Quantitation of Human Uptake of the Anticarcinogen Phenethyl Isothiocyanate after a Watercress Meal[1]

F-L. Chung,[2] M. A. Morse, K. I. Eklind, and J. Lewis

Division of Chemical Carcinogenesis, American Health Foundation, Valhalla, New York 10595 [F-L. C., M. A. M., K. I. E.], and Department of Food Molecular Biochemistry, AFRC Institute of Food Research, Norwich Research Park, Colney Lane, Norwich, NR4 7UA, England [J. L.]

Abstract

Our previous studies showed that phenethyl isothiocyanate (PEITC), a cruciferous vegetable constituent, inhibited the lung tumorigenesis induced by a potent tobacco-specific carcinogenic nitrosamine in animals. These results implicate dietary PEITC as a risk-reducing factor of lung cancers induced by smoking. To define the effect of dietary PEITC on human cancers, a method of measuring its uptake is needed. Since watercress is rich in gluconasturtiin, a glucosinolate precursor of PEITC, it was chosen to be the source of PEITC. Four individuals were asked to eat watercress as part of a breakfast meal, and 24-h urine samples were collected. A urinary metabolite was found, and its identity was confirmed as the *N*-acetylcysteine conjugate of PEITC by comparison with the synthetic standard using nuclear magnetic resonance and mass spectrometry. A dose-dependent excretion of this conjugate was observed. These results clearly showed that PEITC was released in the human body upon ingestion of watercress and suggest that the *N*-acetylcysteine conjugate of PEITC may be a useful marker for quantitating human exposure to this anticarcinogen as a tool for epidemiological investigations.

Introduction

Despite the well-established inverse relationship between vegetable consumption and the incidence of various cancers in humans, the exact nature of the ingredients which might be responsible for the protection is still not well defined. Fibers found in many vegetables have been identified as a risk-lowering factor for certain types of cancer such as colon cancer (1). However, relatively few experimental data regarding the protection against lung cancers by vegetable ingredients are available. Vitamin A and its precursor, β-carotene, have been identified as constituents which may possibly contribute to the protective effects of vegetables against lung cancer in humans (2). However, as stated in a report by the National Academy of Sciences Committee on Diet, Nutrition, and Cancer, "because the indices of vitamin A intake in these studies were derived from foods that also contain other natural inhibitors of carcinogenesis, it is also possible that dietary constituents other than preformed vitamin A or β-carotene are relevant risk-reducing factors" (2).

A more recent population-based study of diet and lung cancer suggested other ingredients in cruciferous vegetables appear to have a stronger inverse association with risk than β-carotene (3). Our previous studies showed that PEITC[3] and indoles, both of cruciferous origin, inhibited tobacco-specific NNK-induced lung carcinogenesis in laboratory animals (4, 5). NNK is a nicotine-derived nitrosamine which is believed to be involved in the development of lung cancer in smokers because of its carcinogenic potency and organ specificity for the induction of lung tumors in laboratory animals (6, 7). NNK-treated rats fed diet containing PEITC developed significantly fewer lung tumors than NNK-treated rats fed a control diet (4). More recently, we have demonstrated that pretreatment with PEITC and other structurally related aromatic isothiocyanates effectively blocks the lung tumorigenic activity of NNK in A/J mice (8, 9). The anticarcinogenic potential of PEITC has been also demonstrated in many other studies. Wattenberg showed that pretreatment with PEITC inhibited the mammary and lung tumorigenesis of 7,12-dimethylbenz(a)-anthracene (10). Feeding PEITC in the diet also inhibited the formation of esophageal tumors in rats treated with *N*-nitrosobenzylmethylamine (11). Therefore, it appears that PEITC possesses a broad spectrum of anticarcinogenic activity. Since some of the carcinogens examined in these studies may be involved in the etiology of human cancers, it is possible that dietary exposure to PEITC may be beneficial to humans in the reduction of risk of certain cancers, such as tobacco-related lung cancer. To assess the potential role of dietary PEITC in human cancers, a method for measuring its uptake is needed. Watercress is rich in gluconasturtiin, a glucosinolate of PEITC, which releases PEITC upon hydrolysis by myrosinase, an enzyme activated by chopping or chewing this vegetable. We chose to use watercress as a dietary source for PEITC. This study describes the development of an assay for the quantitation of dietary uptake of PEITC in humans after a watercress meal.

