insignificant, although oxaluria has been posed as a potential problem (Briggs, 1976). The possibility of oral scurvy occurring due to sudden withdrawal of megadosing can be overcome by gradual withdrawal and maintaining with a supplemental dose of 100 mg of ascorbic acid (Siegel et al, 1982).

Woolfe et al (1980) concluded that vitamin C supplementation would be of benefit when combined with local periodontal therapy. Removal of local irritation promotes the reattachment of epithelium and connective tissue with the optimum gingival ascorbate for the repair and healing process via collagen synthesis, including proper neutrophil function. Therefore, it is a valuable adjunct to therapy to use ascorbate supplementation during the course of periodontal treatment.

The data obtained from the guinea pig experiment provided information only partially relevant to man because of the differences with regard to ascorbate metabolism. Also, the clinical study provided limited information because of the shortage of patients and time. Further investigation of this problem in an animal model closer to the human, e.g. a monkey, seems justified, as does further clinical study. However, the present study does show that chronic vitamin C deficiency has the potential to be associated with periodontal disease.

Conclusions

- the gingival ascorbate level correlated well with the plasma and lymphocyte ascorbate concentrations in guinea pigs;

- the histopathological investigation of chronic vitamin C deficient guinea pigs showed that the periodontium is vulnerable to chronic inflammation and progressive ligature-induced periodontitis;

- in contrast, the human gingival ascorbate concentration was independent of the plasma ascorbate levels, suggesting vitamin C deficiency develops locally in the affected area;

- gingival ascorbate levels both in guinea pigs and in humans were found to be approximately ten-fold higher than the plasma ascorbate levels, suggesting a high requirement for the vitamin in the gingivae. An adequate vitamin C intake might be expected to provide a better gingival ascorbate content in chronic periodontal patients, provided that circulation is not totally compromised;

- The value of vitamin C in the treatment of periodontal disease relies mainly on improved host response mechanisms via neutrophil function, promotion of repair and healing via collagen synthesis, and improved microvascular integrity.

References

Aleo, J.J. (1981):
Diabetes and Periodontal disease: Possible role of vitamin C deficiency: An hypothesis.
J. Perio. 52: 251-254

Alfano, M.C. (1978):
Effect of acute ascorbic acid deficiency on the DNA content and permeability of guinea pig oral mucosal epithelium.
Arch. Oral Biol. 23: 929-932

Alfano, M.C., Drummond, J.F. and Miller, S.A. (1975):
Effect of ascorbic acid on the biosynthesis of basement membrane collagen.
J. Dent. Res. 54A: L107 (Abs. No. L428)

Alfano, M.C., Drummond, J.F. and Miller, S.A. (1975a):
Technique for studying the dynamics of oral mucosal permeability in vitro.
J. Dent. Res. 54: 194

Alfano, M.C., Drummond, J.F. and Miller S.A. (1975b):
Localization of rate-limiting barrier to penetration of endotoxins through non- keratinized oral mucosa in vitro.
J. Dent. Res. 54: 1143-1148

Alfano, M.C., Miller, S.A. and Drummond, J.F. (1975c):
Effect of ascorbic acid on the permeability and collagen biosynthesis of oral mucosal epithelium.
Ann. N.Y. Acad. Sci. 258: 253-263

Alvares,O., Altman,L., Springmeyer,S., Ensign, W.
and Jacobson, K. (1981):
The effect of subclinical ascorbate deficiency on periodontal health in non-human primates.
J. Perio. Res. 16: 628-636

Alvares, O., Ensign, W., Springmeyer, S. and Altman, L. (1980):
Periodontal disease and neutrophil (PMN) function in subclinical ascorbic acid deficiency.
J. Dent. Res. 59A: 296 (Abs. No. 114)

Alvares, O., and Siegel, I. (1981):
Permeability of gingival sulcular epithelium in the development of scorbutic gingivitis.
J. Oral. Path. 10: 40-48

