KINETICS OF BETA-CAROTENE ACCUMULATION AND RETENTION IN EXFOLIATED CELLS FROM SUPPLEMENTED INDIVIDUALS

By

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ABSTRACT

Human cancer intervention trials have found beta-carotene to be effective in reducing the genotoxic damage to oral mucosa cells that resulted from carcinogen exposure. Design of intervention trials using beta-carotene has so far lacked an important component, knowledge of the accumulation and retention of this putative chemopreventive agent in the tissues of interest. The factors of dose, timing of administration of supplements, and the effects of confounding factors are aspects of trial design that demand an understanding of the kinetics of beta-carotene in human tissues.

The oral mucosa is the only tissue so far that has been investigated for beta-carotene levels in exfoliated cells. A non-invasive technique for sample collection, suitable for sampling populations, in combination with a highly sensitive assay for beta-carotene, is appropriate for use with other epithelial sites that may be targets of intervention trials; one such site is the uro-genital tract.

This thesis describes preliminary investigations towards design of an intervention trial with beta-carotene directed at cells of the uro-genital tract.

Initial studies established the feasibility of measuring beta-carotene in uro-genital tract cells, and verified that the technical variation in the assays of oral mucosa cells and uro-genital tract cells was less than the variation between individuals in the study population.

A short-term (four-day) supplementation trial compared the effects of three doses of beta-carotene, given orally, on the beta-carotene content of exfoliated oral mucosa cells. Ingestion of 360 mg, 180 mg, and 90 mg of beta-carotene resulted in a rise in median cell beta-carotene levels from 1.8, 1.5, and 1.0 ng/10^6 cells to 9.2, 7.7, and 3.9 ng/10^6 cells, respectively, one week after the loading. Due to variation in response, the effects of the three doses were not significantly different from one another. The elevation in beta-carotene levels persisted for 2 weeks after the loading.

The kinetic profile of the accumulation and retention of beta-carotene in uro-genital tract cells differed markedly from that of oral mucosa cells, in individuals who were supplemented for four weeks with a field trial dosage of beta-carotene. Median beta-carotene levels in uro-genital tract cells of beta-carotene-supplemented individuals increased from 0.6 ng/10^6 cells to 2.8 ng/10^6 cells during the loading period, a level significantly higher than that of the placebo group, but declined immediately after the end of the loading to levels that were not significantly elevated. In contrast, beta-carotene in exfoliated oral mucosa cells remained at significantly elevated levels until four weeks after the cessation of loading.

Tissue-specific features of beta-carotene accumulation in response to its administration need to be taken into consideration when designing intervention strategies that use beta-carotene.

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LIST OF ABBREVIATIONS

C.V. coefficient of variation

SE standard error of the mean

SD standard deviation

h hours

u micron (micrometer, 10^{-6} m)

ul microliter (10^{-6} l)

ng nanogram (10^{-9} g)

nm nanometer (10^{-9} m)

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INTRODUCTION

Thomas Adams, 17th C.: "Hee is a better physician that keepes diseases off us, than hee that cures them being on us. Prevention is so much better than healing, because it saves the labour of being sick."

1. Background

1.1 Chemoprevention and beta-carotene

Beta-carotene and vitamin A have recently enjoyed considerable attention because of substantial laboratory and epidemiological evidence that they act to inhibit carcinogenesis (Peto et al. 1981, Peto 1983, Wolf 1982, Hennekens et al. 1986). Large-scale clinical trials have used these compounds as probable chemopreventive agents (Stampfer et al. 1985). They are dietary items whose intake could easily be augmented by dietary supplementation; beta-carotene is non-toxic even in large doses. Yet, to what extent they accumulate in human tissues is almost entirely unknown.

The kinetics of beta-carotene in tissues is important to understand, for many reasons. Low baseline levels of beta-carotene in human tissues, possibly due to a dietary deficiency or to an inability to absorb or store the compound, may indicate an increased susceptibility of the tissue to carcinogen injury. Variation in ability to accumulate beta-carotene could affect the likelihood that a significant chemopreventive effect would result from beta-carotene supplementation of a study population; normal variation in tissue levels of beta-carotene are unknown. Tissues may show specific patterns of beta-carotene accumulation that should be taken into account in attempts to test beta-carotene for its ability to interrupt carcinogenesis. A chemopreventive effect may occur only with a certain dose range of beta-carotene; the dose-response characteristics of human tissues to beta-carotene supplementation have not been investigated. Thus, based on the non-human evidence, it appears essential to understand the

relationship of dose of beta-carotene, and the spacing of its administration, to the maintenance of any protective effect that is seen in a tissue for a prolonged period, before planning long-term intervention strategies aimed at reducing cancer incidence in human populations (Rosin et al. 1987). These are some reasons why it is crucial to understand the kinetics of chemopreventive substances in human tissues.

Evidence that beta-carotene can prevent, halt, or reverse carcinogenesis (Wattenberg 1985, Greenwald et al. 1987) has come from three sources: epidemiological studies, *in vitro* studies, and *in vivo* experimental carcinogenesis studies.

A low consumption of green and yellow vegetables, the principal dietary source of beta-carotene, has been associated with an elevated risk of cancer, in many epidemiological studies. The protective effect of dietary beta-carotene may be site-specific (e.g. lung), and lifestyle-specific (e.g. high-risk group such as smokers). Low serum levels of beta-carotene have also been associated with an elevated risk of lung cancer.

In experimental carcinogenesis, beta-carotene, and the retinoids, seemed to act to suppress the progression of precursor lesions into fully malignant lesions (Alavanja et al. 1987). Progression leads to selection for invasiveness and metastases (Ames et al. 1987). The protective effect of beta-carotene appeared to be species- (and strain of species-), sex-, tissue- or organ-, dose-, timing-, and route of administration-specific.

Thus, there is considerable evidence that beta-carotene may be a compound that suppresses the development of a tumour from an initiated cell (Wattenberg 1985).

Tests of this claim in human populations are urgently needed. *In vitro*, *in vivo*, and animal test systems cannot provide direct answers to the question of a chemopreventive effect of beta-carotene in humans. Intervention trials can provide much-needed information relatively quickly. The present study investigated the response of beta-carotene levels in uro-genital tract cells to conditions similar to those of an intervention trial. This study represents the first time that beta-carotene levels have been assessed in uro-genital tract cells, cells which have shown evidence of carcinogen exposure.

1.2 Evidence for Beta-carotene as a Chemopreventive Compound

1.2.1 Epidemiological studies

Epidemiological studies of two kinds have provided evidence that beta-carotene may be a chemopreventive substance. One approach has been to investigate the effect of diet, particularly the ingestion of carotenoid-containing vegetables, on the incidence of cancer. Second, blood levels of beta-carotene in cancer patients have been compared to those of controls.

An inverse association between consumption of beta-carotene-rich vegetables and cancer risk has been suggested by most studies investigating this topic (reviewed by Peto et al. 1981, Wolf 1982, Hennekens et al. 1986). Lung, oral and esophageal, and gastro-intestinal tissues in particular have been implicated as being at an elevated risk of cancer when consumption of these food-items was low (Colditz et al. 1987, Winn et al. 1984, Ziegler et al. 1981); this association has also been detected for the gastro-intestinal tract, bladder, endometrium, and cervix (Modan et al. 1981, Mettlin and Graham 1979, Marshall et al. 1983, La Vecchia et al. 1986). Even among individuals who smoke, drink, and eat meat daily, a protective effect has been observed (Hirayama 1985). Because of the body of supporting evidence that beta-carotene is a chemopreventive substance, interest in certain foods such as green-and-yellow vegetables has focussed on their beta-carotene content, although it may be that other constituents in these foods that are protective, or that consumption of these food-items is correlated with a low intake of some other food which is carcinogenic (Modan et al. 1981, Hirayama 1985, Colditz et al. 1985). Nevertheless, the association for beta-carotene is much more consistent than for intake of other micro-nutrients, such as preformed vitamin A, and a lower risk of cancer.

Low serum beta-carotene in blood samples taken before the onset of disease was associated with an elevated risk of lung cancer, in three out of four studies (Stahelin et al. 1984, Nomura et al. 1985, Menkes et al. 1986, and Willett et al. 1984b). The association was seen for smokers and non-smokers; furthermore, low serum beta-carotene increased the risk for heavy smokers (Nomura et al. 1985). In contrast, no significant association was found between serum beta-carotene levels and the risk of colon, stomach, rectum or bladder cancers (Nomura et al. 1985). A protective effect of dietary vegetable intake among current smokers, suggested by the results of two lung cancer studies, suggests that beta-carotene may be able to interrupt a late stage of carcinogenesis (Pisani et al. 1986, Ziegler et al. 1986).

Conclusions from epidemiological studies will be broadened when specific biochemical markers such as beta-carotene are identified and their association with cancer incidence further clarified.

1.2.2 Experimental studies

Evidence from animal studies and *in vitro* studies supports the hypothesis that beta-carotene is a chemopreventive substance.

In animal models of carcinogenesis, high doses of beta-carotene protected against experimental carcinogenesis induced by chemicals, UV-B radiation, and a chemical followed by radiation (Alam and Alam 1987, Alam et al. 1984, Rettura 1983, Dorogokupla et al. 1973, Suda et al. 1986, Mathews-Roth 1982, Santamaria et al. 1983). Use of beta-carotene led to the development of smaller tumours, fewer tumours, an increased latent period, and enhanced regression of tumours, even when it was applied after the tumours had been induced. Beta-carotene also protected experimental rodents against liver damage due to aflatoxin or due to dimethylnitrosamine (Newberne et al. 1974, Yee et al. 1985).

In vitro, beta-carotene has shown anti-mutagenic effects (Renner 1986, Belisario et al. 1985, Manoharan and Banerjee 1985) and anti-clastogenic effects (Raj and Katz 1985, Renner

1986, Stich and Dunn 1986). Experiments with CHO cells pre-treated with beta-carotene before exposure to 4NQO or to MMS revealed an inhibitory effect of beta-carotene on carcinogen-induced chromosome damage (Stich and Dunn 1986).

In some systems beta-carotene was demonstrated to be active in its own capacity rather than as converted to vitamin A. Beta-carotene inhibited the chemically-induced transformation of mouse mammary epithelial cells in culture (Som et al. 1984), an effect apparently due to the action of beta-carotene itself because the authors verified that retinol was not present nor was it produced by the cells.

It is important to note that the effects of beta-carotene, just as for the effects of most carcinogens, were observed to be dose-, timing-, route of administration-, tissue- or organ-, species-, strain-, and sometimes sex-specific. The null effect of beta-carotene on 1,2-DMH-induced colorectal cancer in the rat (Colacchio and Memoli 1986) may well be challenged if the tumour model is refined in one of those aspects.

Thus, beta-carotene has been effective against initiation and against promotion of carcinogenesis. Proposed mechanisms of action of beta-carotene as an anti-mutagenic, anti-clastogenic, and anti-carcinogenic substance include the following: 1) beta-carotene may be effective against tumours by protecting cells against oxygen radicals which initiate and promote carcinogenesis at a number of stages (Krinsky and Deneke 1982, Ames 1983, Mathews-Roth 1986); and 2) beta-carotene may protect cells by its anti-oxidant activity (Burton and Ingold 1984).

The experiment designed to reveal if beta-carotene inhibition of chemical carcinogenesis in the oral mucosa occurred at the initiation or the promotion stage was unable to resolve the question because beta-carotene inhibited at both stages (Suda et al. 1986). Retinol and the retinoids, on the other hand, involved in cell growth and differentiation, seem to be effective against the progression stage of carcinogenesis. The mode of action of the different retinoids seems to be one of modulating epithelial differentiation, which

manifests as an inhibition of neoplastic transformation in experimental systems (Sporn and Roberts 1983).

1.3 The necessity for human trials

1.3.1 Limitations of other approaches

Epidemiological studies and laboratory studies are an inadequate basis upon which to make recommendations regarding human cancer prevention. Dietary epidemiologic studies may lack specificity; there is imprecision in the dietary recall method; dietary studies rely on food composition tables that may apply to only some of the food items eaten; and controls may also have an abnormal dietary pattern due to their (non-malignant) disease. Serum epidemiological studies also need to address the question of the effect of the disease itself on serum levels of beta-carotene; also, the preservation of the sample must be verifiable. In addition, in any epidemiological study, there may be an inherent bias in the criteria used for selection of the study group.

Experimental studies investigating the effect of beta-carotene also have limited direct applicability to the human situation. Only one *in vivo* study supplied carotene in a natural form: animals were fed a diet supplemented with "an unlimited amount of red carrots" (Dorogokupla et al. 1973). The doses of beta-carotene that have proved effective (e.g. 100 mg/kg diet) are in most cases far higher than that provided to a man by a normal diet (approximately 2 mg/day); to extrapolate from very high to very low doses inevitably means there will be large variation in the estimate of the substance's protective effect. The genetic heterogeneity of human populations, varied diet, and highly variable exposure to carcinogens are in contrast to experimental conditions (Alavanja et al. 1987, Abelson 1987). Differences such as these, however, need not preclude consideration of the results for testing in human experimental settings.

1.3.2 Intervention trials

In view of these limitations, intervention trials in human populations offer a promising experimental approach. An intervention trial monitors the response of an endpoint to the administration of a chemopreventive substance. The largest intervention trial employing beta-carotene as a chemopreventive agent is the ongoing U.S. Physicians Study (Stampfer et al. 1985) which has as its endpoints overall cancer morbidity and mortality after 5 years. This requires observation of a huge cohort for a minimum of 5 years, with the ensuing demands of record-keeping and expense. In contrast, from an intervention trial knowledge can be gained relatively quickly about the effectiveness of the chemopreventive substance against an intermediate stage of human carcinogenesis.

An intermediate endpoint or short-term indicator of carcinogen-exposure, is the frequency of micronucleated exfoliated cells. This indicator can be tested for its response to chemopreventive agents. The elevated frequency of micronucleated cells from the oral mucosa of betel quid chewers (who are at risk for oral cancer) was reduced when the chewers ingested supplemental beta-carotene (Stich et al. 1984a,b). These results were duplicated with snuff-dippers and tobacco chewers in Canada (Stich et al. 1985).

An elevated frequency of micronucleated cells in the oral mucosa is associated with the habits of chewing of tobacco and betel quid and snuff-dipping, and may indicate an elevated risk for oral cancer (IARC 1985).

The suggestion has been made that low levels of beta-carotene in the tissues, presumably due to a meagre dietary supply of the provitamin, could confer increased susceptibility to carcinogenic injury upon the tissue, possibly due to deficiency of an essential function (De Luca 1983, Stich et al. 1986a).

To capitalize on the potential of intervention trials as a tool for cancer research, we need to know more about the interactions of chemopreventive agent, carcinogen, and genetic material, at the cellular level.

1.4 Beta-carotene in humans

1.4.1 Blood

Plasma, serum,or whole blood is uninformative for studies of beta-carotene acting in tissues as a cancer preventive substance. Serum levels of beta-carotene reflect recent dietary intake (Goodwin 1984). Fluctuations in blood level of beta-carotene could confound the interpretation of blood carotene levels; fluctuations may not be a concern if exfoliated cells are examined rather than blood.

1.4.2 Tissues

Different tissues may have different preferred levels of beta-carotene. Evidence from animal studies indicates organ-specific kinetics of accumulation of carotene, referring to its uptake, time to reach a plateau, and retention time (Mathews-Roth et al. 1977, Shapiro et al. 1984). Little is known about beta-carotene in human tissues, and the kinetics of its uptake and depletion in response to supplementation with beta-carotene.

Furthermore, just as the retinoids show tissue-specific chemopreventive effects (Olson 1985), so may the chemopreventive effect of beta-carotene be tissue-specific. Carcinogens are species-, tissues-, organ-, dose-, and timing-specific (Wattenberg 1985), and carcinogen inhibitors are likely to be also.

1.4.3 Loading studies

Despite the limitations of using serum as the focus of investigation of beta-carotene, the kinetics of beta-carotene in serum will be relevant to the kinetics of beta-carotene in cells. Previous studies have provided data on normal levels of beta-carotene in blood, normal levels of beta-carotene in human tissues, and changes in blood, and possibly tissue, levels of beta-carotene resulting from oral supplementation with beta-carotene.

Normal ranges of total plasma carotene or plasma beta-carotene have been established for many populations (Philips et al. 1970, Vuilleumier et al. 1983, Willett et al. 1983a, West and Ash 1984, Herbeth et al. 1986). Human skin, brain, and liver have been analyzed for beta-carotene content (Vahlquist et al. 1982, Mathews-Roth et al. 1976). Daily supplementation of individuals with carotene has resulted in characteristic features of a rapid rise in blood level of carotene to a plateau level (Urbach 1952, Mathews-Roth and Gulbrandsen 1974), elevated levels that could be maintained by smaller daily doses of carotene (Urbach 1952), and after cessation of treatment, a gradual decline in blood carotene (Urbach 1952, Bieri et al. 1985). The relationship of serum levels of beta-carotene to tissue levels could remain a stumbling-block in our understanding of beta-carotene in humans, if it were not for ways to approximate the levels of beta-carotene in tissues, as will be described next.

1.4.4 Exfoliated cells

Beta-carotene in the basal layer of cells can be expected to be reflected in the beta-carotene level in exfoliated cells. The foremost advantage to studying exfoliated cells is that there is the opportunity to investigate, in parallel, genotoxic damage (such as elevated micronucleated cell frequency) and level of protective agent (beta-carotene) in cells from the

same site. Furthermore, it may be possible to correlate *in vitro* assay results with those of *in vivo* (human) studies (e.g. Stich and Dunn 1986).

Exfoliated cells are easily collected by a non-invasive technique that is not traumatic to the donor, requires a minimum of simple equipment, and is suitable for examination of population groups; furthermore, the tissue is rapidly renewed. The small quantity of cells obtained is adequate for use with the present highly-sensitive detection method: the HPLC assay (described in Materials and Methods) is capable of detecting picogram amounts of beta-carotene per million cells (Vuilleumier et al. 1983).

Initial studies by our lab revealed differences between populations in their beta-carotene levels in exfoliated cells: individuals from a detoxification centre and individuals consuming a communal vegetarian diet differed in their profile of beta-carotene levels in exfoliated oral mucosa cells (Stich et al. 1986a). Beta-carotene levels increased in exfoliated oral mucosa cells when individuals consumed beta-carotene supplements (Stich et al. 1986b); there were indications that the increase was not continuous. Furthermore, the examination of beta-carotene in exfoliated cells may reveal other factors that are affecting tissue beta-carotene level; among women in the Philippines who practised inverted smoking, those who received beta-carotene together with vitamin A, in an intervention trial, showed higher cell levels of beta-carotene than those who ingested beta-carotene alone (Stich et al. 1986b).

Beta-carotene level in tissues may become a useful marker for the recognition of persons or groups at elevated risk of carcinogen injury (Stich and Dunn 1986, Alavanja et al. 1987). Oral mucosa cells are the target cells of carcinogens from tobacco and betel quid, therefore studies of beta-carotene in these cells are highly relevant to investigations of the etiology of oral cancer. Fundamental to this kind of investigation must be an understanding of basal levels, baseline variation, and the response to supplementation shown by this beta-carotene marker in the partiular tissue-of-interest.

1.5 Beta-carotene in uro-genital tract cells

No previous investigations have monitored beta-carotene levels in exfoliated cells other than in oral mucosa cells. Investigations of exfoliated cells from the uro-genital tract could be highly appropriate to an understanding of uro-genital tract carcinogenesis. Uro-genital tract cells have displayed markers of carcinogen-induced injury: an increased micronuclei frequency in these cells has been detected with bilharzial infection, radiation exposure, possibly pesticide exposure, and in cells from persons who are heavy smokers and drink large quantities of coffee (Raafat et al. 1984, Stich et al. 1983, Stich and Rosin 1983a,b, 1984). Micronucleated cells may be etiologically related to tumours of the uro-genital tract, including tumours of the urinary bladder, vulva, and vagina (Stich and Rosin 1984).

Uro-genital tract tissue is therefore an appropriate candidate for an intervention trial testing beta-carotene for evidence of chemopreventive activity.

Favourable results, in experimental animals and in humans, have been obtained in preliminary studies of prevention of urinary bladder carcinogenesis with retinoids (Alfthan et al. 1983); research was limited, however, because of toxic effects of the drugs. Tests of beta-carotene, a non-toxic substance, could be attempted in those experiments.

To evaluate the suitability of uro-genital tract cells as target cells of an intervention trial using beta-carotene, it will be valuable to establish the kinetic profile of beta-carotene in these cells after beta-carotene supplementation. Normal levels of beta-carotene, their variation, and the possible correlation of beta-carotene levels in two tissues (oral mucosa and uro-genital tract) may reveal tissue-specific characteristics of beta-carotene accumulation.