Received 1/10/92.
[1] Supported by Grant CA46535 from the National Cancer Institute.
[2] To whom requests for reprints should be addressed, at Division of Chemical Carcinogenesis, American Health Foundation, 1 Dana Road, Valhalla, NY 10595.

[3] The abbreviations used are: PEITC, phenethyl isothiocyanate; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPLC, high-performance liquid chromatography.

Materials and Methods

PEITC was purchased from Aldrich Chemical Co. (Milwaukee, WI). The conjugates of PEITC were prepared as previously described (12). Gluconasturtiin was synthesized by a published method (13). Myrosinase was isolated from mustard seeds according to a published procedure (14). Watercress was obtained from a local grocery store.

Mouse Study. Seven-week-old female A/J mice, three in each group, were fed AIN-76 diet containing gluconasturtiin (10 or 50 μmol/g diet) and supplemented with myrosinase (5 or 25 mg/g diet, respectively). After 24 h on this diet, mice were fed AIN-76 diet for an additional 48 h. Seventy-two-h urine samples were collected at 24-h intervals during the experiment and stored in a dry ice bath until analysis of PEITC metabolites. The collected urine samples were processed by adding an equal volume of methanol to the pooled urine of each group to precipitate solids. Filtered samples were concentrated *in vacuo* to obtain final samples for the analysis by reverse-phase HPLC as described previously (12).

Human Study. Four volunteers (two males and two females) ingested 57 g (Experiment 1) or 30 g (Experiment 2) of watercress at a breakfast with croissants or bagels and cream cheese. All participants were told to avoid cruciferous vegetables and mustard in the diet during the experiment. For the control diet, all participants ate the same food except watercress 2 days prior to the experimental diet. Subject 3 of Experiment 1 did not participate in Experiment 2 and was replaced by another volunteer of the same sex. Urine samples were collected at 2, 4, 8, and 24 h after breakfast and were stored frozen until analysis. Upon thawing, samples were adjusted to pH 3 with phosphoric acid. Three 2-ml aliquots of each urine sample were placed in 7-ml vials. Two g of ammonium sulfate and 3 ml of ethyl acetate were added to each vial. Each vial was vortexed for 20 s and centrifuged to separate the ethyl acetate layer. This extraction was carried out twice. The extract was reconstituted in 0.5 ml of HPLC mobile phase and analyzed by reverse-phase HPLC as described previously (12). This method resulted in quantitative recovery of the *N*-acetylcysteine conjugate of PEITC. A significant amount of *N*-acetylcysteine conjugate was excreted in the urine; therefore, it was isolated in sufficient quantity by repetitive HPLC runs. The identity of this urinary metabolite as the *N*-acetylcysteine of PEITC was confirmed by comparing the 360 MHz proton nuclear magnetic resonance and mass spectra with those obtained from the synthetic standard.

Analysis of Gluconasturtiin Content of Watercress. The watercress was freeze-dried in New York; approximately 300 g were sealed in a polyethylene bag containing $CaCl_2$ and sent to Norwich. The analysis of gluconasturtiin content of the freeze-dried watercress was performed following a published method of Heaney and Fenwick (15). Briefly, the freeze-dried watercress was ground to a fine powder and extracted for 15 min in 100 ml 70% methanol to inactivate myrosinase. Two ml (in duplicate) of the extract was added to a solution of glucotropaeolin (internal standard), and 100 μl of 1:1 (v/v) mixture of barium acetate (0.5 M) and lead acetate (0.5 M) in solution was added. The mixture was centrifuged, and the supernatant was applied to a microcolumn of DEAE Sephadex A25 (15). After washing with distilled water and 0.02 M pyridinium acetate solution, the column (containing the intact glucosinolates) was treated with sulfatase, and the desulphoglucosinolates were eluted with water. HPLC analysis was carried out using the method of Spinks *et al.* (16).

