Metabolic Transformations of Disugran by Rumen Fluid of Sheep Maintained on Dissimilar Diets

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Disugran (methyl 3,6-dichloro-*o*-anisate) is rapidly degraded during *in vitro* incubation with ovine rumen fluid. Metabolites characterized by glc–mass spectral studies included methyl 3,6-dichlorosalicylate, 3,6-dichlorosalicylic acid, and small amounts of 3,6-dichloro-*o*-anisic acid. Direct incubation of 3,6-dichloro-*o*-anisic acid with rumen fluid resulted in no detectable degradation, indicating that the major pathway of disugran metabolism is through initial cleavage of the methyl ether, then subsequent hydrolysis of the ester. Although at least four unidentified disugran metabolites were observed in these studies, the three identified compounds comprised approximately 95% of the total quantity of metabolites generated. The extent of disugran metabolism and relative metabolite distribution differed between sheep provided low- and high-energy diets. Rumen fluid from animals fed a low-energy ration degraded disugran faster and more extensively than did rumen fluid from sheep fed high-energy diets.

Disugran (Racuza, methyl 3,6-dichloro-*o*-anisate) is a growth regulant currently under development by Velsicol Chemical Corp., Chicago, Ill. This compound has shown potential for increasing recoverable sugar in sugarcane and sugarbeets, for effecting early ripening of grapefruit, and for increasing yields of grapes.

Disugran is the methyl ester of the herbicide dicamba (Banvel, 3,6-dichloro-*o*-anisic acid), which is extensively used for postemergence weed control on small grains, pasture lands, and turf grass and for certain types of brush control. Although metabolic pathways have not been reported for disugran, studies have been published relative to the metabolism of dicamba by animals and plants and its breakdown in soil. Dicamba was eliminated rapidly in the urine after oral or subcutaneous administration to rats (Tye and Engel, 1967). About 80% of the doses were excreted unmetabolized, and the rest were eliminated as dicamba glucuronide. Wheat and bluegrass metabolized dicamba to conjugates of the parent compound, 5-hydroxy-3,6-dichloro-*o*-anisic acid, and 3,6-dichlorosalicylic acid (Broadhurst *et al.*, 1966). These products were also observed after dicamba treatment of barley, but wild mustard and "Tartary" buckwheat yielded a conjugate of the 5-hydroxy derivative as the single metabolite (Chang and Vanden Born, 1971). In a heavy clay soil, dicamba was converted to 3,6-dichlorosalicylic acid and unidentified products resulting from loss of the carboxyl moiety as carbon dioxide (Smith, 1973).

Potential use patterns of disugran are such that residues may appear in products comprising livestock feed, in addition to possible environmental contamination of water and forage. Information is therefore needed to evaluate the extent to which disugran may pose hazards to livestock. The Mrak report (1969) has emphasized the need for studies to determine the effects of nutritional and other factors on body responses to foreign compounds, and thus the current work was initiated to investigate the nature and distribution of metabolic products generated after incubation of disugran with rumen contents of sheep fed dissimilar diets.

MATERIALS AND METHODS

Chemicals. Samples of disugran-*carboxyl*-^{14}C (5.02 mCi/mmol) and dicamba-*carboxyl*-^{14}C (5.02 mCi/mmol) were provided by Velsicol Chemical Corp., Chicago, Ill. Both preparations were purified before use by thin-layer chromatography (tlc) to a radiochemical purity of >99%. Unlabeled samples of disugran, dicamba, and certain other compounds considered as potential metabolites were also provided by Velsicol.

Animals and Incubation Procedures. Six adult ewes (mixed breed, weight approximately 40 kg each) were randomly divided into two groups of three each and were housed outdoors in small pens. One group was fed twice daily with a high-energy sheep ration consisting of chopped corn (29%), crimped oats (28%), cottonseed meal (20%), alfalfa leaf meal (20%), bone meal (2%), and salt (1%). Chopped alfalfa hay (5% w/w) was added to the feed to provide additional roughage. The animals were provided appropriate mineral supplements and water. The other group was fed an all-roughage diet consisting solely of "Coastal" bermuda grass hay *ad libitum*, with water and mineral supplements.

