Absorption, Metabolism, and Excretion of Tritium-Labeled Famphur in the Sheep and Calf

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The metabolism of famphur, *O,O*-dimethyl *O,p*-(*N,N*-dimethylsulfamoyl)phenyl phosphorothioate, in the sheep and calf involves rupture of P—O—methyl, P—O—phenyl, and *N*-methyl bonds to yield water-soluble metabolites which are eliminated in the urine. Phenolic metabolites are excreted primarily as glucuronide derivatives. Marked differences in metabolism and excretion patterns occur depending on the method of administration, intramuscular injection providing more sustained famphur levels, and lower famoxon levels in blood than intravenous administration.

The active ingredient in famphur (Warbex, American Cyanamid Co.), *O,O*-dimethyl *O,p*-(*N,N*-dimethylsulfamoyl)phenyl phosphorothioate, has been found to be an effective agent against a variety of livestock pests. It is an anthelmintic agent with activity against a wide range of gastrointestinal nematodes of ruminants (Drudge and Szanto, 1963; Wood *et al.*, 1961). The compound is highly effective against the cattle grub (hypoderma) when fed (Drummond, 1963c), injected intramuscularly (Drummond, 1963b; 1963c), or following pour-on and oral drench treatments (Kohler and Rogoff, 1962; Neel *et al.*, 1963). The insecticide also shows promise in the control of certain fleas (Hill *et al.*, 1963) and hornflies (Drummond, 1963a).

To evaluate the possible toxicological hazards associated with the use of this material, its metabolic degradation in biological systems has been investigated.

O'Brien *et al.* (1961) studied the metabolism of famphur-H^3 in several insects and in the mouse and found that the degradation rates in the various animals differed markedly, although the compound was essentially equitoxic when injected into the animals studied. Among the metabolic products identified were the oxygen analog (famoxon) and *N*-demethyl famphur, the latter compound being present to a very minor extent in the mouse.

The metabolism of famphur-P^{32} in sheep has been investigated by Bourne (1963), who reported the identification of radioactive famoxon, *O*-demethyl famphur, mono- and dimethyl phosphoric acid, and dimethyl phosphorothioic acid in the urine of sheep treated intramuscularly with famphur-P^{32}. It was concluded that hydrolysis of the P—O—phenyl and P—O—methyl linkages were the main routes of detoxification.

To elucidate further the metabolic fate of famphur in mammals, particularly the phenolic portion of the molecule, studies were initiated in these laboratories using the sheep, calf, and rat as test animals and employing famphur labeled with tritium as shown in Figure 1. Certain aspects of this work have been discussed in a preliminary report by Gatterdam *et al.* (1963).

EXPERIMENTAL

Chemicals. The compounds used in this study are listed in Table I, together with chromatographic properties in selected solvent systems. Famphur, *O*-demethyl famphur [*O*-methyl *O,p*-(*N,N*-dimethylsulfamoyl)phenyl phosphate, cyclohexylamine salt] and *p*-(*N,N*-dimethylsulfamoyl)phenol, containing, respectively, 241 and 330 μc. per mg. of tritium were synthesized according to the reactions shown in Figure 1. *O,N*-Bisdemethyl famphur [*O*-methyl *O,p*-(*N*-methylsulfamoyl)phenyl phosphorothioate, dicyclohexylammonium salt] was prepared according to the method of Curry *et al.* (1967) by reaction of the dicyclohexylammonium salt of *O,O*-diisopropyl phosphorodithionate with *O,O*-dimethyl *O,p*-(*N*-methylsulfamoyl)phenyl phosphorothionate, which in turn was made from the *p*-(*N*-methylsulfamoyl)phenol. Famoxon [*O,O*-dimethyl *O,p*-(*N,N*-dimethylsulfamoyl)phenyl phosphate] was prepared by reaction of the *p*-(*N,N*-dimethylsulfamoyl)phenol with dimethylphosphoryl chloride in acetone. *p*-Hydroxybenzenesulfonic acid was obtained from Distillation Products Industries. β-Glucuronidase was obtained from the California Foundation for Biochemical Research.

Administration of Compounds. Two white-faced western ewes and one heifer calf were treated with tritiated famphur. The manner, route of administration, and dose are presented in Table II. Diethyl succinate was used as the

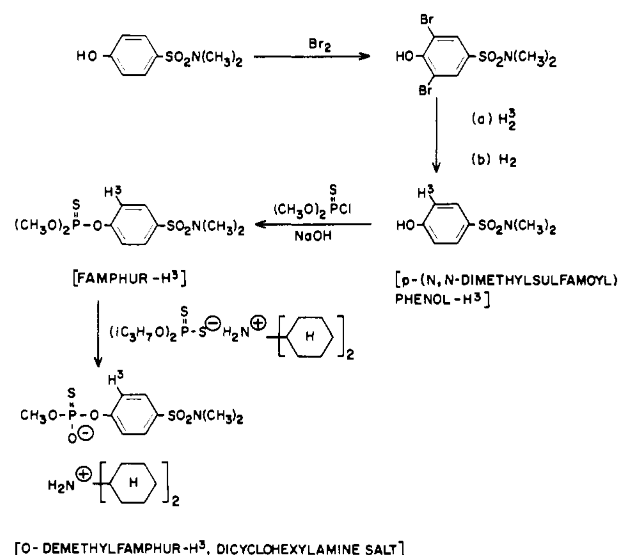

Figure 1. Synthesis of tritiated famphur, *p*-(*N,N*-dimethylsulfamoyl) phenol, and *O*-demethyl famphur, dicyclohexylamine salt

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Table I. Chromatographic Behavior of Famphur Metabolites and Related Compounds

