vide a means of transferring information from one series to another.

This scheme should not be looked upon as a means for identifying the best single compound. Data from larger series of analogs would be expected to suggest a few desirable substituents at more than one position. The rank order of related substituents within a position would be expected to have meaning. Some solutions should suggest untried substituents as good leads.

The proposed models should not be criticized as ignoring the combination of several substituents that produces a biological response far in excess of the additive estimation. Such results will appear in some analog series. Such situations might be identified by a graph of the individual differences of "estimated response minus actual response" for all compounds.

Successful solutions can provide reasonable estimates of inherent variation within the testing system. These may not otherwise be available without repeated testing of the same compounds. Solutions that fail can suggest that the substituents may not be altering the desirable performance characteristics of the analogs.

The suggested mathematical models do not compensate for the three dimensionality of compounds, pH, pK_a, or other similar physical properties. Perhaps, in time, these can be built into the models for better estimation.

The Metabolic Fate of Thiabendazole in Sheep[1]

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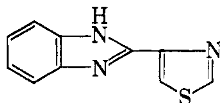
The synthesis of C^{14}- or S^{35}-labeled thiabendazole is described. Following oral administration of this anthelmintic to sheep (50 mg./kg. of body weight), the physiological disposition and metabolic fate of the compound have been investigated. The animals were sacrificed from 6 hr. to 30 days after dosage and the distribution of the drug was studied in urine, feces, plasma, and tissues using liquid scintillation or gas-flow counting. Sheep excrete approximately 75% of the dose in the urine and 14% in the feces in 96 hr. Although thiabendazole is distributed throughout most tissues of the body, only fractional parts per million were detectable in tissue after a few days. The major metabolites were isolated from urine and identified as 5-hydroxythiabendazole which exists either free or conjugated as the glucuronide or sulfate.

Thiabendazole [2-(4′-thiazolyl)benzimidazole], a compound having the following chemical structure



is a new and highly effective drug used in the treatment of helminthiases. The compound has a broad anthelmintic spectrum affecting numerous gastrointestinal roundworms and certain tapeworms.[2,3] More recently the effectiveness of this drug on trichinosis in mice, rats,[4] and swine[5] has been reported.

The present report concerns itself with the absorption, excretion, metabolic transformation, tissue distribution, and retention of thiabendazole in sheep. Radioisotopically labeled drug has been utilized as a guide in the isolation of the various metabolites and to follow its physiological disposition. It is shown that the drug is metabolized in part to a compound which is hydroxylated in the benzimidazole ring. Further metabolism of this hydroxylated product results in the formation of its glucuronide and sulfate ester.

Experimental

Materials and Methods. Synthesis of C^{14}-Labeled Thiabendazole.—Starting material for the synthesis of thiabendazole labeled with C^{14} in the benzene ring portion of the molecule was uniformly ring-labeled aniline[6] (I). Aniline hydrochloride reacted smoothly with oxalyl chloride in boiling benzene to give oxanilide[7] (II). Using a modification of a procedure disclosed in the patent literature,[8] the oxanilide was sulfonated, nitrated, and hydrolyzed to give crude *o*-nitroaniline (III). After purifying the *o*-nitroaniline by crystallization and sublimation, this intermediate was caused to react with 4-thiazolecarbonyl chloride to give the corresponding nitroanilide[9] (IV). Catalytic reduction of the *o*-nitroanilide gave the corresponding aminoanilide (V), which, upon refluxing with acid, cyclized to the hydrochloride of thiabendazole. The free base, thiabendazole (benzene ring carbon-14) (VI), was liberated by treatment of the hydrochloride in water with sodium bicarbonate.

Oxanilide Ring C^{14} (II).—To 76 ml. of azeotropically dried benzene was added 5.30 g. (0.04 mole) of I (30.0 mc. of C^{14})

(1) A preliminary report was presented before the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 14–18, 1962.

(2) H. D. Brown, A. R. Matzuk, I. R. Ilves, L. H. Peterson, S. A. Harris, L. H. Sarett, J. R. Egerton, J. J. Yakstis, W. C. Campbell, and A. C. Cuckler, *J. Am. Chem. Soc.*, **83**, 1764 (1961).

(3) A. C. Cuckler, *J. Parasitol.*, **47**, 37 (1961).

(4) W. C. Campbell, *ibid.*, **47**, 37 (1961).

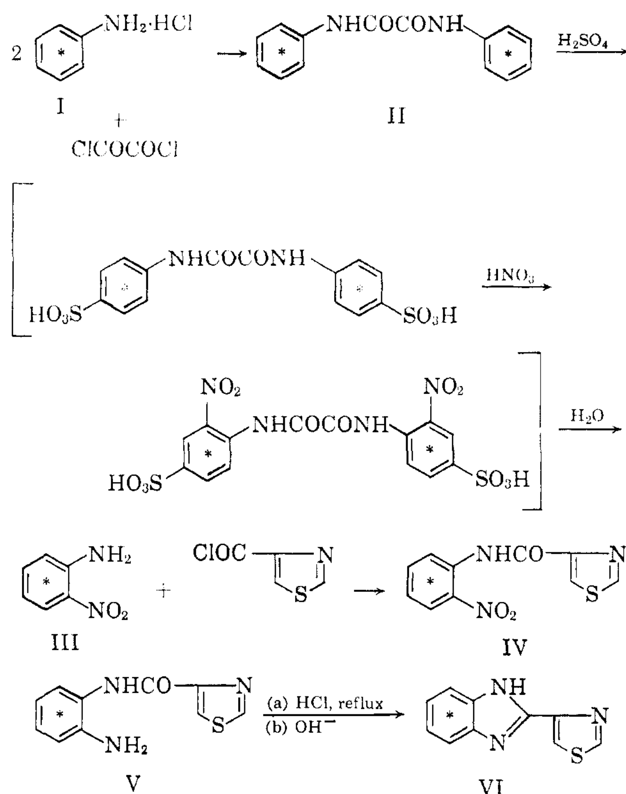
(5) W. C. Campbell and A. C. Cuckler, *Proc. Soc. Exptl. Biol. Med.*, **110**, 124 (1962).

