MODULATION OF THE RISK TO ORAL CANCER

by

Antony Paul Hornby
B.Sc., University of Wales, 1978
M.Sc., University of British Columbia, 1981

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Department of **Pathology**

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Millions of people use smokeless tobacco, such as snuff, chewing tobacco, nass or nasswar (a mixture of tobacco, slaked lime, ash and oil), Khaini tobacco (tobacco and slaked lime), or as part of a simple betel quid (areca nut, tobacco, lime, betel leaf) or a complex "pan" (betel quid with catechu, seeds, perfumes and silver foils). These habits which are involved in the etiology of oral cancer, have been of concern since snuff dipping is becoming popular among teenagers of Canada and the United States. To prevent the development of oral cancer among snuff dippers, it appeared necessary to trace the etiological factors, to develop markers which would identify individuals at elevated risk for oral cancer, and to test the usefulness of these "intermediate endpoints" in following the response of the oral mucosa to the administration of chemopreventive agents.

The population groups chosen for this study included Inuit, Canadian Indians and East Indians. Approximately 57.0% of Inuit males from Gjoa Haven (Northwest Territories) used snuff, 62.6% of native Indians of La Loche (Saskatchewan) dipped snuff daily, and 54.0% of East Indian fisherman from along the coast of Kerala (India) chewed tobacco in the form of a betel quid. The chewing patterns were as follows: number of dips per day for the Inuit were 8.03 at 25.2 min per dip, for the native Indians 9.1 dips per day at 20.3 min per dip and for the East Inians 17.2 chews per day at 15.2 min per chew.

N-Nitroso compounds were found in the saliva of snuff dippers and betel quid chewers. They are considered to be the most probable etiological factors in the development of oral cancers, since they are the only known carcinogens present in mg/kg quantities in the various tobacco mixtures.
High levels of nitrite, a precursor to nitrosamines, were found in tobaccos used by the Canadian natives. Up to 1040 mg/kg nitrite was detected in tobacco samples used by this population. High levels of nitrite were also detected in the saliva of Inuit and Canadian Indian snuff dippers: up to 0.25 mg/ml of nitrite appeared in the saliva within 5 to 10 min of a snuff dipping session. Nitrite was also detected in the saliva of East Indian betel quid chewers, averaging 36.27 µg/ml.

Since nitrite can serve as a precursor to nitroamine reactions, the in vitro nitrosation capacity of saliva from a snuff dipper was tested. After the addition of 200 mg proline to the saliva of a snuff dipper at pH 2.5, an 18fold increase in nitrosoproline (NPRO) was observed over control levels. Moreover, NPRO was observed in the urine of chewers at a fivefold increased level over non-chewers. These results indicate an elevated level of nitrosation within individuals who dip snuff. This increased endogenous nitrosation reaction in snuff dippers can lead to the formation of carcinogenic N-nitrosamines.

Snuff dippers and tobacco chewers are also exposed to tobacco-specific nitrosamines (TSNA). Levels from 3200 ppb for N-nitrosonornicotine (NNK) to 170,000 ppb for N-nitrosoanatabine (NAT) were found in commonly used brands of snuff which were commercially available in the Northwest Territories. In addition, relatively high levels of these carcinogenic nitrosamines were detected in the saliva of chewers (up to 980 µg/ml of saliva).

The second objective of this study was the development of markers which indicate early changes in a human tissue exposed to carcinogens. Two markers were used to detail the damage occurring in the oral mucosa of users of smokeless tobacco. The first was micronuclei which was applied to exfoliated cells from the oral mucosa.
This assay is a quantitative indicator of chromosomal breakage. An elevated frequency of micronucleated cells was observed in the oral mucosa of Inuit snuff dippers, native Canadian Indians and East Indian chewers of tobacco-containing betel quids, as compared to corresponding individuals who did not use smokeless tobacco. The second marker was oral leukoplakia, a preneoplastic lesion commonly found in the oral mucosa of betel quid chewers. By using these two markers to quantify carcinogen-induced damage during the preneoplastic stage, it appeared feasible to identify individuals at elevated risk for developing oral cancer.

The study explored the possibility of using the above-mentioned markers to follow the response of smokeless tobacco users to the administration of beta-carotene and vitamin A. The administration of beta-carotene (180 mg/week) for ten weeks significantly reduced the level of micronucleated cells in the oral mucosa of a group of Inuit snuff dippers who continued to use their usual amount of snuff during the trial period. Similarly, the levels of micronucleated cells in the oral mucosa of chewers of tobacco-containing betel quids (East Indians) were significantly reduced after three months on a regime of either 180 mg/week of beta-carotene or 180 mg/week of beta-carotene plus 100,000 IU of vitamin A.

The reduction of micronucleated oral mucosal cells occurred more rapidly than the remission of leukoplakia in the East Indian group as did the inhibition of newly formed leukoplakia following the administration of the two chemopreventive agents. The remission of established oral leukoplakia was significant (P = 0.004) only after six months in the betel quid chewers receiving beta-carotene plus vitamin A compared to the group receiving a placebo.
The treated group also showed a significant reduction in the appearance of new oral leukoplakia ($P = 0.08$). The administration of vitamin A alone (200,000 IU/week) to betel quid chewers produced a highly significant remission of established leukoplakia ($P = 0.0000089$) plus an inhibition of the formation of new leukoplakia ($P = 0.024$) as compared to the placebo group. The East Indians continued to chew betel quids throughout the administration of chemopreventive agents.

Many different scientific disciplines are necessary to obtain understanding of the etiological factors involved in the development of a particular cancer, and to recognize early changes in the target tissue to carcinogens. Only a profound understanding of these events will help in the early identification of individuals at elevated risk to cancer, the design of largescale chemopreventive trials, and the selection of the most effective chemopreventive agents.
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<tr>
<td>DMNM</td>
<td>Nitroso-cis-2,6-dimethylmorpholine</td>
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<td>MNC</td>
<td>Micronucleated Cell</td>
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<td>MNNG</td>
<td>N-Methyl-N'-nitro-nitrosoguanadine</td>
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<td>NAB</td>
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<td>NAT</td>
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<tr>
<td>NIC</td>
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</tr>
<tr>
<td>NNK</td>
<td>4-(Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone</td>
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<td>N'-Nitrosonornicotine</td>
</tr>
<tr>
<td>NPIC</td>
<td>N-Nitrosopiperidine-2-carboxylic acid</td>
</tr>
<tr>
<td>NPRO</td>
<td>Nitrosoproline</td>
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<tr>
<td>TSNA</td>
<td>Tobacco-specific nitrosamines</td>
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Other abbreviations which are widely used without definition in the biochemical literature (eg. DNA, IU) are not specifically defined here.
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. H.F. Stich for guidance, suggestions, discussions, and unfailing encouragement during the course of the investigations reported in this thesis. I would also like to thank Dr. Bruce Dunn for excellent technical and scientific instruction, and the following members of the research team involved in the chemopreventive trials for their assistance: Dr. K.D. Brunnemann, Naylor Dana Institute for Disease Prevention, American Health Foundation, for analysis of TSNA in saliva and snuff samples; Mrs. H.F. Stich, B.C. Cancer Research Centre, for scoring of exfoliated buccal mucosal cells for micronuclei; Dr. Babu Mathew and Dr. R. Sankaranarayanan, Regional Cancer Centre, Trivandrum, India, for diagnosis of leukoplakia.

I would like to dedicate this thesis to my wife Darlene, who has offered unfailing moral support and encouragement throughout the course of my studies.
INTRODUCTION

1. History of Tobacco Usage

Tobacco has been chewed for as long as it has been smoked. But partly because it is not a very visible habit, this oral use is seldom recognized as a common practice or as a health hazard. A rough estimate puts the number of people in the world using tobacco orally on a daily basis at more than 600 million (IARC, 1985).

Tobacco use can be traced back more than 7000 years, when it appears to have been first cultivated. It was probably used during this period for such purposes as alleviating hunger and whitening the teeth, and as a disinfectant when the juice was expectorated onto wounds. The first people to smoke, chew, and snuff tobacco were probably American Indians. Reports from the 1400s, such as those of Amerigo Vespucci in 1499, speak of Indians on Margarita Island, off the coast of Venezuela, chewing a green herb that was carried in gourds around their necks. Vespucci felt that the green herb, tobacco, was used to quench thirst, because of the scarcity of water on the island and the fact that chewing the leaf increased salivary flow. Indeed, a number of reports from explorers during this period, including Samuel de Champlain, the founder of Quebec, and Columbus, mention that the use of chewing tobacco by natives reduced hunger, thirst and fatigue among its users.

In the Americas tobacco chewing became popular among the non-native population. During the 1860s tobacco was chewed either in the form of a plug or as twists. Of the 348 tobacco factories listed in the 1860 Census for Virginia and North Carolina, only seven manufactured smoking products (Heimann, 1960). Tobacco chewing reached an all-time high in America by 1890, when some three pounds (about 1.5 kg) of plug, twist or fine-cut chewing tobacco were chewed annually per capita in the United States (Heimann, 1960). This remained the
dominant form of tobacco usage until the expansion of the cigarette industry in 1918 (Maxwell, 1980). Its decline was hastened by the "germ theory of disease" which made the habit of spitting in public places socially unacceptable. Anti-spitting laws were passed in New York and Philadelphia in 1896 and in Toronto in 1904 (Kozlowski, 1981).

During the latter half of the 1960s and through the 1970s, there was a resurgence in the use of smokeless tobacco in North America. This renaissance was probably due to an increased awareness of the harmful effects of tobacco smoking coupled with a lack of awareness of those resulting from its oral use. It has become particularly popular among adolescent males, promoted by sports figures and the Western "macho" image. It can be practised in areas where smoking is hazardous, such as in the steel, coal or petroleum industries, and is often said to be cheaper than smoking. In addition, people who require the use of their hands while working or playing can chew tobacco much more conveniently than they can smoke.

Since the 1960s, the U.S. production of smokeless tobacco has increased by approximately 60%. The total U.S. consumption of such products was approximately 132 million pounds (60 million kg) per year during the period from 1980 to 1982 (Tobacco Institute, 1981, 1982, 1983). On 1 January 1982, some types of chewing tobacco were reclassified as snuff (U.S. Department of Agriculture, 1983). Under this classification, consumption of chewing tobacco in 1982 was 88 million pounds (40 million kg) (Tobacco Institute, 1983).

In other parts of the world tobacco is often chewed in combination with other ingredients, in particular with areca nut, betel leaf and slaked lime, as in India and Southeast Asia. It is estimated that more than 450 million people practise this habit in these regions.
The chewing of betel quid without tobacco is a habit of great antiquity. The first mention of betel quid dates from 504 B.C., when it was recorded in the "Mahawamsa," a register of events in Sri Lanka written in Pali, that a princess made a gift of betel to her nurse (Krenger, 1942). The chewing of areca nut is also mentioned in Sanskrit manuscripts, Sushruta Samhita, believed to have been written around 600 B.C. near Benares. The Sanskrit name for the leaf of the betel vine, "tambula," persists in modern Hindi (Gode, 1961) as "tambuli," and is unchanged in Arabic and Persian (Nair and Kirk, 1960). In 1298, Marco Polo (Raghavan and Baruah, 1958) wrote in his travelogues that "the people of India have a habit of keeping in their mouth a certain leaf called the "tembuli" (Krenger, 1942).

Although the chewing of betel quid is practised in a number of different ways in various countries, the major components are relatively consistent. These are:

Areca nut (betel nut) is the fruit of the Areca catechu L. tree. Areca is a small genus comprising about 20 species of slender palms in the Palmaceae family. The areca palm is native to South and Southeast Asia and to several Pacific islands. The fruit grows in large bunches at the base of the leaves and varies in size and shape. It is orange-yellow in colour and is generally the size of a small egg. The fibrous pericarp of the fruit is separated from the seed or endosperm which is then used fresh or after sun-drying or curing (Arjungi, 1976).

Betel leaf (Piper betle L.) has been used since ancient times. Betel vines are cultivated in hot and humid climatic conditions in different parts of India, Indonesia, Malaysia and Sri Lanka.

Lime, known colloquially in India as chuna or chunam, is prepared either from the calcareous or silicious covering of marine invertebrates (sea shells)

...
harvested along the coastline of India, or from quarried stone in central India. It is manufactured on an industrial scale and is sold as a paste mixed with water in order to release calcium hydroxide.

Tobacco is often added to the abovementioned ingredients. The tobacco is usually only sun-dried, cut into strips and chewed with no further processing. A typical betel quid is shown in Figure 1.
Figure 1: A typical betel quid showing betel leaf, a portion of sun-dried tobacco and a quarter of a betel nut. Lime is shown on the right fingertip.
2. **Tobacco-Specific N-Nitroso Compounds**

Since tobacco and a few areca nut components are the only known constituents of any of the above mixtures which can give rise to carcinogenic compounds, this thesis focuses on these components as the most likely causes of oral cancer in users. Of the carcinogenic compounds found in chewing mixtures, the N-nitrosamines far outweigh any other. A large number of studies have shown that during the ageing, curing, fermentation and processing of tobacco, nicotine and other alkaloids give rise to carcinogenic N-nitrosamines (Hoffmann, et al. 1984). The concentration of these nitrosamines exceeds by at least a hundredfold the concentrations found so far in other consumer products.

The known carcinogens contained in snuffs and chewing tobaccos include the tobacco-specific nitrosamines (TSNA), such as 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosonornicotine (NNN), N-nitrosoanabasine (NAB) and N-nitrosoanatabine (NAT); plus the volatile nitrosamines, N-nitrosomorpholine (NMOR) (formed from packaging materials in some snuffs), N-nitrosodimethylamine (NDMA) (found in some snuffs) and N-nitrosopyrrolidine (NPYR) (found in some snuffs).

It has been estimated (National Research Council, 1981) that in the U.S., tobacco smoke gives rise to a twentyfold increase in consumption of N-nitroso compounds over other consumer products. However, since the concentration of TSNA is much higher in chewing tobacco than in cigarette smoke, and since the average chewer consumes 10 g of tobacco versus less than 1 g of tar inhaled by a smoker per day, tobacco chewing appears to be the greatest exogenous source of exposure to N-nitroso compounds (Hoffmann and Hecht, 1985).

In addition to the N-nitroso compounds, tobacco may often contain high levels of nitrate which, when converted to nitrite through ageing or reduction
in the saliva, can supply the precursor elements for nitrosation reactions. This is important when considering the alkaloid content of the areca nut.

In vitro experiments with arecoline and nitrite have shown that this areca nut alkaloid can give rise to at least four N-nitrosamines: N-nitrosoguvacoline, 3-(methylisourosamino)-propionitrile, 3-(methylisourosamino)-propionaldehyde (Wenke and Hoffmann, 1983) and N-Nitrosoguvacine (Nair et al., 1985). N-nitrosoguvacoline has been detected in the saliva of betel quid chewers together with TSNA when the quid contains tobacco (Wenke et al., 1984). Recently, N-nitrosoguvacoline and N-nitrosoguvacine have been found together in the saliva of betel quid chewers (Nair et al., 1985).

A further important consideration is the interaction of alcohol with N-nitrosamines. Of particular relevance to the chewing/alcohol problem is the observation that alcohol changes the distribution pattern of carcinogenic nitrosamines (Swann, 1982). In rats, alcohol diverted nitrosamines from the liver to other organs (e.g., the esophagus) which are more sensitive to these carcinogens. This change in pharmacokinetics may explain the threefold increase in esophageal carcinomas when diethylnitrosamine and ethanol are administered, compared to diethylnitrosamine plus drinking water (Gibel, 1967). Equally important appears to be the observation that N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) induces a high frequency of esophageal cancer when applied concurrently with alcohol (Yioris et al., 1984). Normally MNNG is carcinogenic only in the stomach of rats.