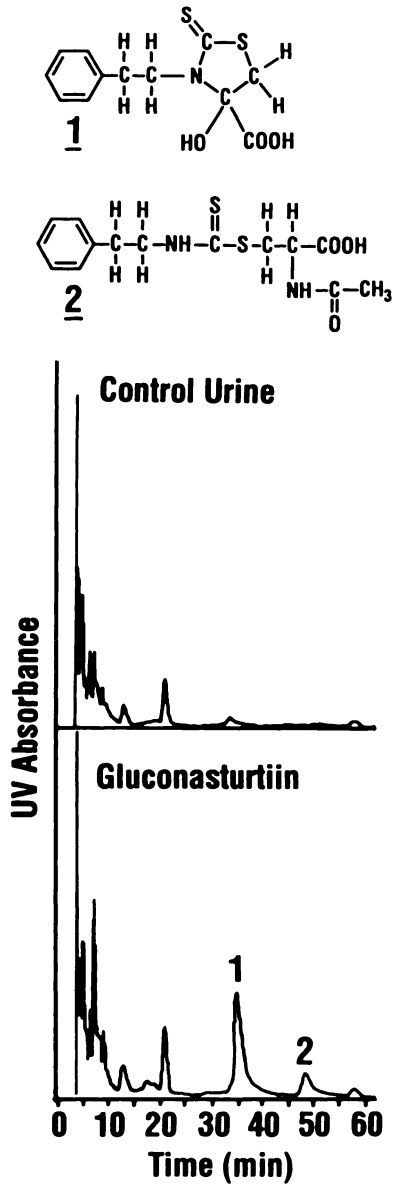

Fig. 1. Reverse-phase HPLC chromatogram obtained from analysis of urine from mice fed diet containing gluconasturtiin and myrosinase. *Peaks 1* and *2* are identical to those found in the urine of mice treated with PEITC (12), and their structures are shown.

Results

As a prelude to the human study, we first examined whether A/J mice fed gluconasturtiin diet supplemented with myrosinase would excrete these metabolites in the urine. As shown in Fig. 1, two major metabolites, identical to the conjugates extracted in the urine of mice treated orally with PEITC, were found in the urine of mice fed a

Table 1 Estimated conversion of dietary gluconasturtiin to PEITC in A/J mice

Gluconasturtiin (μmol/g diet)	Diet consumed (g)	PEITC excreted (μmol)[a]			Estimated % conversion[b]
		0–24 h	24–48 h	48–72 h	
10	0.8 ± 0.2	1.9 ± 0.8	ND[c]	ND	21.6 ± 8.4
50	0.6 ± 0.3	5.6 ± 1.6	0.5 ± 0.2	ND	21.2 ± 3.1

[a] Measured as total amount of metabolites II and III.
[b] Based on 25% and 10% of PEITC excreted as metabolites II and III (12).
[c] ND, not detectable.

gluconasturtiin-containing diet, whereas they were not detected in urine of mice fed control diet (12). Based on the amounts of these metabolites found in the urine of mice treated with PEITC, it was estimated that at least 20% of gluconasturtiin was converted to PEITC in mice after eating a diet containing gluconasturtiin and myrosinase (Table 1). Interestingly, less than 1% conversion was found in mice fed the diet without myrosinase (data not shown), suggesting that the endogenous enzyme activity for the hydrolysis of gluconasturtiin is low.

