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THE METABOLISM OF 1-(*N*,*N*-DIMETHYLCARBAMYLMETHYL)-2,4-DINITROPYRROLE IN MICE

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Abstract—The fate of orally administered 1-(*N*,*N*-dimethylcarbamylmethyl)-2,4-dinitropyrrole was studied in mice. Twenty-four per cent of the drug was recovered from the urine as dinitropyrrole derivatives. An ethyl acetate extract of the urine contained a compound of greater antimicrobial activity than the parent compound. Thin-layer chromatography of the urine extract yielded three fractions in addition to small amounts of the parent compound. The two fractions (I and III), which exhibited antimicrobial activity, were identified as 1-(*N*-methylcarbamylmethyl)-2,4-dinitropyrrole and 1-((*N*-hydroxymethyl)carbamylmethyl)-2,4-dinitropyrrole respectively. Fraction II, the inactive material, was not identified.

OF THE large number of nitroheterocyclic compounds which possess excellent antibacterial activity *in vitro*, only the nitrofuran derivatives have gained widespread use as urinary antiseptics. Such nitrofuran derivatives are eliminated rapidly, produce a urine with high antibacterial activity, and are active against many Gram-negative bacteria. We have noted that derivatives of 1-(carbamylmethyl)-2,4-dinitropyrrole possess similar properties. One compound in particular, ORF-1744, 1-(*N*,*N*-dimethylcarbamylmethyl)-2,4-dinitropyrrole, attracted our attention because mice receiving this drug produce a urine of greater antibacterial activity than a sample containing an equivalent amount of the parent compound. The fate of ORF-1744 was, therefore, studied to determine the nature of the metabolite(s) that has the enhanced activity.

The study of the metabolites of ORF-1744 was purposely limited to those compounds that retained the dinitropyrrole configuration, since not only were these more likely to retain antimicrobial activity, but also their characteristic absorption spectra permitted rapid detection.

METHODS

Administration and collection of the drug

Male Swiss Webster mice weighing 18–22 g were given the drug, prepared as a suspension in 0·25% agar, by means of a stomach tube attached to a syringe. Urine collections were made either by aspirating directly from the bladder at autopsy or through the use of a metabolism cage.

Assay of antibacterial activity

For assays of activity against *Escherichia coli*, 0128:B12:H2, seeded plates were prepared by first pouring a base layer of 6 ml of melted Difco antibiotic agar no. 2 into each Petri plate. The seed layer was prepared by adding 0·5 ml of a washed suspension

of *E. coli* having a 60 per cent T at 660 mμ (Evelyn colorimeter) to 100 ml of melted and cooled (50°) antibiotic agar no. 2. Four ml of this seed agar was dispensed uniformly over the surface of hardened base layer. The plates were stored at +5° until used. Because the length of storage affected the zone size from day to day, comparisons were made either from a single plate or, if from several plates, each with a reference standard.

One-tenth ml urine was applied to a standard paper-disc (Schleicher and Schuell, no. 740-E). The disc was dried and then placed on the surface of a seeded agar plate. The plates were incubated overnight at 37° and the diameter of the zone of inhibition was read with a Fisher–Lilly zone reader.

Estimation of dinitropyrroles

In the urine. Dinitropyrroles were recovered from the urine by extraction with equal volumes of ethyl acetate. This extraction procedure was repeated 2–3 times on the same urine sample. The solvent then was removed by evaporation *in vacuo*. The residue was dissolved in ethanol and the concentration of the dinitroheterocycle was determined spectrophotometrically. The intense characteristic absorption of the dinitropyrrole in the u.v. range enabled determinations to be made on even such relatively crude extracts as those obtained with ethyl acetate. Quantitative determinations were made on the purified preparations in the same manner, by using ORF-1744 as the standard and reading O.D. at 315 mμ with a spectrophotometer.

Chromatography

Thin-layer. All thin-layer chromatograms (TLC) were run on commercially prepared alumina plates (Analtech Inc., Wilmington, Del., U.S.A.). They were developed in a solvent composed of isopropanol–hexane (6:4). The fractions were detected as dark spots by viewing the chromatograms under u.v. light at 260 mμ.

