FRACTIONATED IRRADIATION OF SALIVARY GLANDS
Loss and Protection of Function

Ulrika Funegård

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Loss and Protection of Function

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ABSTRACT


Radiotherapy of malignancies in the head and neck often involves the major and minor salivary glands in the radiation field. Adverse effects, such as dry mouth symptoms, are common after such therapy. The aim of this thesis was to study longitudinal effects of fractionated irradiation to the head and neck on salivary gland function in man and in the rat and to test radioprotection from antioxidant vitamins (retinol, α-tocopherol and β-carotene).

A sharp decrease in parotid saliva flow rate was seen after one week of irradiation in cancer patients, but in some patients recovery was seen two months after completed treatment. Loss and recovery of salivary gland function were dependent on the total dose given. Irradiation with doses of ≥65 Gy to the parotid glands led to permanent loss of function in the majority of parotid glands, while recovery could be seen after irradiation with doses of ≤52 Gy. Concentrations of some proteins and electrolytes in saliva were increased during irradiation but no changes remained 18 months after radiotherapy. However, large inter-individual differences were seen.

The irradiation effects observed in rats did not greatly differ from those seen in humans, but no recovery of salivary gland function was seen. On the one hand the impairments of saliva flow and composition were dose and time dependent, but on the other hand the response pattern differed between salivary components. Morphological alterations were not seen in the rat salivary glands after two or five weeks but 26 weeks after irradiation. No single model seems to be optimal for studying all parameters. Therefore, to study effects on salivary glands after irradiation the rat model must be adjusted to meet the questions addressed.

Supplementation with vitamin A provided no radioprotection, whereas it was found that supplementation with α-tocopherol (3.4 mg/day) and β-carotene (6 mg/day) during irradiation reduced the degree of inflammation and partly preserved salivary gland function. It did not, however, lead to morphometrically detectable differences in proportions of acinar or ductal cells or stroma.

Keywords: Irradiation, salivary glands, saliva composition, antioxidants, radioprotection.

Ulrika Funegård, Department of Cariology, Umeå University, S-901 87 Umeå, Sweden
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Umeå 1995
To Maria, Peter and Anna
ABSTRACT


Radiotherapy of malignancies in the head and neck often involves the major and minor salivary glands in the radiation field. Adverse effects, such as dry mouth symptoms, are common after such therapy. The aim of this thesis was to study longitudinal effects of fractionated irradiation to the head and neck on salivary gland function in man and in the rat and to test radioprotection from antioxidant vitamins (retinol, α-tocopherol and β-carotene).

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Ulrika Funegård, Department of Cariology, Umeå University, S-901 87 Umeå, Sweden
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PREFACE

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


INTRODUCTION

X-rays were identified by Roentgen in 1895 and radioactivity by Becquerel in the following year. It was recognised early on that normal tissues were affected by radiation. The recognition of these effects on normal skin was shortly followed by the suggestion that malignancies could be treated with X-rays. Radiotherapy as a way of treating malignancies was thus developed only a short while after the discovery of ionizing radiation. Radiotherapy was first used in the treatment of a cancer patient as early as in January 1896 (Grubbé 1933). The development of fractionated schemes for irradiation came in the early 1930s (Coutard 1934). In order to increase the antitumoural effects or therapeutic ratio various radiotherapy schedules have developed during recent years. One fraction per day, five days per week is routinely used but today there are alternative schedules such as treatment twice a day, thus shortening the overall treatment time (Parsons et al. 1988; Mittal 1991; Peters et al. 1992).

The application of irradiation in the treatment of head and neck cancer leads to adverse effects including salivary gland dysfunction. In the present thesis such side effects have been studied in irradiated patients and in experimental situations in the rat.

Cancer of the head and neck

Cancer of the head and neck includes cancers of the lips, oral cavity, the oro-, naso-, and hypopharynx, the larynx, the nasal and paranasal sinuses, the neck, ear, and salivary glands, as well as those of regional soft tissues and supporting bones. Squamous cell carcinoma occurs in over 90% of the cases but other neoplasms like sarcomas of any type and adenocarcinomas of major or minor salivary glands may occur. The five-year survival rates for patients with cancer in the head and neck region without any metastatic tumours vary between 50% and 90%, depending on the site and size of the tumour.

More than 40,000 cancer cases are diagnosed in Sweden each year (Swedish Cancer Registry 1991). Approximately 2.5% of these are malignant tumours in the head and neck region. Cancers in the oral cavity and pharynx together represented 1.8% (2.6% in men and 1.0% in women) of all cancers reported during the period 1960–1989 (Östman et al. 1995), which corresponded to approximately 570 diagnosed oral and pharyngeal cancers in Sweden per year (Östman et al. 1995). The relative frequency of oral cancer varies from 0.1% to over 40% in
different populations (Pindborg 1977). The lowest relative frequency is reported in Japan and Western Europe and the highest in India.

The prevalence of oral cancer increases with age. In the industrialised countries 98% of patients with oral cancer are over 40 years of age (Krolls and Hoffman 1976; Langdon et al. 1977). Squamous cell carcinomas of the head and neck are approximately twice as common in men as in women, although an increasing incidence has been reported for women. The male/female ratio for cancer incidence in the lip is 3:1, while the incidence for tongue cancer is equal for both genders (Swedish Cancer Registry, 1991). The aetiology of oral cancer is mostly unknown but lifestyle factors such as the use of tobacco and abuse of alcohol as well as nutritional deficiencies enhance the risk of developing oral malignancies (Mc Laughlin et al. 1988). Individuals who abuse both tobacco and alcohol are up to 100 times more likely to develop squamous cell carcinomas in the aerodigestive tract than non-users (Brugere et al. 1986).

Apart from solid tumour cells which grow together and form a solid mass, others which are able to move freely in blood or in the lymphatic system, such as in leukaemias and lymphomas, may be manifested in the head and neck region. Approximately 200 patients with Mb Hodgkin and 1,300 with non-Hodgkin lymphoma are reported yearly (Swedish Cancer Registry, 1991). In more than 80% of patients with Mb Hodgkin the cervical lymph nodes are involved and mantle field irradiation is the treatment given.

**Classification of cancer**

The TNM system is widely used to classify the extent of the tumour, condition of the regional lymph nodes and the distant metastasis at various cancer sites (Rubin et al. 1992b). T refers to the extent of the primary tumour, N to the condition of the regional lymph nodes and M to the presence of distant metastases. T0 to T4 defines a primary tumour according to surface spread, depth of invasion, and size. E.g. in the classification of solid tumours T0 means no extent of tumour, T1 usually means a localised mobile tumour smaller than 2 cm in diameter, T2 a partial mobile tumour 2 to 4 cm in diameter, T3 a fixed tumour smaller than 10 cm and T4 a massive destructive lesion that is bigger than 10 cm in diameter and is not confined to the region. The condition of the regional lymph nodes (N) is described by size, firmness, capsular invasion, depth of invasion, mobility or fixation, involvement of single or multiple nodes, ipsilateral, contralateral, bilateral or distant nodes. N0 stands for no evidence of disease in lymph
nodes and N1 to N4 for involvement of lymph nodes. The presence or absence of distant metastases are indicated by M1 and M0, respectively.

Cancer management

Cancer therapy is concerned with the removal or killing of the cancer cells and the halting of further proliferation. The main forms of treatment are surgery, chemotherapy, radiotherapy and immunotherapy which can all be used alone or in combination. The intention may be either curative or palliative.

Irradiation is an important treatment modality for malignancies in the head and neck (Rubin and Doku 1976). Today more than 50% of all diagnosed cancers are considered for radiotherapy which is given to 50% with a curative intent and to 50% with a palliative intent (Hoskin 1994), e.g. to restrict the tumour and decrease the pain from cancers that cannot be cured. More than 1,400 patients are treated with radiotherapy each year in Umeå and of these about 100 patients per year receive radiation to the oral region. The majority of the malignancies treated with radiotherapy to the head and neck are squamous cell carcinoma, Mb Hodgkin and non-Hodgkin lymphoma.

There are in general two principal routes for the administration of radiotherapy; external beam therapy and brachytherapy. External beam treatment is the most common form of radiotherapy. It is normally performed with photon beams which are usually high energy X-rays produced by a linear accelerator, but gamma ray beams from cobalt units and lower-energy X-rays within the 50—300 kV energy range may also be used. In addition, megavoltage electron beams with an improved geometrical precision compared to photons are used to treat relatively superficial tumours.

Radiotherapy of malignancies in the head and neck will include the major and minor salivary glands to various degrees depending mainly on the location of the tumour (Wang 1992). The radiation dose affecting the salivary glands is also of most importance (Franzén et al. 1988). Tumours in the head and neck are usually treated with fractionated irradiation 1.8—2 Gy per day, five days a week, and with a total dose to the tumour of 64—68 Gy which is the maximal acceptable dose by the normal tissue. Lower total doses, e.g. 47—52 Gy, have been used for preoperative treatment or when radiotherapy is given in combination with chemotherapy. In addition, patients with Mb Hodgkin and non-Hodgkin lymphomas are treated with mantle field including all the major lymph node
regions above the diaphragm, as well as all major salivary glands (Kaplan 1985). The total dose is 40 to 44 Gy, given in five fractions per week with a daily target dose of 1.8–2.0 Gy per day.

Effects on healthy tissue
Irradiation treatment also includes influences on healthy tissues. The tumour mass is surrounded by healthy tissues through which it may be necessary to direct the radiation beam. Normal tissue effects may be dose-limiting.

All cells can be killed by ionizing radiation, but the dose required to achieve a particular level of cell death varies (Fig. 1). Thus, radiosensitivity differs among cell types and proliferating tissues are more radiosensitive than quiescent cells (for a review see Denekamp 1986). The radiation effects depend on dose and time factors (Thames et al. 1990). Fractionating the total dose will spare most tissues.

Figure 1. Dose-response curves for tumour and normal tissues.
There are no morphological features of radiation damage. However, radiation effects on epithelial cells in the mucosa (Fajardo 1992) are seen a few hours after irradiation. Mitotic activity decreases or stops and cell necrosis occurs progressively. The cell becomes swollen and vacuoles may be seen. Repopulation follows six to eight hours after irradiation and atypical mitosis is usually observed. Later, fatty tissues will be replaced by collagen and fibroblasts (Rubin et al. 1992a). Vascular lesions are first seen in the capillaries and sinusoids. Dilatation and asymmetry can be observed by light microscopy. More subtle alterations may be observed by electron microscopy e.g. swelling of the cytoplasm, obstructing of the lumen, detachment of endothelial cells and rupture of the walls (Fajardo and Berthrong 1988).

Mechanisms for irradiation damage

Radiation treatment of malignancies aims to kill the tumour cells. For this purpose electromagnetic radiation (X-, and $\gamma$-rays) or particle radiation ($\alpha$-, $\beta$-particles, protons and neutrons) can be used. Photons or electrons are most commonly used. Energy is transferred via photons or particles during irradiation. The energy in ionizing radiation is sufficient to eject an electron and ionize an atom. The events leading to cell damage are caused by both the direct and indirect effects of irradiation. The direct effect is caused by ionization or excitation of atoms that are parts of biological structures and the indirect effect by free radicals generated after interaction with the molecules in the cell, particularly water (Whitters 1994). It is suggested that approximately 70% of the damaging effect is caused by water radicals (Michaels and Hunt 1978). Two different types of cell death can be identified after irradiation; reproductive (mitotic) death and interphase death (Altman et al. 1970).

The main lethal effect causing reproductive death is thought to be the induction of double strand breaks (Radford 1986), but other targets such as the cell membranes and the microtubular system may also play a role in the cytotoxicity induced by irradiation (Alper 1977; Stephens et al. 1989a). The energy from X-rays will be distributed within the cell, absorbed equally in the nucleus, cytoplasm and membrane (Wärters et al. 1977).

DNA damage occurs through absorption of ionizing radiation in the DNA molecule itself and by free radicals produced near the sensitive targets. Single strand breaks are of little consequence to the cell since these can be efficiently repaired.
(Powell and McMillan 1990). However, if the radiation dose is high enough, two close single strand breaks can result in a double strand break.

The radiation sensitivity of a particular tissue depends on several different factors, and thus large variations in sensitivity can occur depending on e.g. the vascularisation, oxygen tension and the rate of proliferation. The sensitivity varies also within the cell cycle (Sinclair 1972). Cells in mitosis are generally most sensitive and those in S-phase (DNA synthetizing phase) are most resistant while cells in G₁ (the phase between mitosis and S-phase) and G₂ (the resting phase just before mitosis) have intermediate sensitivities. Salivary gland cells do not normally divide continuously and it is therefore suggested that they die in interphase following irradiation. Intermitotic cell death by apoptosis may thus be the cause of the early death of the secretory cells in salivary glands observed after irradiation (Stephens et al. 1986b, 1989a, 1989b, 1991).