In the human study, two experiments were performed. In both experiments, four healthy individuals were asked to eat a breakfast containing a known amount of watercress. Urine samples from each individual were collected after the breakfast at time intervals up to 24 h. After adjusting the pH of the collected urine to 3.0 followed by extraction with ethyl acetate, the extracts were analyzed by reverse-phase HPLC. A peak eluting at 10.7 min corresponding to the retention time of the synthetic standard of *N*-acetylcysteine conjugate of PEITC was found (Fig. 2). The identity of this metabolite was further verified by the comparison of its proton nuclear magnetic resonance and mass spectra with those obtained from the synthetic marker (Fig. 3). The limits of detection of the *N*-acetylcysteine conjugate in the urine using the method described is in the nanogram range. These results clearly demonstrated that dietary gluconasturtiin is converted to PEITC upon ingestion.

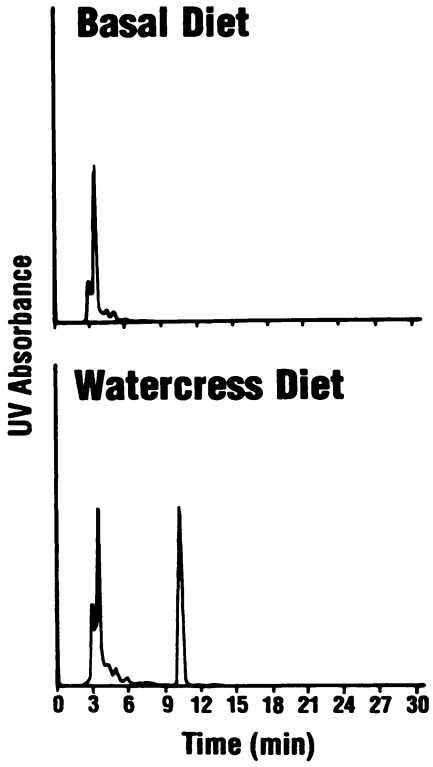

Fig. 2. Reverse-phase HPLC chromatogram obtained from analysis of human urine before and after the watercress meal. Peak eluting at 10.7 min is identified as the *N*-acetylcysteine conjugate of PEITC.

The cumulative excretion of the *N*-acetylcysteine conjugate from these individuals after the watercress meals from these experiments is presented in Table 2. In Experiment 1, individuals excreted the *N*-acetylcysteine conjugate ranging from 24.0 to 30 mg during a 24-h period after consuming 57 g of watercress. In Experiment 2, the amount of excretion ranged from 4.6 to 10.2 mg after ingesting 30 g of watercress. In general, the peak of excretion was reached between 2 and 4 h after ingestion. In both experiments, the *N*-acetylcysteine conjugate of PEITC was completely excreted within 24 h of ingestion. The total amount of conjugate excreted corresponds to 12–15 mg and 2.3–5.1 mg of PEITC in these experiments, respectively. These results demonstrated an uptake-dependent excretion of its *N*-acetylcysteine conjugate after a watercress meal.

To better delineate the uptake-dependent excretion, the freeze-dried watercress obtained from the batch in Experiment 2 was analyzed for gluconasturtiin content and shown to contain 9.79 mg/g dry weight or 0.72 mg/g wet weight. A typical chromatogram obtained from the analysis shows that gluconasturtiin is the major glucosinolate present in watercress, constituting more than 30% of the total glucosinolate content (Fig. 4). Since each individual consumed 30 g of fresh watercress, this corresponds to a total intake of 21.6 mg of gluconasturtiin/person or 7.6 mg of PEITC/person, assuming quantitative hydrolysis to PEITC. Therefore, as shown in Table 3, the minimal percentage of conversion from gluconasturtiin to PEITC ranges from 30% to 67%. The rate of conversion reflects the efficient hydrolysis of gluconasturtiin to PEITC and its conjugate formation, which resulted in significant excretion of *N*-acetylcysteine conjugate in the human urine.

