Fate of the Plant Regulator 2,3,5-Triiodobenzoic Acid (TIBA) in the Bovine

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No residues of TIBA were found in the milk or feces of a dairy cow fed 5 p.p.m. of the compound in the feed. Partial urinary excretion of free TIBA occurred. A compound characterized by retention time as 2,5-diiodobenzoic acid was eliminated freely and as a conjugate in the urine. The compound was also produced when TIBA was briefly exposed to sunlight. When TIBA was fed to steers, urinary excretion of 2,5-diiodobenzoic acid and traces of other partially deiodinated benzoic acids were indicated. TIBA was stable when incubated with fresh rumen fluid, thyroid, or liver from a cow or pork liver. Electron affinity detection and atomic iodine emission spectrometry with gas chromatography were used for analysis.

TIBA (2,3,5-triiodobenzoic acid) is a promising growth-regulating chemical in the culture of soybeans and several other crops. Its ability to produce a more compact soybean plant permitting a greater number of plants per acre and potentially higher yields is under vigorous investigation. The use of soybean meal in cattle feed prompted a study of the fate of this compound in the dairy cow and in steers.

In studies with 2(^{131}I),3,5-triiodobenzoic acid in rats, four iodine-containing metabolites were found using thick-layer chromatography (Ice *et al.*, 1966). Only one of the four metabolites was identified (iodide ion). The compounds, 2,5-diiodobenzoic acid, 3,5-diiodobenzoic acid, and TIBA were identified in a number of organs in rats, chickens, and pigs that ingested TIBA (Barker *et al.*, 1967). The herbicide Ioxynil (3,5-diiodo-4-hydroxybenzonitrile) when fed to a cow underwent deiodination with production of 3-iodo-4-hydroxybenzonitrile and excretion of the latter as a urinary conjugate as indicated by gas chromatographic data (Fisher *et al.*, 1965). Using neutron activation analysis, iodide ion concentration in the cow's urine was found to rise sharply following ingestion of Ioxynil. Urinary excretion of iodide ion has been observed in other animals after administration of various iodoorganic compounds (Williams, 1959). The metabolism of TIBA has not been previously studied in the bovine.

Experimental Procedure

A Holstein cow was catheterized and fed 5 p.p.m. (based on a daily ration of 50 pounds) of pure analytical grade TIBA for four days. The compound in absolute ethanol was thoroughly mixed with the grain. Morning and evening subsamples of the total mixed milk were taken one day prior to feeding (control sample), daily throughout the feeding period, and for five days thereafter. The total daily urine and feces samples were similarly collected, weighed, separately mixed, and subsampled during the same test period. All samples were immediately frozen and held for analysis. All analyses of daily milk were made on a composite of the morning and evening subsamples.

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Four Holstein steers were also used in feeding studies. TIBA, 100 mg., was fed once to each of two of the animals. A third received a single 1-gram dose. A fourth received one 200-mg. dose of 2,5-diiodobenzoic acid, a suspected metabolite. Only steer urine was collected. Since both of these compounds (as pure standards in acetone or ethanol) were unstable in sunlight, they were rapidly weighed out in gelatin capsules and immediately administered to the animals with a bolus gun. Again to minimize and to study possible photolytic decomposition of the compounds in urine, urine from the first steer which received 100 mg. of TIBA was collected directly from the animal in a clean plastic pail and was extracted and analyzed within 5 minutes following its secretion. Urine from the other steers was completely collected daily through urine and feces collection stalls. Owing to inevitable contamination of this urine with fecal matter, 8 p.p.m of TIBA was added to a separate sample of this urine taken before feeding (control) and the solution allowed to stand in daylight for 12 hours before analysis to study possible decomposition of TIBA.