After the animals had been maintained on their respective diets for 8 weeks, samples of rumen fluid were obtained by stomach tube and immediately placed in glass bottles in a 39° water bath. The samples were quickly flushed with carbon dioxide and tightly capped. In the laboratory, the fluid was pooled according to group, and particulate matter was removed by filtering through several layers of cheesecloth. At all times, attempts were made to maintain samples at 39° and under a carbon dioxide atmosphere.

The appropriate radiochemical in a small amount of acetone was added to a 50-ml culture tube, and the solvent was evaporated with a gentle stream of nitrogen. Disugran samples required particular care during this procedure to avoid volatility loss. Five milliliters of rumen fluid was then pipetted into the tube, and the tube was flushed with carbon dioxide, sealed with a Teflon-lined cap, and incubated with shaking at 39°. The incubations, in duplicate for each analysis interval and rumen fluid group, contained disugran or dicamba concentrations equivalent to 5.0×10^{-6} *M*. At appropriate time intervals, samples were removed from incubation, acidified with 0.1 ml of 4 *N* HCl, and immediately frozen until analysis.

In experiments designed to obtain larger metabolite quantities for characterization studies, disugran-^{14}C was diluted with unlabeled disugran and incubated with 50-ml portions of rumen fluid from sheep fed the low-energy diets. The incubations were conducted in stoppered erlenmeyer flasks under carbon dioxide and contained disugran equivalent to 1×10^{-4} *M*. After incubation for 24 hr, the samples were acidified and frozen for subsequent analysis.

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Table I. Metabolites of Disugran-^{14}C after Incubation with Rumen Fluid of Sheep Maintained on Dissimilar Diets

Incubation, hr	Radiocarbon as indicated product, %							
	Met 1	2-Hydroxy dicamba	Dicamba	Met 4	Met 5	Met 6	2-Hydroxy disugran	Disugran
				Low-Energy Diet				
0	0.4	1.0	0.8	0.3	0.2	3.6	5.9	87.8
1	1.0	33.5	3.6	1.0	0.5	5.6	49.4	5.4
4	1.2	74.9	3.1	1.1	0.5	4.5	13.8	0.9
12	1.1	87.1	2.9	1.4	0.6	3.2	3.1	0.6
24	1.0	87.7	3.3	1.9	0.9	2.6	2.5	0.1
24 (boiled fluid)	1.2	3.0	1.4	0.8	0.5	0.7	9.6	82.8
				High-Energy Diet				
0	0.6	1.1	1.1	0.4	0.2	1.3	4.5	90.8
1	1.7	4.9	2.6	0.3	0.9	2.2	51.2	36.2
4	1.6	12.3	3.6	0.4	1.7	3.3	66.5	10.6
12	0.9	10.0	3.4	0.4	1.9	3.3	73.5	6.6
24	1.0	17.3	4.9	0.4	2.2	2.9	65.5	5.8
24 (boiled fluid)	2.2	4.0	1.7	0.7	0.5	0.4	6.6	83.9

Metabolite Isolation and Characterization. Acidified samples were exhaustively extracted with ether until >98% of the radiocarbon present was recovered in the organic phase. Acidification before extraction was necessary to obtain the desired recoveries, and experiments were conducted that indicated acid treatment did not result in further degradation of disugran or its metabolites. Selected nonacidified samples were analyzed by freeze drying, and tlc studies on methanol extracts of the residue indicated the same metabolite patterns in both acidified and nonacidified samples. The disugran in these samples was lost through volatility during the freeze drying process, but studies with the pure compound indicated it to be acid stable also.

The ether extracts were dried over sodium sulfate, concentrated with a gentle stream of nitrogen, and spotted on silica gel precoated thin-layer plates (Silplate F-22, 0.25-mm gel thickness, Brinkman Instruments, Westbury, N. Y.). Predevelopment of the plates in hexane, followed by benzene–acetic acid–ether (32:4:1) (solvent 1) gave satisfactory resolution of all metabolites except methyl 3,6-dichlorosalicylate, which overlapped with the parent compound. For this reason, a part of each extract was resolved on plates developed in hexane–acetone (9:1) (solvent 2), which gave good separation of these two products. Radioactive compounds on the developed plates were visualized by radioautography, and the appropriate gel regions were scraped and quantitated by liquid scintillation counting. Unlabeled metabolite standards were detected by ultraviolet light visualization or by diphenylamine spray reagent (Ivie and Casida, 1971).