Metabolite No.	Structure	Text Designation	Chromatographic Behavior[a] System A	System B	System C
1[b]	$(CH_3O)_2P(S)OC_6H_4SO_2N(CH_3)_2$	Famphur	0.89	0.90	0.85
2	$(CH_3O)_2P(O)OC_6H_4SO_2N(CH_3)_2$	Famoxon	0.87	0.90	0.52
3[b]	$HO(CH_3O)P(S)OC_6H_4SO_2N(CH_3)_2$	*O*-Demethyl famphur	0.52	0.70	0.12
4	$HO(CH_3O)P(S)OC_6H_4SO_2NHCH_3$	*O,N*-Bisdemethyl famphur	0.46	0.74	0.10
5	$HOC_6H_4SO_2OH$	*p*-Hydroxybenzenesulfonic acid	0.06	0.06	0.08
6	$Glu\text{-}OC_6H_4SO_2N(CH_3)_2$	*N,N*-Dimethylsulfamoylphenyl glucuronide	0.30	0.43	...
7	$Glu\text{-}OC_6H_4SO_2NHCH_3$	*N*-Methylsulfamoylphenyl glucuronide	0.18	0.28	...
8[b]	$HOC_6H_4SO_2N(CH_3)_2$	*N,N*-Dimethylsulfamoylphenol	0.85	0.60	0.00
9	$HOC_6H_4SO_2NHCH_3$	*N*-Methylsulfamoylphenol	0.82	0.52	0.00

[a] System A = Whatman No. 1 paper; isoamyl alcohol–acetic acid–water (70:20:20).
System B = Whatman No. 1 paper; acetonitrile–ammonium hydroxide–water (75:13:12).
System C = Whatman No. 1 paper; hexane-toluene (3:1).
[b] Indicates tritiated compounds.

Table II. Dosage and Administration of Tritiated Famphur to Sheep and Calf

Animal	Sex	Body Weight, Kg.	Administration	Dose, Mg./Kg.	Specific Activity, μc./Mg.	Duration of Experiment, Days
Sheep	Female	26.3	Intravenous	22.3	39	4
Sheep	Female	37.3	Intramuscular	55.1	7.2	3
Calf	Female	168	Intramuscular	60.7	25.1	3

vehicle for the intramuscular injections and dimethyl formamide for the intravenous treatment. The intramuscularly treated sheep was given a single dose in the right muscle, and the calf was injected at four sites in the gluteal muscle. Radioactive famphur was diluted with nonradioactive famphur to the specific activity indicated in Table II and dissolved in the vehicle at a concentration of about 25%. In tests designed to isolate glucuronides from urine, sheep were treated daily for 5 days with 5-gram intramuscular doses of weakly radioactive *p*-dimethylsulfamoyl phenol. In certain metabolism characterization experiments, female albino rats were used as test animals. Tritiated dimethylsulfamoyl phenol in water was injected subcutaneously at doses of 20 mg. per kg. of body weight. Tritiated *O*-demethyl famphur in diethyl succinate was injected subcutaneously into rats at dosage levels of 23 mg. per kg. body weight.

Sample Collection and Radioanalysis. Sheep were placed in metal metabolism cages 2 days before treatment. The calf was stanchioned in an adjustable enclosed stall lined with polyethylene sheets. Treated rats were held in metabolism cages (Acme Metal Products, Inc., Chicago, Ill.) that prevented cross-contamination of the excretory products. All animals were allowed food and water *ad libitum*. Blood samples from sheep and the calf were obtained from the jugular vein, transferred to bottles containing heparin, and refrigerated for later radioanalysis. Urine from the calf and sheep was collected by means of retention urethral catheters leading into plastic bottles enclosed in dry ice. Feces from the sheep were collected in plastic bags attached to the cages and in a polyethylene-lined box situated at the rear of the metabolism stall of the calf. Samples of blood, urine, and feces were taken at periodic intervals after treatment. The animals were sacrificed by exsanguination (calf), an overdose of nembutal (sheep), or chloroform vapor (rats).

Total tritium in all biological samples except fat was determined by the Schöniger combustion technique (Kelly *et al.*, 1961). Fat was radioassayed by homogenizing a sample of about 1 gram with 4 ml. of chloroform and counting an aliquot in a modified polyether 611 phosphor (Davidson and Feigelson, 1957). Organophilic radioactivity was extracted from blood and urine samples adjusted to pH 2.0 with hydrochloric acid, using three equal volumes of carbon tetrachloride. The mixture was centrifuged, the carbon tetrachloride extracts were combined and dried over anhydrous sodium sulfate, and aliquots were taken for radioassay and chromatography. Recoveries ranging from 90 to 95% were obtained from control urine and blood fortified with known amounts of tritiated famphur.

Characterization of Metabolites. EXTRACTION FROM BLOOD. Samples of whole blood were adjusted to pH 2.0 with hydrochloric acid and mixed violently with an acetone–95% ethyl alcohol mixture (50:50, v./v.) in a Virtis Model 45 microhomogenizer. The homogenized mixture was centrifuged at 10,000 r.p.m. in a refrigerated Spinco ultracentrifuge for 30 minutes. The pellet was returned to the homogenizer, and the extraction was repeated twice. The supernatant phases were pooled, concentrated to near dryness, and aliquots were taken for radioassay and chromatography. The extraction procedure recovered 70 to 85% of the total blood radioactivity at all time intervals.

CHROMATOGRAPHY. Aliquots of blood extracts and urine were applied to Whatman No. 1 filter paper strips and developed by ascending chromatography in glass chambers (45 × 15 cm.) containing 500 ml. of solvent mixture. The chromatograms were dried and the radioactive areas located by passing the strips through a scanner (Nuclear-Chicago Model C Actigraph II, equipped with a 4 π detector, Model 1036).

