Dietary Modification of Xenobiotic Metabolism: Contribution of Indolylic Compounds Present in *Brassica oleracea*

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Indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33′) are constituents of brassicaceous vegetables, possess anticarcinogenic activity, and are inducers of monooxygenases in rodents. A comparison of the effects of dietary cauliflower (CF, 25%) and I3C (250 ppm) on monooxygenase activities in the rat and mouse demonstrated that these indoles are not the only inducing agents present in these vegetables. Using liquid-phase partitioning, gel permeation, and silica gel chromatography, 12 unique fractions and subfractions of a dichloromethane–methanol CF extract were generated. Hepatic monooxygenase activities were increased in mice fed all but two of these fractions ($p \leq 0.05$). Feeding I3C, IAN, I33′, and indole-3-carbaldehyde at levels greater than or equal to the levels present in these fractions indicated that these indoles are not directly responsible for the induction of hepatic monooxygenases in mice fed dried vegetable material. Additionally, this paper reports the isolation of 1-methoxyindole-3-carbaldehyde from *Brassica oleracea*. This 1,3-disubstituted indole was demonstrated to be a more potent inducer of monooxygenase activity than any of the 3-substituted indoles tested.

Consumption of vegetables from the family Cruciferae, most notably those of *Brassica oleracea* (genus and species), is associated with an increase in the level of hepatic and extrahepatic xenobiotic metabolizing enzymes in experimental animals and man (Bradfield and Bjeldanes, 1984; Bradfield et al., 1985; Pantuck et al., 1979, 1984; Sparnins et al., 1982; Wattenberg, 1972). The alterations in xenobiotic metabolism, resulting from exposure to these vegetables, may explain the inhibitory effects of dietary Cruciferae on chemically induced carcinogenesis observed in rodents (Stoewsand et al., 1978; Wattenberg, 1983) and may contribute to a decreased cancer incidence of the colon and rectum in human populations (Graham, 1983; National Research Council, 1982). The constituents putatively responsible for these activities are indole-3-carbinol (I3C), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33′) (Figure 1; Loub et al., 1975; Wattenberg and Loub, 1978). These indoles are generated, in crushed plant material, via a thioglucosidase-mediated autolysis of a parent glucosinolate commonly referred to as glucobrassicin (GB, indolylmethyl glucosinolate; Virtanen, 1965).

Despite the potency of the major GB autolysis product, I3C, as an inducer of xenobiotic metabolizing enzymes (Bradfield and Bjeldanes, 1984; Loub et al., 1975; Miller and Stoewsand, 1983), this compound's instability in the plant's autolytic milieu suggests that its presence in dried vegetable diets is less than 50 μg/g (Bradfield and Bjeldanes, 1987a). This level would yield experimental diets (typically 25% dried vegetable) below the no-effect level for induction of hepatic and intestinal monooxygenase activity in the rat (Bradfield and Bjeldanes, 1984). Thus, the exact identity of the compound(s) responsible for the modification of cytochrome P-448 dependent monooxygenase activity observed in rodents fed dried cruciferous vegetable diets requires further investigation.

The purpose of this study was to determine the significance of I3C with regard to the induction of hepatic cytochrome P-448 dependent monooxygenase activity observed in mice fed dried cauliflower (Bradfield et al., 1985) and to further describe the chemicals present in cauliflower that might contribute to this inductive phenomenon. Experiment 1 compares the effects of I3C and cauliflower on xenobiotic metabolism in the rat and mouse, and experiment 2 uses hepatic induction of monooxygenases, in the mouse, as a bioassay to further describe inducing agents present in freeze-dried cauliflower.

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MATERIALS AND METHODS

Chemicals. Organic solvents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and biochemicals from Sigma Biochemicals (St. Louis, MO). Unless otherwise indicated, all other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). NMR (^{1}H) spectra were determined at 200 MHz in $CDCl_3$ on an U.C.B.-200 FT-NMR spectrometer (University of California Department of Chemistry, Berkeley, CA). Chemical shift values were recorded relative to tetramethylsilane for all spectra. Low-resolution mass spectra were recorded on a Finnigan 4000 mass spectrometer (Finnigan Instruments, San Jose, CA); high-resolution mass spectra were recorded on a Kratos MS-50 (Ramsey, NJ). 7-Ethoxyresorufin was synthesized by the method of Mayer et al. (1977), with the exception of the substitution of *N*,*N*-dimethylformamide for dimethyl sulfoxide as the reaction solvent. Indole-3-carbinol (I3C), indole-3-carbaldehyde (I3CHO), indole-3-acetonitrile (IAN), and 3,3′-diindolylmethane (I33) were prepared as previously described (Bradfield and Bjeldanes, 1987a).