Anderson, R., Oosthuizen, R., Maritz, R., Theron, A.
and Van Rensberg, A. (1980):
The effect of increasing weekly doses of ascorbate on certain cellular and humoral immune functions in normal volunteers.
Am. J. Clin. Nutr. 33: 71-76

Anderson, R. (1982):
Effects of ascorbate on normal and abnormal leucocyte function:
in vitro and in vivo.
Int. J. Vit. Nutr. Res. Suppl 23: 23-34

Anderson, T.N. (1975):
Large scale trials of vitamin C.
Ann. N.Y. Acad. Sci. 258: 498-503

Anonymous (1979):
The role of ascorbic acid in the hydroxylation of peptide bound proline.
Nutr. Rev. 37: 26-28

Attstrom, R. (1971):
Study on neutrophil polymorphonuclear leucocytes at the dentogingival junction in gingival health and disease.
J. Perio. Res. Suppl 8: 7-15

Aurer-Kozelj, J., Kralj-Klobucar, N., Buzina, R. and Bacic, M. (1982):
The effect of ascorbic acid supplementation of periodontal tissue ultrastructure in subjects with progressive periodontitis.
Int. J. Vit. Nutr. Res. 52: 333-341

Baehni, P., Tsai, C., Norman, M., Stoller, N., McArther, W.H. and Taichman, N.S. (1979):
Studies of host responses during experimental gingivitis in humans.
J. Perio. Res. 14: 279-288

Baird, I., Hughes, R., Wilson, H., Davies, J. and Howard, A. (1979):
The effect of ascorbic acid and flavonoids on the occurence of symptoms normally associated with the common cold.
Am. J. Clin. Nutr. 32: 1686-1690

Banic, S. (1982):
Immunostimulation by vitamin C.
Int. J. Vit. Nutr. Res. Suppl No. 23: 49-52

Barnes, M.J. (1975):
Function of ascorbic acid in collagen metabolism.
Ann. N.Y. Acad. Sci. 258: 264-277

Barness,L.A. (1975):
Safety considerations with high ascorbic acid dosage.
Ann. N.Y. Acad. Sci. 258: 523-527

Basu, T.K. (1982):
Vitamin C - Aspirin interactions.
Int. J. Vit. Nutr. Res. Suppl 23: 83-90

Beisel, W.R. (1982):
Single nutrients and immunity - vitamin C.
Am. J. Clin. Nutr. Suppl 35(2): 423-428

Bissada, N.F., Manouchehr-Pour, M. and Spagnuolo, P.J. (1982):
Neutrophil function activity in juvenile and adult onset diabetic patients with mild and severe periodontitis.
J. Perio Res. 17: 500-502

Blockly, C.H. and Baenziger, P.E. (1942):
An investigation into the connection between vitamin C content of the blood and periodontal disturbances.
Brit. Dent. J. 3: 57-62

Bonjour, J.P. (1979):
Vitamins and alcoholism I. ascorbic acid.
Int. J. Vit. Nutr. Res. 49: 434-441

Briggs, M. and Briggs, M. (1972):
Vitamin C requirements and contraceptives.
Nature. 238: 277

Briggs, M. (1976):
Vitamin C-induced hyperoxaluria.
The Lancet 1: 154

Brook, M. and Grimshaw, J.J. (1968):
Vitamin C concentration of plasma and leukocytes as related to smoking habit, age and sex of humans.
Am. J. Clin. Nutr. 21: 1254-1258

Burr, M.L., Elwood, P.C., Hole, D.J., Hurley, R.J. and Hughes, R.E. (1974):
Plasma and leukocyte ascorbic acid levels in the elderly.
Am. J. Clin. Nutr. 27: 144-151

Burrill, D. Y. (1942):
Relationship of blood plasma vitamin C level to gingival and periodontal disease.
J. Dent. Res. 21: 353-363