3. **Precancerous Oral Lesions and Cancer**

Many reports of case series have emphasized the relatively high frequency of tobacco chewing and snuff use among oral cancer patients (IARC, 1985). The clinical characteristics of cancer patients who use smokeless tobacco products
have also been described (IARC, 1985), especially the propensity of these cancers to occur in the presence of leukoplakia, to often have a verrucous appearance, and to be slow-growing, well-differentiated, squamous-cell carcinomas. Patients with cancer and with a chewing tobacco or snuff habit are frequently described as having cancer at the site or on the side where the quid is most frequently placed. Much epidemiological evidence indicates a highly significant increase in oral cancer among chewers, versus non-chewers, of betel quid (IARC, 1985). Areas of India and Southeast Asia, where the chewing of betel quid is common, have the highest incidence of oral cancer and oral preneoplastic lesions (leukoplakia) of any region in the world. In Kerala, India, where some of the studies presented in this thesis were carried out, oral leukoplakia (see Figure 2) among the Christian fishermen occurred in approximately 40% of chewers of betel quid plus tobacco, compared to a prevalence of 0.2% in people without such a habit (IARC, 1985). In addition, cancer of the mouth is the most prevalent cancer observed in this area. In males, cancers of the mouth, tongue and oropharynx were responsible for 27.2% of all occurring cancers; these sites accounted for 14.8% of tumors in females. The great majority were cancers of the buccal mucosa. Generally, the incidence of oral cancer is much higher where tobacco is added to the quid or where quid chewing occurs concurrently with smoking (IARC, 1985).
Figure 2: A homogeneous leukoplakia in the left buccal mucosa from a male age 30.
4. **Micronucleated Cells**

Beyond preneoplastic lesions, the search for a marker to detect damage at the cellular level led to the development of the micronucleus test. When applied to exfoliated cells of the buccal mucosa, this test permits the detection of localized genotoxic damage to that tissue. It is based on the hypothesis that an increase of micronuclei, which result from chromosome and chromatid fragments, should be found in the early stages of carcinogenesis. Three observations appear to suggest this. Firstly, most chemical carcinogens are active clastogens and should induce a variety of chromosome aberrations in human tissue exposed to carcinogenic mixtures. Secondly, many, if not all, carcinogens seem to produce altered chromosome numbers and chromosomal rearrangements, some of which involve particular regions (Rowley, 1983; Mitelman, 1985). These abnormal karyotypes in transformed cells must evolve from chromosomal aberrations during the preneoplastic stage. The third argument is based on a more theoretical consideration. Amplification and transposition of oncogenes seem to be mechanisms involved in neoplastic transformation and in progression of a malignant phenotype. These oncogene changes result from chromatid breaks and exchanges that can be indirectly detected by the appearance of micronucleated cells at preinvasive stages.

With this working hypothesis in mind, we applied the micronucleus test to exfoliated cells of individuals at elevated risk of cancer of the oral cavity (Stich, et al., 1982a,b; Stich and Rosin, 1983b), esophagus (Zaridze et al., 1985), urinary bladder (Raafat et al., 1984) and cervix (Stich, H.F., unpublished data). A significant increase in the frequency of micronuclei in exfoliated human cells (micronucleated cells or MNC) was found in virtually all individuals. Moreover, an elevated frequency of MNC was also observed in various tissues of individuals afflicted with a cancer-predisposing syndrome
such as Bloom's syndrome or ataxia-telangiectasia (Rosin and German, 1985; Rosin and Ochs, 1986).

There are considerable advantages to applying the micronucleus test directly to tissues of individuals exposed to carcinogens.

1.) The frequency of MNC can be readily estimated in smear preparations of exfoliated cells or in cell suspensions obtained from biopsies following treatment with pronase and/or collagenase. Thus genotoxic damage can actually be measured in tissues which are the targets of carcinogens.

2.) Exfoliated cells and biopsies can also be used to estimate the levels of retinol, beta-carotene, or a number of other chemopreventive agents. Thus a localized deficiency in protective agents, which could increase sensitivity towards the action of carcinogens, can be estimated in tissues in which carcinogen-induced genotoxic damage can also be quantified.

3.) Since exfoliated cells can be sampled from small areas, the distribution of MNC within a tissue can be mapped. Thus it should be possible to link foci with high frequencies of MNC to regions in which preneoplastic lesions, carcinoma in situ, or carcinomas preferentially develop.

4.) The scoring of MNC, which is currently a labour-intensive and time-consuming task, lends itself to automation, so that thousands of cells can be screened within minutes for the presence of micronuclei.

Since both the incidence of preneoplastic lesions and the incidence of micronucleated cells are elevated among chewers of tobacco and/or betel quid, these two factors were used to assess carcinogenic risk in the populations studied in this thesis, and to determine the populations' response to chemopreventive agents.
5. **Chemoprevention**

Having people give up chewing various tobacco-containing mixtures would obviously be the most effective way to prevent the oral cancers this produces. This is not an easy task, however. In India, the practice of chewing betel quid has been around for many thousands of years and is deeply integrated into Hindu culture. In addition, in the countries where these habits are practised, tobacco is often a major cash crop that cannot simply be replaced by another commodity. Also, the addiction to nicotine can often lead to the more dangerous habit of cigarette smoking if an individual is encouraged to give up the chewing habit. Other means of prevention are currently needed.

Considerable attention is currently being given to the use of retinoids and carotenoids as chemopreventive agents (Peto, 1983; Peto et al., 1981). There are several reasons for this. Epidemiological evidence points to an inverse relationship between the intake of beta-carotene-containing green or yellow vegetables and the incidence of cancers at various sites (Hirayama, 1981; Kvale et al., 1983; Marshall et al., 1982; Ziegler et al., 1984; Menkes et al., 1986), including oral cancer (Winn et al., 1984). Animal studies show beta-carotene and vitamin A to have a marked protective effect against a variety of carcinogens (Santamaria et al., 1983; Mathews-Roth, 1982), and in vitro experiments have revealed an antimutagenic (Belisario et al., 1985) and antitransformation (Som et al., 1984) activity. Whether these protective effects of beta-carotene are due to its potential for scavenging radicals (Krinsky and Deneke, 1982; Burton and Ingold, 1984), to its ability to interfere with the activation of carcinogens, or to its conversion into vitamin A, which is the actual chemopreventive agent, is at present difficult to assess. Several large-scale intervention trials have been initiated to prove or
disprove the usefulness of beta-carotene and vitamin A in preventing the
development of carcinomas. However, clinical trials using cancer as an endpoint
are expensive, require a relatively large number of participants, and last for
a long time. Tests that could provide more rapid results would be invaluable in
assessing treatment protocols before long-term, manpower-intensive intervention
trials are initiated. The two "intermediate endpoints" discussed earlier,
precancerous lesions and micronucleated cells, may be the answer to this need.

Preneoplastic lesions, including dysplasia (Cai, 1982), polyps (DeCosse et
al., 1975), leukoplakia (Ryssel et al., 1971), and esophagitis (Munoz et al.,
1985), have been successfully applied to track the response towards
chemopreventive agents. In our pilot trial on betel quid chewers, we examined
the effect of beta-carotene, beta-carotene plus vitamin A, and vitamin A alone
on the remission of established oral leukoplakias and the development of new
ones.

The usefulness of micronucleated cells as markers in chemoprevention
trials has been explored (Stich et al., 1984a,b, 1985). Micronucleated cells
appear to be good indicators of carcinogen-induced injuries to chromosome
complements (Stich and Rosin, 1985). Increased frequencies of micronucleated
cells were observed in tissues at elevated risk of developing cancer, including
the oral mucosa of betel quid chewers (Stich et al., 1982a), snuff dippers
(Stich et al., 1985), users of various tobacco-containing mixtures, including
Khaini tobacco (Mirvish, 1982) and "nass" (Zaridze et al., 1985), and cigarette
smokers (Stich and Rosin, 1983a; Stich, 1987). Since micronuclei reveal the
"pathobiologically effective dose," which is more indicative than a mere
"exposure dose," they should provide information on the protective action of
chemopreventive agents.
6. **Objectives**

The objectives of this thesis were fivefold:

1) To determine the snuff dipping and chewing patterns in three communities (Inuit in the Northwest Territories, native Canadian Indians in northern Saskatchewan and East Indians in Kerala, India).

2) To determine the amounts of nitrosamine precursors (nitrite) and tobacco-specific nitrosamines in samples of smokeless tobacco used by members of the abovementioned communities.

3) To determine the amounts of preformed and endogenously formed nitrosamines in users of smokeless tobacco.

4) To determine the pattern of oral lesions (micronucleated cells, leukoderma and precancerous leukoplakia) in the three population groups, which differ in their chewing patterns.

5) To explore the effects of beta-carotene and vitamin A on reducing the frequency of precancerous oral lesions.
MATERIALS AND METHODS

1. USE OF SMOKELESS TOBACCO

1.1 Inuit in the Northwest Territories

The community of Gjoa Haven was chosen for study because of the large percentage of individuals who dip snuff and because of the low consumption of alcohol resulting from liquor prohibition since 1978.

By the 1984/1985 census (Northwest Territories Data Book 1984 - 85) this community has a population of 523, of which 95% are Inuit. The sex distribution is 50% male and 50% female, with an age distribution of: 0-4, 17%; 5-14, 33%; 15-64, 48%; 65 and over, 2%. The major economic activities are trapping, hunting and fishing, the last two also being the major sources of food. The community has two small stores which supply canned and packaged foods plus intermittent supplies of fresh fruit and vegetables.

The snuff dipping habits of individuals in this community were examined by door to door questionnaire.

No oral carcinomas were seen among the 180 cancers diagnosed between 1949 and 1974 in the Inuit of the Canadian Arctic (Schaefer et al., 1975).

1.2 Native Canadian Indians in Saskatchewan

La Loche was chosen as a study site because of its large number of snuff dippers and because of a relatively high consumption of alcohol.

By the 1983/1984 census carried out by the local band office the community of La Loche has a population of 1847, 90% of which are Chippewa, Metis Indians. La Loche has a sex distribution of 53% male and 47% female, and an age distribution of: 0-4, 15%; 5-14, 25%; 15-64, 57%; 65 and over, 3%. The major
economic activities include trapping, hunting, fishing and some mining. La Loche has a moderate-sized grocery store, plus smaller outlets providing fresh fruit and vegetables and packaged and canned food products year round; a small government liquor store is also present.

The frequency of oral cancer is the same as that found in the rest of the Canadian population that do not use snuff.

The brands of tobacco used by the Canadian natives were checked by requesting to see the cans of snuff or bags of chewing tobacco. The weight of tobacco was determined using a small scale and immediately weighing the snuff after it had been "pinched" by the individual being interviewed.

1.3 **East Indians in Kerala, India**

Fishermen living along a coastal strip near Trivandrum, Kerala, India, were chosen for study because of the large numbers of betel quid chewers, the relatively high consumption of alcohol and the frequently observed precancerous lesions (leukoplakia) and oral cancers. In this area, cancer of the mouth is the most prevalent cancer. In males, cancers of the mouth, tongue and oropharynx were responsible for 27.2% of all cancers; these sites accounted for 14.8% of tumors in females. The great majority were cancers of the buccal mucosa. Generally, the oral cancer incidence is much higher where tobacco is added to the quid or where chewing occurs concurrently with smoking (IARC, 1985).

No seeds, spices, sugar or flavourings, which are ingredients commonly used in betel quid of northern India, were added to the quids used by the fishermen. Weights of quid ingredients were obtained by asking individuals to give a sample of their usual quid and later weighing each portion on an analytical balance.
Demographic data was unavailable for the fishermen of Kerala at the time of writing this thesis.

2. **Nitrite in Smokeless Tobacco Samples**

2.1 **Snuff and Chewing Tobacco**

The following smokeless tobacco samples were used in this study: 5 different commercial brands of snuff (packed in Canada), labelled C, B, SL, SW and K (4 samples each); one "chewing" tobacco (U.S. brand), labelled R; homemade "nass" from Samarkand, the Soviet Union (4 samples); and Khaini tobacco from Bengal, India (4 samples). The snuff, chewing tobacco and Khaini tobacco containers or boxes were freshly opened prior to chemical analysis and prior to being used by the volunteers. During interviews we took particular care to distinguish snuff (commercially available, finely ground tobacco) from chewing tobacco (commercially available strands of tobacco). We checked the brands by requesting to see the cans of snuff or bags of chewing tobacco used by the volunteers. The weight of tobacco was determined with a small scale. The snuff was weighed immediately after it had been "pinched" by the individual being interviewed.

2.2 **Betel Quid**

The East Indian fishermen surveyed in this study all chewed a simple betel quid consisting of areca nut (*Areca catechu* L.), tobacco stems (*Nicotiana tabacum* L.), slaked lime (calcium hydroxide) from marine shells, and betel leaf (*Piper betle* L.). Only the tobacco portion of the quid was analyzed for nitrite.
2.3 **Determination of Nitrite**

Water extracts of tobacco were prepared by suspending 1 g of tobacco in 10 ml of distilled water and shaking vigorously in a 37°C water bath for 30 min. This was followed by centrifugation (2000 rpm for 2 min) to remove tobacco particles.

The nitrite concentration of both saliva and water extracts of tobacco was determined by the same procedure. Colour reagent was prepared by dissolving 1 g of sulfanilic acid and 0.1 g of N-1-naphthylethylenediamine dihydrochloride in 100 ml of 20% acetic acid; 25 µl of either saliva or water extract of tobacco were added to 2 ml of the colour reagent in a Nalgene test tube and allowed to react for 15 min. Absorbance was read at 540 nm on a Bausch and Lomb mini Spectronic 20 against a blank of unreacted colour reagent. Concentrations of nitrite were determined from a standard curve. A known amount of sodium nitrite was carefully weighed out on an analytical balance and dissolved in a precise volume of distilled water. Serial dilutions were made and the standard curve was prepared by the procedure described above.

The average recovery of nitrite added to saliva at levels ranging from 10 to 30 ppm was 95%. Large variations were seen in the nitrite samples: analysis of 56 East Indian saliva samples gave a mean of 36.3 ppm with a standard deviation of 35.06 and a coefficient of variation of 96.7%. These variations could have been due to the varying dietary intake of nitrate and to the oral hygiene of the individuals. Snuff samples purchased in different locations also showed large variations in nitrite concentration. For example, 4 Copenhagen snuff samples purchased in different areas of Canada (NWT, N. Saskatchewan and Vancouver) had a mean nitrite concentration of 735 ppm with a standard deviation of 235 and a coefficient of variation of 32%. These variations were
most probably due to different storage times. However, 10 individual
determinations of nitrite from the same sample of tobacco gave a mean nitrite
ccentration of 434 ppm, a standard deviation of 71.4 and a coefficient of
variation of 16%. All nitrite determinations were done by myself. The procedure
for nitrite determination is outlined in Figure 3.
Figure 3: Procedure for Nitrite Determination

25 μl water extract of tobacco or saliva

→ into 2 ml colour reagent (1 gm sulfanilic acid + 0.1 gm N-1-naphthylethylenediamine dihydrochloride in 100 ml 20% acetic acid)

→ determine absorbance at 540 nm (mini Spectronic 20) against unreacted colour reagent as blank

→ determine nitrite concentration from standard curve
3. Nitrite in the Saliva of Users of Smokeless Tobacco

3.1 Snuff Dippers

Saliva was collected for nitrite determination in the Canadian populations (64 Inuit and 20 Indian) by having individuals expectorate 2-4 ml of saliva into Nalgene test tubes. The samples were analyzed immediately after. Saliva samples were not collected during a chewing period.

For nitrite determination during chewing, seven snuff dipping Inuit males in Gjoa Haven, ranging from 14 to 20 years of age, donated their saliva.

3.2 Betel Quid Chewers

Saliva samples were collected by the same procedure as that described above from 50 different betel quid chewers in Kerala, but not during a chewing period.

4. Nitrosation Capacity of Saliva Samples

For estimating the nitrosation capacity, saliva was collected from one volunteer using 1 g of common North American brands of snuff (Copenhagen, Skoal or Big Horn). During a 20-min period of keeping the tobacco in the lower gingival groove, the volunteer expectorated the saliva into a Nalgene test tube.

The formation of NPRO was quantified by mixing, in vitro, the saliva of the snuff user with proline (200 mg), and incubating the mixture for 30 min at 37°C.

5. N-Nitrosoproline in the Urine of Snuff Dippers

Before, during and after the use of snuff, urine was collected in bottles containing 300 mg of solid NaOH. The urine was stored at -20°C until
nitrosoproline (NPRO) analysis, which took place within one day of sampling. Where urine samples were collected from individuals in a population, the same method of storage and analysis was used.

In the Inuit settlement of Gjoa Haven, urine samples were collected from 14 regular snuff users and 15 non-users who were matched by sex and age. In addition, urine samples from Indians in the community of La Loche were collected from 47 snuff users and 27 non-users of comparable age and sex.

5.1 Determination of Nitrosoproline

Nitrosoproline (NPRO) and N-nitrosopiperidine-2-carboxylic acid (NPIC) were synthesized and purified using published procedures (Lijinsky et al., 1970). Extraction columns, unbuffered, style TE 3020, were obtained from Fisher Scientific. All other chemicals were reagent or equivalent grade.

Samples of urine were preserved by the addition of 300 mg solid NaOH per 100 ml, and were stored frozen until analysis. Samples were analyzed by the procedure of Sen et al. (1983) with minor modifications. Samples of urine (15 ml) were acidified with 3 ml 20% ammonium sulfamate in 1.8 M $\text{H}_2\text{SO}_4$, and 10 $\mu$l internal standard solution in methanol containing 150 ng NPIC was added. Urine samples were loaded onto 20-ml capacity disposable extraction columns (Extube, Analytichem International), and allowed to absorb. Columns were then eluted with 4 x 20 ml ethyl acetate, which was dried over 20 g anhydrous NaSO$_4$, prior to use. The organic extracts were decanted with washings through a cotton wool plug in a funnel into round-bottomed flasks, and evaporated until dry using a rotary evaporator. Residues in the flasks were dissolved in a total of 4 ml methanol, and transferred to 5-ml conical-bottomed sample vials.