Synthesis of 1-Methoxyindole-3-carbaldehyde. 1-Methoxyindole-3-carbaldehyde (NCHO) was synthesized by the method of Acheson et al. (1978, 1984). Briefly, 25 g (0.18 mol) of 2-nitrotoluene was added to a 100-mL solution of *N*,*N*-dimethylformamide containing 28 g (0.27 mol) of *N*,*N*-dimethylformamide dimethyl acetal. The solution was refluxed at 140 °C for 32 h under an atmosphere of nitrogen to generate (*E*)-*N*,*N*-dimethyl-2-(2-nitrophenyl)ethenamine (ENN). The solvent was removed from a 25-mL aliquot of the ENN (0.026 mol) solution with the use of a rotary evaporator (60 °C). The residue was redissolved in 100 mL of diethyl ether, and 4.0 g of ammonium chloride and 15 mL of water were added. After nitrogen was bubbled through this solution for 15 min, 30 g of powdered zinc was added. The solution was vigorously stirred under nitrogen for 20 min. The ether solution was dried ($MgSO_4$) and treated consecutively with 1.4 g of sodium methoxide and 3.7 g (0.054 mol) of iodomethane. The solution was stirred for 20 h and washed twice with

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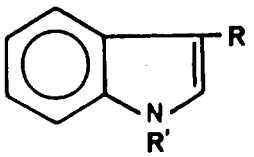

Figure 1. Structures of 3-substituted and 1,3-disubstituted indoles. Key: indole-3-carbinol (I3C), R = CH_2OH, R′ = H; indole-3-acetonitrile (IAN) R = CH_2CN, R′ = H; indole-3-carbaldehyde (I3CHO) R = CHO, R′ = H; 3,3′-diindolylmethane (I33′) R = CH_2-indole, R′ = H; 1-methoxyindole-3-carbaldehyde (NCHO) R = CHO, R′ = OCH_3.

10% NaCl in water and the ether layer evaporated to dryness. The residue was placed on silica (200 mesh, column size 1 × 6 in.) and eluted with petroleum ether. The first 100 mL of eluate contained pure NCHO as a light yellow oil, 1.2 g, 7.0 mmol. The ^1H NMR spectrum was recorded in $CDCl_3$ and was identical with previously reported values (Acheson et al., 1978). High-resolution mass spectra yielded a molecular ion in agreement with the molecular formula for NCHO (i.e., 175.0635, calcd 175.1875). The low-resolution mass spectrum showed peaks (more than 20% of the base peak) at *m/e* 175 (100%), 132 (69%), 116 (88%), 115 (21%), 89% (49%), 77 (25%), 63 (31%), 57 (24%), and 55 (21%).

Analysis of Indoles. Indolylic compounds present in the extracted fractions were quantified by a modification of the high-performance liquid chromatographic method previously described (Bradfield and Bjeldanes, 1987a). In this method fractions were diluted in CH_3OH to a concentration of approximately 1 mg/mL, and a 20-μL aliquot was injected onto the reversed-phase system (column Ultrasphere 4.6 mm × 250 mm ODS, C-18, particle size 5 μm; Altex Scientific, Inc., Berkeley, CA) using a gradient of 30–90% CH_3OH in water over 40 min. Indoles were detected by their absorbance at 280 nm.

Animals and Diets. Male Sprague–Dawley rats were purchased from Bantin and Kingman (Hayward, CA), and male C57BL/6 mice were obtained from a colony maintained by the University of California's Department of Microbiology. All animals were 3–4 weeks old at initiation of the feeding regimens. Animals were equilibrated for 7 days on semipurified diets (Bieri et al., 1977), followed by assignment to experimental diets for a period of 10 (experiment 1, rats and mice) or 7 days (experiment 2, mice only). Purified indoles were added, by trituration, into progressively larger volumes of the diet. Cauliflower (*Brassica oleracea* L. var. botrytis subvar. cauliflower DC, grown in Salinas, CA) was freeze-dried and added to the powdered diets in experiment 1 as described previously (Bradfield et al., 1985). In the fractionation experiment (2), the freeze-dried cauliflower extract, or fraction thereof, was dried under reduced pressure to constant weight (Rotovapor-R; Brinkman Instruments, Westbury, NY) and added to the semipurified diet at the indicated gram-equivalent levels (1 GE/kg = extract obtained from 1 gram of freeze-dried material added/kg of purified diet). In experiment 2, all diets were "pelleted" by addition of 13% water (v/w), followed by air-drying at 4 °C for 24 h.

