Distribution and Metabolism of Carboxyl-^{14}C-2,3,5-triiodobenzoic Acid and 2,3(^{125}I),5(^{125}I)-Triiodobenzoic Acid in the Rat

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Rats receiving oral doses of carboxyl-^{14}C- or 2,3(^{125}I),5(^{125}I)-triiodobenzoic acid excreted 72–75% of the radioactivity in urine and 24–28% in feces during the 4 days following drug administration. Peak levels of ^{14}C were observed in brain, thyroid, liver, lungs, heart, spleen, kidneys, and carcass 4 or 8 hr after dosing and rapidly decreased thereafter. Levels of ^{125}I were significantly higher than ^{14}C levels in the brain and thyroid. ^{125}I in thyroid increased with time. Thin-layer chromatograms of ether extracts of urine (50–80% of the radioactivity was extracted) revealed the presence of: 2,5-diiodobenzoic acid, as the free acid and a conjugate, accounting for 66% of the extractable radioactivity (39.6% of dose); unchanged TIBA, 9.5% (5.7% of dose); 2-hydroxy-3,5-diiodobenzoic acid, 2.3% (1.4% of dose); and 3,5-diiodobenzoic acid, 0.7% (0.4% of dose). Thin-layer chromatograms of feces extracts revealed a metabolic pattern similar to urine.

A plant growth regulator used on soybeans is 2,3,5-triiodobenzoic acid (TIBA). The compound, when properly applied, affects morphology and flowering response, increases pod set and decreases lodging, thereby increasing soybean production.

Due to the possible incorporation of TIBA or its metabolic products into the human food chain it was necessary to establish its safety in a variety of animal species. The toxicity of TIBA has been studied in a number of species as reported elsewhere (Ebert, 1970). As part of the safety evaluation the distribution and metabolism of carboxyl-^{14}C-TIBA and 2,3(^{125}I),5(^{125}I)-TIBA in the rat were studied.

REAGENTS

Radioactive Compounds. Carboxyl labeled ^{14}C-2,3,5-triiodobenzoic acid (^{14}C-TIBA) with a specific activity of approximately 10.5 μCi/mg (5248 μCi/mmol) was obtained from J. E. Christian and associates, Bionucleonics Department, Purdue University, Lafayette, Ind. The compound was synthesized and shown to be radiochemically pure on the basis of paper chromatography in three solvent systems by Spitznagle *et al.* (1968). A sample of 2,3(^{125}I),5(^{125}I)-triiodobenzoic acid (^{125}I-TIBA) was obtained from the Pharmaceutical Division, Nuclear Consultants Corp., St. Louis, Mo. The specific activity of this material was 3.35 μCi/mg (1674 μCi/mmol).

Chromatography Solvent Systems. Five paper and thin-layer chromatographic systems were used in these studies and are shown in Table I. Systems A, B, and C were used for determining the radiochemical purity of TIBA. Systems D and E were used to study the radioactive metabolic products in urine, feces, and tissue extracts.

EXPERIMENTAL DETAILS AND RESULTS

Administration of the Drugs. The sodium salt of carboxyl labeled ^{14}C-TIBA or ^{125}I-TIBA was prepared by the addition of a 10 *M* excess of Na_2CO_3 solution to provide a TIBA concentration of *ca.* 1 mg/ml. The solution was orally administered by feeding tube to several groups of rats (1 or 2 of each sex) (Sprague-Dawley or Holtzman) weighing 118–280 g. Within any group of rats the weight range did not exceed 75 g.

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The dose of ^{14}C-TIBA or ^{125}I-TIBA was 6 mg/kg (63 μCi ^{14}C/kg) or 20 mg/kg (67 μCi ^{125}I/kg), respectively. The exact dose of radioactivity was determined by counting a portion of the solution administered.

Collection of Excreta and Tissue Samples. Each rat was placed in a metabolism cage where urine and feces were separately collected. Food and water were offered *ad libitum*. The urine was collected in glass or plastic containers surrounded by Dry Ice and remained frozen until used. Excreta were collected at 4 and 8 hr after dosing, daily thereafter up to 4 days, and stored at −15° C.

At the same intervals, two or three rats were anesthetized with ether and sacrificed by exsanguination. Brain, thyroid, liver, lungs, heart, spleen, and kidneys were removed for assay of radioactivity. All tissues and organs, except the thyroid, were homogenized in 4 vol of 0.1 *N* HCl using Teflon and glass tissue grinders or a high-speed homogenizer. The remainder of the animal, defined as carcass, was homogenized in 4 vol of 0.1 *N* HCl using a Waring Blendor equipped with a high shear stainless steel blade. Homogenates and intact thyroids were frozen and stored at −15° C until assayed.

