Excretion and Distribution of Thiazesim-^{14}C with Its Biotransformation *In Vivo* and *In Vitro*

By J. DREYFUSS, J. R. SWOAP, C. CHINN, and S. M. HESS

Thiazesim-^{14}C [5-(2-dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one] was demonstrated to have been completely absorbed after administration to rats. After an i.p. dose of 30 mg. of thiazesim-^{14}C per kg. the drug was found in all the tissues that were examined; it was most concentrated in the livers, lungs, and kidneys. The half-life in the brains was about 20 min. The drug was converted in the rat to three major metabolites, two of which were present in unconjugated form and also as sulfate and glucuronide conjugates. Unchanged thiazesim-^{14}C and the three metabolites accounted for 68 and 59 percent of the radioactivity in rat urine and feces, respectively, in the first 24 hr. after administration. More than 90 percent of an oral dose was excreted by rats or by dogs in 3 days; the agent was completely eliminated in 4–6 days. Thiazesim-^{14}C was metabolized by a microsomal enzyme system derived from rat liver. The system required oxygen, NADPH, and nicotinamide. Two major products were obtained by the incubation of the drug with rat liver preparations *in vitro;* they were absent in the excreta of treated rats. Metabolism of thiazesim-^{14}C by liver preparations from the dog, cat, or rabbit was also examined. Although the metabolites appeared to have been similar in all the species examined, the preparations obtained from rat livers metabolized thiazesim-^{14}C twice as fast as microsomes obtained from the other species.

THIAZESIM[1] is an antidepressant agent in humans (1). The compound has been shown to have a specific depressant effect on parts of the amygdalae of various animals; these portions of the brain appear to have been implicated in antidepressant activity (2). Thiazesim depressed the hyperirritability of rats with lesions in the septal area. In addition, the drive by selected aggressive rats to kill mice when they were placed together in a cage was abolished. Thiazesim also blocked the after-discharge produced by stimulation of the amygdaloid nuclei of cats. Afterdischarges due to stimulation to other areas of the limbic system were not affected (3).

This investigation was designed to study the absorption, metabolism, and excretion of thiazesim-^{14}C after administration to animals. The characterizations of the metabolites isolated after treating the animals or after incubation with liver preparations *in vitro* have been described elsewhere in this journal (4).

EXPERIMENTAL

Thiazesim-^{14}C with a specific activity of 0.25 μc./mg. was synthesized by Dr. John Krapcho of The Squibb Institute for Medical Research. The structure of thiazesim-^{14}C is shown in Fig. 1. The purity of the compound was 98% as assayed using infrared spectroscopy and by thin-layer chromatography with the two benzene–ammonia–dioxane systems, as noted in the section on thin-layer chromatography; reference standards of authentic nonradioactive thiazesim were used for comparison. The nonradioactive compound was visualized after chromatography by either the absorption of UV light (254 mμ) against a fluorescent background or by a positive color reaction with Dragendorf's reagent (5).

Extraction Procedure—Except where otherwise noted, male rats of the Holtzman strain weighing 100–250 g. were used. The plasma samples were diluted first with 4 vol. of methanol. The resulting turbid solutions were adjusted to pH 9 with 1 *M* borate buffer, pH 9.4 and 1 *N* NaOH; they were shaken for 15 min. with 2 vol. of benzene and then centrifuged. The benzene was removed and saved. The remaining lower layers and residues were similarly extracted again with a volume of chloroform–methanol (1:1) equal to that of the benzene used above. The combined organic layers were evaporated under reduced pressure at 50° to 5% of the original volume for subsequent use.

Samples of tissues or feces were homogenized with a Potter-Elvehjem or Virtis homogenizer in 4 vol. of 0.1 *N* HCl. Homogenates of tissue and feces or samples of urine were adjusted to pH 9 with 1 *M* borate buffer, pH 9.4, and sufficient 1 *N* NaOH and

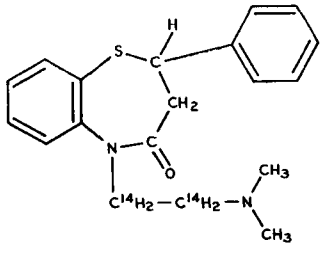

Fig. 1—Structure of thiazesim-^{14}C.

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During these experiments Mr. Michael Rispoli provided excellent technical assistance, and Mr. Octavian Kocy performed the electrophoretic separations.

[1] Marketed as Altinil, E. R. Squibb & Sons, Inc., also known as SQ 10496

extracted with 2 vol. of benzene. The benzene layer was transferred to a clean glass-stoppered bottle, and the lower layer extracted twice with volumes of chloroform–methanol (1:1) equal to that of the benzene used above. In the case of feces only, a fourth extraction was performed with 2 vol. of methanol.

The extracts were combined, acidified with 1 *N* HCl, and twice the volume of *n*-heptane was added. After shaking for 20 min., the lower layer was removed and concentrated to 5% of the original volume by evaporation under reduced pressure at 50–60°. These procedures extracted 99% of the unchanged thiazesim-^{14}C added to control samples and 75–100% of the radioactivity due to metabolites present in tissues and excreta.

Alumina Column Chromatography—Aluminum oxide was used for the initial separation of metabolites. In order to remove UV-absorbing contaminants, all aluminum oxide was washed with chloroform–methanol (5:1) before use and then dried at 100°. Enough neutral aluminum oxide (Woelm) was added to the evaporated extracts, obtained as described above, to make a thick paste (for 5 ml. of concentrate about 9 g. was added). The paste was taken to complete dryness by warming overnight in a vacuum oven at 50°. The dried sample was placed on top of a column (3 × 15 cm.) of neutral aluminum oxide to which 6% water had been added (6). Fractions of 5 ml. were collected; the column was developed in a stepwise manner with chloroform–methanol (5:1) 80 ml., designated Fraction 1; MeOH–H_2O (1:1) 80 ml., designated Fraction 2; and finally H_2O–NH_3 (20:1) 180 ml., designated Fraction 3 (see Fig. 2). Changes of solvent were made after the first 2 vol. noted above had been collected. Care was taken to maintain a small solvent head during the initial elution procedure until the effluent had appeared. Each of the fractions were assayed for radioactivity by counting a suitable sample in a Packard Tri-Carb liquid scintillation spectrometer. Quenching was determined by the addition of internal standard or with an automatic external standardization system. When nonradioactive thiazesim was used, Fraction 1 was measured by the use of its characteristic absorption at 280 mμ.