Extraction and Fractionation of Cauliflower. The washed vegetable was chopped finely and allowed to stand for 24 h at 4 °C prior to freeze-drying. The freeze-dried vegetable was extracted in 1:1 dichloromethane–methanol (CH_2Cl_2–CH_3OH) with stirring at room temperature for 24 h, followed by an additional extraction for 48 h with fresh solvent. The extracts were pooled, and a portion was evaporated to dryness and fed to mice at a level of 200 GE/kg, (total, 70 g/1000 GE). The remaining extract was partitioned three times between 10% aqueous CH_3OH and *n*-hexane (HEX, 30 g/1000 GE). Water was added to the aqueous CH_3OH fraction to make it 30% aqueous. It was then partitioned three times with CH_2Cl_2, yielding an upper aqueous–methanol phase (MEOH, 20 g/1000 GE) and a lower dichloromethane phase (DCM, 15 g/1000 GE). These three fractions were evaporated to dryness and fed to mice at a level of 600 GE/kg. A total of 500 GE portions of DCM were further separated on LH-20 resin (Sigma Biochemicals, St. Louis, MO) with CH_3OH as a mobile phase (column size 70 cm × 7 cm). The eluate was collected and pooled into three fractions (A, 4.6 g/1000 GE; B, 4.6 g/1000 GE; C, 1.2 g/1000 GE) according to similarity of constituents as monitored by TLC (silica gel, ethyl acetate mobile phase, visualized with *p*-(dimethylamino)benzaldehyde). These fractions were fed at 1800 GE/kg. Fractions B and C were pooled and placed on the same LH-20 column. Again three fractions were collected according to similarity of constituents and fed at 1800 GE (D, 3 g/1000 GE; E, 0.9 g/GE; F, 0.1 g/GE). Fraction E was placed on silica (60–200 mesh; Baker Chemical Co., Jackson, TN; column size 40 cm × 5 cm) and eluted in three steps [15% 2-propanol in hexane (HEXSIL, 0.6 g/1000 GE), followed by 60% 2-propanol in hexane (PROPSIL, 0.6 g/1000 GE), and finally 100% methanol (MEOHSIL, 1.5 g/1000 GE)]. These three fractions were fed at 3600 GE/kg diet.

Tissue Preparation and Enzyme Assays. Hepatic postmitochondrial supernatant and intestinal cytosolic and microsomal fractions were prepared as described previously (Bradfield and Bjeldanes, 1984). Protein was determined by the method of Bradford (1976) using a commerically available dye-binding assay, with bovine serum albumin serving as a standard (BioRad, Richmond, CA). Low- and high-K_m ethoxycoumarin *O*-deethylase activities (ECOD) were determined by the method of Greenlee and Poland (1978). Aryl hydrocarbon hydroxylase activity (AHH) was determined by the method of Nebert and Gelboin (1968). Assay conditions and the modifications of the above protocols employed in this laboratory have been reported previously (Bradfield and Bjeldanes, 1984). Ethoxyresorufin *O*-deethylase activity (EROD) was determined by a modification of method of Burke and Mayer (1974). In this assay 100–200 μg of hepatic protein was added to 2.0 mL of 0.05 M KH_2PO_4–K_2HPO_4, pH 7.8 buffer, followed by the stepwise addition of 10 μL of 0.1 M ethoxyresorufin in ethanol and 10 μL of 0.05 M $NADPH_2$. The generation of resorufin was measured fluorometrically on a Perkin-Elmer 650-10s fluorescence spectrofluorometer (Perkin-Elmer, Norwalk, CN) set at 510-nm excitation and 586-nm emmission with slit widths set at 10 nm.

Statistics. One-way analysis of variance was performed on an HP-83 microcomputer. Dunnett's test was used to determine differences at $p \leq 0.05$ and $p \leq 0.01$.