Butler, A.M. and Cushman, M. (1940):
Distribution of ascorbic acid in the blood and its nutritional significance.
J. Clin. Invest. 19: 459

Castelli, A., Martorana, G.E., Meucci, E. and Bonetti, G. (1982):
Vitamin C in normal human mononuclear and polymorphonuclear leukocytes.
Acta. Vitaminol. Enzymol. 4: 189-196

Cianciola, L.J., Genco, R.J., Patters, M.R., McKenna, J. and Van Oss, C.J. (1977):
Defective polymorphonuclear leucocyte function in a human periodontal disease.
Nature, 265: 445-447

Chalmers, T.C. (1975):
Effects of ascorbic acid on the common cold: an evaluation of the evidence.
Am. J. Med. 58: 532-536

Chatterjee, I.B. (1978):
Ascorbic acid metabolism.
Wld. Rev. Nutr. Diet. 30: 69-87

Chatterjee, I.B., Das Gupta, S., Majumder, A.K., Nandi, B.K.and Subramanian, N. (1975a):
Effect of ascorbic acid on histamine metabolism in scorbutic guinea pigs.
J. Physio. 251: 271-279

Chatterjee, I.B., Majumder, A.K., Nandi, B.K. and Subramanian, N. (1975b):
Synthesis and some major functions of vitamin C in animals.
Ann. N.Y. Acad. Sci. 258: 24-47

Chatterjee, I.B., Kar, N.C., Ghosh, N.C. and Guha, B.C. (1961):
Aspects of ascorbic acid biosynthesis in animals.
Ann. N.Y. Acad. Sci. 92: 36-56

Clarke, N.G., Shephard, B.C. and Hirsch, R.S. (1981):
The effects of intra-arterial epinephrine and nicotine on gingival circulation.
OS.OM.OP. 52: 557-568

Clemetson, C.A.B. (1980):
Histamine and ascorbic acid in human blood.
J. Nutr. 110: 662-668

Cloutier, R.M. (1933):
The aetiology of periodontal disease with a special consideration of the dietetic aspect.
Aust. Dent. J. 5: 784-788

Crandon, J.H., Lund, C.C. and Dill, D.B. (1940):
Experimental human scurvy.
New Eng. J. Med. 223: 353-369

De Chatelet, L.R., Cooper, M.R. and McCall, C.E. (1972):
Stimulation of the HMP's in human neutrophils by ascorbic acid: Mechanism of action.
Antimicrob. Agents. Chemotherapy. 1: 12-16

De Chatelet, L.R., McCall, C.E., Cooper, M.R. and Shirley, P.S. (1974):
Ascorbate levels in phagocytic cells.
Proc. Soc. Exp. Biol. Med. 145: 1170-1173

Dreizen, S., Levy, B.M. and Bernick, S. (1969):
Studies on the biology of the periodontium of marmosets. The effect of vitamin C on the marmoset periodontium.
J. Perio. Res. 4: 274-280

Egelberg, J. (1965):
Local effect of diet on plaque formation and development of gingivitis in dogs. I. Effect of hard and soft diets.
Odont. Revy. 16: 31-41

Evans, R.M., Currie, L. and Campbell, A. (1982):
The distribution of ascorbic acid between various cellular components of blood in normal individuals and its relation to the plasma concentration.
Brit. J. Nutr. 47: 473-482

Fazio, V., Flint, D.M. and Wahlquist, M.L. (1981):
Acute effects of alcohol on plasma ascorbic acid in healthy subjects.
Am. J. Clin. Nutr. 34: 2394-2396

Food and Nutrition Board. (1980):
Recommended dietary allowances. 9th ed. Washington, D.C. National Academy of Sciences.