The acidic isoamyl alcohol system (Table I) provided a means of separating *O*-demethyl famphur, *O,N*-bisdemethyl famphur, *p*-hydroxybenzenesulfonic acid, and the two glucuronides from each other and from famphur and famoxon. The hexane-toluene system was useful for separating famphur from famoxon, and the basic acetonitrile system was used principally to separate the phenolic products resulting from hydrolysis of certain of the metabolites. To isolate the metabolites for further study, the chromatographic strips were cut crosswise to separate the radioactive areas from each other. Each section was then cut to a point at one end and the solutes were concentrated at the tip by means of the upward-washing technique described by Menn *et al.* (1960). Carbon tetrachloride was used to elute famphur and famoxon, and methanol-water (1 to 3) was used for the other metabolites. After the sections were dried, the solutes were transferred directly to melting-point capillaries by placing the capillaries horizontally in contact with the tip of the section and allowing fresh solvent to pass over the strip until approximately 100 μl. had entered the capillary. In this manner, essentially complete transfer of the radiometabolites was achieved as indicated by the absence of radioactivity in the washed papers.

Identification of Metabolites. The principal methods used in the identification of famphur metabolites in blood extracts and urine were as follows.

COCHROMATOGRAPHY. The blood extracts or urine were chromatographed in solvent systems A or B (Table I) or both in sequence, and the radiometabolites were isolated as described above. Tentative identifications were based on comparisons of chromatographic behavior with that of the authentic reference compounds. Supporting evidence was obtained by mixing the isolated radiometabolite with the suspected reference compound and chromatographing the mixture in the appropriate solvent. The position and shape of the radiopeak were compared with the position and shape of the reference spot detected by ultraviolet light or chromogenic reaction with 2,6-dibromo-*N*-chloro-*p*-quinoneimine (Menn *et al.*, 1957) for evidence that the two materials chromatographed identically.

HYDROLYSIS OF ISOLATED METABOLITES WITH ACID AND ALKALI. The radiometabolites were isolated and transferred to 2.0 × 100 mm. melting point capillaries as described above and 100 μl. of 2*M* sodium hydroxide or 2*M* hydrochloric acid added. The ends of the tubes were sealed by heating in a flame, and the tube contents were mixed and incubated for 24 hours in an oven at 75° C. Following hydrolysis, the tube ends were severed and the contents applied directly to chromatographic paper for development in the appropriate solvent system. Identification was based on the coincidence of the radioactive area and reference compound hydrolyzed in similar fashion, either alone or mixed with the radiometabolite.

COCRYSTALLIZATION WITH AUTHENTIC COMPOUNDS. Sections of paper chromatograms containing approximately 5 nanocuries of tentatively identified radiometabolites were placed in 50-ml. Erlenmeyer flasks containing 0.600 to 1.00 gram of the appropriate reference compound. A suitable recrystallization solvent and a pinch of decolorizing carbon were added. The mixture was heated to dissolve the carrier compound and equilibrate the labeled material on the paper section with the carrier. The solutions were then slowly cooled to allow crystallization to occur. A small portion of the crystallized product was saved for specific activity determination and the balance of the product was recrystallized repeatedly. *O*-Demethyl and *O,N*-bisdemethyl famphur were recrystallized three times from isopropyl alcohol, *p*-hydroxybenzenesulfonic acid three times from water, and dimethylsulfamoylphenol and methylsulfamoylphenol successively from water, nitromethane, and ether. Attainment of constant specific activity upon repeated recrystallization was taken as evidence that the metabolite was identical to the reference compound.

Detection of Glucuronic Acid Conjugates by Means of the Chromogenic α-Naphthoresorcinol Reaction. The procedure described by Hawk *et al.* (1954) was used to test for the presence of glucuronic acid conjugates in the urine of treated and control sheep and the calf.

To detect glucuronic acid conjugates on paper chromatograms, the chromatograms were sprayed with 4% α-naphthoresorcinol in 95% ethanol, dried, and resprayed with 1*N* hydrochloric acid. While still damp, the sprayed chromatograms were subjected to temperatures of 150° C. for 3 minutes in an autoclave. The presence of glucuronic acid conjugates was indicated by the appearance of green spots on a yellow background.

Special confirmatory tests were also used. On the basis of their chromatographic behavior before and after chemical hydrolysis and of their response to the chromogenic glucuronide test, radiometabolites 6 and 7 were suspected of being conjugation products of the mono- and dimethylsulfamoylphenols and glucuronic acid. Therefore, the isolated radiometabolites were subjected to the action of bovine liver glucuronidase under controlled conditions of pH and temperature (Talalay *et al.*, 1946). The reaction was followed by paper chromatography.

The presence of *p*-hydroxybenzenesulfonic acid was verified by forming its acetyl derivative [$CH_3C(O)$-$OC_6H_4SO_3$—Na^+]. The tentatively identified radiolabeled compound and authentic compound were placed in separate capillary tubes. An acetic acid–acetic anhydride mixture (1 to 1, v./v.) was added to the tubes and the contents heated to 75° C. for 24 hours. Cochromatography of the incubated mixtures in solvent systems A and B in Table I was used as a basis for identification.

Isolation of the *p*-(*N,N*-Dimethylsulfamoyl)phenyl Glucuronide from Sheep Urine. Since classical methods for the isolation of glucuronides failed in the present case, the procedures developed for the isolation of the glucuronide of the dimethylsulfamoylphenol are given in some detail.

Urine, collected as described above from sheep, was pooled and lyophilized to dryness. The dry powder was extracted four times with hot methanol, the methanol removed on a rotating evaporator, and the residue taken up in water. The solution was acidified to pH 1 with concentrated hydrochloric acid and extracted with four equal volumes of water-saturated 1-butanol. The butanol solutions were extracted three times with saturated aqueous sodium bicarbonate solution. The aqueous phase was acidified and extracted five times with 1-butanol. The butanol was removed under vacuum to yield about 30 ml. of brown, viscous oil.