The cell membranes are of special interest since they have been postulated as primary targets for the damage leading to interphase death (Alper 1977; Altman et al. 1970; Stephens et al. 1989a; Vissink et al. 1992; Wills and Wilkinson 1966). Hence, it is claimed that parotid acinar cells are primarily lethally damaged by disturbances in cell membrane and not directly by injury to DNA (Farber and Baserga 1969). Signs of cell membrane injury, intracellular oedema, sequestered degenerative cell membranes and an accumulation of intramitochondrial particles was seen two hours after irradiation (El Mofty and Kahn 1981). Lidocain, which is known to stabilize cell membranes, protects the acinar cell from irradiation damage in vitro (Stephens et al. 1989a).

It has been known for a long time that cells irradiated under anoxic or hypoxic conditions are less sensitive to ionizing radiation than they are in the presence of air or oxygen (Gray et al. 1953). Oxygen is the most potent chemical modifier of radiosensitivity (Gray et al. 1953) and it is claimed that radiation is approximately three times more effective when oxygen is available than without it (Littbrand and Révész 1969; Modig et al. 1974).

Salivary glands and saliva secretion

Salivary gland morphology
The salivary glands in man consist of three pairs of major glands, the parotid, the submandibular and sublingual glands, and a large number of minor salivary
glands distributed in the oral mucosa. Salivary gland morphology is briefly described by Hand (1986) and Riva et al. (1990).

The major salivary glands contain secretory end-pieces (acini) and a duct system (Fig. 2). The acini are separated from each other by connective tissue, often referred to as the interlobular stroma, which is richly vascularized and carries nonmyelinated nerve fibres, plasma cell fibroblasts and lymphocytes (Young and van Lennep 1978). A basal lamina separates the interlobular stroma from the epithelial secretory cells in the acini. An acinus consists of a spherical group of cells, connected to each other by tight junctions. They form a lumen which is the beginning of the duct system. The epithelial cells in acinus are serous or mucous secretory cells. Thus, there are serous or mucous secretory units which deliver different types of saliva. The acini are supported by contractile myoepithelial cells.

Figure 2. Schematic drawing of the parotid gland.
The acinar cell is pyramidal, contains a large nucleus, many mitochondria, extensive rough endoplasmic reticulum in a parallel arrangement in the basal half of the cell, and many densely staining secretory granules and lysozomes in the apical half of the cell (Fig. 3). The Golgi complex is usually located in the apical half of the cell or lateral to the nucleus. In the luminal part of the cell granules and vacuoles are seen. The mucous cells contain larger secretory granules which occupy much of the cell but the morphological features do not differ significantly from those in the serous cell.

Figure 3. Schematic drawing of a serous acinar cell.
The duct system consists of the intercalated ducts which are short narrow ducts connecting the acini and the striated ducts. In rodents, the duct system in the submandibular gland also contains the granular convoluted tubule, situated between the intercalated and striated ducts. Interlobular ducts drain into a system of extralobular or excretory ducts. The intercalated duct cells are smaller and cubic with centrally placed nuclei. The cells contain many mitochondria but have less endoplasmic reticulum. The membrane configuration is similar to that of the acinar cells, with small processes on the luminal wall. The striated duct consists of cylindrical epithelial cells with centrally placed nuclei. The basolateral membranes are extensively folded around a large number of mitochondria.

Mitotic activity in rat salivary glands is high during the prenatal and early postnatal periods, but in adult rats mitotic activity is rarely seen (Chang 1974; Chang and Barka 1974; Zajicek et al. 1985). The intercalated duct cell seems to supply the acinus and granular convoluted tubule with new cells during the development of the gland (Chang 1974; Zajicek et al. 1985). The progenitors for salivary gland cells seem to be the intercalated duct cells. Regenerative growth is proposed after the application of different stimuli including irradiation (Schneyer 1970). The life span of acinar and duct cells in rats is reported to be 60 to 120 days (Zajicek 1985, 1989; Schwartz-Arad et al. 1988).

**Saliva secretion**

The major salivary glands contribute about 90-95% of the total amount of saliva secreted (500-1000 ml/day), the rest being produced by the minor salivary glands (Dawes and Wood 1973). The contribution from individual glands depends on the type of stimulation. The proportion of saliva secreted by the parotid gland increases with stimulation. There are indications that the minor salivary gland cells produce a mucous secretion which forms an insoluble gel. The portion of the secretion from the minor glands can not therefore be included in the fluid secretion and their estimated contribution of 5-10% of the total secreted fluid is thus probably an overestimation.

From the acinar cells an isotonic primary saliva is secreted into the acinar lumen. Passing through the duct system Na$^+$ and Cl$^-$ are reabsorbed in the striated duct cells and small amounts of K$^+$ and HCO$_3^-$ are secreted. Some proteins are also secreted by duct cells (Hand 1990; Baum 1993; Turner 1993; Turner et al. 1993). The secondary saliva secreted into the mouth is therefore hypotonic and
contains only a third of the electrolytes in plasma and approximately 1 mg protein per ml saliva, which can be compared to 70 mg per ml in serum.

Saliva secretion is evoked by transmitters of the sympathetic and parasympathetic nerve systems as well as biologically active peptides (Baum et al. 1993). Smell and taste constitute primary stimulation of the olfactory epithelium and of taste buds which in turn trigger the stimulation of the receptors in the salivary glands described below. Touch and irritation of the oral mucosa and mechanical stimulation by chewing and speaking also stimulate secretion.

**Salivary components and their functions**

The relative proportions of different salivary constituents vary in saliva from different secreting units such as the parotid, submandibular, sublingual and minor salivary glands. The saliva contains a large number of different proteins synthesized in the acinar cells. There is normally no filtration of serum proteins into saliva. Together the secretory components have several important functions in the mouth (Mandel 1987; Kaplan and Baum 1993). Some are based on the physical properties of the protein, such as lubrication of oral tissues and the bolus during the chewing of food, facilitating speech and swallowing food. Others are based on biochemical properties of the proteins and provide biological systems for the protection of the oral mucosa and teeth against pathological changes. Among these systems are constituents with antibacterial properties which regulate both bacterial adhesion and metabolism such as acidic proline-rich proteins, immunoglobulins, non-immunoglobulin agglutinins, salivary peroxidase, lysozyme and lactoferrin. Other constituents are involved in the maintenance of ionic equilibria which protect dental hard tissues from being dissolved (Moreno and Zahradnik 1979). Such Ca-binding proteins occur with varying degrees of binding strength. Proline-rich proteins, statherin and amylase are examples of strong Ca-binders (Lamkin and Oppenheim 1993). The efficiency of these systems is dependent on a properly functioning biosynthesis and secretory mechanism.

**Salivary gland physiology**

Salivary glands are richly vascularized. The blood supply is important for their function and blood vessels follow the ducts and split so each lobulus has its own capillary blood supply. The vessels are affected by the neural system e.g. acetylcholine causes vascular dilatation and noradrenaline causes vaso-
constriction. As in other organs several other modulators of blood vessels, such as bradykinin, are of importance in the regulation. When the peripheral circulation diminishes there is an immediate loss of activity in the acinar cells, which are exclusively dependent upon the energy, water and nutrients from the blood supplied by the capillaries. Among those conditions which decrease basal metabolism we find hypothyreosis or malnutrition which will decrease the secretory capacity.

Salivary secretions are primarily regulated through the autonomic nervous system (Baum 1987). From a traditional view this system consists of the sympathetic and the parasympathetic nerves, both stimulating secretion although with different effects on the volume and composition of saliva. Acetylcholine, the classical neurotransmitter in the parasympathetic nervous system, and muscarinic-cholinergic receptors have their major influence on the release of fluid and electrolytes. In the sympathetic nervous system noradrenaline stimulates protein secretion via β-adrenergic receptors and fluid- and electrolyte secretion via α-adrenergic receptors (Henriksson 1982; Sundström et al. 1988). Other neurotransmitters which influence salivary secretion in the parasympathetic nerve system are for example vasoactive intestinal polypeptide (VIP) and substance P (Gallacher 1983; Inoue et al. 1985).

The function of the secretory cells is well controlled and efficiently regulated by the various receptors and by the prerequisite that there is an adequate supply of blood available for the cells. The well-balanced system guarantees the high degree of alertness of the secretory system under normal conditions. On the other hand this efficient system is also very vulnerable when the neurotransmitters, receptors, and blood supply carrying water, energy and nutrients to the secretory cells are impaired.

**Effects on the salivary glands by irradiation**

Salivary glands are usually involved in the treatment volume when tumours in the head and neck region are subjected to radiotherapy. The acinar cells do not normally divide, the majority of them occur in interphase, and should therefore be relatively radioresistant. However, they are reported to be very sensitive to irradiation (Shannon et al. 1978; Stephens et al. 1986a, 1986b, 1986c, 1989b, 1991; Peter 1994) while other highly differentiated glandular cells, e.g. in the pancreas, are suggested to be more radioresistant (Rubin and Casarett 1972).
The results from various studies show that 1) serous cells are more radiosensitive than mucous cells (Stephens et al. 1986a), 2) acinar cells are more sensitive than duct cells (Cherry and Glucksmann 1959; Kashima et al. 1965; Sholley et al. 1974; Abok et al. 1984) and, 3) parotid glands are more radiosensitive than submandibular and sublingual glands (El-Mofty and Kahn 1981).

It has been suggested that the sensitivity of acinar cells is due to their heavy metal granule content (Abok et al. 1984; Norberg and Lundquist 1989; Kim et al. 1991), which would increase the lipid peroxidation. These authors also report that the sensitivity of acinar cells decreases if degranulation is performed before irradiation. More recent studies (Peter et al. 1994b; 1995) could not find any protective effects from degranulation of the acinar cell and Ahlner et al. (1994) found less damage if the gland function was inhibited during irradiation.

Clinically, swelling and pain of the salivary glands are observed (Kashima et al. 1965; Parsons 1984), and it has been shown that irradiation affects both the secretory system and the vascular supply to the gland. Because of their radiosensitivity (Stephens et al. 1986a; 1986b; 1986c; Parsons 1984), the radiation dose affecting the salivary glands is of crucial clinical importance (Marks et al. 1981). It is however, reasonable to assume that the degree of damage occurring is related not only to the radiation dose but also to the volume of salivary glands that are located in the target area (Cheng et al. 1981; Mira et al. 1981; Makkonen and Nordman 1987; Karlsson 1987).

Sialographic measurements have shown a reduced parotid gland volume in irradiated patients eight months after treatment but no changes could be observed immediately after radiotherapy (Eneroth et al. 1971, 1972a).

**Saliva**

One of the most common adverse effects of irradiation to the head and neck region is the sensation of a dry mouth (Mossman and Scheer 1977; Mossman 1983; Baum et al. 1985). Dryness is already experienced during the first week of treatment (Shannon et al. 1977; Wescott et al. 1978). The decrease in flow rate during and after irradiation was evaluated in 42 patients with oral cancer (Dreizen et al. 1977a). Irradiation was given with a tumour dose of 2 Gy per day, five days per week (total dose not reported). The flow rate of stimulated whole saliva had decreased with 57% after the first week of treatment (10 Gy), with 76% after six weeks (60 Gy) and with 95% three years after radiotherapy.
Even though some patients reported a subjective improvement in the sensation of oral dryness at the 3-year follow-up, no increase in flow rate could be detected (Dreizen et al. 1977a). A decrease in saliva flow rate is seen even after a comparatively low dose to the salivary glands (10.2–17.7 Gy) after whole body irradiation to bone marrow transplant patients. Their unstimulated whole saliva flow rate decreased with 45% from 0.56 ml/min to 0.31 ml/min, and stimulated saliva flow rate with 51% from 1.89 ml/min to 0.92 ml/min (Jones et al. 1992).

A rapid and dose-dependent decrease in parotid saliva flow rate has been reported by Eneroth et al. (1972a) and reduced parotid saliva flow rate have been observed in irradiated patients up to nine years after radiotherapy (Eneroth et al. 1971; Leslie and Dische 1991; Valdez et al. 1993). A recovery of whole saliva flow rate starting 3 to 6 months after irradiation with doses ≤50 Gy are reported by Makkonen et al. (1986a). Furthermore, there are some reports which suggest that patients with high initial saliva flow rate tolerate higher radiation doses before they lose their secretion (Eneroth et al. 1972b; Wescott et al. 1978; Mira et al. 1982).

**Saliva composition**

Knowledge about irradiation effects on saliva composition is sparse. Dreizen et al. (1976) reported a significant increase in protein, sodium and calcium concentrations in whole saliva during radiotherapy. This is supported by Brown et al. (1976b, 1981) who found increased concentrations of total protein, lysozyme, IgG, IgA and albumin in saliva during the treatment period and three months after irradiation. However, three months after irradiation the secretion of total protein per minute amounted to only 6.5% of the pre-irradiation value. The concentrations of lysozyme, lactoferrin and chloride were found to be higher in irradiated patients than in controls, when evaluated two years after irradiation (Valdez et al. 1993).