Assay of Radioactivity. The total ^{14}C or ^{125}I content of tissues and excreta was determined by counting the radioactive combustion products of the material being assayed. Combustions were accomplished using a modification of the Schöniger flask method (Ebert and Hess, 1965). Samples (0.5 ml) of the tissue homogenates (equivalent to 100 mg of wet tissue) were placed in containers constructed of Visking dialysis casing and dried overnight at 52° C in a vacuum oven. In a similar manner samples of urine (0.1 to 0.5 ml) were dispersed on a small piece of ashless filter paper, placed in a Visking casing container, and dried. Samples (50 mg) of previously dried powdered feces were used for combustion.

The dried samples, including the Visking bag, were wrapped in ashless filter paper (Schöniger Sample Wrappers No. 6471-F, Arthur H. Thomas Co., Philadelphia, Pa.), supported on a platinum grid, and ignited by applying a flame to a protruding paper wick. The sample was quickly inserted into a flask containing an atmosphere of 100% oxygen. After combustion was complete the flask was cooled in an ice bath to create a partial vacuum. A suitable solution for absorbing the radioactive products was added through a funnel attached to a stopcock affixed to the grid support. In the experiments with ^{14}C-TIBA, $^{14}CO_2$ was formed and absorbed over a 30-min period in 15 ml of a mixture of freshly distilled phenethyl-

amine, anhydrous methanol, and toluene (27:27:toluene to make 100 ml) (Woeller, 1961). A 5-ml aliquot was removed and added to 10 ml of a scintillation solvent composed of 4 g of PPO and 0.5 g of POPOP dissolved in enough toluene to make 1 l.

The radioactive combustion products of ^{125}I-TIBA were quantitatively recovered by using 20 ml of an absorber composed of Hydroxide of Hyamine 10X (Packard Instrument Co., Inc., Downers Grove, Ill.) and absolute ethanol (1:19). A 5-ml aliquot was removed and added to 10 or 15 ml of Bray's solution for counting (Bray, 1960). Both ^{14}C and ^{125}I were quantitatively recovered by these procedures from tissues spiked with radioactive standards.

All samples were counted in a liquid scintillation spectrometer, corrected for background, and the absolute disintegration rate was calculated by use of internal standards. A computer program, written in basic language and using the General Electric Time-Sharing Service, was used to calculate the percent of the dose of radioactivity and standard deviations for each organ and tissue sample studied (Barker, 1968). The program corrected the experimentally-determined absolute disintegration rates for isotope decay in the samples containing ^{125}I.

Distribution of Radioactivity. The highest levels of radioactivity in all the organs and tissues studied were observed at 4 or 8 hr after dosing. The levels of ^{14}C and ^{125}I in the various organs and tissues are shown in Table II. In all cases except brain and thyroid the levels of ^{14}C and ^{125}I were closely parallel. Throughout the entire course of the study, the major portion of either radioisotope was found in the carcass. Of the organs assayed, the liver contained the most radioactivity. Levels dropped from a high of 4 to 5% of the dose 4 hr after drug administration to less than 0.1% of the dose by the fourth day. Radioactivity in the kidneys followed a similar decline, from about 1.5 to 0.06% of the dose. Levels of radioactivity in the lungs, heart, spleen, brain, and thyroids represented very small amounts of the drug, ranging from about 1 to 2 $\times$ 10^{-4}% of the dose. The lower value, corresponding to approximately 50 DPM, was the lower limit of sensitivity for the assay.

The only statistically significant differences between organ levels of ^{125}I and ^{14}C occurred in the brain and thyroids. In both cases ^{125}I levels were higher than ^{14}C. This phenomenon could be accounted for by a deiodination of the drug in the 3 and/or 5 positions. Only in the thyroids did the level of ^{125}I increase with time.

Table I. Chromatography Solvent Systems

System	Mobile phase	Stable phase
A	Ethyl acetate:methanol:2 *N* NH_4OH (18:2:2)	Paper
B	2-Propanol:35% NH_4OH:H_2O (10:1:1)	Paper
C	1-Butanol saturated with 0.005 *M* NH_4OH	Paper
D	Petroleum ether (30–60°):propionic acid (10:1)	Tlc plates of Silica Gel G (0.25 mm)
E	Benzene:methanol:propionic acid (10:2:1)	Tlc plates of Silica Gel G or alumina

Table II. Distribution of Radioactivity in Organs and Tissues after Dosing with Labeled TIBA