Fraser, R.C., Pavlovic, S., Kurahara, C.G., Murata, A., Peterson, N., Talor, K. and Feigen, G. (1980):
The effect of variations in vitamin C intake on the cellular immune response of guinea pigs.
Am. J. Clin. Nutr. 33: 839-847

Ganguly, R., Durieux, M.F. and Waldman, R.H. (1976):
Macrophage function in vitamin C deficient guinea pigs.
Am. J. Clin. Nutr. 29: 762-765

Garry, P.J., Goodwin, J.S., Hunt, W.C. and Gilbert, B.A. (1982):
Nutritional status in a healthy elderly population: Vitamin C.
Am. J. Clin. Nutr. 36: 332-339

Ginter, E. (1978):
Marginal vitamin C deficiency, lipid metabolism and atherogenesis.
Adv. Lipid Res. 16: 167-215

Ginter, E. (1979):
Chronic marginal vitamin C deficiency: Biochemistry and Pathophysiology.
Wld. Rev. Nutr. Diet. 33: 104-141

Ginter, E., Bobek, P. and Ovecka, M. (1968):
Model of chronic hypovitaminosis C in guinea pigs.
Int. Z. Vit. Forschung. 38: 104-113

Glickman, I. (1948a):
Acute vitamin C deficiency and periodontal disease
I. The periodontal tissues of the guinea pig in acute vitamin C deficiency.
J. Dent. Res. 27: 9-23

Glickman, I. (1948b):
Acute vitamin C deficiency and periodontal disease
II. The effect of acute vitamin C deficiency upon the response of the periodontal tissues of the guinea pig to artificially induced inflammation.
J. Dent. Res. 27: 201-210

Glickman, I., and Dines, M.M. (1963):
Effect of increased ascorbic acid blood levels on the ascorbic acid level in treated and non-treated gingiva.
J. Dent. Res. 42: 1152-1158

Goetzl, E.J., Wasserman, S.I. Gigli, I. and Austen, K.F. (1974):
Enhancement of random migration and chemotactic response of human leukocyte by ascorbic acid.
J. Clin. Invest. 53: 813-818

Golub, L.M. (1973):
The effect of ascorbic acid on the turnover of collagen in bone in tissue culture.
J. Perio. Res. 8: 71-78

Harper, A.E. (1975):
The recommended dietary allowances for ascorbic acid.
Ann. N.Y. Acad. Sci. 258: 491-495

Hausmann, E., Raisz, L.G., and Miller, W.A. (1970):
Endotoxin: Stimulation of bone resorption in tissue culture.
Science. 168: 862-864

Hornig, D. (1975):
Distribution of ascorbic acid, metabolites and analogues in man and animals.
Ann. N.Y. Acad. Sci. 258: 103-117

Hughes, R.E., Hurley, R.J. and Jones, P.R. (1971a):
Retention of ascorbic acid by the guinea pig eye-lens.
Exp. Eye. Res. 12: 39-43

Hughes, R.E., Hurley, R.J. and Jones, P.R. (1971b):
The retention of ascorbic acid by guinea pig tissues.
Brit. J. Nutr. 26: 433-438

Hunt, A.M. and Paynter, K.J. (1959):
The effects of ascorbic acid deficiency on the teeth and periodotal tissues of guinea pigs.
J. Dent. Res. 38: 232-243

Kallner, A.B., Hartmann, D. and Hornig, D.H. (1981):
On the requirements of ascorbic acid in man: steady state turnover and body pool in smokers.
Am. J. Clin. Nutr. 34: 1347-1355

Keith, M.O., and Pelletier, O. (1974):
Ascorbic acid concentrations in leukocytes and selected organs of guinea pigs in response to increasing ascorbic acid intake.
Am. J. Clin. Nutr. 27: 368-372

Kelstrup, J. and Theilade, E. (1974):
Microbes and periodontal disease.
J. Clin. Perio. 1: 15-35

Kenney, E.B., Kraal, J.H., Saxe, S.R. and Jones, J. (1977):
The effect of cigarette smoke on human oral polymorphonuclear leukocytes.
J. Perio. Res. 12: 227-234