The methanol was evaporated to a volume of approximately 0.1 ml using a heating block at 50°C and a stream of nitrogen; 0.5 ml BF$_3$ reagent
(14% methanol) was added, and the samples were capped and incubated for either 4 hr at 50°C or overnight at room temperature. Dichloromethane (0.5 ml) and 2 ml water were added, and the samples were shaken vigorously to extract the esterified nitrosamines into the chlorinated solvent. Calibration samples were prepared by esterifying 150 ng NPIC or NPRO in 0.5 ml BF₃ methanol in a similar manner.

Gas-chromatographic (GC) analysis of 10-μl aliquots of the dichloromethane solution was performed on a Perkin Elmer 3920 gas chromatograph, equipped with a nickel column (2 m x 2 mm i.d.) packed with 10% Carbowax 20 M on 80-100 mesh Chromosorb W. Chromatographic conditions were: injector 250°C, column 190°C, carrier helium at 50 ml/min. Under these conditions, NPIC and NPRO retention times of 3.5 and 4.2 min, respectively, were achieved and were nearly baseline resolved.

Nitrosamines were detected using a thermal energy analyzer (TEA) (Model 502A; Thermal Electron Corp., Waltham, MA) operating at a chamber pressure of 0.55 torr, and using a trap cooled with dry ice. NPRO was quantified using daily calibration samples and an internal standard program on a Spectra Physics SP4100 computing integrator (Spectra Physics Corp., Santa Clara, CA). Practical detection limits were in the order of 0.1 ng NPRO per injection, corresponding to approximately 0.5 ng NPRO/ml urine. Recoveries from samples spiked with standard NPRO were generally in the 95-100% range. Analysis of 9 separate samples taken over a 17-hr period from the same individual produced a mean NPRO value of 16.09 ng/ml, with a standard deviation of 2.31 and a coefficient of variation of 14.4%.
In order to determine the reproducibility and the rate of elimination of NPRO from the human body in in vivo experiments, urine samples were analyzed for NPRO after ingestion of variable amounts of nitrate. In five experiments, 65, 130, 195, 260 and 325 mg nitrate were ingested together with 500 mg proline. In each case, a similar pattern of NPRO excretion was obtained, and the amount of NPRO returned to the background level within 24 hr, indicating that NPRO was totally eliminated into the urine within that period. Therefore the NPRO analyzed in urine collected over 24 hr after dosing was an indicator of daily nitrosation in vivo. Nitrosamines in saliva and extracts were determined in a manner similar to that stated above by scaling down extraction volumes. The procedure for NPRO determination is outlined in Figure 4.
Figure 4: Procedure for N-nitrosoproline (NPRO) Determination

Urine preserved with 300 mg solid NaOH/100 ml

15 ml urine

Acidify with 3 ml 20% ammonium sulfamate in 1.8 M H₂SO₄

add 10 μl (150 ng NPIC) internal standard in MeOH

Load onto 20-ml capacity extraction columns

elute 4 x 20 ml ethyl acetate
(dried over 20 gm anhydrous NaSO₄)

Collect organic extracts through cottonwool plug into round-bottomed flask

Evaporate to dryness with rotary evaporator

Redissolve residue in 4 ml MeOH and transfer to 5-ml conical-bottomed vial

Evaporate to 0.1 ml using heating block at 50°C under N₂

Add 0.5 ml BF₃ (14% MeOH)

Cap and incubate for 4 hr at 50°C or overnight at room temperature

Add 0.5 ml dichloromethane and 2 ml water, and shake vigorously

Gas chromatographic/Thermal energy analyzer analysis
6. **Tobacco-Specific Nitrosamines in Different Brands of Tobacco**

Two brands of snuff (Copenhagen and Skoal) commonly chewed by Canadian natives were analyzed for the presence of TSNA. Water extracts were prepared by suspending 1 g of tobacco in 10 ml of distilled water and shaking vigorously in a 37°C water bath for 30 min. 0.5 ml of the extract was used for TSNA analysis.

After adding internal standards (N-nitrosodimethylamine-\(^{14}\)C and N'-nitrosonornicotine-\(^{14}\)C), each sample was extracted four times with 20 ml ethyl acetate, using a prewetted pretube. The organic fractions were pooled and concentrated to approximately 3 ml, and then subjected to column chromatography on 65 g basic alumina. After elution with 200 ml freshly distilled dichloromethane (fraction I, containing volatile nitrosamine), the samples were chromatographed with 200 ml dichloromethane-acetone (4:1) (fraction II, containing the TSNA). After being concentrated to 1 ml, the samples were analyzed by GC-TEA. For the volatile nitrosamines, a 12 ft x 1/4 in (2 mm i.d.) glass column packed with 10% Carbowax 20 M on Chromosorb WNAW (100-120 mesh) at an oven temperature of 175°C was used. For the TSNA, a 12 ft x 1/4 in (2 mm i.d.) glass column packed with 10% UCW-98 on Gas Chrom Q (80-100 mesh) at an oven temperature of 200°C was used (Adams et al., 1983). An aliquot of both fractions was used for scintillation counting to determine the recovery rate.

Several blank samples, using 5 ml water, were also assayed to ensure that there was no contamination during analysis, since the largest sources of error in all nitrosamine analyses result from contamination by nitrosamines or precursors from other sources. Detection limits for TSNA are 2.4 ppb and acceptable levels of DMNM are those below 0.9%. Recoveries from saliva spiked with TSNA were generally 80-90%. Table I gives the means and precision of three analyses.
The following four TSNA were estimated in the saliva of the snuff dippers: N'-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB), and 4-(methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK).
Table I Analysis of three of the same tobacco samples for TSNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compound</th>
<th>Mean (μg/g) and Precision (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>N-Nitrosonornicotine (NNN)</td>
<td>45.6 ± 7%</td>
</tr>
<tr>
<td></td>
<td>4-(Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)</td>
<td>7.4 ± 8%</td>
</tr>
<tr>
<td></td>
<td>N-Nitrosoanatabine (NAT)</td>
<td>47.7 ± 8%</td>
</tr>
<tr>
<td></td>
<td>N-Nitrosoanabasine (NAB)</td>
<td>1.8 ± 12%</td>
</tr>
</tbody>
</table>
7. **Tobacco-Specific Nitrosamines in the Saliva of Snuff Dippers**

Subjects from whom saliva samples were collected were asked to take their usual quantity of snuff and to expectorate into a tube at intervals when they would normally spit. Collection of saliva took place over a period of 15 minutes (before, during and after the chewing period). One ml of citrate-phosphate buffer (pH 4.5) containing 3.25 mg NaN₃ (nitrosation inhibitor) and 1.00 mg *cis*-2,6-dimethylmorpholine (serving as monitor amine) was added to all saliva samples, which were then frozen. The amount of saliva collected varied between 3.0 and 6.5 ml. All participants in this study used the same brand of snuff (made in the U.S.A. for National Tobacco Co., Ltd., Montreal, Canada) and none were smokers.

After the saliva samples had been allowed to thaw, 0.5 ml was removed for the radioimmunoassay of nicotine and cotinine. The remainder was used for the analysis of TSNA and nitroso-*cis*-2,6-dimethylmorpholine (DMNM) by the method described above.

8. **Determination of Nicotine and Cotinine**

Nicotine and cotinine were determined from 0.5-ml thawed saliva samples by radioimmunoassay according to the method of Langone et al. (1973) with specific antisera produced by injection into rabbits of *trans*-3-succinylmethylnicotine and *trans*-4-carboxycotinine bound to albumin.

Recoveries of 98-104% relative to the internal standard were obtained on standard samples. By suitable dilution of a standard solution the minimum detectable level was found to be 5 ng, i.e., equivalent to a concentration of 300 nmole/litre.
Replicate analyses of a smoker's urine showed a coefficient of variation of 7.3% for nicotine and 9.0% for cotinine. The coefficient of variation of the calculated nicotine:cotinine ratios was 11.1%.

9. **Frequency of Micronuclei in the Oral Mucosa of Snuff Dippers and Tobacco Chewers**

Exfoliated cells of the oral mucosa were obtained by swabbing with a moistened wooden tongue depressor, smearing the cells onto a clean microscope slide, air-drying, and fixing in 80-85% ethanol. If exfoliated cells from smaller areas of the gingival groove were desired, split wooden popsicle sticks were used. The fixed cell preparations were stained with Feulgen reaction following a 10 min hydrolysis with N-HCl, and counterstained with fast green. The Feulgen-stained slides were screened for various nuclear anomalies, including micronuclei, whose size can vary from small Feulgen-positive bodies to larger, nucleus-like structures with membranes and clearly visible chromatin. The pros and cons of this technique have been recently reviewed (Stich, 1987). Micronuclei were scored only in intact epithelial cells. We followed well-established criteria for estimating the frequency of human exfoliated cells with micronuclei (Stich and Rosin, 1983b, 1984a). The usefulness of this technique in following the response to chemopreventive agents is discussed by Stich and Rosin (1985). An elevated frequency of micronucleated cells was found to be a simple marker indicating an elevated risk of developing cancer (Table II). The micronucleated cell frequencies were determined in this study by Mrs. H.F. Stich, an expert in this field.

Anucleated exfoliated cells are common in the palate, but rare in the buccal mucosa of non-chewers of tobacco and non-smokers. At least 300 cells per sample were counted to estimate the percentage of anucleated cells. Feulgen-stained smear preparations were used.
TABLE II
Frequency of Micronuclei in Human Tissues at Elevated Risk for Cancer

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Suspected Carcinogenic Factors</th>
<th>Elevation of Micronucleated Cells (Fold)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral mucosa:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gingival groove</td>
<td>Snuff</td>
<td>4.2</td>
<td>Stich et al. (1985)</td>
</tr>
<tr>
<td>Inner lip</td>
<td>Khaini tobacco</td>
<td>4.4</td>
<td>Stich et al. (1982b)</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>Nass</td>
<td>8.2</td>
<td>Stich (1987)</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>Betel quid</td>
<td>9.4</td>
<td>Stich et al. (1982a)</td>
</tr>
<tr>
<td></td>
<td>Smoking and drinking</td>
<td>4.6</td>
<td>Stich and Rosin (1983a)</td>
</tr>
<tr>
<td></td>
<td>Smoking</td>
<td>3.4</td>
<td>Fontham et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Curry</td>
<td>1.5</td>
<td>Picker and Fox (1986)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>Unknown (esophagitis)</td>
<td>3.4</td>
<td>Stich and Zaridze (unpublished data) Mandard et al. (1987)</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Schistosoma haematobium</td>
<td>13.5</td>
<td>Raafat et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>Smoking</td>
<td>6.0</td>
<td>Fontham et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Smoking and coffee drinking</td>
<td>9.4</td>
<td>Stich (1987)</td>
</tr>
<tr>
<td></td>
<td>Smoking</td>
<td>Shift to higher values</td>
<td>Reali et al. (1987)</td>
</tr>
<tr>
<td>Bronchial epithelium</td>
<td>Smoking</td>
<td>2.6</td>
<td>Fontham et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Smoking</td>
<td>2.2</td>
<td>Stich (unpublished data)</td>
</tr>
<tr>
<td>Cervical epithelium</td>
<td>Smoking</td>
<td>5.1</td>
<td>Fontham et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Unknown (dysplasia)</td>
<td>3.0</td>
<td>Stich (1987)</td>
</tr>
<tr>
<td>Blood lymphocytes</td>
<td>Smoking</td>
<td>1.5</td>
<td>Högstedt et al. (1983a)</td>
</tr>
<tr>
<td></td>
<td>Styrene</td>
<td>1.5</td>
<td>Högstedt et al. (1983b)</td>
</tr>
<tr>
<td></td>
<td>Styrene</td>
<td>9.0</td>
<td>Meretoja et al. (1978)</td>
</tr>
<tr>
<td></td>
<td>Styrene</td>
<td>4.0</td>
<td>Nordsenson and Beckman (1984)</td>
</tr>
<tr>
<td></td>
<td>X-ray contrast media</td>
<td>1.6-4.3</td>
<td>Parvez et al. (1987)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Antileukemia agents</td>
<td>&gt;34.0</td>
<td>Abe et al. (1984)</td>
</tr>
<tr>
<td>Spermatids</td>
<td>Smoking</td>
<td>1.4</td>
<td>Lähdetie (1986)</td>
</tr>
</tbody>
</table>

*Elevated frequencies were observed in only 5% of patients examined.*
10. Precancerous Lesions and Cancer of the Oral Cavity of Users of Smokeless Tobacco

10.1 Leukoderma

Oral lesions in users of snuff and chewing tobacco have been classified, according to their appearance, into four groups by Axell et al. (1976) and into three categories by Greer and Poulson (1983). To avoid any subjective bias in subdividing these lesions, we recorded only two types of anomalies: (1) whitish to yellowish wrinkled patches of the mucosa with or without furrows (corresponding to categories 2 and 3 of Greer and Poulson [1983]), which are the most commonly observed anomalies, and (2) definite leukoplakias, as described by the World Health Organization (1980). There is a certain ambiguity in properly defining the many whitish lesions of the oral mucosa. The whitish wrinkled areas which we observed at the sites where the snuff or chewing tobacco came in close contact with the mucosa are not comparable to the preleukoplakias, leukoplakias or erythroplakias so commonly seen in betel quid chewers (IARC, 1985) or users of "nass" (a mixture of tobacco, slaked lime, oil and ash) (Zaridze et al., 1986). The whitish areas can disappear within days or weeks, whereas remission of other mucosal abnormalities requires several months following the cessation of snuff or betel quid use. The precancerous state of leukoplakias, particularly those with reddish areas, appears well-established, whereas there is no evidence to consider the whitish wrinkled patches of snuff dippers a preneoplastic stage. The presence or absence of these latter lesions were recorded for all the Canadian Inuit and Indians surveyed.
10.2 **Leukoplakia**

A precancerous lesion is defined as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart. This condition is, therefore, a generalized state associated with a significantly increased risk of cancer (WHO Collaborating Centre for Oral Precancerous Lesions, 1978; Axell et al., 1984). Examples of oral precancerous lesions are leukoplakia and erythroplakia; oral precancerous lesions also include sideropenic dysphagia, submucous fibrosis, and possibly lichen planus.

The concept of leukoplakia as a precancerous lesion is based on findings that a significant number of oral carcinomas are associated with pre-existing leukoplakia, and that some leukoplakias appear to undergo malignant transformation (Pindborg et al., 1975; Silverman et al., 1984). However, studies of oral leukoplakia are often difficult to compare because of a lack of uniformity in the histological definition of leukoplakia.

In this thesis leukoplakia are classified according to the WHO Collaborating Centre for Oral Precancerous Lesions (1978), as outlined in Table III.
### Table III

Classification of Oral Precancerous Lesions (WHO Collaborating Centre for Oral Precancerous Lesions, 1978)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description of Lesion</th>
<th>Histopathology</th>
<th>Predisposition to Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukoplakia</td>
<td>White, whitish/yellow, or grey patch affecting very small to large areas of the mucosa; smooth or wrinkled surface often with cracks; may present as homogeneous, nodular or speckled</td>
<td>Hyperorthokeratosis; hyperparakeratosis; atrophy; diffuse chronic inflammation in lamina propria (lymphocyte and plasma cells); some dysplasia in nodular type</td>
<td>6-10% become malignant</td>
</tr>
<tr>
<td>Erythroplakia</td>
<td>Bright red, velvety plaques; irregular, well-defined outline; occasional nodular surface with white or yellow spots</td>
<td>Marked epithelial atrophy; variable epithelial dysplasia; heavy chronic inflammation in subepithelial connective tissue (mainly plasma cells)</td>
<td>Greater than 10% become malignant</td>
</tr>
<tr>
<td>Leukoderma</td>
<td>Whitish to yellowish wrinkled patches; with or without furrows</td>
<td>Epithelium hyperplastic; microbial colonization of irregular surface; superficial epithelial cells large and angular, pyknotic nuclei, cytoplasm lightly stained or empty</td>
<td>None known</td>
</tr>
</tbody>
</table>
11. Intervention Strategies

11.1 Nitrite Trapping Agents

The inhibitory effect of ascorbate and caffeic acid on nitrosamine formation was studied using NPRO as a model in both in vitro and in vivo (ascorbate only) systems.

The in vitro method was done by having a volunteer (myself) chew 1 g of three different brands of snuff and expectorate the saliva (10 ml) into a Nalgene test tube. To the saliva 200 mg proline was added either alone (control) or with either 200 mg ascorbate or 200 mg caffeic acid. The solutions were then taken to pH 2.5 using small amounts of normal HCl and allowed to incubate at 37°C for 30 min. NPRO analysis was then made by the procedure described above.