	Percent of dose ± standard deviation			
	Brain		Thyroid	
Time	^{14}C	^{125}I	^{14}C	^{125}I
4 hr	0.060 ± 0.007	0.064 ± 0.006	0.010 ± 0.009	0.038 ± 0.013
8 hr	0.065 ± 0.044	0.068 ± 0.016	0.022 ± 0.016	0.038 ± 0.022
1 day	0.012 ± 0.006	0.033 ± 0.005	0.003 ± 0.0007	0.103 ± 0.009
2 day	0.012 ± 0.004	...	0.0008 ± 0.0003	0.129 ± 0.048
3 day	0.002 ± 0.0003	0.014 ± 0.013	0.0002 ± 0.00005	0.113 ± 0.043
4 day	0.002 ± 0.001	0.015 ± 0.004	0.0003 ± 0.0001	0.096 ± 0.012
	Liver		Lung	
Time	^{14}C	^{125}I	^{14}C	^{125}I
4 hr	3.72 ± 0.14	4.84 ± 1.20	0.65 ± 0.28	1.04 ± 0.46
8 hr	2.39 ± 1.56	3.38 ± 0.75	0.80 ± 0.54	0.48 ± 0.05
1 day	1.14 ± 0.77	1.90 ± 0.19	0.18 ± 0.07	0.56 ± 0.28
2 day	0.39 ± 0.11	0.81 ± 0.44	0.12 ± 0.03	0.088 ± 0.023
3 day	0.087 ± 0.009	0.20 ± 0.07	0.015 ± 0.007	0.022 ± 0.003
4 day	0.050 ± 0.019	0.10 ± 0.03	0.011 ± 0.01	0.012 ± 0.0008
	Heart		Spleen	
Time	^{14}C	^{125}I	^{14}C	^{125}I
4 hr	0.61 ± 0.46	0.25 ± 0.10	0.12 ± 0.03	0.134 ± 0.012
8 hr	0.30 ± 0.14	0.30 ± 0.024	0.055 ± 0.073	0.082 ± 0.016
1 day	0.086 ± 0.11	0.21 ± 0.032	0.030 ± 0.018	0.071 ± 0.014
2 day	0.037 ± 0.007	0.044 ± 0.021	0.015 ± 0.002	0.046 ± 0.042
3 day	0.005 ± 0.003	0.011 ± 0.003	0.0022 ± 0.0004	0.018 ± 0.019
4 day	0.004 ± 0.004	0.003 ± 0.001	0.036 ± 0.061	0.0042 ± 0.0015
	Kidney		Carcass	
Time	^{14}C	^{125}I	^{14}C	^{125}I
4 hr	1.24 ± 0.54	1.43 ± 0.54	61.50 ± 28.12	65.95 ± 3.18
8 hr	1.26 ± 0.66	1.30 ± 0.54	25.75 ± 3.59	57.19 ± 14.74
1 day	0.62 ± 0.22	0.58 ± 0.13	9.52 ± 7.50	37.60 ± 4.02
2 day	0.37 ± 0.10	0.35 ± 0.14	2.98 ± 0.72	9.22 ± 4.18
3 day	0.09 ± 0.03	0.10 ± 0.015	2.76 ± 2.12	3.03 ± 0.46
4 day	0.059 ± 0.027	0.062 ± 0.010	2.17 ± 2.53	2.50 ± 0.23

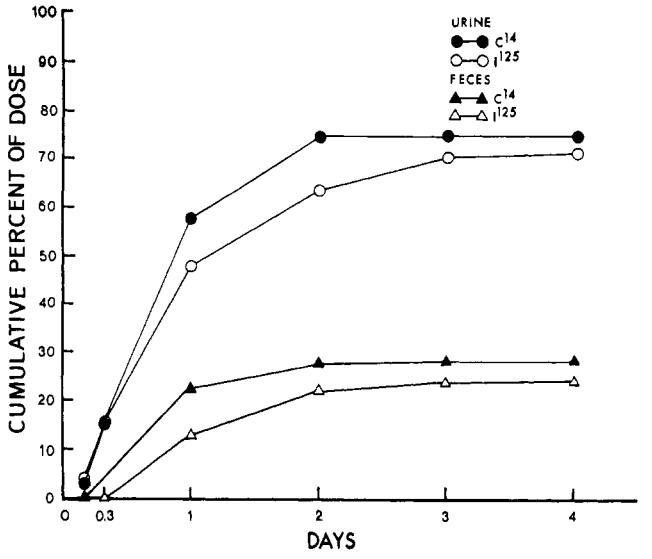


Figure 1. Cumulative excretion rates of ^{14}C and ^{125}I

Rate and Route of Excretion of Radioactivity. Results of assays for radioactivity excreted by animals dosed with the ^{125}I or ^{14}C labeled compound are shown in Figure 1. Both isotopes were excreted at similar rates. Most of the radioactivity (72 to 75%) was excreted in the urine during the 4 days following drug administration, while 24 to 28% was excreted in the feces. Thus the total radioactivity recovered in the excreta was 103% of the ^{14}C dose and 96% of the administered ^{125}I.

NATURE OF THE RADIOACTIVITY

Extraction of Urine. Ether extracts were prepared of a number of the urine samples. Portions of each extract were counted in a liquid scintillation spectrometer. Although the exact amounts of the urine and reagents used for each extraction varied, the following procedure was typical.

Urine (100 ml) was made acidic by the addition of 3 ml of concentrated HCl and exhaustively extracted by shaking with five 50-ml vol of ether. The ether extracts were combined, concentrated *in vacuo*, and reconstituted to 25 ml in ethanol.