Kornman, K.S., Manti, F. and Goldschneider, I. (1982):
Peripheral blood lymphocyte populations in ligature-induced periodontitis.
J. Perio. Res. 17: 469-471

Kraal, J.H. and Kenney, E.B. (1979):
The response of PMN leukocytes to chemotactic stimulation for smokers and non smokers.
J. Perio. Res. 14: 383-389

Kramer, G.M., Fillios, L.S. and Bowler, E.C. (1979):
Ascorbic acid treatment on early collagen production and wound healing in the guinea pig.
J. Perio. 50: 189-192

Krasner, N., Dow, J., Moore, M.R. and Goldberb, A. (1974):
Ascorbic acid saturation and ethanol metabolism.
Lancet. II: 693-695

Lee, C.J. (1978):
Nutritional status of selected teenagers in Kentucky.
Am. J. Clin. Nutr. 31: 1453-1464

Lee, N., Hameruyik, M., Raisys, V.A. and Labbe, R.F. (1982):
Ascorbic acid in lymphocytes: cell preparation
and liquid chromatographic assay.
Clin. Chem. 28: 2165-2169

Leibovitz, B., and Siegel, B. (1978):
Ascorbic acid, neutrophil function and the immune response.
Int. J. Vit. Nutr. Res. 48: 159-164

Listgarten, M.A. and Ellegaard, B. (1973);
Experimental gingivitis in the monkeys.
J. Perio. Res. 8: 199-214

Loe, H.S., Theilade, E. and Jensen, S.B. (1965):
Experimental gingivitis in man.
J. Perio. 36: 177-187

Loe, H.S. and Wilson, C.W.M. (1971):
Relationship between leucocytes and plasma ascorbic acid
concentrations.
Brit. Med. J. 3: 733-755

Lowry, O.H., Bassey, O.A., Brook, M.J. and Lopez, J.A. (1946):
The inter-relationship of dietary, serum, white blood cell and total body ascorbic acid.
J. Biol. Chem. 166: 111-119

McCall, C.E., De Chatelet, L.R., Cooper,M.R. and Ashburn, T. (1971):
The effects of ascorbic acid on bacteriocidal mechanisms of neutrophils.
J. Infect. Dis. 124: 194-198

McLeroy, V.J. and Schendel, H.E. (1973):
Influence of oral contraceptives on ascorbic acid concentrations in healthy sexually mature women.
Am. J. Clin. Nutr. 26: 191-196

Mc.Mullen, J.A., Van Dyke, T.E., Horoszewicz, H.U. and Genco, R.J. (1981):
Neutrophil chemotaxis in individuals with advanced periodontal disease and a genetic predisposition to diabetes mellitus.
J. Perio. 52: 167-172

Mallek, H. and Miller, S.A.M. (1979):
Role of ascorbic acid in barrier function of human crevicular gingiva.
J. Dent. Res. 58(A): 351 (Abs. No. 1039)

Mergenhagen, S.E. (1970):
Complement as a mediator of inflammation: Formation of biologically active products after interaction of serum complement with endotoxins and antigen-antibody complexes.
J. Perio. 41: 202-204

Messer, H.H. (1972):
Dentin and bone formation in scorbutic monkeys.
J. Dent Res. 51: 1106-1107

Navia, J.M. and Hunt, C.E. (1976):
Nutrition, nutritional disease and nutritional research applications. "The biology of the guinea pig." eds. Wagner, J.E. and Manning, P.J. Acad. Press. N.Y., Sanfrancisco, London.

Navia, J.M. and Lopez, H. (1973):
A purified gel diet for guinea pigs.
Lab. Anim. Sci. 23: 111-114

Norman, M.E., Baehni, P.C., Stoller, N., McArthur, W.P. and Taichman, N.S. (1979):
Studies of host responses during experimental gingivitis in humans.
II Changes in acute phase reactants, serum immunoglubulins and complement during the development of gingival inflammation.
J. Perio. Res. 14: 361-369

Nisengard, R.J. (1977):
The role of immunology in periodontal disease.
J. Perio. 48: 505, 1977.

