Photolysis of Thiabendazole

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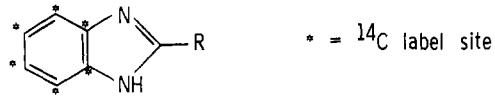
Thiabendazole labeled with carbon-14 has been employed to study the degradation of this fungicide on sugar beet leaves. Only unchanged thiabendazole was recovered from plants grown in artificial light, whereas with plants grown in sunlight only 78% of the recovered radioactivity was identified as starting compound. As the degradation appeared to arise from photolysis and not plant-induced metabolism, experiments were carried out by exposing thin films of thiabendazole on glass plates to sunlight and by irradiating aqueous methanolic solutions of thiabendazole with strong ultraviolet light. Benzimidazole and benzimidazole-2-carboxamide were shown by a combination of radiochemical, chromatographic, and spectrometric techniques to be produced by all three photolytic systems.

The anthelmintic thiabendazole (TBZ, I) possesses fungicidal properties (Robinson et al., 1969). TBZ has been shown to be effective against various fungi responsible for diseases in plants, animals, and man as well as certain strains of fungi known to produce mycotoxins. It shows little activity against bacteria, actinomyces, and yeasts. TBZ is both fungistatic and fungicidal. TBZ is active against pathogenic fungi, particularly the dermatophytes, *Microsporum* and *Trichophyton*. Also among the susceptible fungi were species of *Cladosporium, Phialosphora, Fonsecaea, Madurella, Pyrenochacta, Leptosphaera,* and *Hormodendrum*. An investigation into the chemical nature of radioactive residues on sugar beet leaves was undertaken in connection with a study on the use of this compound against fungal growth on sugar beet plants.



I, R = 

II, R = $C-NH_2$ (O)

III, R = H

As TBZ was recovered unchanged from sugar beet plants grown in a Phytotron, the plants do not metabolize the compound. However, the 20% of the recovered radioactivity was found to consist of substances other than TBZ when the plants were grown in sunlight. The degradation of TBZ on glass plates exposed to sunlight and in an aqueous methanol solution exposed to high intensity ultraviolet irradiation (xenon–mercury lamp) was used to provide a source of photolysis products for characterization. Such approaches have been employed recently in photodecomposition studies of other bioactive compounds [Archer et al., 1972; Cheng et al., 1972; Mazzocchi and Rao, 1972; Moilanen and Crosby, 1972; Powers, 1971; Rosen and Siewierski, 1972; Su and Zabick, 1972]. We wish to discuss the use of complementary radiometric, chromatographic, and spectrometric techniques in the identification of two TBZ degradation products, and the demonstration of the presence of these compounds on sugar beet leaves by use of a reverse isotope dilution assay (RIDA).

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EXPERIMENTAL SECTION

Sugar Beets Sprayed with ^{14}C-Labeled TBZ and Grown in a Phytotron and Outdoors. Materials. The radioactive compounds used in this study were synthesized in the Merck Sharp & Dohme Research Laboratories. The sugar beet (*Beta vulgaris* L. Commercial va. HH8, Holly Sugar Corporation, Colorado Springs, Colo.) was used in this study.

Experimental Design. The sugar beets were planted in 5-oz paper cups (3 seeds/cup) in an artificial soil mixture (W. Atlee Burpee Co., Philadelphia, Pa.). The sugar beet seedlings were subsequently transplanted into 12-oz cups and finally into 4.5 in. plastic flower pots. In both transplanting operations, 1 plant per cup or pot was made. The plants were watered as needed and fertilized with a fluid fertilizer (Miracle-Gro 15–30–15, 1 tablespoon per gal of water, Stern's Nurseries, Inc., Geneva, N.Y.).

Except where noted, the plants were grown in a Phytotron (Model PT 80, Percival Refrigeration and Manufacturing Company, Inc., Des Moines, Iowa).

Ten plants were selected for treatment. The plants were then randomized. Each plant was assigned a number from 1 to 10. The plants numbered 2 to 10 were sprayed with ^{14}C-labeled TBZ in Triton X-100. Plant number 1, the control, was sprayed with 100 ppm of Triton X-100. Plants numbered 2 and 3 were harvested 30 min after the spray was applied, and plants numbered 4 to 10 were harvested 34 days later.

Plants numbered 5, 6, 7, 8, and 10 were placed outdoors on 16 days for periods ranging from 4 to 9 hr each day, equivalent to 14 8-hr days of sunlight. The plants were only set out on days in which the Weather Bureau indicated a 20% or less probability of rain. The plants were placed in an open area and were not shaded during exposure to sunlight. In order to simulate natural conditions, the plants were not placed under glass or plastic sashes. Plants numbered 4 and 9 were not placed outdoors but were retained in the Phytotron.