Possible residues of free TIBA were extracted from milk, urine, and feces by blending 25 grams of the sample with 70 ml. of acetone containing 1 ml. of orthophosphoric acid. The mixture was filtered, and the filter was rinsed with acetone to a total volume of 100 ml. One milliliter of the acetone extract was evaporated to dryness in a 10-ml. volumetric flask, the residue was esterified with diazomethane (Kirkland, 1961), and the methyl ester of TIBA was analyzed for by gas chromatography. The column used for most separations was 6 feet long, containing 10% DC-200 on 80- to 100-mesh Gas Chrom Q. For analysis of urine from the steer fed 1 gram of TIBA, a column of the same dimensions (and with the same operating parameters) packed with 0.75% diethylene glycol succinate (DEGS) plus 0.25% ethylene glycol succinate (EGS) on 80- to 100-mesh acid-washed Chromosorb W was used. Ebert and Thompson (1966) later found this column to separate more effectively 2,5-diiodobenzoic acid and 3,5-diiodobenzoic acid than the DC-200 column. The gas chromatograph used was a Barber-Colman Model 10 with a battery-operated 6-cc. electron affinity detector containing 56 μc. of radium-226. The operating temperatures for the column, flash heater, and detector were

200°, 265°, and 235° C., respectively, and nitrogen (60 cc. per minute) was the carrier gas. Electrometer gains of 3000 and 10,000 were used.

Possible formation of conjugated derivatives (for instance with glycine to form hippuric acid or phenolic glucuronides resulting after ring hydroxylation with or without partial deiodination) was investigated. Hydrolysis of the evaporated acetone extracts was conducted by refluxing with 85% orthophosphoric acid for 30 minutes followed by diazomethane esterification to yield the corresponding methyl esters and ethers. The resulting hexane (benzene is also suitable) solutions were chromatographed on the DC-200 column. Phosphoric acid is an effective agent for cleavage of glycine conjugates of benzoic acids (Hickenbottom, 1957) and would also hydrolyze phenolic conjugates. Phosphoric acid hydrolysis of milk, urine, or feces directly caused the production of many interfering peaks in the chromatograms.

Stability of TIBA when incubated with rumen fluid was studied. One milligram of TIBA in 1 ml. of absolute ethanol was thoroughly mixed with 100 ml. of filtered rumen fluid freshly sampled from a Holstein steer, and the mixture was held at the normal body temperature (38° C.). At measured intervals, the fluid was again mixed, and 5 ml. were removed and diluted to 25 ml. with acetone. One milliliter of the solution was evaporated, and the residue was methylated and chromatographed as described.

Possible metabolism of TIBA in the presence of liver and thyroid from a freshly slaughtered cow was studied. The compound (50 μg.) in 0.1 ml. of ethanol was added to 1 gram of thinly sliced liver or thyroid in a 100-ml. beaker with 10 ml. of 0.9% sodium chloride solution. The contents were maintained at 38° C. in a shaking water bath. At specific intervals, a sample beaker was removed, and the contents were blended with acetone and filtered and rinsed with acetone to a total volume of 50 ml. Two milliliters of this solution were evaporated, methylated, and analyzed as described.

The analysis of urine for TIBA and the metabolite was also performed using emission spectrometry and gas chromatography (Bache and Lisk, 1966). Measurement of the 2062-A. atomic iodine emission provided a sensitive, selective method of detecting and determining organic iodine-containing compounds. The selectivity ratio of this detector for iodine compounds versus noniodine-containing hydrocarbons of equal molecular weight is about 40 to 1. Therefore, other peaks representing compounds not necessarily containing iodine did appear in the chromatograms. The method of extraction and isolation follows.