Column. A column (45 × 150 mm) was packed dry over a sand base with alumina, neutral, grade I (M. Woelm, Eschwege, Germany). The column was overlayered with sand, after which it was washed with isopropanol until the effluent showed no absorption at 315 mμ. The urine extracts, which had been evaporated to dryness, were dissolved in a minimum volume of isopropanol and put on the column. A gradient elution system was produced by the gradual addition of methanol to a mixing chamber containing isopropanol. The effluent from the column was monitored continuously at 315 mμ with a Beckman DK-1 spectrophotometer which had been modified and fitted with a quartz flow cell. The flow rate was set at 125 drops/min with 400 drops (approximately 10·2 ml) being collected in each tube.

Spectral analysis

Infrared spectral determinations were made with a Beckman IR-5 spectrophotometer with KBr wafers. Nuclear magnetic resonance determinations were made in deuterated acetone with a Varian A-60.

EXPERIMENTAL

Effect of dose level

ORF-1744 was administered to groups of nine mice each at 3 dose levels: 0·5 mg, 2 mg, and 5 mg/mouse. The mice were placed in metabolism cages where the urine and feces were collected for 48 hr.

The urine from each dose level was pooled separately and extracted twice with equal volumes of ethyl acetate. Two ml of each sample was evaporated to dryness, dissolved in 10 ml ethyl alcohol, and the u.v. spectrum was determined. Only the 5-mg dose level produced a spectrum which clearly resembled that of the parent drug Fig. 1).Based on the spectral data (Table 1), there appears to be a threshold value below which only small amounts of dinitropyrroles are excreted in the urine.

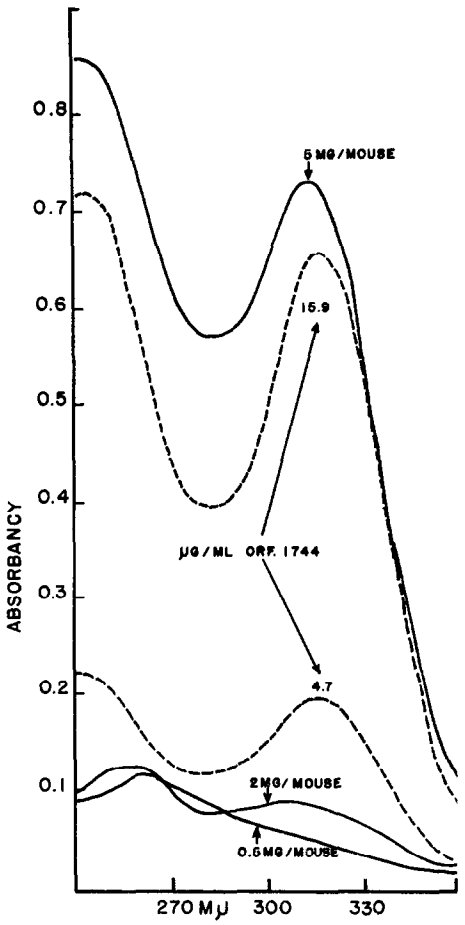

FIG. 1. The effect of dose levels on the recovery of dinitropyrrole from the urine of mice. Ethanolic solutions of ethyl acetate extracts of 2 ml pooled mouse urine (——). ORF-1744 administered orally at three levels: 0·5 mg, 2 mg, and 5 mg/mouse. Ethanolic solutions of ORF-1744 for estimating concentrations (– – – –).

The fecal samples were extracted with ethyl acetate (Soxhlet). The resulting solutions absorbed so intensely throughout the u.v. range that no determinations could be made on them. When the sample was diluted to a point where a reasonable scan was produced, the dinitropyrrole had been diluted to a point where it no longer could be detected.