Initial indications of possible metabolite identity were obtained by comparison of chromatographic behavior of radioactive metabolites with standards of known structure. If metabolites were generated in sufficient quantity, they were isolated by tlc and reacted with diazomethane to generate methyl esters or ethers. The nature of the derivatives was studied by combined gas–liquid chromatography/mass spectrometry. The instrument used was a Varian-MAT-CH-7 spectrometer coupled with a Varian 2700 chromatograph. The compounds were resolved on a 6-ft stainless steel column packed with 1.5% OV-17 + 1.95% QF-1 on 80–100 mesh Supelcoport. Operating parameters were as follows: injector, 220°; column, 175°; separator and inlet, 250°; ion source, 260°; ionizing voltage, 70 eV. Helium flow rate through the column was maintained at 50 ml/min. Before the glc–mass spectral studies, derivatized metabolites were subjected to cleanup either by tlc or on small Florisil columns eluted with a 2:1 mixture of ether–hexane. The products were then transferred to acetone solution for glc–mass spectral analysis.

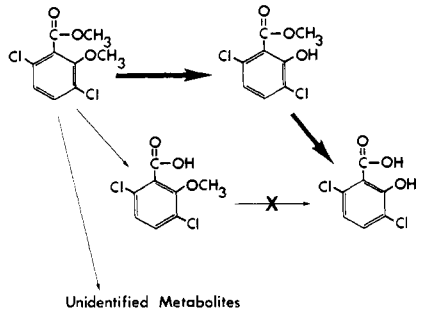

Figure 1. Metabolic pathway for disugran in the ovine rumen.

RESULTS AND DISCUSSION

Disugran-^{14}C undergoes rapid degradation when incubated with rumen fluid from sheep fed both low- and high-energy diets (Table I). However, animals fed the low-energy diet yielded rumen fluid that catalyzed disugran breakdown at a considerably faster rate than did that from sheep fed the high-energy diet. Incubations from the low-energy group resulted in almost total disugran breakdown within 4 hr, but significant amounts of unmetabolized disugran were observed in the high-energy samples even after incubation for 24 hr.

Seven radioactive metabolites were observed after tlc analysis of the extracted rumen samples. The following information was obtained on these products.

Metabolite 1 (R_f 0.0, solvent 1) consisted of those products that remained at the origin after solvent development of the chromatoplates. These materials were generated in only minor quantity, and no attempts were made to determine their chemical nature.

Metabolite 2 (R_f 0.10, solvent 1) exhibited the same tlc behavior as 3,6-dichlorosalicylic acid, and diazomethane converted the metabolite to a product identical in tlc and glc–mass spectral behavior with disugran. An intermediate corresponding to methyl 3,6-dichlorosalicylate was observed in the methylation of metabolite 2 to disugran. This is consistent with the structural assignment of the compound as 3,6-dichlorosalicylic acid (2-hydroxy dicamba, Table I and Figure 1).

Metabolite 3 (R_f 0.20, solvent 1) was identified as dicamba (3,6-dichloro-*o*-anisic acid) on the basis of tlc behavior, and its methylation to a product identified as disugran by tlc and glc–mass spectra.

Metabolites 4 and 5 were minor, diffuse products at R_f values (solvent 1) of approximately 0.25 and 0.31, respectively. The chromatographic properties of these products suggested that each might consist of more than one com-

ponent, but the low amounts generated did not permit detailed studies of their chemical nature.

Tlc behavior of the unidentified metabolite 6 (R_f 0.36, solvent 1) indicated it to be considerably less polar than dicamba, and thus it seemed likely that the methyl ester remained intact. The larger scale incubations of disugran with rumen fluid failed to produce this product in sufficient amounts for glc–mass spectral studies, and thus no definitive information is available relative to its chemical nature. The metabolite did react with diazomethane to produce a single derivative of higher tlc R_f, and this product differed slightly in tlc behavior from both disugran and methyl 5-methoxy-3,6-dichloro-*o*-anisate (prepared by methylation of authentic 5-hydroxy-3,6-dichloro-*o*-anisic acid). If metabolite 6 is generated by ring hydroxylation, which seems likely on the basis of the available information, then reaction must have occurred at position 3, 4, or 6 because the product is not 5-hydroxy disugran. Reaction at positions 3 or 6 would require replacement of chlorine by hydroxyl.