Experimental Procedures. *Preparation of TBZ Spray.* A suspension of TBZ was prepared by grinding the required amount of the radioactive and nonradioactive compound in a Teflon–glass tissue grinder containing an aqueous solution of 100 ppm of Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). A fine suspension was achieved with most TBZ particles less than 10 μ in size. The concentration of TBZ in the suspension was 980 ppm and the radioactivity was 1.9 μCi/g of suspension. The specific activity of TBZ was 1.9 μCi/mg.

Application of TBZ Spray. A hand atomizer with a squeeze bulb was used to spray both surfaces of the sugar beet leaves. The quantity of spray applied was estimated by recording the weight loss of the sprayer as the spray mix was used. Two to 2.2 g of suspension was applied to each

plant. The pot and soil of each plant were completely covered with aluminum foil. Also, absorbent cotton was wadded between the petioles of the sugar beet leaves to prevent coalesced spray droplets from running down the stem and onto the tap root. Approximately 10 min was required to spray a plant, and the droplets appeared to evaporate within 20 min.

Harvest and Storage of Plants and Samples. The plants were either harvested immediately after the TBZ application or 34 days later. Old, dead leaves were removed from each of the 34-day plants and stored in polyethylene bags. The number of leaves removed per plant ranged from 0 to 4 leaves. These leaves were removed as a safeguard against possible loss due to wind action. At harvest the leaves of each plant were cut off individually and placed in a plastic bag.

Radioactivity Measurements. Radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrophotometer. Solutions were counted in a scintillation solution containing Liquifluor in a 70:30 toluene–ethanol mixture. The counts per minute/disintegrations per minute counting efficiency was 76%. Solids were analyzed by combustion to $^{14}CO_2$ by the Peterson procedure [Peterson et al., 1969; Peterson, 1969].

Preparation of Samples for Reverse Isotope Dilution Assay (RIDA). The tops from the two plants which had been grown only in the Phytotron were added to about 1 l. of a Dry Ice–acetone slurry contained in a 1-gal Waring Blendor. The slurry was homogenized at high speed for about 2 min. After homogenization, the slurry was allowed to stand at room temperature until most of the CO_2 had evolved. It was then transferred to a round-bottomed flask, and the acetone evaporated at reduced pressure (water aspirator) and 40–50°. When nearly all the acetone had been removed, the flask was pumped at about 1 mmHg at 30° for 2 days. The product, which had fused slightly during drying, was readily reduced to a fine powder with a mortar and pestle. Tops from the five plants, which had been placed outside for 16 days, were combined and processed in a similar manner.

The total radioactivity of the powder was determined by a combustion method. Samples weighing 20–40 mg were burned in an atmosphere of pure oxygen; the liberated $^{14}CO_2$ was trapped in Hyamine T-X and the radioactivity of an aliquot was determined by scintillation counting.

RIDA for TBZ. The reverse isotope dilution assay (RIDA) for the TBZ content of sugar beet tops grown only in the Phytotron was run according to the following procedure.

Forty milliliters of 1.0 *N* HCl and 79 mg of pure TBZ were added to 367 mg of powdered tops containing 7.74×10^4 cpm of radioactivity (equivalent to about 24 μg of [^{14}C]TBZ) and the mixture was refluxed overnight. To the cooled suspension was added 160 ml of methanol, which was then filtered. The filtrate and washes were combined, concentrated at reduced pressure to near dryness, taken up in water, and neutralized with aqueous NaOH. The solution was then extracted with 3 × 75 ml of ethyl acetate. The ethyl acetate solution was back extracted with 3 × 30 ml of 0.1 *N* HCl; the combined HCl extract was concentrated in vacuo to dryness and then taken up in 25 ml of 0.1 *N* HCl. The solution was neutralized with 1 *N* NaOH and extracted three times (50 + 25 + 25 ml) with ethyl acetate. The ethyl acetate extracts were combined and extracted three times (10 + 5 + 5 ml) with 0.1 *N* HCl. The combined extract was neutralized with aqueous 1 *N* NaOH to yield a crystalline precipitate, which was filtered and dried in a vacuum oven. The yield of recovered TBZ was 57.5 mg.

The specific activity of the recovered TBZ was determined by dissolving two samples (3.95 and 4.48 mg) in methanolic HCl and measuring the radioactivity by scintillation counting. The average specific activity of these samples was 968 cpm/mg. Recrystallization of the isolated TBZ by neutralization of an acid solution after treatment with charcoal gave a product with the same specific activity. Based on this assay, TBZ accounted for 98% of the total radioactivity in the plant tops.