Discussion

We reported previously that two major urinary metabolites, an *N*-acetylcysteine conjugate and a cyclic mercaptopyruvate conjugate (structures in Fig. 1), were isolated and identified from mice treated orally with PEITC (12). These urinary metabolites account for, respectively, approximately 25% and 10% of the PEITC administered. The relatively high levels of excretion of these conjugates suggested the possibility of using them as markers for

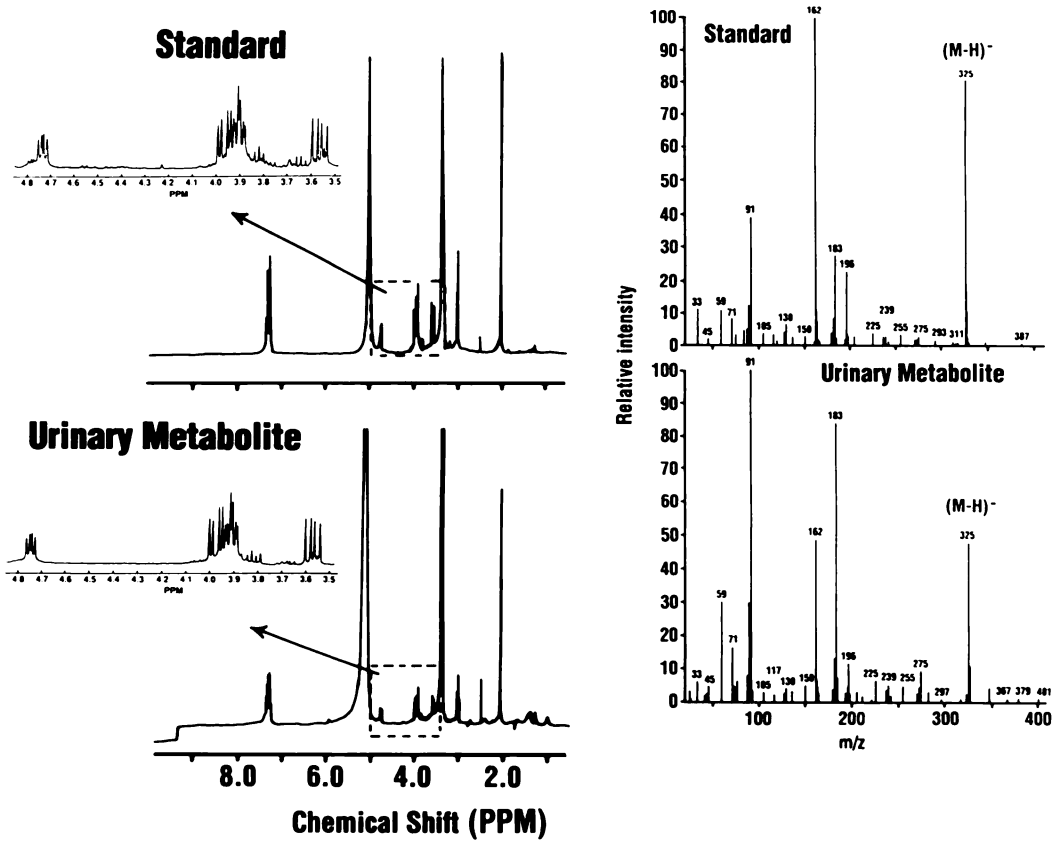

Fig. 3. 360 MHz proton nuclear magnetic resonance and mass spectra of the synthetic standard of the *N*-acetylcysteine conjugate of PEITC and the peak isolated from human urine after the watercress meal.