For the in vivo determination of endogenously formed NPRO a volunteer (myself) chewed 1 g of snuff (2 times at 60 min intervals) plus ingested simultaneously 200 mg of proline (control). This experiment was repeated with ingestion of 500 mg of chewable ascorbic acid 30 min prior to chewing the snuff and ingesting proline and 30 min following snuff chewing and proline ingestion. The same experiment was repeated with a larger ingestion of ascorbate at 30 min prior to the snuff and proline, 30 min after and again 30 min, 60 min and 90 min after the final ingestion of proline and snuff chewing. All urine passed over the next 17 hr was collected for NPRO analysis. The experimental protocol is shown in Figure 5.
Figure 5: Experimental Protocol for Endogenous NPRO Formation

(A) SNUFF + PROLINE

(B) SNUFF + PROLINE

(C) SNUFF + PROLINE

-30 0 30 60 120 180 240 MIN
11.2 Administration of Beta-carotene and Vitamin A

Beta-carotene was supplied by Hoffmann-La Roche as water-dispersal beadlets consisting of approximately 20% starch, 20% dextrose, 30-50% gelatin, 10-11% beta-carotene, and trace amounts of alpha-tocopherol and ascorbate palmitate. The butylated hydroxyanisole and butylated hydroxytoluene present in some forms of beta-carotene were absent from our preparations.

Vitamin A capsules were purchased from Stanley Drug Company Ltd., Vancouver. Each capsule contained vitamin A derived from fish liver oils equivalent to 15 mg retinol.

Placebo capsules supplied by Hoffmann-La Roche contained dextrose.

11.3 Inuit: Beta-carotene

Three beta-carotene capsules (30 mg beta-carotene per capsule) were administered twice weekly for 10 weeks under the strict supervision of myself and an Inuit interpreter who spent the entire trial period in Gjoa Haven. Treatment with beta-carotene was initiated with 27 snuff dipping individuals, plus 10 receiving placebo. Individuals were selected who took more than 6 snuff dips per day. Three boys lost interest in cooperating. The participants who received beta-carotene, placebo capsules or no treatment (18 individuals) were chosen from the subjects previously interviewed. To the best of our knowledge, neither the number of tobacco plugs, the site, nor the length of time that the tobacco was kept in the mouth changed during the 10-week trial period.

11.4 East Indians: Beta-carotene and Beta-carotene plus Vitamin A

A total of 130 betel quid chewers with oral leukoplakias were divided into three groups. One (35 participants) received placebo capsules, a second (35 participants) received beta-carotene, and a third (60 participants) received
beta-carotene plus vitamin A. Individuals were selected on the basis of having established leukoplakia plus the location in which they lived to make the distribution of capsules easier to people in the same vicinity. To ensure that all trial participants received the desired doses, the placebo, beta-carotene and vitamin A capsules were administered twice weekly for 6 months under the strict supervision of myself, a local nurse and a staff member of the Regional Cancer Centre, Trivandrum.

The following were given twice weekly to each person, depending on his group: (1) 3 beta-carotene capsules, each containing 30 mg beta-carotene; (2) 3 beta-carotene capsules plus one vitamin A capsule (50,000 IU), and (3) 3 placebo capsules containing dextrose and water-dispersal beadlets. Of the original participants, 5 in Group 1, 4 in Group 2, and 9 in Group 3 were lost due to death, illness or emigration. Endpoints were examined at 3 months and again at 6 months.

11.5 **East Indians: Vitamin A**

Betel quid chewers with well-established leukoplakias (diagnosed by Drs. Mathew and Sankaranarayanan according to the WHO Collaborating Centre for Oral Precancerous Lesions [1978]) were distributed into two groups based on the area of the beach in which they lived: one group of 35 participants received placebo capsules (dextrose), and the second group of 30 participants received vitamin A (200,000 IU/week), given twice weekly as 2 capsules of 50,000 IU of vitamin A each. In the placebo group, 33 out of 35 chewers completed the trial, and in the vitamin A-treated group, 21 out of 30 chewers remained on the vitamin A regime. In all the intervention studies careful attention was taken by myself or a staff member of the Regional Cancer Centre, Trivandrum to make sure that the pills were actually swallowed by each of the trial participants.
12. **Determination of Beta-carotene and Retinol**

To determine the existing levels of beta-carotene and retinol in the Inuit population studied, serum samples were collected from 110 subjects including 70 non-users of snuff and 40 users.

Blood samples were collected in the community of Gjoa Haven using vacutainers (No. 6421) containing a clot activator. Samples were stored on ice for approximately 1 hr after collection, then spun in a benchtop centrifuge (700 g for 5 min). Serum was collected with a Pasteur pipet into 2-ml cryotubes, and stored in liquid nitrogen until analysis.

Aliquots of serum (200 µl) were placed in 1.5-ml polypropylene microcentrifuge tubes, and 200 µl aliquots of 5% KOH in methanol were added. Samples were digested for 1 hr at 50°C, and then 600 µl of hexane were added. Samples were extracted by vortex mixing for 1 min, and the hexane was separated from the sample by centrifugation. Aliquots of hexane (400 µl) were removed from the samples, and dried under reduced pressure in 1.5-ml polypropylene microcentrifuge tubes in a centrifugal evaporator (Speed Vac Model SVC100H, Savant Instruments, Hicksville, NY). Residues were redissolved in 20 µl methylene chloride, and then diluted to a total of 200 µl with methanol.

Retinol and carotene were measured by isocratic high-pressure liquid chromatography (HPLC) (Miller et al., 1984). For retinol, 50-µl aliquots of sample were injected into a Brownlee Spheri 5 reversed-phase column (5 micron packing, 2.1 mm internal diameter x 22 cm) coupled to a 2.1 mm internal diameter x 3 cm guard column with the same packing. Solvent consisted of 99% methanol and 1% water at a flow rate of 0.5 ml/min. For carotene, 50-µl aliquots of sample were injected into a Vydac 201 TP reversed-phase column (10 micron packing, 3.2 mm internal diameter x 25 cm). Solvent consisted of 95%
methanol and 5% methylene chloride at a flow rate of 1.0 ml/min. All solvents contained 0.1% phosphoric acid to protect against possible column degradation by alkaline samples.

Retinol and carotene were detected by a variable wavelength UV/VIS HPLC absorbance detector using a tungsten lamp, which was set at 325 nm for retinol and then switched to 450 nm for carotene. Samples were quantified by an electronic computing integrator which had been calibrated by injection of retinol and carotene standards in methanol. Calibration standards were prepared by dissolving crystalline retinol or carotene (Sigma, St. Louis, MO) in methanol, and determining the concentration of solutions by their absorbance, using molar extinction coefficients in methanol of 52,500 (retinol, 325 nm) and 130,000 (carotene, 450 nm).

Within-day precision was 5.6% for beta-carotene in 10 samples from a pooled single specimen of normal serum (mean = 57 ng/ml; SD = 3.2 ng/ml). Day-to-day precision during 27 days was 5.9% (mean = 57 ng/ml, SD = 3.4 ng/ml) for the same pool. The efficiency of extraction of beta-carotene from serum appeared to exceed 95%: the absorbance at 450 nm after a second extraction was less than 3% of that for the original extract.

Retinol analysis of the same plasma on 10 consecutive days gave a mean value of 450 ng/ml (SD = 19.8 ng/ml), and a coefficient of variation of 4.4%. Precision for the same-day analysis of 10 aliquots of a second plasma gave a mean of 440 ng/ml (SD = 18.0 ng/ml), and a coefficient of variation of 4.1%. The procedure for the determination of serum retinol and beta-carotene is outlined in Figure 6.
Figure 6: Determination of Serum Beta-carotene and Retinol

Serum (200 μl)

- into 1.5-ml polypropylene microcentrifuge tubes

- add 200-μl aliquots of 5% KOH in MeOH

- digest for 1 hr at 50°C

- add 600 μl hexane

- extract by vortex mixing for 1 min

- separate hexane from sample by centrifugation

- remove 400-μl aliquots of hexane and dry under reduced pressure in 1.5-ml polypropylene microcentrifuge tubes in centrifugal evaporator

- redissove in 20 μl methylene chloride and dilute to 200 μl with MeOH

- inject 50 μl into HPLC
13. **Questionnaire**

The Inuit surveys were conducted during the summer of 1986 in the communities of Gjoa Haven, NWT, and the native Indian surveys during the summer of 1987 in the community of La Loche, N. Saskatchewan. A total of 300 native Indians and 200 Inuit were interviewed, and their oral cavities were screened for lesions. In a settlement, sampling was carried out in houses, schools, sports grounds and native band offices.

The questionnaire used (Table IV) included questions on patterns of snuff dipping or tobacco chewing, smoking and alcohol consumption; localization of tobacco within the oral cavity; ingestion of soft drinks, coffee or tea; sex, age and band affiliation. I carried out the interviews accompanied by a native social worker, nurse or translator. To avoid any peer pressure, the interviews of individual participants were conducted in the absence of any interfering onlookers.

A similar questionnaire (Table V) about chewing habits and alcohol consumption was administered house to house among the East Indian fishermen in Kerala. In all more than 500 fishermen were interviewed.
**TABLE IV**

**Questionnaire Used in the Northwest Territories and Saskatchewan**

<table>
<thead>
<tr>
<th>Study code:</th>
<th>Date of interview:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Document code:</td>
<td>Interviewer:</td>
</tr>
<tr>
<td>Name:</td>
<td>Place of interview:</td>
</tr>
<tr>
<td>Age:</td>
<td></td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
</tr>
<tr>
<td>Current address:</td>
<td></td>
</tr>
<tr>
<td>Ethnic origin:</td>
<td></td>
</tr>
<tr>
<td>Place of birth:</td>
<td></td>
</tr>
</tbody>
</table>

### Chewing Pattern

<table>
<thead>
<tr>
<th>Type of tobacco:</th>
<th>Smoking Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number per day:</td>
<td>Type (cigarette, cigar, etc.):</td>
</tr>
<tr>
<td>Weight of pinch:</td>
<td>Brand:</td>
</tr>
<tr>
<td>Location in mouth:</td>
<td>Number per day:</td>
</tr>
<tr>
<td>Time per chew:</td>
<td>Years of smoking:</td>
</tr>
<tr>
<td>Years of chewing:</td>
<td>Inhale:</td>
</tr>
</tbody>
</table>

### Alcohol Use

<table>
<thead>
<tr>
<th>Type</th>
<th>Wine:</th>
<th>Bottles per week:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whiskey</td>
<td>Other:</td>
<td></td>
</tr>
<tr>
<td>Home brew:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tea:</th>
<th>Cups/day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee:</td>
<td>Cups/day:</td>
</tr>
</tbody>
</table>

### Vitamins

<table>
<thead>
<tr>
<th>Type:</th>
<th>Dose:</th>
</tr>
</thead>
</table>
**TABLE V**

**Questionnaire Used in Kerala, India**

<table>
<thead>
<tr>
<th>Interview no.</th>
<th>Patient no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date:</td>
<td></td>
</tr>
</tbody>
</table>

**Smoking**

- No. of bidi per day:
- No. of cigarettes per day:

**Alcohol (ml per day)**

- Toddy: Local:

**Chewing**

- No. of quids per day:
- Duration of chew:
- Nut+leaf+to tobacco+lime:
- Nut+leaf+lime:
- Use tobacco leaf:
- Use tobacco stem:

**Appearance of Lesion**

1. No change:
2. New lesion:
3. Complete remission:
4. Partial regression:
   a) Decrease in lesion size:
   b) Patient has multiple lesions
   c) Change in lesion appearance:
   d) Lesion changes classification:
5. Progression of lesion:
   a) Lesion increases in size:
   b) Lesion changes classification:

**Smears:**
**Brushings:**
**Photos:**
14. **Statistical Analysis**

Tests for significant difference in outcome were performed either by Student's T-test or by Fisher's exact test. A P value of < 0.05 was considered significant. Fisher's exact test was performed under the direction of Dr. J. Spinnelli, Department of Epidemiology, Biometry and Occupational Oncology, B.C. Cancer Research Centre.
RESULTS

1. **Use of Smokeless Tobacco**

The objectives of this section were to examine by questionnaire the prevalence of oral tobacco use in two northern Canadian native settlements (Inuit and Indian) and one East Indian community (Kerala, India), to record other oral habits which may exert a modifying effect on carcinogenesis, such as cigarette smoking and the drinking of alcoholic beverages, and to examine the various patterns of snuffing and chewing tobacco.

In the two Canadian settlements examined, the dipping of snuff (finely cut tobacco commercially available in cans) far exceeds the actual chewing of tobacco plugs and twists. The placement of finely ground tobacco into the oral cavity is designated by native peoples with a variety of labels, including "snuff dipping," "using snoose," "chewing snoose," "taking a pinch," or "taking a chew." The East Indian fishermen use chewing tobacco only as part of the betel quid.

Inuit, Indians and East Indians use smokeless tobacco in conjunction with other oral habits, including cigarette smoking and alcohol drinking. Two of these habits can take place concurrently, (e.g., snuff dipping plus alcohol drinking, or snuff dipping plus cigarette smoking). The prevalence of individuals with one or more habits is shown in Table VI. The 114 snuff dipping Indians examined represent approximately 10% of the total La Loche population. The 152 Inuit examined represent approximately 15% of the native population of Gjoa Haven. The 300 East Indian betel quid chewers examined represent approximately 10% of the population of fishermen living close to Trivandrum, India.
The alcoholic beverages consumed by Indians in La Loche are mainly beer and wine. The East Indian fishermen of Kerala drink fermented, distilled sap from palm trees (approximately 40% ethanol). A more detailed evaluation of the drinking habits of the East Indian population in a neighbouring area (Ernakulam, Kerala) has recently been completed (Gupta, 1984). Canadian Indians living in northern Saskatchewan did not report the drinking of Lysol, shoe polish or Chinese cooking wine. These types of alcoholic items, however, are consumed by some Indians and non-Indians living in Vancouver.
TABLE VI

Snuff Dipping, Tobacco Chewing, Cigarette Smoking, and Drinking of Alcoholic Beverages by Native Indians, Inuit and East Indians

<table>
<thead>
<tr>
<th>Location</th>
<th>Sex</th>
<th>Number Examined</th>
<th>Snuff Dipping (%)</th>
<th>Tobacco Chewing (%)</th>
<th>Cigarette Smoking (%)</th>
<th>Alcohol Drinking (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>La Loche</td>
<td>M</td>
<td>55</td>
<td>62.6</td>
<td>0.0</td>
<td>27.2</td>
<td>47.2</td>
</tr>
<tr>
<td>(Indian)</td>
<td>F</td>
<td>59</td>
<td>32.2</td>
<td>0.0</td>
<td>50.8</td>
<td>44.1</td>
</tr>
<tr>
<td>Gjoa Haven</td>
<td>M</td>
<td>107</td>
<td>57.0</td>
<td>10.3</td>
<td>32.7</td>
<td>0.0</td>
</tr>
<tr>
<td>(Inuit)</td>
<td>F</td>
<td>45</td>
<td>0.0</td>
<td>0.0</td>
<td>91.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Kerala</td>
<td>M</td>
<td>120</td>
<td>0.0</td>
<td>54.0</td>
<td>14.6</td>
<td>36.6</td>
</tr>
<tr>
<td>(East Indian)</td>
<td>F</td>
<td>110</td>
<td>0.0</td>
<td>39.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Information on the length of time that snuff users or chewers retain tobacco in their mouth proved to be more difficult to obtain than anticipated, because of a general lack of interest in and lack of feeling for "time." The results shown in Table VII were obtained, partly, by interviews and, partly, by actually measuring the snuff dipping period. As a rule, younger snuff dippers usually start with a smaller amount of snuff and keep it in the mouth for less than 15 min. They stated that larger quantities of snuff held for longer periods of time made them "dizzy." The brands of snuff and chewing tobacco used by the native Indians of Saskatchewan and the Inuit during the study period were Copenhagen, Skoal and Redman.

The amount of tobacco in a "pinch" of snuff varies according to the size of a person's fingers and the number of fingers used. Knowledge of how long a tin of snuff will last an individual can be misleading when calculating the amount of tobacco used daily, since virtually all native Indians and Inuit share their snuff. These difficulties were overcome by weighing snuff pinches taken by different snuff dippers. The average weight of tobacco chewed by 60 Inuit was 0.61 g compared to an average of 1.5 g chewed by 51 Indian males and 1.3 g chewed by Indian females. The amount of chewing tobacco used by East Indians in one betel quid was 1.1 g (SD = 0.6). The weight of the entire quid was 5.9 g (SD = 0.63).
TABLE VII

Means and Ranges of Various Snuff-Related Factors Among Inuit and Native Indians

<table>
<thead>
<tr>
<th>Location</th>
<th>Sex</th>
<th>Number Examined</th>
<th>Number of Dips per Day</th>
<th>Length of Dip (min)</th>
<th>Number of Years of Use</th>
<th>Snuff Kept in Mouth per Day (min)</th>
<th>Weight of Tobacco per Pinch (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gjoa Haven (Inuit)</td>
<td>M</td>
<td>60</td>
<td>8.03 (1-20.0)</td>
<td>25.2 (1-180.0)</td>
<td>4.1 (0.5-20.0)</td>
<td>208.8 (3-1140.0)</td>
<td>0.61 (0.45-0.83)</td>
</tr>
<tr>
<td>La Loche (Indian)</td>
<td>M</td>
<td>51</td>
<td>9.1 (1-30.0)</td>
<td>20.3 (3-60.0)</td>
<td>8.5 (1-25.0)</td>
<td>174.3 (10-600.0)</td>
<td>1.5 (0.3-3.5)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>24</td>
<td>6.2 (1-25.0)</td>
<td>11.9 (3-30.0)</td>
<td>5.7 (1-20.0)</td>
<td>57.8 (3-150.0)</td>
<td>1.3 (0.5-2.6)</td>
</tr>
</tbody>
</table>
Approximately 90% of the 114 adult male and female snuff dippers examined in La Loche place the tobacco into the lower gingival groove and hold it either to the left or the right of centre. Less than 5% of Indians keep it in the centre behind the lower lip during a dipping session. It was stated repeatedly that they feel pain in the frenulum when the snuff is kept in the centre for a long time. Adolescent males and younger adult females attempt to conceal their snuff taking by placing the tobacco far back in their cheeks. Virtually all snuff users change the location of the snuff each week due to irritation if they keep the tobacco too long in the same place. Some (approximately 6%) of snuff dippers actively move the snuff around the oral cavity with the tongue.