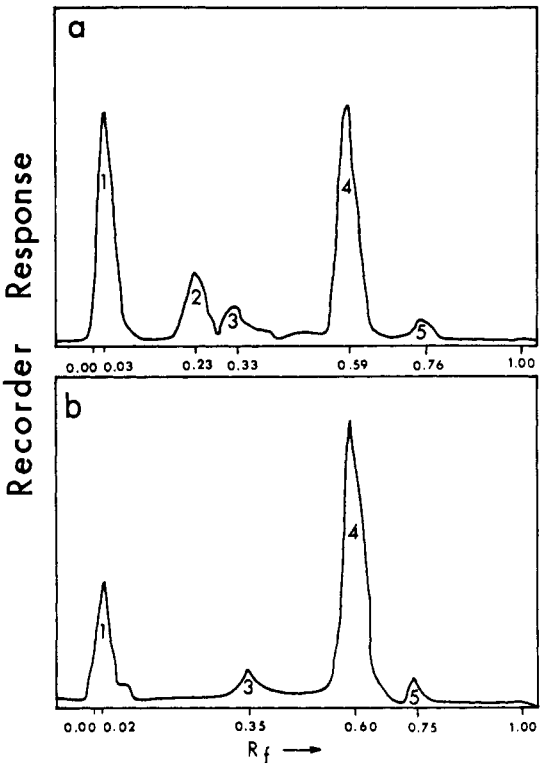


Figure 2. Thin-layer chromatogram scans of: (a) ether extract and (b) acid hydrolyzed ether extract of day 1 urine from rats dosed with ^{14}C-TIBA. Solvent system: D on Silica Gel G plates. Peak 1, unknown, 2-OH-3,5-DIBA, conjugate of 2,5-DIBA; Peak 2, unknown; Peak 3, TIBA; Peak 4, 2,5-DIBA; Peak 5, 3,5-DIBA

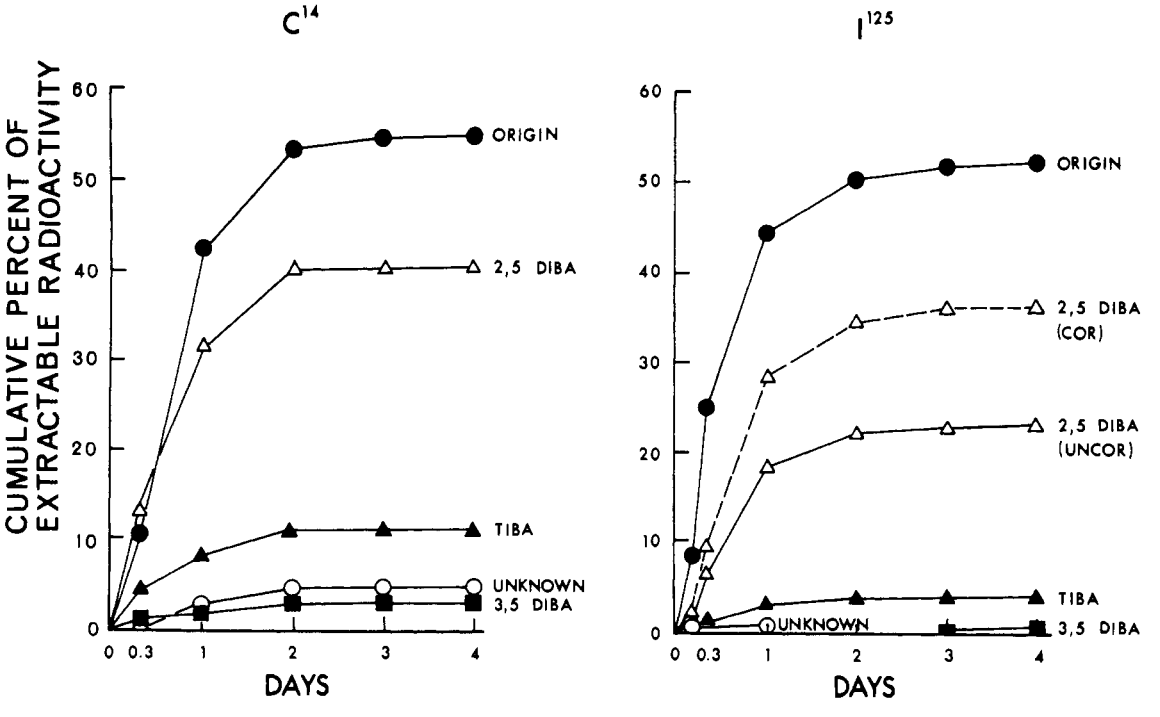


Figure 3. Cumulative percent excretion of ether extractable radioactive metabolites