Thin-Layer Chromatography—The contents of the tubes in each of the three fractions were pooled and evaporated to about 2% of the original volume.

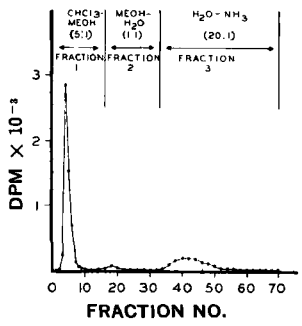

Fig. 2—Alumina column chromatography of a kidney extract containing thiazesim-^{14}C and metabolites. The tissue samples were obtained from rats 30 min. after an i.p. dose of 30 mg. of thiazesim-^{14}C per kg.

Aliquots of the evaporated Fraction 1 were chromatographed on a thin-layer plate with Silica Gel HF (Brinkmann); the plate was developed in benzene–ammonia–dioxane (BAD) (10:10:80 or 60:5:35) (7). Ninety-six percent of the spotted radioactivity obtained from control tissue samples to which thiazesim-^{14}C had been added migrated to the same relative position as authentic thiazesim-^{14}C (R_s = 1). The identity of thiazesim-^{14}C was confirmed by infrared spectroscopy of the eluted sample and by a comparison of the R_s values of the unknown samples with authentic thiazesim-^{14}C in the four solvent systems mentioned below. Other systems that were also used for the chromatography of Fraction 1 or hydrolyzed Fractions 2 and 3 were dioxane–methanol–ammonia–benzene (DMAB) (6:1:2:1) and water–ammonia–dioxane (WAD) (50:1:10).

Enzymatic Hydrolysis—A portion of the evaporated Fraction 2 was hydrolyzed with glucuronidase Mixture A[2] for 18 hr. at 37° to cleave glucuronides. A 0.25-ml. sample was mixed with 0.01 ml. of glucuronidase Mixture A and 0.05 ml. of 1 *M* acetate buffer, pH 5. Alternatively, a 0.25-ml. sample of Fraction 2 was hydrolyzed by mixing it with 0.05 ml. of 1 *M* acetate buffer, pH 5, and 25 units of aryl sulfatase, Type III (Sigma Chemical Co.), followed by incubation for 18 hr. at 37°. The hydrolyzed Fraction 2 was then chromatographed on Silica HF with BAF (10:10:80). A portion of the evaporated Fraction 3 of urine, 0.25 ml., was hydrolyzed by mixing it with 0.025 ml. of glucuronidase Mixture B and 0.05 ml. of 1 *M* acetate buffer, pH 5, followed by incubation for 18 hr. at 37°. The sample of hydrolyzed Fraction 3 was chromatographed on Silica Gel HF in DMAB (6:1:2:1).

Electrophoresis—The evaporated sample of Fraction 3 of feces was spotted on Whatman No. 1 filter paper and subjected to electrophoresis for 6 hr. at 500 v. with a buffer, pH 1.9, which was prepared by mixing 0.6 *M* formic acid with 1 *M* acetic acid.

Combustion Procedure—Tissue or feces samples were combusted in an oxygen atmosphere by the method of Kelly *et al.* (8), as described by Ebert and Hess (9).

Preparation of Subcellular Liver Fractions—All experiments, except those involving enzyme localization and cofactor requirement studies, were performed with the supernatant fraction from liver homogenates that was obtained at 9000×*g*. Livers from male Holtzman rats 250–300 g., male albino rabbits 1.9–2.1 kg., cats of either sex 2–2.2 kg., or male beagle dogs were used. Dogs were sacrificed by a rapid intravenous injection of 5 ml. of saturated KCl; other animals were killed by either stunning or decapitation, or both, and exsanguination. Livers were quickly excised, chilled in ice-cold 1.15% KCl, blotted on filter paper, and weighed. Subsequent operations were performed at 4°. Livers were minced and homogenized in 2 vol. of 1.15% KCl using a Potter-Elvehjem tissue grinder with a Teflon pestle. The homogenate was centrifuged at 9,000×*g* for 15 min. at 4°; the supernatant fraction was used for incubation. Washed microsomes were prepared by centrifuging such a supernatant fraction at 105,000×*g*

[2] Glusulase, Endo Labs, Garden City, N. Y.
[3] Ketodase, Warner-Chilcott, Morris Plains, N. J.

for 60 min.; the pellet was washed twice by resuspending it in a volume of 1.15% KCl equal to that of the supernatant material removed, followed by centrifuging at 105,000×g for 60 min. The pellet obtained at 9,000×g was similarly washed twice; the microsomes or the pellet were finally resuspended in a volume of 1.15% KCl equal to that of the homogenate from which the cell fractions were derived.

Total nitrogen was determined by the micro-Kjeldahl method.

Incubation Conditions In Vitro—Enzyme activity determinations were carried out in either open beakers or conical flasks at 37° on a Dubnoff metabolic shaker. The incubation mixture consisted of 50 μmoles of phosphate buffer, pH 7.4, 2 ml. of supernatant obtained at 9,000×g, 2 μmoles of thiazesim-^{14}C, 5.6 μmoles of glucose-6-phosphate, 0.39 μmole of NADP, 5 μmoles of $MgCl_2$, and 60 μmoles of nicotinamide; the final volume was 4 ml. After 5 min. of incubation at 37°, the substrate was added to initiate the reaction.