Note. *Induction* is used to denote a relative increase in enzyme activities measured in vitro as described above and does not necessarily imply an increase in de novo protein synthesis in relation to these enzymes.

RESULTS AND DISCUSSION

Experiment 1. Rat intestinal monooxygenases have proven in our hands to be quite sensitive to dietary I3C (Bradfield and Bjeldanes, 1984) and thus serve in experiment 1 as a bioassay for this indole in freeze-dried vegetables. Table I shows that rats fed I3C at 250 ppm exhibit marked induction of intestinal ECOD and AHH, whereas rats fed dietary cauliflower show no increases in these enzymatic activities. This result is consistent with the demonstration that I3C is unstable in the plant's autolytic milieu (Bradfield and Bjeldanes, 1987a) and thus is un-

Table I. Effect of Dietary Indole-3-carbinol and Cauliflower on Intestinal Monooxygenases in the Rat

	ECOD: pmol 7-OH-coumarin formed/mg protein per min	AHH: pmol 3-OH-B[a]P formed/mg protein per min
control	34 ± 17	74 ± 31
cauliflower (25%)	36 ± 10 (1.0)	95 ± 28 (1.3)
indole-3-carbinol (250 ppm)	709 ± 634[a] (20.8)	1598 ± 1499[a] (21.6)

[a] Statistically different from control at $p \leq 0.05$. Values are means ± SD, with the ratio of the group mean to the control group mean in parentheses. $N = 4$ for each group, intestines were pooled by twos.

likely to be present in significant quantities in chopped, freeze-dried vegetables. In contrast, mouse hepatic low-K_m ECOD is insensitive to dietary I3C (250 ppm) but is increased 2-fold by the cauliflower diet (Table II). This observation suggests that unidentified inducers of murine hepatic monooxygenases exist in these vegetables.

Experiment 2. Hepatic monooxygenase activity in the mouse liver was used as a bioassay to identify inducing agents present in freeze-dried cauliflower for the following reasons: (1) Results from experiment 1 suggested that monooxygenase levels in the mouse liver are responding to a dietary component other than I3C. (2) Previous results from this laboratory suggested that the murine liver may serve as a useful system for the identification of dietary inducing agents (Bradfield et al., 1985). (3) The low food intake of the mouse minimizes the mass of plant isolate required in feeding studies. (4) The genetics of xenobiotic metabolism in the murine system is well described (Nebert et al., 1981).

Figure 2 shows the time course of induction of hepatic monooxygenases in mice fed a diet supplemented with cauliflower extract at 250 GE/kg. These results indicated that 7 days of feeding led to the greatest increase in the monooxygenase activities tested. The drop in monooxygenase activities from day 7 to day 10 may be the result of increased metabolism of the inducing agent. Alternatively, this result could be explained by decomposition of inducing agents in these diets. However, feeding this same diet after 6 months of storage yielded similar day 10 results (Bradfield and Bjeldanes, 1987b).

The results of the fractionation study (experiment 2) indicate the presence of a number of different inducing agents in cauliflower, as feeding all but two of the fractions, led to significant increases in both ECOD and EROD activities. Fraction HEXSIL was chosen for a more rigorous attempt at identification of responsible constituents, as this fraction appeared to contain the simplest mixture of compounds (as monitored by TLC). The two major compounds in this fraction were purified by semipreparative HPLC (silica, 10 mm × 250 mm, 2-propanol/hexane gradient) and were found to represent 20% of the mass of this fraction. The compounds identified were I3CHO (112 mg/1000 GE) and NCHO (24 mg/1000 GE). The identities of NCHO and I3CHO were verified by comparison

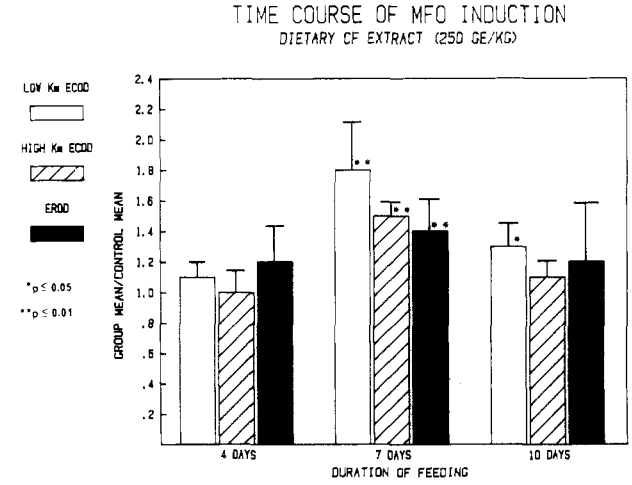

Figure 2. Time course of induction of hepatic mixed-function oxidase activity in mice fed cauliflower extract at 250 GE/kg. Control values = picomoles of product formed/milligram of protein per minute ± 1 SD. $N = 5$ animals/group.