Twenty-five grams of urine were thoroughly blended with 100 ml. of acetone and 5 ml. of 19.6% orthophosphoric acid. The mixture was vacuum-filtered, and the filter was rinsed with 25 ml. of acetone. The combined filtrates were concentrated to about 50 ml. using suction and a rotating evaporator in a water bath at 30° C. The resulting aqueous solution was successively extracted with 50 and 25 ml. of chloroform. The combined chloroform solutions were dried over sodium sulfate and evaporated to dryness using the evaporator as described. The residue was dissolved in 50 ml. each of chloroform and 2% sodium bicarbonate, and the combined solutions were partitioned. The chloroform layer was drawn off, and the aqueous solution was extracted with 25 ml. of chloroform. The organic layer was discarded, and the aqueous solution was adjusted to pH 2 with 5*N* hydrochloric acid. The solution was extracted with three successive 25-ml. portions of chloroform, and the combined chloroform extracts were again dried over sodium sulfate and evaporated to about 2 ml. using the evaporator. The remaining solution was quantitatively transferred to a 10-ml. volumetric flask using ether, and the solution was evaporated to dryness in the flask using a gentle air stream. The residue was dissolved in 3 ml. of 10% methanol in anhydrous ether and was methylated using diazomethane (Schlenk and Gellerman, 1960). Following esterification, the solution was evaporated to dryness in the 10-ml. volumetric flask. The residue was dissolved in 1 ml. of ethyl acetate, 9 ml. of saturated sodium chloride solution was added, and the contents were thoroughly shaken. Up to 6 μl. of the upper ethyl acetate layer were injected into the column. The column was identical to that described above except that it was operated at 210° C. The operating parameters of the gas chromatograph and emission detector, were the same as described previously (Bache and Lisk, 1966). The retention time for TIBA methyl ester was 36 minutes. A column temperature of 185° C. was used for analysis of the metabolite. Its retention time was 12.8 minutes. This lower column temperature was used in the case of the metabolite to improve its separation from other compounds with similar retention times. At this lower temperature, however, the retention time for TIBA methyl ester was unduly long.

Results and Discussion

Dairy Cow. Table I lists the recoveries of TIBA and other possible metabolites added to the control sample of milk, urine, etc. TIBA was not detected in milk or feces. Figure 1 shows chromatograms (using electron affinity detection) of the recovery of 0.2 p.p.m. of TIBA added to milk before extraction and an equivalent amount of control milk. The peak height of TIBA in both Figures 1 and 2 represents approximately 30% of the full recorder scale (10 inches). The method was sensitive to about 0.05 p.p.m. of TIBA. Phosphoric acid hydrolysis of evaporated acetone extracts of milk and feces to release possible acid (glycine) or phenolic (glucuronide) conjugates of TIBA or other metabolites was performed but no peaks to indicate their production were observed.

TIBA and a metabolite were excreted in the urine. Using electron affinity detection, the totals of TIBA for the third (third day after feeding began) through the seventh day of the experiment were, respectively, 10.9, 14.9, 17.4, 15.5, and 2.6 mg. This represented 13.5% of the total (454 mg.) TIBA fed. Figure 2 shows chromatograms (using electron affinity detection) of free TIBA and the metabolite found in unhydrolyzed urine on the fourth day and of control urine. Equivalent amounts of sample and control urine are represented in

Table I. Recovery of TIBA and Possible Metabolites

Compound	Milk P.p.m. added	Milk %	Urine P.p.m. added	Urine %	Feces P.p.m. added	Feces %	Liver P.p.m. added	Liver %	Thyroid P.p.m. added	Thyroid %	Rumen Fluid P.p.m. added	Rumen Fluid %
TIBA (EA)[a]	0.2	60	5.0	114	2.0	90	50	78, 82	50	75	10	75
	2.0	85										
TIBA glycine conjugate (EA)	4.0	70	0.4	102	4.0	89						
			1.0	101								
			4.0	92								
TIBA (MW)[b]			0.2	103								
2,5-Diiodobenzoic acid (EA)			0.4	97.5, 100								
2,5-Diiodobenzoic acid glycine conjugate (EA)			0.4	72.5								

[a] Electron affinity detection.
[b] Microwave emission spectrometry.