This oil was dissolved in a small volume of methanol, and methylene chloride was added gradually until the methanol–methylene chloride ratio was 1 to 3. Several crops of white, needle-shaped crystals were precipitated, removed, and discarded during this process. Further additions of methylene chloride did not result in further precipitation. The solvents were removed under vacuum and the residue taken up in water. Seven volumes of absolute ethanol were added, and the precipitate which formed on standing was removed by filtration. Approximately 45% of the residue remaining after removal of solvents was the desired metabolite.

This residue, in aliquots of 3.5 ml., was chromatographed on 6.5 × 30 cm. silica gel columns using a linear solvent gradient generated by a Buchler Varigrad and ranging from 25% methanol in toluene to 100% methanol. Fractions of 60 ml. were collected automatically and each fraction was assayed for radioactivity. The radioactivity emerged from the column in three distinct peaks, the second of which was the desired metabolite and was radiochemically pure. However, its absolute purity, based on ultraviolet and chromatographic analyses, was only about 46%. After removal of solvents, the residue was taken up in methanol and the large amount of methanol-insoluble material was removed by filtration. The methanol-soluble material was approximately 72% pure.

Further purification was effected by preparative paper chromatography in acetonitrile–ammonium hydroxide–water (75:13:12). Sheets of Whatman No. 17 paper were suspended from the solvent troughs by means of double wicks of Whatman 3 MM paper sewn the width of the sheet. After thorough washing with methanol and water, approximately 150 mg. of the sample was applied as a streak to each sheet, and the chromatograms were developed at right angles to the machine direction of the paper. The radioactive bands were cut out and eluted with methanol to yield 80% pure metabolite. Rechromatography of this material at 75 mg.-per-sheet loading yielded material of 90% purity. Recrystallization from a mixture of methanol and diethyl ether resulted in a final yield of 100 mg. of a powdery, cream-colored solid (ammonium salt) of 95% purity.

This sample was subjected to elemental analysis and infrared and mass spectrometry.

RESULTS AND DISCUSSION

Total Radioactivity Levels in Plasma, Urine, and Tissues. The variation with time of the total radioactivity levels, expressed as famphur equivalents, in plasma and urine are shown in Figures 2 and 3, respectively.

From the steep slopes of the curves for the sheep treated intravenously, it appears that famphur introduced directly into the circulatory system is eliminated from the body very rapidly. Over 50% of the administered dose was recovered in the excreta within 6 hours, and approximately 98% was recovered within 48 hours. Nearly all of the radioactivity was excreted in the urine, fecal elimination accounting for less than 3% of the administered dose. Less than 1% of the urinary radioactivity was extractable with carbon tetrachloride, showing that famphur and its oxygen analog are not excreted as such to any considerable extent and that extensive metabolism must have occurred. Urine

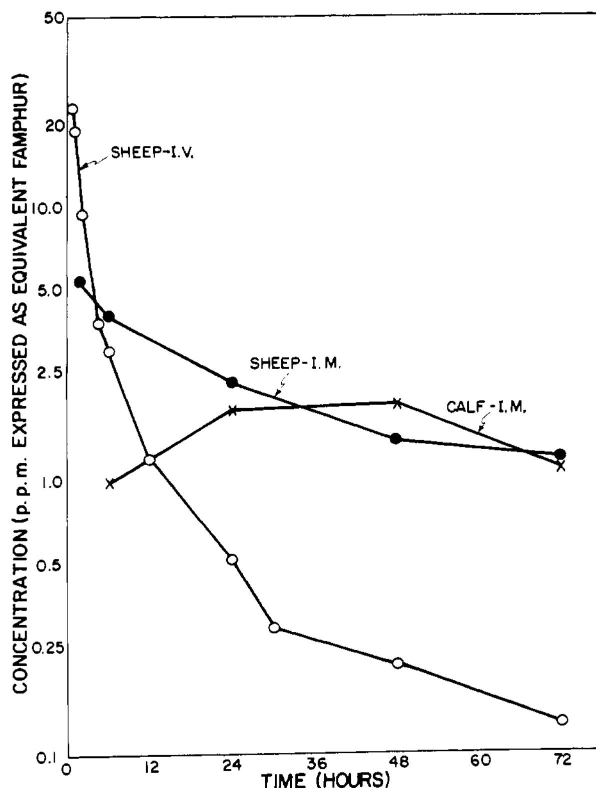

Figure 2. Radioactivity in plasma of sheep and calf following treatment with tritiated famphur

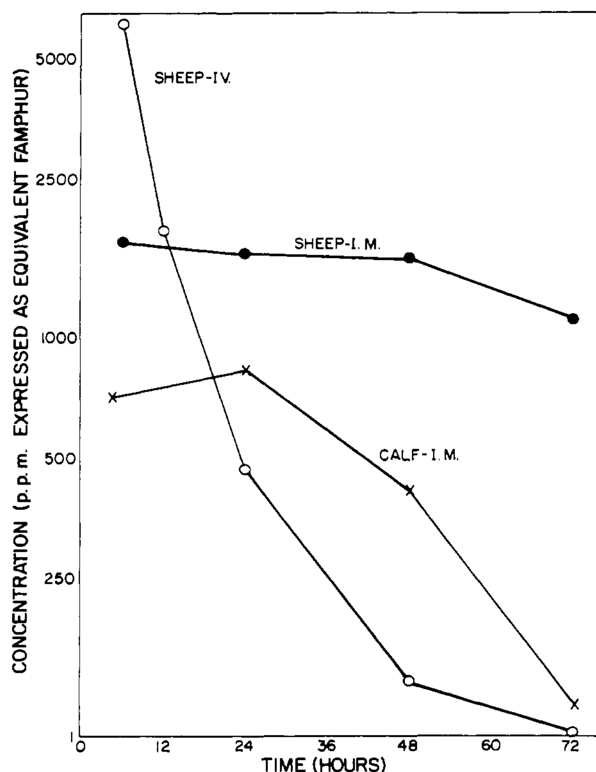

Figure 3. Radioactivity in urine of sheep and calf following treatment with famphur-H^3

samples collected up to 3 days after treatment with famphur-H^3 contained about 1% volatile tritiated material as determined by vacuum distillation, indicating that the exchange of tritium within the animal body is of minor importance.