After irradiation, Makkonen et al. (1986b) observed a reduction in the total protein content and Cowman et al. (1983) found qualitative but unspecified changes in protein composition in whole saliva. Mossman et al. (1981) reported a 60% decrease in the protein content of parotid saliva after the first week of irradiation (10 Gy). Whole saliva collected from irradiated patients with inflammatory soft tissue changes is also likely to contain non-secreted components, such as albumin (Brown et al. 1976b).
Irradiation with single doses of ≥10 Gy has been observed to decrease the parotid and whole saliva flow rate in the rat as early as three days after exposure (Rice et al. 1982; Bodner et al. 1984; Vissink et al. 1990a, 1990b). However, Bodner et al. (1984) found a decreased parotid saliva secretion in vivo, but no significant changes in the acinar cells were found in vitro. The function of the parotid gland has been shown to be impaired earlier and after lower irradiation doses than that of the submandibular gland. A single dose of 15 Gy reduced rat parotid saliva flow rate as early as 24 h after the exposure but no effect was seen in the submandibular saliva secretion rate (Nagler et al. 1993a). The reduction in saliva flow was paralleled by an increased phosphate concentration three days after irradiation with 10 Gy (Vissink et al. 1990b) and a decrease in sodium and an increase in potassium concentrations 1 to 30 days after irradiation with 15 Gy (Vissink et al. 1991a; Nagler et al. 1993b). Giving the radiation dose as two fractions (7.5 Gy x 2) had less effect on the saliva flow rate, sodium and potassium concentrations than one single dose of 15 Gy (Vissink et al. 1991a).

Phillips (1970) followed the effects of a well-defined single dose of 21 Gy to one parotid gland in the rat. He found a progressive decrease in parotid saliva flow rate up to four days after exposure. After eight days an increase in saliva flow occurred which remained 16 and 42 days after irradiation. A spontaneous secretion of parotid saliva was observed by Phillips (1970) five minutes after irradiation but this could not be confirmed by Nagler et al. (1993a) after a single dose of 15 Gy. Kohn et al. (1992) studied the effect of ionizing radiation on sympathetic nerve function in rat parotid gland, and their results indicated that acinar cell responsiveness to norepinephrine was reduced without affecting nerve function. In a later study Forsgren et al. (1992) found an enhanced expression of substance P in the parasympathetic innervation of the submandibular gland.

Vasculatory changes
It has been claimed that the damage seen in salivary glands after irradiation is mainly secondary to changes in the fine vasculature leading to increased permeability, interstitial oedema and perivascular inflammation (Rubin and Casarett 1972). However, the results are not consistent. Several investigators have not been able to observe any sign of capillary damage in irradiated rat salivary glands using light microscopy (Sodicoff et al. 1974; Sholley et al. 1974; Savage et al. 1985), whereas endothelial swelling and cytoplasmic vacuoles were seen
using electron microscopy three days after fractionated irradiation with 5 Gy (Savage et al. 1985). Further, no effect on blood flow could be observed three days after irradiation with 15 Gy in the parotid and submandibular gland in the rat (Hiramatsu et al. 1994) or in the submandibular gland in the rabbit four and ten months after exposure (Ahlner and Lind 1994).

Morphological changes

The effects of irradiation on salivary gland morphology are mainly studied in animals and most studies have been made with single dose irradiation. However, in order to be able to give higher total doses, Shafer (1953) compared fractionated irradiation with target doses of 5.0 or 7.5 Gy and total doses of 45 to 80 Gy in the rat. The histological changes evaluated 21 days after irradiation were similar after fractionated and single dose exposure. The conclusions drawn from animal studies are supported by one light microscopy study on human parotid and submandibular glands, showing that serous cells exhibited degenerative changes and necrosis while mucous cells were unaffected (Kashima et al. 1965).

A dose related destruction and regeneration of the salivary glands was reported early (Shafer 1953; Cherry and Glucksmann 1959). Single dose irradiation with doses between 4.8 and 35 Gy induced increasingly severe alterations in the structure of the salivary glands. The reactions were characterised by inflammation and degeneration of the parenchymal cells. The submandibular glands were more resistant than the parotid glands and displayed only minor changes 21 days after irradiation (Shafer 1953). However, the reactions in all glands were generally degenerative changes with loss of acinar cells. Regeneration was also seen with repair most marked in the sublingual glands and least evident in the parotid glands. No regeneration was detected after doses exceeding 30 Gy (Cherry and Glucksmann 1959). Later Ahlner et al. (1993) found that degenerative changes in serous tubuli of rabbit submandibular gland, present four months after irradiation, began to regenerate six months later.

Isoproterenol-induced cell proliferation in the parotid gland decreased after irradiation. The DNA content in the parotid gland decreased in a dose-dependent way after exposure (Furuno et al. 1974). This was claimed to be related mainly to inhibition of cell multiplication, although cell death could not be excluded. The reaction pattern of the parotid gland in the rat has been described by Phillips (1970), who found a reduced parotid gland weight after irradiation with 21 Gy. The acinar cells were atrophic and necrosis as well as degeneration of cell nuclei
were seen one day after exposure. The destruction was progressive with a maximum four days after irradiation. On the 8th and the 16th day after irradiation acinar cells of normal size appeared, but after 42 days all acinar cells became atrophic again. Quantitative measurements in the rat submandibular gland displayed that the proportion of acinar cells was decreased three, five and nine months after irradiation with 20 Gy (Espinal and Cabrini 1983).

At a dose of 15 Gy to the rat only minor morphological changes could be found in acinar and ductal cells one day after exposure using light microscopy (Vissink et al. 1991b). The maximal effect was seen three days after exposure. Both the cisterns of the endoplasmic reticulum and the mitochondria appeared swollen in electron microscopy. In light microscopy pyknotic nuclei, oedema in the serous cells and depletion of serous granules were observed. The glands were invaded by mononuclear cells and macrophages. The morphological changes observed were almost reversed ten days after irradiation.

Recent studies show that morphological alterations can already be found within a few hours after exposure (Stephens et al. 1991; Vissink et al. 1991b). Signs of cell membrane injury (intracellular oedema, sequestered degenerative cell membranes, and an accumulation of intramitochondrial particles) were seen in the rat parotid gland as early as two hours after irradiation with 20 Gy (El-Mofty and Kahn 1981). Twenty-four hours after irradiation necrosis of acinar cells was observed even after low doses as 2 Gy in the rat parotid gland (El-Mofty and Kahn 1981) and 2.5 Gy in the submandibular gland of the monkey (Stephens et al. 1986b).

Late effects seen in salivary glands in the rat (Cherry and Glucksmann 1959), rabbit (Ahlner et al. 1993) and monkey (Stephens et al. 1986b) are atrophy and replacement of parenchyma by fibrous tissue (El-Mofty and Kahn 1981).

Effects of irradiation on the oral mucosa and oral function

Oral mucositis is a common side effect of irradiation. Several authors have given broad presentations of acute and late effects on oral mucosa after head and neck irradiation (Rubin and Doku 1976; Dreizen et al. 1977b; Mossman and Scheer 1977; Parsons 1984; Baker 1982; Al-Tikriti et al. 1984; Kuten et al. 1986; Pyykönen et al. 1986; Maciejewski et al. 1990).
During the first or second weeks of radiotherapy either a white discoloration or redness and swelling of the gingiva and oral mucosa may occur. During the second and third weeks of treatment erythema and pseudomembranes are seen. Ulcerations occur during the third or fourth week of irradiation (Al-Tikriti et al. 1984). A scoring method based on signs of local mucositis has been suggested; 1) white discoloration, 2) erythema, 3) pseudomembranes, and 4) ulceration (Spijkervet et al. 1989). Mucosal recovery usually follows within one month but irradiation will lead to a sensitive and hypotrophic oral mucosa which is less resistant to traumatic injury.

Histologically, the mucosa initially becomes hyperkeratotic. This is followed by destruction of the basal cell layer with acute cell necrosis and loss of available replacement cells (Parsons 1984). Submucosal capillaries become engorged, which result in oedema and infiltration of leukocytes (Rubin and Doku 1976). Superficial erosions covered by a pseudomembrane of fibrin may be found during and after irradiation. Late morphological changes are submucosal fibrosis and atrophy of the squamous epithelium.

Gustatory dysfunction is commonly reported by patients during and after head and neck irradiation (Mossman and Henkin 1978; Mossman et al. 1982; Mossman 1983). The taste sensation of individual food items may be heightened, suppressed or altered (Shatzman and Mossman 1982). This may reduce the appetite, change the selection of foods, and lead to weight loss. A recovery in taste acuity is often achieved within two months after irradiation (Conger 1973).

Head and neck irradiated patients may also have difficulties in swallowing due to pain and dryness. This may affect food intake even in the longer perspective (Chencharick and Mossman 1983; Bäckström et al. 1995). Furthermore, eating ability may be reduced due to limited jaw opening following muscle fibrosis in response to radiation injury (Rubin and Doku 1976). Generally trismus develops three to six months after irradiation.

Yeasts are frequently isolated in low numbers from the oral cavity of healthy individuals (Arensdorf and Walker 1979), but during radiotherapy to the head and neck both the number of carriers and the number of colonies increase (Chen and Webster 1974). The number of patients with positive fungal cultures is reported to increase from 20-37% before radiotherapy to 49-80% after irradiation (Chen and Webster 1974; Silverman et al. 1984; Makkonen et al. 1989). In some patients increased levels are still reported eight years after radiotherapy.
Accordingly, candidiasis is the most commonly occurring oral infection during or after irradiation.

The connection between irradiation of malignancies in the head and neck region and rampant caries was recognised more than 40 years ago (Frank et al. 1965). The often dramatically enhanced caries activity is mainly a result of the impaired protection provided by saliva and a parallel shift in the oral microflora (Frank et al. 1965; Llory et al. 1972; Dreizen et al. 1976; Brown et al. 1978; Shannon 1981). The numbers of *Streptococcus mutans* and lactobacilli are reported to be enriched while *Streptococcus sanguis* decreases both in saliva and dental plaque (Llory et al. 1972; Brown et al. 1975). Preventive programmes, including fluoride application, have successfully been used to inhibit the rampant caries development (Dreizen et al. 1977a; Brown et al. 1976a; Joyston-Bechal et al. 1994).

Vascular changes will reduce the blood supply to the soft tissues, which then have a decreased vitality and ability to withstand trauma and infection. Secondary to vascular changes necrosis may occur in soft tissues, cartilage or bone, predominantly in the mandible after infection or trauma (Beumer et al. 1972). In addition, gingival recession is reported to increase after irradiation (Markitziu et al. 1992) and periodontal destruction may occur in the irradiated area (Yusof and Bakri 1993).

**The rat model**

Since the middle of the 19th century animals have frequently been used in the study of salivary gland physiology and the rat has become a commonly used experimental model. Much of the modern cell biology related to secretion is based on studies in the rat. Even though there are discrepancies between the salivary glands of man and the rat, the traits they have in common have led to an increased understanding of the basics of the mechanisms involved in secretion. Standardised techniques for collecting whole (Johansson et al. 1989) as well as gland specific secretion (Vissink et al. 1990a, 1990b; Nagler et al. 1993a, 1993b) have been described in the rat and subsequently used. One difference between man and the rat is that saliva flow in the rat must be stimulated by pharmacological or electrical means, whereas stimulation in man is normally achieved by chewing or gustatory stimulation.
Even though the reported effects of irradiation on rat salivary gland are generally consistent, there are contradictory results concerning morphological changes which may relate to experimental variations. The data from animal studies are obtained from a variety of species. The radiation dose and method of irradiation as well as the time interval until examination varies. In the vast majority of these studies irradiation has been given as a single dose. However, various fractionation schemes have also been used (Nicolatou 1981; Petrovic' et al. 1982; Norberg and Lundquist 1988). Sodicoff et al. (1977) used a radiation schedule, fractionated similar to that schedule used in the treatment of cancer in man. He found less damage on parotid gland function in the rat after fractionated irradiation than after a single exposure with the same total dose.

Free radicals

Free radicals are highly reactive metabolites produced as by-products of normal metabolism, lipid peroxidation, or exogenous exposure, e.g. smoking or radiation (Halliwell and Gutteridge 1989). A free radical is a molecule with at least one unpaired electron. It may be positively or negatively charged or neutral and its reactivity varies. Since most radicals exist only briefly because of their reactivity they normally exist in very low concentrations, $10^{-4}$ to $10^{-9}$ mol/l (Pryor 1976).

The radiation-induced reactions with intra- and extracellular water result in formation of reactive species. There are essentially three different free radicals formed in the radiolysis of water, the hydroxyl radical ($\text{OH}^\cdot$), the solvated electron ($e^-_{aq}$) and the H-atom (von Sonntag 1987).

The $\text{OH}^\cdot$ radical appears to be the most reactive, but most organic radicals react at high rates (Halliwell and Gutteridge 1989). The mean distance an $\text{OH}^\cdot$ radical would travel in a mammalian cell before reacting with its target is 6-9 nm (Roots and Okada 1975). The lifetime and range of free radicals are prolonged by oxygen (von Sonntag 1987).