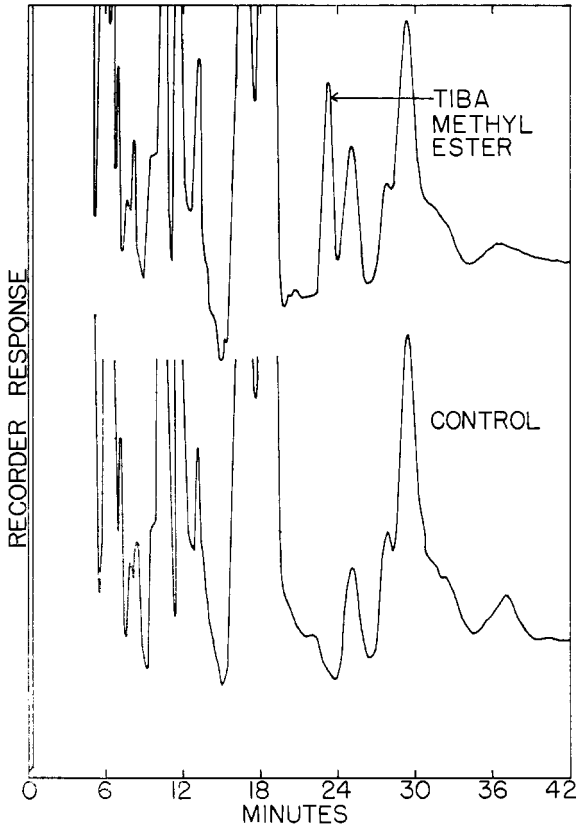

Figure 1. Chromatograms of the recovery of 0.2 p.p.m. of TIBA added to milk and the control milk

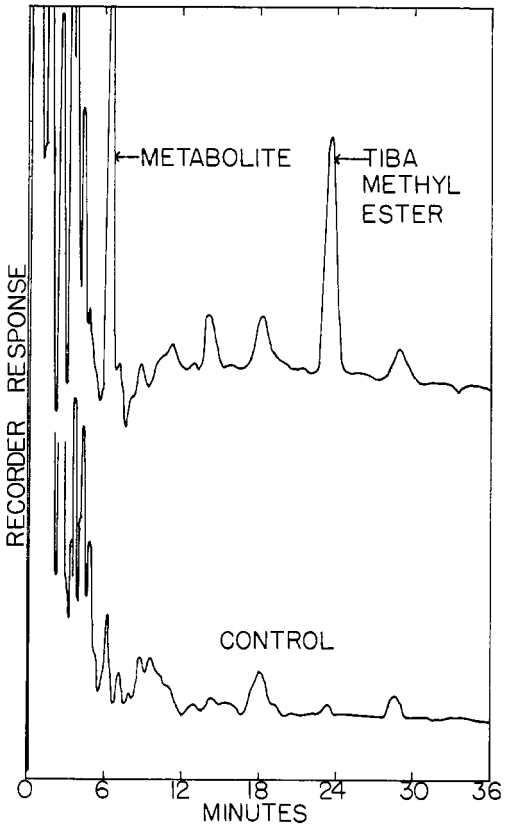

Figure 2. Chromatograms showing the excretion of TIBA and the metabolite in unhydrolyzed cow urine 4 days after feeding began and the control urine

the chromatograms. Using microwave emission detection, the totals of TIBA for the third through the seventh day of the experiment were, respectively, 7.1, 12.2, 13.9, 11.0, and 1.7 mg. The metabolite compound showed a retention time of about 6.4 minutes. Pure TIBA standard in acetone or ethanol and in daylight rapidly decomposed to yield a yellow solution which, when evaporated, methylated, and chromatographed, showed a peak with the same retention time as the urinary metabolite. Since methylation was required to chromatograph the decomposition product on the nonpolar DC-200 substrate, the compound was suspected of being an acid or phenol. The strong response of the compound to both electron affinity and atomic iodine emission detection indicated that it contained iodine. Its much shorter retention time as compared with TIBA methyl ester could have been caused by a loss in molecular weight from partial deiodination. TIBA added to uncontaminated urine and allowed to stand in daylight for 12 hours did not decompose. Therefore, the metabolite in urine might simply be the decomposition product of TIBA which presumably formed in the alcohol solution which stood for 2 days prior to being added to the cow's grain.

Seven iodobenzoic acid analogs of TIBA (2-iodobenzoic acid, 3-iodobenzoic acid, 2,3-diiodobenzoic acid, 2,5-diiodobenzoic acid, 3,5-diiodobenzoic acid, 2-hydroxy-5-iodobenzoic acid, and 2-hydroxy-3,5-diiodobenzoic acid) were methylated and chromatographed to compare their retention times with that of the unknown metabolite found in urine. Only the methyl ester of 2,5-diiodobenzoic acid had a retention time identical with that of the methyl ester of the metabolite. A solution of this compound in ethanol or acetone is yellow. 2,5-Diiodobenzoic acid, also decomposes in acetone solution in daylight, but at a much slower rate than TIBA. The solution changes from yellow to red over a period of about 2 weeks. Methylation and chromatography simply show a progressive decrease in peak height of the compound during this period, but possible monoiodo decomposition products are not discernible because of their very short retention times.