Table 2 Cumulative amounts of the *N*-acetylcysteine conjugate of PEITC in human urine 24 h after ingestion of watercress

Subject	Time interval (h)	Experiment 1		Experiment 2	
		PEITC conjugate (mg)	Total excretion (mg)	PEITC conjugate (mg)	Total excretion (mg)
1	0-2	4.9 ± 0.1[a]		0.6 ± 0.0	
	2-4	7.7 ± 0.2		1.1 ± 0.0	
	4-8	6.6 ± 0.1		2.2 ± 0.0	
	8-24	4.8 ± 0.3	24.0	0.7 ± 0.0	4.6
2	0-2	2.4 ± 0.0		1.1 ± 0.1	
	2-4	11.2 ± 0.2		0.2 ± 0.0	
	4-8	5.8 ± 0.1		3.8 ± 0.0	
	8-24	3.5 ± 0.9	22.9	0.8 ± 0.1	5.9
3[b]	0-2	6.5 ± 0.4		1.6 ± 0.1	
	2-4	9.1 ± 0.3		3.3 ± 0.1	
	4-8	5.6 ± 0.1		2.3 ± 0.2	
	8-24	8.8 ± 0.8	30.0	0.5 ± 0.0	7.7
4	0-2	7.3 ± 0.1		1.1 ± 0.1	
	2-4	10.0 ± 0.2		4.4 ± 0.1	
	4-8	4.9 ± 0.1		3.5 ± 0.2	
	8-24	3.9 ± 0.1	26.1	1.3 ± 0.1	10.3

[a] Mean ± SD of three separate determinations.
[b] Subject 3 participated in Experiment 1 but was replaced by another individual of the same sex in Experiment 2.

measuring exposure to dietary PEITC in humans. The main source of human exposure to PEITC is through the consumption of Chinese cabbage, radishes, and watercress, cruciferous vegetables which are rich in its glucosinolate precursor, gluconasturtiin (17-19). PEITC is one of the major products formed from the hydrolysis of gluconasturtiin mediated by myrosinase, an enzyme released during chewing or chopping of cruciferous vege-

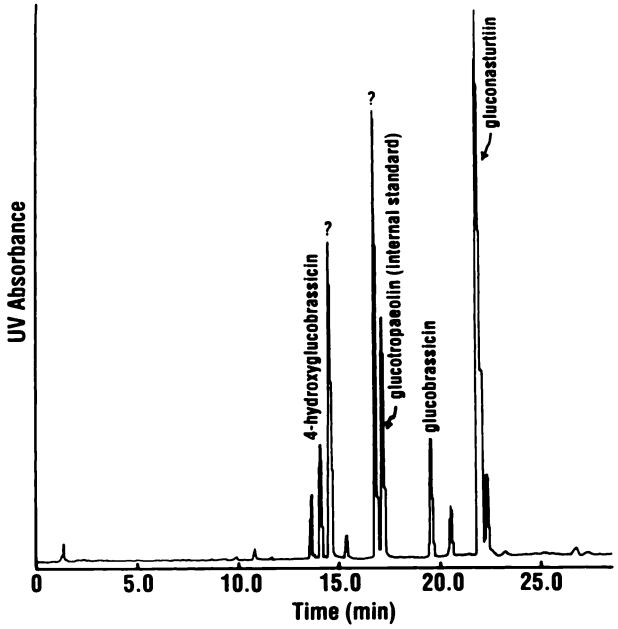

Fig. 4. Reverse-phase HPLC chromatogram of major glucosinolates obtained from analysis of the freeze-dried watercress.

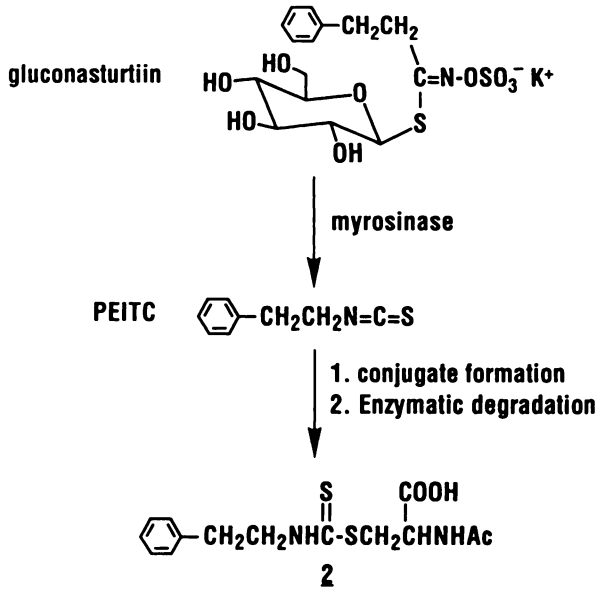

Fig. 5. Scheme depicting the conversion of gluconasturtiin to PEITC followed by conjugation to form major metabolites found in the human urine.