The ir, uv, and TLC of the isolated product indicated that the sample was essentially pure TBZ as required.

In a similar manner, a 378.1-mg sample of powdered tops from a composite of the plants grown outdoors and containing 6.50×10^4 cpm of radioactivity (equivalent to about 21 μg of [^{14}C]TBZ) was mixed with 81.4 mg of TBZ and processed as described. The yield of isolated TBZ was 63.7 mg with a specific activity of 628 cpm/mg. Based on this assay 78% of the radioactivity in these plant tops was present as TBZ.

RIDA for Benzimidazole. A 3.15-g sample of acetone-desiccated leaf powder from plants grown in sunlight was mixed with 114.4 mg of benzimidazole in about 500 ml of 80% methanol–20% water and heated in a steam bath. The slurry was filtered, the filtrate evaporated in vacuo to dryness, and the residue taken up in a small volume of 0.25 *N* HCl. The extract was diluted with a few milliliters of water, the pH adjusted to 1.0, and the aqueous solution extracted with ethyl acetate. The ethyl acetate solution was discarded. The pH of the aqueous phase was adjusted to 7.0 and the solution extracted with ethyl acetate. This extract was evaporated to dryness and the crude benzimidazole crystallized from benzene after treating with charcoal. The specific activity of the isolate was 121 cpm/mg. By repeated crystallization from benzene and by preparative scale TLC, a product with a constant specific activity of 37 cpm/mg was obtained. This is equivalent to a benzimidazole content of 0.84% of the total radioactivity in the sugar beet leaves.

The uv spectrum of a TLC eluate (R_f 0.37 in benzene–ethanol (80:20)) established that the radioactive isolate was essentially pure benzimidazole.

RIDA for Benzimidazole-2-carboxamide. A 1.07-g sample of acetone-desiccated leaf powder from plants grown in sunlight was mixed with 96.2 mg of benzimidazole-2-carboxamide in about 500 ml of 80% methanol–20% water and warmed on a steam bath. The slurry was filtered and the filtrate evaporated to dryness in vacuo. The residue was taken up in a small volume of ethyl acetate and washed with water. The ethyl acetate extract was then evaporated to dryness and the crude benzimidazole-2-carboxamide crystallized from acetonitrile.

The specific activity of the first crystalline product was 544 cpm/mg. After four more crystallizations from acetonitrile with the use of charcoal, the specific activity of the product dropped to a constant 21 cpm/mg, equivalent to 1.17% of the original radioactivity in the beet leaves present as benzimidazole-2-carboxamide.

Photolysis of [^{14}C]TBZ on Glass Plates. *Preparation of TBZ Coated Plates.* A methanol solution was prepared containing 5.0 mg of ^{14}C-labeled TBZ per ml with a specific activity of 0.065 μCi/mg or 1.07×10^5 cpm/mg. Four milliliters of this solution containing 20 mg of thiabendazole (or 2.14×10^6 cpm) was spread over the surface of a 10 in. × 16 in. glass plate. The methanol was allowed to evaporate and the plates were laid out in a conventional greenhouse with the roof closed. Periodically, some of the glass plates were removed and the TBZ and photolysis products recovered by extraction.

A control plate, coated in the same manner as the others, was covered with aluminum foil and placed in the greenhouse.

Solvent Extraction of the Glass Plates. The glass plates were carefully flooded with methanol and wiped with glass wool wet with methanol in order to recover the TBZ and photolysis products. The plates were then wiped with glass wool wet with a solution of 2 vol of methanol/1 vol of 1 *N* HCl. The radioactivity in each solution was measured by

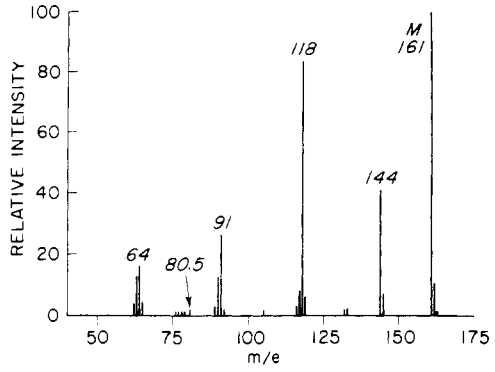

Figure 1. Mass spectrum (direct inlet) of benzimidazole-2-carboxamide isolated from the thiabendazole glass plate photolysis reaction product.

scintillation counting and the total recovery of TBZ plus photolysis products calculated.