Analysis of urine for the metabolite was performed using both detectors, and the results were quantitated using a standard curve developed from 2,5-diiodobenzoic acid. Using electron affinity detection, the totals of the compound for the second through the seventh day were, respectively, 18.1, 22.3, 17.4, 17.4, 6.3, and 0.5 mg. On an equivalent basis, this represented 24.1% of the total TIBA fed. Using emission detection, the totals of the compound for the second through the sixth day were 13.7, 18.0, 19.4, 16.7, and 4.8 mg. The methods were sensitive to about 0.05 p.p.m. of the compound (2,5-diiodobenzoic acid).

Acid hydrolysis of urine extracts to test for the presence of conjugates of TIBA or the metabolite was performed. The concentrations of TIBA found in the urine following hydrolysis were, within experimental error, identical to those determined without hydrolysis. The concentrations of the metabolite, however, were considerably greater. The totals of the metabolite quantitated as 2,5-diiodobenzoic acid for the first through the ninth day were, respectively, 0.8, 35.5, 39.2, 42.5, 42.5, 15.4, 3.0, 1.8, and 1.0 mg. The difference between the total of these values (181.7 mg.) and that obtained by electron affinity detection for the metabolite before hydrolysis (82 mg.) is 99.7 mg. and represents the conjugated metabolite. Thus, 67% of the total TIBA fed was accounted for based on elimination in the urine of free TIBA (13.5%), the free metabolite (24.1%), and the conjugated metabolite (29.4%). TIBA did not decompose in rumen fluid in 6 hours and was stable in the presence of beef or pork liver and beef thyroid for 30 minutes. Ioxynil was also stable in rumen fluid (Fisher *et al.*, 1965).

Steers. Urine collected directly from the first steer fed 100 mg. of TIBA and immediately analyzed showed substantial amounts of TIBA and the metabolite 3.5, 7, 15, and 25 hours after dosing. This indicated that the animal (as well as sunlight) was capable of causing this conversion. Fourteen per cent of the TIBA (100 mg.) fed to the second steer was eliminated unconjugated in the urine. An additional 38% of equivalent TIBA was eliminated as the unconjugated metabolite in this steer. Interestingly, no residues of the conjugated metabolite were found in the urine of this animal, although analysis for it was repeated several times.

The third steer was fed 1 gram of TIBA to determine if a single large dose would reveal the presence of traces of other metabolites in the urine. Figure 3 illustrates chromatograms (using electron affinity detection) representing equivalent amounts of this steer's urine (unhydrolyzed) collected 24 hours after dosing and control urine. Peaks numbered 1, 2, 3, and 5 in the upper chromatogram had retention times on the DEGS-EGS column corresponding, respectively, to the methyl esters of 3,5-diiodobenzoic acid, 2,5-diiodobenzoic

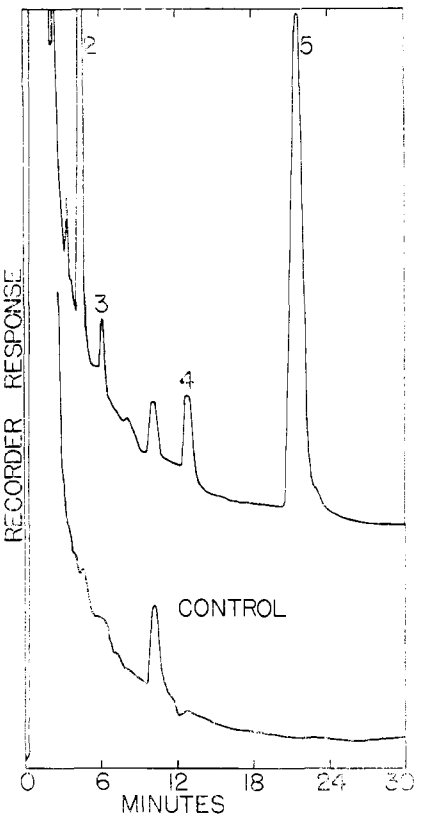

Figure 3. Chromatograms showing the excretion of TIBA and metabolites in unhydrolyzed steer urine 1 day after feeding 1 gram of TIBA and the control urine