(6) Aniline-C^{14} (uniform) was supplied by Merck & Co., Limited, Montreal, Canada.

(7) M. J. Th. Bornwater, *Rec. Trav. Chim.*, **31**, 105 (1912).

(8) Anilinoel-Fabrik A. Wulfung, Elberfeld, German Patent 65,212; Friedl., III, 44 (1894).

(9) The procedure for the sequence of reactions from *o*-nitroaniline to thiabendazole was furnished by E. E. Harris and R. B. Currie of the Chemical Division of Merck & Co., Inc.



and then 1.86 ml. (2.67 g., 0.021 mole) of oxalyl chloride. The resulting mixture was refluxed for 22 hr. Evolution of HCl was complete by this time. The mixture was cooled, and crystalline II was collected, washed with benzene, and then with water. After air-drying the product weighed 4.698 g. (0.0195 mole) (97.7% yield), m.p. 254.5–255°.[10] The product had a specific radioactivity of 1.56 × 10^9 c.p.m./mmole.

***o*-Nitroaniline-C^{14} (III). A. Sulfonation.**—To 250 ml. of concentrated sulfuric acid (*d* 1.83) was added 4.46 g. (0.0186 mole) of II. The resulting mixture was stirred and heated in a boiling water bath for 15 min. The oxanilide dissolved as the mixture became warm. This was followed by prompt precipitation of the disulfonic acid.

B. Nitration.—The sulfonation mixture was cooled to 40°, and a solution of 2.11 ml. of nitric acid [77% HNO_3 (*d* 1.435)] in 2.1 ml. of concentrated sulfuric acid was added dropwise, with stirring and with the temperature held in the range of 36–40°. Addition time was 5 min. The resulting slurry was stirred for 15 min. more at 40°, then was allowed to stand for 1 hr., during which time it cooled to about 25°.

C. Hydrolysis.—Addition of 28 ml. of water to the mixture with stirring caused the temperature to rise to about 80°. The mixture was then heated to 130–135° at which temperature foaming and gas evolution began. After about 20 min. foaming stopped, and the mixture boiled smoothly at 138°. Refluxing was continued for 7 hr. The mixture was then cooled and poured onto 250 g. of ice. After aging, the crude *o*-nitroaniline was collected, washed with water, and air-dried. A benzene extract of the aqueous mother liquor was combined with the benzene soluble part of the first crop crude material. The resulting solution deposited 3.97 g. (77.5% yield) of crude *o*-nitroaniline, m.p. 52–60, upon evaporation.

The crude material was purified by vacuum sublimation, chromatography over alumina, and recrystallization from water to give 2.90 g. (54%) of *o*-nitroaniline ring (uniform) C^{14} (III), m.p. 70.5–71°, λ_{max} 230 mμ (ε 16,570), and 277 mμ (4750); λ_{max} 397–400 mμ (ε 5190); specific radioactivity 7.93 × 10^8 c.p.m./mmoles.

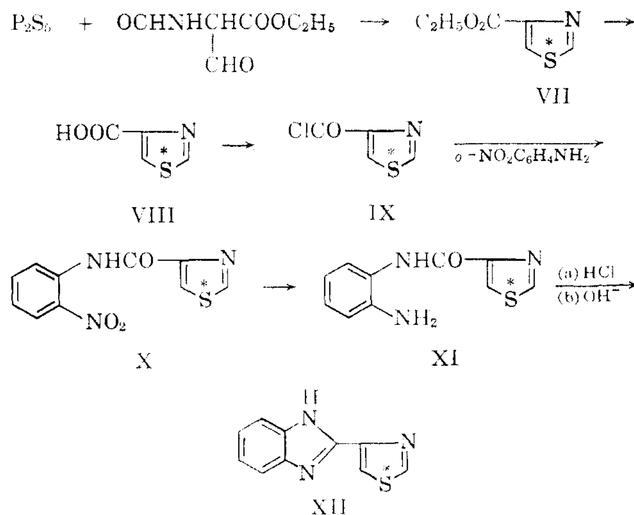
N-(*o*-Nitrophenyl)-4-thiazolecarboxamide (Benzene Ring (Uniform) C^{14}) (IV).—To a solution of 1.73 g. (0.0125 mole) of III in 7 ml. of anhydrous pyridine was added in small portions and with stirring 1.85 g. (0.0125 mole) of 4-thiazolecarbonyl chloride. The resulting mixture was stirred and heated to 75° for 2 hr., then allowed to cool to about 25° with some crystallization, and was poured into about 200 g. of ice and water. The product was collected after standing overnight, and was washed with a little cold water. The crude product weighed (dry) 2.69 g., m.p. 143.5–145.5°. The crude material was dissolved in absolute alcohol (50 ml./g.), and treated while hot with decolorizing carbon (Nuchar C-1000 N). After filtering, the solution was boiled down to about 60 ml. and an equal volume of hot water was added. In this manner was obtained 2.57 g. of pure IV, m.p. 145.5–146.5°; specific radioactivity 7.22 × 10^8 c.p.m./mmole.

N-(*o*-Aminophenyl)-4-thiazolecarboxamide (Benzene Ring (Uniform) C^{14}) (V).—A solution of 2.50 g. (0.010 mole) of IV in 100 ml. of methanol with 2.0 g. of 5% palladium-on-Darco catalyst was shaken under hydrogen at 2.81 kg./cm.^{2} (gauge) at room temperature for 5 hr. After standing for 9 more hr. under hydrogen, the mixture was filtered to remove the catalyst. The filtrate was concentrated to dryness under vacuum. The residual solid was dissolved in 8 ml. of hot methanol, and 20 ml. of hot water was added. The aminoamide crystallized as the solution cooled. After refrigeration, the product was collected, washed with water, and air-dried. The product (V) weighed 1.49 g. (0.00678 mole) (67.8% yield), and melted at 106.5–109°; λ_{max} 302 (ε 5080), and 232 mμ (18,900); specific radioactivity 7.45 × 10^8 c.p.m./mmole.