2. Rationale

2.1 Kinetics

Oral mucosa cells show genotoxic damage (increased frequency of micronucleated cells) in persons who chew carcinogenic mixtures; this damage was found to diminish, in an intervention trial, when beta-carotene supplements were ingested by the chewers. Little is known about the accumulation and retention of beta-carotene in the dividing layer of cells, where the events of carcinogenesis are thought to take place. As a marker for beta-carotene in the cells of interest, we can monitor the level of beta-carotene in exfoliated cells. To better design intervention strategies directed at reducing genotoxic or carcinogen damage in epithelial cells by means of elevating the cell level of beta-carotene, a proposed chemopreventive agent, it was proposed to observe the response of beta-carotene in these cells to different short-term, oral doses of beta-carotene.

2.2 Uro-genital Tract Cells

It is important to gain an understanding of the kinetics of the tissue's accumulation and retention of beta-carotene after its administration. There is evidence that tissues show characteristic basal levels of beta-carotene and tissue-specific patterns of accumulation and retention of beta-carotene after its administration. Tissue-specificity implies that the potential of beta-carotene as a chemopreventive agent is specific to the tissue. Carcinogenesis in the uro-genital tract could be investigated, as has been done for the oral mucosa, with an intervention trial using beta-carotene. Better design of intervention trials employing beta-carotene as a chemopreventive substance could be achieved with a better understanding of the accumulation of beta-carotene specific to each tissue.

3. Objectives

The primary objective of this research was to investigate the kinetics of beta-carotene accumulation and retention in exfoliated cells in individuals receiving oral supplementation with beta-carotene. Specifically, two aspects of the kinetics were examined: maximum beta-carotene levels, and duration of elevated levels of beta-carotene. The studies were done on cells collected from the oral mucosa and from urine. The specific objectives were:

1) to establish a procedure for collecting and assaying exfoliated uro-genital tract cells for the determination of beta-carotene levels; a procedure for determining beta-carotene levels in oral mucosa cells had already been established and its use had been validated.

2) to determine the extent to which technical artifacts contribute to variation in measurements obtained for beta-carotene in the aforementioned cell samples.

3) to establish baseline levels of beta-carotene, and baseline variation in beta-carotene in exfoliated oral mucosa cells and uro-genital tract cells from each individual in the study population, and to compare the beta-carotene levels, and their variation, in cells from the oral mucosa and from the uro-genital tract, in each individual.

4) to determine the effect of short-term (4-day) oral supplementation with three doses of beta-carotene on the level of beta-carotene in exfoilated oral mucosa cells.

5) to determine the effect of long-term (4-week) supplementation with beta-carotene at a field-trial dose on levels of beta-carotene in uro-genital tract cells and exfoliated oral mucosa cells.

This represents the first time that beta-carotene levels were determined in uro-genital tract cells of beta-carotene-supplemented individuals.

4. Statistical Analysis

Intra-individual variation was calculated with the formulae for SD and C.V. given in Appendix C. Inter-individual variation was represented by the interquartile range about the median. Beta-carotene levels in the two cell types were compared by the Wilcoxon ranked pairs method, and the variations in beta-carotene in the two cell types were compared by Kendall's Tau rank correlation method.

Post-loading beta-carotene levels shown by the three dose-groups in the short-term study were compared by the nonparametric Kruskal-Wallis test. Beta-carotene levels between the beta-carotene group and the placebo group in the long-term study were compared by the nonparametric Mann-Whitney U test.

For details of these statistical methods, see Zar (1984).

MATERIALS AND METHODS

1. Study Group

Exfoliated cells from individuals were obtained from healthy males and females located at either the British Columbia Cancer Research Center or the Vancouver General Hospital School of Nursing. It was essential that individuals be located close to the Cancer Research Center for repeated sampling to be feasible, for ease of distribution of supplements, and for long-term access to individuals. Samples of exfoliated oral mucosa cells were obtained from 73 individuals of both sexes; samples of exfoliated uro-genital tract cells were collected from 62 females. Age-range for both groups was 18-65 years, median age 27 years. A questionnaire was employed to determine the consumption of beta-carotene- and vitamin A-containing foods and vitamin supplements and to assess the general dietary habit. A blank copy of the questionnaire is given in Appendix A.

2. Beta-carotene Capsules

Beta-carotene was supplied by Hoffman-La Roche as water-dispersal beadlets consisting of approximately 20% starch, 20% dextrose, 30 - 40% gelatin, 10 - 11% beta-carotene, and trace amounts of alpha-tocopherol and ascorbate palmitate. Each capsule contained 30 mg beta-carotene (50,000 IU of vitamin A activity). The butylated hydroxyanisole and butylated hydroxytoluene present in some forms of beta-carotene were absent from this preparation. Dextrose was supplied by Quest Vitamin Supplies, Ltd., Vancouver, B.C.

3. Collection and Isolation of Exfoliated Cells

Samples of exfoliated cells were protected from light as soon as they were obtained, and were processed, extracted, and analyzed for beta-carotene under yellow light. The steps in the preparation of samples for beta-carotene quantitation are outlined in Figure 1.

3.1 Oral Mucosa Cells

Collection of exfoliated oral mucosa cells was performed by the subjects. The individual rinsed his mouth thoroughly with tap water and discarded the rinse, then rinsed his mouth again. With a moistened soft toothbrush the subject brushed the entire buccal mucosa and labial mucosa twice, rinsed his mouth with 25 ml water, and collected the rinse in a 30 ml plastic centrifuge tube. The toothbrush was then shaken in the rinse-water in the tube.

Oral mucosa cells were isolated from the rinse by the following steps: the sample, shaken well, was strained through two layers of cheesecloth into a second 30 ml tube and was centrifuged at 700g for 5 min (Silencer H-103NA desk-top centrifuge). The resulting cell pellet was transferred into a 1.0 ml cryovial, and cells were re-suspended in water. The aliquot was frozen at -70 degrees C. until analysis.

3.2 Uro-genital Tract Cells

Subjects were asked to collect the first urine void and subsequent voids for the next 3 hours and to protect the urine sample from light. The 3 hr collection time was long enough that the sample would be likely to contain cells, but not so long that crystals would tend to precipitate in the sample. Urine was collected no matter what stage of the monthly cycle the person was in. Samples were brought to the laboratory within 2 hours of collection.

Fig.1 Protocol for processing urine samples or brushings of the oral mucosa to prepare the cell sample for beta-carotene analysis.

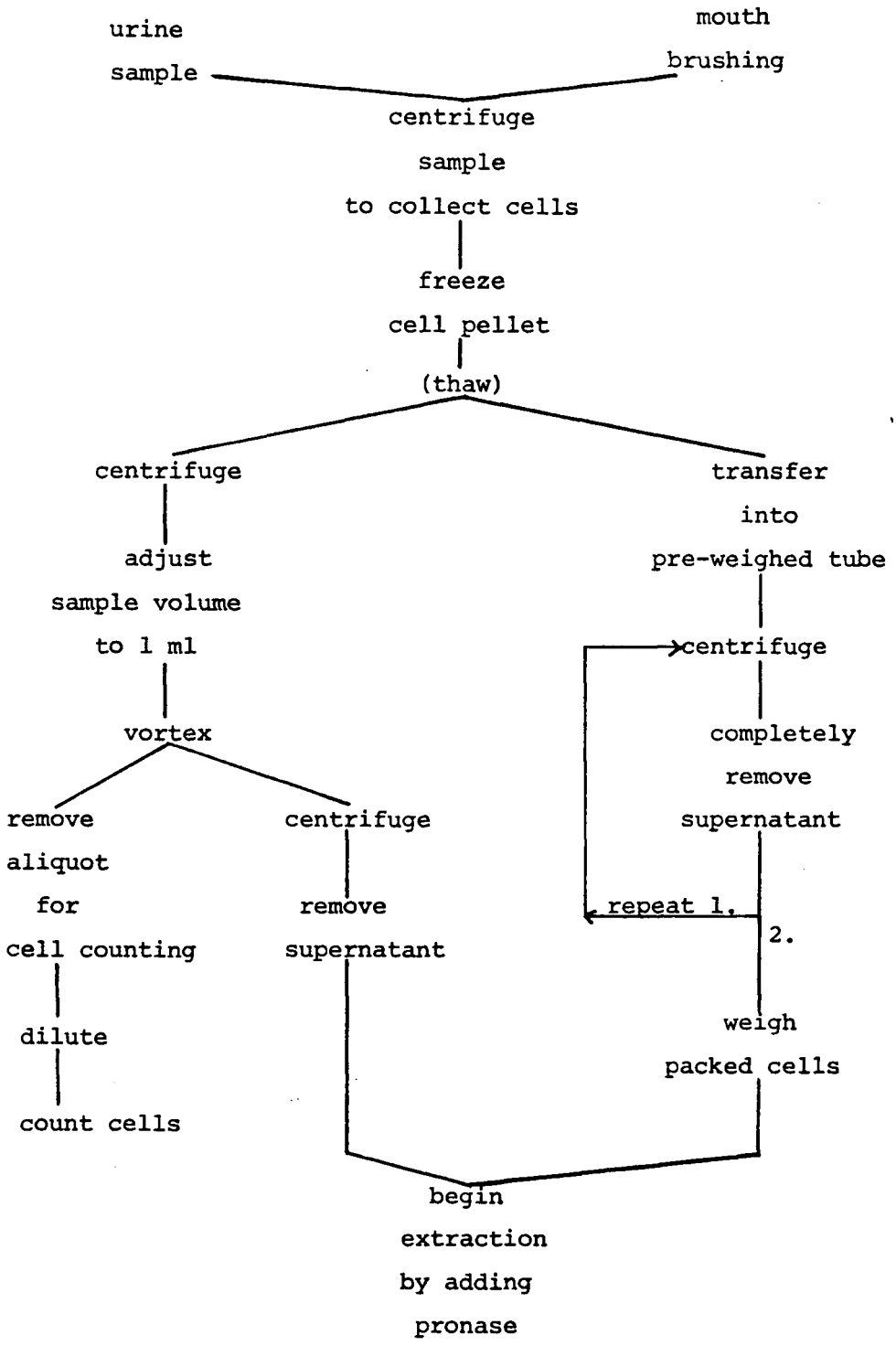
urine sample
mouth brushing
centrifuge sample to collect cells
freeze cell pellet
(thaw)
centrifuge
adjust sample volume to 1 ml
vortex
remove aliquot for cell counting
dilute
count cells
centrifuge
remove supernatant
transfer into pre-weighed tube
centrifuge
completely remove supernatant
repeat 1.
2.
weigh packed cells
begin extraction by adding pronase

Fig.1

Centrifugation of urine in 250 ml bottles followed by repeated centrifugation of the sediment proved to be the most efficient way to isolate uro-genital tract cells from several urine samples at once. Urine samples, transferred into 250 ml glass bottles, were centrifuged at 630g for 5 min (I.E.C. Universal model UV centrifuge). The resulting sediment was transferred into a 5 ml conical-tip glass tube and centrifuged at 700g for 5 min (Silencer H-103NA desk-top centrifuge). The supernatant was poured off.

If the sediment was chalky with crystals it was treated in the following way. First, the pH of the sample was determined, using graded pH paper (pHydrion). Second, attempts were made to identify the crystals by microscopic examination of a 50 ul aliquot of sediment, with reference to textbooks of urinalysis (Spencer and Pedersen 1976, Freeman and Beeler 1983). On the basis of the pH and the kind of crystals, drops of 0.1N HCl or 1.0N NaOH were added, with mixing, to the sample, to the limits of pH 5.0 or pH 8.0, respectively. If uric acid crystals were present the sample was warmed in a water bath (50 deg.C.) for 5 min. If a dense concentration of crystals persisted in spite of the treatments, the sample wasn't analyzed further. In most cases the treatment dissolved the crystals sufficiently to allow the uro-genital tract cells to be counted (see below). Samples that were visibly bloody weren't analyzed for beta-carotene.

4. Extraction and Measurement of Beta-Carotene

Oral mucosa cells were transferred into a tared 1.5 ml microcentrifuge tube and centrifuged at 3000g for 5 min (Silencer H-25FI desk-top microcentrifuge); the supernatant was removed, the aliquot re-centrifuged, and residual liquid was completely removed with the aid of a 50 ul syringe. The aliquot was weighed ("wet weight" of cells).

The uro-genital tract cell suspension was centrifuged at 3000g for 2 min, then the volume of the aliquot was adjusted to 1.0 ml. Cells were resuspended and clumps of cells were dispersed by repeated pipetting of the sample within the tube. The aliquot was vortexed and

a 20 ul aliquot, added to 80 ul of water, was removed for cell counting purposes (sec.5). The parent aliquot was centrifuged at 3000g for 5 min (Silencer H-25FI desk-top microcentrifuge), and the supernatant was removed.

The beta-carotene extraction procedure is outlined in Figure 2.

Cells were digested in two steps. The aliquot of exfoliated cells plus 200 ul of freshly-prepared 10% pronase (protease, Sigma, St.Louis, MO) in pH 7.5 phosphate buffer (0.05M K_2HPO_4, KH_2PO_4), was vortexed and incubated at 37 degrees C. for 1 hour. 200 ul of 5% KOH in methanol was added (stock solution stored at -20 degrees C.), and the sample was incubated at 50 degrees C. for 1 hour. The aliquot was cooled to room temperature on ice.

To extract the beta-carotene 750 ul hexane plus a drop of 1% phenol red in methanol (to demarcate the aqueous from the hexane layers) was added, and the mixture was thoroughly vortexed at high speed for 1 minute. The aliquot was centrifuged at 3000g for 5 minutes, then most of the lower, aqueous layer was carefully removed and discarded, using a fine-tipped plastic pipette. The tube was re-centrifuged, and 600 ul of the hexane layer was transferred into a second microcentrifuge tube. The hexane was evaporated off by centrifugation of the extract under reduced pressure (10 min at room temperature) in a Savant spin-evaporator. Thirty ul of 100% (anhydrous) ethanol was added immediately to the extraction residue, in the case of oral mucosa cells, and the tube was gently vortexed for 1 minute then centrifuged at 1350g for 1 minute. Ten ul of water followed by the entire 30 ul ethanol extract was drawn into a 50 ul HPLC injection syringe for injection into the HPLC column. In the case of uro-genital tract cells the extraction residue was reconstituted with 50 ul of ethanol and the top 40 ul was injected.

Fig.2 Procedure for extracting beta-carotene from exfoliated cells.

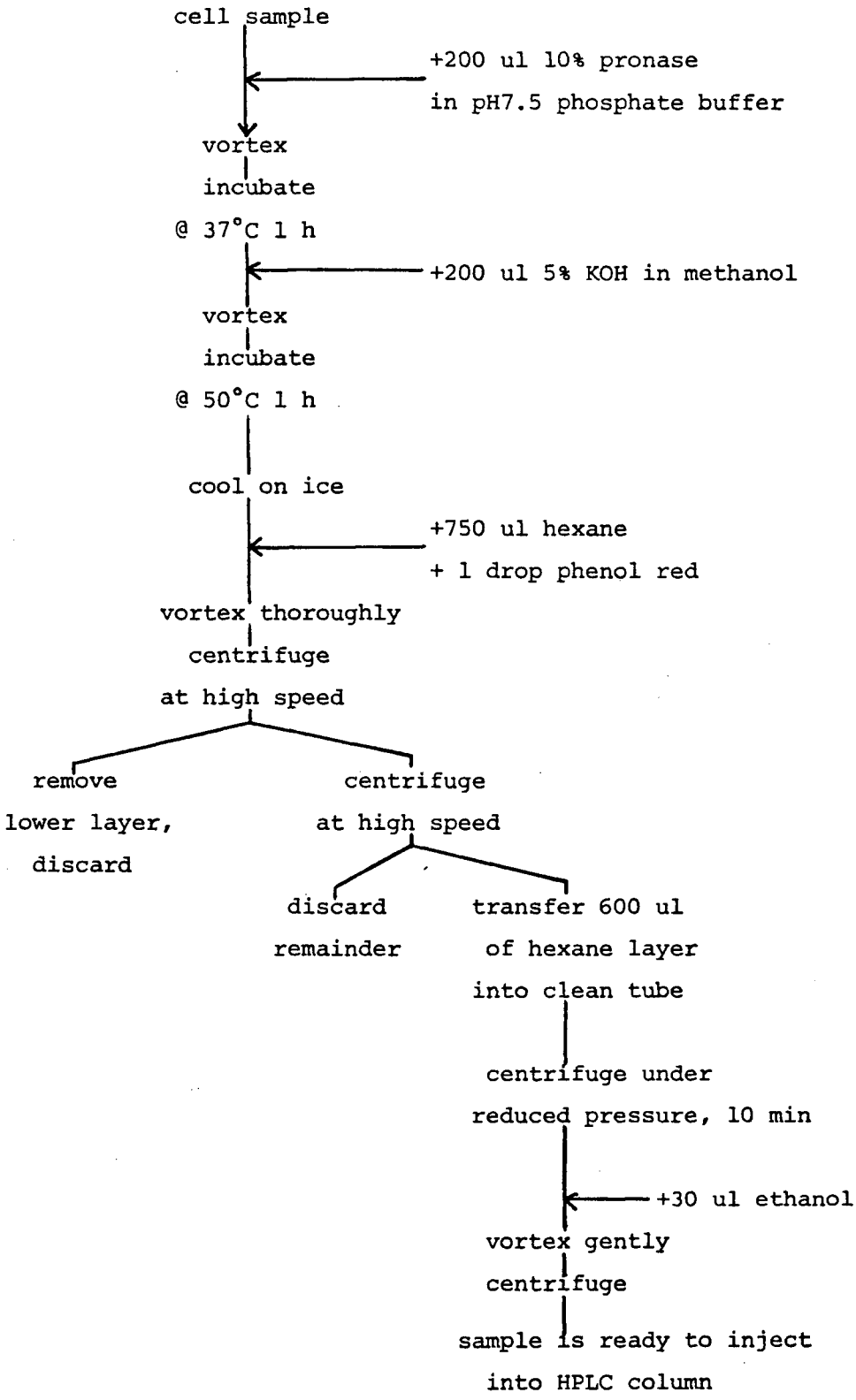
cell sample
+200 ul 10% pronase
in pH7.5 phosphate buffer
vortex
incubate
@ 37°C 1 h
+200 ul 5% KOH in methanol
vortex
incubate
@ 50°C 1 h
cool on ice
+750 ul hexane
+ 1 drop phenol red
vortex thoroughly
centrifuge
at high speed
remove
lower layer,
discard
centrifuge
at high speed
discard
remainder
transfer 600 ul
of hexane layer
into clean tube
centrifuge under
reduced pressure, 10 min
+30 ul ethanol
vortex gently
centrifuge
sample is ready to inject
into HPLC column

Fig.2

The ethanol extract was injected into a Vydac 201 TP reversed-phase chromatography column (internal diameter 2.1 mm, length 150 mm; 5 micron octadecylsilane packing). Running solvent was methanol (glass-distilled, Vancouver, B.C.) and 4% methylene chloride (HPLC-grade, Fisher Scientific Ltd., Vancouver, B.C.), delivered at a flow rate of 0.40 ml/min under approximately 1000 psi pressure by a Spectra-Physics SP-8700 solvent delivery system. Spectrophotometric detection of beta-carotene at 450 nm was performed by a Spectroflow 757 absorbance detector (ABI Analytical Kratos Division) with a tungsten lamp, linked to a Spectra-Physics SP4100 computing integrator and printer.

To calibrate the assay, two 30 ul injections of beta-carotene standard (Sigma, St.Louis, MO) in methanol (66.7 ng/ml, 1 ng/injection), injected without the 10 ul bolus of water, preceded each batch of sample injections.

5. Units of Beta-Carotene Level

The level of beta-carotene in the sample was expressed in units of nanograms beta-carotene per million cells. From the chromatogram, beta-carotene was quantitated by comparing the heights of the beta-carotene peaks of sample and standard; when the sample beta-carotene peak went off the chart the integrated areas under the peaks were compared. The beta-carotene measurement was calculated from the sum of two peaks: the principal beta-carotene peak, identified as the *trans-* isomer, retention time approximately 4.9 minutes, and a lesser peak, thought to be a *cis-* peak, eluting approximately 0.6 minutes after the *trans-* peak. Spontaneous isomerization towards an equilibrium mixture of the *cis-* and *trans-* forms of beta-carotene, reported by Zechmeister and Polgar (1943), was demonstrated with the HPLC technique by Tsukida et al. (1982).

The number of oral mucosa cells in the original aliquot was determined from the wet weight of the cells and comparison with a previously-established calibration curve (see Appendix B). It wasn't suitable to use the wet weight short-cut method to determine the

number of uro-genital tract cells in the sample because these cells didn't pack down into a defined pellet after centrifugation; not all the supernatant could be separated from the cells. Instead, to determine the number of uro-genital tract cells in the original aliquot the cell counting method was preferred. Twenty ul of the 100 ul aliquot of the diluted uro-genital tract cell suspension was applied to a Reichert Bright-Line Hemacytometer, a minimum of 200 cells were counted within a grid that lies over a defined volume, and the count was corrected for dilution. Only intact squamous epithelial cells (approximately 95% of the cells present) were counted.