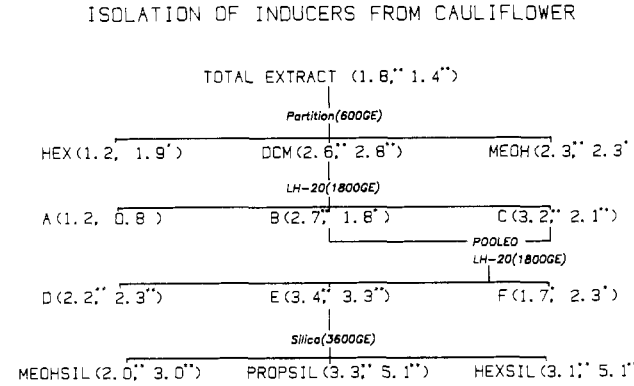

Figure 3. Hepatic mixed-function oxidase activity in mice fed various fractions of CF extract. $N = 4$–5 animals/group. Values are picomoles of product formed/milligram of protein per minute ± 1 SD.

of chromatographic characteristics (HPLC and TLC) and ultraviolet, ^{1}H NMR ($CDCl_3$), and mass spectra with authentic samples.

The identification of these indolylglucosinolate autolysis products, as well as TLC data indicating the presence of additional indolylic compounds in fraction E, led us to analyze this fraction for other known glucobrassicin autolysis products. Results from this analysis indicated the presence of I3C (10.4 mg/1000 GE), IAN (6.2 mg/1000 GE), and I33′ (4.6 mg/1000 GE). Thus, all the indoles identified account for 12% of the total mass of fraction E.

The isolation of NCHO and I3CHO from HEXSIL and the presence of IAN, I33′, and I3C in fraction E led us to compare the effects of dietary exposure of these indoles on hepatic EROD and low-K_m ECOD activities. All indoles tested were fed at 2.8 mmol/kg, a level equivalent to the estimated level of I3CHO in 3600 GE/kg (approximately 400 ppm), except I33′, which was fed at 1.4 mmol/kg (in

Table II. Effect of Dietary Indole-3-carbinol and Cauliflower on Hepatic Monooxygenases in the Mouse

	ECOD: pmol 7-OH-coumarin formed/mg protein per min		AHH: pmol 3-OH-B[a]P formed/mg protein/per min, 80 μM
	5 μM[a]	500 μM	
control	165 ± 38	365 ± 91	1071 ± 279
cauliflower (25%)	327 ± 82[b] (2.0)	583 ± 122[b] (1.6)	1028 ± 167 (0.8)
indole-3-carbinol (250 ppm)	214 ± 62 (1.3)	439 ± 141 (1.2)	891 ± 191 (0.9)

[a] Substrate concentration. [b] Statistically different from control at $p \leq 0.05$. Values are means ± SD with the ratio of the group mean to the control group mean in parentheses. $N = 4$ for each group, intestines were pooled by twos.

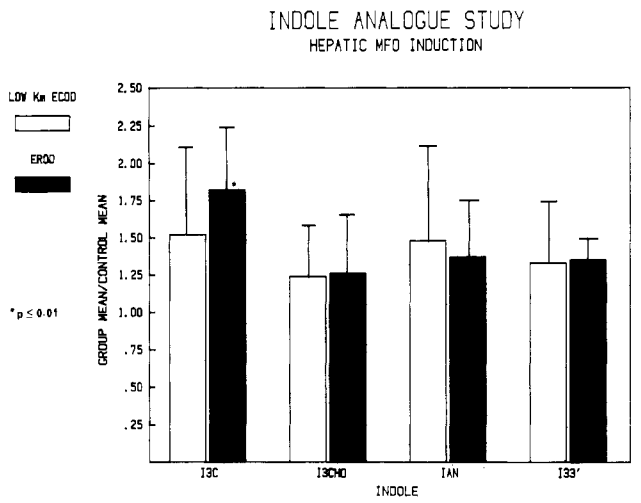

Figure 4. Effect of dietary I3C, IAN, I33′, and I3CHO on hepatic mixed-function oxidase activity in the mouse (indoles fed at 2.8 mmol/kg except I33′, which was fed at 1.4 mmol/kg). Control values are picomoles of product formed/milligram of protein per minute. Error bars represent ±1 SD. $N = 5$.