The methanol solutions were examined directly by TLC and MS. Aliquots of the methanol solutions were evaporated to dryness and the residues partitioned between ethyl acetate–water followed by a partition between 1-butanol–water. The ethyl acetate and butanol solutions were also examined by TLC and MS.

Thin Layer Chromatography (TLC) and Autoradiography. Two-dimensional TLC analyses were run on 8 in. × 8 in. Analtech GF (silica gel) plates, 250 μ in thickness. A spot containing about 280 μg equiv of TBZ was applied in one corner. The plate was developed in one direction with benzene–dioxane–ammonium hydroxide (10:80:10, v/v) and, after drying, in the second direction with 1-butanol–water–acetic acid (65:25:10, v/v). The plates were examined under ultraviolet light for uv absorbing compounds which showed up as dark spots. A typical plate showed about five–eight distinct spots of differing intensities. The autoradiography was carried out by placing the TLC plate against an 8 in. × 10 in. sheet of medical X-ray film (e.g., Kodak-No Screen no. NS54T) and storing in the dark for about 1 week. The film was removed and developed by the standard process. The zones of radioactivity on the TLC plate appeared as gray to black spots on the X-ray film, the size and darkness of the spot indicating the relative amount of radioactivity in the zone. A tight spot on the TLC containing the equivalent of about 1 μg of TBZ (107 cpm) could be detected on the X-ray film after 1 week's exposure to the TLC plate.

Isolation and Identification of Benzimidazole-2-carboxamide. A glass plate which had been exposed for 128 days in the greenhouse was washed with methanol. An aliquot of the methanol containing 5.5×10^5 cpm (equivalent to 5.2 mg of TBZ) was concentrated to 3 ml of methanol, transferred (with 2 ml of methanol wash) to a separatory funnel, diluted with 50 ml of water, and extracted six times with equal volumes of carbon tetrachloride. The extraction removed essentially all the TBZ present. The aqueous phase, which contained 28% of the original radioactivity, was then extracted with two equal volumes of ethyl acetate. This extract contained 11% of the original radioactivity.

The ethyl acetate solution was taken to dryness and triturated successively with isooctane and carbon tetrachloride. The residue was extracted into methylene chloride. A crystalline product was formed on reduction of the methylene chloride volume and was found to possess the following spectral properties: uv $E_{290\ nm}^{1\%,1\ cm}$ (MeOH) 348 nm; ir 1680 (carbonyl), 1620 (NH_2 bending), 1580, 1520, and 1480 cm^{-1} (unsaturated ring). This product was identified as benzimidazole-2-carboxamide on the basis of infrared and mass spectroscopic (vide infra; see Figure 1) data.

Table I. Volatility of Thiabendazole and Benzimidazole-2-carboxamide[a]

Temp, °C	Conditions	Time, hr	Percentage loss of compound: Thiabendazole	Percentage loss of compound: Benzimidazole-2-carboxamide
110	Forced air	1	95	70
		3	96	96
		6	97	97
53	Vacuum (~1 mmHg)	16	93	28
50	Atmospheric pressure	16	4	5
		126	22	8

[a] Measured by loss in radioactivity which occurred on exposing 1 μg of the ^{14}C-labeled compounds on a micro glass slide.

High Intensity Ultraviolet Irradiation. A 3.2-mg sample of ^{14}C-labeled TBZ was dissolved in 1.0 l. of 80% water–20% methanol. The total radioactivity was 3.4×10^6 cpm. The solution was exposed to a high intensity xenon–mercury lamp and the course of photolysis followed by a uv monitor. After 1-hr exposure, the uv spectrum of the solution showed a substantial change with little of the TBZ absorption peak at 300 nm remaining.

A portion of the reaction solution was evaporated to dryness and the residue partitioned between ethyl acetate and water. The ethyl acetate solution contained 52% of the radioactivity of the original sample.

Examination of the ethyl acetate extract by TLC suggested the presence of benzimidazole-2-carboxamide and TBZ. The amount of benzimidazole-2-carboxamide was estimated by elution of the radioactive zone from the TLC plates to be 25–30% of the total TBZ originally used. The amount of remaining TBZ was estimated to be 10–15% based on TLC data.

Combined gas–liquid chromatography/mass spectrometry of a trimethylsilylated aliquot of the reaction mixture demonstrated not only the presence of TBZ and benzimidazole-2-carboxamide but also a small amount (~1%) of benzimidazole.