Beta-carotene level, in units of nanograms per million cells, was found from the chromatogram beta-carotene value (ng/injection) corrected for the volumes transferred in the extraction, divided by the number of cells in the aliquot.

RESULTS

1. Feasibility Studies With Uro-genital Tract Cells

Since this study represents the first usage of uro-genital tract cells for beta-carotene quantitation, a series of studies were done to determine the following: 1) the most efficient way to collect and isolate uro-genital tract cells; 2) the stability of beta-carotene in uro-genital tract cells; and, 3) technical variation in the beta-carotene measurement in these cells.

1.1 Sampling procedure

A study was done to determine the number of uro-genital tract cells necessary to ensure a detectable level of beta-carotene in the sample, for the majority of unsupplemented individuals. Urine samples collected from a group of student nurses were analyzed for beta-carotene in the cells. Beta-carotene was detected in 42 out of 46 samples (91%) when a minimum cell number of 0.5×10^6 cells was used as the cut-off point. Subsequent to this investigation, subjects were asked to donate urine samples on 2 or 3 consecutive days, in order to ensure collection of this number of cells. Each day, the cells were isolated and frozen. Cell samples from consecutive days were pooled immediately prior to beta-carotene analysis.

1.2 Stability of beta-carotene in cells suspended in urine

Processing of urine samples in order to isolate uro-genital tract cells took 3 to 5 hours. The question of whether the prolonged contact of cells with urine altered the beta-carotene level in cells was addressed as follows. Uro-genital tract cells were isolated from urine samples collected between 9 and 11 a.m. by six females; the cells were pooled, and the pool

was divided into three aliquots. The first aliquot was processed immediately, and the cells were frozen. Urine (removed after the cells had been centrifuged) was returned to the other two aliquots, they were vortexed, and one aliquot was left under yellow light at room temperature for 4 h and the other for 8 h, then the urine supernatants were again removed and cells were isolated and frozen. The three aliquots of cells were analyzed for beta-carotene in the same HPLC batch. By microscopic examination of the sediments, the uro-genital tract cells were found to be intact at 0, 4, and 8 h. Analysis of the aliquots for beta-carotene (Table 1) revealed that the variation between the three beta-carotene levels was 12% (C.V.), which is within the technical variation of the measurement (sec.1.3 below). Beta-carotene levels were not significantly altered by 4 h or 8 h of exposure of the cells to urine.

1.3 Technical variation in measurements of beta-carotene in uro-genital tract cells

To determine the amount of technical variation inherent in the beta-carotene assay of uro-genital tract cells, pooled samples of uro-genital tract cells were analyzed for beta-carotene. Two approaches were used. Cells from urine from eight individuals were pooled and multiple aliquots of the pool were assayed for beta-carotene. The variation in six aliquots was 38% (Table 2). In the second approach, cells from urine from each of 14 individuals were dividied into two samples and assayed individually on two occasions. A similar variation in beta-carotene levels was observed (median variation was 28%, interquartile range 12 - 60%) (Table 3).

2. Technical Variation in the Measurement of Beta-carotene in Oral Mucosa Cells

Samples of oral mucosa cells donated by 13 individuals at the Cancer Research Centre were pooled. The variation in beta-carotene levels in ten aliquots of the pooled sample was 22% (Table 4).

TABLE 1

STABILITY OF BETA-CAROTENE IN EXFOLIATED URO-GENITAL TRACT CELLS SEPARATED FROM URINE AT DIFFERENT TIMES AFTER URINE COLLECTION[1]

Beta-carotene ($ng/10^6$ cells)			Variation (%C.V.)
0 h	4 h	8 h	
2.0	1.6	2.0	12

[1]For details, see Results sec.1.2.

TABLE 2

REPRODUCIBILITY OF THE ASSAY FOR BETA-CAROTENE IN UROGENITAL TRACT CELLS[1]

Source	Beta-carotene ($ng/10^6$ cells)			Variation
	Samples	Mean	SD	%C.V.
Cell pool	0.3, 0.6, 0.7, 0.7, 0.9, 1.1	0.7	0.3	38

[1]Cells from urine samples from eight individuals were pooled, and the pool was divided into six aliquots.

TABLE 3

REPRODUCIBILTY OF THE ASSAY OF BETA-CAROTENE IN DUPLICATE ALIQUOTS OF URO-GENITAL TRACT CELLS[1]

Subject	Beta-carotene ($ng/10^6$ cells)				Variation
	Samples 1	2	Mean	SD	%C.V.
A08	0.1	0.1	0.10	0.00	0
B22	0.1	0.4	0.25	0.21	84
A03	0.2	0.5	0.35	0.21	60
B24	0.3	0.2	0.25	0.07	28
A03	0.5	0.4	0.45	0.07	16
A03	0.5	0.2	0.35	0.21	60
A02	0.6	0.5	0.55	0.07	13
B09	0.7	0.3	0.50	0.28	56
B08	0.9	0.6	0.75	0.21	28
A05	1.0	2.6	1.80	1.13	63
L09	1.5	1.1	1.30	0.28	22
B25	1.5	1.7	1.60	0.14	9
L08	7.9	12.7	10.30	3.39	33
B22	10.3	11.8	11.05	1.06	10
a[2]	0.7				28
b	(0.3 - 1.5)				(12 - 60)

[1]A single sample of uro-genital tract cells from each individual was divided into two; each aliquot was assayed individually for beta-carotene.

[2]a = median, b= interquartile range.

TABLE 4

REPRODUCIBILITY OF BETA-CAROTENE ASSAY WITH EXFOLIATED ORAL MUCOSA CELLS[1]

Source	Beta-carotene ($ng/10^6$ cells)			Variation
	Samples	Mean	SD	%C.V.
Cell pool	1.1, 1.4, 1.4, 1.4, 1.5, 1.5, 1.7, 1.8, 2.1, 2.2	1.6	0.4	22

[1]Cells were collected from 13 individuals (collection procedure described in text), pooled, and the pool was divided into ten aliquots.

3. Establishing Basal Levels and Within-subject Variation of Beta-carotene Levels in Unsupplemented Individuals

The median (50th percentile) was chosen as the measure of central tendency instead of the mean, in this and in subsequent sections, for two reasons: 1) beta-carotene levels tend to show a right-skewed distribution rather than a normal distribution on a frequency distribution plot, therefore the mean tends to be falsely elevated by a few high outlier values and the standard deviation (SD) will also be larger than it would be if the data were normally distributed; and 2) two means should only be compared when the variances of the two sets of data are equal, which usually requires transformation of the data, but then the values are less-readily understood. The interquartile range, the range from the 25th percentile to the 75th percentile, was chosen to describe the spread of the data about the median.

3.1 Oral mucosa cells

Individuals who were not taking beta-carotene supplements donated two samples of oral mucosa cells each, with a time-lag of three days (Table 5) or one week (Table 6) between samples, in order that the variation in beta-carotene level between two samples could be estimated. Samples in Table 5 were obtained in January, 1986, and samples in Table 6 in July and August, 1986. The variation in beta-carotene in the two samples from each person is identified as the within-subject variation (%C.V.).

The median of variation within subjects was 18% in the three-days-apart samples (interquartile range 11% - 40%) and 32% in the one-week-apart samples (interquartile range 12% - 57%), which was not a significant difference (p=.36, Mann-Whitney U test, a=.05). When all subjects were considered together median variation in repeated oral mucosa cell

TABLE 5

BASELINE DATA OF BETA-CAROTENE IN EXFOLIATED ORAL MUCOSA CELLS FROM UNSUPPLEMENTED INDIVIDUALS[1]

Subject	Beta-carotene ($ng/10^6$ cells)				Variation
	Samples 1	2	Mean	SD	%C.V.
21	0.4	0.5	0.5	0.1	16
14	0.4	1.2	0.8	0.6	71
25	0.6	0.7	0.7	0.1	11
22	0.6	0.9	0.8	0.2	28
20	0.9	0.5	0.7	0.3	40
27	0.9	2.1	1.5	0.8	57
09	1.1	0.5	0.8	0.4	52
08	1.1	0.9	1.0	0.1	14
26	1.1	0.9	1.0	0.1	14
07	1.1	1.6	1.4	0.3	26
19	1.2	1.5	1.4	0.2	16
01	1.7	0.2	0.9	1.1	112
04	1.8	1.8	1.8	0.0	0
10	1.9	1.9	1.9	0.0	0
03	2.2	3.1	2.6	0.6	24
24	2.7	2.6	2.6	0.1	3
18	3.3	3.0	3.1	0.2	7
23	8.4	10.9	9.7	1.8	18
02	8.8	6.5	7.7	1.6	21
a[2]	1.1				18
b	(0.9 - 2.2)				(11 - 40)

[1]Samples were collected three days apart.

[2]a = median, b = interquartile range.

TABLE 6

ADDITIONAL BASELINE DATA OF BETA-CAROTENE IN EXFOLIATED ORAL MUCOSA CELLS FROM UNSUPPLEMENTED INDIVIDUALS[1]

Subject	Beta-carotene ($ng/10^6$ cells)				Variation
	Samples 1	2	Mean	SD	%C.V.
L03	0.2	0.3	0.2	0.1	28
A01	0.2	0.3	0.2	0.1	28
L12	0.3	0.3	0.3	0.0	0
L15	0.3	0.3	0.3	0.0	0
L11	0.4	0.2	0.3	0.1	47
L19	0.4	0.2	0.3	0.1	47
A03	0.4	0.9	0.7	0.3	54
L09	0.5	0.5	0.5	0.0	0
L04	0.5	0.8	0.7	0.2	32
A17	0.6	0.1	0.3	0.3	100
A16	0.6	0.5	0.6	0.1	13
L05	0.6	1.1	0.8	0.3	41
L01	0.7	0.7	0.7	0.0	0
L07	0.7	1.1	0.9	0.3	31
L16	0.7	1.8	1.2	0.8	62
L13	0.8	0.5	0.7	0.2	32
A13	0.9	0.7	0.8	0.1	18
A02	0.9	0.9	0.9	0.0	0
A06	1.0	0.4	0.7	0.4	60
A04	1.2	0.7	0.9	0.3	37
A08	1.2	2.2	1.7	0.7	42
A05	1.8	0.6	1.2	0.8	71
A11	2.3	1.8	2.0	0.3	17
A22	2.5	0.8	1.6	1.2	73
A14	2.7	1.6	2.1	0.8	36
A15	2.9	0.9	1.9	1.4	74
L06	3.1	3.6	3.4	0.3	10
L14	3.5	3.3	3.4	0.1	4
L18	4.2	4.3	4.2	0.1	2
A09	4.4	1.3	2.9	2.2	77
L08	5.2	4.3	4.8	0.6	13
A07	5.4	4.5	5.0	0.6	13
A12	9.3	2.8	6.0	4.6	76
a[2]	0.9				32
b	(0.5 - 2.9)				(12 - 57)

[1] Samples were collected one week apart.

[2] a = median, b = interquartile range.

samples was 27% (interquartile range 11% - 51%); i.e., for three-quarters of the study population the variation between two samples was less than 51%.

The median level of beta-carotene in the oral mucosa cell samples collected three days apart was 1.1 ng/10^6 cells (interquartile range 0.9 - 2.2 ng/10^6 cells). In the one-week-apart samples the median was 0.9 ng/10^6 cells (interquartile range 0.5 - 2.9 ng/10^6 cells). For all subjects taken together, median beta-carotene was 1.1 ng/10^6 cells (interquartile range 0.6 - 2.7 ng/10^6 cells).

3.2 Uro-genital tract cells

Twelve females from the School of Nursing and the Cancer Research Centre contributed samples of uro-genital tract cells on two occasions one week apart, in August, 1986, so that an estimate could be made of the variation of beta-carotene in repeated samples of uro-genital tract cells. Median variation between two samples was 81% (interquartile range 45% - 122%) (Table 7).

The median level of beta-carotene in uro-genital tract cells from this group was 0.5 ng/10^6 cells (interquartile range 0.1 - 0.9 ng/10^6 cells).

To summarize sections 3.1 and 3.2, the median level of beta-carotene was lower in uro-genital tract cells than in oral mucosa cells (0.5 ng/10^6 cells vs. 1.1 ng/10^6 cells), and the variation in beta-carotene from two samples tended to be greater for uro-genital tract cells than for oral mucosa cells (approximately 81% vs. approximately 27%).

It is important to note that these samples of oral mucosa cells and uro-genital tract cells were not taken at the same time from the same individuals, as they were for the studies that are described in the next section (sec.3.3).

TABLE 7

VARIATION IN BETA-CAROTENE LEVELS IN SAMPLES OF URO-GENITAL TRACT CELLS FROM UNSUPPLEMENTED INDIVIDUALS[1]

Subject	Beta-carotene ($ng/10^6$ cells)				Variation
	Samples 1	2	Mean	SD	%C.V.
A09	0.0	0.8	0.4	0.5	138
A03	0.0	0.5	0.3	0.3	122
A02	0.0	0.6	0.3	0.4	123
L05	0.1	0.2	0.2	0.1	47
A16	0.2	0.2	0.2	0.0	20
A11	0.4	2.3	1.4	1.4	99
A21	0.6	0.6	0.6	0.0	0
A15	0.7	9.3	5.0	6.1	122
A13	0.8	1.9	1.4	0.7	55
A05	0.9	2.2	1.5	1.0	62
A07	0.9	1.8	1.3	0.6	44
L08	2.1	13.0	7.6	7.7	102
a[2]	0.5				81
b	(0.1 - 0.9)				(45 - 122)

[1]Samples were obtained one week apart.

[2]a = median, b = interquartile range.

3.3 Comparison of variation in beta-carotene level in oral mucosa cells with variation in beta-carotene level in uro-genital tract cells from the same individual

In order to investigate whether the level of beta-carotene in one tissue (exfoliated cells from the oral mucosa) was proportional to the beta-carotene level in another (exfoliated cells from the uro-genital tract), samples of cells from the two sites were collected on the same day from 36 individuals, and cells were analyzed for beta-carotene (Table 8). Beta-carotene levels in uro-genital tract cells fell within a range of 0.1 - 3.5 ng/10^6 cells. In contrast, beta-carotene levels in oral mucosa cells, with one exception, fell within a wider range of 0.1 - 5.4 ng/10^6 cells. As indicated in Table 9, the level was higher in oral mucosa cells than in uro-genital tract cells for 75% of examined individuals, but for most of those individuals the level did not exceed eight times the level in uro-genital tract cells. (In agreement with the findings of sections 3.1 and 3.2, median beta-carotene levels in the two cell types were significantly different [p=.004, Wilcoxon ranked-pairs test, a=.05]). The oral cell and uro-genital tract cell beta-carotene levels showed a weak, but significant, correlation (r=.23, Kendall's Tau rank correlation method, p=.03).

The question of a possible correlation between the two sites in their variation in beta-carotene levels was addressed in the following way. Fourteen individuals contributed oral mucosa cell samples and uro-genital tract cell samples on two occasions one week apart. Beta-carotene levels and within-subject variation (%C.V.) of beta-carotene in each cell type are shown in Table 10. Median variation in uro-genital tract cell beta-carotene was significantly greater than the median variation in oral mucosa cell beta-carotene (p=.01, Wilcoxon ranked-pairs test, a=.05). Variation of beta-carotene in exfoliated oral mucosa cells and variation of beta-carotene in uro-genital tract cells from the same individual showed only a weak, non-significant correlation (r=.22, Kendall's Tau rank correlation method, p=.15).

TABLE 8

BETA-CAROTENE IN EXFOLIATED CELLS FROM TWO TISSUES IN EACH INDIVIDUAL[1]

Cell sample	Beta-carotene ($ng/10^6$ cells)					
	A[2]	B	C	D	E	F
a[3]	0.9	0.9	1.2	1.9	1.0	5.4
b	0.6	0.5	0.1	0.9	0.1	0.9
	G	H	I	J	K	L
a	2.2	1.3	2.3	9.3	0.9	2.9
b	0.6	0.8	0.4	1.0	0.8	0.7
	M	N	O	P	Q	R
a	0.6	0.6	0.5	0.4	0.8	0.6
b	0.2	0.8	0.1	0.6	3.5	0.7
	S	T	U	V	W	X
a	0.7	4.2	2.2	1.5	0.7	1.3
b	0.6	2.5	3.4	0.5	0.5	3.1
	Y	Z	AA	BB	CC	DD
a	1.0	0.1	1.3	3.6	0.8	0.6
b	1.5	0.1	0.3	1.0	0.2	2.1
	EE	FF	GG	HH	II	JJ
a	0.6	3.1	1.3	5.2	0.6	3.8
b	0.1	0.6	0.2	2.1	1.3	0.2

[1]Table shows one pair of beta-carotene values per person.

[2]Letters denote 36 individuals.

[3]a = oral mucosa cells, b = uro-genital tract cells.

TABLE 9

BETA-CAROTENE LEVEL IN ORAL MUCOSA CELLS RELATIVE TO LEVEL IN URO-GENITAL TRACT CELLS: DISTRIBUTION OF THIS RATIO IN THE EXAMINED POPULATION[1]

Percentage of individuals in various ratio groupings			
<1	1 - 3.9	4 - 8	>8
25	39	25	11[2]

[1]Data shown in Table 8 (n=36).

[2]For 3 of the 4 individuals the uro-genital tract cell beta-carotene level was 0.1 or 0.2 ng/10^6 cells; the fourth individual showed a high oral mucosa cell beta-carotene level (9.3 ng/10^6 cells).

TABLE 10

COMPARISON OF BASELINE DATA OF BETA-CAROTENE IN EXFOLIATED CELLS FROM THE ORAL MUCOSA AND URO-GENITAL TRACT[1]

Subject	Oral mucosa cells			Uro-genital tract cells		
	Beta-carotene ($ng/10^6$ cells)		Variation[2] (%C.V.)	Beta-carotene ($ng/10^6$ cells)		Variation (%C.V.)
	Sample 1	Sample 2		Sample 1	Sample 2	
A02	0.9	0.9	1	ND[3]	0.6	138
A03	0.4	0.9	59	ND	0.5	138
A05	1.9	0.6	73	0.9	2.2	62
A07	5.4	4.5	13	0.9	1.8	44
A09	4.4	1.3	77	ND	0.8	138
A11	2.3	1.9	15	0.4	2.3	99
A13	0.9	0.7	13	0.8	1.9	55
A15	2.9	0.9	75	0.7	9.3	122
A16	0.6	0.5	11	0.2	0.2	20
B12	1.5	0.8	42	0.5	0.3	34
B15	0.7	0.4	48	0.5	0.2	62
B22	3.6	0.9	86	1.0	0.3	66
L05	0.6	1.1	42	0.1	0.2	47
L08	5.2	4.8	6	2.1	13.0	102

Note: Median variation in beta-carotene in uro-genital tract cells was significantly greater than in oral mucosa cells ($p=.01$, Wilcoxon ranked-pairs test, $a=.05$). Variation of beta-carotene in exfoliated oral mucosa cells and uro-genital tract cells from the same individual showed a weak, non-significant correlation ($r=.22$, Kendall's Tau rank correlation method, $p=.15$).

[1]Samples were collected one week apart.
[2]Variation determined from data to two decimal places.
[3]ND = none detected, i.e. below analytical limits.

4. Dose-response Study of Beta-carotene in Exfoliated Oral Mucosa Cells Following a Short-term Loading

4.1 Study design

To investigate the relationship of beta-carotene dose to the amount of beta-carotene accumulated and retained in cells, a short term loading study was undertaken in which 27 individuals from the British Columbia Cancer Research Centre participated. Ages ranged from 23 to 65 years, with a median age of 32 years. Individuals consumed beta-carotene supplements (30 mg beta-carotene per capsule) in one of three total doses: 360 mg (3 capsules/day for 4 days), 180 mg (2 capsules/day for 3 days), and 90 mg (1 capsule/day for 3 days). There were 8, 9, and 10 individuals in the three groups, respectively. Participants donated two samples of oral mucosa cells before the beta-carotene loading and a total of three samples after the loading, at weekly intervals from the last day of loading. The treatment and sampling schedule is shown schematically in Figure 3. Cell samples were collected before the individuals ate their noon meal.

The regimen of 360 mg of beta-carotene given in four days has been used in short-term field studies (Stich et al. 1986b). A weekly dose of 180 mg has been used in several intervention trials (Stich et al. 1984a,b).

Data were summarized by determining medians and interquartile ranges of beta-carotene levels. Post-loading beta-carotene levels in the three groups of individuals were compared by the Kruskal-Wallis test. The first of the two pre-loading values was used when a calculation required a measure of pre-loading beta-carotene level.

Fig.3 Sampling and supplementation schedule of the short-term (four-day) loading study. Individuals received one of three doses of beta-carotene in a four-day loading period. Samples of exfoliated oral mucosa cells were collected twice before the loading, three days apart, and at three times after the loading, one week, two weeks, and three weeks from the last day of supplementation.