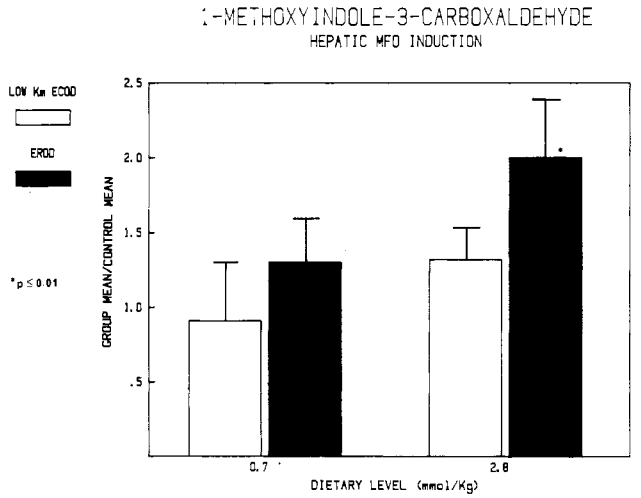

Figure 5. Effect of dietary NCHO on hepatic mixed-function oxidase activity in the mouse (fed at 0.7 or 2.8 mmol/kg). Control values are picomoles of product formed/milligram of protein per minute. Error bars represent ±1 SD. $N = 5$, except for 2.8 mmol/kg of NCHO, where $N = 4$.

consideration of the fact that I33′ contains 2 mmol of indole rings/mmol of I33′). Of the nonmethoxylated indoles, only I3C led to a significant increase in either of the monooxygenase activities tested (1.8-fold increase in EROD; Figure 4). It is of interest that although NCHO feeding did not significantly induce low-K_m ECOD, exposure to this indole at 2.8 mmol/kg led to a 2.0-fold induction in EROD activity (Figure 5), a level of induction greater than that caused by I3C at this level. The apparent lack of increased EROD activity in animals fed NCHO at 0.7 mmol/kg (the estimated level of NCHO in 3600 GE) suggests that this indole is not the major inducing agent in fraction HEXSIL. The relative potency of NCHO compared to the nonmethoxylated analogues suggests the importance of other 1-methoxyindoles (e.g., 1-methoxyindole-3-carbinol, 1-methoxyindole-3-acetonitrile, and 1,1′-dimethoxy-3,3′-diindolylmethane). It should be noted that relative "potency" as measured here is probably a function of the compound's stability in a pelleted purified diet, as well as its intrinsic ability to induce monooxygenase activity.

To our knowledge, this is the first reported isolation of NCHO from plant material. As I3CHO appears to be an oxidative product of I3C (Bradfield and Bjeldanes, 1987a; Fetizon et al., 1976), it would seem likely that NCHO arose in a similar fashion from 1-methoxyindole-3-carbinol, the putative autolysis product of 1-methoxy-indolylglucosinolate (neoglucobrassicin; Gmelin and Virtanen, 1962), which exists in a number of brassicaceous vegetables at levels as high as 180 μg/g (Sones et al., 1984).

The results presented here, as well as the previous demonstration that carbinols are the major autolysis products of indolylglucosinolates in fresh plant material, and the observation that these alcohols are oxidized to aldehydes over extended time periods (Bradfield and Bjeldanes, 1987a) are not in agreement with the suggestion by the National Research Council (1982) that IAN is the most abundant indole produced in cruciferous vegetables. This statement by the National Research Council's Committee on Diet, Nutrition and Cancer would appear to be based on the indole isolation work by Loub et al. (1975). These investigators isolated IAN in quantities greater than I3C, I3CHO or, I33′.