Extraction Procedure In Vitro—Samples of the incubation mixtures were removed at suitable intervals and transferred to 45-ml. centrifuge tubes containing 8 ml. of water-saturated butanol and 2 g. of NaCl. The mixture was shaken for 15 min. and centrifuged for 10 min. The butanol layer was transferred to a small round-bottom flask and the residue was extracted once more with 8 ml. of water-saturated butanol. The butanol extracts were combined and evaporated to dryness under reduced pressure; the resulting residue was dissolved in 1 ml. of methanol. After removing the alcohol-insoluble solids by centrifugation, 10 μl. of the supernatant was spotted on plates coated with 250 μ of Silica Gel HF. The plates were developed in BAD (60:5:35 or 10:10:80). The former mixture of solvents provided a means for the rapid separation of unchanged thiazesim; the latter mixture allowed a better resolution of the metabolites. The quantities of unmetabolized thiazesim-^{14}C and its metabolites were determined by measuring the radioactivity found in the sectioned chromatogram. This technique effected recoveries in the range of 75–90% of the radioactive substances in the incubation mixtures.

Excretion Experiments—Male and female rats were placed in cylindrical glass cages that allowed the animals access to condensed milk–water (1:1) but allowed only minimal freedom of movement. To collect feces, a small plastic bottle with the bottom removed and a hole drilled in the cap was used. The rat's tail was threaded through the cup (wide end first) and positioned in such a way that the anus was covered but not the urethra. The bottle cap was affixed and tape was used to immobilize the bottle. With this technique, feces were collected uncontaminated by urine. Urine was collected directly in a vessel cooled over dry ice.

Measurement of Binding to Plasma Proteins—Thiazesim-^{14}C was added to dog plasma. The plasma samples were placed in dialysis bags and tied. The bags were then supported in test tubes lined with wire screens of about 100 squares/cm.[2] The tubes were centrifuged for 45 min. at about 1,000×g in a Sorvall model SS-1 centrifuge or until 0.3 ml. of filtrate had accumulated in the bottom of tubes (10). The radioactivity present in the plasma before centrifugation was measured by dissolving 0.2 ml. of plasma in 1 ml. of NCS solubilizer (Nuclear Chicago), with the subsequent addition of 15 ml. of toluene scintillation fluid. The plasma filtrate was counted directly in Bray's scintillation fluid (11).

Biliary Excretion—Male rats were anesthetized with ether, the common bile duct was exposed and catheterized with a polyethylene tube that passed through the abdominal incision. The incision was sutured and the animal placed in a restraining cage. Bile was collected in tubes for 24 hr.

RESULTS AND DISCUSSION

Absorption and Excretion—Data are given in Table I for the distribution of drug after rats had received oral doses of either 30 or 100 mg. of thiazesim-^{14}C per kg. Peak levels of radioactivity were observed in the blood, brains, and livers within 30 min. These levels of radioactivity declined rapidly thereafter. The amount of radioactivity is expressed in terms of thiazesim-^{14}C equivalents, that is the radioactivity present was assumed to have had the molecular weight and specific activity of thiazesim-^{14}C.

The levels of total radioactivity found in rat brains as a function of time after animals had been dosed i.p. with 30 mg. of thiazesim-^{14}C per kg. are shown in Fig. 3. Radioactivity rapidly disappeared from the brains. Within 10 min. after an i.p. injection, the peak levels in the brains had already been reached; the half-life during the initial linear portion of the curve was about 20 min. No detectable radioactive substances were present in the brains after 2 hr.

The distribution of thiazesim-^{14}C in various organs of the rat was measured during a period of 6 hr. after an i.p. dose of 30 mg. of the drug per kg. (see Table II). The levels of thiazesim-^{14}C equivalents were highest in the livers, lungs, and

TABLE I—DISTRIBUTION OF RADIOACTIVITY AFTER A SINGLE DOSE OF THIAZESIM-^{14}C, EXPRESSED AS THIAZESIM-^{14}C EQUIVALENTS

Time, hr.	Dose, mg./kg.[a]	Blood, mcg./ml.	Brain, mcg./g.	Liver, mcg./g.
0.5	30	6.1 ± 0.9	5.0 ± 1.9	93.5 ± 6.9
1.0	30	3.6 ± 0.4	<1.5	59.6 ± 6.0
3	30	2.0 ± 0.4	<1.5	33.7 ± 3.2
0.5	100	16.8	23.5	178
1	100	12.7	11.6	149
3	100	5.2	4.2	64.0

[a] Rats received p.o. either 30 mg. or 100 mg. of thiazesim-^{14}C per kg.; three rats were used in each experiment at the lower dose level and the values for these are given ± the standard error. Two animals were used at the higher dose; the values represent the mean.

kidneys; intermediate levels were found in the spleens, mesenteric fat, and testes. Relatively low levels were found in the hearts, brains, thigh muscle, and blood. Six hours after the injection the liver contained 17 mcg. of radioactive substances per g., while the other tissues had much lower concentrations. It was interesting to note that the kidney, which might have been expected to have concentrated the drug and its metabolites in the urine, contained only about 0.33 as much as were found in the liver.

Figure 4 summarizes the excretion of radioactivity by male and female rats after single oral doses of 100 mg. of thiazesim-^{14}C per kg. Ninety-three percent of the dose administered to male rats was excreted in 2 days. After 4 days, 99.9 ± 3% of the dose had been excreted; at this time, 1.3% of the dose was left in the whole animal. Thirty-seven percent of the dose was excreted in the urine and 63% of the dose in the feces. Excretion by female rats similarly dosed was also rapid, although 6.7% of the dose still remained in the whole animals after 4 days (see Fig. 4). Male dogs dosed with 5 mg. of thiazesim-^{14}C per kg. excreted the entire dose in 6 days; the excreted dose appeared equally in the urine and feces (see Table III).