Therapeutic doses of irradiation induce such high levels of free radicals that most cellular components will be damaged, such as the unsaturated bonds in membrane lipids, sulphur containing enzymes, nucleic acids, carbohydrates and DNA (Halliwell and Gutteridge 1989). Since radiotherapy is accompanied by tissue damage it is obvious that the natural free radical scavenger system is not sufficiently active.
Antioxidants

Antioxidants may restrict the damage that reactive free radicals can do to cells and cellular components. Thiols are reported to reduce some radiation damage (Alexander and Charlesby 1955) and both exogenously administered as well as cell-produced thiols protect against free radicals. The standard diet may provide antioxidative vitamins and provitamins, such as tocopherols, ascorbic acid and β-carotene (Seifert et al. 1988; Di Mascio et al. 1991; Sies et al. 1992) which also act as scavengers of free radicals.

Vitamin A

Vitamin A is a fat soluble vitamin, present in animal food. Retinol and its active metabolite, retinoic acid, have a limited capacity to scavenge free radicals in general (Burton and Ingold 1984) but a more specific function as a potent radical scavenger is reported (Daquino et al. 1989). Vitamin A is also an important regulator of cell differentiation and protein expression (Peck et al. 1977; Goodwin 1984; Ross and Ternus 1993).

Carotenoids

Carotenoids are fat soluble substances that function as provitamin A and/or scavenger of free radicals. Carotenoids are present in plants. β-Carotene predominates quantitatively and also has the highest potential vitamin A activity (6 mg β-carotene is equal to 1 mg retinol). β-Carotene is an effective membrane-associated antioxidant. It can react with the superoxide anion radical directly or quench singlet oxygen (Burton and Ingold 1984; Liebler 1993).

Vitamin E

Vitamin E is a joint expression of eight fat soluble tocopherols and tocotrienols. All forms of vitamin E have antioxidant effects but α-tocopherol is the most common form and has also the highest vitamin E effect. Vitamin E is present in all membranes with approximately one tocopherol molecule per 5,000 lipid molecules (Halliwell and Gutteridge 1989). Vitamin E can interact directly with a variety of free radicals, for example OH·, and O₃⁻, and also with singlet oxygen but their main role as antioxidants is to break the lipid peroxidation chain by reacting with lipid peroxyl radicals (Machlin 1980; Machlin and Bendich 1987). Palozza and Krinsky (1991) showed that α-tocopherol and β-carotene inhibited radical-induced lipid peroxidation additively in an in vitro system. α-Tocopherol was 40-50 times more efficient than β-carotene. The tocopheryl radical is formed.
by donation of a hydrogen atom (Burton and Ingold 1984). It can be reduced and
thereby recycled by vitamin C, glutathione and ubiquinol (Packer et al. 1979;
Borek 1987).

Protection against radiation effects
Thiols (Modig et al. 1971, 1977), vitamin A (Harapanhalli et al. 1994), vitamin
E (Empey et al. 1992) and WR–2721 (Pratt et al. 1980) have been found to
protect normal tissue against some radiation effects. Isoproterenol (Hall 1974,
Schneyer et al. 1969), pilocarpine (Kim et al. 1991), and WR–2721 (Pratt et al.
1980; Asami and Furuno 1981; Sodicoff and Conger 1983) have been found to
protect the rat parotid gland from some radiation damage. The differential pro-
tection of normal and malignant tissues by WR–2721 was studied by Yuhas
(1979) and his result indicated a selective protection of normal tissue.
The aims of the present thesis were:

1. To describe the longitudinal effects of fractionated irradiation on human salivary gland function
2. To describe the longitudinal effects of fractionated irradiation on rat salivary gland function.
3. To study any possible radioprotective effects of antioxidants on salivary gland function.
MATERIALS AND METHODS

HUMAN STUDIES (PAPERS I AND II)

Patients

The participants in studies I and II were patients who were to receive radiotherapy for malignancies in the head and neck region at the Department of Oncology, Umeå University Hospital, Umeå, Sweden. Prior to radiotherapy, the patients were subjected to oral examination and dental prophylactic treatment at the Department of Cariology, Umeå University. Twenty-five consecutive patients in whom the radiation field included one or both parotid glands were included in the studies, provided that they were not on medication, received no other cancer treatment during the study period, had no disease of the salivary glands and suffered no obvious effects on their general condition. The number of the patients by age and gender, diagnoses and radiation doses are presented in Table 1. Both studies were approved by the Ethics Committee for Human Experiments at Umeå University.

Table 1. Patients' characteristics

<table>
<thead>
<tr>
<th>Treatment and diagnosis</th>
<th>Age</th>
<th>Gender</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bilateral irradiation field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Hodgkins disease</td>
<td>25,42,56,60,61</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>1 malignant lymphoma</td>
<td>52</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td><strong>Unilateral irradiation field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 thyroid carcinoma</td>
<td>55</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>1 malignant lymphoma</td>
<td>58</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td><strong>Bilateral irradiation field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hypopharynx carcinoma, T3/N0/M0</td>
<td>54</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>2 oral carcinoma, T3/N0/ M0</td>
<td>54,62</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td><strong>Unilateral irradiation field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 oral carcinoma, T2-3/N0/M0</td>
<td>47,48,55,66,67</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>Bilateral irradiation field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 nasopharyngeal carcinoma, T3-4/N0-1/M0</td>
<td>21,48,53</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>1 hypopharyngeal carcinoma, T3/N1/M0</td>
<td>62</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>1 oral carcinoma, T4/N0/M0</td>
<td>58</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td><strong>Unilateral irradiation field</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 oral carcinoma, T2-3/N0-1/M0</td>
<td>35,45,58,66</td>
<td>–</td>
<td>4</td>
</tr>
</tbody>
</table>
Radiotherapy

The radiation treatment was performed with linear accelerators 4 MV (Warian, USA) or 6 MV (BBC, Switzerland), with opposed lateral, posterior-anterior, or oblique fields with fixed SSD (source to source distance) or isocentric techniques. Patients with lymphoma were treated with mantle field. The target doses were between 1.45 and 2.37 Gy daily with a dose rate of 2.2 Gy/min, and a focus to skin distance of 80 cm (4 MV) or 100 cm (6 MV). Five fractions were delivered per week. Electrons with energies of 10-18 MeV (Microtron, Scanditronix, Sweden) were used in order to avoid doses exceeding 42 Gy to the spinal cord. The dose plan for each patient and simulation films were studied before the patients were included in the study. In all patients the parotid glands were exposed to at least 95% of the prescribed target dose. The radiation fields were regularly verified using portal films in a simulator. A typical field size, and dose plan used for radiotherapy with photons and electrons of a tongue cancer is shown in Fig. 4.

Oral examination and basic treatment of patients

A baseline oral examination, including soft tissues and teeth, was performed prior to radiotherapy. The patients were examined weekly at the dental clinic by the same dentist (UF) during the radiation period and at 2, 4, 6, 9, 12 and 18 months after radiotherapy.

At baseline all dental treatments called for, e.g. tooth extractions, treatment of caries lesions and adjustment of ill fitting dentures, were carried out. Furthermore, individual prophylactic programs were designed, including fluoride administration. Weekly professional cleaning of the teeth and the oral mucosa during the radiation period and at each control appointment was performed. All patients were informed about the adverse effects of irradiation, including symptoms related to hyposalivation. Saliva substitutes, local anaesthetics, antibiotics and anti-fungal drugs were prescribed when needed. In cases of severe mucositis, a gel containing hydrocortisone and neomycin was used for oral rinses. Saliva was collected and the subjective feeling of a dry mouth was recorded on a three level scale (lack of, slight, and severe dryness).
Saliva collection in humans

Prior to radiotherapy parotid saliva stimulated by sucking on a mildly acid lozenge (SST, Salix Pharma, Sweden), and whole saliva stimulated by chewing on a 1 g piece of paraffin were collected. Parotid saliva was collected weekly during irradiation treatment and at 2, 4, 6, 12 and 18 months after completion of radiotherapy using Lashley cups (Lashley 1916). Aliquots of approximately 1 ml were collected into ice-chilled test tubes from the right and left parotid glands separately. The appointment times for saliva collection varied between 9 and 12 a.m. but for each individual all collections were performed within the same hour and the collection time did not exceed 20 minutes. At bilateral irradiation the flow from both glands was evaluated but in case of unilateral treatment only the flow from the irradiated gland was used for evaluation. Flow rates were determined gravimetrically.

**RAT STUDIES (PAPERS III, IV, V AND VI)**

**Rats and their treatment**

White albino rats of the Sprague-Dawley strain were used in all animal studies. The rats were kept at the Central Animal Laboratory at Umeå University Hospital, at an ambient temperature of 21°C and with a 12-hour light period starting at 6.00 a.m. After arrival the rats were left for one week to accommodate to the new environment. *Ad libitum* fed rats (studies III, IV and VI) were housed in plastic cages on beddings of wooden chips. Four to six rats were kept in each cage. In study V the rats were kept in single steel cages in an automated feeding machine (König et al. 1968). Tap water was always given *ad libitum*. The body weight gain was registered by weighing the rats individually. The animal studies were approved by the Ethics Committee for Animal Experiments at Umeå University.

Since the study design for the animal studies varied according to the specific aims their basic design are described separately.

**Study III** - Eight-week-old female Sprague-Dawley rats, weighing approximately 200 g (ALAB, Södertälje, Sweden) were randomly divided into five equal groups and fed a standard pellet diet (R3, ALAB, Södertälje, Sweden) *ad libitum*. The rats were given fractionated irradiation with various total doses (20, 30, 35, 40 and 45 Gy). Ten days after irradiation the salivary glands were removed and used for *in vitro* studies of acinar cell function.
Study IV - Eight-week-old female Sprague-Dawley rats, weighing approximately 200 g (ALAB, Södertälje, Sweden) were randomly divided into six groups and fed a standard pellet diet (R3, ALAB, Södertälje, Sweden) ad libitum. Various total doses (20, 25, 30, 35 and 40 Gy) of irradiation were given in five fractions to the rats. The effects on saliva flow and composition were measured in vivo. Thus, whole saliva was collected before irradiation and at 2, 15 and 26 weeks after completed irradiation.

Study V - Twenty-one-day-old male Sprague-Dawley rats (ALAB, Södertälje, Sweden) were randomly divided into six equal groups. Semi-synthetic, powdered diets derived from diet MIT 200 (Navia 1968) were prepared by Ewos AB (Södertälje, Sweden). A basic vitamin A deficient diet was supplemented with retinyl acetate (ACO, Stockholm, Sweden) to achieve three levels of dietary vitamin A. Throughout the experiment two groups were given the basic vitamin A deficient diet (0.2 IU/g diet), two groups a diet with an adequate vitamin A content (11.5 IU/g diet), and two groups the diet with adequate vitamin A content from age 21 to 41 days and a diet with a high vitamin A content (105 IU/g diet) from age 42 to 70 days. The age period 42 to 70 days corresponds to three weeks before irradiation and six days after completed irradiation when whole saliva was collected. During the initial 3-week period (age 21-41 days) the rats were given diets ad libitum. The rats were then moved to individual single steel cages placed in a programmed feeding machine ad modum König-Hofer (König et al. 1968). Thus the availability of food could be controlled. The transferral of the rats to the feeding machine coincided with the switch to "high vitamin A" diet for two groups. The other groups maintained their diets: "vitamin A adequate" or "vitamin A deficient" diets.

Blood and liver samples, used to determine the vitamin A status of the rats, were collected at age 41 days (the day of transfer to the feeding machine) and at age 70 days (the day saliva was collected). The retinol concentration was determined using a reversed phase HPLC-technique (Epler et al. 1993).

Study VI - Eight-week-old female Sprague-Dawley rats, weighing approximately 200 g (Møllegaard Breeding Center Ltd, Ejby, Denmark) were randomised into four equal groups. A semi-synthetic, powdered, basic diet (Diet MIT 200; Navia 1968) was given ad libitum during the whole experimental period. The basic diet lacked β-carotene, but contained 1.5 mg retinol and 30 mg α-tocopherol per kg diet. Based on an estimated intake of basic diet of 20 g per day the average daily
intake from the basic diet corresponded to 30 µg retinol and 600 µg α-tocopherol, an intake considered optimal for adult rats (Nutrient requirements of the laboratory rats, 1978). The rats were given a supplementation of 3.4 mg α-tocopherol (Roche AB, Helsingborg, Sweden) mixed in 100 µl of corn oil and 6 mg starch-coated β-carotene (Dry β-Carotene 10% Water-soluble, gift from Roche AB, Helsingborg, Sweden) mixed in 300 µl of water. The two supplements were administered separately within 5 min by pipetting the emulsions into the back of the mouths. The α-tocopherol and β-carotene supplementation started two weeks prior to irradiation and lasted until 12 days after irradiation exposure was completed. Two groups of rats (one with and one without supplementation) were irradiated. Saliva was collected at 2, 4 and 26 weeks after irradiation and the salivary glands were removed for morphological analyses. The details of irradiation, saliva collection and salivary gland extirpation are described later.