Odumosu, A. (1982):
Ascorbic acid and cortisol metabolism in hypovitaminosis C guinea pigs.
Int. J. Vit. Nutr. Res. 52: 175-184

Odumasu, A. and Wilson, C.W.M. (1971):
Metabolic availability of ascorbic acid in female guinea pigs.
Brit. J. Pharmacol. 42: 637P-638P

Ostergaard, E., and Loe, H. (1975):
The collagen content of skin and gingival tissues in ascorbic acid deficient monkeys.
J. Perio. Res. 10: 103-114

Page, R.C., and Ammons, W.F. (1974):
Collagen turnover in the gingiva and other mature connective tissues of the marmoset: saguinus oedipus.
Arch. Oral. Biol. 19: 651-658

Pauling, L. (1970):
Evaluation and need for ascorbic acid.
Proc. Nat. Acad. Sci. 67: 1643-1648

Pauling, L. (1976):
For the best of health - how much vitamin C do you need?
Penn. Dent. J. 43: 53

Pelletier, O. (1968):
Smoking and vitamin C levels in humans.
Am. J. Clin. Nutr. 21: 1259-1267

Pelletier, O. (1975):
Vitamin C and cigarette smokers.
Ann. N.Y. Acad. Sci. 258: 156-167

Perlitsh, M.J., Nielson, A.G. and Stanmeyer, W.R. (1961):
Ascorbic acid plasma levels and gingival health in personnel wintering over in Antarctica.
J. Dent. Res. 40: 789-799

Peterkofsky, B. (1972a):
Regulation of collagen secretion by ascorbic acid in 3T3 and chick embryo fibroblasts.
Biochem. and Biophy. Res. Comm. 49: 1343-1350

Peterkofsky, B. (1972b):
The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts.
Arch. Biochem. Biophy. 152: 318-328

Priest, R.E. (1970):
Formation of epithelial basement membrane is restricted by scurvy in vitro and is stimulated by vitamin C.
Nature. 225: 744-745

Prinz, W., Bortz, R., Bregin, B. and Horsch, M. (1977):
The effect of ascorbic acid supplementation on some parameters of the human immunological defence system.
Int. J. Vit. Nutr. Res. 47: 248-257

Ringsdorf, W.M. and Cheraskin, E. (1982):
Vitamin C and human wound healing.
Oral. Surg. 53: 231-236

Ringsdorf, W.M., Cheraskin, E. and Medford, F.H. (1978):
What does vitamin C have to do with the utilisation and tolerance of drugs?
J. Oral. Med. 33: 132-133

Ringsdorf, W.M., Cheraskin, E. and Medford, F.H. (1980):
Vitamin C and antibiotics.
J. Oral. Med 35: 14-17

Rippin, J.W. (1976):
Collagen turnover in the periodontal ligament under normal and altered functional forces I. young rat molars.
J. Perio. Res. 11: 101-107

Rippin, J.W. (1978):
Collagen turnover in the periodontal ligament under normal and altered functional forces. II. Adult rat molars.
J. Perio. Res. 13: 149-154

Rivers, J.M. and Devine, M.M. (1972):
Plasma ascorbic acid concentration and oral contraceptives.
Am. J. Clin. Nutr. 25: 684-689

Roe, J.A. and Kuether, C.A. (1943):
The determination of ascorbic acid in whole blood and urine through the 2,4 dinitrophenylhydrazine derivative of dehydroascorbic acid.
J. Biol. Chem. 147: 399-407

Russell, A.L. (1963):
International nutrition surveys: A summary of preliminary dental findings.
J. Dent. Res. 42: 233-244