TABLE 1. DINITROPYRROLE RECOVERIES FROM URINE

Dose (mg/mouse)	Urine concn. (μg/ml)	Per cent recovery
5	17·6	24·2
2	2·1	9·5
0·5	(Undetectable)	

Chromatography of urine extracts

Thin-layer chromatography. The urine extract was spotted on an alumina TLC plate and the chromatogram was developed in the isopropanol–hexane solvent system. When viewed under u.v. light (260 mμ), four spots were observed. A subsequent chromatogram was run on which a group of known 2,4-dinitropyrroles (Fig. 2),

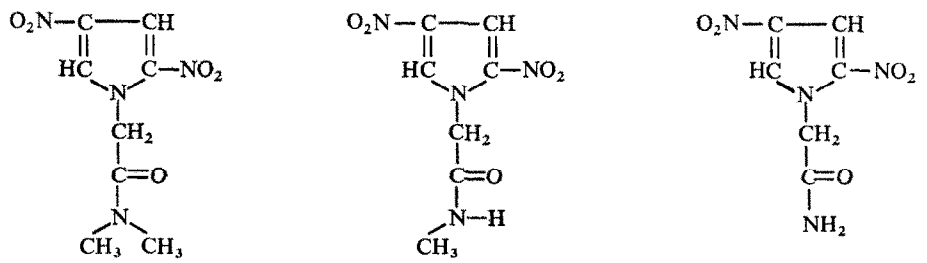

FIG. 2. Structural formulae of carbamylmethyl-2,4-dinitropyrroles. ORF-1744 = 1-(*N*,*N*-dimethylcarbamylmethyl)-2,4-dinitropyrrole; ORF-1743 = 1-(*N*-methylcarbamylmethyl)-2,4-dinitropyrrole; ORF-1594 = 1-(carbamylmethyl)-2,4-dinitropyrrole.

considered to be possible metabolites, were run along with the urine extract. The results (Fig. 3) indicate that only a small part of the original ORF-1744 was excreted as such in the urine. Fraction I, the major component, had an R_f very similar to that of ORF-1743, which is 1-(*N*-methylcarbamylmethyl)-2,4-dinitropyrrole. Neither Fraction II nor Fraction III had an R_f which coincided with that of ORF-1594 (1-[carbamylmethyl]-2,4-dinitropyrrole).

The three spots were eluted from the TLC plate with methanol and the concentrations were determined spectrophotometrically with ORF-1744 as the standard and it was found that the u.v. spectra of all three fractions appeared to be almost identical to that of ORF-1744. Fraction I comprised approximately 41 per cent, Fraction II 36·5 per cent, and Fraction III 22·5 per cent of the total. Although these three fractions were always present, both the relative and total concentrations were found to vary from one experiment to another.

Column fractionation. For identification, large amounts of the several fractions were required. Accordingly, 200 mice were each given a single 10-mg dose of ORF-1744; the urine was collected daily for 3 days and then pooled. The pooled urine was extracted twice with 2 vol. of ethyl acetate; the combined extract was concentrated *in vacuo* to half the starting (urine) volume and filtered through Whatman no. 12

filter paper. The filtrate was evaporated to dryness *in vacuo* and the residue was taken up in acetone (approximately $\frac{1}{6}$ the urine volume). The acetone-soluble portion was evaporated to dryness and the residue was dissolved in a minimum volume of isopropanol and placed on the column.

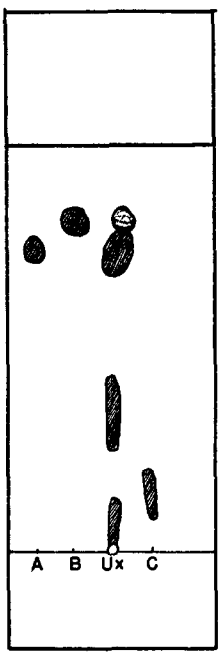

FIG. 3. An alumina TLC of an extract of the urine of mice treated with ORF-1744 and some known dinitropyrroles. Solvent system, isopropanol–hexane (6:4). A = ORF-1743; B = ORF-1744; Ux = urine extract; C = ORF-1594.