Table 3 Minimal percentage of conversion of gluconasturtiin to the *N*-acetylcysteine conjugate of PEITC in humans after a watercress meal[a]

Subject	Total conjugate excreted (mg)	PEITC equivalent (mg)	% of conversion
1	4.6	2.3	30
2	7.7	3.9	51
3	5.9	3.0	39
4	10.3	5.2	67

[a] Based on 9.79 mg/g dry weight of gluconasturtiin in the watercress consumed in Experiment 2.

tables (17). A crude estimate of glucosinolate intake in humans was obtained from the analysis of the glucosinolate content in vegetables and the average consumption of these vegetables (18). The data from this type of study would not provide a measure of the actual uptake of PEITC in each individual. A urinary PEITC conjugate would provide a more accurate marker for quantitating the uptake of PEITC in individuals.

The conversion of gluconasturtiin to the *N*-acetylcysteine conjugate of PEITC is depicted in Fig. 5. The released PEITC subsequently serves as a substrate for glutathione *S*-transferase, followed by enzymatic degradation to release the *N*-acetylcysteine conjugate in the urine. Unlike mice, humans do not excrete the cyclic mercaptopyruvate conjugate of PEITC in urine after ingesting gluconasturtiin. Approximately 50% of benzyl isothiocyanate, a homologue of PEITC, was excreted as its *N*-acetylcysteine in human urine after oral administration (20). Therefore, the amount of PEITC excreted in the urine after the watercress meal represents the minimal amount of PEITC released in the body. The actual internal exposure to PEITC would most likely be greater than these values. Possible factors which could contribute to the variability in excretion are differences in vegetable crops, individual differences in chewing pattern, and rate of glutathione conjugation.

This study is the first to demonstrate the quantitation of human uptake of PEITC, a dietary compound with potential for protection against human cancers. A great deal of information has been generated from laboratory studies regarding the potential roles of some dietary compounds in modulating the carcinogenic effects of various environmental agents. The development of methods for quantitating the internal uptake of these dietary components in individuals would perhaps provide epidemiologists valuable means of assessing the role of these dietary compounds in modifying the incidence of certain human cancers.

Acknowledgments

We thank all individuals who participated in this study, Mark Kagan for the mass spectra, and Dr. Roger Fenwick of the Agriculture and Food Research Council of Norwich for his consultation on the use of watercress for this study.

References

1. Pilch, S. M. (ed.). Physiological Effects and Health Consequences of Dietary Fiber. Washington, DC: Center for Food Safety and Applied Nutrition, Food and Drug Administration, Department of Health and Human Services; Bethesda, MD: Life Sciences Research Office, Federation of American Societies for Experimental Biology, 1987.

2. Committee on Diet, Nutrition, and Cancer, Assembly of Life Sciences, National Research Council. Diet, Nutrition, and Cancer. Washington, DC: National Academy Press, 1982.

3. Marchand, L. L., Yoshizawa, C. N., Kolond, L. N., Hankin, J. H., and Goodman, M. T. Vegetable consumption and lung cancer risk: a population based case-control study in Hawaii. J. Natl. Cancer Inst., *81:* 1158-1164, 1989.

4. Chung, F-L., Morse, M. A., and Eklind, K. I. New potential chemopreventive agents for lung carcinogenesis of tobacco-specific nitrosamine. Cancer Res., *52* (Suppl.): 2719s-2723s, 1992.