Russell, A.L., Leatherwood, E.C., Consolazio, C.F. and Van Reen, R. (1965):
Periodontal disease and nutrition in South Vietnam.
J. Dent. Res. 44: 775-782

Sandler, J.A., Gallin, J.I. and Vaughan, M. (1975):
Effect of serotonin, carbamylcholine and ascorbic acid on leukocyte cyclic GMP and chemotaxis.
J. Cell. Biol. 67: 480-484

Sauberlich, H.E. (1975):
Vitamin C status: Methods and findings.
Ann. N.Y. Acad. Sci. 258: 438-449

Schorah, C.J., Zemroch, P.J., Sheppard, S. and Smithells, R.W.(1978):
Leukocyte ascorbic acid and pregnancy.
Br. J. Nutr. 39: 139-149

Schorah, C.J., Tormey, W.P., Brooks, G.H., Robertshaw, A.M., Young, G.A., Talukder, R. and Kelly, J.F. (1981):
The effect of vitamin C supplements on body weight, serum proteins and general health of an elderly population.
Am. J. Clin. Nutr. 34: 871-876

Shannon, I.L. (1973):
Significant correlations between gingival scores and ascorbic acid status.
J. Dent. Res. 52: 394

Sharma, S.G., Molloy, A., Walzman, M. and Bonnar, J. (1981):
Levels of total ascorbic acid and histamine in the blood of women during the third trimester of pregnancy.
Int. J. Vit. Nutr. Res. 51: 266-273

Shephard, B.C. and Clarke, N.G.
The effects of epinephrine and nicotine upon gingival blood flow.
In Press

Shilotri, P.G. (1977a):
Glycolytic, hexose monophosphate shunt and bactericidal activities of leukocytes in ascorbic acid deficient guinea pigs.
J. Nutr. 107: 1507-1512

Shilotri, P.G. (1977b):
Phagocytosis and leukocyte enzymes in ascorbic acid deficient guinea pigs.
J. Nutr. 107: 1513-1516

Shilotri, P.G. and Bhat, K.S. (1977)
Effect of mega doses of vitamin C on bacteriocidal activity of leukocytes.
Am. J. Clin. Nutr. 30: 1077-1081

Siegel, B. (1974):
Enhanced interferon response to Murine leukemic virus by ascorbic acid.
Infect. Immun. 10: 409-410

Siegel, B. (1975):
Enhancement of interferon production by poly (rI) . poly(rC) in mouse cell cultures by ascorbic acid.
Nature. 254: 531-532

Siegel, B. and Morton, J.I. (1977):
Vitamin C and the immune response.
Experimentia. 33: 393-395

Siegel, C., Barker, B. and Kunstadter, M. (1982):
Conditioned oral scurvy due to megavitamin C withdrawal.
J. Perio. 53: 453-455

Snedecor, G.W. and Cochran, W.G. (1969):
Statistical methods. 6th ed.
The Iowa State Uni. Press, Ames, Iowa.

Snook, J.T., London, C.P. and DeLany, J.P. (1983):
Supplementation freuency and ascorbic acid status in adult males.
Am. J. Clin. Nutr. 37: 532-539

Socransky, S.S. (1970):
Relationship of bacteria to the aetiology of periodontal disease.
J. Dent. Res. 49: suppl no 1., 203-222

Srikantia, S.G., Mohanram, M. and Krisnaswamy, K. (1970):
Human requirement of ascorbic acid.
Am. J. Clin. Nutr. 23: 59-62

Stankova, L., Gerhardt, N.B., Nagel, L. and Bigley, R.H. (1975):
Ascorbate and phagocyte function.
Infect. Immun. 12: 252-256

Sulkin, N.M. and Sulkin, D.F. (1975):
Tissue changes induced by marginal vitamin C deficiency.
Ann. N.Y. Acad. Sci. 258: 317-327