2. **Nitrite in Smokeless Tobacco Samples**

Tobacco has been found in all chewing mixtures that increase the risk of developing oral cancer (IARC, 1985). It has been concluded that smokeless tobacco must contain either precursors of carcinogens or carcinogenic agents themselves. Since snuff and chewing tobacco are not exposed to excessive heat, they do not contain carcinogenic pyrolysis products, unlike cigarettes and cigars. This section focuses on the capacity of smokeless tobacco to supply nitrite, which could lead to the formation of carcinogenic N-nitrosamines in snuff dippers and tobacco chewers.

2.1 **Snuff and Chewing Tobacco Brands**

Nitrite was estimated in aqueous extracts of several tobacco samples, including five different brands of snuff which were commercially available in Canada, four "nass" (tobacco mixed with slaked lime, ash and oil) samples from Samarkand in the Soviet Union, and four Khaini tobacco specimens from Bengal, India. Each tobacco sample was analyzed three times. The figures given in Table
VIII represent their averages. Tobacco samples 1 to 4 in Table VIII represent four different cans or boxes.
TABLE VIII

Nitrite Content in Aqueous Extracts of Smokeless Tobacco Samples

<table>
<thead>
<tr>
<th>Tobacco Type</th>
<th>Brand&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Origin</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snuff</td>
<td>C</td>
<td>Canada</td>
<td>1040</td>
<td>800</td>
<td>550</td>
<td>550</td>
</tr>
<tr>
<td>Snuff</td>
<td>B</td>
<td>Canada</td>
<td>500</td>
<td>400</td>
<td>305</td>
<td>95</td>
</tr>
<tr>
<td>Snuff</td>
<td>SL</td>
<td>Canada</td>
<td>820</td>
<td>750</td>
<td>500</td>
<td>70</td>
</tr>
<tr>
<td>Snuff</td>
<td>SW</td>
<td>Canada</td>
<td>75</td>
<td>18</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>Snuff</td>
<td>K</td>
<td>Canada</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chewing</td>
<td>R</td>
<td>U.S.</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Niss</td>
<td></td>
<td>Uzbekistan</td>
<td>20</td>
<td>10</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Khaini, dry</td>
<td></td>
<td>India</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Khaini, dry</td>
<td></td>
<td>India</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chewing</td>
<td>Parijat</td>
<td>India</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chewing</td>
<td>Gundi</td>
<td>India</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Chewing</td>
<td>Sun-dried</td>
<td>India</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup>C, Copenhagen; B, Big Horn; SL, Skoal; SW, Skoal Wintergreen; K, Kodiak; R, Redman

<sup>b</sup>ND, not detectable
2.2 **East Indian Tobacco**

The tobacco used by the East Indian fishermen in Kerala, to make betel quids, is a sun-dried variety. The nitrite levels in the samples of this tobacco were too low to be detected by the analytical method used (Table VIII). Two other East Indian chewing tobaccos which are used to make a betel quid were also examined, with similar negative results (Table VIII).

3. **Nitrite in the Saliva of Users of Smokeless Tobacco**

3.1 **Snuff Dippers**

Saliva was collected from seven Inuit volunteers prior to snuff dipping and after they had placed 0.5 g of tobacco (Copenhagen) into the lower gingival groove. Saliva was collected at 5 min intervals for the next 30 min, and the nitrite determined in these saliva samples. Considerable quantities of nitrite were released from the snuff into the saliva (Figure 7). No active "chewing" of the tobacco occurred during the experimental period. Once the snuff was removed from the oral cavity, the nitrite levels of the saliva approached control levels within approximately 10 min. The mean nitrite levels are given in Table IX for individuals not during a chewing period.

3.2 **Betel Quid Chewers**

Saliva samples were collected from 50 different betel quid chewers in Kerala, but not during a chewing period. The results are shown in Table IX.
Figure 6: Nitrite in the Saliva of Seven Inuit Snuff Dippers

Brackets represent the Standard Deviation of the Mean
<table>
<thead>
<tr>
<th>Population</th>
<th>Number Examined</th>
<th>Tobacco Type</th>
<th>Mean Nitrite (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inuit (Gjoa Haven)</td>
<td>64</td>
<td>Snuff</td>
<td>5.02 (SD=7.07, CV=140%)</td>
</tr>
<tr>
<td>Indian (La Loche)</td>
<td>20</td>
<td>Snuff</td>
<td>6.87 (SD=3.68, CV=53%)</td>
</tr>
<tr>
<td>East Indian (Kerala)</td>
<td>56</td>
<td>Sun-dried</td>
<td>36.27 (SD=35.06, CV=96.7%)</td>
</tr>
</tbody>
</table>
4. **Nitrosation Capacity of Saliva Samples**

The objective of this part of the study was to show whether the nitrite released from tobacco into the saliva of snuff dippers can actually lead to nitrosation. The formation of N-nitrosoproline (NPRO) was used as an assay. The experiment was carried out on one volunteer holding 1 g of snuff in the lower gingival groove. Saliva was collected over a 20-min period. The formation of NPRO was quantified by mixing, in vitro, the saliva of the snuff user with proline (200 mg), and incubating the mixture for 30 min at 37°C. Considerable amounts of NPRO were formed during the incubation period at pH 2.5 (Table X). The use of Copenhagen, Skoal or Big Horn brands of snuff, resulted in saliva samples that showed comparable levels of NPRO formation in this in vitro test system.
TABLE X

In Vitro Nitrosation Capacity of Saliva in a User of Three Different Brands of Smokeless Tobacco (Snuff)

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>pH</th>
<th>No Tobacco</th>
<th>Snuff C&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Snuff SL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Snuff B&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva</td>
<td>7.5</td>
<td>&lt;1</td>
<td>771</td>
<td>647</td>
<td>283</td>
</tr>
<tr>
<td>Saliva + 200 mg proline</td>
<td>7.5</td>
<td>&lt;1</td>
<td>672</td>
<td>821</td>
<td>149</td>
</tr>
<tr>
<td>Saliva</td>
<td>2.5</td>
<td>32</td>
<td>791</td>
<td>506</td>
<td>334</td>
</tr>
<tr>
<td>Saliva + 200 mg proline</td>
<td>2.5</td>
<td>60</td>
<td>14,476</td>
<td>11,797</td>
<td>13,496</td>
</tr>
</tbody>
</table>

<sup>a</sup>C, Copenhagen; SL, Skoal; B, Big Horn
5. **N-Nitrosopropylene in the Urine of Snuff Dippers**

The increased intake of nitrite from snuff could lead to an elevated level of nitrosation in snuff dippers. This issue was analyzed by estimating levels of NPRO in the urine of snuff dippers and of individuals who do not use smokeless tobacco. The results are shown in Table XI. The NPRO in the urine of Inuit snuff users was five times greater than for non-users, and for the Indian snuff users the NPRO level was 2.9 times greater than in non-users. The results indicate that exposure to high levels of nitrite occurs in users of smokeless tobacco and that the nitrite can react to form N-nitroso compounds endogenously.

6. **Tobacco-Specific Nitrosamines in Different Brands of Tobacco**

The amounts of TSNA found in samples of smokeless tobacco purchased in the Northwest Territories are shown in Table XII. Compared to smokeless tobaccos from other countries, the levels of TSNA measured in the two moist brands of Canadian snuff are by far the highest observed. This result could be due to continuous nitrosation during the extended storage of the snuff samples in the small shops of the northern communities.
TABLE XI

NPRO in the Urine of Two Snuff Dipping Populations (Inuit and Indian)

<table>
<thead>
<tr>
<th>Habit</th>
<th>Population</th>
<th>Number Examined</th>
<th>Mean Number of Dips per Day</th>
<th>Mean Length of Dip (min)</th>
<th>Snuff (gm/Chew)</th>
<th>Mean a ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snuff users</td>
<td>Inuit</td>
<td>14</td>
<td>6.4 ± 3.4</td>
<td>28.6 ± 14.8</td>
<td>1.0 ± 0.7</td>
<td>7.5 ± 4.9</td>
</tr>
<tr>
<td>Snuff users</td>
<td>Indian</td>
<td>47</td>
<td>7.2 ± 5.2</td>
<td>19.5 ± 16.7</td>
<td>1.4 ± 0.8</td>
<td>4.3 ± 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4.3)</td>
</tr>
<tr>
<td>Non-users</td>
<td>Inuit</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.4)</td>
</tr>
<tr>
<td>Non-users</td>
<td>Indian</td>
<td>27</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.5)</td>
</tr>
</tbody>
</table>

For the calculation of means, two values for "not detectable" were assumed: 0.2 ng/ml, maximum non-detectable value; 0.0 ng/ml, lowest possible value (figures in parentheses).

Inuit snuff users vs. Inuit non-users: P<0.001
Indian snuff users vs. Indian non-users: P<0.001
Total snuff users vs. total non-users: P<0.001

Non-parametric by Mann-Whitney U test:
Inuit snuff users vs. Inuit non-users: P=0.0001
Indian snuff users vs. Indian non-users: P=0.001
Total snuff users vs. total non-users: P=0.000001
### TABLE XII

**TSNA in Various Samples of Canadian and Foreign Tobacco Brands**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Tobacco Product</th>
<th>NNN (ppb)</th>
<th>NAT (ppb)</th>
<th>NAB (ppb)</th>
<th>NNK (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>Plug</td>
<td>2090</td>
<td>1580</td>
<td>100</td>
<td>240</td>
</tr>
<tr>
<td>Canada</td>
<td>Snuff</td>
<td>79,000</td>
<td>152,000</td>
<td>4000</td>
<td>5800</td>
</tr>
<tr>
<td>Canada</td>
<td>Snuff</td>
<td>50,400</td>
<td>170,000</td>
<td>4800</td>
<td>3200</td>
</tr>
<tr>
<td>USA</td>
<td>Chewing Tobacco</td>
<td>3500-8200</td>
<td>500-7000</td>
<td>-</td>
<td>100-3000</td>
</tr>
<tr>
<td>India</td>
<td>Chewing Tobacco</td>
<td>2400</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>USA</td>
<td>Snuff</td>
<td>800-89,000</td>
<td>200-4000</td>
<td>-</td>
<td>200-8300</td>
</tr>
<tr>
<td>Sweden</td>
<td>Snuff</td>
<td>2000-6700</td>
<td>900-2400</td>
<td>-</td>
<td>600-1500</td>
</tr>
<tr>
<td>Bavaria</td>
<td>Snuff</td>
<td>6000-6800</td>
<td>3900-4400</td>
<td>-</td>
<td>1500-1600</td>
</tr>
<tr>
<td>Denmark</td>
<td>Snuff</td>
<td>4500-8000</td>
<td>2600-6200</td>
<td>-</td>
<td>1400-7000</td>
</tr>
</tbody>
</table>

---

*NNN, N'-nitrosonornicotine; NAT, N-nitrosoanatabine; NAB, N-nitrosoanabasine; NNK, 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone*

*From Hoffmann and Adams (1981)*
7. **Tobacco-Specific Nitrosamines in the Saliva of Snuff Dippers**

7.1 **Appearance of TSNA in the Saliva**

Saliva was collected from two individuals (Inuit G21 and G60) prior to, during and after snuff dipping. The results on subject G60 show a steady increase in NNN and NAT+NAB with the length of time that the snuff was kept in the gingival groove (Table XIII). It is interesting to note that the saliva of the two dippers contained measurable amounts of NNN prior to taking snuff, although they had rinsed their mouth, and that the NNN and NAT+NAB do not disappear completely within 5 min after removal of the tobacco from the oral cavity. These results indicate that a snuff dipper is exposed to TSNA for a longer period than the actual use of tobacco itself.
### TABLE XIII

**TSNA in the Saliva of Two Individuals Prior to, During and After Snuff Dipping**

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Time of Sampling</th>
<th>Nicotine (ppm)</th>
<th>Cotinine (ppm)</th>
<th>TSNA (ppb)</th>
<th>DMNM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NNN</td>
<td>NAT+NAB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G21</td>
<td>Prior to dip</td>
<td>14.7</td>
<td>0.21</td>
<td>64.9</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15 min into dip</td>
<td>228.0</td>
<td>1.50</td>
<td>959.0</td>
<td>1160.0</td>
</tr>
<tr>
<td></td>
<td>After dip</td>
<td>19.3</td>
<td>0.30</td>
<td>67.2</td>
<td>33.4</td>
</tr>
<tr>
<td>G60</td>
<td>Prior to dip</td>
<td>2.3</td>
<td>0.55</td>
<td>30.6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>5 min into dip</td>
<td>44.8</td>
<td>0.69</td>
<td>216.0</td>
<td>357.0</td>
</tr>
<tr>
<td></td>
<td>10 min into dip</td>
<td>132.0</td>
<td>1.66</td>
<td>499.0</td>
<td>624.0</td>
</tr>
<tr>
<td></td>
<td>15 min into dip</td>
<td>164.0</td>
<td>1.89</td>
<td>650.0</td>
<td>943.0</td>
</tr>
<tr>
<td></td>
<td>After dip</td>
<td>30.4</td>
<td>0.76</td>
<td>129.0</td>
<td>130.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>ND, not detectable

Controls (non-chewers, non-smokers) had ND levels of TSNA in their saliva (n=60)
7.2 Variations in Salivary TSNA of Snuff Dippers

To learn about the extent of variation of amounts of TSNA in the saliva of snuff users, 20 dippers were asked to take their regular amount of snuff, which varied between approximately 0.5 and 1.5 g per dip, and keep it in the mouth in their usual manner for 15 min. Saliva samples were then collected, and the levels of nicotine, cotinine and TSNA were determined (Table XIV).

The results show considerable variations in the levels of TSNA despite the fact that the chewing times were kept constant. The threefold difference between the amounts of snuff used can hardly explain the approximately 22-fold difference in the amount of NNN or the 37-fold difference for NAT+NAB found between the two extreme cases (G4 and G9). In addition, the saliva was analyzed for nitroso-cis-2,6-dimethylmorpholine (DMNM)( Table XIV), in order to monitor for unintended nitrosation during handling and work-up. In all examined cases, the amount of DMNM was even lower than the usually acceptable amounts.