The excretion of radioactivity in the bile of rats was more rapid than that in the feces after an i.p. dose of 30 mg. of thiazesim-^{14}C per kg. Within 24 hr. after the injection, 52% of the dose was found in the feces. During the same period other rats, similarly dosed, excreted 70% in the bile (see Fig. 5); the major portion, 66% had been excreted in the bile in the first 6 hr. after the dose. The delay in the excretion of radioactivity in the feces, as compared with the outpouring in the bile, suggested

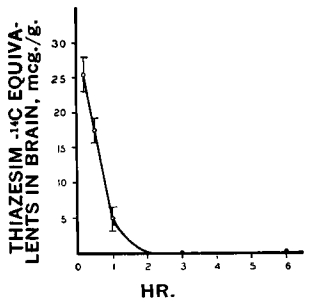

Fig. 3—Thiazesim-^{14}C equivalents present in brains as a function of time. Rats were dosed i.p. with 30 mg. of thiazesim-^{14}C per kg.

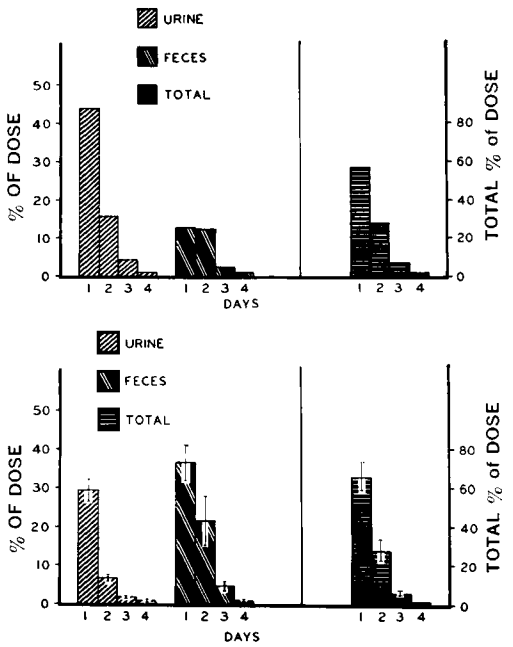

Fig. 4—Excretion of radioactivity measured as percent of dose in male rats (lower) and female rats (upper) dosed p.o. with 100 mg. of thiazesim-^{14}C per kg. Data for male rats are given plus or minus the standard error; n is equal to 3. Data for female rats are the averages of two animals.

TABLE III—EXCRETION OF THIAZESIM-^{14}C BY MALE DOGS[a]

Time, Days	Urine, % of Dose	Feces, % of Dose[b]	Cumulative Excretion, % of Dose
0–1	40.3	13.3	53.6
1–2	7.6	23.3	84.5
2–3	2.2	10.8	97.5
3–4	0.4	2.0	99.9
4–6	—	1.2	101.1
	50.5	50.6	

[a] Dogs received p.o. 5 mg. of thiazesim-^{14}C per kg. All numbers are the averages of values from two beagle dogs. [b] One dog did not excrete any feces during the first day.

that thiazesim and its metabolites may have been absorbed from the intestine.

Separation of Metabolites—In order to isolate and identify metabolites, the total radioactivity

TABLE II—DISTRIBUTION OF THIAZESIM-^{14}C AND METABOLITES IN TISSUES MEASURED AS THIAZESIM-^{14}C EQUIVALENTS, mcg./g.[a]

Tissue	0.5 hr.[b]	1 hr.[b]	3 hr.[b]	6 hr.[b]
Liver	91.4 ± 7.2	44.4 ± 3.7	19.1 ± 2.2	17.0 ± 2.0
Lung	69.2 ± 3.8	23.1 ± 3.3	4.9 ± 0.5	4.1 ± 1.2
Kidney	59.2 ± 5.8	27.7 ± 4.8	6.3 ± 2.1	5.3 ± 0.7
Spleen	46.7 ± 1.5	15.4 ± 1.5	5.3 ± 1.7	2.8 ± 0.9
Mesenteric fat	38.1 ± 2.1	11.0 ± 1.9	4.1 ± 0.5	1.7 ± 0.2
Testes	33.7 ± 2.9	11.3 ± 3.2	3.7 ± 0.5	1.7 ± 0.2
Heart	20.0 ± 2.9	9.3 ± 1.5	2.1 ± 0.8	1.8 ± 0.4
Brain	17.4 ± 1.8	4.8 ± 1.7	<1.5	<1.5
Thigh muscle	16.7 ± 5.2	7.3 ± 1.9	2.0 ± 0.1	<1.5
Blood	8.2 ± 0.5	4.9 ± 0.6	1.5 ± 0.2	1.0 ± 0.3

[a] Rats received i.p. 30 mg. of thiazesim-^{14}C per kg. The values given are ± the standard error; *n* is equal to 3 or 4. [b] Blood levels are in mcg./ml.

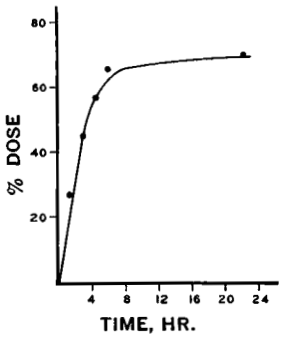

Fig. 5—Excretion of radioactivity in the bile as a function of time. Rats were dosed i.p. with 30 mg. of thiazesim-^{14}C per kg.

extracted from tissues or excreta of the rat and dog was separated grossly into three fractions, as described previously. Unmetabolized thiazesim-^{14}C and metabolites with minor structural alterations were eluted in Fraction 1. Metabolites that were conjugated as sulfate esters were eluted in Fraction 2. Metabolites that were present as glucuronide conjugates, as well as other polar metabolites, were eluted in Fraction 3.

Table IV shows the distribution of conjugated and unconjugated metabolites in various tissues and excreta of the rat and dog in typical experiments. Brains of rats contained primarily unconjugated metabolites and a trace of materials that was isolated in Fraction 2; glucuronide conjugates, Fraction 3, were absent from the brains. Rat plasma contained radioactivity in all three fractions. Livers and kidneys, and the feces of rats, as well as that of dogs, contained primarily unconjugated metabolites. By contrast, rat urine and bile, as well as dog urine, contained primarily conjugated metabolites.