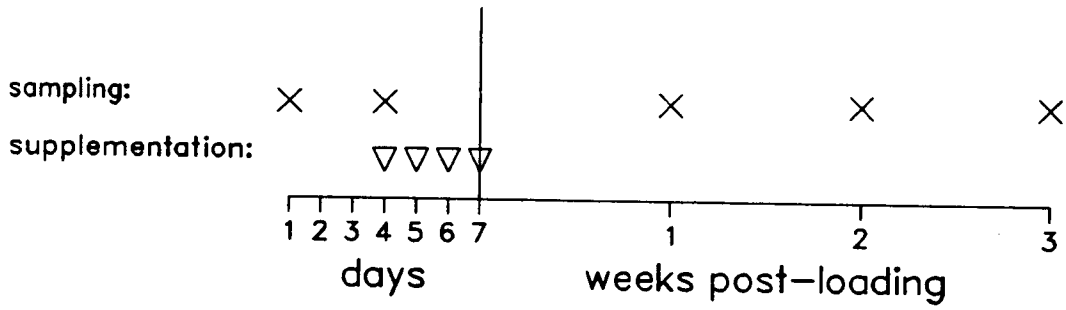
sampling:
supplementation:
1 2 3 4 5 6 7
1
2
3
days
weeks post-loading

Fig.3

4.2 Pre-loading beta-carotene levels

Twenty-three of the original study group of 27 individuals completed the supplementation regimen. Pre-loading beta-carotene levels of the three groups of individuals were not significantly different, by the nonparametric Kruskal-Wallis test, at a = .05 (Table 11).

The same table shows that the median beta-carotene level in exfoliated oral mucosa cells of all participants was 1.1 ng/10^6 cells (interquartile range 0.9 - 2.7 ng/10^6 cells). Data from individuals who contributed two pre-loading samples three days apart comprised the data (excluding subject 23) in Table 5 of section 3.1. Beta-carotene levels of pre-loading samples are plotted as Figure 4. The levels of beta-carotene were in agreement with those of one-week-apart samples taken from the larger study population.

4.3 Beta-carotene levels in response to loading

Post-loading beta-carotene levels in exfoliated oral mucosa cells from individuals who received beta-carotene supplements are shown in Figure 5. One week after the loading, the 360 mg dose group showed a median beta-carotene level (9.2 ng/10^6 cells) approximately two and one-half-fold greater, and that of the 180 mg group a median (7.7 ng/10^6 cells) approximately two-fold greater, than the median level of the 90 mg group (3.9 ng/10^6 cells). Elevated levels persisted for at least two weeks after a short-term loading of 360 mg or of 180 mg, and at least one week after a short-term loading of 90 mg of beta-carotene. Apparent differences between median beta-carotene levels of the three groups were not significant, however, according to the nonparametric Kruskal-Wallis test (a = .05), on week 1, 2, or 3. The plots also indicate the variation in response.

TABLE 11

PRE-LOADING BETA-CAROTENE LEVELS IN EXFOLIATED ORAL MUCOSA CELLS FROM INDIVIDUALS PRIOR TO SHORT-TERM SUPPLEMENTATION WITH THREE DOSES OF BETA-CAROTENE[1]

Dose	n	Beta-carotene ($ng/10^6$ cells)			
		Samples	Mean (SD)	Median	(Interquartile Range)
360 mg	6	1.1, 1.1, 1.7, 1.8, 2.2, 8.8	2.8 (3.0)	1.8	(1.1 - 3.8)
180 mg	8	0.4, 0.4, 1.1, 1.1, 1.9, 3.1, 3.1, 10.6	2.7 (3.4)	1.5	(0.6 - 3.1)
90 mg	9	0.4, 0.6, 0.6, 0.9, 0.9, 1.1, 1.2, 2.7, 3.0	1.3 (0.9)	1.0	(0.6 - 2.8)
all participants			2.2 (2.5)	1.1	(0.9 - 2.7)

[1]Pre-loading beta-carotene levels of the three groups were not significantly different (Kruskal-Wallis test, a = .05).

Fig.4 Pre-loading levels of beta-carotene in two samples of exfoliated oral mucosa cells from participants in the short-term loading study. Each point represents one sample of exfoliated oral mucosa cells. Sample number 1 represents one sample from each participant. Sample number 2 represents a second pre-loading sample from 18 of 23 individuals who contributed sample number 1. Sample number 2 was collected three days after sample number 1. Bar = median.

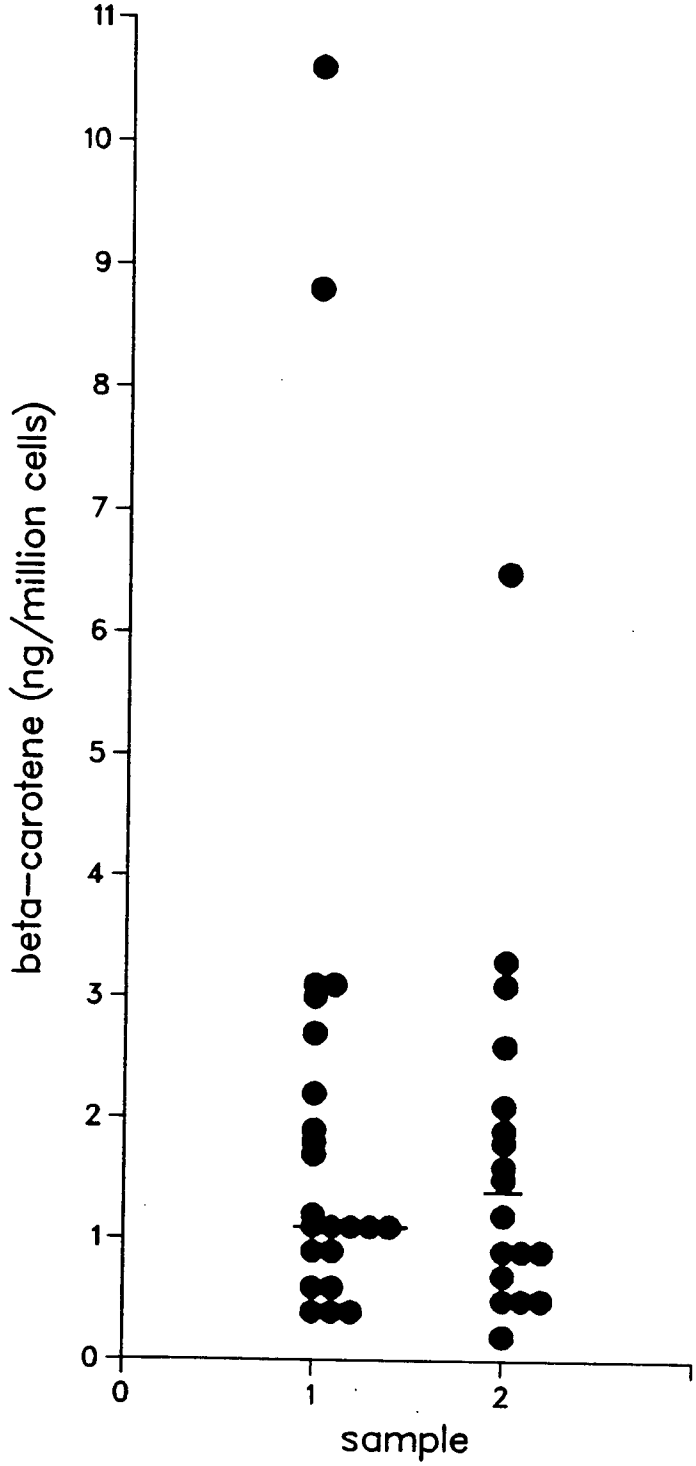

Fig.4

Fig.5 Post-loading beta-carotene levels in exfoliated oral mucosa cells from individuals after they had ingested a short-term dose of beta-carotene. Individuals received 360 mg, 180 mg, or 90 mg of beta-carotene in a four-day period. Markers are offset at each time-point for illustration purposes only; dots = medians, bars = interquartile ranges, of 4 - 9 individuals. Dashed range for 90 mg group on week 3: data from three individuals.

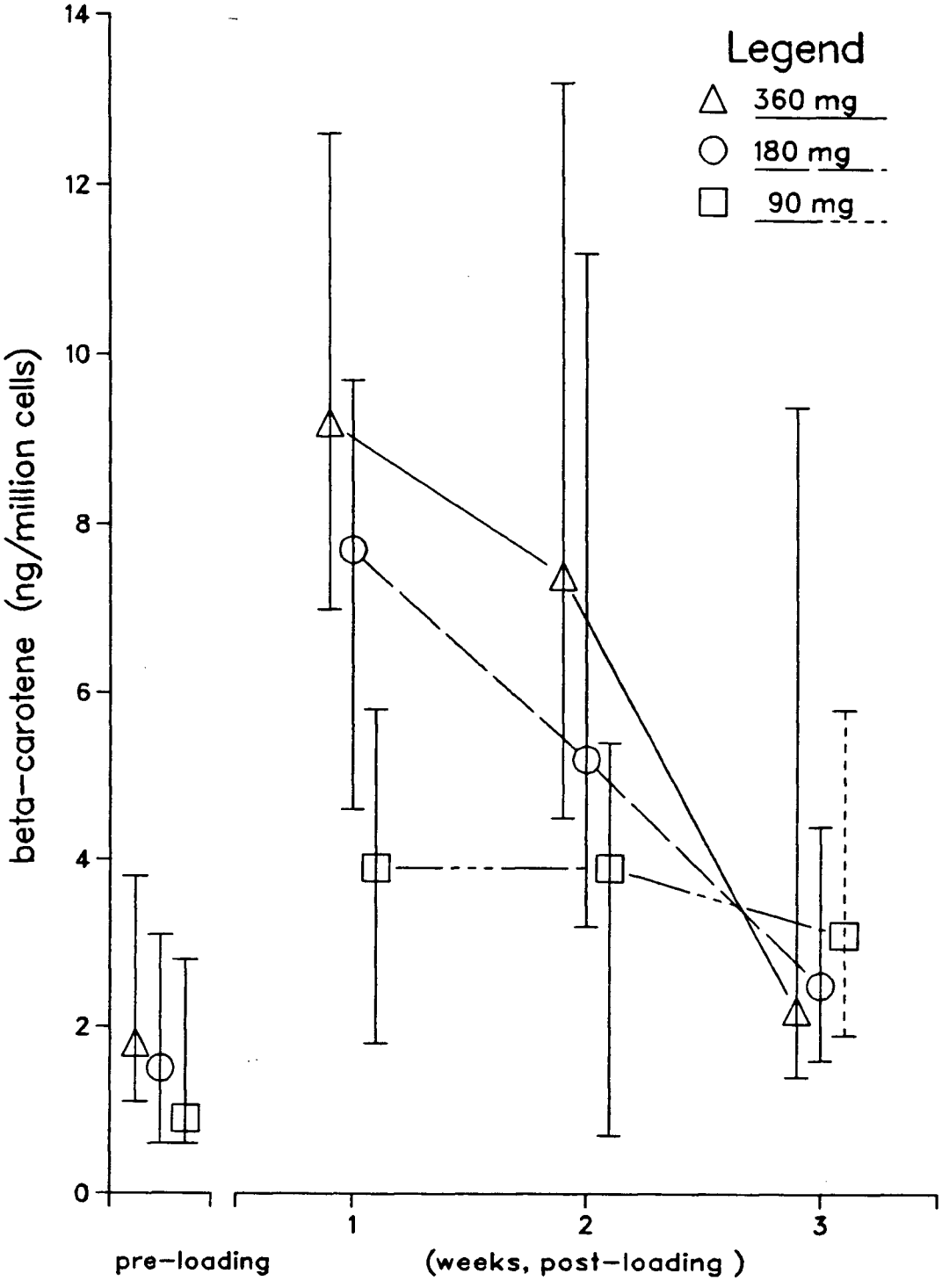
Legend
360 mg
180 mg
90 mg
beta-carotene (ng/million cells)
14
12
10
8
6
4
2
0
1
2
3
pre-loading
(weeks, post-loading)

Fig.5

4.4 Maximum beta-carotene levels in response to loading

Maximum beta-carotene levels were determined for the 17 individuals who had provided cell samples on both week 1 and week 2 in the post-loading period.

The median of maximum beta-carotene levels after the 360 mg dose was approximately three-fold greater, and the median of maximum levels following the 180 mg dose approximately two and one-half-fold greater, than the median of maximum levels after the 90 mg dose (Figure 6, Table 12).

The maximum post-loading level was observed one week after the loading, for 12 of the 17 individuals, and two weeks after the loading for the other 5 individuals.

When post-loading beta-carotene was related to the pre-loading level (Figure 7), the three doses didn't differ significantly in their effects, at week 1, 2, or 3.

5. Long-term (Four-week) Beta-carotene Loading of Uro-genital Tract Cells and Exfoliated Oral Mucosa Cells

5.1 Study design

The four-week loading study was designed with the following two objectives: 1) to determine whether uro-genital tract cells will accumulate beta-carotene after a four-week supplementation period, and 2) to determine the kinetics of beta-carotene accumulation and retention in these cells. As a control for changes in beta-carotene level, oral mucosa cells, previously shown to accumulate beta-carotene in response to supplementation, were donated concurrently by supplemented individuals. The dose chosen was that known to produce a substantial increase in cell beta-carotene levels in high-risk, carcinogen-exposed populations and to result in a chemopreventive effect (reduced micronuclei frequency) in the study populations (Stich et al. 1984, 1985, 1986b).

Fig.6 Pre-loading and maximum levels of beta-carotene in exfoliated oral mucosa cells from three groups of individuals who received different short-term doses of beta-carotene. Each point represents one cell sample from one of 17 individuals who provided samples on both weeks 1 and 2 after the loading; a = pre-loading beta-carotene, b = maximum beta-carotene; bars = medians, of 5 - 7 individuals.

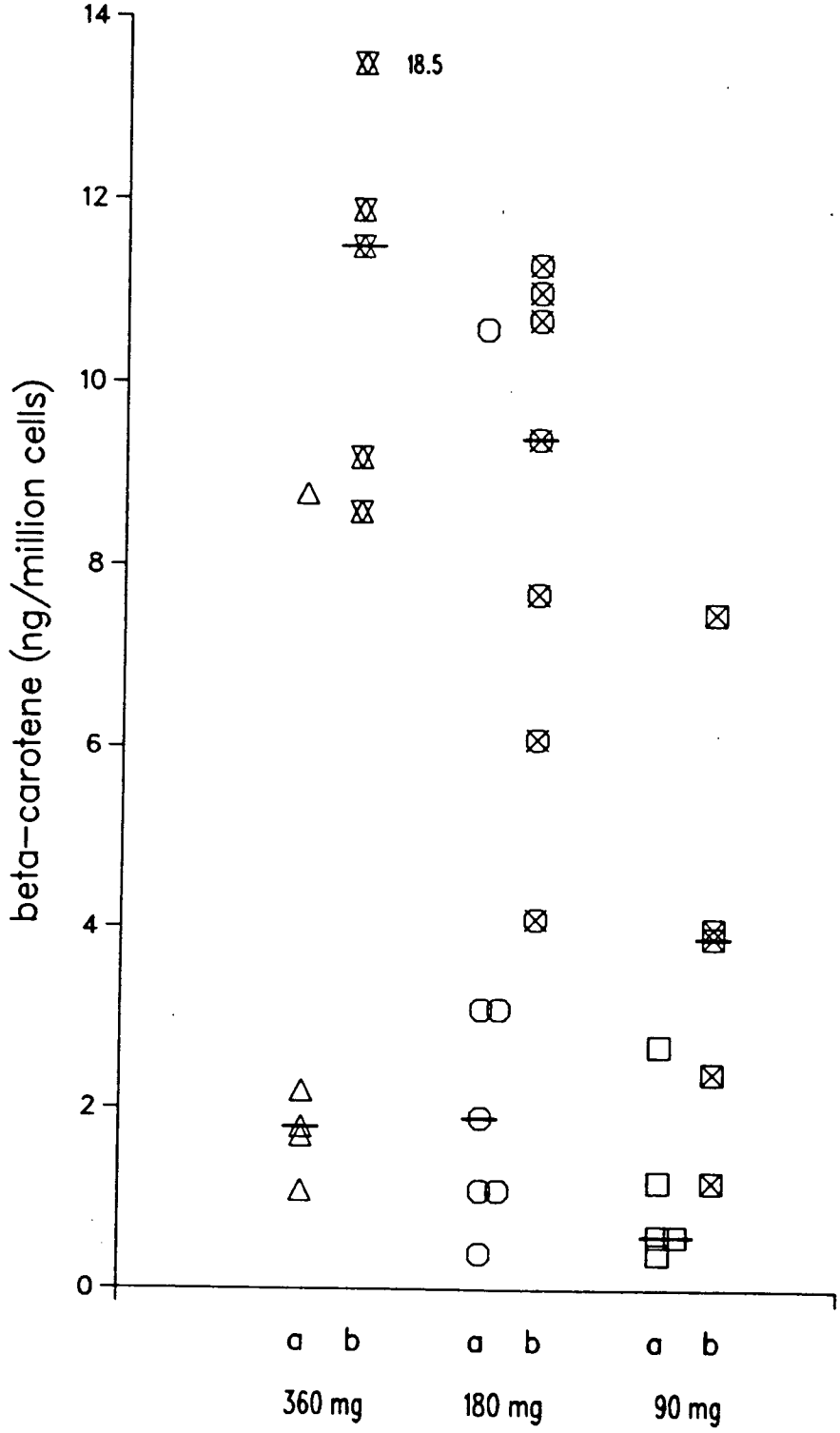
14
12
10
8
6
4
2
0
18.5
beta-carotene (ng/million cells)
a b
a b
a b
360 mg
180 mg
90 mg

Fig.6

TABLE 12

MAXIMUM BETA-CAROTENE COMPARED TO PRE-LOADING BETA-CAROTENE LEVELS IN EXFOLIATED ORAL MUCOSA CELLS FROM HUMAN SUBJECTS AFTER SHORT-TERM SUPPLEMENTATION WITH THREE DOSES OF BETA-CAROTENE

Dose (mg)	n	Beta-carotene ($ng/10^6$ cells)			
		Samples	Mean (SD)	Median	(Inter-quartile range)
360	5	a[1] 1.7, 2.2, 1.8, 1.1, 8.8	3.1 (3.2)	1.8	(1.4-3.3)
		b 9.2, 11.9, 8.6, 11.5, 18.5	11.9 (3.9)	11.5	(8.9-15.2)
180	7	a 1.1, 1.9, 0.4, 3.1, 1.1, 3.1, 10.6	3.0 (3.5)	1.9	(1.1-3.1)
		b 6.1, 11.3, 11.0, 9.4, 7.7, 4.1, 10.7	8.6 (2.7)	9.4	(6.1-11.0)
90	5	a 1.2, 0.4, 0.6, 2.7, 0.6	1.1 (0.9)	0.6	(0.5-2.0)
		b 4.0, 1.2, 3.9, 7.5, 2.4	3.8 (2.4)	3.9	(1.8-5.8)

[1]a = pre-loading, b = maximum.

Fig.7 Post-loading beta-carotene as a percent of pre-loading beta-carotene level in exfoliated oral mucosa cells from individuals who ingested a short-term dose of beta-carotene. Dots = medians, bars = interquartile ranges, for 4 - 9 individuals. Dashed bars for 90 mg group on week 3: data from three individuals.