In the sheep treated intramuscularly, the radioactivity in both plasma and urine decreased much more slowly than in the intravenous treatment, only 64% of the administered dose being recovered in excreta after 72 hours.

That this may be due to a depot effect is suggested by the fact that even 72 hours after intramusuclar treatment the muscle tissue at the injection site contained approximately 20 times as much radioactivity as muscle taken from sites removed from the injection area (Table III). Thus, the difference in rate of decrease between intravenous and intramuscular treatments is probably due to the formation of a depot of relatively insoluble material at the injection sites. The famphur in this depot should be mobilized and released into the general circulation relatively slowly, thus accounting for the sustained levels of total plasma and urinary radioactivity in the case of intramuscular treatment. This effect is more strikingly evident in the calf treated intramuscularly, in which case about 24 hours were required for the plasma and urine radioactivity levels to reach maximum values. Whether this is due to a true difference between the animals in their ability to mobilize the famphur in the depot, or to accident of placement of the depot in relation to the vascular systems, cannot be evaluated on the basis of present evidence.

The radioactivity present in selected tissues from intravenously and intramuscularly treated sheep are presented in Table III, expressed as parts per million of equivalent famphur. Evidently, very little radioactivity entered the tissues in the intravenous treatment, and the small amounts present are probably due to the presence of blood in the tissues. The levels of residual radioactivity in the tissues resulting from the intramuscular treatment were significantly greater, presumably because of a slower and more sustained release into the blood stream of the radioactivity derived from famphur. The highest levels of radioactivity, exclusive of the injection site, were in kidney and bile. Thigh muscle, cerebrospinal fluid, and brain were low, while blood and other major organs were intermediate in radioactivity content.

Table III. Residual Radioactivity in Selected Tissues of Sheep Following Intravenous and Intramuscular Administration of Tritiated Famphur

Tissue	Famphur Equivalents Based on Initial Specific Activity, P.P.M.	
	Sheep I.V. (sacrificed at 96 hours)	Sheep I.M. (sacrificed at 72 hours)
Kidney	0.3	8.0
Bile	0.05	6.6
Fat	0.02	5.0
Muscle (injection site)	...	15.0
Liver	0.3	2.3
Spleen	0.3	1.7
Lung	0.4	1.6
Blood	1.4	2.0
Brain	0.1	0.9
Muscle (thigh)	0.03	0.7
Cerebrospinal fluid	0.6	0.7

Identification of Metabolites. With the exception of the glucuronides of dimethylsulfamoylphenol and methylsulfamoylphenol, all of the metabolites reported here were available as pure, authentic products of chemical synthesis. Cochromatography of metabolites isolated from blood or urine with authentic compounds gave results which were consistent with the identifications indicated in Table I. In addition, acid and basic hydrolysis of the *O*-demethyl and *O,N*-bisdemethyl metabolites and of the corresponding authentic compounds yielded the same products (the corresponding substituted sulfamoylphenols) as determined by cochromatography and cocrystallization. The presence of *p*-hydroxybenzenesulfonic acid was shown by similar techniques. Identical chromatographic behavior of the authentic acetyl derivative of *p*-hydroxybenzenesulfonic acid and the acetyl derivative of the corresponding metabolite lent support to this identification.

The two glucuronide metabolites could not be identified by cochromatography, since authentic samples were not available through chemical synthesis. However, the excretion of a wide variety of phenolic compounds as conjugates with glucuronic or sulfuric acid is well known (Williams, 1959; Dutton, 1966). Sulfonamides and their derivatives having unsubstituted nuclear amino groups are excreted partly unchanged (Smith and Williams, 1948), partly acetylated (Williams, 1959), and partly oxidized to phenolic compounds which are conjugated with glucuronic or sulfuric acid (Scudi, 1940).

Application of the naphthoresorcinol test to the urine of famphur-treated and control animals indicated the presence of larger amounts of glucuronic acid in the treated animals than in the controls. Metabolites 6 and 7 were highly resistant to the action of dilute alkali but were hydrolyzed to the corresponding sulfamoylphenols by heating with dilute acids. This behavior is characteristic of ether linkages such as would be expected in the glucuronide conjugates of phenols. The metabolites were slowly cleaved (40% in 2 days) by β-glucuronidase to yield the corresponding phenols.

Characterization by several methods of the sample of the ammonium salt of metabolite 6 isolated from sheep urine strongly supports the proposed identification. Anal. Calcd. for $C_{14}H_{22}N_2O_9S$: C, 42.64; H, 5.58; N, 7.10; S, 8.12. Found: C, 42.31; H, 6.01; N, 6.84; S, 8.12. The infrared spectrum, presented in Figure 4, shows absorption bands as follows: OH and/or NH stretching (3 microns), carboxylate anion and phenyl ring deformation (6.26 microns), H out-of-plane bending modes of a 1,4-substituted phenyl ring (12 microns), SO_2 stretching modes (7.5 and 8.64 microns), and phenyl C—O stretching (8.02 microns). Mass spectrometry indicated a molecular weight of 394, in exact agreement with the proposed structure, and, in addition, showed several predictable fragments.