Irradiation of rats

Fractionated irradiation was given with daily fractions of 4 to 9 Gy for five consecutive days in studies III, IV and VI. Irradiation was given in a field including the salivary glands but limited to the head and neck region of the rat. Close parts of the body, including the nose (VI), were shielded by 80—100 mm lead. In study III irradiation was restricted to the right side of the head and neck with daily doses of 4, 6, 7, 8 or 9 Gy, (total doses 20, 30, 35, 40 and 45 Gy). In study IV, both sides of the head and neck were irradiated with daily doses of 4, 5, 6, 7, or 8 Gy, (total doses 20, 25, 30, 35 or 40 Gy). In study VI, fractions of 7 Gy were given over five consecutive days (total dose 35 Gy) to both sides of the head and neck. A linear accelerator 6 MV with a dose rate of 2.19 Gy/min and a focus-to-skin distance of 100 cm was used. The total radiation field, in which two rats were irradiated simultaneously, was 8 x 20 cm. The animals were immobilised during irradiation by intravenous injection of 0.1 ml Brietal® containing 10 mg/ml of the active substance methohexitol (Lilly, Indianapolis, IN). The rats in the non-irradiated groups were anaesthetised following the same schedule. The anaesthetised rats were placed in a plastic mould to hold them firmly in position during irradiation. A TV-camera was used to observe the rats during radiation exposure. To reduce the effect of desiccation in rats irradiated with total doses of 35 Gy or more, subcutaneous injections of 5-6 ml Ringer solution with 2.5% glucose (Kabi Pharmacia, Uppsala, Sweden) were adminis-
tered once or twice daily starting at day four of the irradiation period and lasting for two weeks.

**Single dose irradiation** with 25 Gy to the head and neck region of the rat was used only in study V. The animals were anaesthetised with 50 mg/kg body weight of sodium pentobarbital. Six rats at a time were placed in a ring on a 20 cm thick polystyrene box filled with paraffin to give full backscatter. The bodies of the rats were covered with a 5 mm thick lead-shield. The irradiation was given with a focus-to-scatter-block distance of 40 cm. The radiation quality was 195 kV X-rays with filter of 0.5 mm copper and 1 mm aluminium. The dose to the parotid glands was 25 Gy with a dose rate of 1.4 Gy/min.

**Rat salivary gland extirpation and morphometry**

The parotid and submandibular glands were extirpated 10 days (III) or 5 and 26 weeks (VI) after the final irradiation. Food, but not drinking water, was removed the night before extirpation. The rats were anaesthetised with Mebumal® (III) or Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) in combination with Stesolid® (Dumex A/S, Copenhagen, Denmark) (VI). Parotid and submandibular glands were removed and carefully freed from adjacent tissue, sliced, and fixed in glutaraldehyde. After rinsing in 0.2 mol/l phosphate buffer (pH 7.4) the salivary gland specimens were postfixed for 2 hours in 1% osmium tetroxide in the same buffer. After a cold buffer rinse, the specimens were dehydrated in stepwise increasing concentrations of ethanol solutions and embedded in Epon 812. Semithin (1 µm) sections were stained with toluidine blue, and used for morphometry. An ocular with 10 x 10 grid mesh was used and "hits" in the crossings were counted (Weibel 1979). One to five hundred hits per section were counted.

In study III, the salivary glands were also used for **in vitro** comparisons of protein (amylase) and electrolyte (86Rubidium) secretion in irradiated and non-irradiated glands. The irradiated and the contralateral non-irradiated parotid glands were rapidly excised and immersed in separate containers with basal medium consisting of a HEPES-buffered Krebs-Ringer solution supplemented with 5.0 mmol/l fumarate, 5.0 mmol/l glutamate, 5.0 mmol/l pyruvate and 1.0 mmol/l ascorbic acid. The pH was set to 7.40 and the gas phase was ambient air. The glands were dissected free of connective tissues under a stereo microscope, then cut into ten pieces, each weighing approximately 100 µg (wet weight). The
pieces were pre-incubated in basal medium for 30 min at 37° C and preloaded in basal medium supplemented with 28 μmol/l $^{86}$RbCl (10-15 T bq/mol) for 120 min. Specimens of irradiated and control glands were processed separately but in parallel.

The release of amylase and $^{86}$Rb$^+$ in response to continuous noradrenaline perfusion was studied. After preloading, the pieces were rapidly washed in nonradioactive basal medium for 2 minutes and transferred to a perfusion chamber. The device consisted of polycarbonate membranes (8 μm pore size) in a Nucleopore 13 mm Pop-Top holder (Nucleopore Corp., Pleasanton, CA). The peristaltic pump (Pharmacia Fine Chemicals, Uppsala, Sweden) and the media reservoirs were enclosed in a human infant incubator maintained at 37° C. The flow rate was 1.0 ml/min and fractions of the effluent were collected directly into scintillation vials. The medium was changed without interrupting the perfusion by using a three-way valve (Pharmacia Fine Chemicals, Uppsala, Sweden). The time taken for new medium to reach the chamber was 60 sec. For details see Danielsson et al. (1988) and Sundström et al. (1985).

Blood collection in rats

Blood was collected from a tail vein from all rats before radiation exposure. The blood samples were protected from light and air exposure and left in room temperature for 1 h. Serum was prepared by centrifuging at room temperature for 15 min. Aliquots of serum were then immediately frozen and kept under nitrogen gas at −80°C.

Saliva collection in rats

Food, but not drinking water, was removed the night before saliva collection. In study VI standardisation was further improved by administration of a subcutaneous injection of 5 ml Ringer solution with 2.5 % glucose (Kabi Pharmacia, Uppsala, Sweden) at the time when the food was removed. Saliva was collected between 9 a.m. and 11 a.m. the following morning. For saliva collection the rats were anaesthetised either with 0.02 ml of Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) - containing the active substances fluanisone (10 mg/ml) and fentanyl (0.2 mg/ml) - in combination with 0.02 ml of Stesolid® (Dumex A/S, Copenhagen, Denmark) containing 5 mg/ml of the active component diazepam (IV, VI) or with 30 mg/kg of Mebumal® containing sodium pentobarbital (V).
The Hypnorm® and Stesolid® preparations were given as two separate intramuscular injections in the thigh and the Mebumal® as an intraperitoneal injection. During saliva collection the rats were placed on a table that inclined 10° with their mouths placed over a plastic cup in a way that prevented direct contamination from nasal secretion and tears. Saliva secretion was stimulated by a subcutaneous injection of 5 mg/kg body weight of pilocarpine (Sigma Chemical Co., St. Louis, MO) singly in study V. In studies IV and VI 2.5 mg/kg body weight of pilocarpine in combination with 2.5 mg/kg body weight isoproterenol (Sigma Chemical Co., St Louis, MO) was given. Fresh solutions of the sialogogues were mixed every 30 min. Saliva was collected for 15 min. The saliva samples were kept ice chilled. The volumes needed for the various analyses were pipetted into analytical test tubes and then immediately frozen and stored at -20°C until analysed. After saliva collection 0.05 ml atropine (Kabi Pharmacia, Helsingborg, Sweden) and 0.5 ml of the Hypnorm® antidote Narcanti® (Du Pont, Stevenage, UK), were given (IV, VI) to stop the sialogogue activity and enhance recovery from anaesthesia (Johansson et al. 1989). Flow rates were determined gravimetrically.

**CHEMICAL ANALYSES**

The concentrations of *calcium, sodium, potassium and chloride* were determined by atomic absorption spectrophotometry (Varian Techtron AA6, Varian associates, Instrument Group, Palo Alto, CA). Prior to analysis chloride was precipitated as silver chloride.

*Total protein* concentrations were determined by a Coomassie Brilliant Blue method (Spector 1978) using bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, CA).

*Amylase* activities were measured as the degradation of an insoluble blue-coloured starch polymer (Phadebas® kit, Pharmacia AB, Uppsala, Sweden). The absorbance was read at 620 nm after the reaction had been halted with 0.5 M NaOH. The procedure was carried out according to the manufacturer’s instructions.

*Secretory IgA* concentrations were measured with the Immuno-Fluor Technique (Bio-Rad Laboratories, Richmond, CA). A rabbit anti-human antibody preparation and a lyophilised gamma globulin standard prepared from clarified human serum were used.
Salivary peroxidase (SPO) activities were measured at 400 nm by following the peroxidation of pyrogallol by 1M H₂O₂ spectrophotometrically (Gothefors and Marklund 1975).

Myeloperoxidase (MPO) activity was measured by following the oxidation of 5-thio-2-nitrobenzoic acid (Nbs) by OCl⁻ (Mansson-Rahentulla et al. 1986).

Salivary bacteria agglutinating activities - defined by Ericson et al. (1975) as BAGP, a glycoprotein with the ability to aggregate a serotype c strain (TH16) of Streptococcus mutans, (Rundegren and Ericson 1981) - were measured by following the decrease in turbidity of a bacteria suspension of OD 1.5 spectrophotometrically (Beckman DU50, Beckman Instruments Inc., Glenrothes, UK) for 60 min.

Albumin concentrations were measured by rocket electrophoresis as described by Laurell (1972) using rabbit anti-rat-albumin as antiserum (Nordic Immunologic Laboratory, Tilburg, Netherlands) and rat serum albumin (Sigma Chemical Co., St Louis, MO) as a standard.

Sialic acid concentrations were analysed according to Warren (1959). N-acetyl neuraminic acid was used as a standard. The absorbances were measured at 513 and 549 nm.

Concentrations of hexosamines were assessed after lyophilization of the hydrolysate by an Elson-Morgan method modified after Blix (1948).

Concentrations of fucose were measured using a method by Gibbons (1955). L-fucose (Sigma Chemical Co., St. Louis, MO) was used as a standard.

The concentrations of α- and γ-tocopherol, α- and β-carotene and retinol were measured simultaneously using reversed phase high performance liquid chromatography, HPLC (Epler et al. 1993). Throughout all procedures the samples were protected from light and air. After extraction with n-hexane (Merck, Darmstadt, Germany), the samples were centrifuged (Beckman J-6B Centrifuge, Galway, Ireland) and 800 µl of the hexane phase was evaporated to dryness under nitrogen. The samples were then dissolved in ethanol at room temperature for 30 min. A stainless steel column (200 mm x 4.6 mm) with Spherisorb ODS-2, C-18, 5 µm (HPLC Teknik AB, Umeå, Sweden) was used. Initially (V) the mobile phase for retinol was methanol and water (9:1, v:v), the flow rate 0.5 ml/min and the temperature 30°C. In order to separate carotenoids the following changes were made. The mobile phase was 95% methanol and 5% tetrahydro-
fluran (Merck, Darmstadt, Germany) with an addition of butylated hydroxytoluene (Sigma Chemical Co., St Louis, MO), 0.15 g/l mobile phase. The separation was made at 30°C and with a flow rate of 1.5 ml per min (LKB 2150 HPLC Pump, Bromma, Sweden). A variable wavelength UV detector (LKB, Bromma, Sweden) was used to measure the absorbancies at 325 nm (retinol), at 292 nm (tocopherols) and at 450 nm (carotenes). Tocol (gift from Roche AB, Helsingborg, Sweden) in ethanol (Merck, Darmstadt, Germany) was used as an internal standard. Standards from Sigma Chemical Co., St Louis, MO were used for α-tocopherol, γ-tocopherol, α-carotene, β-carotene and retinol. The concentrations of the standards were determined using molar extinction coefficients after the purity of the commercially available standards had been tested by HPLC separation.

**STATISTICAL PROCEDURES**

Descriptive statistics and univariate test methods were used according to the specific study design. All tests were two-sided and p-values below 0.05 were considered statistically significant.

In the prospective human studies (I and II) with repeated sampling differences between values at baseline and at various times after irradiation were tested with a paired t-test (Snedecor and Cochran 1976).

**Study IV** - Data are expressed as means ± SD of crude data or the percentage of the pre-irradiation value for each rat. Differences between values at baseline and at later collections were tested with a paired t-test. Rats from which saliva could not be collected at least before and two weeks after irradiation were excluded.

**Study V** - Differences between groups were analysed by a one-way analysis of variance (ANOVA). When the ANOVA test rejected the null hypothesis that all groups were samples from the same population Dunnett’s t-test was applied to test whether any treatment differed significantly from the non-irradiated group. Student’s t-test was used when differences between two groups were tested.

**Study VI** - Differences between means were tested using Student’s t-tests after the null-hypothesis that the four groups were samples from the same population was rejected by ANOVA.
RESULTS

HUMAN STUDIES (PAPERS I AND II)

Twenty-five patients were included in a prospective evaluation of salivary gland function after irradiation of malignancies in the head and neck.