Table III. Summary of Enzyme Hydrolysis Studies

Sample no.	Reagents	Treatment	Results
1	Urine from ^{14}C-TIBA dosed rats β-Glucuronidase in acetate buffer	Urine (1 ml) boiled; 1000 units β-glucuronidase added. Incubated 24 hr at 37° C. Extracted and chromatographed	No change in chromatographic pattern from untreated urine
		Urine (1 ml) boiled; 4 ml of acetate buffer added. Incubated 24 hr at 37° C. Extracted and chromatographed	Same as above
		Urine (1 ml) boiled; 1000 units β-glucuronidase added. Incubated 24 hr at 37° C. Additional 1250 units β-glucuronidase. Incubated 24 hr at 37° C. Extracted and chromatographed	Same as above
		Urine (1 ml) boiled; 4 ml of acetate buffer added. Incubated 24 hr at 37° C. Additional 5 ml of acetate buffer. Incubated 24 hr at 37° C. Extracted and chromatographed	Same as above
2	0.01 *M* Phenolphthalein-glucuronic acid solution (PGA). β-Glucuronidase in acetate buffer. Water. 5% Trichloroacetic acid solution (TCA)	Water (0.9 ml) plus 0.1 ml PGA; 625 units β-glucuronidase added. Incubated 24 hr at 37° C. 1 ml TCA added. pH adjusted to 10–10.5	Red solution
		Water (0.9 ml) plus 0.1 ml acetate buffer; 625 units β-glucuronidase added. Incubated 24 hr at 37° C. 1 ml TCA added. pH adjusted to 10–10.5	Colorless solution
3	0.01 *M* Phenolphthalein-glucuronic acid solution (PGA). β-Glucuronidase in acetate buffer. Urine from untreated rats. 5% trichloroacetic acid solution (TCA)	Urine (0.9 ml) plus 0.1 ml PGA; 625 units β-glucuronidase added. Incubated 24 hr at 37°C. 1 ml TCA added. pH adjusted to 10–10.5	Red solution
		Urine (0.9 ml) plus 0.1 ml of acetate buffer; 625 units β-glucuronidase added. Incubated 24 hr at 37° C. 1 ml TCA added. pH adjusted to 10–10.5	Colorless solution

Seventy-seven to 85% of the urinary ^{14}C was extracted by this method. From 50 to 80% of the ^{125}I was ether extractable, the amount extractable decreasing with time after dosing. This decrease in extracted radioactivity might be explained by the deiodination of TIBA. The iodide ion thus produced would not be ether soluble.

Chromatography. The ether extracts of urine were chromatographed on thin-layer plates of Silica Gel G or alumina and developed in solvent systems D or E. Detection of radioactive zones was achieved using a Packard Model 7200 gas flow (isobutane:helium, 0.95%:99.05%) chromatogram strip scanner. The thin-layer chromatograms resolved the radioactivity in the urine extracts from rats dosed with ^{14}C-TIBA into five radioactive spots. A large portion of the extractable radioactivity remained near the origin of the chromatogram (Figure 2a). Similar results were obtained from chromatograms of extracts of urine from rats dosed with ^{125}I-TIBA.

Chromatographic comparison of the urine extracts with known compounds, visualized on the chromatogram as quenched spots under uv light, indicated that a major urinary metabolite was 2,5-diiodobenzoic acid (2,5-DIBA). Another radioactive compound was identified as unchanged TIBA. A minor spot accounting for about 2% of the extractable radioactivity migrated with the same R_f value as 3,5-diiodobenzoic acid (3,5-DIBA). 2-Hydroxy-3,5-diiodobenzoic acid (2-OH-3,5-DIBA) migrated at the same rate as the peak near the origin.

Ether extracts were made of urine from rats dosed with either ^{14}C-TIBA or ^{125}I-TIBA collected at intervals of 8 hr, 1, 2, 3, and 4 days after drug administration. Chromatograms of these extracts were prepared, and the cumulative percent radioactivity was calculated for each spot at each collection interval (Figure 3). The excretion patterns for the two labeled compounds were essentially the same. Levels of 2,5-DIBA in the ^{125}I studies are shown as corrected and uncorrected. The correction was necessary because the formation of this metabolite resulted from the loss of ^{125}I from the number 3 position, thus decreasing the specific molar activity of the molecule by one-half. Also, it was discovered that 2,5-DIBA was present as a minor impurity in the ^{125}I-TIBA used in this experiment. The line designated 2,5-DIBA (cor) reflects the appropriate corrections.

CONJUGATED RADIOACTIVITY

Acid Hydrolysis. To determine if ^{14}C-TIBA or any of its metabolites were excreted in urine as a conjugated derivative, a 10-ml sample of whole urine from treated rats was hydrolyzed by the addition of 10 ml of 2 *N* HCl and refluxing 20