Concentrations of Famphur Metabolites in Plasma and Urine. Results of the fractionation of plasma radioactivity by extraction with carbon tetrachloride and chromatography in system C are given in Table IV. The extensive hydrolytic degradation undergone by famphur

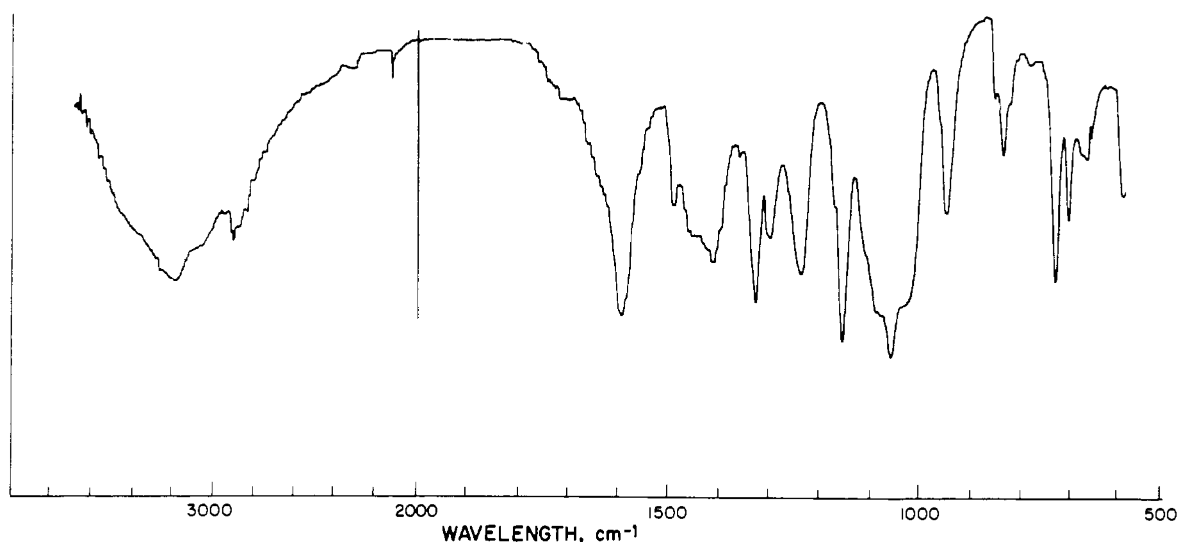

Figure 4. Infrared spectrum of purified p-(N,N-dimethylsulfamoyl) phenyl glucuronide

Table IV. Plasma Levels of Famphur and Famoxon as Determined by Extraction with Carbon Tetrachloride and Chromatography in System C

Animal	Route	Time of Sampling, Hr.	Organophilic, %	Famphur, P.P.M.	Famoxon, P.P.M.	Famoxon/Famphur
Sheep	I.V.	2	27.0	0.600	5.600	9
		24	2.8	0.002	0.012	6
Sheep	I.M.	2	21.0	0.900	0.100	1/9
		72	5.6	0.056	0.009	1/6
Calf	I.M.	4	41.0	0.400	0.000	...
		72	20.2	0.180	0.045	1/4

and famoxon is indicated by the relatively small fraction of the total plasma radioactivity which was extracted into carbon tetrachloride. Within 2 hours of intravenous administration, more than 70% of the radioactivity was due to water-soluble metabolites. In the case of intramuscular administration, the hydrolysis to water-soluble metabolites seems to be somewhat slower, since 72 hours post-treatment the organophilic activity accounted for 5.6% of the total plasma activity as compared with only 2.8% after 24 hours in the sheep treated intravenously. The calf appears to be less efficient in hydrolytic cleavage of famphur or the oxygen analog than is the sheep, 20% of the total plasma activity being due to these two compounds after 72 hours.

Comparison of the famoxon to famphur ratios for the sheep shows that following intravenous administration, several times as much famoxon as famphur are present in the blood, while in the intramuscular treatment the reverse is true. That this is due to a higher rate of conversion of famphur to famoxon in the intravenously treated sheep and not to a failure in subsequent famoxon-degrading metabolism is suggested by the facts that the ratios did not change greatly with time and that relatively little undegraded famoxon was in the urine.

Probably, the difference in the conversion rate is related to the relatively high concentration of famphur introduced suddenly into the blood stream by intravenous treatment, as compared with the rather slow and sustained release of famphur from the depot formed by intramuscular administration. If the sites of conversion were very active but relatively few in number and highly localized on a microscopic scale, their total activity should be considerably higher when large concentrations of famphur were present in the blood. The possibility also exists that some activation mechanism is involved, whereby the presence of large concentrations of famphur or famoxon stimulates the conversion system to higher activity.

The concentrations of the various metabolites in calf plasma at several intervals post-treatment are shown in Table V. The maximum concentration of famphur occurred at 24 hours, whereas the famoxon concentration increased from 0.017 p.p.m. at 24 hours and appeared to level off at about 0.05 p.p.m. between 48 and 72 hours. Both the O-demethyl and O,N-bisdemethyl compounds reached maximum concentrations at 48 hours.

Because of the relatively low radioactivity levels in blood and technical difficulties with the chromatography of the crude blood extracts, metabolites 6 and 7 (Table I) were not resolved sufficiently to give meaningful individual values for their respective concentrations. Their combined concentration was calculated from the area of the overlapping peaks. These two compounds appeared to reach a plateau at about 0.17 p.p.m. and remained close to this level from 24 through 72 hours.