In this study, a longitudinal evaluation of parotid flow and oral dryness was performed before and during radiotherapy, and 6, 12 and 18 months after the end of treatment. Three different groups were outlined, one receiving doses not exceeding 45 Gy, another 47–52 Gy and a third group ≥65 Gy. Five patients died before termination of the 18-month follow-up period. These patients were included in the earlier observations.

Appearance of dryness
Subjective experience of a dry mouth was reported already after one week of irradiation by 10 patients and by 20 of 24 patients after five weeks of irradiation compared to only one patient before radiotherapy. Unilateral radiation treatment with doses ≤45 Gy caused no or only slight subjective dryness during or following radiotherapy. All patients subjected to bilateral irradiation with the low doses (≤45 Gy) experienced dry mouth symptoms, at least during some part of the treatment, and two out of six patients had persistent dryness. Radiotherapy with doses between 47 and 52 Gy caused dry mouth problems from the third week of irradiation in all patients irrespective of whether a unilateral or bilateral technique was used. The problem was more pronounced however in the bilateral group. All patients irradiated with doses of ≥65 Gy experienced dry mouth symptoms and radiotherapy involving both parotid glands led to severe discomfort from dryness for the patients. There was no correlation between flow rate of whole saliva stimulated by chewing, at baseline and the development of a dry mouth sensation at the end of the treatment period, regardless of dose.
Saliva flow

Initially, a sharp decrease in parotid saliva secretion rate was already encountered in all patients within the first week of treatment, i.e. a delivered dose of 7.25–11.85 Gy. At the end of the treatment (≤45 Gy) 5 out of 14 irradiated parotid glands displayed detectable saliva secretion. Six months after the end of radiotherapy 12 out of these 14 parotid glands showed measurable secretion. The saliva flow was already partially restored in some patients within 2 months after the end of radiotherapy and the secretory capacity then continuously improved in many patients during the follow-up period.

In the 11 parotid glands treated with 47–52 Gy the mean salivary secretion rate was calculated as 21% and 25% of the initial values at 6 and 12 months, respectively, after the end of radiotherapy. At 18 months the mean secretion rate for the five glands with secretory capacity was 62% of the initial value.

The vast majority of parotid glands irradiated with doses ≥65 Gy lost their secretory capacity totally. In the parotid glands treated with maximal acceptable dose only 3 out of 14 had measurable secretion at the follow-up at 6, 12 and 18 months after radiotherapy. Although it is evident that there are great inter-individual variations, a relationship was seen between the total dose delivered and gland function after irradiation.


Parotid saliva flow and salivary composition were studied before, during and up to 18 months after the irradiation period in 16 cancer patients treated for malignancies in the head and neck region. In order to obtain aliquots for analyses in study II, only those patients from study I who maintained a measurable saliva flow were included. This coincides mainly with patients treated with doses ≤52 Gy, thus 15 glands could be followed during and after radiotherapy.

Saliva flow

The secretion rates decreased rapidly during the first week of irradiation, but 72% of all irradiated glands still produced some saliva at the end of the first week. After the whole irradiation period of five weeks 40% of the parotid glands produced measurable volumes of saliva. In the postirradiation period the number
of active glands gradually increased. Twelve months after the end of irradiation 80% of the parotid glands produced measurable volumes of saliva and after 18 months 88%. The secretion rates increased gradually during the follow-up period and returned to a mean of 72% of their preirradiation values after 18 months. However, large interindividual variations were observed and in a paired t-test the decrease was significant up to one year after the end of treatment.

**Salivary composition**

The concentrations of total protein, sialic acid, potassium and calcium and the activity of salivary peroxidase increased in the first week of radiotherapy (approximately 10 Gy). The values for these variables, with the exception of calcium, were still significantly increased at the end of the treatment period. Total protein, salivary peroxidase, salivary IgA and potassium were still increased six months after radiotherapy. Twelve months after irradiation therapy, amylase activity and calcium and sodium concentrations were significantly decreased. At the 18-month observation all concentrations had returned to levels which were not significantly different from the baseline values.

The outputs per minute of protein, amylase, peroxidase, hexosamine, potassium and calcium were significantly decreased during the radiotherapy and up to six months after the end of treatment. The output of total protein, hexosamine and amylase were still significantly decreased 18 months after end of radiotherapy. There were increased outputs of salivary IgA at the observations at 4, 6, 12 and 18 months but significance was observed only at the 6-month control.

Radiation doses of 40 to 52 Gy caused reversible changes in 60% of the glands with an almost restored function in some patients within 6—18 months following the end of radiotherapy.

**Conclusions from human studies**

At doses of ≥65 Gy to the head and neck region a permanent loss of parotid flow occurred. At doses ≤52 Gy there was first a loss of secretory function but two months after irradiation a slow recovery started. A normalisation of the variables studied was seen in most patients 18 months after radiotherapy. Large individual variations in the degree of the effect of irradiation on parotid flow and composition were observed.
RAT STUDIES (PAPERS III, IV, V AND VI).


Radiation was given to one side of the head and neck in the rat according to a five-day schedule with 4, 6, 7, 8 or 9 Gy per day. The parotid glands were extirpated and the effects on amylase and potassium efflux were measured in vitro 10 days after the final irradiation treatment.

Morphology of salivary glands

The morphology was not obviously altered after irradiation with any of the doses. No inflammatory cells appeared, and no oedema was seen in the parotid parenchyma. The cells were not swollen and no acute cell death was detected. Morphometric measurements revealed no significant alterations regarding proportions of various cell or tissue components, such as proportions of acinar cells, ductal cells and stroma, even though a slight increase was seen in the proportion of stroma in the irradiated parotid gland with increasing radiation dose.

Amylase and potassium efflux

There was no significant impact on the capacity of in vitro noradrenaline induced release of amylase at any of the irradiation doses as compared with the contralateral, non-irradiated gland. However, the noradrenaline stimulated potassium secretion traced by $^{86}$Rubidium release was reduced by irradiation. In the lowest dose, 4 Gy x 5, the $^{86}$Rb-efflux was only slightly lower than in the contralateral parotid gland, whereas after the highest dose, 9 Gy x 5, the $^{86}$Rb-efflux in the irradiated parotid gland was only 15% of that seen in the contralateral non-irradiated gland.

Conclusion

Fractionated irradiation of the rat parotid gland caused early damage in the noradrenaline stimulated potassium efflux whereas the exocytotic amylase release and gland morphology remained without significant changes. The results suggest that the mechanism regulating potassium efflux in salivary gland acinar cells is a sensitive target in the early events of irradiation induced damage.

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The aim of this study was to investigate the effect of various doses of fractionated irradiation on the salivary gland function in the rat. Saliva was collected from non-irradiated rats and rats irradiated in the head and neck with 4, 5, 6, 7 or 8 Gy per day on five consecutive days.

**General effects**

Doses of 5 Gy x 5 or more induced loss of hair, mucositis, erythema and dermatitis corresponding to the radiation field. Some rats irradiated with 6 Gy x 5 and 7 Gy x 5 developed cataract in the eyes during the 4th month after exposure. The degree of adverse effects increased with radiation dose and the time following irradiation.

The average body weight increased for the non-irradiated animals during the experimental period. All irradiated rats lost weight after irradiation but began to regain weight 1 to 2 weeks after irradiation. At the saliva collections 15 and 26 weeks after irradiation exposed rats weighed significantly less than non-exposed rats but the average body weights were similar in all exposed groups.

**Salivary flow**

The secretion rate decreased by radiation dose and duration of post-irradiation period indicating a successively increasing effect on salivary gland function. Thus, irradiation with 4 Gy x 5 did not affect the secretion rate significantly during the first 15 weeks, but it was then reduced. The rats exposed to 5 Gy x 5 maintained their flow rate 2 weeks after irradiation but their flow rates were significantly reduced at 15 and 26 weeks. Radiation doses equal to or higher than 6 Gy x 5 led to marked reductions in the secretion rates as early as two weeks after irradiation and throughout the study period.

**Salivary constituents**

Different response patterns to irradiation were seen for the salivary constituents as exemplified by amylase, potassium and salivary peroxidase. The output of amylase followed the same general pattern as the secretion rate. Another pattern was seen for potassium, where no changes occurred in the output after the two lowest radiation doses but at 6 Gy x 5 and higher, the output decreased with increasing radiation dose and postirradiation time. Yet another pattern was seen
for salivary peroxidase. The output increased significantly at the lowest irradiation dose but for all other exposures a dose- and time-related rapid decrease was seen.

The effect of time for all components analysed in saliva collected from rats given various doses of irradiation can be seen in Fig. 2, Paper IV. The output of protein follows that of amylase and the glycoconjugate markers fucose, sialic acid and hexosamine were not reduced two week after irradiation with 4 Gy x 5, but an increase was seen at 15 weeks. A more pronounced effect at the 5 Gy x 5 dose was already seen after two weeks with even more obvious effects after 26 weeks. There were no effects on the output of a bacteria aggregating glycoprotein at the two-week observations, regardless of dose. At the later observations (15 and 26 weeks) BAGP output was already strongly reduced at a dose of 5 Gy x 5.

The effects of irradiation on the output of inorganic components varied between the electrolytes. As mentioned above the output of potassium was not affected by the two lowest doses of irradiation but decreased at higher doses. Calcium output followed the same pattern. Thus, essentially no effect was seen on calcium output by the two lowest doses at the two-week observation but a significant decrease was observed at 15 and 26 weeks with the 5 Gy x 5 dose. Sodium output increased with the lowest dose at the two-week observation and a non-significant increase persisted at 15 and 26 weeks. At higher doses the outputs of all electrolytes were reduced to very low levels.

In general, in addition to the effect of radiation dose, the reduction in output became more evident for all salivary components with the length of the observation period after irradiation.

Conclusion

Fractionated irradiation of rats showed dose-related effects on salivary gland function. At total doses of 20 and 25 Gy only minor changes were observed after two weeks. Clearer effects were seen after 15 and 26 weeks. At doses ≥30 Gy significant dose-related changes already occurred after two weeks and were strongly accentuated after 15 and 26 weeks. No recovery of function occurred. The selection of doses and observation times have to be adjusted to the question asked.

The effects on saliva secretion of a single dose of 25 Gy given to the head and neck of rats with varying vitamin A status were studied six days after exposure. A strong reduction in retinol content in serum was observed in the animals fed the vitamin A deficient diet whereas rats given a diet with adequate or high levels of vitamin A had similar serum levels but the latter group had higher stores in the liver.

A weight loss was observed in all rats regardless of vitamin A intake during the six days after irradiation. Irradiation of rats fed a diet adequate in vitamin A, reduced the saliva secretion rate and the salivary concentrations of hexosamine, sodium and calcium. Irradiation also reduced the activity of a bacteria aggregating glycoprotein (BAGP).

Irradiation of rats deficient in vitamin A caused a lower protein content than in the irradiated rats given a diet adequate in vitamin A. The difference was significant when compared to non-irradiated rats given a diet adequate in vitamin A. The secretion rate in the irradiated rats, deficient in vitamin A was higher than in irradiated rats receiving a diet with normal vitamin A content. In comparison with rats fed a diet adequate in vitamin A, a deficiency in vitamin A gave significantly lower concentrations of hexosamine in non-irradiated rats.

Excess supplementation of vitamin A did not affect saliva composition either in non-irradiated or in irradiated rats.

Conclusion
Excess of vitamin A provided no radioprotection for the rat.

The aim of this study was to evaluate whether supplementation with two antioxidants, α-tocopherol and β-carotene, could reduce the effects of irradiation on salivary glands.

Rats given fractionated irradiation (7 Gy x 5) began to lose weight at the end of the irradiation period but regained weight after another week. At the end of the experiment (26 weeks after irradiation) the irradiated non-supplemented rats were slightly smaller than non-irradiated supplemented rats.

In serum samples collected five days before irradiation supplemented rats had significantly higher levels of α-tocopherol and β-carotene, whereas the γ-tocopherol levels were lower than in non-supplemented. The retinol levels were similar in supplemented and non-supplemented rats. α-Carotene was not detected in any group.

Saliva flow
Salivary secretion rates were significantly reduced after irradiation in both supplemented and non-supplemented rats. However, irradiated supplemented rats had significantly higher flow rates two weeks after irradiation than non-supplemented (43 ± 17 μl/min vs. 27 ± 8 μl/min). At the collection four weeks after irradiation flow rates were still higher in supplemented than in non-supplemented rats (41.5 ± 15.8 μl/min vs. 29.2 ± 9.6 μl/min) but the group sizes were smaller and the difference was not statistically significant. At the collection 26 weeks after irradiation saliva secretion rates had decreased further in all irradiated rats but the decrease was less accentuated in supplemented rats than in non-supplemented rats. No difference was seen between supplemented and non-supplemented non-irradiated groups.