Metabolite 7 (R_f 0.62, solvent 1; R_f 0.28, solvent 2) was characterized as methyl 3,6-dichlorosalicylate (2-hydroxy disugran, Table I and Figure 1) on the basis of its tlc behavior and methylation of the metabolite to disugran as confirmed by tlc and glc–mass spectra.

Small levels of each of the seven disugran metabolites were detected even with 0 time incubations and in samples incubated for 24 hr with boiled rumen fluid (Table I). In these samples, the products are possibly formed nonenzymatically during sample work-up and analysis; alternatively, they may be generated at least in part by very rapid enzymatic action before acidification and freezing of the 0 time samples and by incomplete inactivation of enzyme activity of the boiled samples.

Although the same metabolites were generated, regardless of the diet provided by test animals, the relative metabolite distribution varied considerably between the two nutritional groups (Table I). Rumen fluid from animals fed the low-energy diet produced 2-hydroxy dicamba as by far the major metabolite, and this compound comprised about 90% of the total quantity of metabolic products after incubation for 12 hr. Animals fed high-energy diets, however, yielded 2-hydroxy disugran as the major metabolite, and levels of 2-hydroxy dicamba observed were much lower than those in the low-energy group. Metabolite distribution relative to incubation time (Table I) strongly indicated 2-hydroxy disugran to be a precursor to 2-hydroxy dicamba, which would indicate that cleavage of the methyl ether was the first major step in the rumen metabolism of disugran. Although ester hydrolysis of disugran to dicamba, followed by cleavage of the ether, is also a potential route to 2-hydroxy dicamba, studies with dicamba indicated this pathway to be of negligible importance in the rumen. Direct incubation of dicamba-^{14}C with rumen fluid surprisingly yielded no detectable metabolites, and thus the 2-hydroxy dicamba observed does not involve dicamba as a precursor.

These studies demonstrate several interesting examples of enzyme specificity. Although an aryl-*O*-demethylase capable of degrading disugran is very active in the rumen of sheep maintained on both dietary regimens, comparable enzymes for metabolizing the free acid, dicamba, are apparently inactive or nonexistent. Also, esterases capable of hydrolyzing disugran to dicamba are not appreciably active in the ovine rumen, as indicated by only very small levels of dicamba generated. However, once the methyl ether is cleaved, the disugran molecule becomes susceptible to ester hydrolysis, particularly so in the low-energy samples. Thus, the major metabolite observed in the high-energy samples is 2-hydroxy disugran, whereas this compound is further degraded to yield 2-hydroxy dicamba as the major metabolite in the low-energy samples. In either case, the major disugran metabolites generated in the rumen are of such chemical nature that, if absorbed by the animal, they will most likely be rapidly excreted in the urine either unchanged or as conjugates. Thus, there seems little likelihood that potentially toxic residues would accumulate in the body.

It was not surprising that the two nutritionally different groups of ruminants differed considerably both in the extent of rumen metabolism of disugran and in relative metabolite distribution. That microbial rumen populations vary in nature and number with the nutritional regimen provided the animal is well known (Hungate, 1966). Also, recent studies have shown that sheep fed an all-concentrate, high-energy diet yielded rumen fluid that was more efficient in the degradation of DDT than was rumen fluid obtained from animals fed an all-roughage diet (Sink *et al.*, 1972). The studies with DDT might suggest that animals receiving excellent nutrition would tend to exhibit a greater capacity for rumen degradation of foreign compounds than would those fed marginal diets. However, regardless of the diet fed to an animal, the rumen will maintain appropriate microbial populations for energy utilization, and its capacity to metabolize a given foreign compound will be related only to the enzymatic mechanisms required for metabolism and the extent to which these processes are active within the rumen. Thus, while the findings that disugran is metabolized faster and more extensively by rumen fluid resulting from a low-energy diet additionally indicate that dietary composition can significantly affect the rumen metabolism of foreign compounds, they further show that these effects are likely to be unpredictable and may vary considerably with individual chemicals.

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