As shown in Table VI, both the sheep and the calf eliminate the O-demethyl and O,N-bisdemethyl metabolites

Table V. Concentration of Radiometabolites in Calf Plasma Following Intramuscular Administration of Tritiated Famphur (P.p.m. Famphur-H^3 equivalents)

Post-treatment, Hr.	Famphur	Famoxon	*O*-Demethyl Famphur	*O,N*-Bisdemethyl Famphur	Combined Glucuronide	Activity Unextractable
4	0.40	...	0.19	0.05	0.06	0.28
24	0.61	0.02	0.35	0.14	0.15	0.51
48	0.51	0.04	0.46	0.41	0.19	0.30
72	0.18	0.05	0.21	0.25	0.17	0.25

Table VI. Concentration of Radiometabolites in Urine of Sheep and Calf Following Administration of Tritiated Famphur (P.p.m. famphur-H^3 equivalents)

Post-treatment, Hr.	*O*-Demethyl Famphur	*O,N*-Bisdemethyl Famphur	*N,N*-Dimethylsulfamoylphenyl Glucuronide	*N*-Methylsulfamoylphenyl Glucuronide	*p*-Hydroxybenzenesulfonic Acid	Unknowns
			Sheep, Intravenous Administration			
0–6	825	1956	2035	904	92	299
6–12	229	779	525	292	Trace	45
12–24	42	159	166	94	Trace	6
			Sheep, Intramuscular Administration			
0–6	310	430	650	85	98	149
6–24	330	575	522	139	24	27
24–48	513	487	420	152	Trace	20
			Calf, Intramuscular Administration			
0–4	303	122	226	43	19	...
4–24	262	159	384	29	Trace	...
24–48	103	65	232	16	Trace	...
48–72	28	5	80	5	Trace	...

as such in the urine in fairly high concentrations, especially in the first few hours post-treatment.

Hydrolysis of the *O*-demethyl and *O,N*-bisdemethyl compounds to the corresponding transitory dimethylsulfamoyl- and methylsulfamoylphenols followed by elimination as glucuronide conjugates is apparently a major route of detoxification. The rapidity with which this occurs is indicated by the high levels of glucuronides in the earliest urine taken from the sheep. In the calf, the maximum urinary glucuronide level occurred at the 1-day interval, which is not surprising since the maximum levels of total radioactivity in urine and blood of the calf were not reached until 24 and 48 hours, respectively, after administration of famphur.

In the calf, *O*-demethyl famphur is present at considerably higher levels than the *O,N*-bisdemethyl metabolite, and the dimethylsulfamoylphenyl glucuronide predominates over the monomethyl glucuronide at all sampling periods. In contrast, the *O,N*-bisdemethyl metabolite was predominant in the sheep urine, although in this case the dimethylsulfamoylphenyl glucuronide was also present in higher concentration than the monomethyl conjugate.

The relative contribution of the *N*-demethylation mechanism may be evaluated by comparing the sums of the concentration of the *N,N*-dimethyl metabolites with those for the *N*-methyl metabolites at each time interval; the ratios of dimethyl to monomethyl range from 0.70 to 1.00 in the sheep treated intravenously, from 1.12 to 1.85 in the sheep treated intramuscularly, and from 3.20 to 9.26 in the calf. Thus, the *N*-demethylation mechanism appears to be much less important in the calf than in the sheep and appears to be favored somewhat by intravenous administration as compared with intramuscular treatment.

In addition to the *O*-demethyl and *O,N*-bisdemethyl metabolites and the two glucuronide conjugates, early urine samples contained small amounts of *p*-hydroxybenzenesulfonic acid and, in the case of the sheep, moderate amounts of two unidentified metabolites. Preliminary chromatographic evidence suggests that the unknowns may be the sulfates of the mono- and dimethylsulfamoylphenols, but these identifications must be considered tentative. In any case, the concentrations of these materials dropped rapidly and were found in later urine samples at very low levels.

Metabolism of *O*-Demethyl Famphur and *N,N*-Dimethylsulfamoylphenol in Rats. To elucidate further the mechanisms by which the *O*-demethyl metabolite is eliminated, this compound, radiolabeled as described previously, was administered subcutaneously to rats. One day after treatment more than 50% of the urinary radioactivity was due to the unchanged *O*-demethyl compound. The major metabolite was the dimethylsulfamoylphenyl glucuronide, which accounted for approximately 30% of the urinary radioactivity. About 12% of the radioactivity occurred as *O,N*-bisdemethyl famphur, and about 7% was due to the methylsulfamoylphenyl glucuronide. A trace of *p*-hydroxybenzenesulfonic acid was also detected.

Following subcutaneous administration of radioactive dimethylsulfamoylphenol to rats, both the dimethyl and monomethyl glucuronides were detected in the urine, but the relative amounts of the two varied markedly from ex-

periment to experiment. Small amounts of *p*-hydroxybenzenesulfonic acid were found, suggesting that this compound arises from the dimethyl- or monomethylsulfamoylphenols rather than from less extensively degraded metabolites.

Proposed Metabolic Scheme for Famphur in Mammals. Based upon the studies described herein and those reported by others (Bourne, 1963; O'Brien, 1965), the scheme shown in Figure 5 is suggested to account for the known facts concerning the metabolism of famphur in mammals.

According to this scheme, the parent compound may undergo any of three reactions: oxidation of the P=S bond to yield famoxon, hydrolysis at the P—O—phenyl bond to yield the transitory dimethylsulfamoylphenol which is immediately conjugated to form the corresponding glucuronide (reaction F), or hydrolysis at one of the P—O—methyl bonds to yield the *O*-demethyl metabolite. The products of reaction B, dimethylsulfamoylphenol and its conjugation product, the dimethylsulfamoylphenyl glucuronide, may also arise by hydrolysis of famoxon (reaction D) or the *O*-demethyl compound (reaction E).