Volatility of Thiabendazole and Benzimidazole-2-carboxamide. Two microliters of methanol solutions containing about 1 μg of ^{14}C-labeled TBZ and about 1 μg of ^{14}C-labeled benzimidazole-2-carboxamide, respectively, was applied to 1.5-cm micro cover glasses. The coated glasses were exposed to the test conditions (see Table I) and then immersed in a phosphor solution for scintillation counting. From the results (also shown in Table I) it is obvious that both TBZ and benzimidazole-2-carboxamide are significantly volatile.

Mass Spectrometric Examination of Samples. An LKB Model 9000 instrument was employed for these studies. Aliquots (approximately 1 μg of mass based on the TBZ radioactivity) of the appropriate samples were subjected to direct probe mass spectrometry in the usual fashion and identification made on the basis of molecular ion and fragmentation pattern. Analysis was also carried out by combined gas–liquid chromatography/mass spectrometry with a 4 ft × 3 mm i.d. glass column containing 6% F-60 stationary phase on 80–100 mesh acid washed and silanized Gas-Chrom P. For these experiments, the samples were treated with bis(trimethylsilyl)acetamide (60°; 30 min) to form the trimethylsilyl derivatives. An aliquot of the reaction mixture was then injected into the combined instrument and mass spectra obtained for the component(s) as eluted from

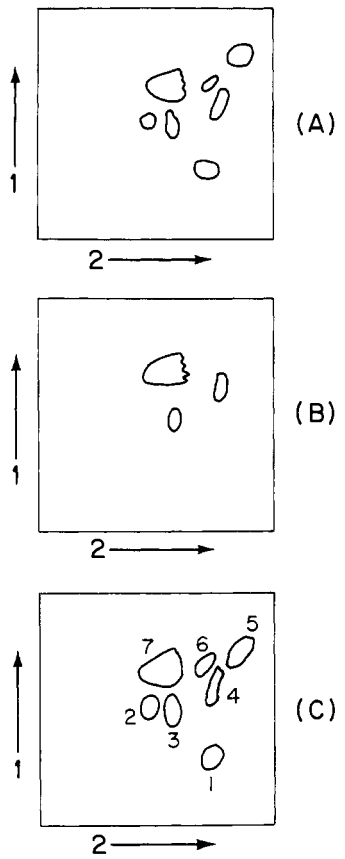

Figure 2. Thin-layer plate resulting from two-dimensional chromatography of ethyl acetate extract of TBZ glass plate photolysis reaction product: (A) uv visualization; (B) X-ray film visualization; (C) plate following scraping of zones showing spot size and identifying numbers of fractions listed in Table I.

the column (temperature programmed from 100 to 200° at 5°/min). Identification was made by inspection of the spectra and by direct comparison with standards.

RESULTS AND DISCUSSION

Sugar beet plants were sprayed with an aqueous suspension of [^{14}C]TBZ and grown in a Phytotron under incandescent and fluorescent illumination for 34 days. The plants were harvested and the leaves separated and analyzed for TBZ by the reverse isotope dilution procedure (RIDA). Of the total radioactivity on the leaves (about 95% of that applied), 97–98% was accounted for as unchanged TBZ. Thus, in the absence of sunlight there is essentially no photolytic decomposition of TBZ, nor do growing sugar beet plants translocate or metabolize TBZ supplied to the leaf.

In an experiment similar to that described above, sugar beet plants were sprayed with ^{14}C-labeled TBZ and then exposed to sunlight for the equivalent of 14 8-hr days. A reverse isotope dilution assay of the leaves accounted for 78% of the total radioactivity present as TBZ (total radioactivity recovered was about 76% of that applied). The remaining 22% represented chemically altered products which appeared to be induced by exposure to direct sunlight.

Preliminary attempts to isolate TBZ transformation products from the leaves of plants exposed to sunlight proved unsuccessful. As the formation of TBZ-related substances on the leaves was dependent upon irradiation with sunlight and not on metabolism by the growing plant, the transformation was investigated using glass plates coated with thin films of [^{14}C]TBZ exposed to sunlight in a conventional greenhouse. Unchanged TBZ was by far the major component in the plate residues. The percentage of TBZ decreased with exposure from 95% at 7 days to 60% at 128 days. The film of organic matter from one plate, exposed for 128 days, was dissolved in methanol. Selective extraction procedures yielded finally an ethyl acetate extract containing radioactive transformation products. Further fractionations of this crude material yielded a product, II, with infrared characteristics suggesting a primary amide function.