Thiabendazole-C^{14} (VI).—To a suspension of 1.38 g. (0.0063 mole) of V in a mixture of 19 ml. of ethyl alcohol (BBA, denatured), and 11 ml. of water was added 4.1 ml. of concentrated hydrochloric acid. The resulting solution was refluxed for 5 hr., then was cooled. The bulk of the ethanol was removed under vacuum. Refrigeration of this solution caused the product to crystallize. Crude hydrochloride (1.483 g.) was collected. Further concentration of the mother liquor gave a small second crop (0.089 g.).

The combined crude hydrochloride was dissolved in 23.6 ml. of hot water, and was then stirred with 0.2 g. of decolorizing carbon (Nuchar C-1000 N) and filtered hot. The solution was cooled, and 0.55 g. (0.0066 mole) of sodium bicarbonate was added slowly. The resulting suspension was aged in the refrigerator and then filtered. The product was washed well with water, then air-dried and vacuum-dried at 56° yielding 1.023 g. (5.08 mmoles, (80.8%)) of 2-(4′-thiazolyl)benzimidazole (benzene ring (uniform) C^{14}), m.p. 301–302°; λ_{max} 311 (ε 16,390), 298.5 (23,300), and 242.5 mμ (15,230); radioactivity 7.78 × 10^8 c.p.m./mmole. This material gave a single spot on a paper strip chromatogram (*n*-butyl alcohol–acetic acid–water, 4:1:1). The spot was cut into sections and eluted with methanol. The eluted material showed uniform specific radioactivity within the spot and in agreement with that of the product before paper stripping.

Synthesis of S^{35}-Labeled Thiabendazole.—Sulfur-35 labeled phosphorus pentasulfide furnished radioactive sulfur for the preparation of S^{35} (thiazole moiety) labeled thiabendazole. Phosphorus pentasulfide reacted with ethyl formamidomalonaldehydate[11] in pyridine.[12] One-fifth formula weight of phosphorus



(10) All melting points were measured in open capillaries with calibrated total immersion, Anshutz-type thermometers, and may be considered corrected.

(11) R. G. Jones, *J. Am. Chem. Soc.*, **71**, 383 (1949).

(12) The procedure for this reaction was kindly furnished by R. A. Firestone of these laboratories, private communication.

pentasulfide per mole ester was sufficient to give a good yield of ethyl 4-thiazole-S^{35}-carboxylate (VII) thus permitting efficient utilization of the radioactive reagent. The ester was hydrolyzed to the acid (VIII) which in turn was converted to the acid chloride (IX) by treatment with thionyl chloride. Reaction of the acid chloride with *o*-nitroaniline gave the nitroanilide, which, as in the previously described sequence, was reduced to the aminoanilide and then cyclized to give thiabendazole-S^{35}.

A critical radiometric evaluation was made for each of these preparations, and in each case the material was found to be pure as evidenced by all the physical properties measured, particularly by a paper strip chromatography with minute analysis of the single radioactive spot.

Ethyl 4-Thiazole-S^{35}-carboxylate (VII).—A mixture of 0.252 g. (1.135 m.f.w.) of phosphorus pentasulfide-S^{35} (specific radioactivity, May 10, 1960, 2.69 × 10^{10} c.p.m./m.f.w.), 0.488 g. (2.2 m.f.w.) of unlabeled phosphorus pentasulfide, and 2.868 g. (16.85 mmoles) of ethyl formamidomalonaldehydate in 15 ml. of pyridine was stirred and heated in an oil bath held at 100–105° for 16 hr., during which time a gummy oil layer separated from the originally clear yellow solution. Ether was added to the cooled mixture, and the solution of product was decanted from the viscous oil. The solvents were removed under vacuum. The resulting residue was extracted with ether. Ether was removed under vacuum to leave 2.47 g. (15.7 mmoles, 93.3%) of crude, crystalline VII.

A small sample of VII was removed and sublimed *in vacuo*. This sublimed material melted at 51.5–53°; specific radioactivity 1.98 × 0^9 c.p.m./mmole.[13]

4-Thiazole-S^{35}-carboxylic Acid (VIII).—To 2.47 g. (15.7 mmoles) of VII was added 4.4 ml. of 4 *N* sodium hydroxide (17.6 mequiv.). The mixture became quite warm, and the ester dissolved, leaving a few tiny particles of sulfur-like material. This mixture was filtered and then cooled in ice–water. To the clear, cold solution was added 2.5 ml. (30 mequiv.) of concentrated hydrochloric acid, to give a suspension, after thorough shaking, with pH *ca.* 2.5. The mixture was aged at 0–5° overnight, and the crude acid was collected, washed with water, and dried, yielding 1.14 g. (8.85 mmoles, 56.3% yield).

The combined aqueous mother liquor and washings were evaporated to dryness to leave 2.15 g. of residue. This was extracted with three 10-ml. portions of hot acetone. Removal of the acetone from this extract left 0.65 g. (5.03 mmoles, 32.1% yield) more of crude acid.

A small sample of the first crop of crude acid was sublimed *in vacuo*, with near quantitative recovery, to give an analytical sample of VIII for radioactivity measurement; specific radioactivity 2.15 × 10^9 c.p.m./mmole.

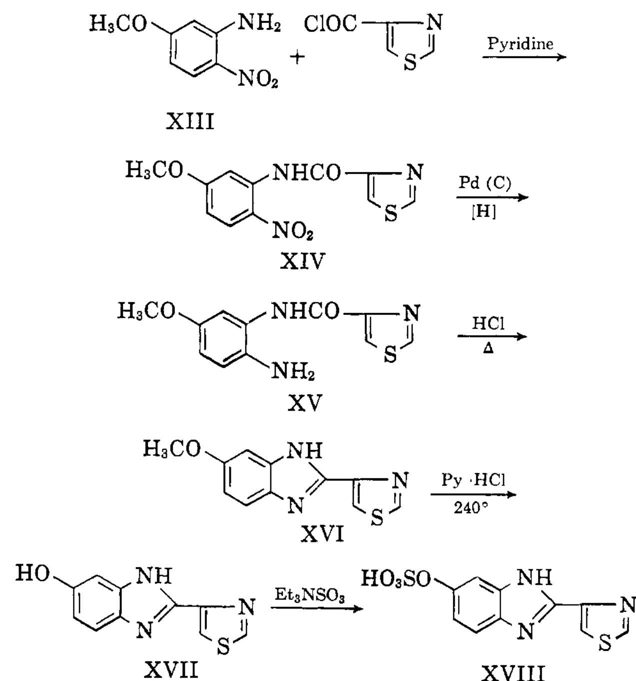
4-Thiazole-S^{35}-carboxylyl Chloride (IX).—A mixture of 1.79 g. (13.8 mmoles) of crude VIII and 10 ml. of thionyl chloride was refluxed with stirring for 2 hr. The mixture was then allowed to come to room temperature and stand overnight. The remaining thionyl chloride was removed under vacuum, and the residue was flushed with two 5-ml. portions of benzene. The crude product was purified by vacuum sublimation [bath temperature 70–80° (0.1–0.2 mm.)] to give 1.056 g. (7.15 mmoles, 51.5%) of IX; m.p. 87–89°; specific radioactivity 2.02 × 10^9 c.p.m./mmole.