Table II. Retention Times of TIBA and Various Analogs on Two Columns

Compound	Retention Time, Min. DC-200	DEGS-EGS
TIBA	23.2	21.0
2,5-Diiodobenzoic acid	6.4	3.9
2,3-Diiodobenzoic acid	7.5	5.7
3,5-Diiodobenzoic acid	6.8	3.0
2-Iodobenzoic acid	1.4	0.7
3-Iodobenzoic acid	1.5	0.9
2-Hydroxy-3,5-diiodobenzoic acid	8.9	9.1
2-Hydroxy-5-iodobenzoic acid	4.0	8.7

Table III. Urinary Metabolites in the Cow and Steers

Animal	Compound	Dose, Mg.	Products Excreted, % of Dose: TIBA	2,5-Diiodobenzoic acid	2,5-Diiodobenzoic acid conjugate	Other
Cow	TIBA	454	13.5	24.1	29.4	
Steer 1	TIBA	100	Substantial	Substantial	n.d.[a]	
Steer 2	TIBA	100	14	38	0	
Steer 3	TIBA	1000	Substantial	Substantial	n.d.	2,3-Diiodobenzoic acid (trace) 3,5-Diiodobenzoic acid (trace) Unknown compound
Steer 4	2,5-Diiodobenzoic	200		60.9	0	

[a] n.d., not determined.

acid, 2,3-diiodobenzoic acid, and TIBA. Peak No. 4 was not identified. Characterization of the major metabolite as 2,5-diiodobenzoic acid in cow urine was therefore based on the retention time of its methyl ester (6.4 minutes) on the DC-200 column and in steer urine based on this latter retention time as well as a retention time of 3.9 minutes on the DEGS-EGS column. These chromatograms (Figure 3) are quite free of other interfering peaks owing to the sample-dilution required to keep TIBA on scale. Gas chromatographic analysis of massive amounts of the TIBA standard (which was fed) assured the absence of these other iodine-containing benzoic acid compounds as trace impurities in TIBA. The DEGS-EGS column was used for this analysis.

The steer which received 2,5-diiodobenzoic acid (the suspected metabolite) eliminated 60.9% of the 200 mg. fed as unconjugated 2,5-diiodobenzoic acid. No decomposition of TIBA added to the urine containing fecal material and exposed to daylight for 12 hours was observed. Table II lists the retention times in minutes for TIBA and the available analogs which were studied on both columns using electron affinity detection. A summary of the urinary metabolites (as characterized by their retention times) resulting from the feeding studies with the cow and steers is tabulated in Table III.

Conclusions

TIBA or conjugates of it did not appear in the milk or feces of a cow fed 5 p.p.m. of the compound in its feed. The cow urine contained a major metabolite characterized by retention time on a DC-200 column as 2,5-diiodobenzoic acid. This metabolite was found in the urine freely and as a conjugate in about equal amounts. In steers, TIBA was excreted in the urine freely and as the unconjugated metabolite. At a high feeding level of TIBA, the presence of TIBA, the major metabolite, and traces of three other metabolites in steer urine was indicated. Two of these additional compounds had retention times on a DEGS-EGS column corresponding to 3,5-diiodobenzoic acid and 2,3-diiodobenzoic acid. The other compound was not identified. When the suspected major metabolite (2,5-diiodobenzoic acid) was fed to a steer, 60.9% was eliminated freely in the urine.

Other pathways of degradation may involve further deiodination to monoiodo derivatives. These would be difficult to chromatographically separate from other early emerging compounds as is evident in the chromatograms (Figures 1 and 2). The retention times on the DC-200 column of the 2- or 3-iodobenzoic acid methyl esters were 1.4 and 1.5 minutes, respectively, at 200° C. These compounds are also of very low electron affinity and are therefore only detectable at very high concentrations. Elimination of iodide ion in the urine following deiodination may have occurred but analysis was not made for it. TIBA rapidly decomposed when exposed to sunlight but was stable when incubated with rumen fluid, beef or pork liver, and beef thyroid.

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