The amounts of thiazesim-^{14}C (expressed as percents of the total radioactivity) present in various tissues and excreta of the rat and dog are shown in Table V. Rat brain contained primarily thiazesim-^{14}C. Rat liver, urine, feces, and bile contained very little unmetabolized thiazesim-^{14}C. Excreta of the dog contained several times more thiazesim^{14}C than was found in the excreta of the rat.

TABLE IV—DISTRIBUTION OF TOTAL EXTRACTED RADIOACTIVITY IN VARIOUS TISSUES AND EXCRETA OF THE RAT AND DOG

Sample[a]	% of Radioactivity		
	Fraction 1	Fraction 2	Fraction 3
Rat brain	93.8	6.2	0.0
Rat plasma	35.5	18.1	46.4
Rat liver	62.0	10.9	27.1
Rat kidney	64.6	4.4	31.0
Rat urine[b]	10.9	11.2	78.0
Rat feces[b]	62.0	8.9	29.1
Rat bile	9.4	18.7	71.8
Dog urine[c]	28.3	42.6	29.9
Dog feces[c]	62.0	14.1	23.9

[a] Male rats were given a dose i.p. of 30 mg. of thiazesim-^{14}C per kg.; organ samples were taken 30 min. after injection of the animals. Excreta samples were taken 24 hr. after dosing the animals. [b] The values cited for rat urine and feces are the results of pooling samples from 2 animals. [c] The values cited for dog urine and feces and for rat bile are the results of averaging values from 2 animals.

TABLE V—FRACTION OF RADIOACTIVITY PRESENT AS THIAZESIM-^{14}C IN VARIOUS TISSUES AND EXCRETA OF THE RAT AND THE DOG

Sample[a]	Amount of Thiazesim Present	
	%	mcg./g.
Rat brain	76.3	13.3
Rat plasma	22.5	3.7
Rat liver	3.9	3.6
Rat kidney	15.0	8.9
Rat urine	1.5	—
Rat feces	5.3	—
Rat bile	1.7	—
Dog urine	9.0	—
Dog feces	15.3	—

[a] Organ samples were taken after 30 min; samples of excreta were collected after 24 hr. Rats received i.p. 30 mg. of thiazesim-^{14}C per kg.; dogs received p.o. 5 mg. of thiazesim^{14}C per kg.

Figure 6 summarizes the results of separating by TLC the radioactive products in Fractions 1 and 2 of excreta and bile of rats and dogs that had received thiazesim-^{14}C. Four metabolites and thiazesim were distinguished. Fraction 2 was hydrolyzed with glucuronidase Mixture A prior to chromatography. The material of Fraction 2 remaining at the origin represents, in part, unhydrolyzed material. After the i.p. administration to rats of 30 mg. of thiazesim per kg., the metabolites in Fraction 1 of the bile were found to have been similar to the metabolites in the urine of rats that had received the same dose either i.p. or p.o. Dogs metabolized thiazesim-^{14}C differently from rats since in the dog greater fractions of the dose in the excreta exhibited R_s values of 0.64 and 1; also the urine of dogs contained a greater proportion of the dose in Fraction 2 than did the urine of rats.

The results of the separation of the components of Fraction 3 of urine after enzymatic hydrolysis are shown in Fig. 7. Five components were separated. With chromatography in BAD (10:10:80), DMAB, and WAD, it was demonstrated that the compounds with R_S values of 0.52 and 0.88 in BAD (10:10:80) (Fig. 6) were identical with the compounds with values of 0.83 and 0.95, respectively, in DMAB (Fig. 7). These components accounted for the bulk of the metabolite radioactivity in the rat. No radioactivity was detected at R_s values of 0.06, 0.28, or 0.45 in the urine of female rats.

The radioactivity present in Fraction 3 of feces and bile was separated into two components by electrophoresis (Fig. 7).

It may be seen from Figs. 6 and 7 that the pattern of excretion was qualitatively and quantitatively similar after i.p. or oral doses of 30 mg. of thiazesim per kg.; this suggested strongly that the drug was completely absorbed after oral administration.

It is noteworthy that the electrophoretic separation of extracts of the feces of rats demonstrated the presence of a much smaller amount of the component at −10.9 cm. than did extracts of bile (Fig. 7). Furthermore, Fraction 3 of bile was hydrolyzed with β-glucuronidase to the extent of 60–70% as opposed to the fraction from feces that was hydrolyzed to the extent of only 30%. An explanation for this quantitative difference between bile and feces, may be that intestinal microorganisms hydrolyzed glucuronide-conjugated metabolites. An alternative explanation may be that glucuronide conjugates were absorbed in the in-