N-(*o*-Nitrophenyl)-4-thiazole-S^{35}-carboxamide (X).—As previously described for the C^{14}-labeled analog 1.05 g. (7.12 mmoles) of IX reacted with 0.98 g. of *o*-nitroaniline to give 1.26 g. (5.05 mmoles, 71.3%) of X, m.p. 144–146°; specific radioactivity 2.05 × 10^9 c.p.m./mmole.

N-(*o*-Aminophenyl)-4-thiazole-S^{35}-carboxamide (XI).—Hydrogenation of X (1.26 g., 5.05 mmoles), as previously described, gave XI. The specific radioactivity of this intermediate was determined on an aliquot of the reduction solution after filtering from the catalyst; specific radioactivity 1.95 × 10^9 c.p.m./mmole. The total crude amide was carried on into the cyclization step.

Thiabendazole-S^{35} (XII).—The alcohol was removed *in vacuo* from the total product of the aforedescribed reduction. The resulting crude XI was converted to the desired thiabendazole-S^{35} as previously described. The product was purified by sublimation [190° (0.1 mm.)] to yield 717 mg. (3.57 mmoles, 82.8%) of material; m.p. 300–301.5°; specific radioactivity 2.09 × 10^9 c.p.m./mmole. A paper strip chromatogram (*n*-butyl alcohol–acetic acid–water, 4:1:1) showed a single ultraviolet-absorbing radioactive spot. Specific radioactivity was constant within the spot, and in agreement with that of the compound as measured directly.

Synthesis of 5-Hydroxythiabendazole and its Sulfate Ester.—The synthesis of 5-hydroxy-2-(4′-thiazolyl)benzimidazole (XVII), a major metabolite of thiabendazole, was accomplished by acylation of 5-methoxy-2-nitroaniline (XIII) with 4-thiazolecarbonyl chloride followed by catalytically reducing the intermediate nitroanilide (XIV) with hydrogen over palladium-on-carbon catalyst. The resulting *o*-aminoanilide (XV) was subsequently cyclized to 5-methoxy-2-(4′-thiazolyl)benzimidazole (XVI). Cleavage of the 5-methyl ether with pyridine hydrochloride at 240° gave the desired phenol (XVII).



5-Hydroxythiabendazole was converted to its sulfate ester (XVIII) by reaction with triethylamine–sulfur trioxide in alkali, following the method of Hardy and Scalera.[14]

N-(5-Methoxy-2-nitrophenyl)-4-thiazolecarboxamide (XIV).—To 1.7 g. of XIII in 35 ml. of pyridine was added 1.4 g. of 4-thiazolecarbonyl chloride. The mixture was protected from moisture and heated at 70° for 6 hr. Following quenching in 300 ml. of water, the precipitate was filtered and washed with dilute hydrochloric acid, sodium bicarbonate, and finally was recrystallized by dissolving in 800 ml. of alcohol, chilling, and subsequent filtration. The yield of bright yellow XIV was 2.1 g., m.p. 200–201°.

Anal. Calcd. for $C_{11}H_9N_3O_4S$: C, 47.32; H, 3.25. Found: C, 47.76; H, 3.76.

5-Methoxy-2-(4-thiazolyl)benzimidazole (XVI) Hydrochloride.—A solution of 2.1 g. of XIV in 500 ml. of absolute ethanol was reduced over 1 g. of platinum-on-charcoal. The reaction continued overnight at which time 1.47 kg./cm.^{2} drop in hydrogen pressure was observed (theory: 1.68 kg./cm.^{2}). The colorless solution of XV was filtered from the catalyst and 50 ml. of concentrated hydrochloric acid admixed with 10 ml. of water was added to the filtrate and refluxed for 4 hr. The reaction mixture was concentrated *in vacuo* to about 50 ml. The HCl salt was chilled and filtered; it melted with decomposition and loss of HCl at 280–300°.

5-Hydroxythiabendazole (XVII).—Compound XVI (400 mg.) obtained by neutralization of the hydrochloride in methanol was mixed thoroughly with 6 g. of anhydrous pyridine hydrochloride and heated to 220–240° for 45 min. The light straw-colored melt was cooled and 10 ml. of phosphate buffer (pH 6.5) was added. The aqueous solution was then extracted with two 15-ml. portions of benzene and similarly with methylene chloride. The combined organic extracts were filtered and concentrated *in vacuo*. After adding 25 ml. of benzene the solution was again

(13) The specific radioactivities for the intermediates containing the short-lived S^{35} isotope have been corrected for decay to the date of the first measurement (that for $P_2S_5{}^{35}$).

(14) W. B. Hardy and M. Scalera, *J. Am. Chem. Soc.*, **74**, 5212 (1952).

concentrated to aid in the removal of pyridine. The residue was finally recrystallized 3 times from small volumes of ethyl acetate with the aid of Darco. The yield of XVII was 210 mg., m.p. 283–286°.

Anal. Calcd. for $C_{10}H_7NO_3S$: C, 55.30; H, 3.25; N, 19.35. Found: C, 55.75; H, 3.79; N, 18.70.

Further characteriziation was made by means of the n.m.r. spectrum in which three aromatic proton signals were observed whose spin–spin coupling patterns were characteristically those of a 1,2,4-trisubstituted benzene ring. The thiazole ring protons remained unchanged as would be expected. Moreover the acidic phenolic OH band was also present.