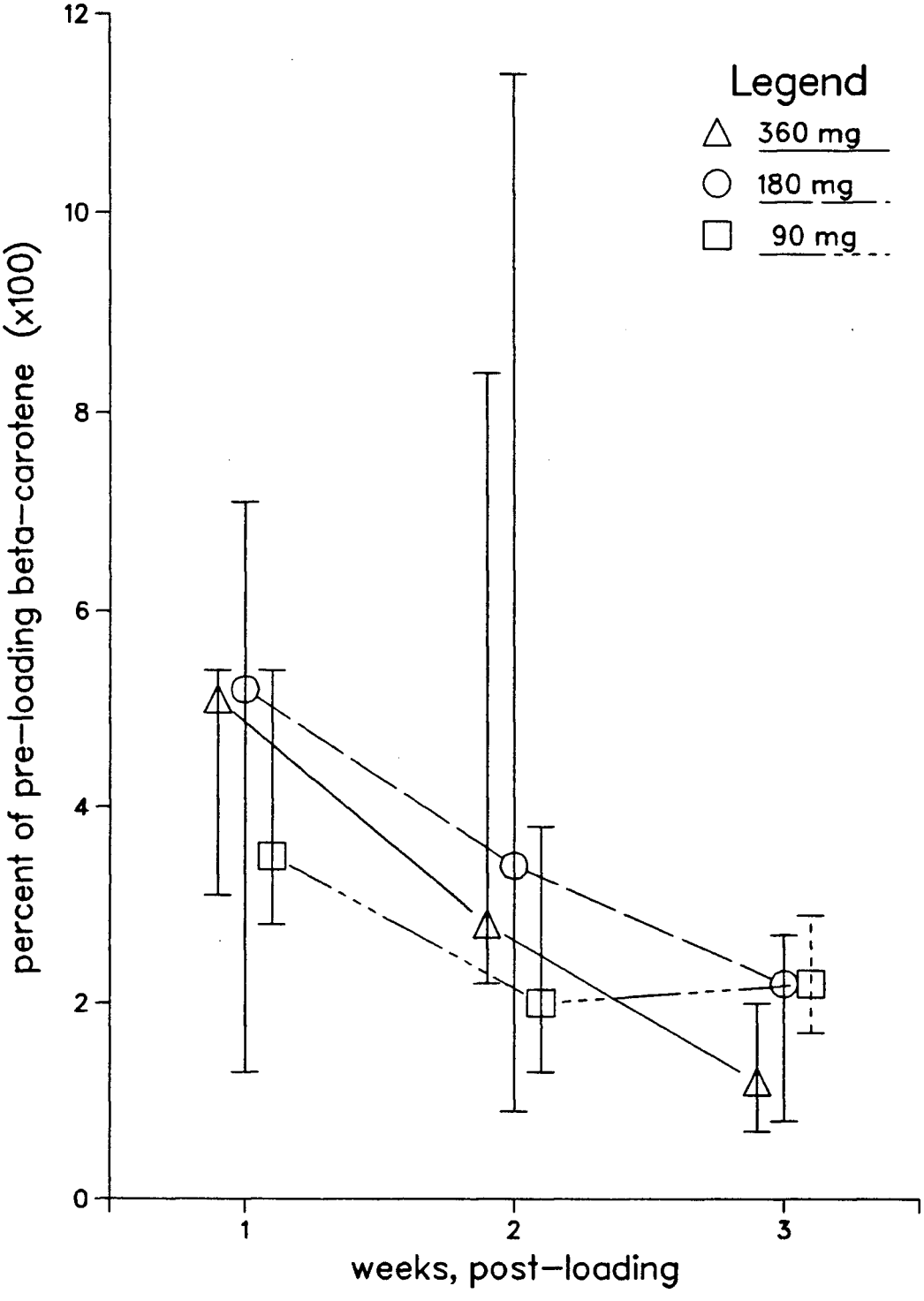
Legend
360 mg
180 mg
90 mg
percent of pre-loading beta-carotene (x100)
0
2
4
6
8
10
12
1
2
3
weeks, post-loading

Fig.7

Eighteen student nurses from the Vancouver General Hospital School of Nursing, of whom 17 completed the study, were randomly assigned to receive either beta-carotene or placebo supplements. Individuals received either 3 capsules of beta-carotene (30 mg/capsule, equivalent to 50,000 IU vitamin A) twice weekly (total dose 180 mg/wk), or 3 capsules of dextrose placebo, twice weekly, for a four-week period. Supplements were distributed between 9 a.m. and 12 p.m.

The schedule of supplementation of participants and collection of samples is shown in Figure 8. Individuals donated samples of exfoliated oral mucosa cells and samples of uro-genital tract cells at the start of the loading period and after 10 or 14 days and 17 or 21 days of supplementation. After the last day of supplementation, individuals donated cell samples on days 3, 7, 10, 14, 21, and 28 or 31 of the post-loading period.

Data were summarized by determining medians and inter-quartile ranges of beta-carotene levels.

5.2 Pre-loading beta-carotene levels

Median beta-carotene level in pre-loading samples of oral mucosa cells from all participants was 1.6 ng/10^6 cells (interquartile range 0.7 - 2.6 ng/10^6 cells). The median beta-carotene level in uro-genital tract cells was 0.6 ng/10^6 cells (interquartile range 0.2 - 0.7 ng/10^6 cells). These levels were comparable to the beta-carotene levels seen in the larger study population of females from both the School of Nursing and the Cancer Research Center (see sections 3.1 - 3.3).

Pre-loading beta-carotene levels in oral mucosa cells, shown in Figure 9, were not significantly different between the beta-carotene group and the group receiving placebo (p=.59, nonparametric Mann-Whitney U test, a=.05). Similarly, pre-loading beta-carotene levels in uro-genital tract cells, shown in Figure 10, were not significantly different between the two groups (p=.20, Mann-Whitney U test, a=.05).

Fig.8 Sampling and supplementation schedule of the long-term (four-week) beta-carotene loading study. Seventeen individuals ingested beta-carotene (180 mg/wk) or placebo supplements twice weekly for four weeks. Samples of exfoliated uro-genital tract cells and exfoliated oral mucosa cells were collected before the loading, during the loading period, and, beginning three days after the last day of supplementation, for four weeks after the loading.

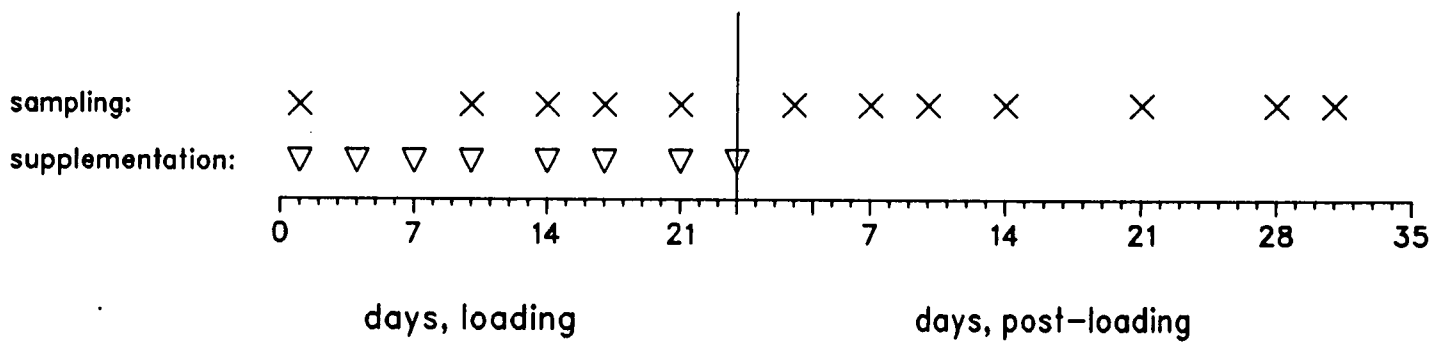
sampling:
supplementation:
0
7
14
21
7
14
21
28
35
days, loading
days, post-loading

Fig.8

Fig.9 Pre-loading and maximum post-loading beta-carotene in exfoliated oral mucosa cells from participants who ingested supplements for four weeks. a = pre-loading beta-carotene, b = maximum post-loading beta-carotene; bars = medians, of 7 (placebo) and 10 (beta-carotene) individuals. * Beta-carotene one month before the start of loading.

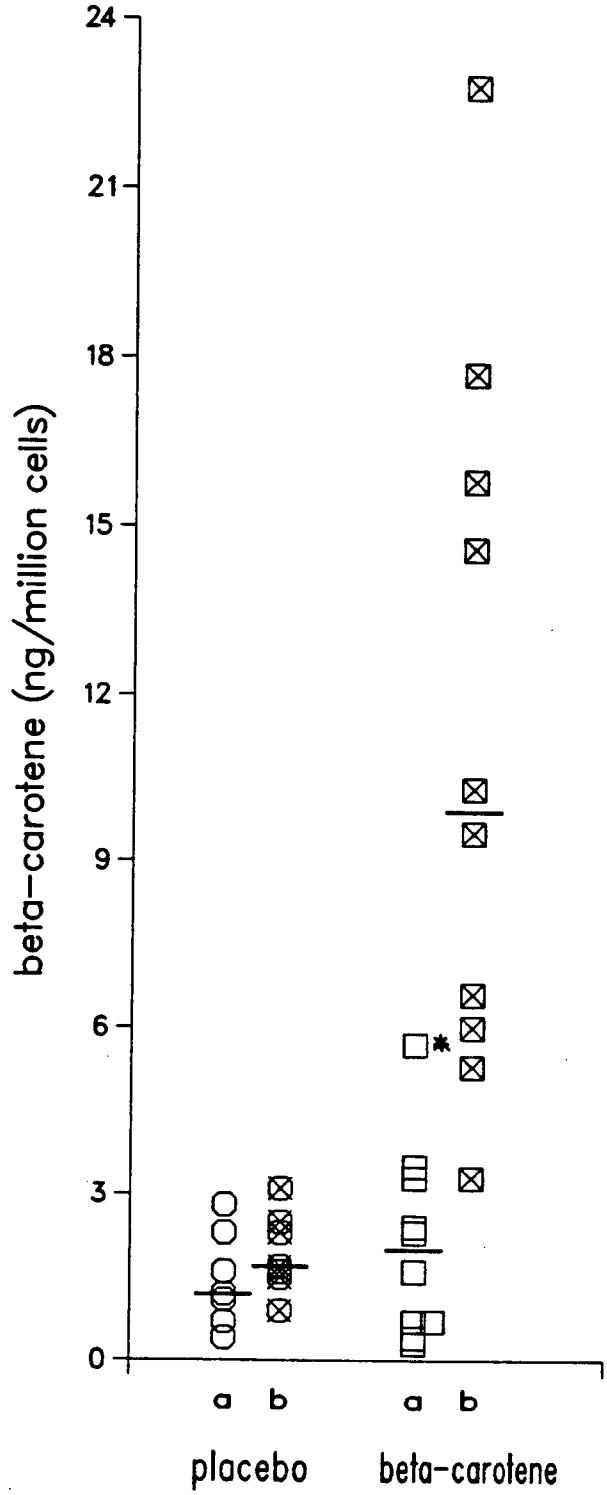
24
21
18
15
12
9
6
3
0
beta-carotene (ng/million cells)
a
b
a
b
placebo
beta-carotene

Fig.9

Fig.10 Pre-loading and maximum post-loading beta-carotene in exfoliated uro-genital tract cells from individuals who ingested supplements for four weeks. a = pre-loading beta-carotene, b = maximum beta-carotene; bars = medians, of 6 and 9 individuals. Two maxima, 0.3 ng/10^6 cells (in the placebo group) and 2.7 ng/10^6 cells (in the beta-carotene group), were not included, because pre-loading data from the two individuals were not available. For one person in the placebo group and four persons in the beta-carotene group the first available uro-genital tract cell samples were obtained on days 10 - 14 of the loading period.

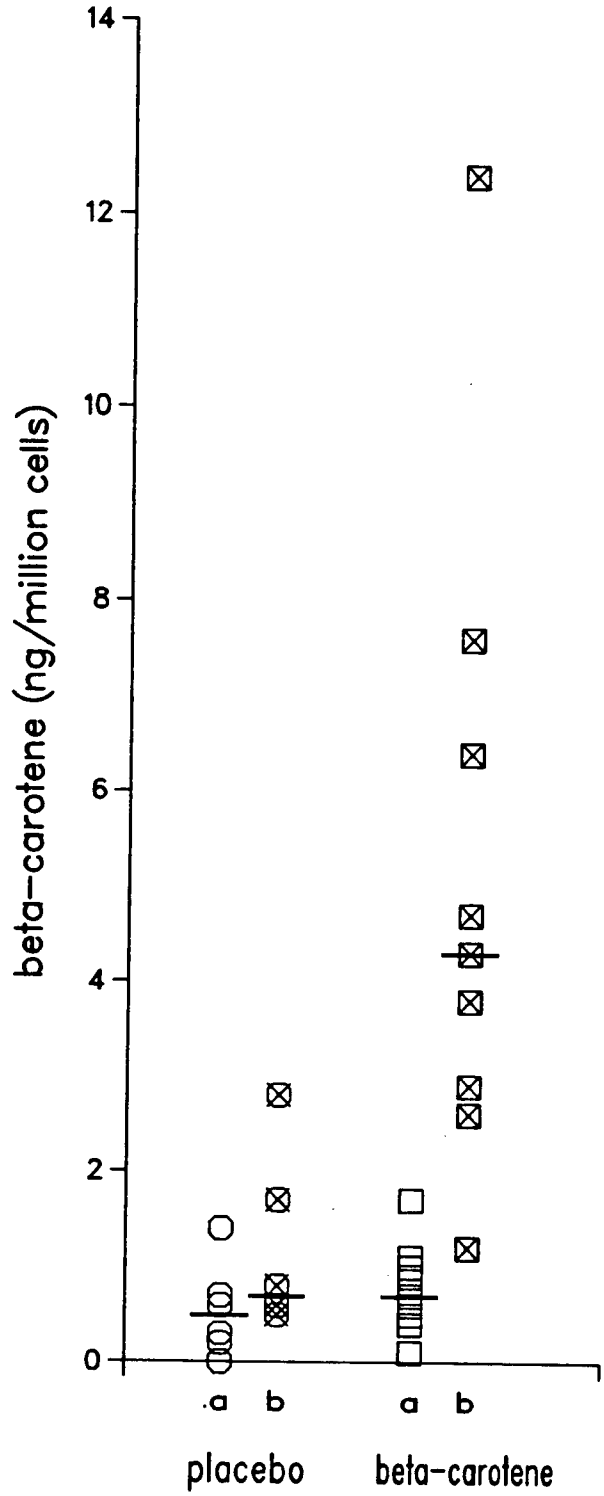
14
12
10
8
6
4
2
0
beta-carotene (ng/million cells)
a
b
a
b
placebo
beta-carotene

Fig.10

5.3 Beta-carotene levels in response to loading

Beta-carotene in oral mucosa cells from the beta-carotene group rose to higher levels than those in the placebo group on days 10 - 14 of the loading period, and remained higher on days 3, 7, 10, 14, and 21 of the post-loading period (Figure 11); the differences were significant ($p<.05$) on all of those occasions (nonparametric Mann-Whitney U test, at $a=.05$). In the post-loading period, median beta-carotene level in oral mucosa cells in the group that received beta-carotene ranged from approximately five-fold greater, on day 3 post-loading, to approximately three and one-half-fold greater, on days 28 - 31 post-loading, than the median beta-carotene level in the group that received placebo. Pre-loading levels and levels on days 28 - 31 post-loading were not significantly different between the two groups ($p=.59$ and $p=.17$) . On days 17 - 21 of the loading, beta-carotene levels in oral mucosa cells from three beta-carotene-supplemented individuals were higher than their day 10 - 14 levels; however, more data would be necessary to determine the median beta-carotene level for the group at that time.

On days 17 - 21 during the loading period was the only occasion, during or after the loading, when beta-carotene levels in uro-genital tract cells were significantly different between the two groups ($p<.05$, nonparametric Mann-Whitney U test, $a=.05$) (Figure 12). On that occasion, the median beta-carotene level in those cells in individuals taking beta-carotene supplements was approximately four-fold higher than the median level in the placebo group.

5.4 Maximum beta-carotene levels

The median of maximum beta-carotene levels in oral mucosa cells (in the post-loading period) was approximately five-fold greater in beta-carotene-supplemented individuals than in placebo-supplemented individuals (Figure 9). Furthermore, for the beta-carotene group,

Fig.11 Loading and post-loading levels of beta-carotene in exfoliated oral mucosa cells from individuals who ingested beta-carotene supplements for four weeks. Dots = medians, bars = interquartile ranges, of data from 4 - 10 individuals. At every time-point between the start of loading and 28 days after the loading, cell beta-carotene levels were significantly greater from beta-carotene-supplemented individuals than from placebo-supplemented individuals ($p<.05$, $a=.05$).

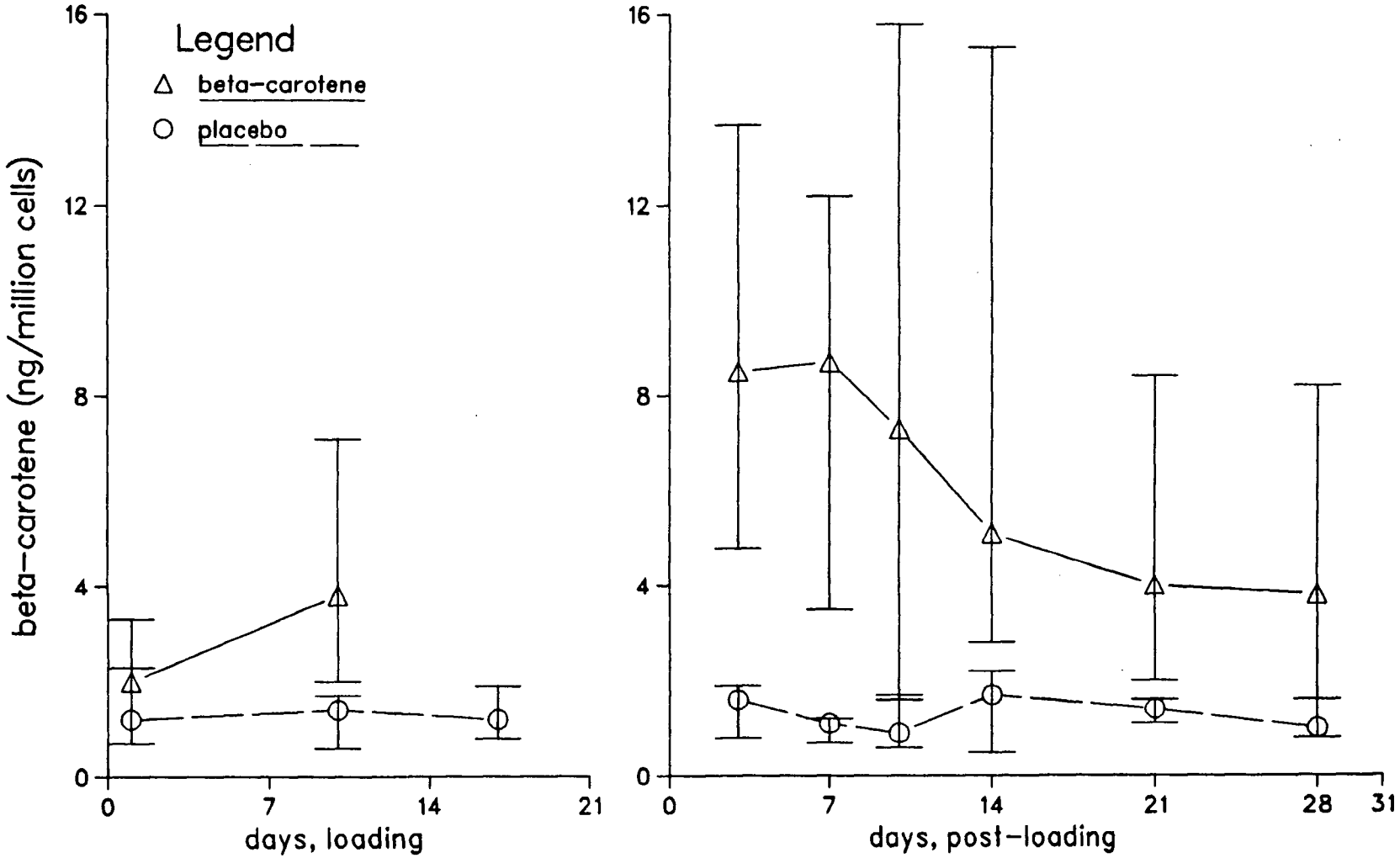
Legend
beta-carotene
placebo
beta-carotene (ng/million cells)
16
12
8
4
0
0
7
14
21
days, loading
16
12
8
4
0
0
7
14
21
28
31
days, post-loading

Fig.11

Fig.12 Loading and post-loading levels of beta-carotene in uro-genital tract cells during and after a four-week supplementation period. Dots = medians, bars = interquartile ranges, of data from 5 - 10 individuals. Beta-carotene-supplemented individuals showed significantly higher beta-carotene levels than those of the placebo group on days 17 - 21 of the loading period ($p<.05$, $a=.05$).