Table II. Ethyl Acetate Extract of TBZ-Coated Glass Plate, 128-Day Exposure. Radioactivity Recovery from Two-Dimensional TLC[a]

Zone	cpm	% distribution of radioact. eluted from TLC plate	Identification
1	0	0	
2	0	0	
3	575	2.1	Benzimidazole
4	1,600	5.9	Benzimidazole-2-carboxamide
5	0	0	
6	0	0	
7	24,650	90.6	TBZ
Origin	380	1.4	

[a] Ninety-one percent of the applied radioactivity was recovered from the TLC plate.

The mass spectrum of II is presented in Figure 1. The molecular weight of the compound appears to be 161, and the presence of a primary amide group, suggested by ir, is supported by the signal at *m/e* 144 (M − 17). On the basis of these data II was identified as benzimidazole-2-carboxamide. Authentic amide was subsequently synthesized and the assignment of this structure to the photolysis product verified. The synthetic sample was then used as a carrier in reverse isotope dilution assays to determine the amount of II produced in the sugar beet leaves (14 days sunlight) and glass plate (128 days) experiments. Benzimidazole-2-carboxamide was found to account for 1.2% of the total radioactivity in the sugar beet experiment and for 3.6% in the glass plate experiment.

As no transformation products other than the carboxamide were obtained by classical isolation procedures from the glass plate experiment, another approach was investigated. A portion of the methanol extract from the 128-day exposure plate was taken to dryness and the residue partitioned between ethyl acetate and water. Of the total radioactivity in the methanol extract, 58% was recovered in the ethyl acetate. An aliquot of the ethyl acetate solution (equivalent to about 280 μg of thiabendazole) was spotted on silica gel phosphor TLC plates and developed in two dimensions. Detection of the uv absorbing spots and the autoradiography of the TLC plate have already been described.

A drawing of the TLC plate viewed under uv light and resulting from analysis of the ethyl acetate extract is seen in Figure 2A; the zones indicate the presence of uv-absorbing compounds. The corresponding X-ray film (Figure 2B) shows that all the radioactive zones on the TLC plate show a corresponding uv spot, suggesting that all the photolysis products are benzimidazole like. A number of uv-absorbing spots are not radioactive, suggesting these to be uv-absorbing impurities deposited on the glass plates during the long exposure in the greenhouse. A third drawing (Figure 2C) shows the TLC plate after the uv-absorbing spots had been scraped off. The silica gel from these areas was extracted successively with methanol and methanolic hydrochloric acid, and the eluates examined. The amount of radioactivity contained in each eluate (number corresponding to the number on the plate) is listed in Table II. The eluates were

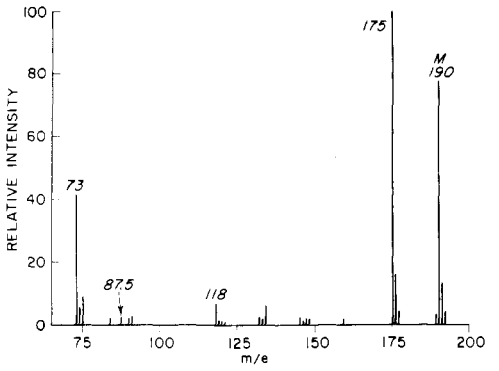

Figure 3. Mass spectrum (GLC–MS) of benzimidazole (as the trimethylsilyl derivative) from the ethyl acetate extract of the thiabendazole glass plate photolysis reaction product.

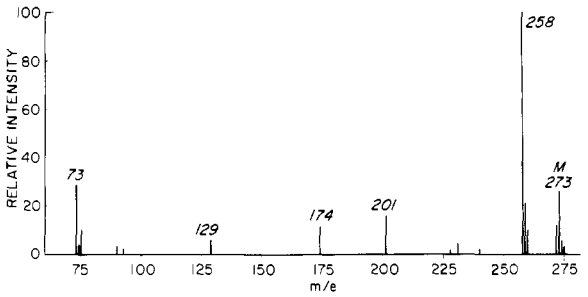

Figure 4. Mass spectrum (GLC–MS) of thiabendazole (as the trimethylsilyl derivative) from an aqueous methanolic solution of thiabendazole irradiated by a xenon–mercury lamp.