5-Hydroxythiabendazole Sulfate (XVIII).—A solution of 108 mg. of 5-hydroxythiabendazole in 2.0 ml. of pyridine was added to 100 mg. of triethylamine–sulfur trioxide complex. Following an overnight incubation at room temperature, 2 ml. of water and 0.5 ml. of *N* sodium hydroxide were added and the faintly alkaline solution was extracted several times with ether. The aqueous phase was lyophilized to yield 165 mg. of pale amber solid which was redissolved in 2 ml. of warm, aqueous (50%) ethanol. A flocculent gray deposit which appeared after brief cooling in ice was removed by centrifugation and discarded. The supernatant solution was evaporated *in vacuo* on a rotating concentrator (bath about 50°) to yield 143 mg. of nearly white sodium salt of XVIII. In water solution the substance had an inflection at 235 mμ (ε 15,325) and a maximum at 302 mμ (ε 21,948); these characteristics were not altered by addition of sodium hydroxide. Chromatography on Whatman No. 1 paper (ascending technique) in the system *n*-butyl alcohol–acetic acid–water (4:1:1) gave a single ultraviolet absorbing spot of R_f 0.27. Following 1-hr. hydrolysis at 100° in 1 *N* hydrochloric acid, or incubation with glusulase (Sörenson buffer, pH 6.5) at 37° for 8 hr., the sulfate ester was transformed into a substance of R_f 0.59, the same as that of 5-hydroxythiabendazole. The hydrolyzed product isolated from the paper strip with 0.1 *N* HCl had an absorption maximum at 318 mμ which shifted to 335 mμ in alkaline solution, as observed with known 5-hydroxythiabendazole.

Animal Studies.—Eight lambs, ranging in weight from 15 to 25 kg., were dosed orally with gelatin capsules containing 50 mg. of radioactive thiabendazole per kg. of body weight. Five lambs received the C^{14}-labeled drug and three the S^{35} modification. Thiabendazole-C^{14} was ring labeled in the benzene nucleus, with S^{35} in the thiazole moiety. The specific activity of the compounds was approximately 0.54 μc./mg.

Intervals between dosing and sacrifice ranged from 6 hr. to 30 days. Urine and feces were collected daily for the entire duration of each experiment. Blood was drawn for analysis at suitably frequent intervals during the first 8 days. Measurement was made on intact blood, or plasma from citrated blood. Tissue samples were kept frozen until assayed.

Determination of Radioactivity in Biological Fluids and Tissues. Procedure for Urine.—A 10-fold aqueous dilution of urine (0.1 ml.) was added to 17 ml. of a scintillator solution consisting of 0.3% diphenyloxazole (PPO) and 0.01% of *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in a solvent of 30% ethanol in toluene. Radioactivity was measured by liquid scintillation counting in a Packard Tri-Carb scintillation spectrometer operated at tap 7 and a 10–100 channel. Standards were prepared by adding quantities of labeled thiabendazole to urine samples or to water. Since no quenching was observed, calculations were based on aqueous standards. Control urines were treated similarly to obtain background activity.

Procedure for Plasma and Feces.—Samples were plated directly in cupped planchets of 2.5-cm. diameter, and counted in a gas flow counter with a micromil window. All counts were corrected for self-absorption and calculations were based on standards carried throughout the procedure. Either 0.5 ml. of plasma or whole blood or 1 ml. of a 20-fold aqueous dilution of feces were plated directly in the planchets, evaporated to dryness, and counted.

Procedure for Tissues.—One gram of tissue was homogenized with 3 ml. of formamide in a Virtis homogenizer. One ml. of this homogenate, equivalent to 0.25 g. wet weight of tissue, was added to 17 ml. of the toluene–ethanol–phosphor solution and counted in the liquid scintillation spectrometer (counting efficiency was 50% with S^{35} and C^{14}). An internal standard technique employing C^{14}-labeled benzoic acid was used to compensate for the quenching of scintillations by tissue and tissue pigments. A correction factor (range for all tissues except spleen, 1.0–1.3; spleen, 1.7) was obtained by dividing the radioactivity measurement (c.p.m.) of a vial containing only the labeled benzoic acid standard by the c.p.m. of the benzoic acid in the presence of tissue. Tissues from control animals were processed in identical fashion, and served for measurement of background radioactivity, which was subtracted from the activity of tissues from medicated animals.

Fractionation and Isolation Procedures (Paper Chromatography).—Urine samples from sheep dosed with C^{14}- or S^{35}-labeled thiabendazole were chromatographed on Whatman No. 1 or No. 4 paper with *n*-butyl alcohol–acetic acid–water (4:1:1) as the ascending solvent. Two methods of detecting the spots were used: (1) Radioautographs were prepared by exposure on Eastman type KK industrial X-ray film. (2) The radioactivity on the chromatograms was counted by a gas-flow counting integral scanning instrument. With this instrument it is possible to locate the spots and determine the relative concentrations of radioactivity in each spot on the paper.

Column Chromatography.—In the course of chromatography of urine samples on a strong acid cation-exchange resin (CG-120),[15] the metabolites of thiabendazole were eluted sequentially by the use of a series of buffers of increasing pH values, followed by alkali. The components were detected in the effluent fractions by radioactivity and ultraviolet absorption.

All buffers were 0.2 *M* anion strength, adjusted to the desired pH with NH_4OH. Buffers used were ammonium tartrate and ammonium acetate.

The chromatographic system consisting of 70 ml. of resin in a 9-mm. o.d. glass column and a bed height of 70 cm. was equilibrated with pH 3.0 ammonium tartrate buffer prior to chromatography.

Results and Discussion

Recovery of Administered Radioactivity.—Following a single oral dose of thiabendazole (50 mg./kg. of body weight), 8 lambs excreted approximately 90% of the dose in their urine (range 73–77%) and feces (range 13–16%) in 48 hr. The results of one lamb (typical of the others) are presented graphically in Fig. 1. Chromatography of whole urine indicated that nearly all of the radioactivity was in the form of thiabendazole metabolites. Of the total urinary radioactivity approximately 2% remained as unchanged thiabendazole.