5. Morse, M. A., LaGreca, S. D., Amin, S. G., and Chung, F-L. Effects of indole-3-carbinol on lung tumorigenesis and DNA methylation induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and on the metabolism and disposition of NNK in A/J mice. Cancer Res., *50*: 2613–2617, 1990.

6. International Agency for Research on Cancer. Tobacco smoking. *In*: Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 38. Lyon: IARC, 1986.

7. Rivenson, A., Hoffmann, D., Prokopczyk, B., Amin, S., and Hecht, S. S. Induction of lung and exocrine pancreas tumors in F344 rats by tobacco-specific and Areca-derived *N*-nitrosamines. Cancer Res., *48*: 6912–6917, 1988.

8. Morse, M. A., Amin, S. G., Hecht, S. S., and Chung, F-L. Effects of aromatic isothiocyanates on tumorigenicity, O^6-methylguanine formation, and metabolism of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mouse lung. Cancer Res., *49*: 2894–2897, 1989.

9. Morse, M. A., Eklind, K. I., Hecht, S. S., Jordan, K. G., Choi, C-I., Desai, D. H., Amin, S. G., and Chung, F-L. Structure-activity relationships for inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) lung tumorigenesis by arylalkyl isothiocyanates in A/J mice. Cancer Res., *51*: 1846–1850, 1991.

10. Wattenberg, L. W. Perspectives in cancer research: chemoprevention of cancer. Cancer Res., *45*: 1–8, 1985.

11. Stoner, G. D., Morrissey, D., Heur, Y-H., Daniel, E., and Galati, A. Inhibitory effects of phenethyl isothiocyanates on *N*-nitrobenzylmethylamine carcinogenesis in the rat esophagus. Cancer Res., *51*: 2063–2068, 1991.

12. Eklind, K. I., Morse, M. A., and Chung, F-L. Distribution and metabolism of the natural anticarcinogen phenethyl isothiocyanate in A/J mice. Carcinogenesis (Lond.), *11*: 2033–2036, 1990.

13. Benn, M. H. The synthesis of gluconasturtiin. J. Chem. Soc., 4072–4074, 1962.

14. Miller, K. N., Boyd, J. N., Babish, J. G., Lisk, D. J., and Stoewsand, G. S. Alteration of glucosinolate content, pattern, and mutagenicity of cabbage (*Brassica oleracer*) grown on municipal sewage sludge-amended soil. J. Food Saf., *5*: 131–143, 1983.

15. Heaney, R. K., and Fenwick, G. R. Glucosinolates in *Brassica* vegetables: analysis of 22 varieties of brussels sprouts. J. Sci. Food Agric., *31*: 785–793, 1980.

16. Spinks, E. A., Sones, K., and Fenwick, G. R. The quantitative analysis of glucosinolates in cruciferous vegetables, oilseeds, and forage crops using high performance liquid chromatography. Fette Seifen Anstrichm., *86*: 228–231, 1984.

17. Tookey, H. L., VanEtten, C. H., and Daxenbichler, M. E. Glucosinolates. *In*: I. E. Liener (ed.), Toxic Constituents of Plant Foodstuffs, pp. 103–142. New York: Academic Press, 1980.

18. Sones, K., Heaney, R. K., and Fenwick, G. R. An estimate of the mean daily intake of glucosinolates from cruciferous vegetables in the UK. J. Sci. Food Agric., *35*: 712–720, 1984.

19. Carlson, D. G., Daxenbichler, M. E., Van Etten, C. H., Tookey, H. L., and Williams, P. H. Glucosinolates in crucifer vegetables: turnips and rutabagas. J. Agric. Food Chem., *29*: 1235–1239, 1981.

20. Mennicke, W. H., Görler, K., Krumbiegel, G., Lorenz, D., and Rittmann, N. Studies on the metabolism and excretion of benzyl isothiocyanate in man. Xenobiotica, *18*: 441–447, 1988.