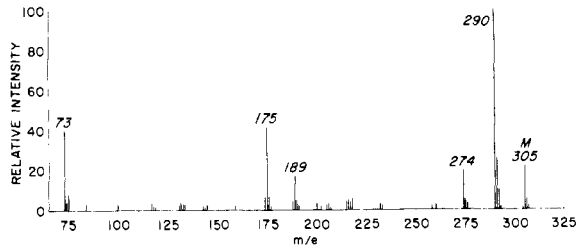

Figure 5. Mass spectrum (GLC–MS) of benzimidazole-2-carboxamide (as the bis(trimethylsilyl) derivative) from thiabendazole irradiated by a xenon–mercury lamp.

also examined by direct probe mass spectrometry. Not unexpectedly, benzimidazole-2-carboxamide and TBZ were positively identified (as zones 4 and 7, respectively). The compound in the eluate from zone 3 was found to be benzimidazole. The presence of these three compounds in the ethyl acetate extract of the plate residue was demonstrated by combined gas–liquid chromatography/mass spectrometry of an aliquot of a trimethylsilylation mixture of this extract. Peaks were observed at the appropriate retention times, and the corresponding mass spectra matched those of the trimethylsilylated reference standards. The mass spectrum of the trimethylsilyl derivative of the benzimidazole produced during the glass plate experiments is presented in Figure 3. The spectrum is dominated by the molecular ion (*m/e* 190) and the M − 15 ion (loss of a methyl from the Me_3Si group) at *m/e* 175. A doubly charged M − 15 ion is found at *m/e* 87.5. The ion of *m/e* 73 is the familiar $[(CH_3)_3Si]^+$.

Radioactivity measurements indicated a gradual loss of TBZ (and related compounds) from the glass plates on continued exposure in the greenhouse. To verify the possibility of loss by sublimation, the volatility of ^{14}C-labeled TBZ and benzimidazole-2-carboxamide was measured. The data in Table I show that even at 50° the losses due to volatilization are significant. Therefore, the lowered radioactivity recovery results from evaporation on long storage.

Table III. Products Formed in the Photolysis of TBZ

% of total recovered radioact. present as	Sugar beet plants, 14-days sunlight[a]	Glass plates, 128-days sunlight[a]	High-intensity uv, 1 hr[a]
Thiabendazole	78[b]	~60[c]	10–15[c]
Benzimidazole	0.8[b]	~1[d]	~1[d]
Benzimidazole-2-carboxamide	1.2[b]	3.6[b]	25–30[c]
Polar products and polymer	20	N.A.[e]	N.A.[e]

[a] Exposure time. [b] RIDA. [c] TLC and GLC-MS. [d] GLC-MS. [e] N.A., not assayed.

The photochemistry of TBZ was further investigated by irradiating an aqueous methanolic solution of this compound with strong ultraviolet light. Examination of the photolysis products showed the presence of unreacted TBZ, benzimidazole-2-carboxamide, and a trace of benzimidazole (see Figures 4 and 5). The base peak in the mass spectrum of the Me_3Si derivative of TBZ (Figure 4) is found at *m/e* 258, and arises via loss of a Me_3Si methyl group from the molecular ion (*m/e* 273). An $[M - 15]^{2+}$ is again observed (*m/e* 129). The ion of *m/e* 201 is probably the molecular ion of TBZ, a small amount of which can be formed during chromatography by replacement of the Me_3Si group by a hydrogen atom. Loss of HCN from the molecular ion of TBZ would yield the ion of *m/e* 174. The mass spectrum of the bis(trimethylsilyl) derivative of benzimidazole-2-carboxamide exhibits a molecular ion at *m/e* 305, with the base peak at *m/e* 290 (loss of a Me_3Si methyl). The ions of *m/e* 189 and 175 are M − $CONHMe_3Si$ and M − $[CH_3 + CONMe_3Si]$, respectively. Labeling experiments with normal and perdeuterio BSA have demonstrated that the ion of *m/e* 274 is M − $(CH_3 + CH_4)$, loss of two Me_3Si methyl groups and one non-Me_3Si hydrogen. A metastable ion is observed at *m/e* 258.5 for the transition M − 15 → M − [15 + 16].

The photolysis of TBZ in three different systems—on sugar beet leaves and glass plates exposed to sunlight, and in aqueous methanolic solution exposed to high intensity uv radiation—is summarized in Table III. These results demonstrate the practical utility of combining complementary techniques, such as radioactive labeling, TLC, autoradiography, gas chromatography (GC), and MS in the determination of reaction products at the microgram level.

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Electron-Donor and Affinity Constants and Their Application to the Inhibition of Acetylcholinesterase by Carbamates

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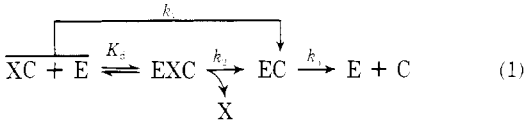
Twenty-three methylcarbamates were studied, and the effects of variations in their hydrophobicity and ability to form charge-transfer complexes upon their affinity, reactivity, and overall potency for acetylcholinesterase were explored. Variations in these factors accounted for most of the variation in enzymic effects, but the compounds fell into two distinct classes with respect to the relation between, for instance, reactivity and affinity.