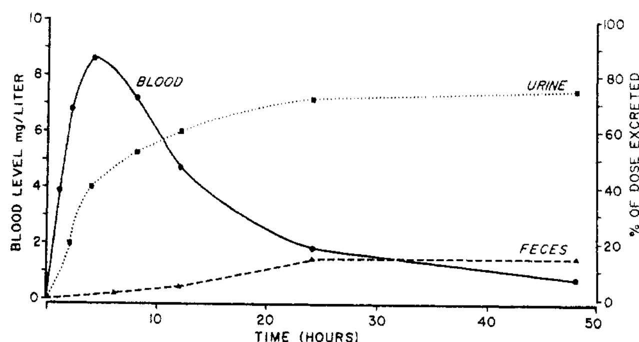


Fig. 1.—Distribution of radioactivity following oral administration of thiabendazole to a lamb. The ordinates are calculated from the specific activity of thiabendazole (120,600 c.p.m./mmole).

Drug concentrations in plasma of one of the lambs are reported (Fig. 1) as a function of time up to 48 hr. after dosing. Absorption apparently occurred fairly rapidly, peak drug levels in plasma being attained within several hours. Thereafter, the drug concentration in plasma dropped continuously and disappeared in 3 days.

Tissue residues of radioactivity in 8 lambs receiving 50 mg. of thiabendazole (C^{14}- or S^{35}-labeled) per kg. of

(15) Chromatographic grade resin, 100–200 mesh, was washed with 2.5 *N* NaOH, water, 3 *N* HCl, water, and then 2 *N* NH_4OH to convert to ammonium cycle. The excess was washed off with water.

TABLE I

DISTRIBUTION OF RADIOACTIVITY (γ/G.) IN TISSUES OF LAMBS SACRIFICED AT VARIOUS TIMES FOLLOWING A SINGLE ORAL DOSE OF LABELED THIABENDAZOLE (50 MG./KG.)

	Duration and isotope label							
Organ or tissue	6 hr. C^{14}	5 days C^{14}	8 days C^{14}	8 days S^{35}	16 days S^{35}	24 days C^{14}	30 days C^{14}	30 days C^{14}
Abomasum	5.1	0.0	0.09	0.20	0.0	0.0	0.0	0.0
Brain	1.0	0.09	0.12	0.16	0.0	0.0	0.0	0.0
Cecum	34.4	0.0	0.0	0.0	0.16	0.0	0.0	0.0
Fat	2.8	0.0	0.0	0.08	0.0	0.0	0.0	0.0
Heart	2.7	0.15	0.12	0.16	0.08	0.0	0.0	0.0
Kidney	13.9	0.28	0.15	0.28	0.0	0.0	0.0	0.0
Large intestine	4.6	0.0	0.0	0.20	0.0	0.0	0.0	0.0
Liver	9.6	0.62	0.52	0.72	0.15	0.0	0.0	0.0
Lung	2.4	0.0	0.0	0.08	0.08	0.0	0.0	0.0
Muscle	2.0	0.0	0.0	0.12	0.13	0.0	0.0	0.0
Pancreas	2.6	0.18	0.18	0.08	0.0	0.0	0.0	0.0
Plasma	7.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Skin	3.2	0.0	0.17	0.20	0.0	0.0	0.0	0.0
Small intestine	33.6	0.0	0.0	0.32	0.0	0.0	0.0	0.0
Spleen	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0

body weight are compiled in Table I. Only minute quantities of labeled substance were retained by lambs sacrificed 5, 8, or 16 days after dosing, and no detectable residue in tissue appeared after 24 to 30 days. Values recorded as 0.0 are equal to or less than 0.06 γ of thiabendazole/g. of tissue. At the approximate peak plasma level (6 hr. after dosage) concentrations of isotope in tissue ranged from 1 to 34 γ/g. Those tissues apparently not actively engaged in metabolism or excretion contained 1 to 3 γ/g.

Paper Chromatographic Study of Thiabendazole Metabolites.—When urine from sheep receiving C^{14}- or S^{35}-labeled thiabendazole was examined by radioautography of paper chromatograms, a total of six radioactive spots was revealed. Reported in Table II are the R_f values of each component found in 24-hr. sheep urine and the approximate per cent of the total radioactivity of each component as determined by the flow-gas counting integral scanning instrument.

TABLE II

PAPER CHROMATOGRAPHY OF URINARY METABOLITES OF THIABENDAZOLE[a] (24-HR. LAMB URINE)

Component	R_f value	Total radioactivity, %	Identity
1	0.73	2	Thiabendazole
2	0.59	10	5-OH thiabendazole
3	0.42	2	Unknown
4	0.27	14	Ethereal sulfate of spot 2
5	0.20	2	Unknown
6	0.12	70	Glucuronide of spot 2

[a] Urine samples from sheep dosed with C^{14}- or S^{35}-labeled thiabendazole were chromatographed on Whatman No. 1 paper with *n*-butyl alcohol–acetic acid–water (4:1:1) as the ascending solvent. The spots were detected by radioautographs prepared by exposure on Eastman type KK industrial X-ray film. The radioactivity on the chromatograms was counted by a gas-flow counting integral scanning instrument.

Component 1 appeared to be intact thiabendazole. It had the same R_f as the parent compound and showed similar fluorescent characteristics. The compound, eluted from the paper with 0.1 *N* HCl, fluoresces maximally at 370 mμ following excitation at 310 mμ using an Aminco-Bowman spectrophotofluorometer.

Components 4 and 6, accounting for approximately 84% of the total radioactivity, were converted enzymatically to component 2. Thus when 1 ml. of urine was incubated with 250 units of β-glucuronidase (Sigma), component 6 did not appear but rather there was a quantitative addition to component 2. In like manner when 1 ml. of urine was similarly incubated with 0.01 ml. of glusulase,[16] both components 4 and 6 were converted to component 2. Elution of component 2, before or after enzymatic treatment, with 0.1 *N* HCl yielded a fluorescent material which after excitation at 325 mμ fluoresced maximally at 425 and 525 mμ.