Fig. 4 represents a typical column fractionation, and shows three well separated fractions. Although there was no peak representing the parent drug (ORF-1744), thin-layer chromatograms routinely revealed the presence of small amounts of this compound. The peak portion of each fraction was pooled and evaporated to dryness. The residues were recrystallized from ethyl ether and a portion of each was subjected to a purity check by TLC. Fractions I and III each gave a single, well defined spot; Fraction II, on the other hand, gave two spots, one with an R_f approximating that of Fraction I and the other with an R_f characteristic of Fraction II.

The infrared spectrum for each of the three fractions was determined. Fractions I and III produced spectra with well defined peaks, but the spectrum of Fraction II was poorly defined. The infrared spectra for Fractions I and III were compared with those of several 2,4-dinitropyrroles in the carbamylmethyl series. Because of the similarities between the thin-layer R_f values of Fraction I and of ORF-1743, these spectra were compared at the outset and found to be identical. On recrystallizing, Fraction I gave an m.p. of 167–169°; a second recrystallization gave an m.p. of 168–169°. ORF-1743 had an m.p. of 169–171°. The production of identical i.r. spectra by Fraction I and ORF-1743 and the melting point data plus the chromatographic

and u.v. evidence were considered sufficient to justify suggesting the identification of Fraction I as 1-(*N*-methylcarbamylmethyl)-2,4-dinitropyrrole.

Additional purification of Fraction II

Fraction II was subjected to further purification by alumina column chromatography. Two fractions were isolated and the elution pattern exactly duplicated those obtained previously for Fractions I and II.

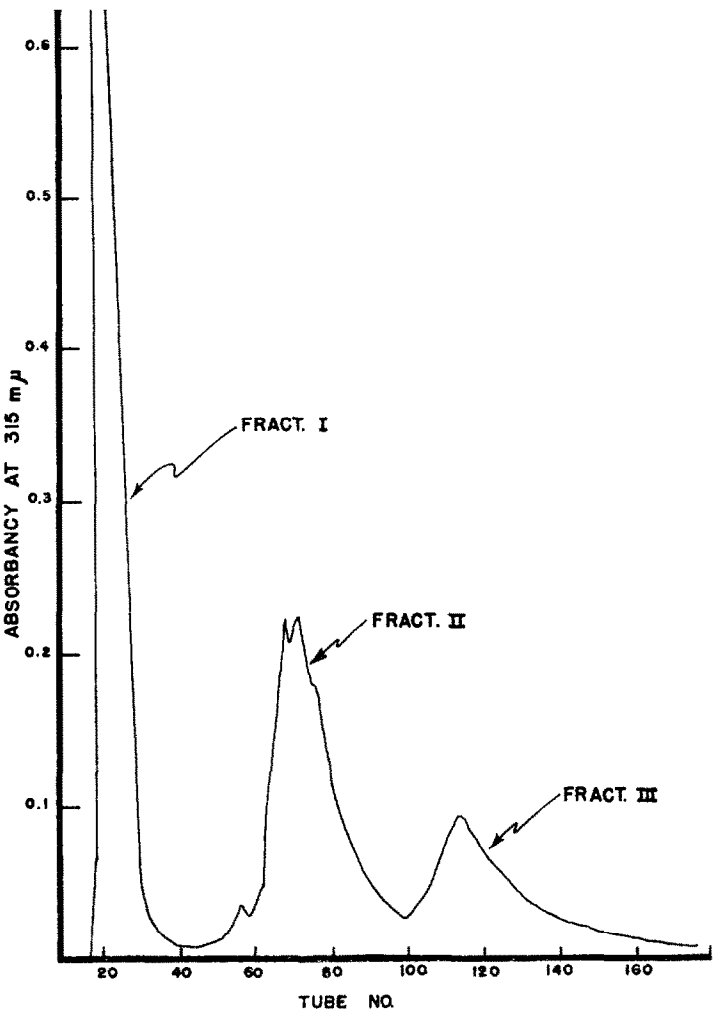

FIG. 4. Column fractionation of a urine extract from mice receiving 10 mg of ORF-1744 orally. Column 45 × 150 mm Woelm alumina, neutral, grade I. A gradient elution with isopropanol–methanol at 125 drops/min, 10 ml/tube, was used. The effluent was monitored at 315 mμ.