Table IV. Cumulative Percent of Extractable Radioactivity from Various Tissues

Tissue	Metabolite	Hours following ^{14}C-TIBA administration 4	8	24	48
Liver	Unknown	6.95	15.00	18.65	20.12
	TIBA	20.53	45.28	50.40	50.62
	2,5-DIBA	11.11	20.25	23.68	27.03
	3,5-DIBA	0	0	1.06	2.17
Lung	Unknown	2.92	6.66	8.42	9.29
	TIBA	22.38	54.95	59.18	60.31
	2,5-DIBA	9.78	21.32	22.48	26.06
	3,5-DIBA	0	2.12	2.44	4.29
Kidney	Unknown	4.70	13.84	17.42	19.03
	TIBA	13.27	42.85	48.41	55.09
	2,5-DIBA	7.64	18.75	20.86	20.86
	3,5-DIBA	0	3.94	3.94	4.43
Spleen	Unknown	6.40	6.40	8.60	
	TIBA	30.50	46.50	56.70	
	2,5-DIBA	17.50	27.40	31.50	
	3,5-DIBA	2.10	3.10	3.10	
Heart	Unknown	7.60	10.00	12.20	
	TIBA	37.90	53.00	58.70	
	2,5-DIBA	14.30	26.30	28.20	
	3,5-DIBA	0	0.50	0.90	
Carcass	Unknown	1.40	4.53	4.83	5.33
	TIBA	34.09	63.44	69.81	70.87
	2,5-DIBA	11.82	17.86	21.04	23.91
	3,5-DIBA	1.26	2.44	3.22	3.41

hr. The sample was then extracted by shaking with five 50-ml portions of ether. This procedure extracted about 70% of the total urinary radioactivity.

The ether extracts were combined, concentrated, and reconstituted to 5 ml in acetone. Scans of thin-layer chromatograms of these samples revealed that the amount of radioactivity remaining at the origin of the chromatograms had markedly decreased, as illustrated in Figure 2b. Levels of TIBA and 3,5-DIBA were not affected by acid hydrolysis. However, there was a marked increase in the radioactivity associated with 2,5-DIBA on hydrolysis, indicating the presence of an acid hydrolyzable conjugate of 2,5-DIBA in whole urine. In addition, the unknown material which migrated with an R_f value of 0.23 in extracts of nonhydrolyzed urine was not detected in the hydrolysate.

One hydrolysis experiment was carried out using the urine of animals dosed with ^{125}I-TIBA. In this experiment 65 to 70% of the urinary ^{125}I was extractable. A chromatographic pattern similar to the studies with ^{14}C was observed.

Enzymic Hydrolysis. To investigate the nature of the conjugated metabolite revealed by acid hydrolysis of urine, 1-ml samples of whole urine were incubated with β-glucuronidase (Nutritional Biochemicals Corp., Cleveland, Ohio) according to the method of Bergmeyer (1963). As illustrated in Table III, four 1-ml samples of urine from rats dosed with ^{14}C-TIBA were boiled for 5 min to inactivate enzyme inhibitors. β-Glucuronidase in acetate buffer (4 ml), pH 4.7 (250 units/ml), was added to each of two samples. To the two remaining urine samples 4 ml of acetate buffer alone was added. The samples were incubated at 37° C in a water bath shaker. After 24 hr incubation one experimental and one control sample were removed, made acidic with HCl, and extracted three times with 40-ml ether.

The remaining experimental and control samples were treated with an additional 5 ml of enzyme solution and 5 ml of acetate buffer, respectively. These two samples were reincubated 24 hr, then made acidic and extracted with ether. The ether extracts were concentrated and chromatographed on thin-layer plates, as described previously.

A positive control was run to test the activity of the β-glucuronidase. Samples containing 0.1 ml of 0.01 *M* phenolphthalein-glucuronic acid plus 0.9 ml of water or 0.9 ml of urine from rats dosed with nonradioactive TIBA were incubated with 2.5 ml of enzyme solution (625 units) for 24 hr. After incubation 1 ml of 5% trichloroacetic acid was added to each sample and the sample centrifuged. Three milliliters of 0.1 *M* alkaline glycine solution were added to bring the pH of the solution to 10–10.5, optimal for phenolphthalein detection. A red color developed in all the samples which had contained phenolphthalein-glucuronic acid plus enzyme. No color was observed in control samples which had contained only enzyme with no substrate or substrate with no enzyme. Therefore, the presence of urine in the incubation mixture did not inhibit enzyme activity.

Chromatograms of the ether extracts of enzyme-hydrolyzed urine from rats dosed with ^{14}C-TIBA did not differ from chromatograms of extracts of controls (urine of rats dosed with ^{14}C-TIBA treated with acetate buffer alone) nor from chromatograms of extracts of unhydrolyzed urine. These data indicated that neither TIBA nor its metabolites were excreted in the urine as glucuronide conjugates.

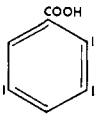
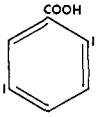
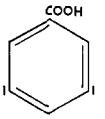
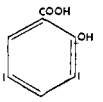
Extraction of Feces. A 2-g sample of feces, collected 1 day after dosing from rats treated with ^{14}C-TIBA, was dried and pulverized. The powdered feces were extracted first with 75 ml and then 50 ml of acidic ether. The ether extracts were combined, evaporated *in vacuo*, and reconstituted in acetone to 5 ml. Thin-layer chromatograms of the feces extract revealed a metabolic pattern similar to that of urine. Most of the extractable radioactivity remained at the origin. Unchanged TIBA accounted for approximately 18% of the extracted radioactivity; 13% of the fecal radioactivity was in the form of 2,5-DIBA, markedly lower than the 32% found in urine at this time interval. A spot was also found migrating with the same R_f value as 3,5-DIBA and represented about 3% of the extracted radioactivity.