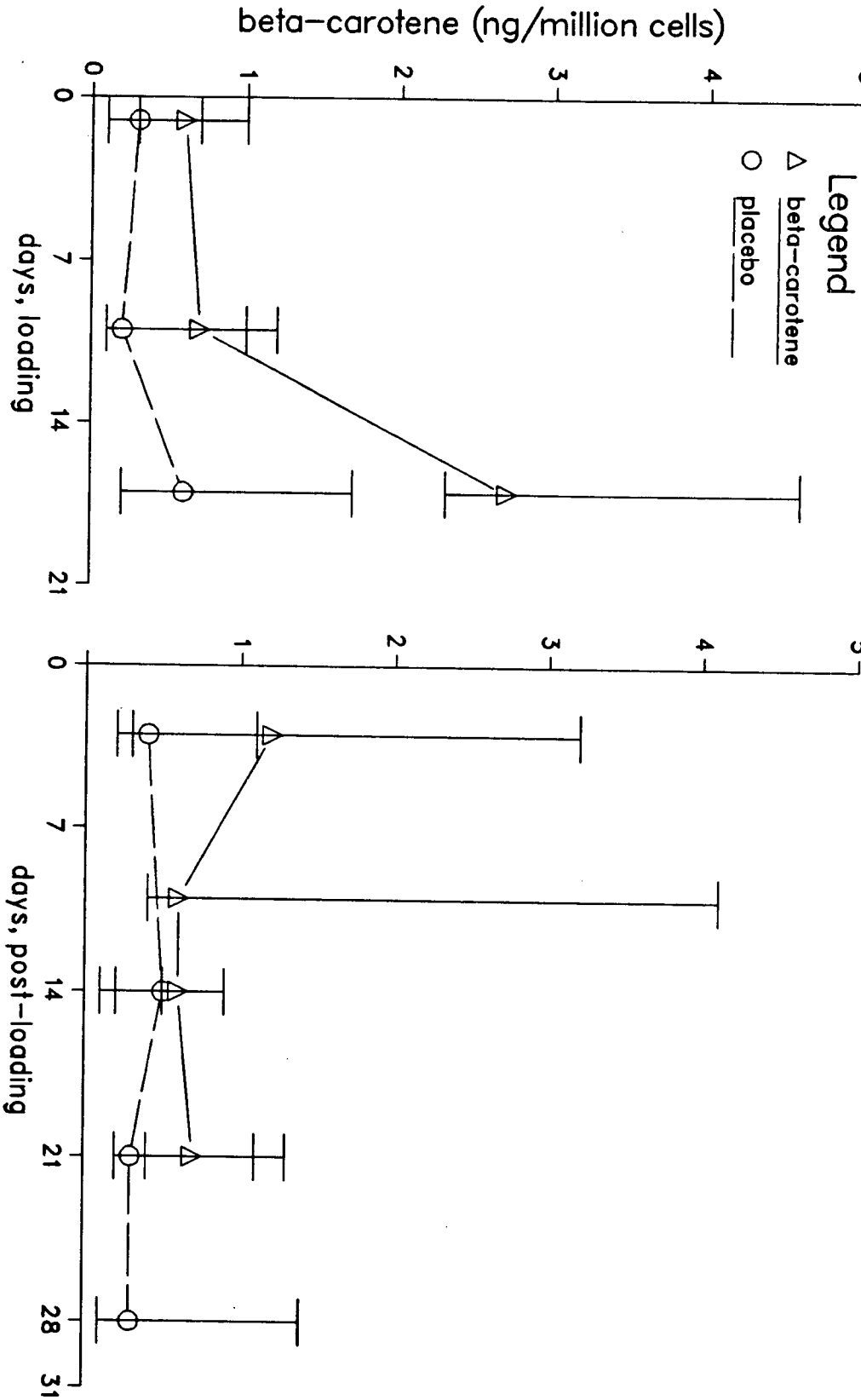
beta-carotene (ng/million cells)
0
1
2
3
4
5
Legend
beta-carotene
placebo
0
7
14
21
days, loading
0
7
14
21
28
31
days, post-loading

Fig.12

the median of the maximum level showed a five-fold increase over the median pre-loading level; in the placebo group this difference was one-half-fold.

The median of maximum beta-carotene levels in uro-genital tract cells (during or after the loading period) in the beta-carotene-supplemented group was approximately six-fold greater than the median of those in the placebo-supplemented group (Figure 10). The same fold difference in medians was seen between maximum levels and pre-loading levels in the provitamin group, whereas the difference was one-half-fold for the placebo group.

Maximum levels of beta-carotene in the two cell types from each individual are shown in Figure 15.

Lower pre-loading levels of beta-carotene, in oral mucosa cells from beta-carotene-supplemented individuals, were associated with greater fold increases to the maximum (Table 13). For uro-genital tract cells, pre-loading beta-carotene levels and the fold increase to maximum levels appeared to be unrelated (Table 14). When the two sites for each individual were compared (Table 15), the fold increase to maximum beta-carotene level in the two types of cell appeared to be somewhat related. For half of the individuals in the beta-carotene group, oral mucosa cell level increased to a greater extent than did uro-genital tract cell level; for the other half of the group the contrary was true.

5.5 Post-loading beta-carotene levels as a percent of maximum levels: Retention

In order to estimate the half-life of beta-carotene in oral mucosa cells in subjects who ingested beta-carotene, post-loading beta-carotene levels were expressed as a percent of maximum levels (Figure 13). Beta-carotene was 50% or less of the maximum level in approximately 25% of individuals, ten days after the end of the loading (determined by the lower limit of the interquartile range); this was the case for 50% of individuals approximately 21 days after the loading. The beta-carotene level in oral mucosa cells declined from the maximum to 50% of maximum in 7 to 10 days, when subjects were considered individually.

TABLE 13

RELATION OF FOLD-INCREASE-TO-MAXIMUM-BETA-CAROTENE-LEVEL WITH PRE-LOADING LEVEL, IN EXFOLIATED ORAL MUCOSA CELLS FROM INDIVIDUALS SUPPLEMENTED FOR FOUR WEEKS

Beta-carotene-supplemented individuals			Placebo-supplemented individuals		
Pre-loading beta-carotene ($ng/10^6$ cells)	Subject	Fold increase to maximum	Pre-loading beta-carotene ($ng/10^6$ cells)	Subject	Fold increase to maximum
<1.0	A01	11.0	< 1.0	B18	3.8
	B08	13.6		B23	1.3
	B09	13.2			
	B21	8.6			
1.0 - 2.0	B20	14.2	1.0 - 2.0	B24	2.3
				B01	1.3
				A05	1.1
> 2.0	A03	4.5	> 2.0	B19	1.0
	A12	4.4		A08	1.1
	B22	2.8			
	B25	4.5			
	L08	3.1			

TABLE 14

LACK OF A RELATION OF FOLD-INCREASE-TO-MAXIMUM, WITH PRE LOADING LEVEL, OF BETA-CAROTENE IN URO-GENITAL TRACT CELLS DURING AND AFTER A FOUR-WEEK LOADING PERIOD

Beta-carotene group			Placebo group		
Pre-loading beta-carotene ($ng/10^6$ cells)	Subject	Fold increase to maximum	Pre-loading beta-carotene ($ng/10^6$ cells)	Subject	Fold increase to maximum
< 0.5	A03	2.4	< 0.5	B18	1.7
	B20[1]	7.3		B24	8.5
	B22	47[2]		B23	( )[3]
0.6 - 1.0	A01	4.3	0.6 - 1.0	A05	1.1
	B25[1]	5.4		B01	1.0
	B21	4.3			
> 1.0	A12[1]	5.8	> 1.0	B19[1]	2.0
	B09	8.4			
	L08[1]	7.3			

[1]First sample collected on day 10 - 14 of loading.

[2]B22: pre-loading, 0.1, maximum, 4.7, $ng/10^6$ cells.

[3]B23: pre-loading, ND (none detected), maximum, 0.6, $ng/10^6$ cells.

TABLE 15

COMPARISON OF FOLD-INCREASE-TO-MAXIMUM-BETA-CAROTENE IN THE TWO EXAMINED SITES IN SUPPLEMENTED INDIVIDUALS

Supplement	Subject	Fold increase to maximum beta-carotene	
		Oral mucosa cells	Uro-genital tract cells
Beta-carotene	B22	2.8	47[1]
	L08	3.1	7.3
	A12	4.4	5.8
	B25	4.5	5.4
	A03	4.5	2.4
	B21	8.6	4.3
	A01	11.0	4.3
	B09	13.2	8.4
	B08	13.6	- [2]
	B20	14.2	7.3
Placebo	B19	1.0	2.0
	A05	1.1	1.1
	A08	1.1	- [2]
	B01	1.3	1.0
	B23	1.3	- [2]
	B24	2.3	8.5[3]
	B18	3.8	1.7

[1]Pre-loading, 0.1, maximum, 4.7, ng/10^6 cells.

[2]No beta-carotene detected in pre-loading sample (B23), or no pre-loading sample (B08, A08).

[3]Pre-loading, 0.2, maximum, 1.7 ng/10^6 cells.

Fig.13 Beta-carotene in the post-loading period as a percent of maximum beta-carotene, in exfoliated cell samples from two tissues from individuals who ingested beta-carotene supplements for four weeks. Dots = medians, bars = interquartile ranges, of data from 7 - 10 individuals (oral cells) and 5 - 10 individuals (uro-genital tract cells).

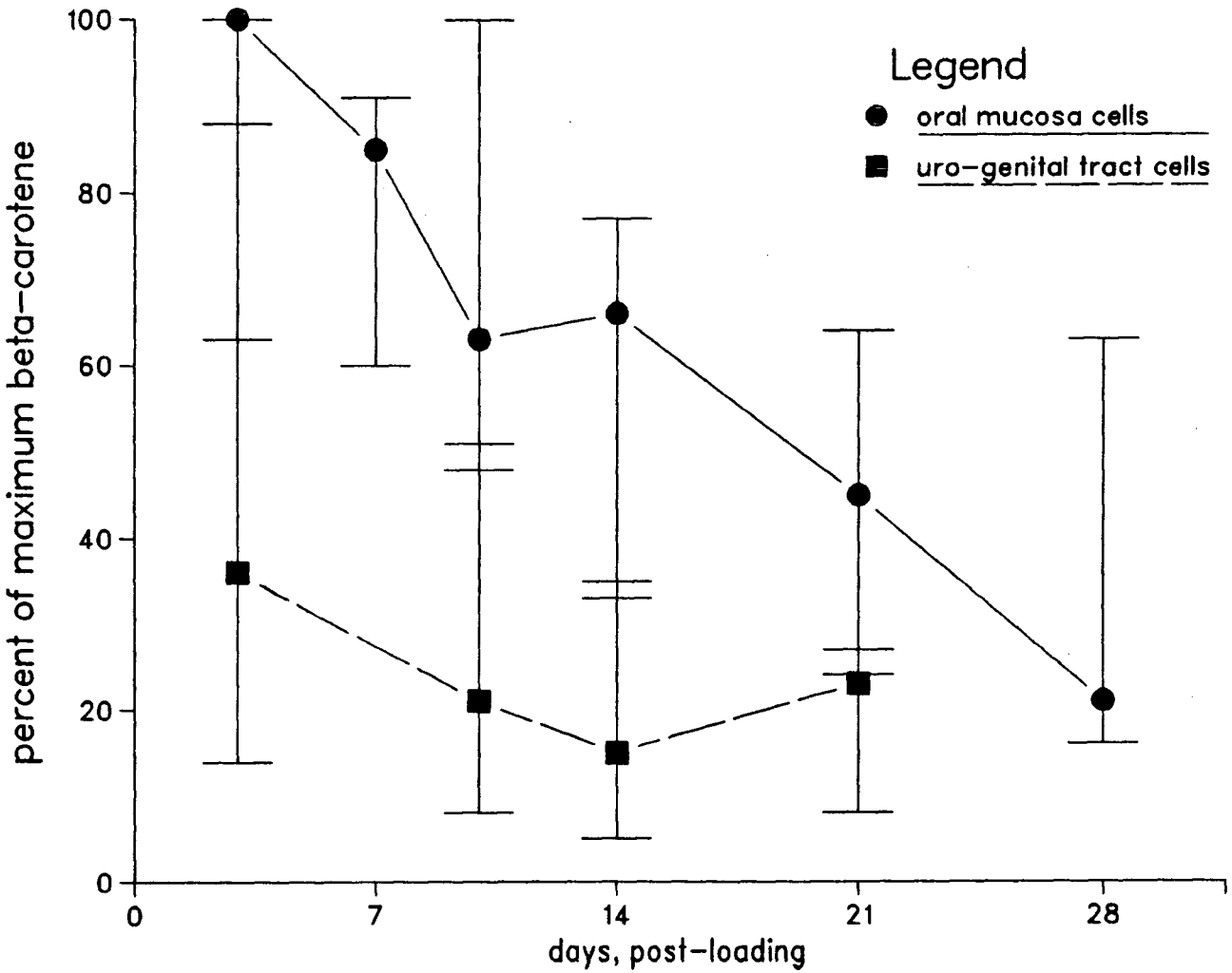
Legend
oral mucosa cells
uro-genital tract cells
percent of maximum beta-carotene
100
80
60
40
20
0
0
7
14
21
28
days, post-loading

Fig.13

Maximum beta-carotene levels in uro-genital tract cells of beta-carotene-supplemented individuals occurred during the loading period for several subjects, hence post-loading levels relative to the maximum levels in these cells tend to be low (Figure 13). Beta-carotene level in uro-genital tract cells was 50% or less of the maximum level, ten days after the loading, in 75% of individuals.

5.6 Post-loading beta-carotene levels relative to pre-loading levels

Seven days after the end of the loading the median beta-carotene level in oral mucosa cells, for the beta-carotene supplementd group was 5.4 times the pre-loading level, and from there it declined to 1.7 times the pre-loading level, on days 28 - 31 post-loading (Figure 14). A similar relationship between the medians was observed during the post-loading period, when the beta-carotene group was compared to the placebo group (Figure 11).

Three days after the end of the loading, median beta-carotene level in uro-genital tract cells was three times the pre-loading level, but declined quickly to equivalence with pre-loading level by day 10 post-loading. These findings are in agreement with the relationship that was observed between uro-genital tract cell levels in the beta-carotene group and in the placebo group (Figure 12).

Fig.14 Beta-carotene in exfoliated cells from beta-carotene-supplemented individuals after the four-week loading period, as a percent of pre-loading beta-carotene. Dots = medians, bars = interquartile ranges, of data from 7 - 10 individuals (oral cells) and 5 - 10 individuals (uro-genital tract cells).

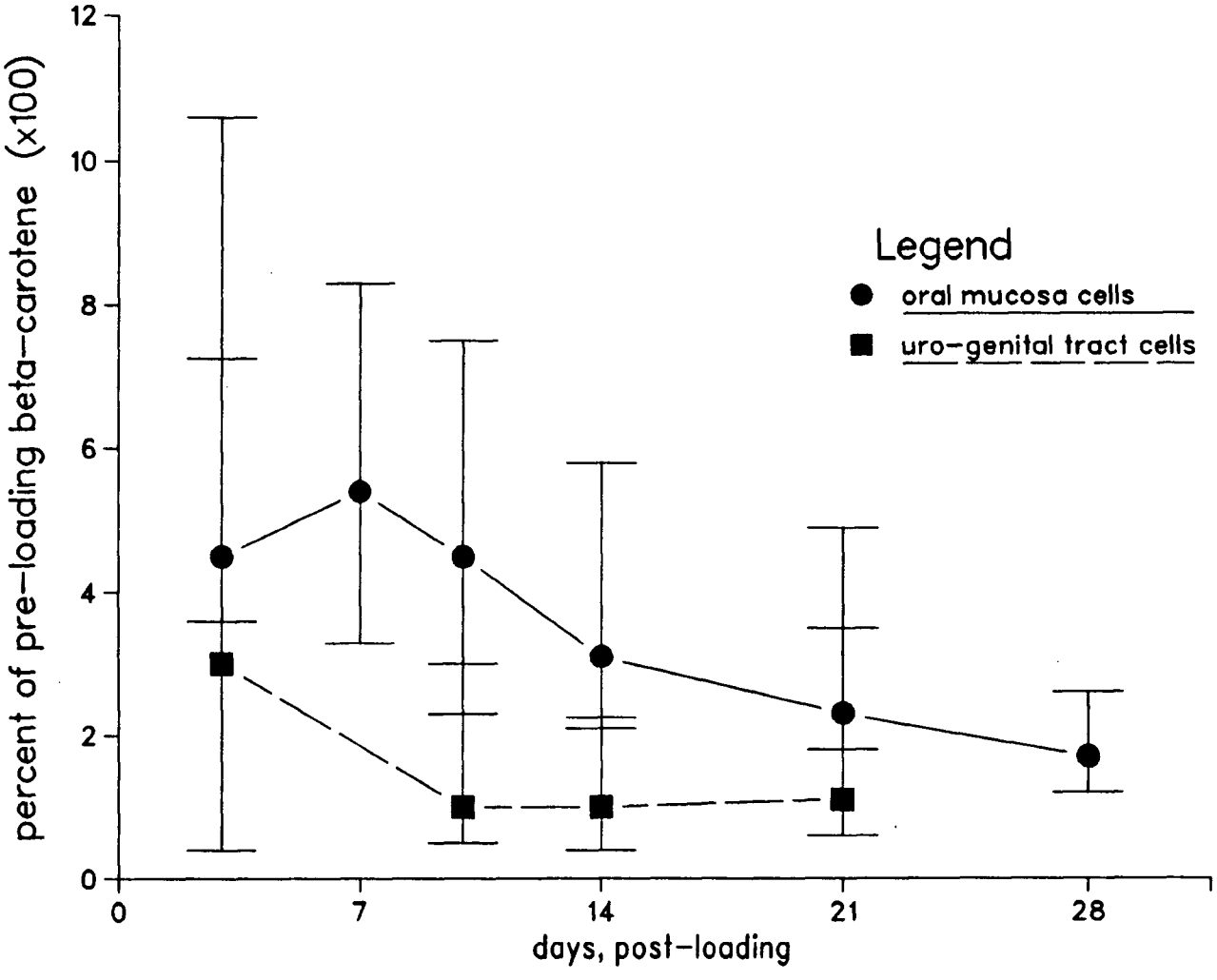
percent of pre-loading beta-carotene (x100)
12
10
8
6
4
2
0
0
7
14
21
28
days, post-loading
Legend
oral mucosa cells
uro-genital tract cells

Fig.14

Fig.15 Maximum measured beta-carotene levels in exfoliated cells from two sites, from individuals who received beta-carotene supplements for four weeks. Maximum beta-carotene levels in urogenital tract cell samples occurred during or after the supplementation. Maximum levels, in oral mucosa cell samples, which occurred after the end of the supplementation, are shown.

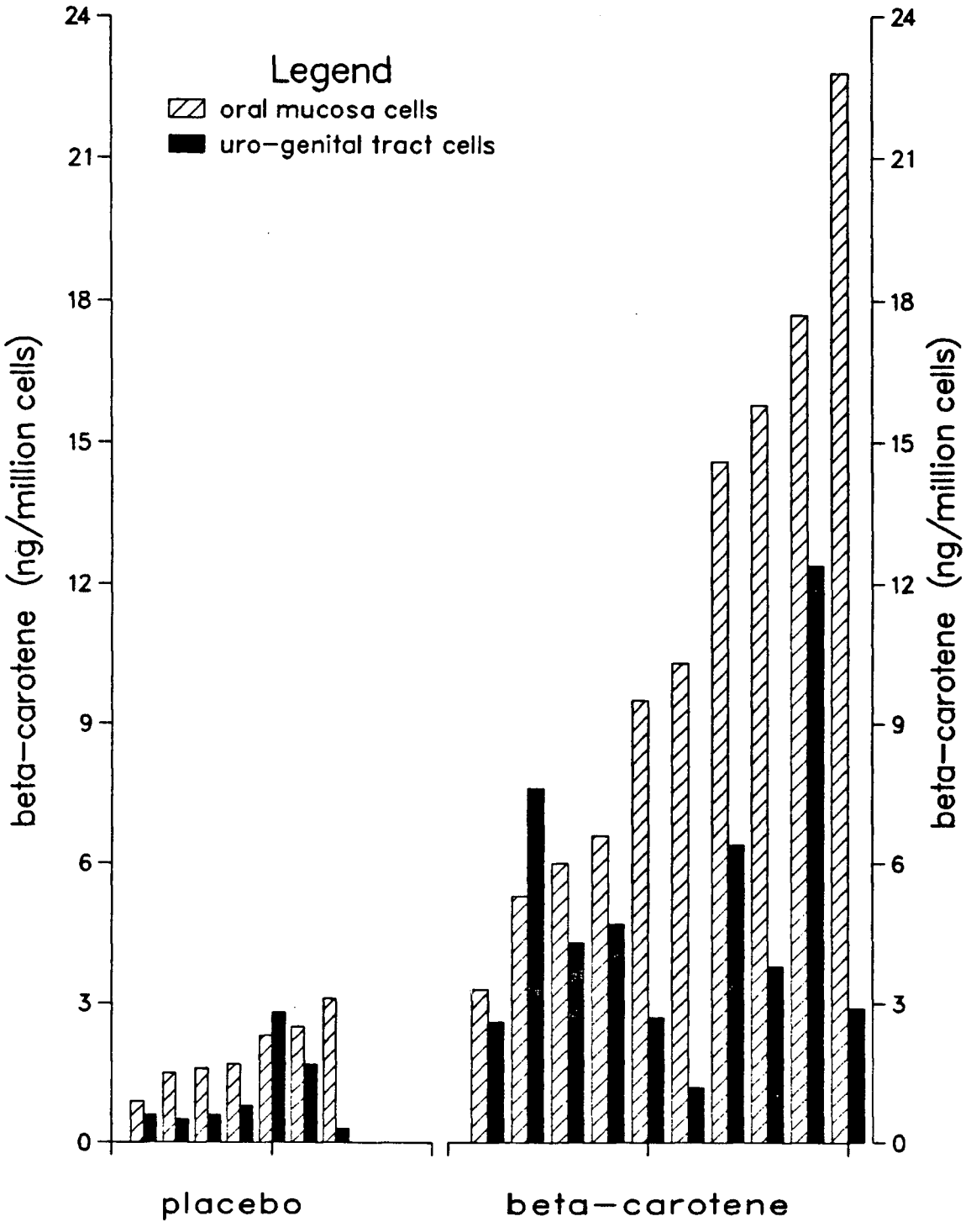
Legend
oral mucosa cells
uro-genital tract cells
beta-carotene (ng/million cells)
24
21
18
15
12
9
6
3
0
placebo
beta-carotene

Fig.15

DISCUSSION

This study represents the first time that beta-carotene levels have been determined in uro-genital tract cells. Not only was beta-carotene detectable, but level of beta-carotene in uro-genital tract cells showed a distinctive profile in response to supplementation.