The results show that exposure to known carcinogenic agents (TSNA) present in smokeless tobaccos occurs at relatively high levels. The exposure of the oral mucosa to the carcinogenic and mutagenic agents contained in these tobaccos may result in the increased levels of micronucleated cells seen in the oral mucosa of snuff dippers.
### TABLE XIV
TSNA in the Saliva of Snuff Dipping Inuit (Gjoa Haven)

<table>
<thead>
<tr>
<th>Code</th>
<th>Nicotine (ppm)</th>
<th>Cotinine (ppm)</th>
<th>TSNA (ppb)</th>
<th>DMNM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NNN</td>
<td>NAT+NAB</td>
</tr>
<tr>
<td>No.</td>
<td>(ppm)</td>
<td>(ppm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>512</td>
<td>2.90</td>
<td>2610</td>
<td>4560</td>
</tr>
<tr>
<td>G8</td>
<td>566</td>
<td>2.08</td>
<td>1730</td>
<td>2100</td>
</tr>
<tr>
<td>G9</td>
<td>320</td>
<td>0.84</td>
<td>115</td>
<td>123</td>
</tr>
<tr>
<td>G13</td>
<td>808</td>
<td>3.94</td>
<td>2570</td>
<td>4030</td>
</tr>
<tr>
<td>G20</td>
<td>123</td>
<td>1.61</td>
<td>491</td>
<td>731</td>
</tr>
<tr>
<td>G21</td>
<td>171</td>
<td>1.19</td>
<td>610</td>
<td>840</td>
</tr>
<tr>
<td>G23x</td>
<td>802</td>
<td>3.00</td>
<td>2200</td>
<td>3070</td>
</tr>
<tr>
<td>G23y</td>
<td>202</td>
<td>1.16</td>
<td>683</td>
<td>1130</td>
</tr>
<tr>
<td>G24</td>
<td>695</td>
<td>2.33</td>
<td>295</td>
<td>253</td>
</tr>
<tr>
<td>G26</td>
<td>255</td>
<td>1.02</td>
<td>363</td>
<td>617</td>
</tr>
<tr>
<td>G31</td>
<td>869</td>
<td>3.11</td>
<td>607</td>
<td>670</td>
</tr>
<tr>
<td>G37</td>
<td>350</td>
<td>1.64</td>
<td>443</td>
<td>569</td>
</tr>
<tr>
<td>G42x</td>
<td>170</td>
<td>1.83</td>
<td>407</td>
<td>437</td>
</tr>
<tr>
<td>G42y</td>
<td>330</td>
<td>2.75</td>
<td>855</td>
<td>795</td>
</tr>
<tr>
<td>G46</td>
<td>482</td>
<td>2.14</td>
<td>2150</td>
<td>2570</td>
</tr>
<tr>
<td>G47</td>
<td>1300</td>
<td>3.55</td>
<td>357</td>
<td>377</td>
</tr>
<tr>
<td>G48</td>
<td>1150</td>
<td>3.66</td>
<td>2260</td>
<td>2890</td>
</tr>
<tr>
<td>G49</td>
<td>223</td>
<td>1.19</td>
<td>515</td>
<td>697</td>
</tr>
<tr>
<td>G50</td>
<td>239</td>
<td>1.28</td>
<td>423</td>
<td>618</td>
</tr>
<tr>
<td>G60</td>
<td>142</td>
<td>1.27</td>
<td>272</td>
<td>418</td>
</tr>
<tr>
<td>G61</td>
<td>70</td>
<td>0.54</td>
<td>407</td>
<td>527</td>
</tr>
<tr>
<td>JER</td>
<td>450</td>
<td>2.04</td>
<td>1210</td>
<td>896</td>
</tr>
</tbody>
</table>

a ND, not detectable

b x and y, saliva samples taken at two different times during the same day from the same snuff dipper

Controls (non-chewers, non-smokers) had ND levels of TSNA in their saliva (n=60)
8. **Frequency of Micronuclei in the Oral Mucosa of Snuff Dippers and Tobacco Chewers**

An increased frequency of micronucleated cells (MNC) in the oral mucosa has been observed in individuals at elevated risk for oral cancer, including smokers and alcohol drinkers, Khaini tobacco users from India, and betel quid chewers of Orissa and Meghalaya, India (reviewed in Table II). In this study, we compared the MNC frequency in the oral mucosa of snuff dipping Inuit (Gjoa Haven) with that of snuff dipping Indians (La Loche) and East Indian tobacco chewers (Kerala). The exfoliated cells were collected from areas of the mucosa at which the tobacco had been kept. The results shown in Table II indicate an elevated frequency of MNC in the group of tobacco chewers and snuff dippers.

9. **Precancerous Lesions and Cancer of the Oral Cavity in Users of Smokeless Tobacco**

Two types of anomalies of the oral mucosa were recorded: (a) whitish to yellowish wrinkled patches with or without furrows, corresponding to categories 2 and 3 in the classification of Greer and Poulson (1983), and (b) definite leukoplakias, as described by the WHO Collaborating Centre for Oral Precancerous Lesions (1978) (Table III).
9.1 Precancerous Lesions

Of the 50 smokeless tobacco users examined in Gjoa Haven, 26% showed whitish to yellowish wrinkled patches (Table XV). Approximately one-third also had one or more furrows in the whitish areas of the mucosa. The lesions were located in the lower gingival groove and at the front of the mouth, where the tobacco is kept. No anomalies were detected in regions of the oral cavity not directly exposed to tobacco. The occurrence of the whitish to yellowish patches (leukoderma) in the oral cavity of snuff dipping Indians (La Loche) was higher (Table XV) than among the Inuit. In this connection, it may be of interest to note that the Indian snuff dippers are relatively heavy drinkers of alcoholic beverages. No definite leukoplakias, which are believed to be precancerous lesions, were noted among the examined snuff dippers. However, it should be kept in mind that a high percentage of the subjects used in the study were teenagers who had dipped snuff for too short a time to have already developed precancerous oral lesions.

Leukoplakias, which represent precancerous lesions, were observed in a relatively high frequency in the betel quid chewers examined in Kerala (Table XV). Leukoplakias were diagnosed by myself and confirmed by two experts, B. Mathew, M.D., and R. Sankaranarayanan, M.D. All leukoplakias were photographed and the records stored at the B.C. Cancer Research Centre, Vancouver.
**TABLE XV**

Prevalence of Oral Lesions Among Snuff Dippers and Tobacco Chewers

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Sex</th>
<th>Number of Tobacco Users</th>
<th>Individuals with Whitish Patches %</th>
<th>Individuals with Oral Leukoplakia %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inuit</td>
<td>Gjoa Haven</td>
<td>M</td>
<td>50</td>
<td>26.0</td>
<td>0</td>
</tr>
<tr>
<td>Indians</td>
<td>La Loche</td>
<td>M</td>
<td>49</td>
<td>46.9</td>
<td>0</td>
</tr>
<tr>
<td>Indians</td>
<td>La Loche</td>
<td>F</td>
<td>23</td>
<td>52.2</td>
<td>0</td>
</tr>
<tr>
<td>East Indians</td>
<td>Kerala</td>
<td>M</td>
<td>325</td>
<td>0</td>
<td>59.7</td>
</tr>
</tbody>
</table>
9.2 Oral Cancer

This study focused on three restricted population groups with different patterns of smokeless tobacco use. No attempt was made to evaluate the biological effects of snuff dipping or tobacco chewing on a larger population group. Information on the incidence of oral cancer among snuff dippers and tobacco chewers can, however, be found in several epidemiological studies of population groups comparable to those examined in this thesis.

The relative risk of oral cancer increased with the number of tobacco-containing betel quids chewed per day in India (Table XVI). The study by Orr (1933) was carried out in Kerala, the location of our research project. The risk of developing carcinomas of the oral cavity and pharynx faced by snuff dippers in the southern United States is also considerably increased (Table XVII). Reports of the incidence of oral cancer among Canadian Inuit and Indians differ from those reported for East Indian chewers and for snuff dippers in the United States. No oral cancers were reported in the Inuit over a 20-year period (Schaefer et al., 1975). The incidence of oral cancer in snuff dipping Indians is comparable to that found in the Canadian population not engaging in this habit (Nutrition Canada, 1975b).
<table>
<thead>
<tr>
<th>No. Betel Quids per Day</th>
<th>Relative Risk for Oral Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>India</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>&lt;2</td>
<td>8.5</td>
</tr>
<tr>
<td>3-5</td>
<td>14.5</td>
</tr>
<tr>
<td>&gt;6</td>
<td>18.1</td>
</tr>
<tr>
<td>Retaining quid during sleep</td>
<td>63.7</td>
</tr>
</tbody>
</table>
TABLE XVII

Effect of Snuff Dipping on the Relative Risk for Oral Cancer in the Southern United States (from Winn et al., 1981)

<table>
<thead>
<tr>
<th>Site of Cancer</th>
<th>Years of Snuff Use</th>
<th>Relative Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gum and buccal mucosa</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1-24</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>25-49</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>&gt;50</td>
<td>47.5</td>
</tr>
<tr>
<td>Other mouth and pharynx</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1-24</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>25-49</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>&gt;50</td>
<td>1.3</td>
</tr>
</tbody>
</table>
10. **Intervention Strategies**

Inhibition of carcinogenesis can be obtained by using anti-initiating, anti-promoting, or anti-proliferating agents. A better understanding of the precursors of carcinogens, procarcinogens, carcinogens and promoters involved in neoplastic transformation should help in the selection of chemopreventive agents. Our results and studies from other laboratories point to the N-nitrosamines as the main carcinogenic agents released from smokeless tobacco. They may induce genetic anomalies in the oral mucosa, and they may initiate the development of oral precancerous lesions and oral carcinomas (IARC, 1985). Based on these assumptions, the efficacy of four chemopreventive agents was examined: ascorbic acid and caffeic acid as nitrite trappers, beta-carotene as a free radical scavenger, and vitamin A as an agent with scavenging potential and the capacity to regulate gene function in epithelial cells.
10.1 **Nitrite Trapping Agents**

Relatively large amounts of NPRO appeared in the urine of a volunteer who dipped snuff and ingested proline simultaneously (Figure 7 and 8; Table XVIII). The excreted NPRO could be formed endogenously, or could be preformed and released from the tobacco. The snuff brand used had an NPRO content of 14.0 μg/g tobacco (wet weight). To gain information on the amount of NPRO formed endogenously, the experiment was repeated with the additional ingestion of ascorbic acid. The experimental design is shown in Figure 5. The NPRO released in the urine of a snuff user over an 11 hr period was 26.1 μg when no ascorbate was used, and this was reduced to 7.8 μg when ascorbate was present at a total dose of 1.5 g (Figure 8). With a reduced ingestion of ascorbate (2 x 500 mg), the amount of NPRO excreted in the urine was reduced from 27.3 μg in 11 hrs to 14.9 μg (Figure 9). The efficacy of ascorbate and caffeic acid in reducing the endogenous formation of NPRO is shown in Table XVIII.
Figure 7: The Appearance of NPRO in the Urine of a Volunteer Who Dipped Snuff and Ingested Proline and Ascorbate (for detailed outline, see Figure 5C).
Figure 8: The Appearance of NPRO in the Urine of a Volunteer Who Dipped Snuff and Ingested Proline and Ascorbate (for detailed outline, see Figure 5B).
TABLE XVIII

Inhibitory Effect of Ascorbate and Caffeic Acid on the Formation of NPRO in a Snuff Dipping Volunteer

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>pH</th>
<th>No Tobacco</th>
<th>Snuff C&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Snuff SL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Snuff B&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saliva + 200 mg proline</td>
<td>2.5</td>
<td>60</td>
<td>14,476</td>
<td>11,797</td>
<td>13,496</td>
</tr>
<tr>
<td>Saliva + 200 mg proline + 200 mg ascorbate</td>
<td>2.5</td>
<td>-</td>
<td>5,497</td>
<td>3,237</td>
<td>442</td>
</tr>
<tr>
<td>Saliva + 200 mg proline + 200 mg caffeic acid</td>
<td>2.5</td>
<td>-</td>
<td>910</td>
<td>667</td>
<td>145</td>
</tr>
</tbody>
</table>

<sup>a</sup>C, Copenhagen;  SL, Skoal;  B, Big Horn
10.2 *Inuit Snuff Dippers: Beta-carotene*

Chemopreventive trials on tobacco-areca nut chewers in the Philippines have shown the capacity of beta-carotene to reduce the frequency of micronucleated cells which were used as indicators of genetic damage in the buccal mucosa (Stich et al., 1984a,b). A vitamin A deficiency prevailed in the population group of this intervention trial, as was evident from a relatively high incidence of night blindness and xerophthalmia. The question was raised whether Inuit snuff dippers of Gjoa Haven, who have "normal" serum levels of retinol, would respond in the same way to the oral administration of beta-carotene.

Serum samples from 110 male Inuit were analyzed for retinol and beta-carotene. Subjects were divided into those who used smokeless tobacco (40 individuals) and those who did not use tobacco (70 individuals). No significant difference in serum retinol or beta-carotene was observed between the two groups (Table XIX). The retinol levels of the Inuit males examined were comparable to those found for Canadian males consuming a "normal" Western-type diet (Nutrition Canada, 1975a). The observed beta-carotene levels fall substantially below those observed in a Canadian population. The low levels of serum beta-carotene may result from an insufficient intake of green-yellow vegetables by the Inuit of Gjoa Haven. Serum retinol levels are in the "normal" range due to the relatively large intake of seal and caribou meat and liver.
### TABLE XIX

Serum Levels of Retinol and Beta-carotene in Male Inuit Prior to Initiation of the Prevention Trial

<table>
<thead>
<tr>
<th>Habit</th>
<th>Number of Subjects</th>
<th>Retinol (ng/ml)</th>
<th>Beta-carotene (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-users</td>
<td>70</td>
<td>447 ± 104</td>
<td>57.4 ± 36.2</td>
</tr>
<tr>
<td>Tobacco users</td>
<td>40</td>
<td>463 ± 127</td>
<td>47.0 ± 27.6</td>
</tr>
</tbody>
</table>

Standard deviations (±) are given for number of subjects shown.

P values calculated by Student’s t-test for retinol and beta-carotene (P>0.40)
Since the distribution pattern of MNC within the oral cavity of snuff dippers is unequal (Stich, 1987), it is paramount for any comparative study of MNC frequencies to sample cells from precisely the same locations within the mouth. In this study, exfoliated cells were collected from the area of the mucosa at the site where the tobacco was kept. The results of a 10-week administration of beta-carotene are shown in Figure 10, where the frequency of MNC before treatment is plotted against that following administration of the chemopreventive agent. The results indicate an inhibitory effect of beta-carotene treatment. The average frequency of MNC prior to treatment with beta-carotene was 1.87% ± 0.92% (n = 24), and decreased significantly (P < 0.001) after treatment to 0.74% ± 0.42%. No significant reduction in the frequency of MNC occurred in individuals who received no treatment or who were given a placebo (Figure 10). The average frequencies of MNC prior to and after the 10-week period were 2.0% ± 0.94 (n = 31) and 1.99% ± 0.80, respectively.

MNC frequencies were determined in the oral mucosa of 5 snuff users 8 months and 1 month prior to, at the onset of, and after treatment with beta-carotene (Figure 11). The results indicate that the frequencies of MNC in individuals who use snuff daily are relatively stable. After a 10-week administration of beta-carotene, the frequency of MNC decreased in the mucosa of the snuff dippers. All the snuff users continued their usual habit throughout the course of the study.

Besides the micronuclei, some exfoliated cells from the oral mucosa of snuff users show a loss of nuclei. The frequency of anucleated cells before treatment was compared to that seen after treatment with beta-carotene. As can be seen from Figure 12, no significant changes occurred.
The results show that beta-carotene appears to be an efficient inhibitor of micronuclei, which indicate genetic damage in the oral mucosa of snuff users who do not suffer from a vitamin A deficiency.
Figure 10: Effect of Beta-carotene Treatment on MNC in the Oral Mucosa.

(A) Frequency of MNC at the oral site where the tobacco is located:
●, before and after a 10-week ingestion of placebo capsules: ○, before and after the 10-week trial period (no treatment). (B) Changes in the frequency of MNC before and after a 10-week administration of beta-carotene (180 mg/week).
Figure 11: Changes in the Frequency of MNC at the Oral Site Where the Tobacco is Located Over a Period Preceding the Pilot Trial and After a 10-Week Administration of Beta-carotene (5 different Inuit males using snuff)
Figure 12: Frequency of Anucleated Exfoliated Cells from the Oral Site Where the Tobacco is Located Before and After a 10-Week Administration of Beta-carotene (180 mg/week).
10.3 **East Indian Tobacco Chewers: Beta-carotene and Beta-carotene plus Vitamin A**

Because of the close association of MNC frequencies, precancerous oral lesions and oral cancer with the habit of chewing tobacco-containing betel quids, pilot intervention trials were attempted on a group of East Indian fishermen. The objective was to investigate the protective effect of beta-carotene and beta-carotene plus vitamin A on the levels of micronucleated oral mucosal cells and on the incidence of oral leukoplakias found in this group.

The frequency of MNC was elevated in areas of oral leukoplakias and in normal-appearing mucosa of betel quid chewers who participated in the intervention trial (Table XX). There were no significant differences in MNC frequencies between samples of exfoliated cells taken from normal-appearing areas of the buccal mucosa and those collected from regions with well-established leukoplakias. Swabs of normal mucosa were taken adjacent to areas with leukoplakia. This close sampling pattern was necessary since the frequency of micronucleated cells can differ at various locations of the oral mucosa. It has been shown that the frequency of MNC is highest in areas where the betel quid or tobacco plug comes in close contact with the mucosa (Stich et al., 1982a,b; Stich and Rosin, 1985).