Taichman, N.S., Tsai, C.C., Baehni, P.C., Stoller, N. and McArthur, W.P. (1977):
Interaction of inflammatory cells and oral micro-organisms. IV. In vitro release of lysosomal constituents from polymorphonuclear leucocytes exposed to supragingival and subgingival plaques.
Infect. Immun. 16: 1013-1023:

Ten State Nutrition Survey. (1968-1970):
Dept. of Health, Education and Welfare.
Publication No. (HSM) 72-8131, 72: 8130-8134

Thomas, W. and Holt, P. (1978):
Vitamin C and immunity: an assessment of the evidence.
Clin. Exp. Immunol. 32: 370-379

Thomas, S. and Corden, M. (1970):
Table of composition of Australian foods.
Commonwealth Dept. of Health, Aust. Govt. Pub. Serv. Canberra.

Thomas, A.E., Busby, M.C., Ringsdorf, W.M. and Cheraskin, E. (1962):
Ascorbic acid and alveolar bone loss.
Oral Surg. 15: 555-565

Thyberg, J. and Nilsson, J. (1982):
Effect of nicotine on endocytosis and intracellular degradation of horseradish peroxidase in cultivated mouse peritoneal macrophages.
Acta. Path. Microbiol. Immunol. Scand. Sect. A. 90: 305-310

Vallance, S. (1979):
Leukocyte ascorbic acid and the leukocyte count.
Br. J. Nutr. 41: 409-411

Van Dyke,T.E., Horeszewicz, H.U., Ciancola, L.J.
and Genco, R.J. (1980):
Neutrophil chemotaxis dysfunction in human periodontitis.
Infect. Immun. 27: 124-132

Van Dyke, T.E., Levine, M.J., Al-Hashemi, I.
and Genco, R.J. (1982):
Periodontal diseases and impaired neutrophil function.
J. Perio. Res. 17: 492-494

Veen-Baigent, M.J., Ten Cate, A.R., Bright-See, E.
and Rao, A.V. (1975):
Effects of ascorbic acid on health parameters in guinea pigs.
Ann. N.Y. Acad. Sci. 258: 339-354

Vir, S.C. and Love, A.H.G. (1979):
Nutritional status of institutionalised and non-institutionalised aged in Belfast, Northern Ireland.
Am. J. Clin. Nutr. 32: 1934-1947

Waerhaug, J. (1958):
Effect of C-avitaminosis on the supporting structure of the teeth.
J. Perio. 29:87-97

Weininger, J. and King J.C. (1982):
Effect of oral contraceptive agents on ascorbic acid metabolism in the rhesus monkey.
Am. J. Clin. Nutr. 35: 1408-1416

Wilde, G., Cooper, M. and Page, R.C. (1977):
Host tissue response in chronic periodontal disease VI. The role of cell-mediated hypersensitivity.
J. Perio. Res. 12: 179-196

Woolfe, S.N., Hume, W.R. and Kenney, E.B. (1980):
Ascorbic acid and periodontal disease: A review of the literature.
J. Western Soc. Periodont/Periodont. Abs. 28: 44-56

Zannoni, V.G., Flynn, E.J. and Lynch, M. (1972):
Ascorbic acid and drug metabolism.
Biochem. Pharmacol. 21: 1377-1392

Zannoni, V.G. and Lynch, M. (1973):
The role of ascorbic acid in drug metabolism.
Drug. Metab. Rev. 2: 57-69

Zannoni, V.G., Lynch, M., Goldstein, S. and Sato, P. (1974):
A rapid micromethod for the determination of ascorbic acid in plasma and tissues.
Biochem. Med. 11: 41-48

Zannoni, V.G. and Sato, P.H. (1975):
Effects of ascorbic acid on microsomal drug metabolism.
Ann. N.Y. Acad. Sci. 258: 119-131

Zeman, F.J. and Wilber, C.G. (1965):
Hematology in the normal male guinea pig.
Life Sciences. 4: 871-883