Salivary composition
Two weeks after irradiation the concentrations of calcium, sodium, potassium, total protein and hexosamines and the activities of amylase and salivary peroxidase were significantly increased in irradiated non-supplemented rats. The effect of irradiation was more pronounced in non-supplemented than in supplemented rats. Thus, in supplemented irradiated animals only the sodium concentration and salivary peroxidase activity were elevated.
The effects of irradiation and α-tocopherol and β-carotene supplementation, respectively and in combination, found on saliva composition were similar two and four weeks after radiation exposure. Twenty-six weeks after irradiation saliva composition was further affected in all irradiated rats but the effects were less pronounced in supplemented than in non-supplemented rats.

Albumin concentration in saliva was measured as an indicator of inflammation. Two weeks after irradiation there was a substantial increase in albumin concentration in saliva from irradiated animals but supplemented rats had a significantly lower increase in albumin concentration. Thus, a 30-fold increase in average albumin concentration was observed in the irradiated non-supplemented group as compared to a 15-fold increase in the irradiated supplemented group. A slight increase in albumin concentration persisted in irradiated non-supplemented rats after four weeks whereas it was almost normalised in the irradiated supplemented rats. At 26 weeks albumin concentrations were normalised in all groups.

**Morphology**

The proportions of acinar and duct cells and stroma in parotid and submandibular glands were determined 5 and 26 weeks after irradiation. No obvious morphometric differences were seen between the non-irradiated non-supplemented group and any of the other three groups after 5 weeks. After 26 weeks the proportions of acinar cells were significantly decreased both in irradiated parotid and submandibular glands and the proportions of duct cells and stroma were increased in irradiated parotid glands. Supplementation with α-tocopherol and β-carotene did not alter the morphology of the glands.

**Conclusions**

A combination of α-tocopherol and β-carotene gave some radioprotection measured as less loss of secretion rate and fewer alterations in salivary composition. The degree of inflammation was also reduced by antioxidative supplementation as measured by lower albumin values in saliva.
Primary or secondary reactions in healthy tissues and cells occur as a consequence of radiotherapy of malignancies. The healthy tissues then lose their function entirely or partly. Such effects in the head and neck therefore lead to severe subjective symptoms like a dry and sore mouth as well as an increased risk for dental caries and oral candidiasis (Toljanic and Saunders 1984; Baum et al. 1985; Stephens et al. 1989a).

Methodological aspects

*Human studies*
All patients included in the present studies were given conventional fractionated irradiation e.g. one fraction of approximately 2 Gy per day, five days per week over a period of four to seven weeks (c.f. Table 1). Dose plan and simulation films of each patient were studied before the patient was included in the study, and the radiation dose to the parotid glands were at least 95% of the prescribed target dose.

The ages of the patients included in the present studies were on average 52 ± 12 (SD) years and the proportion of men was 76% (c.f. Table 1). Ageing *per se* in healthy individuals does not affect saliva flow rate or protein content (Baum 1981; Baum et al. 1982). However, saliva flow rate is known to be reduced in elderly people compromised by diseases and medication (Närhi 1992). The participants included in studies I and II did not take any prescribed drugs and did not suffer from any diseases known to affect saliva flow rate or composition. Since the patients were followed longitudinally and all statistical evaluations were based on paired data, confounding variables, such as age and genetic associated variations were taken into account.

At baseline the participants in paper I had an average flow rate for chewing stimulated whole saliva of 1.9 ml/min (range 0.3–4.3 ml/min). The flow rate at baseline was not correlated to the oral dryness noted by the patients or the objective loss of flow during and after irradiation. This observation is not in line with the reports by Eneroth *et al.* (1972b), Wescott *et al.* (1978) and Mira *et al.* (1982), who suggested that patients with high baseline secretion could tolerate higher doses before loss of secretion. This discrepancy may be partly explained by the fact that only non-medicated and otherwise healthy cancer patients were included in the present study (c.f. Leslie and Glaser 1993). None of the other
authors state the medical history of their patients. In the present study, the cancer was diagnosed 7 to 14 days before the saliva collection. The patients were therefore unlikely to be in an acute stress situation when the salivary baseline values were determined.

Thus, the results and conclusions from the present studies on humans are based on the fact that the patients were not using any prescribed drugs and had no additional medical diagnoses. However, many patients had problems chewing food during the early phase after radiotherapy and therefore preferred liquid or mashed food. The lower intake of energy and nutrients from a liquid diet combined with the loss of stimulation from chewing reduces the flow rate within a few days (Johansson and Ericson 1986, 1989). Acute starvation can thus contribute to a reduced saliva flow rate (Johansson and Ericson 1986). The eating problems in the present study led to weight loss in several patients during the period of treatment. However, the extent of the contribution from malnutrition cannot be established.

Collection of parotid saliva
The decision to collect glandular secretion was based on the fact that irradiation induces mucositis in the early phase after exposure and thereby mucosal leakage of serum proteins. Whole saliva, and especially saliva stimulated by chewing, is therefore likely to contain serum proteins and other non-salivary components during and after radiotherapy (Rice et al. 1982; Vissink et al. 1990b). The correctness of the decision to chose glandular saliva was confirmed when it was found that irradiated rats had higher levels of albumin in whole saliva than non-irradiated rats, two and four weeks after irradiation (VI).

Another reason for choosing parotid saliva in the human studies (I, II) was that the parotid gland is reported to be more sensitive to irradiation than the submandibular/sublingual glands. Further, more complicated techniques are needed for collecting submandibular/sublingual saliva. The irradiated patient can only tolerate simple and rapid methods especially when the collections are repeated several times during the irradiation period and are not part of direct cancer therapy.

Animal models
Animals have been used for salivary gland studies for more than a century. Thus, the majority of observations on secretory physiology do not originate from
human studies. The use of an animal model to study the effects of irradiation on biological events in general allows a more systematic evaluation of various irradiation schedules and experimental conditions.

Some differences between man and the rat must, however, be considered and extrapolations can only be made with caution. The salivary glands differ in size and to some extent in structure, especially in the ducts where convoluted granular duct cells are found in the rat submandibular gland (Gresik 1994). Functional differences are indicated by the fact that rat saliva is more viscous, and that the concentrations of proteins and electrolytes are higher. Furthermore, stimulation of saliva secretion has to be done pharmacologically in the rat which yields differences in composition depending on the secretagogues used.

The rat must be anaesthetised during saliva collection. Two types of sedations were used in the present series of papers. Mebumal® was used in study V and Hypnorm® and Stesolid® in studies IV and VI. It could be assumed that the two types of sedation would influence the saliva secretion differently. However, a comparative study shows no significant differences in saliva flow or composition after either of these two sedation regimens (Johansson et al. 1989).

Whole stimulated saliva was collected in the rat studies (IV, V, VI). It might have seemed more straightforward to collect parotid saliva in order to make comparisons with the results from the human studies. There are methods for collecting glandular saliva from the rat parotid ducts by cannulation extraorally (Qwarnström and Hand 1982) or intraorally (Nagler 1993a, 1993b), but since the present rat studies were longitudinal saliva collection had to be repeated several times in the same rats. None of the cannulation procedures were therefore possible, especially when the irradiation effects on epithelial tissue were considered.

Other differences between man and rat to consider were the relative longevity and size of the species. The relative volume irradiated is considerably larger in the rat than in humans. The whole brain (or half brain in Paper III) becomes irradiated which could mean that general neurophysiological and hormonal effects are included. These effects cannot be evaluated from the present papers, nor can they be entirely disregarded.
Salivary variables studied

The salivary variables in this series of papers were chosen to represent different aspects of salivary gland function. Flow rate gives obvious and basic information about the secretion of water. The conditions for this are related to the availability of water by capillary flow and is also coupled to the efflux of electrolytes (Nauntofte 1992). The salivary proteins reflect the biosynthetic processes and the glycoconjugate markers fucose, sialic acid and hexosamine provide information on the degree of protein glycosylation. These processes occur partly in the endoplasmic reticulum and partly in the Golgi apparatus. The ratio of carbohydrates to protein gives some information on the quality of the glycoproteins in general.

Some specific proteins like salivary peroxidase, secretory IgA and BAGP were selected because they relate to the microflora. Amylase was chosen because it has traditionally been used to describe salivary gland function. Apart from being involved in bacterial metabolism, i.e. SPO, peroxidase may also be a candidate for the elimination of hydrogen peroxides in the cells provided that there is a relationship between cytosolic and secreted peroxidase. As such it could have a special assignment during irradiation-induced peroxide production. It is therefore noteworthy that the output, as well as the concentrations, of salivary peroxidase increase initially at the lowest dose 4 Gy x 5 in the rat. This can be interpreted as an attempt of protection.

There are a number of other proteins that could have been selected, such as lactoferrin, statherin, proline-rich proteins (PRPs) or histidine-rich proteins. Because of the limited volumes of saliva available after irradiation, the number of proteins selected had to be restricted. Unfortunately, this limits the possibility to evaluate the concerted effects of salivary proteins after irradiation, but it may still be possible to obtain a broad understanding of salivary gland function.

Loss and recovery of function

One of the aims of the present thesis was to study longitudinal effects of head and neck irradiation on saliva secretion. A concerted evaluation and discussion of early and late radiation effects was therefore made from data in Papers I-VI, which is summarised in Table 2. Adverse effects seen during irradiation and up to two weeks after completed treatment are considered as early effects both in man and in the rat whereas, effects seen six months and later are considered to be late effects. This definition is in accordance with Rubin and Casarett (1968).
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<td><strong>General effects</strong></td>
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a) concentration
b) activity
General effects of irradiation

The early general effects seen in man coincided mainly with the early effects seen in the rat. Thus, humans and rats epithelial tissues, e.g. the skin and oral mucous membranes, were affected early after the initiation of radiotherapy to the head and neck region. Oral mucositis developed during the second to third week of irradiation. Some patients had thus an eating problem and lost weight. However, ulcers were healed and no mucosal pain persisted two weeks after the treatment was completed, although the mucosa still appeared dry and fragile. In the rat a dose-related erythema reaction and loss of hair corresponding to the radiation field was seen after exposure to 25 Gy or more. Thus, irradiation with 20 Gy did not obviously affect the general condition. These rats appeared healthy but developed one extra lower incisor which caused eating problems. Abnormal teeth after irradiation are reported by Cherry and Gluksmann (1959). Irradiation doses ≥30 Gy affected the general condition more seriously and the rats ate and drank poorly for varying periods of time. Their body weights decreased during the late part of the irradiation period and up to two weeks after irradiation. Fluid and energy support was given and the irradiated rats began to gain weight from two to ten days after irradiation.

In humans no acute signs of mucositis or dermatitis remained six months after irradiation although the skin and mucosa still appeared dry, thin and fragile. Six months after irradiation the rats also appeared healthy and showed no erythema, although rats irradiated with ≥30 Gy still had hair loss in the exposed area. In humans recovery of hair can be seen 6 months after irradiation even if the doses exceed 55 Gy (unpublished data). Most rats irradiated with doses of ≥35 Gy became blind during the 4-month period after irradiation (IV, VI).

Another early adverse effect from radiotherapy of malignancies in the head and neck was the subjective experience of a dry mouth. This was reported by 20 out of 24 patients after five weeks of irradiation. The four patients that did not experience dryness had received a total dose of ≤52 Gy (paper I, Table 3). The experience of a dry mouth was paralleled by a sharp decrease in parotid saliva secretion rate in all patients within the first week of treatment, i.e. a delivered dose of 7.25-11.85 Gy. Both the development of a dry mouth sensation and the reduced saliva flow were dose dependent. Irradiation with doses of ≥65 Gy led to severe discomfort from dryness. The vast majority of human parotid glands irradiated with doses of ≥65 Gy totally and permanently lost their secretory capacity whereas flow persisted from glands exposed to doses of ≤52 Gy. However, it has
to be emphasized that there are large interindividual variations in appearance, severity and duration of the general side effects. This points out that individual specific factors, at present unknown, are of importance with regard to radiation induced effects.

The effect of irradiation on the minor salivary glands was not studied in the present thesis but such a study is highly recommended. On the one hand mucous acinar cells, such as in the minor glands, are less sensitive to radiation, but on the other hand the early effects of oral dryness and mucositis could be an expression of radiosensitivity in the minor salivary glands. If they lose their function the production of the gel formed mucous would be affected and the highly viscous gel would not be there to provide for low friction between tissues or to form a protective layer, possibly preventing mucositis (Tabak et al. 1982; Gibbons 1982; Walker et al. 1985). The secretion from the major glands may partly compensate for a loss of the mucous secreted from the minor glands but it appears that minor glands are the most important in preventing oral dryness (Wolff et al. 1990).