Extraction of Tissue. To determine the nature of the radioactivity in the various organs and tissues, portions of liver, lung, kidney, spleen, heart, and carcass homogenates from rats dosed with ^{14}C-TIBA were extracted and studied chromatographically. For each assay a 10-ml sample of the homogenate was extracted with three 50-ml portions of ether. 30% NaCl solution (5 ml) was added to the kidney homogenate before extraction to inhibit the formation of emulsions. Each ether extract was evaporated to dryness, and the residue reconstituted in 10 ml of acetone. Radioactivity was determined by counting a portion of the acetone solution in a liquid scintillation spectrometer.

The radioactive metabolites were identified by comparison with standards using thin-layer chromatography on silica gel G plates, and solvent system D. The cumulative percent distribution of ether extractable radioactivity in the samples at various time intervals after dosing is shown in Table IV.

In all the organs and tissues studied, TIBA was the major radioactive component in the ether extract. Of the radioactivity extracted, 2,5-DIBA accounted for 20–32%; TIBA accounted for 48–70%; and 1–4% was due to 3,5-DIBA. An unknown component accounting for 5–20% of the extracted radioactivity, which did not migrate in chromatography system D, was found to be more predominant in the liver and kidney than in the other tissues.

Table V. Inverse Isotope Dilution of Radioactive Metabolites in Urine from ^{14}C-TIBA Treated Rats

Compound	Structure	Wt of compound added to extract (g)	DPM of extract used	Crystallization solvent	Specific activity of recrystallized material (DPM/mg)	% as ether extractable radioactivity
TIBA		1.96634	2.518×10^6	Chloroform	122	9.5
2,5-DIBA (after hydrolysis)		2.03403 (2.24819)	2.491×10^6 (2.190×10^6)	Benzene (benzene)	567 (643)	46.3 (66)
3,5-DIBA		1.08614	2.384×10^6	Chloroform	16	0.7
2-OH-3,5-DIBA		2.08495	3.156×10^6	Benzene	35	2.3
			Total Radioactivity Identified, before hydrolysis			58.8
			Total Radioactivity Identified, after hydrolysis			78.5

Inverse Isotope Dilution Analysis. Qualitative and quantitative determination of urinary metabolites of TIBA was accomplished using inverse isotope dilution analyses. For these studies, an analytically pure sample was prepared of each compound suspected of being a metabolite of TIBA on the basis of the chromatographic data. A known weight of the compound being studied was added to a known quantity of radioactive ether extract of combined day 1 and day 2 urine from rats dosed with ^{14}C-TIBA. The compound was successively recrystallized from an appropriate solvent to a constant specific activity. To determine the specific activity of each crystallization, a small portion of the crystals was placed in a tared glass counting vial and dried to constant weight under a vacuum at the temperature of refluxing ethanol. The dry crystals were dissolved in 1 ml of *p*-dioxane, diluted with 10 ml of Bray's scintillation solvent, and counted in a liquid scintillation spectrometer. The absolute disintegration rate was determined by the addition of a ^{14}C-toluene internal standard. Results of the isotope dilution studies for the compounds shown to be metabolites of ^{14}C-TIBA are presented in Table V. Unchanged TIBA accounted for 9.5% of the ether extractable radioactivity excreted during the 48 hr following drug administration. The major metabolite was 2,5-DIBA, accounting for 46.3% of the extractable radioactivity. The presence of two minor metabolites, 3,5-diiodobenzoic acid and 2-hydroxy-3,5-diiodobenzoic acid, accounting for 0.7 and 2.3%, respectively, of the extractable radioactivity, was confirmed.

To obtain inverse isotope dilution data on the 2,5-DIBA content of hydrolyzed urine extract, a 1-ml sample of the extract was evaporated to dryness. The residue was then refluxed 4 hr in 10 ml of 1 *N* HCl. Water was removed *in vacuo*, the residue dried by azeotropic distillation (*in vacuo*) with benzene, and nonradioactive 2,5-DIBA added to the flask. Recrystallization to constant specific activity was conducted as before.

Following acid hydrolysis of the ether extract, 2,5-DIBA accounted for 66% of the extractable radioactivity, as shown in Table V. Hence about 20% of the radioactivity extractable from urine was present as a conjugate of 2,5-DIBA.

These results are in agreement with the chromatographic data presented in Figure 3. On the basis of the two methods it can be concluded that 39.6% of the dose of ^{14}C-TIBA was excreted in the urine as 2,5-DIBA and its conjugate; 5.7% was excreted as TIBA; 1.4% was excreted as 2-OH-3,5-DIBA; and 0.4% was excreted as 3,5-DIBA.

An isotope dilution using 4-hydroxy-3,5-diiodobenzoic acid demonstrated that this compound was not a metabolite. To test the possibility that a conjugate of 2-OH-3,5-DIBA was excreted, an isotope dilution with this compound was done using acid hydrolyzed urine extract. No increase in the amount of 2-OH-3,5-DIBA over the value for nonhydrolyzed urine extracts was observed. Thus, five radioactive substances in the urine extracts of rats dosed with ^{14}C-TIBA were identified, accounting for 78.5% of the extractable radioactivity.