Isolation of 5-Hydroxythiabendazole.—A substance which chromatographs and shows similar fluorescent characteristics as does component 2 was isolated from glusulase-treated urine and shown to be identical with synthetically prepared 5-hydroxythiabendazole. Isolation was carried out by extracting 5 times, 200 ml. of urine adjusted to pH 6, with an equal volume of ethyl acetate followed by one extraction with methylene chloride. This extraction procedure served to remove components 1 and 2 leaving behind components 4 and 6 (determined by radioautographs of the two phases). No trace of unidentified components 3 and 5 was seen.

Less than 10% of the urinary radioactivity had been extracted by the above procedure. To the aqueous phase 1 ml. of glusulase was added and incubated overnight at 37°. The urine was again extracted 3 times with equal volume of methylene chloride and the 3 extractants combined. Approximately 50% of the urinary radioactivity was located in the organic phase. The organic phase was evaporated, under reduced pressure at 40°, to a small volume (about 10 ml.), and streaked across a sheet of Whatman No. 4 paper and chromatographed, using the above *n*-butyl alcohol–acetic acid–water system. A single radioactive band appeared, having an R_f of 0.6, which was eluted from the paper with 0.1 *N* HCl. Following adjustment of the HCl eluate to pH 6, the material was extracted into methylene chloride and evaporated to dryness.

The crystals formed were shown to be identical with synthetically prepared 5-hydroxythiabendazole by the

(16) Glusulase, available from Endo Laboratories, New York, N. Y., contains 100,000 units of β-glucuronidase and 50,000 units of sulfatase per ml.

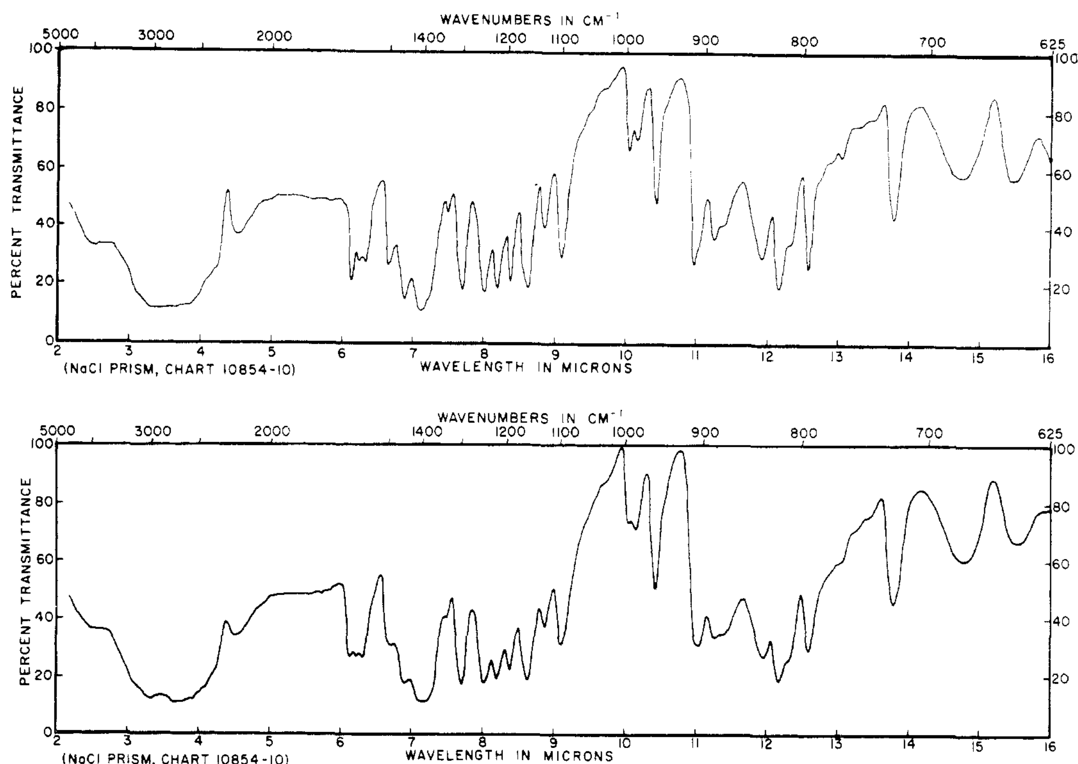


Fig. 2.—Infrared absorption spectra of 5-hydroxythiabendazole (upper) and compound isolated from urine (lower) run in KBr pellets.

following tests. Like the synthetic material it had R_f 0.59 in *n*-butyl alcohol–acetic acid–water (4:1:1) and upon excitation at 325 mμ, it fluoresced maximally in 0.1 *N* HCl at 425 and 525 mμ. Both compounds had identical ultraviolet spectra. In 0.1 *N* HCl, they absorbed maximally at 318 (ε 17,794) and 249 mμ (10,416); in 0.1 *N* NaOH they absorbed at 335 mμ (ε 13,063). The crystals melted at 282° and the melting point was not altered when mixed with authentic 5-hydroxythiabendazole. The infrared spectrum of the metabolite was indistinguishable from the authentic material (Fig. 2).

Isolation of the Glucuronide of 5-Hydroxythiabendazole.—The procedure for the isolation of the major urinary excretion product of thiabendazole metabolism consisted basically of chromatography on a strong cation-exchange resin, CG-120 (ammonium cycle) followed by elution with a series of buffers of increasing pH values.

Sheep urine (45 ml.) containing radioactivity equivalent to 147 mg. of thiabendazole-C^{14} was diluted with water to 250 ml. and adjusted to pH 3.0 with HCl. This solution was then charged to the column and eluted as shown in Table III. Approximately 83% of the radioactivity in the original urine sample was accounted for in the eluates. In the case of each fraction indicated, elution was continued until the radioactivity fell to below 1% of that in the initial stock solution.