1. Feasibility of Measuring Beta-carotene in Uro-genital Tract Cells

The primary requirement for detection of beta-carotene in cells from the urine was that the sample contain enough cells. Designating approximately 0.5×10^6 as the minimum number of cells for an acceptable sample ensured that beta-carotene was detected in most samples and that the reliability of the cell counting was acceptable. To collect this number of cells it was necessary to collect an adequate volume of fresh urine; to this end, participants were asked to collect urine on consecutive days and to collect morning voids. The benefit of this was two-fold: the morning void has a low volume-to-cells ratio, and the morning was the most convenient time for subjects to collect the sample, resulting in better compliance. Collection of urine voids throughout the day could also have provided enough cells for analysis, had it been possible for subjects to comply with this request.

From the outset, then, the two kinds of cell samples differed in a temporal aspect: the oral mucosa cell sample could be obtained at once, whereas the uro-genital tract cell sample had to be obtained over two to three days.

2. Factors Affecting the Reproducibility of the Assay

Determination of cell number may have been the largest single source of uncertainty in the beta-carotene measurement, with both types of cell. The reproducibility of the assay

for beta-carotene was similar for the two cell types: CV=38% for uro-genital tract cells and 22% for oral mucosa cells. These figures probably reflect the inherent variation in the cell counting (CV = 33% - 13%) and weighing (CV = 9% - 4.5%) procedures for determining the number of cells in the sample (see Appendix B).

Minor technical details could have made the extraction of uro-genital tract cells not as precise, and the quantitation of beta-carotene not as complete, as that of oral mucosa cells. The one-hour pronase digestion, adequate for digestion of oral mucosa cell samples, may not have been long enough for digestion of some uro-genital tract cell samples of large bulk. The separation of layers after the hexane extraction of uro-genital tract cells was not as clean as with oral mucosa cell samples. Lastly, a hexane-soluble component in the extraction residue from uro-genital tract cells did not dissolve in ethanol; efforts were made to avoid injecting this into the HPLC column.

3. Steps in Sample Processing as Factors Affecting Variation in Beta-carotene Level

Unique features of each kind of cell sample, apart from anatomical and physiological considerations, may account for some of the intra-individual variation in beta-carotene level.

Diet may have affected cell beta-carotene in the time interval between collection of repeat samples, but probably to a small extent. Samples were collected with as little time between repeat samples as possible. Oral mucosa samples were collected at least three days apart, since that length of time allowed for the mucosal surface to be renewed. Intra-individual variation in oral cell beta-carotene was found to be slightly higher in samples collected one week apart than in samples collected three days apart, but the difference was not statistically significant. Uro-genital tract cell samples were collected roughly one week apart, to allow for the collection of consecutive days' samples. Two reports have suggested that recent diet may not be an important factor affecting blood carotene measurements. A

beta-carotene-rich meal (one that included three portions of carrots), taken by volunteers after they had been on a 10-day low-carotene dietary regime, led to a rise in plasma beta-carotene that was statistically significant only at five hours after the meal; the effect of a single portion of carrots in the meal was not significant (Jensen et al. 1986). Mejia and Arroyave (1983) observed that ingestion of a meal containing beta-carotene didn't dramatically affect blood beta-carotene levels in the succeeding four hours. The minor changes in tissue beta-carotene levels measured three days apart, in the present study, may be a reflection of minor changes in blood carotene level.

Brushing the oral mucosa would be expected to result in a sample containing less-differentiated cells than would be obtained from merely washing the mucosal surface. Therefore, the uro-genital tract cell sample may contain more-mature cells than the oral mucosa cell sample.

Exfoliated oral mucosa cells might be contaminated by food residues (therefore the sample was strained through cheesecloth); it is unlikely that uro-genital tract cells would be contaminated.

Although oral mucosa cells spent less time in the collection medium (water) than uro-genital tract cells spent in contact with urine, before they were frozen, prolonged contact of uro-genital tract cells with urine, from the time the samples were first received in the lab until 8 hours later, was found to have little effect on beta-carotene (Table 1).

There is the question of the effect of prolonged contact with urine on the integrity of the cells themselves. I observed no major visible changes in test samples until after more than 8 hours (visible changes being the appearance of an intermediate-density layer of cell debris, mucus threads, and occasional cells and crystals, when examined under the microscope), and no destruction of cells until after 12 - 24 hours . Both room temperature and time favour the destuction of cells found in urine because they accelerate bacterial action; the action of urea-splitting organisms leads to an increase in urine pH, which may destroy cells (Freeman and Beeler 1983). In all cases, I verified that cells were present in the aliqout by counting the

cells; however, any cells that were destroyed before the cell pellet was isolated would have gone undetected. In addition, an increased pH favours crystal formation; when the crystals can't be dissolved, it may lead to the sample being rejected. Urine pH was unlikely to have affected the extraction of beta-carotene from the pellet of isolated cells because, even to an 800 ul sample (cells plus supernatant) of pH7, for example, the addition of 200 ul 5% KOH in methanol (pH12) would raise the pH of the mixture to approximately 11, still satisfactory for saponification.

The urine samples could have been exposed to light before I received them. The effect of exposure to light is likely to have been small since 24 hours exposure to laboratory light didn't significantly affect beta-carotene in serum (Mathews-Roth and Stampfer 1984) nor does exposure to ambient light during one day significantly affect beta-carotene in exfoliated oral mucosa cells (our lab, unpublished observations). No effect on beta-carotene of repeated freezing and thawing of specimens was also found by both groups of investigators.

Pooling of uro-genital tract cells was a necessity. More often, more than one day's cells had to be combined to make a sample. Typically, the number of cells in the urine sample varied greatly from day to day. Because the yield of cells from a urine sample on any one day was unpredictable the decision was made to pool cells only when necessary, and not to pool when sufficient cells presented themselves in one day's sample.

The minor technical difficulties with the extraction of beta-carotene from uro-genital tract cells, already discussed, could also have lowered the yield of beta-carotene from uro-genital tract cells than what was possible to obtain from oral mucosa cells.

Finally, in the discussion of factors that could account for the difference in basal levels of beta-carotene in the two types of exfoliated cells, mention must be made of the relationship between cell mass, cell number, and beta-carotene measurement. Because of the difficulty of completely removing the supernatant from the centrifuged uro-genital tract cell specimen without losing cells, the wet weight of uro-genital tract cell specimens could not be

determined accurately, and to have related it to the number of cells counted would have been erroneous. The large, flat, squamous cells (20 - 50 u across [Spencer and Pedersen 1976]) from the two cell types appeared, under the microscope, approximately the same size. On that basis, I assumed that the relationship between cell mass and cell number for oral mucosa cells held for uro-genital tract cells as well. The level of beta-carotene in the two cell types could then be compared.

4. Factors Contributing to Inter-individual Variation in Beta-carotene

Intra- and inter-individual variation in level of beta-carotene in exfoliated cells may be due to differences in 1) dietary intake of beta-carotene, 2) the ability of the intestinal mucosa to absorb beta-carotene, 3) the capacity of lipoproteins in serum to transport beta-carotene, and 4) the capacity of tissues to store beta-carotene. Factors that probably affect the tissue level are the rate of accumulation of beta-carotene by major storage reservoirs such as adipose tissue, the rate of beta-carotene catabolism, and the rate of cell proliferation in the tissue.

4.1 Diet

In North America, available good sources of beta-carotene are, in decreasing order of beta-carotene content, carrots, spinach, dark-green leafy vegetables, and broccoli, of which carrots and lettuce (salad) were the items most-preferred by the study population. In North America about half the daily supply of vitamin A comes from carotenoids; for many people in the Third World almost all their vitamin A is supplied by carotenoids. In North America the rest of the vitamin A is supplied by preformed vitamin A from animal sources. Preformed vitamin A was obtained most frequently from milk, and secondarily from meat

and eggs, by this population. Only a small percentage of individuals in a typical North American population ingest more than the RDA of vitamin A daily.

The juice from one 100 g carrot contains approximately 2.5 mg beta-carotene (our lab, unpublished data); one portion of carrots weighing 69.1 g was reported to contain 8 mg of beta-carotene (Jensen et al. 1986). A meal that included one portion (70 g) of cooked carrots or one 100 g raw carrot would present approximately 2.5 mg - 8 mg beta-carotene to the intestinal lining for absorption. If approximately 12% is absorbed and 25% of that is absorbed unchanged, approximately 0.1 - 0.2 mg beta-carotene would enter the blood stream. Normal range of beta-carotene in serum is 30 - 80 ug/100 ml (VGH 1981), so that, in a total blood volume of 4.5 l and with a hematocrit of 45%, there would already be approximately 0.75 - 2.0 mg beta-carotene in the circulation. The addition of 0.1 - 0.2 mg might be significant.

4.2 Absorption

Beta-carotene, carried in the mucopolysaccharide fraction of plant material, is absorbed in the small intestine. It is first transferred into the barrier external to the intestinal villi; from there it crosses the cell wall into the epithelial cell lining the mucosa. An average estimate of the amount of beta-carotene ingested that is absorbed is 12% (Wolf 1982), but estimates of the amount that is excreted vary from 2% to 99% (cited in Stich et al. 1986b). The wide variation of the amount of ingested beta-carotene that is excreted could contribute to interindividual variation in tissue levels.

Of the beta-carotene that is absorbed, some is cleaved by the enzyme 15,15'-dioxygenase, while traversing the cell wall, into retinal, which is reduced to retinol in the cytoplasm, esterified, and passes from the cell into the lymph such that 60 - 70% of absorbed beta-carotene enters the lymph as retinyl esters; 20 - 30% of absorbed beta-carotene is

transferred from the cell into the lymph system as part of a chylomicron, to enter the circulation as unchanged beta-carotene (Wolf 1982).

Bile salts, and nutritional factors including readily-digested dietary lipids, anti-oxidants, particularly tocopherols, in moderate amounts (excessive amounts reduce absorption), and lecithin, facilitate carotenoid absorption in humans (Goodwin 1984). Adequate protein in the diet is necessary for the activity of the enzyme to convert beta-carotene into retinol.

Supplementation with vitamin A or vitamin E may affect beta-carotene absorption. The following might suggest that the enzyme activity is feedback inhibited. As previously described (see Introduction, sec.1.4.4) in an intervention trial in which exfoliated oral mucosa cells were collected from inverted smokers after prolonged supplementation with vitamin A and/or beta-carotene, the group taking vitamin A plus beta-carotene showed significantly higher levels of beta-carotene in cells than did the group taking the same dose of beta-carotene alone (Stich et al. 1986b). However, two serum studies did not detect a change in serum beta-carotene in subjects supplemented with vitamin A (Urbach 1952, Willett et al. 1983b). In cows and chickens, high vitamin A ingestion reduced the level of blood carotene (Urbach 1952).

The enzyme for beta-carotene conversion to retinol is also found in the human lung, liver, and kidney (Goodwin 1984). In cows, the enzyme has been found in those sites and in the adrenal medulla. Cows are the only mammal reported to synthesize beta-carotene; synthesis of beta-carotene was detected in the corpus luteum.

4.3 Transport and distribution

Newly-absorbed beta-carotene bypasses the liver and circulates with the low-density lipoprotein (LDL) complex (cholesterol and triglyceride-rich particles) in the blood. Mathews-Roth and Gulbrandsen (1974) found that, in beta-carotene-supplemented individuals, beta-carotene rose in all three lipoprotein fractions (VLDL, LDL, and HDL)

above pre-supplement levels, but that carotene as percentage of total carotene was increased only in the LDL fraction. Blood levels of carotene reach a plateau after about 1 month of continuous (daily) supplementation, a phenomenon that could be due to saturation of the binding capacity of the lipoprotein for carotene, or to an inability to absorb more carotene (Mathews-Roth and Gulbrandsen 1974). A positive association of serum or plasma beta-carotene with serum or plasma total cholesterol, HDL- or LDL-cholesterol, or plasma lipids has been observed in several epidemiological studies (Russell-Briefel et al. 1985, Willett et al. 1983b, 1984, Nomura et al. 1985, D'Antonio et al. 1986), and no association with serum triglyceride or with plasma lipids was observed in two reports (Bieri et al. 1985, Russell-Briefel et al. 1985).

Vitamin E intake appeared to increase serum beta-carotene in one study (Urbach 1952), and to lower serum beta-carotene in another (Willett et al. 1983). Supplementation with multi-vitamins resulted in a rise in plasma beta-carotene in a population in northern China at high-risk for esophageal cancer (Yang et al. 1984). It is not known if these substances affect the absorption stage, by enzyme inhibition, or if they affect the transport of beta-carotene in the serum.

The principal carotenoids in human serum are beta-carotene, lycopene, and various hydroxylated carotenoids. The C.V.s for carotenoids in blood are usually 30 - 50%; mean concentration depends on diet, and different populations can show quite different means (Olson 1984).

Elevated blood levels of carotenes, or carotenemia, occur in the hypothyroid state, in anorexia, can occur with amennorhea, and can be due to an increased consumption of carrots. Low levels are seen with certain malabsorption syndromes.

Beta-carotene might be expected to be found in higher concentration in cells which have more LDL receptors, such as muscle and adipose tissue. Of total body carotenoid, adipose tissue contains over 80%, the liver contains about 10%, the plasma contains about 1%

or less, and other tissues contain the remainder (Olson 1984). The median amount of total body carotene in an adult on a normal mixed diet may be 100 - 200 mg (Olson 1984).

We can expect the distribution of beta-carotene in other tissues to be affected by their blood flow and by the area of the absorptive surface, and the rate of distribution to be affected by the rate at which beta-carotene equilibrates with the major tissue reservoirs. Body height, weight, and metabolic rate are likely to affect tissue storage capacity for beta-carotene. Ingestion of a fixed supplemental dose of beta-carotene could result in very different tissue levels in different individuals if the dose was not adjusted for body weight.

4.4 Catabolism and excretion

Little is known about the clearance of beta-carotene. It is broken down in the liver where the products are conjugated, and they are excreted in the urine. The length of time it is stored is unknown, nor is the half-life in tissues known.

4.5 Cell proliferation

The question of whether the concentration of beta-carotene changes if exfoliated cells are less mature, or less differentiated, is one that is relevant to beta-carotene measurement in both oral mucosa cells and uro-genital tract cells. If repeated brushing of the oral mucosa stimulates cell proliferation, this increased turn-over of cells may be reflected in an altered beta-carotene content compared to that of normal mucosa. The beta-carotene content of oral mucosa cells may be related to their glycogen content, which increases as the cells migrate to the surface (Weiss and Greep 1978).

Most cells found in urine are probably squamous epithelial cells that originate from the vulva and the lower urinary tract (lower one-third of the urethra). Cervical and vaginal epithelial cells may be present. The vagina is lubricated by cervical mucus and by a

transudate from the lamina propria, that could carry vaginal epithelial cells and possibly cervical epithelial cells towards the exterior; however, the number of these cells in urine, compared to the number coming from the vulva, are probably few. Other cells in the urinary sediment are probably transitional epithelial cells from the bladder and from the lining of the ureters and urethra. None will be from the normal kidney (Weiss and Greep 1978).

In the present study non-squamous epithelial cells in the sample of uro-genital tract cells accounted for only approximately 5% of the total number of cells; the cell sample can be considered to be of constant composition, of squamous epithelial cells, likely originating from the vulva and lower urethra.

The female hormone cycle affects the stage of maturation of exfoliated vaginal epithelial cells (Greenspan and Forsham 1983). A rise in estrogen level stimulates the proliferation and maturation of the epithelial cells; cells swell with glycogen, and more mature cells predominate in a cell scraping of the vaginal wall. The rise in progesterone level is accompanied by increased shedding of cells, and less mature cells predominate. If cells that differ in maturity differ in content of beta-carotene, then the female hormonal cycle would be expected to have an effect on the level of beta-carotene in uro-genital tract cells.

In preliminary investigations conducted before the long-term loading study, I did not find that there were fewer cells in the samples obtained at mid-cycle, when estrogen concentrations are highest, nor were there any more cells obtained at end-of-cycle, when progesterone levels are high.

The many ways in which the amount of beta-carotene available to a tissue can vary makes it obligatory to use a sufficient loading dose of beta-carotene in a loading study to guarantee a measurable boost to the tissue level in most subjects.

5. Dose-Response of Beta-carotene in Exfoliated Cells After Short-term Supplementation

An understanding of the dose-response characteristics of beta-carotene in exfoliated oral mucosa cells could have application to the design of intervention trials that examine these cells.

The effect of short-term oral supplementation with three doses of beta-carotene was to produce what appeared to be different elevations in beta-carotene, however, the variation within each treatment group made the differences indistinct. Beta-carotene levels remained elevated for two weeks after all three loading doses, but returned to initial levels by week 3 (360 mg and 180 mg doses; insufficient data from the 90 mg group).

The variation in beta-carotene levels measured on these sampling days is understandable in light of the variety of serum responses to a loading dose of beta-carotene, including the time to reach maximum level, and the duration of elevated levels. A single oral dose of beta-carotene caused serum carotene to rise to a peak in, variously, 2 - 8 hours (Wennersten and Swanbeck 1974), 5 hours (Jensen et al. 1986), and 4 - 8 hours (Bieri et al. 1985). The doses given were, respectively, 2 - 8 mg/kg body weight, 24 mg from carrots added to a meal, and 30 mg. Subsequent to this peak, the half-time of beta-carotene in serum in the first study was 26 - 96 hours, and, according to the authors, showed no apparent correlation to the dose; the half-time was not determined in the second study, and was only 8 - 14 hours in the third report.

The use of fixed doses of beta-carotene in the present study could have contributed to variation in blood levels of beta-carotene, appearing as variation in tissue levels. In the former study in which dose was adjusted for body weight, the increase in serum carotene was about the same in all subjects despite the variety of doses given, suggesting that a relationship exists between body mass and the proportion of a loading dose that is distributed into extra-vascular tissues.

The choice of sampling days, which may have affected the findings of this study, was made by estimating the optimum times at which to collect cells. A previous short-term loading study done in our lab had resulted in elevated serum levels of beta-carotene at the end of a four day loading period, and elevated oral mucosa cell beta-carotene levels two weeks after the loading (Stich et al. 1986a). Since oral mucosa cells migrate from the basal layer to the surface in as little as 3 - 5 days, we suspected that we would be able to detect an elevation in beta-carotene level in exfoliated oral mucosa cells one week after the end of the four day loading, and that that time might coincide with the time of the peak. The variety of responses, however, indicates that the time of the maximum level varies widely. Taking samples more frequently in the post-loading period would better illustrate the different effects of the three doses.

It is ironic that in some individuals, such as the three individuals in the short-term loading study, dietary supply alone of beta-carotene was enough to achieve levels of beta-carotene in exfoliated oral mucosa cells that were the same (approximately 8.0 ng/10^6 cells) as the level achieved one week after a four-day load of either 180 mg or 360 mg of beta-carotene, and the same as the level that resulted from a four-week loading of 180 mg/wk.

The levels of those 3 individuals are also remarkable when one compares them to the results of other intervention trials: four months of ingestion of 180 mg of beta-carotene per week resulted in a change in mean oral mucosa cells levels from 1.0 to 4.5 ng/10^6 cells, in Indian betel quid chewers (Stich et al. in press); the same dosage for the same length of time resulted in mean oral mucosa cell levels of 2.5 ng/10^6 cells, compared to 0.5 ng/10^6 cells in the placebo group, in Filipino inverted smokers Stich et al. 1986a). Members of a local Vancouver community whose dietary practice is to eat communal, vegetarian meals showed a mean beta-carotene level in oral mucosa cells of 3.8 ng/10^6 cells, considerably higher than the mean of Cancer Research Center controls of 1.8 ng/10^6 cells (this author, unpublished data). Clearly, other variables besides dose, such as diet, ability of the intestinal mucosa to absorb

beta-carotene, and tissue storage capacity, discussed previously, can play a dominant role in determining the level of beta-carotene in tissues.

The results of the present study suggest that different doses of beta-carotene can produce distinct changes in beta-carotene level in exfoliated oral mucosa cells. Examination of a larger study population would be necessary in order to statistically confirm these results.