The contents of the tubes constituting Fraction II were then pooled and concentrated *in vacuo*. Twenty-five μl of the concentrated Fraction II was streaked at the origin of an alumina TLC plate and the chromatogram was developed with isopropanol–hexane. The resulting chromatogram formed three bands (see Fig. 5A). The thin band

with an R_f corresponding to that of ORF-1744 (near the solvent front) probably was an artifact resulting from the large amount streaked. The Fraction II band was scraped from the plate and the alumina was washed alternately with water and ethanol. The wash was filtered and concentrated to dryness and the residue was taken up in ethyl acetate. After concentrating to 0·1 ml, the ethyl acetate solution was spotted on an alumina TLC plate at three concentrations and developed with the usual solvent (see Fig. 5B). The results demonstrated for the third consecutive time that Fraction II, when chromatographed on alumina, consistently yielded significant amounts of Fraction I.

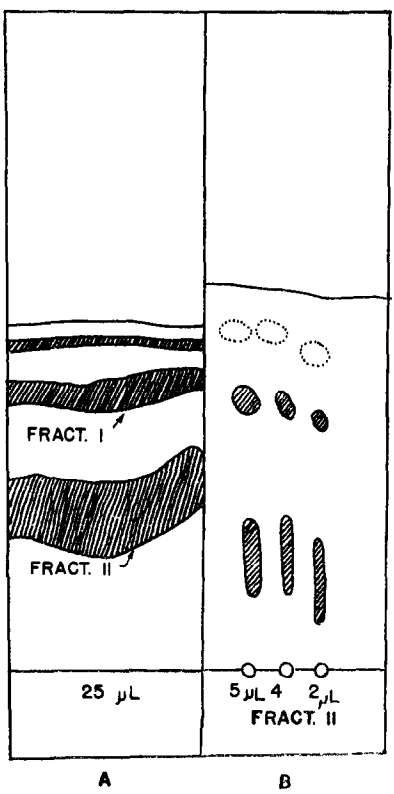

FIG. 5. Thin-layer chromatograms of Fraction II. Both TLC plates were alumina and were developed with isopropanol–hexane (6:4). (A) Chromatogram of 25 µl of a Fraction II concentrate obtained from a column fractionation. (B) Chromatogram of material extracted only from the Fraction II band.

On several occasions, studies with Fraction II showed that this fraction separated into three components on TLC plates; one of these represented Fraction I. One of the column fractionations also produced two distinct peaks in the area normally occupied by Fraction II. Thus, although some of the data obtained with Fraction II varied, such variations occurred from experiment to experiment but not within a single sample.

Identification of Fraction III

The urine of 200 mice treated with ORF-1744 at a dose level of 10 mg/mouse was collected daily for 3 days. The pooled urine was extracted with ethyl acetate and chromatographed on a neutral alumina column as before. Aliquots from all tubes containing Fraction III were chromatographed on alumina TLC plates. Only those tubes which produced a single spot, the R_f of which corresponded to known Fraction III, were pooled. This was designated Fraction IIIa. The pooled liquid was concentrated *in vacuo* and Fraction IIIa was recrystallized from benzene. The infrared spectrum was determined and was found to be identical with that of a Fraction III obtained previously. Because our purified sample consisted of only a small amount of the solid material (169–171° on repeated recrystallization), we relied on micro elemental analysis, i.r., u.v. and NMR for structural elucidation.

An elemental analysis was carried out on a proportion of Fraction IIIa (Midwest Microlab Inc.). The results were as follows: C, 34·49, 34·77; H, 3·64, 3·49; O, 39·44; N, 22·72. These values very closely approximate those of a compound having the formula $C_7H_8O_6N_4$. (Calcd: C, 34·43; H, 3·30; O, 39·32; N, 22·95.) Two of the most likely metabolic products with such an empirical formula would be the *N*-hydroxylamine and the *N*-methylol analogs of 1-(*N*-methylcarbamylmethyl)-2,4-dinitropyrrole.