In the placebo group, the frequency of MNC did not change significantly over the 3-month study period (Figure 13; Table XX). Following the twice-weekly administration of beta-carotene and beta-carotene plus vitamin A, the frequency of MNC was found to be significantly reduced in areas with leukoplakia and areas of normal-appearing mucosa (Table XX). The degree of MNC reduction in leukoplakias and normal-appearing mucosa was comparable. The frequencies of micronuclei in each individual before and after beta-carotene or placebo administration were paired and compared in a paired-sample Student's t-test. The reduction in MNC following the administration of beta-carotene and beta-
carotene plus vitamin A in both leukoplakias and normal-appearing mucosa is statistically significant ($P < 0.001$). The effect of the beta-carotene treatment on the frequency of MNC in areas of leukoplakia of each trial participant is shown in Figure 13. The MNC frequency of at least one tobacco/betel quid chewer did not respond to the 3-month administration of beta-carotene. Comparable results were obtained when beta-carotene and vitamin A were administered for 3 months (Table XX; Figure 13).
Figure 13: Response of MNC in Areas of Leukoplakia to a 3-Month Administration of Beta-carotene and Beta-carotene Plus Vitamin A vs. Placebo
**TABLE XX**

Frequency (%) of Micronucleated Cells in Oral Leukoplakias and in Normal Mucosa of Tobacco/Betel Quid Chewers Before and After the Administration of Chemopreventive Agents for 3 Months

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Individuals</th>
<th>Leukoplakia</th>
<th>Normal Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Placebo</td>
<td>30</td>
<td>3.60±1.22</td>
<td>4.00±1.32</td>
</tr>
<tr>
<td>Beta-carotene</td>
<td>31</td>
<td>4.09±1.01</td>
<td>1.18±0.77</td>
</tr>
<tr>
<td>Beta-carotene + vitamin A</td>
<td>51</td>
<td>4.01±1.05</td>
<td>1.16±0.94</td>
</tr>
</tbody>
</table>

Significant at P<0.01 with Student's t-test:

Beta-carotene treatment: micronucleus frequencies before vs. after supplementation for areas with leukoplakia, and frequencies before vs. after supplementation for areas of normal mucosa; beta-carotene + vitamin A treatment: before vs. after supplementation, values for both leukoplakias and normal mucosa.
Approximately 48% of all examined adult chewers who inhabit the coast of Kerala, near Trivandrum, South India, had well-established leukoplakias, as defined by the WHO Collaborating Centre for Oral Precancerous Lesions (1978). The intraoral distribution of leukoplakias among 160 chewers in Kerala was as follows: buccal mucosa, 53.4%; commissure, 23.3%; retromolar, 9.3%; tongue, 8.4%; lip, 4.4%; others, 1.4%. The frequency distribution of 160 leukoplakias according to their appearance was: homogeneous leukoplakia, 83%; speckled leukoplakia, 2.9%; and verrucous leukoplakia, 5.7%. Only one leukoplakia contained erythematous areas. Since biopsies were not taken, the leukoplakias cannot be associated with a particular histopathological pattern.

The location, size and appearance of each oral leukoplakia were recorded by marking them on a chart and by photographs. The recording of leukoplakias was done prior to the onset of the trial, and thereafter each month during the entire treatment period. As described previously, oral leukoplakias of betel quid chewers have a tendency to change in size and location with time (Gupta et al., 1980, 1986). At present, it is difficult to decide whether these "spontaneous" regressions are due to changes in the holding of the betel quid at different sites within the oral cavity, or to changes in the ingestion of chemopreventive agents caused by seasonal dietary variations.

The remission of oral leukoplakias and the development of new leukoplakias also occurred among the trial participants who received placebo capsules (Tables XXI and XXII). After three months of beta-carotene and beta-carotene plus vitamin A administration, the percentage of leukoplakias that regressed was comparable to that found in the placebo group, whereas the development of new leukoplakias was already significantly reduced. After a 6-month treatment period, a significant difference between the two treated groups (remission and new leukoplakias) and the placebo group became evident (Table XXII).
TABLE XXI

Response of Oral Leukoplakias to the Administration of Beta-carotene or Beta-carotene plus Vitamin A for 3 Months

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Individuals with Oral Leukoplakia</th>
<th>Remission Number (%)</th>
<th>No Change Number (%)</th>
<th>New Leukoplakia Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>33</td>
<td>1 3.0</td>
<td>25 75.8</td>
<td>7 21.2</td>
</tr>
<tr>
<td>Beta-carotene (180 mg/week)</td>
<td>35</td>
<td>3 8.6</td>
<td>25 71.4</td>
<td>7 20.0</td>
</tr>
<tr>
<td>Beta-carotene (180 mg/week) + vitamin A (100,000 IU/week)</td>
<td>51</td>
<td>4 7.8</td>
<td>46 90.2</td>
<td>1 2.0</td>
</tr>
</tbody>
</table>

Test for significant difference in outcome between treatment groups after 3 months of supplementation: chi-square = 10.03; df = 4; P = 0.04
TABLE XXII

Response of Oral Leukoplakias to the Administration of Beta-carotene or Beta-carotene plus Vitamin A for 6 Months

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Individuals with Oral Leukoplakia</th>
<th>Number (%)</th>
<th>Remission</th>
<th>Number (%)</th>
<th>No Change</th>
<th>Number (%)</th>
<th>New Leukoplakia</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>33</td>
<td>1</td>
<td>3.0</td>
<td>25</td>
<td>75.8</td>
<td>7</td>
<td>21.2</td>
<td></td>
</tr>
<tr>
<td>Beta-carotene (180 mg/week)</td>
<td>27</td>
<td>4</td>
<td>14.8</td>
<td>19</td>
<td>70.4</td>
<td>4</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>Beta-carotene (180 mg/week) + vitamin A (100,000 IU/week)</td>
<td>51</td>
<td>14</td>
<td>27.5</td>
<td>33</td>
<td>64.7</td>
<td>4</td>
<td>7.8</td>
<td></td>
</tr>
</tbody>
</table>

Test for significant difference in outcome between treatment groups after 6 months of supplementation: chi-square = 10.1; df = 4; P = 0.38

P values (2-sided) by Fisher's exact test: Placebo vs. beta-carotene: remission, P=0.16; new leukoplakias, P=0.53. Placebo vs. beta-carotene + vitamin A: remission, P=0.004; new leukoplakias, P=0.08. Beta-carotene vs. beta-carotene + vitamin A: remission, P=0.26; new leukoplakias, P=0.44
Prior to, once during, and at the end of the trial, we examined the number of tobacco-containing betel quids chewed per day, the length of the chewing period, and the alcohol consumption and smoking pattern of all participants. These oral habits did not change during the course of the trial. It can therefore be assumed that all the participants were exposed to approximately the same types and levels of carcinogenic and mutagenic agents released from tobacco and other betel quid ingredients.

10.4 **East Indian Tobacco Chewers: Vitamin A**

The capacity of vitamin A to reduce the frequency of micronucleated buccal mucosal cells of tobacco chewers has been previously reported (Stich and Rosin, 1984a). The objective of this study was to determine the effects of vitamin A on the remission of oral leukoplakias. This short-term pilot trial was carried out on fishermen living along the coast near Trivandrum, Kerala. Questionnaires on chewing, smoking and drinking patterns were completed at the onset of the trial, after three months, and at the end of the six-month treatment period. The results permitted us to draw the conclusion that the various oral habits did not change throughout the course of the study. The participants in this study chewed an average of 13.1 (SD = 9.1) tobacco-containing quids per day. Each quid was kept in the mouth for 26.1 (SD = 25.4) min. Exposure to tobacco- and areca nut-related carcinogens amounted to approximately six hours each day.

To ensure that all the trial participants received the desired doses, the placebo and vitamin A capsules were administered twice weekly under the strict supervision of a local nurse and a staff member of the Regional Cancer Centre in Trivandrum or myself, who checked that the capsules were actually swallowed. Due to this approach, an excellent compliance with the vitamin administration regime was achieved. Only 4 of 39 vitamin A or placebo doses (six months) were
missed among 6% of the participants, and 2 of 39 doses were missed by 14% of all trial participants. Despite the continuous exposure to carcinogens, the administration of 200,000 IU of vitamin A, resulted in a pronounced remission of oral leukoplakias (Table XXIII). This amount of vitamin A did not produce any detectable adverse effects during the trial period, such as dryness of the lips and mouth, changes in the skin (scaling of palms), headaches or dizziness.

New oral leukoplakias developed throughout the trial period among chewers of tobacco-containing betel quids. It was therefore possible to examine the effect of vitamin A treatment on the formation of new leukoplakias. In the group receiving the placebo, 7 (21%) new leukoplakias were formed, whereas no new leukoplakias developed in the group receiving vitamin A over the six-month trial period (Table XXIII).
TABLE XXIII

Response of Oral Leukoplakias to the Administration of Vitamin A for 6 Months

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Individuals with Oral Leukoplakia</th>
<th>Remission</th>
<th>No Change</th>
<th>New Leukoplakia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>33</td>
<td>1 3.0</td>
<td>25 75.8</td>
<td>7 21.2</td>
</tr>
<tr>
<td>Vitamin A (200,000 IU/week)</td>
<td>21</td>
<td>12 57.1</td>
<td>9 42.9</td>
<td>0 0.0</td>
</tr>
</tbody>
</table>

Test for significant difference in outcome between treatment groups after 6 months of supplementation: chi-square = 22.27; df = 3; P = 0.000015

P values (2-sided) by Fisher's exact test: Placebo vs. vitamin A: remission, P=0.0000089. Placebo vs. vitamin A: new leukoplakia, P=0.024
DISCUSSION

The objectives of this research were to compare the usage of smokeless tobacco among three communities (Inuit, Canadian Indian and East Indian), to analyze smokeless tobacco samples, and saliva and urine of chewers for various etiological factors, and to investigate possible chemopreventive agents which may reduce the oral lesions caused by the use of snuff or chewing tobacco.

1. Use of Smokeless Tobacco

Although a strict random sampling of each population was not carried out, the data accumulated do indicate a large prevalence of smokeless tobacco use by each group: 86% of the male Canadian Indians examined said they used snuff daily, as did 50% of the females. In the Inuit population, 70% of the males responded that they used snuff daily, as did 4.2% of females, although this habit is socially unacceptable for this latter group. In the East Indian population where betel quid chewing is acceptable for both males and females, roughly 57% of those questioned responded that they practised this habit.

In this study we also attempted to determine the prevalence of other oral habits which may exert a carcinogenic effect, such as alcohol drinking and cigarette smoking. In the Canadian Indian population, approximately 26.3% of males and 10.4% of females combine the drinking of alcohol with snuff dipping, as do 37% of the East Indian males who chew betel quid. Considering the synergistic action between alcohol consumption and cigarette smoking (Walters et al., 1979), the possibility that a similar effect may occur between alcohol and snuff or chewing tobacco cannot be excluded. The combination of snuff dipping, alcohol drinking and cigarette smoking was found to occur in 35.1% of males and 21.5% of females among the Canadian Indians studied. The influence of
smoking can be seen from the observation that the highest prevalence of leukoplakia occurs among individuals who are concurrently using "nass" and smoking cigarettes (Stich and Rosin, 1984).

Also of interest in this study was the relatively consistent weight of tobacco chewed by each group: 0.61 g by the Inuit, 1.5 g by the Canadian Indians and 1.1 g by the East Indians, suggesting that other modifying effects must be at work which influence the different oral cancer rates seen amongst these groups.

2. Precursors of Nitrosamines Contained in Smokeless Tobacco Samples and the Saliva of Their Users

To investigate the probable etiological factors contained in smokeless tobacco, we focused on the N-nitroso compounds and their precursors since these are the only known carcinogenic compounds contained in smokeless tobacco mixtures chewed throughout the world (IARC, 1985).

Nitrite levels were examined in a variety of smokeless tobacco samples, including five common brands of snuff (Canada), one common brand of chewing tobacco (U.S.), four "nass" samples (mixtures of tobacco, slaked lime, ash and oil placed under the tongue by inhabitants in southern regions of the Soviet Union, Afghanistan and Pakistan), four Khaini tobacco preparations widely used in several eastern states of India (e.g., Bihar, Orissa and Bengal), and East Indian tobacco used in the preparation of betel quids. Relatively high levels of nitrite were found in three popular brands of snuff commonly used by northern Canadian natives. The amounts of nitrite in the three Canadian snuff samples, which varied from 95 to 1,040 mg/kg, and in four "nass" samples from Samarkand (Uzbekistan, Soviet Union), which varied from 70 to 200 mg/kg, greatly exceed those found in several food products such as nitrite-cured bacon
(12-42 mg/kg), nitrite-cured ham (16-37 mg/kg), smoked sausages (18-31 mg/kg) (Kawabata et al., 1984), or Chinese cabbage (Walters et al., 1979).

To arrive at an estimation of nitrite which could conceivably enter the digestive system of snuff or nass users, we can assume a saliva flow of 16 ml per 20 min (Anderson and Krinsky, 1973) and a concentration of 0.2 mg nitrite per ml of saliva. Based on these assumptions, an estimated 3.2 mg of nitrite will enter the stomach during a 20-min period of tobacco use if the snuff dipper swallows rather than expectorates the saliva. Furthermore, assuming that the tobacco is kept within the mouth for 3 hrs per day, approximately 29 mg of nitrite would enter the stomach in a 24-hr period. This amount is considerably larger than the 4.2 mg of nitrite ingested by an individual per day from solid food products and beverages (Kawabata et al., 1984).

Considerable concern has been expressed about the possible carcinogenic effects of tobacco-related nitrosamines (Hoffmann and Hecht, 1985) which are formed during tobacco processing (Andersen and Kasperbauer, 1984). The possibility cannot be completely ignored that the relatively large doses of nitrite released into the saliva of snuff dippers could pose an even greater hazard. By using levels of excreted NPRO, information about the nitrosation activity within man can be obtained (Ohshima and Bartsch, 1981; Ohshima et al., 1982). Using this method, relatively high levels of NPRO were found in the urine of several snuff dippers, suggesting an increased endogenous nitrosation capacity. Thus the daily use of nitrite-containing snuff could lead to the endogenous nitrosation of a great many ingested dietary compounds. Several studies have shown the formation of strongly mutagenic, and by implication carcinogenic compounds following the nitrosation of meat products (Marquardt et al., 1977; Tomita et al., 1984), beverages (Lin and Tai, 1980), vegetables (Van Der Hoeven et al., 1984) and spices (Namiki et al., 1983). The tobacco-related
nitrosamines (Hecht et al., 1984; Hoffmann and Hecht, 1985) and possibly other tobacco-derived mutagens (Whong et al., 1985) may represent only one group of N-nitroso compounds which could conceivably be formed in users of nitrite-containing tobacco.

It was recently pointed out that salivary nitrite levels may be only one factor affecting the endogenous formation of N-nitroso compounds (Mirvish, 1985), and that a neglect of the interactions between nitrite and numerous scavenging chemicals could lead to erroneous conclusions (Forman et al., 1985). This argument is undoubtedly applicable to users of nitrite-containing snuff. As expected, the addition of ascorbate to a mixture of proline and saliva of a snuff dipper inhibited the in vitro formation of NPRO. Ascorbate taken by a volunteer both concurrently and shortly after the use of snuff and proline reduces the excretion of NPRO. It could be speculated that the observed differences in urinary NPRO levels between Inuit (Northwest Territories) and Indian (Saskatchewan) snuff users is due to differences in the intake of vegetables and fruits, which are the major source of ascorbic acid and nitrite-scavenging phenolics (Pignatelli et al., 1982; Stich and Rosin, 1984b; Stich et al., 1984c). This is supported by the observation that the percentage of Inuit males in the 10-54 year age range who show severe vitamin C deficiencies is significantly higher than that of Indians of comparable age (Nutrition Canada, 1975b).

Whether the widespread use of smokeless tobacco among inhabitants of Arctic regions, combined with a deficient intake of nitrite-trapping compounds, makes them more prone to the development of oral cancer can only be ascertained by a detailed epidemiological study.
Relatively high levels of TSNA, including NNN, NAT+NAB and NNK, were found in the saliva of Inuit during a 15-min snuff taking period. An estimate, based on the results presented, reveals that the amounts of nitrosamines swallowed by a snuff dipper are by no means negligible. Assuming an average level of 980 μg TSNA per 1 ml of saliva of the Inuit snuff dipper, an average of 6.5 dips per day, an average of 25 min per dipping period, and a saliva flow of 5.83 ml per 5 min, then the amount of NNN ingested should be in the order of 185 μg per day. The same calculation applied to a more intensive snuff user (G13, Table XIV) shows an intake of approximately 899 μg NNN. Most of the saliva produced during snuff taking is probably swallowed, since most Inuit snuff dippers do not, or only occasionally, spit during a dipping session. The extreme salivation and resulting repeated spitting which is so common among Asiatic betel quid chewers does not occur in snuff dippers. To gain a more complete picture of the amount of TSNA ingested, approximately 249 μg of NAT+NAB and 10 μg of NNK must be added to the 185 μg of NNN, making a total of 444 μg. Thus the amount of nitrosamines ingested daily by snuff dippers exceeds by far that swallowing through drinking beer (0.34 μg per day), eating cured meat products (0.17 μg per day), or using cosmetics (0.41 μg per day)(Kawabata et al., 1984).