6. Kinetics of Beta-carotene in Exfoliated Cells After Long-term Supplementation

The most dramatic result of the long-term loading study was the distinctive loading profile shown by uro-genital tract cells. This finding corroborates the claim for the tissue-specificity of beta-carotene, and illustrates the need to examine each tissue-of-interest for its pharmacokinetic characteristics.

The actual profile is not easily explained. Nevertheless, the features were not simply due to normal fluctuation. Verification in a larger group of subjects could make the conclusions more statistically solid.

Unique kinetic features of uro-genital tract cell loading with beta-carotene were the following. First, the rise in beta-carotene began later in uro-genital tract cells than in oral mucosa cells. This could have been due to factors such as blood supply to the tissue, and the rate of exfoliation of uro-genital tract cells. In future intervention trials with this schedule, three weeks after the start of supplementation with this dose it should be possible to verify that a rise in beta-carotene has occurred in uro-genital tract cells, i.e. that participants are taking supplements. In some individuals the increment in beta-carotene was small in response to the loading. In this regard, previous authors have suggested that the distinction between responding and not-responding may be a matter of the quantity of beta-carotene given (Mathews-Roth et al. 1977, Deuel 1959).

Second, it appears that the maximum uro-genital tract cells level occurred during the loading. A similar phenomenon has been observed in an animal study. In beta-carotene-

supplemented rats, the liver showed a slight decline in beta-carotene level during the loading (Shapiro et al. 1984). The authors suggested that variability, or induction of the dioxygenase enzyme in the liver, could explain their findings. In the present study, individuals who were sampled late in the loading period (days 17 - 21) also showed a suggestion of maximal beta-carotene levels in oral mucosa cells at that time. Taking samples at more frequent time-intervals would help to determine when maximum levels in the two tissues occurs.

Loading for four weeks was expected to bring serum levels close to a plateau level. Continuous daily supplementation with 15-180 mg of beta-carotene per day led to a maximum in blood level in 4 - 6 weeks (Mathews-Roth et al. 1972); 17 days of continuous supplementation with 40 mg or 60 mg of beta-carotene with another carotenoid led to plasma beta-carotene approaching a plateau level, however the supplementation did not continue longer than that (Meyer et al. 1985).

The fact that carotenodermia was not reported in the present study does not mean that high levels of beta-carotene in serum had not been achieved. Different individuals on the same diet can vary in the degree of carotenodermia they develop (Mathews-Roth 1982, Meyer et al. 1985).

Third, maximum levels of beta-carotene were markedly different in the two tissues. Different responses in different tissues have been previously reported. In human tissue, the concentration of carotene in epidermis from psoriasis patients who had taken daily beta-carotene-canthaxanthin supplements increased by 150-850% (Rollman and Vahlquist 1985). The percent increase in uninvolved skin was 170% and in involved skin was 610% (medians), however, the explanation for this differential accumulation is unknown. No healthy controls received supplements in that study. In human cerebrum, on the other hand, the amounts of carotene were not altered by supplementation (Mathews-Roth et al. 1976).

In human skin, baseline carotene concentration was similar in epidermis from four locations on the body, and lower in a fifth location; In breast tissue obtained from cadavers there were differences in the amounts of beta-carotene in different layers of the skin

(Vahlquist 1982). Baseline levels of beta-carotene in both uninvolved and involved skin from psoriasis patients, in the afore-mentioned study, was 25 - 50% lower than in epidermis from healthy controls; however, epidermis from patients with several other skin diseases was not different in beta-carotene content from that of controls. Similarly, there can be species differences in carotene concentration: beta-carotene was detected in various tissues in the guinea pig, whereas it was not detected in the same tissues in hairless mice or in the rat (Mathews-Roth et al. 1977, Shapiro et al. 1984) (Table 16).

Each author mentioned considerable variation in the carotene concentrations from individual animals or individuals.

The lack of information about tissue levels of beta-carotene may be righted in the future if HPLC techniques, capable of detecting nanogram amounts of beta-carotene, become widely adopted.

Fourth, the time required for depletion of beta-carotene to half-maximum levels differed significantly between the two tissues. The uro-genital tract cell median beta-carotene declined immediately; oral mucosa cell levels declined in 14 - 21 days. Four weeks after the end of supplementation was not long enough for complete depletion of oral mucosa cell beta-carotene, whereas depletion appeared to be complete after two weeks, in uro-genital tract cells. For comparison, we can ask how fast do serum levels decline. Serum levels show a gradual decline from an elevated level; after 17 days of supplementation, levels had not returned to basal levels 60 days later (Meyer et al. 1986). Even after a single loading dose, the time for serum levels to decline to half their maximum averaged 4.2 days (Bieri et al. 1985).

The following mechanisms can be proposed to explain the loading profile of uro-genital tract cells: 1) cells respond immediately to loading, then by some modulating mechanism, accumulate less beta-carotene as time goes on; however, beta-carotene levels did not rise as soon after the start of loading as did oral mucosa cells levels, suggesting that uro-genital tract cells have a slower turn-over time than oral mucosa cells; 2) cells turn over as rapidly as oral mucosa cells, but high levels of beta-carotene in the blood are needed before

TABLE 16

EPIDERMAL CAROTENE CONCENTRATIONS IN BACK SKIN OF VARIOUS ANIMALS INCLUDING HUMANS[1]

Species		Epidermal carotene (ug/100 g wet weight)	Reference
Man (back)		165 ± 55[1]	Vahlquist 1982
Man (shoulder or buttock)		208 ± 86	Rollman and Vahlquist 1985
Hairless Mouse, female[2]		120 ± 80	Mathews-Roth et al. 1977
	controls	ND	
Guinea Pig[2]		3 ± 2	Mathews-Roth et al. 1977
	controls	2 ± 2	
Sprague-Dawley Rat female[2]		14	Shapiro et al. 1984
	controls	ND	

[1]Mean ± SD

[2]Beta-carotene-supplemented.

the cells accumulate any beta-carotene; perhaps uro-genital tract tissue receives beta-carotene-laden lipids only after other reservoirs of beta-carotene are full; 3) same model as 2), but beta-carotene is lost from the cells before they can be analyzed; or 4) cells show a weak ability to accumulate, and retain, beta-carotene; cells would retain little beta-carotene unless serum levels are quite high.

The reason for using a dose of beta-carotene, and the same timing of its administration, that have been used in past and present intervention trials, is that it is not possible to collect cells frequently for quantitation of beta-carotene, during an intervention trial. This study differed from what would occur in an actual trial in that the oral mucosa of participants was brushed frequently in order to collect exfoliated cells. The effect of this repeated (1-2 times/week) irritation to the epithelium, such as by increasing the rate of cell turnover and causing the cells to exfoliate sooner than normal, on beta-carotene levels in the sample, is unknown.

The tissue-specificity, and variation, in beta-carotene levels has implications for attempts at cancer control using this substance, as will be discussed in the next section.

7. Relevance

The highly-sensitive method for analyzing exfoliated cells for beta-carotene content, as employed in this study, allowed unique characteristics of two tissues to be detected. With this method, we can hope, in the long run, to 1) improve the design of intervention trials specifically in regard to dose and timing of beta-carotene, 2) investigate the protective level of beta-carotene in the basal layer of cells, 3) identify tissues specifically low or high in beta-carotene, enabling further inquiries into the correlation of beta-carotene level to cancer risk, and 4) make recommendations regarding beta-carotene consumption to individuals who would most benefit.

7.1 Improved design of intervention trials

With respect to oral mucosa cells, the elevated levels achieved by the short-term loading with a high dose (360 mg in 4 days) approximated the level achieved by the long-term loading (180 mg/wk for 4 weeks). Clearly, however, the benefit of a longer loading period is the longer time cell levels remain elevated after cessation of loading. The implication is that in a long-term intervention trial or intervention program, administration of small, frequent doses of beta-carotene may be able to maintain cell beta-carotene at a high plateau. Elevated plasma carotene, that resulted from ingestion of 63 mg of beta-carotene daily for two weeks, was maintained by daily ingestions of only 28 mg of beta-carotene, in one study (Urbach 1952). A study of a population of teen-age girls in the southern US found that plasma vitamin A was maintained by small, occasional intake of good sources of vitamin A (Sumner et al. 1987).

Individuals with relatively high initial levels of cell beta-carotene showed the highest levels after loading, in the present study. Once elevated tissue levels have been achieved it may take little input to maintain them at that level.

7.2 Understanding the events of carcinogenesis in cells

Measurement of beta-carotene in cells may allow scheduling of beta-carotene doses so that a steady concentration can be maintained in cells. This may allow determination of the protective concentration, in a given tissue. The protective effect of beta-carotene and vitamin A in reducing micronucleated cell frequency lasted at least 4 months following cessation of treatment, in one study (Stich and Rosin 1985). Was the protection commensurate with the concentration of beta-carotene in the cells? What is the minimum administered dose of beta-carotene that would achieve the desired cell concentration of beta-carotene in the majority of

individuals with whom one is concerned? Answers to these questions may be forthcoming from continued studies with beta-carotene.

7.3 Application to appropriate tissues

Appropriate sites to investigate would be other tissues in which cancer at that site has been linked to lower beta-carotene intake or lower serum level of beta-carotene, and for which there is some intermediate endpoint such as micronucleated cell frequency. These findings could be applied to intervention of carcinogenesis affecting the urinary bladder, uro-genital tract, vulva, or the pelvic area in general (e.g. radiation) (Stich and Rosin 1983a,b, 1984). Radiation of the lower bowel, for instance, in therapy for colon cancer might result in less damage to the epithelium if high amounts of beta-carotene were taken concurrently.

Beta-carotene in oral mucosa cells could be used as a marker for changes in beta-carotene in other cells. Techniques exist for the collection of exfoliated cells from the respiratory tract, the gastro-intestinal tract, and the uro-genital tract, for the purpose of cytological examination. The Pap test program as a screening tool for pre-cancerous cells of cervical cancer (Witte 1983, CCABC 1986), screening of individuals for stomach cancer in Japan (Yamagata et al. 1983), sputum cytology for the detection of bronchial carcinoma, and urine cytology for detection of bladder cancer, are examples in which examination of epithelial cells has led to early detection of abnormal, pre-malignant or malignant cells. A parallel investigation of cytologic and biochemical features of a tissue, specifically its beta-carotene content, could be attempted. Lung cancer and colon cancer are two of the top five fatal cancers of men and women in British Columbia (CCABC 1986). Lung and colon epithelia could be examined with the intention of collection of exfoliated cells for analysis for beta-carotene. Studies are urgently needed of the basal levels, and effects, of putative chemopreventive substances such as beta-carotene in tissues in which the incidence of cancer is high.

In regard to intervention in human tissues at high risk for cancer, Wattenberg (1985), however, warns that beta-carotene has shown relatively specific tumour-inhibiting properties, and it that may not be effective when tested for human cancer prevention because the doses necessary to produce an effect may be unacceptably high.

7.4 Application to appropriate high-risk groups

Measurement of beta-carotene levels in human population may allow identification of groups showing abnormally low tissue levels of this substance. The suggestion has been made that low levels of beta-carotene in the tissues, presumably due to a meagre dietary supply of the provitamin, could confer increased susceptibility to carcinogen injury upon the tissue.

It may be that supplementing the diet with beta-carotene could reduce the risk of particular cancers such as of the esophagus, larynx, and lung, among individuals at high risk of cancer (Smith and Sullivan 1986). The potential value of beta-carotene may be limited to these individuals, who include alcohol consumers and smokers. The results of the present study argue against the giving of a single, massive dose of beta-carotene with the intention of conferring protection to a high-risk group for a long time, as is done with retinol (given with the aim of preventing blindness, and susceptibility to other diseases, to children who lack a dietary supply of vitamin A). The results of the present study would argue in favour of regular maintenance doses of beta-carotene. By the same principle, the dietary intake of regular, modest doses of beta-carotene can be encouraged as maintaining beta-carotene levels for longer, after cessation of intake, than infrequent consumption of single large doses.

8. Recommendations

The following are recommendations for future loading studies.

An increase in the size of the study population would lead to the differences between means becoming more significant. The number of subjects that would be needed can be calculated. For example, in the short-term loading study, the means of beta-carotene levels on week 1 resulting from the 180 mg and the 360 mg doses (6.9 and 8.9 ng/10^6 cells; two outliers excluded) were not significantly different, at a=.10. These means could be called different (in a two-tailed test with a=.05 and a power of .90, and assuming the same SD of approximately 2.4 ng/10^6 cells for each group) if there were 17 subjects in each group instead of approximately 6.

With a limited population size, the minimum difference between two means that can be detected with a certain degree of confidence can be predicted. For example, one week after the short-term loading, the group that received 180 mg of beta-carotene showed a mean level of beta-carotene in exfoliated oral mucosa cells of 6.9 ng/10^6 cells. The minimum mean beta-carotene level that would be called significantly different from the week 1 mean would be 10.1 ng/10^6 cells (assuming the same SD of 2.4 ng/10^6 cells and the same number of subjects [n=6], in a two-tailed test with alpha=.05 and a power of the test of .90).

Use of a higher dose of beta-carotene, administered more frequently, could result in a definite, greater increase in uro-genital tract cell beta-carotene level in most individuals. Use of a second dose that is 10 times the first dose, for example, is a practice in animal studies (e.g. Shapiro et al. 1984) that could be applied to human studies.

Restricting the carotene content of the diet of participants before and during the study could augment the response of cell levels to beta-carotene loading. With information on the height and weight of participants the dosage of beta-carotene could be adjusted for body mass. Volunteers could be screened before the study commenced, to eliminate those with unusually high pre-loading levels; the population could then be subdivided into groups with

equal mean pre-loading levels (e.g. Jensen et al. 1986, Jensen 1987). A pre-trial period of supplementation with placebo could be used to screen out those who could not comply.

A study to compare groups of individuals who differ in their pre-loading levels of beta-carotene for their response to one dosage of beta-carotene should select 2 or 3 groups of individuals who have markedly-different beta-carotene levels, e.g. 0 - 0.5 ng/10^6 cells, 1.0 - 1.5 ng/10^6 cells, and 3.0 - 5.0 ng/10^6 cells.

Probably the most important consideration in the design of loading studies is that they be applicable to, or involve, a population at-risk; in that way, the influence of diet, and of confounding factors such as alcohol and use of tobacco, would be incorporated into the experiment.

SUMMARY

In short-term pilot studies of cancer intervention, beta-carotene was effective in reducing the frequency of micronucleated cells in the oral cavity of betel quid and tobacco chewers. An elevated frequency of micronucleated cells is a marker of exposure to carcinogens and may indicate that the population is at elevated risk for cancer.

There have been few inquiries into the changes in tissue levels of beta-carotene in individuals who ingest beta-carotene supplements as part of an intervention trial.

Our laboratory has developed a method to measure beta-carotene in samples of exfoliated oral mucosa cells. This offers the opportunity to measure both genotoxic damage, and the level of chemopreventive substance, in the same cells.

In the first part of the project I investigated the feasibility of using uro-genital tract cells in an intervention trial, and evaluated the reproducibility of the beta-carotene measurement and the variation in beta-carotene in the population. The second project investigated the change in levels of beta-carotene in exfoliated oral mucosa cells following short-term supplementation with three different doses of beta-carotene. The third project explored the kinetics of the increase and decrease in beta-carotene in uro-genital tract cells and oral mucosa cells following long-term oral supplementation of individuals with beta-carotene.

It was feasible to measure beta-carotene in uro-genital tract cells providing there were enough cells in the sample. This requirement made it mandatory to collect urine samples on consecutive days and pool the cells. The number of cells in the sample was best ascertained by counting the cells, however, this incurred a somewhat wider measure of uncertainty in the resulting beta-carotene measurement, compared to use of the wet weight method with exfoliated oral mucosa cells.

Oral mucosa cells showed indications of specific dose-response patterns in cell beta-carotene after the short-term loading, however the levels produced by ingestion of each dose

were not clearly distinct from one another due to large variation in cell beta-carotene levels within each group. Levels remained elevated for two weeks after the loading.

Markedly different kinetics of beta-carotene accumulation were observed between uro-genital tract cells and exfoliated oral mucosa cells, in individuals who ingested oral beta-carotene supplements in the long-term loading study. This finding is relevant to the prevention of cancer in a particular tissue susceptible to carcinogen injury, such as the uro-genital tract. Uro-genital tract cells can carry a marker for carcinogen injury in individuals who are exposed to certain carcinogens. Beta-carotene, having shown chemopreventive ability in oral mucosa cells showing the same marker, would be a likely chemopreventive agent to test in an intervention trial involving uro-genital tract cells that show the marker.

These studies have suggested that levels, and response to supplementation, of beta-carotene in tissues are tissue-specific. A tissue's baseline amount of beta-carotene - a protective substance - and the tissue's capability to accumulate beta-carotene under conditions of supplementation are unique characteristics of the tissue that need to be considered when intervention trials are designed.

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Appendix A: Carotene and vitamin A questionnaire

name _____ date _____
age _____
sex _____

I a) Describe how often you've eaten these foods during the past month:

	daily	2-4 times per week	1 time /wk.	less than 1x/wk.	never
1 medium portion of cooked carrots or 1 raw carrot, approx. 3/4" x7"					
1 med. portion cooked broccoli or 6 pcs. raw broccoli					
1 med. portion cooked spinach or 1 med. spinach salad					
1 green salad, 1 bowlful					
name any other dark-green leafy vegetable that you eat more than a little of					

b) Compared to this time last year, of the above vegetables are you eating more? ☐ the same? ☐ less? ☐

II

Describe your consumption of:	daily	2-4x /wk.	1x /wk.	less than 1x/wk.	never
Meat -- a meat portion					
Dairy - 1 glass of milk, or 1 portion of yogurt, or cheese, enough for 1 cheese sandwich					
Other - 1 egg					
- 1 serving of liver					

III Describe the vitamin supplements you are taking (those taken for at least one month):

name of vitamin*	how much or how many units in each	how many per day	how often

* if multi-vitamin, write "multi", and specify it's vitamin A content.

Appendix B: Investigation of Appropriate Unit For Expressing Content of Beta-Carotene in Exfoliated Cells

This work was initiated and conducted by Miss Erika Hach, chemist; the present author did the cell counting. The following is a summary of the parts of the report by Erika Hach that are relevant to my thesis project.

Method The question of how to express the beta-carotene content of a sample of exfoliated cells led to the following steps being taken.

Three aliquots were taken from a large pool (30 ml) of exfoliated oral mucosa cells to estimate the concentration of cells in the pool. The mean count was 4.175×10^6 cells/ml. The cell pool was portioned into four groups of 10 tared microcentrifuge tubes each, of volumes 125 ul, 250 ul, 500 ul and 750 ul (containing approximately 0.5, 1, 2 and 3×10^6 cells). The volume of each tube was brought to 1.0 ml, and aliquots for cell counting were taken.

The parent aliquots were centrifuged at 3000g for 5 min. Supernatant was discarded; the last traces of supernatant were removed with a syringe. The aliquots were weighed. Beta-carotene was extracted by the procedure described in the text (see Materials and Methods). Extraction residues were re-dissolved in 30 ul of denatured ethanol, or in a multiple of 30 ul according to the original sample size, to produce approximately the same peak height by all samples.

Results A. Cell Count Method

1. Mean cell counts in each of the four sample-size groups showed a linear relationship.

2. The C.V. was 33% for low cell counts (0.5×10^6 cells) and decreased to 13% as the cell counts increased to 3.6×10^6 cells.

B. Wet Weight Method

1. Mean wet weights of the four sample-volumes showed a linear relationship, but the line did not intercept the origin.

2. The C.V. was 11% for low wet weights and 5% for high wet weights. (With the aliquots used in drawing the calibration curve the C.V.'s were 9% and 4.5% respectively.)

Better reproducibility with the wet weight method made it more favourable than the cell counting method; however, the wet weight didn't increase linearly with the estimated cell count but instead, increased in a curve. Therefore a calibration curve was derived.

Establishing a Calibration Curve for Translating Wet Weight into Cell Count

The mean cell count was determined from the 40 cell counts performed in step A above. The wet weights of aliquots containing $0.6 - 6.0 \times 10^6$ cells, plotted against their theoretical cell counts, resulted in the curve that is used to derive the cell counts of samples from their wet weights. Overall we can say there may be a 20% variation in beta-carotene determinations with the wet weight method, and a 30% variation with the cell counting method.

Appendix C: Formulae

%C.V. = SD/mean SE = SD/n

Interquartile range = range from 25th to 75th percentile

25th percentile = $\frac{n+1}{4}$ th value

Standard deviation: SD (or s)

$s = \sqrt{s^2}$ where:

Variance = $s^2 = \frac{\sum(x - \bar{x})^2}{n-1}$

and mean = $\bar{x} = \sum x / n$

For practical purposes, use:

variance = $\frac{n\sum x^2 - (\sum x)^2}{n(n-1)}$

References:

Erickson and Nosanchuk 1977

Glass and Hopkins 1984

Zar 1984