Saliva
In humans treatment doses of ≤52 Gy decreased the average (SD) flow rate of parotid saliva from 0.53 (0.40) ml/min (baseline) to 0.05 (0.09) ml/min at the end of the radiation period. The proportion of parotid glands producing measurable volumes of saliva decreased to 40% (Paper II). Eighteen months after irradiation there was no significant decrease in flow rate. This recovery of function seems contradictory to the observations by Dreizen et al. (1977a) but is in accordance with Makkonen et al. (1986a) who observed recovery in whole saliva flow rate after radiation doses ≤50 Gy, and Leslie and Dische (1992b) who reported a recovery of parotid function starting about 6 to 9 months after irradiation with 54 Gy and continuing for at least two years. Dreizen et al. (1977a) found no recovery using the same fractionation scheme as in study I but the total doses may have differed. He does not report the total doses, nor the medical history of the patients. Radiation doses of ≥65 Gy decreased the average parotid saliva flow from 0.45 (0.31) ml/min to 0.01 (0.02) ml/min and 86% of the parotid glands permanently lost their capacity to produce detectable volumes of saliva (Paper I). Irradiation to the head and neck of the rat also dose-dependently reduced saliva flow in the early phase (Papers IV, V and VI). Irradiation with total doses of ≥30 Gy decreased the saliva flow rate as early as two weeks after
exposure whereas doses of ≤25 Gy did not affect flow rate during the early phase (Paper IV). There was no recovery of secretion rates in the rat, probably due to the higher fraction doses used in the rat and the higher relative volume irradiated. The shorter life span of the rat may also be a contributory factor.

In man the concentrations or activities of the measured electrolytes and organic components in saliva either increased or remained unaffected during the first week of radiotherapy (Paper II, Table 1). At the end of the 5-week treatment period the concentrations or activities of potassium, total protein, salivary peroxidase and sialic acid were significantly increased whereas the concentrations of sodium, calcium, and sIgA and the activity of amylase and BAGP were unaffected (Paper II). Increased levels of sIgA were observed six months after irradiation. This is in accordance with Brown et al. (1981) who found increased levels of sIgA for nearly three years after irradiation. In contrast the amounts secreted per minute (output) of potassium, calcium, protein, amylase, salivary peroxidase and hexosamines were significantly decreased during the early phase after radiotherapy. This is in accordance with the data of Brown et al. (1976b, 1981), Dreizen et al. (1976), Mossman et al. (1981) and Makkonen and Nordman (1987). Even though secretion rates have an obvious effect on the output of components per time unit there is no simple relationship. There is a simultaneous effect on the biosynthesis of proteins, efflux of electrolytes and water. However, the complicated relationships cannot be reduced to changes in water efflux only.

Irradiation with total doses of 35 Gy to the rat increased the saliva concentration of potassium, sodium, calcium, total protein and hexosamines and the activities of amylase and salivary peroxidase (Paper VI). However, when evaluated on parotid gland slices in vitro noradrenaline stimulated potassium secretion, traced by $^{86}$Rubidium release, was reduced by irradiation, but the release of amylase induced by noradrenaline was unaffected at all irradiation doses tested (20—45 Gy) (paper III). Thus, a lowered release of potassium from the acinar cells was seen whereas saliva entering the mouth had increased concentrations. This may seem contradictory, but when the decrease of potassium efflux in vitro and the increase seen in vivo are compared one has to consider that effects seen in saliva concentrations mirrors the function of the whole secretory system while, in vitro experiments only consider the function of the isolated cells. The radiosensitivity of duct cells is lower than that of acinar cells and exchange of electrolytes and water may still function well. The present data are partly in accordance with the
results from Bodner et al. (1984). They studied potassium and amylase release from enzymatically isolated acinus cells after a single dose of 20 Gy. No significant effects were observed regarding amylase or potassium release after isoproterenol or epinephrine stimulation, respectively. However, a numerically lower potassium release was observed in the irradiated than in the non-irradiated glands when using a higher dose of secretagogue. Other studies show that the activity of amylase in the mouse parotid gland was unaffected 24 h after irradiation with 10 Gy (Sasaki et al. 1980), but decreased in the rat one and three days after irradiation with doses from 4 to 64 Gy (Sodicoff et al. 1977). They observed a tendency of recovery on the seventh day. Increased levels of amylase in serum after irradiation are reported by several authors (van den Brenk et al. 1969; Becciolini et al. 1984; Leslie and Dische 1992a; Dubray et al. 1992). The latter stress the importance of precaution when salivary gland function is to be evaluated based on whole saliva secretion in conditions where mucositis is present.

Recovery of function
Recovery from the dry mouth sensation in humans was paralleled by a recovery of salivary gland function (Paper I). Recovery of parotid gland secretion began two months after radiotherapy for patients treated with doses of ≤52 Gy but no recovery was seen in patients given higher doses. The flow rates improved continuously up to 18 months and the number of secreting glands gradually increased with time. In patients irradiated with ≤52 Gy and having continuously functioning parotid glands, the secretion rate had returned to an average of 72% of the baseline value at the 18-month follow-up. At the 18-month follow-up all concentrations and activities had returned to levels not significantly different from baseline values (II). The secreted amounts per minute (output) of most components had also returned to near baselines values 18 months after radiotherapy. Recovery starting three to nine months after irradiation and continuing for at least two years have been reported by Makkonen et al. (1986a) and Leslie and Dische (1992b). In contrast to what was seen in the late phase in those who regained secretory capacity after doses ≤52 Gy, the impairment in saliva secretion rate in the rat, seen in the early phase, was further pronounced in the late phase. This pattern was followed for all doses except for 8 Gy x 5, where the effect was already maximised two weeks after irradiation. Even after a single dose of 2.5 Gy rat parotid saliva flow rate has been reported to be decreased 12 months after exposure (Nagler et al. 1994).
The recovery of function of the salivary glands in man irradiated with ≤52 Gy calls for some suggested explanations. The loss of function could be due to one or more of several reasons, e.g. loss of membrane function including effects on receptors and secretory granules, destruction of interlobular capillaries and DNA destruction. However, the cells contain repair systems which could restore function in time. Other alternatives to regain function would be regeneration of secretory cells by differentiation of intercalated duct precursor cells. One may speculate that these cells are less radiosensitive than the highly specialised and differentiated acinar cells. The acinar cells which occur in interphase are subjected to subtile changes in function, which ultimately could lead to loss of acinar cells. This might trigger differentiation and migration of the precursor cells with a primary loss of function and, later recovery of function (Peter et al. 1994a, 1995). Signs of regeneration have been observed also after high doses of fractionated irradiation (Gustafsson et al. 1995).

**Morphology and function**

Morphological changes occurring after irradiation were observed in the rat (III, VI) by the use of morphometry and light microscopy. No alterations in salivary gland morphology were seen ten days (III) or five weeks (VI) after fractionated irradiation with 30 to 45 Gy. Thus, no oedema was seen in the parotid parenchyma, no inflammatory cells appeared, and no acute cell death was detected in the parotid glands. This is in accordance with Nishi et al. (1986). Furthermore, morphometric measurements revealed no significant differences in proportions of acinar or ductal cells in the parotid or submandibular glands, even though the proportion of stroma increased slightly with radiation dose (Paper III). In the late phase, however, acinar cells were destroyed after irradiation with a total dose of 35 Gy (VI). The fraction of acinar cells was significantly lower in parotid as well as in submandibular glands in the irradiated rats than in the non-irradiated rats 26 weeks after exposure. Supplementation with antioxidants two weeks before, during and 12 days after irradiation had no detectable saving effect on the acinar cells. A dose dependent decrease in the proportion of acinar cells in the rat parotid gland is also reported 26 weeks after fractionated irradiation with total doses of 30 to 45 Gy (Franzén et al. 1993). The rather dramatic early changes in flow rates and output of some salivary components were not accompanied by visible changes in acinar or duct cells.
However, these gross morphological criteria are not sufficient to allow conclusions to be drawn as to whether histological changes had occurred at this early stage after fractionated irradiation or not. It has been suggested that the earliest changes in the subcellular organelles after irradiation occur in the membranes of the secretory granules (Norberg and Lundquist 1989). By depleting the acinar cells before irradiation diminished changes in saliva flow and composition were seen in parotid but not in submandibular/sublingual saliva in the rat (Peter et al. 1995). The hypothesis was that secretory protein appearing in the cytosol would be toxic to the acinar cell and possibly upset its normal functions. This hypothesis has recently been questioned by Peter (1994b). No clarification on the detailed mechanisms involved in loss of function of salivary acinar cells can be obtained from the data in the present series of papers.

The sequence of changes in salivary gland function seems to involve primarily structures related to the secretion of water. Such structures may be endothelial cells in the capillary system and parts of the membrane of the secretory cell wall. Changes related to protein secretion seem to occur later and involve damage to DNA and membranes of subcellular organelles related to synthesis and secretion of proteins. Whether or not a recovery will occur depends on the irradiation schedule. The damage caused by irradiation with lower total doses may induce replacement of damaged acinar cells by migration and differentiation of intercalated duct cells (van den Brenk 1968).

**Effect of antioxidant supplementation**

Radiation effects on saliva secretion and composition in rats with different vitamin A levels in serum were studied six days after exposure (Paper V). It was found that at an adequate level of vitamin A in the diet, irradiation *per se* significantly reduced the secretion rate and the concentrations of sodium, calcium and hexosamines as well as the activity of a glycoprotein (BAGP) aggregating a serotype *c* strain of *Streptococcus mutans* in pilocarpine stimulated saliva. The concentrations of potassium and the activities of peroxidase and amylase were not significantly affected. Supplementation of excess dietary retinol neither diminished nor increased the adverse effects from irradiation. However, the damage caused by irradiation was slightly enhanced by vitamin A deficiency. Irradiated rats deficient in vitamin A had lower concentrations of total protein and hexosamines in saliva compared with irradiated rats fed a diet with adequate
vitamin A. However, the secretion rates were higher in irradiated vitamin A deficient rats than in the irradiated rats fed a vitamin A adequate diet. Harapanhalli et al. (1994) observed a radioprotective effect of intratesticular injection of soybean oil against radionucleids in the mouse. The effect was enhanced when vitamin A was added to the oil.

The effects of $\alpha$-tocopherol and $\beta$-carotene supplementation on adverse effects of irradiation were studied in Paper VI. Using this combination of vitamin supplementation the reduction of secretion rates after irradiation was lower than in non-supplemented rats at 2, 4 and 26 weeks after irradiation. Furthermore, the changes in saliva composition were less accentuated in irradiated vitamin supplemented rats than in irradiated non-supplemented rats.

Irradiation *per se* also led to significantly higher levels of albumin in saliva during the acute phase. This is interpreted as a leakage from the mucosa and from the salivary glands because of inflammation. Increased levels of albumin were also seen in bronchoalveolar lavage fluid after irradiation (Bjermer et al. 1992; Nilsson et al. 1992). Irradiated rats supplemented with $\alpha$-tocopherol and $\beta$-carotene had significantly lower albumin concentrations in saliva two and four weeks after irradiation than irradiated non-supplemented rats. Twenty-six weeks after irradiation the albumin levels were normalised even in non-supplemented rats. Rats supplemented with $\alpha$-tocopherol and $\beta$-carotene showed the same morphological changes as the non-supplemented rats. The proportions of acinar cells were significantly decreased both in parotid and submandibular glands 26 weeks after irradiation in supplemented and non-supplemented rats. Empey et al. (1992) observed that intestinal fluid absorption in the rat was altered after irradiation with 10 Gy but that the morphology of the mucosa was unchanged. Supplementation with vitamin E for six days before irradiation protected against absorptive injury.
GENERAL CONCLUSIONS

Irradiation treatment of malignancies in the head and neck region usually induced severe subjective dry mouth symptoms. Irradiation with doses of \( \geq 65 \) Gy to the parotid glands led to permanent loss of function in the majority of the parotid glands, while recovery could be seen after irradiation with doses of \( \leq 52 \) Gy. Concentrations of some proteins and electrolytes in saliva were increased during irradiation but no changes remained 18 months after radiotherapy. However, large interindividual differences were seen. The recovery of function may involve an irradiation induced replacement of destroyed acinar cells by intercalated duct cells.

The irradiation effects observed in rats did not greatly differ from those seen in humans. The impairments in saliva flow and composition were dose and time dependent and acute and late effects were different. No recovery of salivary gland function could be seen in the rat. Therefore, to study variations in salivary gland function after fractionated irradiation the rat model must be adjusted to meet the questions addressed. No single model seems to be optimal for studying all parameters.

Supplementation with \( \alpha \)-tocopherol (3.4 mg/day) and \( \beta \)-carotene (6 mg/day) during irradiation reduced the degree of inflammation and partly preserved salivary gland function, but did not lead to morphometrically detectable differences in proportions of acinar or ductal cells or stroma.
I which to express my sincere gratitude to all those who made this study possible and especially to:

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LIST OF CORRECTIONS

The following corrections should be read in:

Paper I:

*Table 2:*
(S.E.) should have been (S.D.).

Paper II:

*Abstract, line 11:*
"salivary peroxidase, hexosamine and salivary IgA were", should have been "salivary peroxidase, sialic acid and potassium were".

*Table 1, column 5:*
n = 16 should have been n = 6.