Most carbamates inhibit acetylcholinesterase (AChE) by reacting with it in two steps (Wilson et al., 1961; O'Brien et al., 1966; O'Brien, 1968). The first step is characterized by the dissociation constant for the reversible complex of AChE and the carbamate (Michaelis complex); the second involves carbamylation of a serine hydroxyl in the AChE active site and is usually characterized by the reaction constant, k_2. If C is the methylcarbamyl group and X is the leaving group, the inhibition process may be represented as follows:



$$XC + E \rightleftharpoons EXC \rightarrow EC \rightarrow E + C \quad (1)$$

where k_3 is the rate constant of enzyme regeneration. The overall potency of the carbamate is measured by k_i, which equals k_2/K_d (Main, 1964).

In para-substituted phenyl methylcarbamates, the variation in potency (for instance, as measured by I_{50}, the concentration of a carbamate to inhibit AChE by 50% in a fixed time) is primarily due to variation in K_d (O'Brien et al., 1966). For 12 such compounds, we showed that their I_{50} values were closely correlated with the ability of their aromatic portions to donate electrons to a model electron acceptor (tetracyanoethylene [TCNE]) and form charge-transfer complexes (CTC) (Hetnarski and O'Brien, 1972). The CTC formation ability is measured by K_x, the association constant for CTC formation. Later we showed (Hetnarski and O'Brien, 1973) that for seven arylmethyl methylcarbamates, which are virtually noncarbamylating, and therefore simple reversible inhibitors, their K_d and K_x values were excellently correlated.

In the present study we have extended this approach to additional para-substituted aromatic methylcarbamates and also to nine meta-substituted compounds. We have also introduced new constants, the electron-donor and affinity factors, to simplify the quantitation of structure–activity relation, and to examine the processes in question from the viewpoint of the linear free-energy relationship. The current work also includes investigations on kinetic constants of the second stage (k_2), and hence covers the whole inhibition process, characterized by k_i.

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METHODS

K_d values were determined by the zero-time method (O'Brien, 1968; Hart and O'Brien, 1973). The assays were performed at 25° by the following procedure, modified from Ellman et al. (1961). A mixture was prepared consisting of 15 vol of buffer (0.2 *M* sodium phosphate, pH 7.6), 0.2 vol of acetylcholine chloride (Sigma) designed to give a final concentration of $0.73K_m$ and $0.88K_m$ ($K_m = 0.29 \times 10^{-3}$ *M*, found under conditions described here) in freshly distilled water, and 1 vol of a 0.014 *M* solution of Nbs_2 [5,5′-dithiobis(2-nitrobenzoic acid)] plus 0.02 *M* sodium carbonate in 0.2 *M* sodium phosphate buffer (pH 7). Of this mixture, 1.62 ml was placed into the cuvette and 1 ml of 1.5 units/ml of bovine erythrocyte AChE (Sterwin) solution in the aforementioned buffer solution (pH 7.6) was added; the final concentration of enzyme was 0.57 unit/ml, of Nbs_2 0.53 m*M*, and of sodium phosphate (the dominant ions) 0.2 *M*. The final pH was 7.56. The reaction was followed over 0.5 min at 412 nm using a Beckman Acta III spectrophotometer, then 0.02 ml of a freshly prepared solution of inhibitor was added, the contents of the cuvette were mixed instantly, and readings were taken at 412 nm (Figure 1). In order to find K_d values we plotted i (inhibitor final concentration) against$(v_c/v_0) - 1$, where v_c and v_0 are the reaction velocities in the absence and presence of an inhibitor, respectively. Four or five concentrations of inhibitor were used. The v_0 data were obtained by extrapolating velocities of the substrate hydrolysis to zero time, using the plot of the logarithm of hydrolysis rate as a function of time (Main, 1967) (Figure 2). The inhibitor concentrations ranged between 10^{-3} and 10^{-6} *M*, depending on solubility and potency; the actual range for each compound is in Table II.

K_x values (association constants of CTC between aryl methylcarbamates and TCNE) were determined by the Benesi–Hildebrand (1949) method in 1,2-dichloroethane at 23° (Hetnarski, 1964, 1965). The method involved the preparation of a series of concentrations of carbamates (acting as donors) in solvent, with mole fractions of from 0.003 to 0.007. Each was made 5×10^{-3} *M* with respect to TCNE. The resultant absorption was measured and obeyed