The analytical data, recognition of the fact that carcinogenic N-nitrosamines can be absorbed from the saliva (Hoffmann and Adams, 1981) and the results of bioassays for carcinogenicity (Peto et al., 1984; Hecht et al., 1983) make it possible to estimate the contribution of N-nitrosamines to the increased risk of snuff dippers for oral cancer. Peto et al. (1984) showed that 1 mg/kg N-nitrosodimethylamine (NDMA) or N-nitrosodiethylamine (NDEA)
administered in drinking water caused liver neoplasms in 23% and 42% of rats, respectively. These incidence rates were significantly higher than those in the controls. In addition, 1 mg/kg NDEA caused eosophageal tumors in 27% of male rats and none in control animals. The total dose administered to the animals during their lifetime was about 37 mg/kg for males and 64 mg/kg for females. This study suggested the existence of a linear dose-response relationship in the dose range of 0.033 to 1 mg/kg. Since NNK levels in moist snuff of the four Canadian brands average greater than 2.5 µg/kg (Table XII), 30 years of exposure for average snuff dippers, who consume about 10 gm of snuff daily, amounts to 270 mg NNK or about 3.9 mg/kg. In addition, snuff dippers are exposed to about 900 mg NNN (±13 mg/kg), 800 mg NAT (±11.4 mg/kg) and 56 mg NAB (±0.8 mg/kg) during a 30-year span. These estimates, together with the dose-response study in rats cited above and the fact that N-nitrosamines are probably formed during snuff dipping, strongly suggest that N-nitrosamines play a major role in the increased oral cancer risk of snuff dippers. This hypothesis is supported by the recent observation that a single administration of 1 mg NNK induces a significant number of tumors in Syrian golden hamsters (Hecht et al., 1983).

4. **The Unfinished Search for the Factors Involved in Oral Carcinogenesis**

The comparative study of tobacco users among Inuit, Indian and East Indian population groups was initiated in the hope of revealing the main carcinogenic factors involved in the development of oral precancerous lesions and oral cancer. However, the complexity of carcinogenesis and its etiological factors prevented us from finding simple answers.
The examined groups at elevated risk for oral cancer engage in a habit that leads to an increased ingestion of TSNA. In all these groups the frequency of micronucleated cells is also increased. These users of smokeless tobacco include snuff dippers in the United States and Canada, "nass" chewers in the Soviet Union, and tobacco chewers in the United States and India. Moreover, TSNA also appear in the saliva of cigarette smokers and "reverse" smokers, who hold the burning end of the cigarette in the mouth. A difficulty arises when quantitative relationships between TSNA levels and oral precancerous lesions or oral cancer are being sought. For example, various groups of snuff dippers can differ significantly in their TSNA levels. Levels of NNN found among Inuit snuff dippers significantly exceed those detected in the saliva of snuff dipping American women (Hoffmann and Adams, 1981). Approximately 65% of the Inuit exceeded 420 ppb NNN, which was the highest level found in the saliva of the American dippers, and 22% of the Inuit reached levels exceeding 2,100 ppb. Levels of NNN in the saliva of snuff dipping Inuit were also higher than the 16.5 to 59.7 µg/ml found in Indian tobacco chewers (Nair et al., 1985), or the 1.2 to 31.0 µg/ml and 1.6 to 14.7 µg/ml in users of tobacco containing betel quids (Nair et al., 1985; Wenke et al., 1984). The incidence of oral cancer, however, does not follow this pattern. The highest incidence is among chewers of tobacco-containing betel quids (IARC, 1985) followed by snuff dippers in the southern United States (IARC, 1985). The incidence of oral cancer among Inuit snuff dippers who have the highest levels of TSNA in their tobacco samples and their saliva must be very low since no oral carcinomas were seen among the 180 cancers diagnosed between 1949 and 1974 in the Inuit of the Canadian Arctic (Schaefer et al., 1975). Moreover, the frequency of micronucleated oral mucosal cells in the snuff dipping Inuit is lower (1.8%) than in other tobacco-using groups: 4.4% in the floor of the mouth of "nass" users in Uzbekistan, 8.4% in
the buccal mucosa of tobacco/betel quid chewers of Orissa, India (Stich et al., 1982a,b), 4.8% in tobacco/betel quid chewers of the Philippines (Stich et al., 1984a,b), and 2.8% in the mucosa of the lower groove of Indian users of Khaini tobacco (Mirvish, 1982).

The lack of any simple quantitative relationship between the amount of carcinogens ingested by a tobacco user and the frequency of genetic lesions (MNC), leukoplakia and cancer in the target tissue points to the involvement of modulating factors. One could speculate that the observed differences in oral lesions are due to different intakes of dietary vitamin A and/or beta-carotene. Many Asiatic populations are afflicted with a vitamin A deficiency, as suggested by the occurrence of xerophthalmia and nightblindness. Subnormal levels of retinol were actually observed in Uzbekis (Zardize et al., 1985), and in Pakistanis and Indians at elevated risk for oral cancer (Ibrahim et al., 1977; Wahi et al., 1965). Another possibility worth considering is that the aforementioned differences in the frequency of preneoplastic changes or carcinomas may result from differences in the composition of the chewing mixtures. Recent studies in our laboratory have shown that aqueous extracts of areca nut enhance the formation of transformed foci in a bovine papilloma virus DNA transformation assay (Stich and Tsang, 1989), and induce melanomas in a strain of platyfish (Stich and Anders, 1989) which responds to tumor promoters rather than to tumor initiators. The promoting activity of the areca nut extract was by no means negligible. In the BPV DNA transformation assay, it was comparable to the activity of approximately 0.5 mg/ml mezerein, a second-stage promoter. This promoting activity of areca nut compounds may be responsible for the exceptionally high frequency of MNC, leukoplakia and oral carcinomas in betel quid chewers. This promotional stage appears to be lacking in individuals
who use only snuff or chewing tobacco, which show only a small activity in several assays for cancer promoters.

5. Exploring Preventive Measures

5.1 Mechanism of Action of Chemopreventive Agents

The ICRDB (Boutwell, 1979) has compiled summaries of 324 cancer research papers concerned with retinol, other retinoids, beta-carotene or other carotenoids. Together, these show that various retinoids can reversibly suppress the malignant behaviour of cultured cells that have been transformed by viruses, chemicals or ionizing radiation; they can delay or prevent the onset of cancer in experimental animals previously treated with DNA-binding carcinogens; they can cause human skin keratoses or oral leukoplakias to regress; they can prevent "tumor promoters" from eliciting papillomas on mouse skin; and they can inhibit some of the very specific biochemical effects of tumor promoters on cells in vivo or in culture. Although inappropriate use of retinoids may do more harm than good (Schroder and Black, 1980), it seems reasonable to hope that exposure of human tissues to retinoid-like activity can be manipulated to reduce cancer risks (Sporn and Newton, 1979).

It has been suggested that retinoid application can indeed promote tumor development in a few model systems (Schroder and Black, 1980). In the short-term studies reported here no increase in leukoplakia was seen in any of the East Indian fishermen given vitamin A; in fact, the opposite was observed. The only harmful effect of vitamin A administration is hypervitaminosis A, but no symptoms of this were observed in any of the subjects. It can be concluded that the doses of vitamin A given to people in these studies produced no harmful side-effects in the time period during which it was administered.
Neoplastic transformation can often be subdivided into "stages," the early stage involving conversion of a normal cell, and the later stage involving conversion of a partially altered cell into a fully neoplastic cell. To be effective, the neoplastic cell must not be eliminated, but must proliferate into a pathological tumor. Early- and late-stage changes are both necessary (halving either may thus be an equally effective way of halving cancer risks), but typically have different causes (Peto, 1977, 1979). The demonstrated protective effects of retinol (and perhaps beta-carotene) suggest that it is the later stages of neoplastic progression which are chiefly affected, together perhaps with the final process of tumor growth. There may often be a latent period of a few decades before a doubling or halving of the early process has any effect on human cancer risk within about 5 years, while effects on the final process (proliferation) may be even more rapidly evident. Thus human cancer risks may be noticeably influenced by the average levels of blood retinol within a period of as little as 5 or 10 years.

In contrast to the retinoids, the question of whether dietary beta-carotene or some other carotenoids can affect cancer risk has not received nearly as much experimental attention. Possible mechanisms to consider include (1) a direct retinoid-like effect of some carotenoids on cellular differentiation in the target tissues, (2) conversion in the target tissue of some carotenoids into molecules with such retinoid-like effects, or (3) protection by carotenoids of the target tissues via mechanisms not in control of cellular differentiation, for example, by affecting immunological function (Rettura, 1975; Felix et al., 1976), or by quenching singlet oxygen, a highly reactive, excited molecular species which occurs as a toxic by-product of many normal processes in both animals and plants.
In the studies presented here, the twice weekly oral administration of beta-carotene, beta-carotene plus vitamin A or vitamin A affected oral mucosal lesions at three different levels: the frequency of micronucleated cells was reduced, well-established leukoplakias regressed, and the development of new leukoplakias was inhibited. The results are noteworthy for several reasons. First, the effective doses are considerably lower than the 1 to 2 mg/kg body weight/day of 13-cis-retinoic acid successfully given to patients with oral leukoplakias (Cordero et al., 1981; Koch, 1981; Hong et al., 1986). If the recommended conversion factor for beta-carotene and vitamin A is applied (FAO/WHO, 1967; Polacchi, 1980), then our dose of retinol equivalents was approximately 0.14 mg/kg body weight/day (0.7 mg from vitamin A, and 0.7 mg from beta-carotene). Second, the administration of beta-carotene alone had a significant effect on the frequency of micronucleated cells, but a lesser one on the remission of leukoplakias. Among the 31 chewers who received beta-carotene alone, only one (3%) failed to show a reduction in micronucleated cells, whereas no remission was seen in 23 (85%) of 27 participants who completed the trial. Third, micronucleated cells respond much more rapidly than leukoplakia to beta-carotene or to beta-carotene plus vitamin A. Fourth, all participants in the intervention trial continued to chew tobacco-containing betel quids in their accustomed manner and at their usual rate. Thus remission of oral leukoplakias, reduced development of new leukoplakias, and reduction of micronucleated cells occurred during daily exposure to various carcinogens released from betel quid ingredients. In this respect, the trials with retinoids on betel quid chewers differ from those conducted by Koch (1981), Shah et al. (1983) or Hong et al. (1986) on patients with oral leukoplakia, who may not have been exposed to carcinogens during the treatment period. Fifth, the doses of beta-carotene and vitamin A administered did not cause any
detectable side-effects, such as dryness of the lips and mouth, scaling and peeling of the skin, or conjunctivitis.

The question must be raised whether there are ways to reduce the dose of beta-carotene and vitamin A to levels that can be readily administered and that produce no undesirable side-effects.

The development of new oral leukoplakias was inhibited, and remission of established leukoplakias induced, by the six-month oral administration of vitamin A at a dose of 0.14 mg/kg body weight per day. This preventive dose was about 13-fold higher than the FAO/WHO (1967) recommended intake of retinoids, and sevenfold higher than the 0.02 mg/kg body weight per day of dietary vitamin A which, according to epidemiological evidence, conveys a protective effect (Marshall et al., 1982). The dose of vitamin A was considerably lower than those reported previously: approximately 1 mg/kg/day (Cordero et al., 1981; Gupta et al., 1980); 1 to 2 mg/kg/day (Hong et al., 1986); 3, 5 or 10 mg/kg/day when administered as a lozenge (Wahi et al., 1965); 10 mg/kg/day (Nair et al., 1980); and 20 to 100 mg/kg/day for several weeks (Ryssel et al., 1971). (To make the retinoid levels comparable, they were recalculated from published results, assuming a body weight of 70 kg for an adult).

One way to reduce the total amount of vitamin A would be to at first administer for several months heavy doses of vitamin A to reduce the frequency of micronuclei and leukoplakia, and then follow this treatment with the non-toxic beta-carotene. Preliminary results obtained on the tobacco/betel quid chewers in Kerala revealed that the protective effect of vitamin A administration can be maintained for at least 5 additional months with beta-carotene. Several other experiments can be mentioned in support of this approach.
Following a relatively short-term administration of higher doses of retinoids (0.6 mg/kg/day), the protective effect was maintained by lower doses in the range of 0.3 mg etretinate/kg/day (Sloberg et al., 1983). The chemopreventive effect induced by 1 mg/kg/day of Ro 10-9359 for 2 to 10 weeks was similar.

Another approach worth considering consists of combining the administration of the somewhat toxic retinoids with the non-toxic beta-carotene. In animal models, beta-carotene is effective in preventing carcinogenesis, including that of the oral cavity (Suda et al., 1986). In human populations, beta-carotene has also exerted a protective effect, as shown by a reduction in the frequency of micronucleated cells in the buccal mucosa of betel quid chewers (Stich, 1987; Stich et al., 1984a,b) and snuff dippers (Stich et al., 1985). The efficacy of replacing doses of vitamin A with beta-carotene can be judged by comparing the extent of remission induced by 200,000 IU of vitamin A per week with that induced by 200,000 IU given in part as vitamin A (100,000 IU/week) and in part, as beta-carotene (amount equal to 100,000 IU retinol). The pilot trials on betel quid chewers revealed that vitamin A treatment induced the remission of oral leukoplakias in 57.1% of participants (n = 21), whereas the vitamin A plus beta-carotene administration resulted in remission in only 27.5% (n = 51) (Stich, 1987). This difference in protective activity between the two treatments may only be apparent, mainly due to the fact that only a fraction of beta-carotene is converted into vitamin A. Thus the doses of the two treatment protocols are not strictly comparable. Theoretically, doses of the non-toxic beta-carotene could be increased to levels matching that of 100,000 IU of vitamin A using one of the current conversion factors (Ibrahim et al., 1977). However, the implementation of this
idea could meet with objections because of the large number of beta-carotene capsules to be swallowed by the trial participants.

6. **Outlook**

Although the risk of oral cancer is not high amongst these peoples (both Inuit and Indian), the use of oral tobacco is a relatively new phenomenon which has increased dramatically since the 1960s (IARC, 1985), and which is practised mainly by young adolescents.

In the United States, the use of smokeless tobacco increases by 10-11% yearly. Given this situation, the question must be raised as to whether one can expect to see in the near future the high incidence of oral leukoplakias and oral cancers among Canadian snuff dippers that is so common among chewers of tobacco-containing betel quids in Asian countries, users of "nass" in southern regions of the Soviet Union (IARC, 1985) or snuff dippers in the southeastern United States. In the latter case, snuff dipping was found to be strongly associated with cancers of the oral cavity, pharynx and larynx. A study by Winn et al (1984) showed that the use of snuff was associated with a fourfold increase in the risk of oral and pharyngeal cancers, and that the relative risk increased markedly to almost fiftyfold for those who had dipped snuff for 50 years or more.

The distribution of beta-carotene or vitamin A to a large population remains an unresolved issue. Because of the expenses and logistical difficulties involved in a large-scale intervention program using pills or capsules, other methods must be sought and implemented. The following examples are practically feasible and warrant closer consideration.

Red palm oil provides a very rich source of beta-carotene, and the introduction of this substance into the diet could provide the protection
needed by increasing both beta-carotene and vitamin A levels in its consumers. In North America, an increased intake of green/yellow vegetables and education on the importance of these products in the diet may lead to a change in the resulting serum levels of beta-carotene and vitamin A, and eventually reduce the risk not only for oral but also for other epithelial cancers. In Taiwan, sweet potato strains which are rich in beta-carotene are being used as potato chips, french fries and flour. In this way, the diet of a population can be enriched with beta-carotene without any major changes in eating habits.
SUMMARY

The only known carcinogens contained in any tobacco mixtures used orally throughout the world are the N-nitroso compounds. An analysis of the levels of these agents, plus their precursors, in the tobaccos used and in the saliva of their users was conducted in three different communities. High levels of nitrite were found in the snuff tobacco used by Canadian Inuit (Gjoa Haven) and Indians (La Loche) and in the saliva of snuff dippers. These high nitrite levels can act as precursors to nitrosation reactions, as evidenced by the higher levels of NPRO detected in the urine of snuff users versus non-users. In addition, relatively high levels of tobacco-specific nitrosamines were detected in tobacco samples and in the saliva of snuff users.

The genotoxic damage induced by exposure to these carcinogens was evident from the elevated levels of micronucleated cells found in the oral mucosa of snuff dippers. Although, at present the Inuit and Indian communities studied, do not have an elevated incidence of oral cancer, these abnormalities of the genome, together with the whitish/yellowish wrinkled patches observed in the mucosa, indicate a risk of developing precancerous lesions and/or cancer over a long enough period of time. The East Indian population studied shows a substantially increased risk for oral cancer, high micronucleated cell counts, plus a large percentage of individuals with preneoplastic oral lesions (leukoplakia).

In the Inuit population studied, it was shown that the MNC level could be reduced by a short-term intervention trial (10 weeks) using 180 mg/week of beta-carotene. In the East Indian group, it was possible to reduce the MNC level and the frequency of leukoplakia using beta-carotene (180 mg/week) plus...
vitamin A (100,000 IU/week) over a six-month period. An even more marked reduction in lesion frequency was observed after a six-month administration of vitamin A alone (200,000 IU/week).

It now appears conclusive that exposure to the carcinogenic N-nitroso compounds contained in smokeless tobacco is involved in the etiology of oral cancer and that of the risk can be reduced by increasing serum levels of beta-carotene and vitamin A via supplementation with pills or by adjusting the diet to increase intake of these compounds.
REFERENCES