In addition to reaction E to form the dimethylsulfamoylphenol, *O*-demethyl famphur can also give rise to the *O,N*-bisdemethyl metabolite by removal of one of the methyl groups of the sulfonamide moiety (reaction G). *O,N*-Bisdemethylfamphur is converted by hydrolysis (reaction H) to the corresponding transitory *N*-methylsulfamoylphenol which is immediately conjugated (reaction I) to yield the corresponding glucuronide.

The very small amounts of oxygen analog in the blood of intramuscularly treated animals suggests that reaction A is of relatively minor importance in the metabolic degradation of famphur administered in this manner. Apparently, this reaction is more important in intravenous administration since, in this case, most of the organophilic radioactivity was due to famoxon.

The relative importance of reactions B and C could not be established by the experiments reported here, but strong evidence for the existence of the direct hydrolysis of famphur to the intermediate phenol has been developed by Bourne (1963), who found considerable amounts of dimethyl phosphorothioic acid in the urine of sheep treated with famphur-P^{32}. This compound could not have originated from the *O*-demethyl compound nor the oxygen analog.

That the *O*-demethyl metabolite is converted to the dimethylsulfamoylphenol is indicated by the results of experiments in which the *O*-demethyl compound was administered directly to rats. The appearance of the glucuronide of the dimethylsulfamoylphenol as the major urinary metabolite provides evidence for this conversion. The appearance of the *O,N*-demethyl metabolite and the glucuronide of *N*-methylsulfamoylphenol indicated that reactions G, H, and I can occur.

Administration of dimethylsulfamoylphenol to rats demonstrated that reaction F was a major excretory pathway for this compound, although a small amount of the methylsulfamoylphenyl glucuronide was also in the urine, showing that reaction J occurs to a limited extent.

While hydrolysis of P—O—phenyl and P—O—methyl bonds in several dialkyl aryl phosphorothioates is well documented (Dauterman *et al.*, 1959; O'Brien *et al.*, 1961;

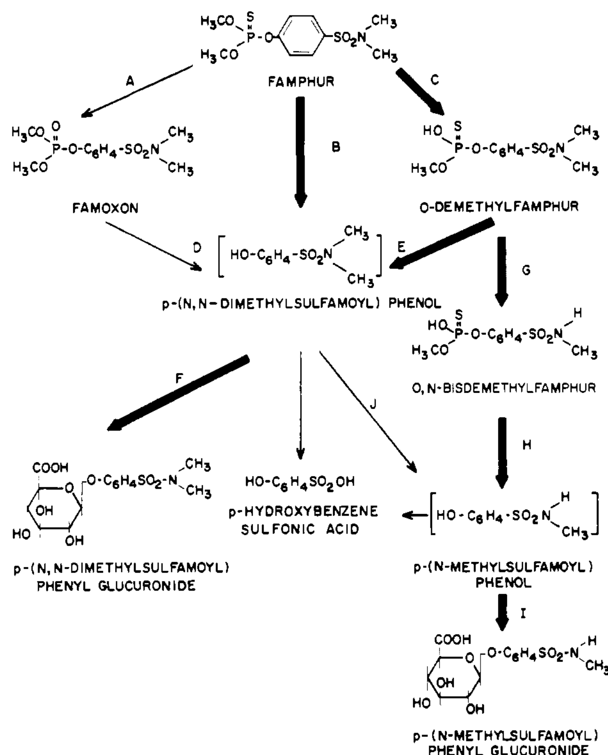

Figure 5. Proposed metabolic scheme for famphur in mammals

Plapp and Casida 1958a; 1958b), the evidence for the in vivo cleavage of the *N*-alkyl group of sulfonamides is meager. O'Brien *et al.* (1965) have found *N*-demethyl famphur, $(CH_3O)_2P(S)OC_6H_4SO_2NHCH_3$, in the excreta from mice and certain insects, although this compound was not found in the present study with sheep, calf, and rat. Maren (1956) has presented evidence for the *N*-dealkylation of isopropyl-, methyl-, and *tert*-butyl–substituted 2-acetylamino-1,3,4-thiadiazole-5-sulfonamides in the dog and rat.

The discovery of small amounts of *p*-hydroxybenzenesulfonic acid in the urine of sheep and calf was surprising in view of reports that the sulfonamide linkage is not easily hydrolyzed enzymatically (Maren, 1956; Williams, 1959). Presumably, this compound could originate from either phenol intermediate by amide hydrolysis.

Toxicological Significance. The acute toxicities of the various metabolites are presented in Table VII. Appar-

Table VII. Acute Oral Toxicity of Famphur and Its Metabolites to Mice

Compound	LD_{50}, Mg./Kg.
Famphur	27
Famoxon	18
O-Demethyl famphur[a]	2270
O,N-Bisdemethyl famphur[a]	860
N,N-Dimethylsulfamoylphenol	2290
N-Methylsulfamoylphenol	2500
p-Hydroxybenzenesulfonic acid	6400
p-(*N,N*-Dimethylsulfamoyl) phenyl glucuronide[b]	>5000

[a] Dicyclohexylammonium salts.
[b] Ammonium salt.

ently, of the famphur metabolites found in this study only the oxygen analog would present any significant health hazard if ingested. However, this compound was not present to any appreciable extent except in the sheep treated intravenously.

The *O,N*-bisdemethyl metabolite was two to three times as toxic as the *O*-demethyl metabolite from which it was derived. However, a similar difference in toxicity of the corresponding phenols was not found. The glucuronide conjugation products have not been isolated in sufficient quantities to allow accurate determination of acute toxicities, but it is reasonable to assume that these products would not be more toxic than the phenols from which they are derived (Williams, 1959).

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