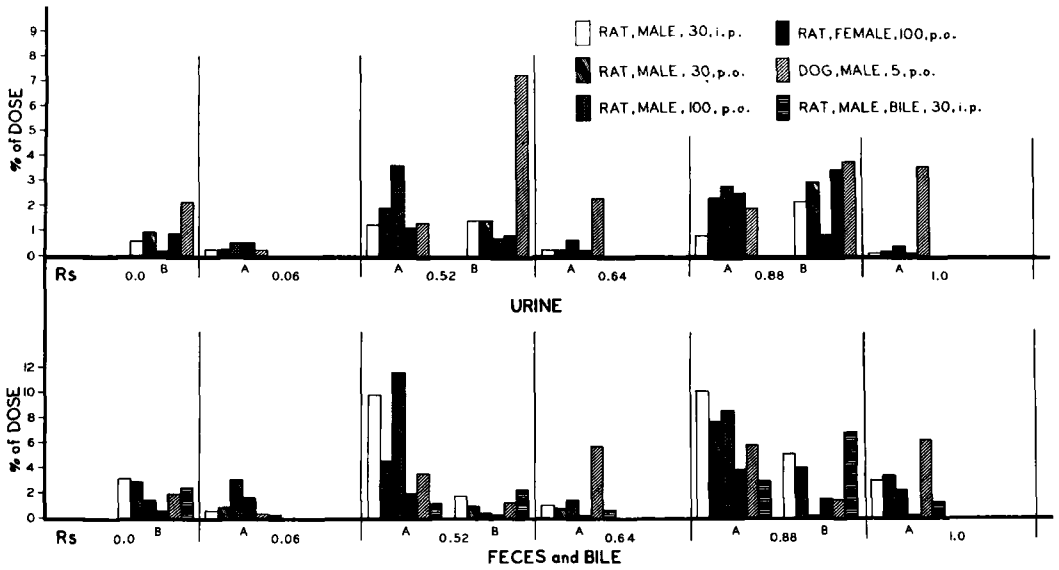

Fig. 6—Distribution of metabolites in Fractions 1 and 2 of rat urine, feces, and bile. Metabolites were located, according to their R_s values relative to thiazesim, on Silica Gel HF plates developed with BAD (10:10:80). Metabolites of Fraction 1 are designated A. *Metabolites of Fraction 2 were chromatographed after enzymatic hydrolysis with glucuronidase mixture A and are designated* B.

testinal tract and subsequently excreted in either the bile or the urine. Williams *et al.* (12) seem to favor the former since they have reported that glucuronide conjugates did not appear to have been absorbed from the intestinal tract, although the hydrolyses of glucuronide conjugates by bacteria have been observed.

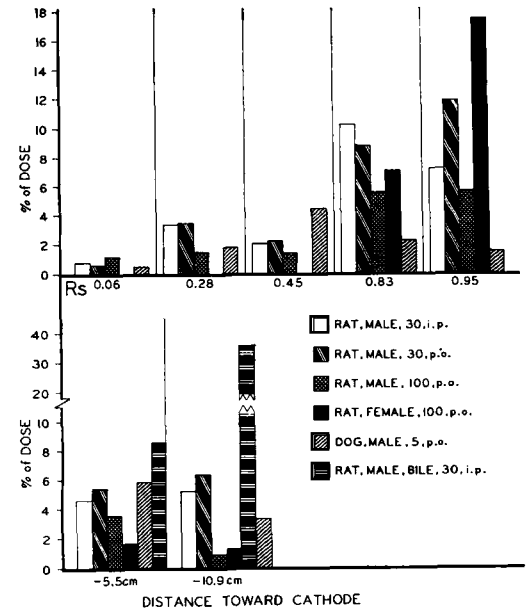

Fig. 7—Distribution of metabolites in Fraction 3 of rat urine, feces, and bile. Components of Fraction 3 of urine (upper) were separated, after enzymatic hydrolysis with glucuronidase mixture B, followed by chromatography on Silica Gel HF plates developed with DMAB (6:1:2:1). Metabolites were located according to their R_s values relative to thiazesim. Components of Fraction 3 of feces or bile (lower) were separated by paper electrophoresis as described in the text.

In order to ascertain whether the metabolites, as isolated from rat feces, had undergone bacterial alterations, bile was collected from rats fitted with indwelling biliary cannulae. The metabolites found in bile in the unconjugated form were the same on the basis of R_s values as those found in feces which suggested that microbial degradation had not produced the metabolites isolated from feces.

The three isolated metabolites, in both their conjugated and unconjugated forms, accounted for 68 and 59% of the amount of radioactivity present in urine and feces, respectively, in 24 hr. A detailed listing of the percent of the dose found in excreta after 24 hr. is shown in Table VI.

The binding of a parent compound to plasma proteins tends to inhibit its metabolism and excretion; in this manner its action in the animal is prolonged. Thiazesim-^{14}C was bound to plasma constituents of the dog. The binding varied between 53–72%, over the range of concentrations (0.33–33 mcg./ml. of plasma) found in a dog that had received i.v. 3 mg. of thiazesim per kg.

Are Microsomal Enzymes Induced By Thiazesim?—Thiazesim did not stimulate the proliferation of drug-metabolizing enzymes in rats. Rats that had been given either 30 mg. of thiazesim per kg. twice daily for 4 days or 98 mg. of thiazesim per kg. per day in the diet for 3 weeks exhibited no decrease in hexobarbital sleep time when compared with a control group that had received no drug. Rats given phenobarbital, a known inducer of drug-metabolizing enzymes (13), demonstrated the expected decrease in the duration of the hexobarbital sleep time.

Metabolism of Thiazesim-^{14}C In Vitro—A microsomal preparation derived from rat liver was capable of catalyzing the metabolism of thiazesim-

TABLE VI—ISOLATED METABOLITES OF THIAZESIM-^{14}C IN RAT EXCRETA IN THE CONJUGATED AND UNCONJUGATED STATES[a]

R_s of Metabolite[b]	% of Dose in 24 hr. Unconjugated Urine	Unconjugated Feces	Sulfate Urine	Sulfate Feces	Glucuronide Urine	Total
0.52	0.8	10.2	1.4	1.7	10.2	24.3
0.64	0.2	1.0	0.0	0.0	0.0	1.2
0.88	1.2	9.8	2.2	5.1	7.1	25.4

[a] Rats received i.p. 30 mg. of thiazesim-^{14}C per kg. [b] R_s values are given relative to that of thiazesim in the solvent system BAD (10:10:80).

^{14}C in the presence of suitable cofactors (Table VII). Specifically, either NADPH or an NADPH-generating system was required. The presence of nicotinamide strongly stimulated the reaction. Incubation in the presence of a nitrogen atmosphere severely inhibited metabolism.