The isolation of 1-methoxyindole-3-carbaldehyde from *B. oleracea* is of interest for two reasons. First is the observation that dietary exposure to this methoxylated indole can lead to alterations in cytochrome P448 dependent monoxygenases. This suggests that other methoxylated indoles present in these vegetables may have similar properties (presumably, 4-methoxylated indoles also exist in these vegetables; Hanley et al., 1985; Truscott et al., 1982, 1983). Second is the presence of indolecarbaldehydes in these vegetables at levels greater than their corresponding carbinols, supporting the earlier observation (Bradfield and Bjeldanes, 1987a) that oxidation of carbinols is occurring to a considerable extent in the autolysis protocols used in this and other laboratories (Bradfield and Bjeldanes, 1984; Loub et al., 1975). This fact is significant because I3C has been demonstrated to be a much more potent inducer of intestinal and hepatic monooxygenases than I3CHO (Loub et al., 1975), and thus the resultant biological effects in animals fed dried or fresh vegetable material could be quite different. Additionally, the results presented in this paper demonstrate that vegetables of *B. oleracea* contain a number of as yet unidentified inducers of hepatic and probably extrahepatic monooxygenases. These unidentified compounds may be important with regard to the alterations in xenobiotic metabolism observed in humans and animals fed these vegetables (Miller and Stoewsand, 1983; Pantuck et al., 1979; Salbe and Bjeldanes, 1985).

Registry No. I3C, 700-06-1; I3CHO, 487-89-8; I33′, 1968-05-4; IAN, 771-51-7; NCHO, 67282-55-7; ECOD, 42613-26-3; EROD, 59793-97-4; AHH, 9037-52-9.

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Analysis of Tissue Residues and Comparative Metabolism of Virginiamycin in Rats, Turkeys, and Cattle

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Liver tissue samples from turkeys, cattle, and rats given [^{14}C]virginiamycin were examined for the presence of metabolites. Extraction of the liver was performed sequentially with methanol and pH 7.4 phosphate buffer. The methanol fraction was further partitioned into chloroform-soluble and water-soluble fractions. The majority of the total liver residue (56–73%) remained intractable following these treatments. The three extracts were fractionated by normal- or reversed-phase HPLC. The results indicated that virginiamycin was metabolized to a large number of fragments and that no single metabolite represented greater than 3.5% of the total liver residue. Due to sample limitations no metabolite identification was possible at this time. Fortification experiments indicated that little, if any, parent virginiamycin was present in the tissue. Additional balance–excretion studies conducted in cattle and rats demonstrated that the majority of the dose (83–94%) was eliminated in the feces.

Virginiamycin is produced by a mutant of *Streptomyces virginiae* and is active against gram-positive bacteria of the gut (DeSomer and Van Dijck, 1955). The antibiotic is composed of two major factors (M_1 and S_1) functioning synergistically when combined in the optimum ratio of 4:1 (M to S). The structures of factors M_1 and S_1 are shown in Figure 1.

Favorable responses in growth and/or feed efficiency have been reported when virginiamycin is supplemented in the diets of broilers (March et al., 1978; Miles, 1982; Miles et al., 1984), turkey poults (Buresh et al., 1984), and swine (Maxwell, 1984). The compound has also been shown to be effective against necrotic enteritis in broilers (Daniels, 1984) and in the management of swine dysentery (Miller et al., 1972).

We have undertaken several studies comparing the virginiamycin metabolic profile in liver tissue from rats, turkeys, and cattle. In conjunction with these studies, additional data concerning the excretion profile in rats and cattle were also obtained. The results of these investigations indicate that the metabolic profile for virginiamycin is similar among the three species studied and that the antibiotic appears to be extensively metabolized, with little, if any, parent compound being present in the tissues.

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Test Substance. The production of [^{14}C]virginiamycin is via fermentation with *S. virginiae* using radiolabeled sodium acetate, glycine, proline, lysine, and phenylalanine as precursors. Both factors become relatively uniformly labeled by this procedure. The fermentation broth is extracted with hexane to remove oils followed by ethyl acetate which contains the crude product. The solvent is evaporated, redissolved in chloroform, and purified by preparative HPLC (Lichrosorb SI-60, 10 μm, 23 mm × 50 cm column, ES Industries; chloroform–methanol–trifluoroacetic acid (98.5:1.5:0.02) mobile phase; 20 mL/min flow rate; 330-nm UV detection). Fractions cooled in dry ice were collected (factor S, 8–10 min; factor M 14–17 min) and flash evaporated. Final purification of the individual factors was achieved by recrystallization from hot methanol

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