The three most highly radioactive portions of the pH 5.0 eluate, containing 30% of the urinary radioactivity, were combined. Removal of acetate was accomplished by passing the solution through a 14-ml. CG-120 hydrogen-cycle column, which retained all the radioactivity and allowed the free acetic acid to be washed away in the effluent. Elution with 2 *N* ammonium hydroxide recovered the radioactivity in better than 95% yield. The excess ammonia in the eluate was distilled *in vacuo* and the remaining water lyophilized, leaving a frothy, off-white solid residue (95 mg.) with radioactivity equivalent to 43 mg. of the original labeled thiabendazole, indicating a 2.2-fold dilution of the molecule, presumably by conjugation. The ultraviolet spectral properties of the conjugate showed only minor alterations of the thiabendazole chromophores except for mass dilution effects, indicating that the conjugating group had no significant ultraviolet character except for end absorption as shown in Table IV.

The infrared spectrum of the conjugate was characterized by a large region of absorption at 9.4 μ where the C–O stretch frequency is known to occur, and in addition showed very strong –OH absorption.

The n.m.r. spectrum of the conjugate showed changes

TABLE III

SEPARATION OF URINARY METABOLITES OF THIABENDAZOLE IN SHEEP BY COLUMN CHROMATOGRAPHY ON CG-120 CATION-EXCHANGE RESIN

Fraction	Vol., ml.[a]	Urinary radioactivity, %[b]
Effluent charge solution	250	4
pH 3.0 (NH_4 tartrate)	900	14
pH 4.0 (NH_4 acetate)	700	4
pH 5.0 (NH_4 acetate)	500	43
pH 6.0 (NH_4 acetate)	300	4
pH 7.0 (NH_4 acetate)	200	2
2 *N* NH_4OH	200	10
1 *N* NaOH	200	2
		83

[a] Collected in 50-ml. portions. [b] The elution pattern shown here is typical but not necessarily quantitatively identical for different urine samples, nor for the same urine with time. It was found that during storage the pH 5 fraction decreased and the 2 *N* NH_4OH fraction increased. This change was shown to be attributable to slow hydrolysis of 5-glucuronide to 5-hydroxythiabendazole.

TABLE IV

Solvent	Thiabendazole λ_{max}	Thiabendazole ϵ	Glucuronide λ_{max}	Glucuronide ϵ
0.1 *N* HCl	301	26,130	308	17,920
	243	13,668	248	10,768
0.1 *N* NaOH	302	20,703	308	16,506
	235 (infl.)	16,381	237 (infl.)	16,506

in the aromatic spin–spin coupling pattern, but no change in the thiazole protons at positions 2′ and 5′ from those of thiabendazole, leading to the inference that the molecule was substituted at the aromatic positions 5 or 6. A large number of active protons and protons on C–O functions were also evident.

The above spectral evidence suggested strongly that a sugar moiety such as glucuronic acid must be attached to the thiabendazole molecule. Enzymatic hydrolysis proved that this conclusion was correct.

β-Glucuronidase Hydrolysis of the Conjugate.—Conjugate (50 mg.) and 1000 units of β-glucuronidase were dissolved in 10 ml. of pH 6.5 Sörenson buffer (20 mg./ml.) and incubated at 37° for 18 hr. Extraction with ethyl acetate removed most of the radioactivity from the aqueous phase. The hydrolysis product was then back-extracted into 0.1 *N* HCl, neutralized to pH 6, and re-extracted into ethyl acetate, from which it was recovered by evaporation.

The ultraviolet spectra of the product were as follows: in 0.1 *N* HCl, λ_{max} 318 (ϵ 17,577) and 251 mμ (10,199); in 0.1 *N* NaOH, λ_{max} 336 (ϵ 12,803) and 254 mμ (infl.) (13,020).

These spectra showed a large shift with pH in contrast with that of the conjugate which showed no change in position in the 300 mμ region. This is in agreement with the expectation that liberation of an ionizable group in the aromatic position of the molecule had taken place.

The n.m.r. spectrum of the hydrolysis product was found to be identical with that of an authentic specimen of 5-hydroxythiabendazole.

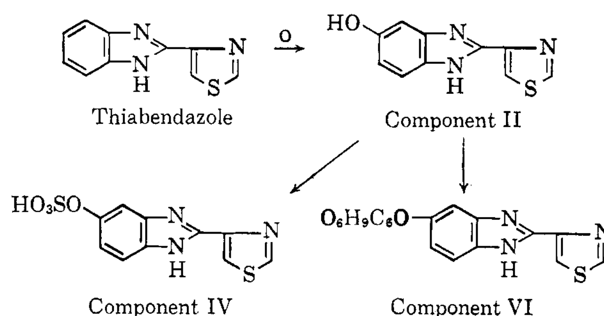
Isolation and Identification of the Sulfate Ester of 5-Hydroxythiabendazole.—The sulfate conjugate of 5-hydroxythiabendazole was isolated from sheep urine by preparative electrophoresis and identified by comparing its physical properties with the synthesized material.

Following oral administration of thiabendazole to a sheep (400 mg./kg.), a 1.2-ml. sample of the animal's first 24-hr. urine was applied to 6–4 cm. wide strips of Whatman No. 3 MM paper. The strips were subjected to electrophoresis (200 v., 16 hr.) in pH 10 glycine–sodium hydroxide, 0.05 *M* in glycine. Authentic sulfate ester of 5-hydroxythiabendazole, run simultaneously with the urine samples, was detected by ultra. violet absorption in a compact spot centered 8 cm. from the load line.

The urine strips showed the presence of broad multiple ultraviolet absorption bands in the region of 2–6 cm. from the load line. These bands were isolated and extracted with ethanol. Paper chromatograms of this ethanol-soluble material were prepared and the ultraviolet absorbing material at R_f 0.27 was eluted with methanol to yield 1.5 mg. of compound. Further purification was not attempted since, by the following tests, the fraction was indistinguishable from the synthetic material. Qualitative tests for sulfate[17] ion on 100-γ amounts of both the synthetic and natural material were negative. After acid hydrolysis (boiling in 6 *N* HCl for 1 hr.), sulfate ion was detected in both materials and conversion to 5-hydroxythiabendazole was demonstrated by paper chromatography.

On the basis of these studies, the scheme presented in Table V is suggested for the metabolic transformation of thiabendazole in sheep.

TABLE V
METABOLISM OF THIABENDAZOLE



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(17) F. Feigl, "Qualitative Analysis by Spot Tests," 3rd Ed., Elsevier Publishing Co., Inc., New York, N. Y., 1946, p. 238.