Table VIII shows the results of an experiment that tested various subcellular fractions of rat liver for their ability to metabolize thiazesim-^{14}C. The data showed that either the supernatant fraction obtained at 9,000×g or the microsomal fraction, together with the supernatant fraction collected at 105,000×g, contained the most activity. When the supernatant material obtained at 9,000×g was fractionated at 105,000×g into microsomes and supernatant material, each had greatly diminished activity, however, when the two fractions were combined, activity was equal to that of the parent supernatant fraction. The lack of glucose-6-phosphate dehydrogenase supplementation to the reaction mixture probably accounted for the low activity of the isolated microsomal fraction. The pellet collected at 9,000×g, as well as the whole homogenate, contained very little activity. Furthermore, the supernatant fraction separated at 9,000×g, reconstituted with the pellet derived at 9,000×g, had no activity (data not shown). The lack of activity in the whole homogenate is notable and suggests the presence of inhibitory properties in the material that sediments at 9,000×g. Indeed, Axelrod (14) has shown that the metabolism of drugs by NADPH-dependent microsomal enzymes can be inhibited by substances present in liver homogenates of rats. Gillette *et al.* (15) have found evidence that pyrophosphatases, which hydrolyze NADPH and NADP, can partially account for the inhibitory effects of rat nuclei. An effort was made to obtain increased metabolic activity in the whole homogenate by supplementing the incubation mixture with ATP and DPN, however, the presence of these additional cofactors did not increase the activity. As pointed out by Gillette (16), the binding of substrates to nuclei and mitochondria, components of the whole homogenate, also can result in much slower rates of metabolism in unfractionated homogenates.

Isolation of the metabolic products produced by incubation of the drug *in vitro* with preparations of rat liver revealed that these substances had the same R_s values as those isolated from the excreta of rats dosed with thiazesim-^{14}C (Table IX). It was at once apparent that although the relative concentrations of the metabolites obtained from the *in vitro* system differed from those obtained from the excreta of the rats, the R_s values suggested that the products were the same. The products of

TABLE VII—COFACTOR REQUIREMENTS FOR THIAZESIM-^{14}C METABOLISM IN RAT LIVER *In Vitro*[a]

Additions	Thiazesim-^{14}C Metabolized[b] %	μmoles
None	5.9	0.12
NADPH, 1 μmole	4.1	0.08
NADPH, 4 μmoles	12.0	0.24
NADPH, 4 μmoles + 60 μmoles nicotinamide	27.5	0.55
NADPH-generating system + 60 μmoles nicotinamide	31.0	0.62
NADPH-generating system[c]	2.5	0.05
NADPH-generating system + 60 μmoles nicotinamide, under N_2 atmosphere	9.1	0.18

[a] The data represent the mean of 2 experiments. The basic reaction mixture contained rat liver microsomes (derived from 1 g. of liver), which had been washed twice with 1.15% KCl; 100 μmoles of phosphate buffer, pH 7.4; and 2 μmoles of thiazesim-^{14}C in a final volume of 4 ml.; incubation was for 30 min. at 37°. [b] The figures were calculated after subtracting the amount of thiazesim-^{14}C found in the boiled blank (4.5%). [c] The NADPH-generating system consisted of 5.6 μmoles of glucose-6-phosphate, 0.39 μmole of NADP, and 8 units of glucose-6-phosphate dehydrogenase.

TABLE VIII—RATES OF THIAZESIM-^{14}C METABOLISM IN SUBCELLULAR FRACTIONS OF RAT LIVER

Cell Fraction	Thiazesim-^{14}C Metabolized[a] %	μmoles
Whole homogenate	2.4	0.05
Pellet obtained at 9,000×g	3.7	0.07
9,000×g-Supernatant fraction	25.1	0.50
Microsomes	8.5	0.17
105,000×g-Supernatant fraction	3.3	0.07
Microsomes + 105,000×g-supernatant fraction	26.0	0.52

[a] Incubation was for 30 min. at 37° with subcellular fractions equivalent to 667 mg. of liver. The values given here represent the data of two experiments; the figures were calculated after subtracting the amount of thiazesim-^{14}C found in the boiled blank (4.3%).

TABLE IX—METABOLITES OF THIAZESIM-^{14}C IN EXCRETA AND IN LIVER PREPARATIONS OF THE RAT

R_s[a] of Metabolite	Liver Preparations % Metabolism[b]	Excreta % of Dose[c]
0.05	2.9	0.2
0.11	2.1	0.0
0.52	2.6	25.2
0.64	33.0	1.2
0.88	7.4	24.3

[a] BAD (10:10:80) on Silica Gel HF; R_s is relative to thiazesim. [b] The incubation was carried out using the 9,000×g supernatant fraction, as described in the text. [c] Rats were dosed i.p. with 30 mg. thiazesim-^{14}C per kg.

TABLE X—DISTRIBUTION OF THIAZESIM-^{14}C AND METABOLITES AFTER INCUBATION *In Vitro* WITH LIVER PREPARATIONS FROM THE RAT, DOG, RABBIT, OR CAT[a]

R_s Compd.[b]	Rat, %[c]	Rabbit, %[c]	Dog, %[c]	Cat, %[c]
1.0	51.8	60.8	60.7	69.1
0.88	7.4	9.3	6.0	3.4
0.64	33.0	20.9	10.4	17.4
0.52	2.6	2.8	1.8	0.9
0.11	2.1	2.7	18.2	4.2
0.05	2.9	3.5	2.9	5.1

[a] Incubation was for 90 min. at 37° using the supernatant fraction of livers obtained at 9,000×g and the conditions described in the section on experimental procedures in the text. Samples were extracted and chromatographed on thin-layer plates prepared with Silica Gel HF and developed with BAD (10:10:80) as described in the text. [b] The R_s value is relative to thiazesim. [c] The percentages in the table refer to the distribution of metabolites on the chromatographic plates; they have been corrected to represent the metabolism per g. of nitrogen in the 9,000×g-supernatant; the figures are averages for two experiments with the liver of one animal in each.

incubation with R_s values of 0.64 and 0.88 were isolated, and an effort was made to characterize them (4). It was surprising to learn that the two major products obtained from the incubations *in vitro* were different from the *in vivo* metabolites that had comparable R_s values. No evidence has yet been uncovered to show that the major products formed *in vitro*, those with R_s values of 0.64 and 0.88, were present in any fractions isolated after the administration of thiazesim-^{14}C to animals.