DISCUSSION

Metabolic fate studies of TIBA have been conducted on a number of animal species and on at least one species of plant. Ice *et al.* (1968), using lactating goats and cows, found the major urinary metabolite of 2(^{131}I),3,5-triiodobenzoic acid to be 2,5-DIBA, agreeing with the work of Gutenmann *et al.* (1967). Trace amounts of 2,3-DIBA, *o*-iodobenzoic acid, and iodide ion were also found in the urine of these animals. These authors also observed a substantial concentration of radioactivity in the thyroid which they identified as iodide ion. McGee *et al.* (1969), using gas chromatographic methods, identified 2-OH-3,5-DIBA as the major metabolite in the milk of cows dosed with 2(^{131}I),3,5-TIBA. Also found in the milk were TIBA and trace amounts of "mono-iodobenzoic acid," 2-hydroxy-5-iodobenzoic acid, and 3,5-DIBA.

Ice *et al.* (1966), in another study using rats dosed with 2(^{131}I),3,5-TIBA, chromatographically resolved the excreted ^{131}I in whole urine into five radioactive spots. Two of the radioactive spots were identified on the basis of R_f values as

TIBA and iodide ion. Chromatographic data from hydrolyzed urine led these workers to believe that one of the remaining four compounds was a conjugate of TIBA. Our data demonstrated that the conjugated metabolite was a derivative of 2,5-DIBA.

One might speculate that if loss of iodine from the number 2 position of TIBA occurred, resulting in the formation of 3,5-DIBA, hydroxylation could follow to yield 2-hydroxy-3,5-DIBA. However, Ice *et al.* were not able to determine the presence or absence of either compound due to the loss of the label.

Such a metabolic route as a major detoxication pathway of TIBA in the rat can now be eliminated because, in our studies using ^{14}C-TIBA, 2-hydroxy-3,5-DIBA was shown to account for only 2.3% of the extractable urinary radioactivity (1.4% of the dose) and 3,5-DIBA for less than 1% (0.4% of the dose). In his study using rats, Ice found that iodide ion accounted for 10.4% of the excreted radioactivity in whole urine. As we found 2–3% of the ^{14}C as products deiodinated in the 2 position, it is possible that about 7–8% of the unidentified urinary radioactivity is due to derivative(s) of TIBA lacking a #2 iodine atom.

A number of other compounds were investigated by us as being possible metabolites of TIBA in the rat. The compounds were obtained commercially or synthesized in the laboratory. These were: 2,3,5-triiodobenzoyl glycine; 2,5-diiodobenzoyl glycine; 4-methoxy-3,5-diiodobenzoic acid; 4-hydroxy-3,5-diiodobenzoic acid; 2,3-diiodobenzoic acid; 2-methoxy-3,5-diiodobenzoic acid; 3-hydroxy-5-iodobenzoic acid; 2-hydroxy-3-iodobenzoic acid; 2-iodo-3-hydroxybenzoic acid; 2-hydroxy-5-iodobenzoic acid; 3-iodobenzoic acid; 2-iodobenzoic acid; salicylic acid; benzoic acid; anthranilic acid; hippuric acid; 2,3,4-trihydroxybenzoic acid; 3,4-dihydroxybenzoic acid; 2,3-dihydroxybenzoic acid; 3,5-dihydroxybenzoic acid; 2,6-dihydroxybenzoic acid. These compounds, which represent some of the more obvious products one might expect from metabolic deiodination and/or ring hydroxylation of TIBA, were not present in the ether extract of urine from rats dosed with radioactive TIBA.

Rowles *et al.* (1970) separated eight radioactive metabolites from the excreta of chickens which had been dosed with ^{14}C carboxyl labeled TIBA. These authors identified four of the compounds as: TIBA; 2,3-diiodobenzoic acid; 2,5-diiodobenzoic acid; and 3,5-diiodobenzoic acid. The excretion of 2,3-diiodobenzoic acid by the chicken provides an example of species specificity in routes of detoxication, as 2,3-diiodobenzoic acid was shown by us to be absent from excreta of rats.

Studies on pigs (Barker *et al.*, 1967) revealed the presence of trace amounts of 2,5-DIBA, 3,5-DIBA, and TIBA in the brain and thryroids of pigs dosed with nonradioactive TIBA.

Spitznagle *et al.* (1969) identified residues of TIBA, 2,5-diiodobenzoic acid, and 3,5-diiodobenzoic acid in the various plant parts and seeds of soybean plants which had been sprayed with ^{14}C-TIBA. Thus it would appear that TIBA is metabolized in essentially the same manner by five species of animals and one plant species in that deiodination with or without conjugation is a common metabolic fate of TIBA; but ring hydroxylation and esterification of the acidic group represent only minor, at best, metabolic routes of this compound.

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