A survey was carried out of the metabolism of thiazesim-^{14}C by several animal species that exhibited somewhat varying sensitivities to the drug. The object of the study was to learn if any correlation existed between the formation of any specific metabolites, or between the overall rate of metabolism and the sensitivity to the drug. The species chosen were the rat, dog, rabbit, and cat. The survey was made by incubating *in vitro* the supernatant fraction obtained by centrifuging at 9,000 × *g*, homogenates of the livers of the animals.

It was observed (Table X) that the metabolites produced *in vitro* exhibited R_s values that corresponded closely to those found *in vivo*. The amounts of each metabolite formed varied somewhat among the species. The only notable difference was found in the incubation mixture prepared with the liver of the dog; the liver of the dog produced about five times more radioactive materials at the R_s of 0.11 than had been produced by the preparations of the other species.

A study of the rates at which thiazesim-^{14}C had been metabolized *in vitro* by the livers of the rat, rabbit, cat, and dog is summarized in Fig. 8. It may be seen that preparations obtained from rats were about twice as active as those obtained from the other species. This difference seemed to be related to the activity of the microsomes, since the quantity of microsomes per mg. of nitrogen in the supernatant fraction obtained at 9,000×*g* was equal in the preparations obtained from the dog and rat. The dog, which was the species most sensitive to the drug,[4] provided incubation mixtures that

[4] Private communication from Dr. J. Poutsiaka of the Toxicology Department of the Squibb Institute for Medical Research.

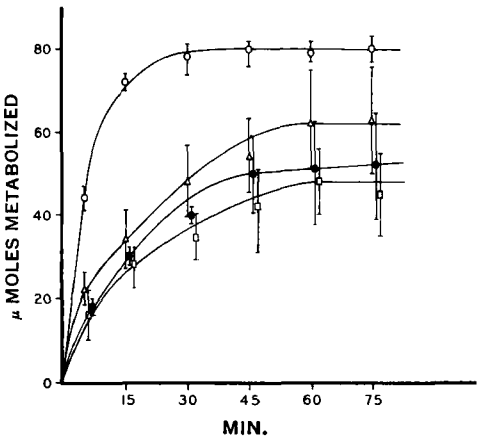

Fig. 8—Rates of thiazesim-^{14}C metabolism by the supernatant fractions (9,000×g) obtained from the liver of various species. Each point represents average values from 2 experiments that had been run in duplicate. Incubation conditions are described in the section on experimental procedure. Metabolism is expressed as μmoles thiazesim-^{14}C metabolized per g. of nitrogen in the 9,000×g-supernatant. Key: ○, rat; △, dog; ●, rabbit; □, cat.

metabolized the drug at a rate that did not differ from the rates exhibited by the rabbit and cat. The LD_{50} values for representatives of the four animal species were determined under fasted conditions. These values for the rat, rabbit, dog, and cat were 560, 220, 70 and 40 mg./kg., respectively. These LD_{50} values corresponded roughly to the rates at which the liver microsomes metabolized thiazesim, however, the correlation was not precise enough to demonstrate whether the rate of thiazesim metabolism was directly related to the degree of toxicity of the compound.

The likelihood that the metabolite isolated at the R_s of 0.11 could account for the varying sensitivities to thiazesim should be considered with caution since the rabbit and cat, which were also more sensitive to the drug than was the rat, produced the same amount of the metabolite as did the rat. In addition, other metabolic alterations, as well as other factors not measured *in vitro*, may have played roles in the varying sensitivities to the drug.

Identification of Metabolites In Vitro and In Vivo—A variety of spectroscopic procedures was used to identify metabolites. The results of these studies are reported in detail in a companion publication (4).

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Keyphrases

Thiazesim-^{14}C—excretion, distribution
Biotransformation, *in vivo*, *in vitro*—thiazesim-^{14}C
Column chromatography—separation
TLC—separation, identity
IR spectrophotometry—identity, purity
Electrophoresis—separation, radioactive metabolites

Metabolism of Thiazesim, 5-(2-Dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one, in the Rat *In Vivo* and *In Vitro*

By J. DREYFUSS, A. I. COHEN, and S. M. HESS

Three metabolites of thiazesim, 5-(2-dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one, were isolated from rat feces. Metabolite 1 contained a hydroxyl group in the 2-phenyl ring. Metabolite 2 was dihydroxylated at unknown positions of the 2-phenyl ring. Metabolite 3 was a derivative of Metabolite 1 and, in addition, appears to have had one carbon and two oxygens added to the side chain. Two metabolites of thiazesim (4 and 5) were isolated from rat liver homogenates incubated with NADPH and nicotinamide. Metabolite 4 has been identified as the sulfoxide derivative of thiazesim. Metabolite 5 has not yet been identified, but it was not similar to those isolated *in vivo*. Hydroxylation of either aromatic ring was not an observed metabolic reaction *in vitro*. Sulfoxidation was not an observed metabolic reaction *in vivo*.

A PAPER ELSEWHERE in this journal (1) has described aspects of the metabolism of the antidepressant agent, thiazesim-^{14}C, 5-(2-dimethylaminoethyl)-2,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one, in the rat and dog that relate to its absorption, distribution, excretion, as well as the techniques developed for the separation of both conjugated and unconjugated metabolites. Also discussed were the results obtained by incubating thiazesim-^{14}C with liver preparations from various animal species.

This paper will describe the identification of the metabolic products of thiazesim-^{14}C formed by the rat *in vivo* or by rat liver preparations *in vitro;* these metabolites have not yet been tested for pharmacologic activity. The results showed that the metabolites formed by the rat *in vivo* were not the same as those produced by rat liver preparations *in vitro*.

EXPERIMENTAL

Isolation of Fecal Metabolites—Three metabolites of thiazesim were isolated from feces in an unconjugated form by the following procedure. Rats were repetitively dosed once a day for 4 days with 100 mg. of thiazesim per kg. and the feces samples combined. The feces were homogenized in 4 vol. of methanol that was 0.12 *N* with respect to HCl, and centrifuged. After centrifugation, the supernatant fraction was removed and saved, and the residue was extracted twice more with 4 vol.

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