The infrared spectrum showed characteristic bands for dinitropyrrole along with an OH band at 2·79 μ and an NH band at 2·98 μ. The NMR in deuterated acetone confirmed the presence of dinitropyrrole by exhibiting the doublets for aromatic protons at 7·68 and 8·17 ppm. The *N*-CH_2-CO resonance appeared as a singlet at 5·30 ppm. A multiplet at 4·76 ppm was attributed to either NH-CH_2-OH or HN-OH grouping. This multiplet which integrated for 2 protons was reduced to a "singlet" by exchanging with D_2O, thus confirming NH-CH_2-OH grouping. We believe that the NMR data coupled with i.r., u.v. and elemental analysis strongly suggest the structure proposed for Fraction III.

Antibacterial activity

A study was made of the antibacterial activity of the extracts of the feces and of the urine from treated mice. No antibacterial activity was detected in the fecal extract. The urine extract not only exhibited good antibacterial activity, but appeared to be substantially more active than a sample containing an equivalent amount of the parent compound (ORF-1744), Table 2a. Since our findings suggested that one of the fractions (1) isolated from the urine of treated mice was ORF-1743, its antibacterial activity was compared with that of ORF-1744 (Table 2b). The zone of inhibition it produced was significantly greater than that produced by ORF-1744. The antibacterial activity of the three fractions isolated from urine was determined. The activities of Fraction I and Fraction III were considered to be good as well as comparable. The antibacterial activity of Fraction II was only marginal by comparison.

DISCUSSION

The results indicate that 1-(*N*,*N*-dimethylcarbamylmethyl)-2,4-dinitropyrrole is metabolized after oral administration to mice. Three metabolites, in addition to the parent compound, were recovered consistently from the urine of treated mice. The amounts of these metabolites recovered were markedly influenced by the dosage of the drug administered. The poor recoveries observed in the case of the low dosage of 0·5

mg/mouse may indicate that the drug was degraded extensively, no longer producing the characteristic dinitropyrrole u.v. spectrum and, therefore, not being detectable. At the higher doses, the animal's capacity to handle the dinitropyrroles in this fashion may have been impeded, resulting in an increased amount of detectable metabolites.

TABLE 2. ANTIBACTERIAL ACTIVITY OF DINITROPYRROLES

Material	Approx. concn. dinitropyrrole/disc (μg)	Diameter of zone of inhibition* (mm)
Assay a		
Urine extract	85	28·4
Fecal extract	120	13·0†
ORF-1744	300	25·8
Assay b		
ORF-1744	58	13·2
ORF-1743	55	20·7‡
Assay c		
Fraction I	50	25·8‡
Fraction II	60	15·4
Fraction III	40	26·8

* *E. coli.*

† 13 mm is the zone diameter produced by placing an untreated disc on the surface of the seeded plate.

‡ Difference probably due to the effect of storage on the assay plates.

Of the three metabolites isolated, all appeared to contain one less *N*-methyl group than the parent compound. Fraction I, identified as 1-(*N*-methylcarbamylmethyl)-2, 4-dinitropyrrole, represents the parent compound minus one methyl group. Fraction III, 1-[(*N*-hydroxymethyl)carbamylmethyl]-2,4-dinitropyrrole may have resulted from the hydroxylation of Fraction I. A summary of the suggested metabolic route is given in Fig. 6. Thus far, attempts to identify Fraction II have been unsuccessful. Although its u.v. spectrum is characteristic of the dinitropyrroles, a well defined i.r. spectrum has not been obtained. The TLC studies, however, did provide some information, since the R_f was midway between that of Fraction I and Fraction III. It is unlikely that we are dealing with a glucuronide, since a polar compound of this type should remain at the origin along with Fraction III. On alumina, TLC or column, Fraction II consistently yielded Fraction I. It seems unlikely that such a reaction would involve the loss of a methyl group; and thus, Fraction II is possibly a derivative of the monomethyl compound similar in structure to Fraction I. Whatever the difference may be between Fraction I and Fraction II, there is a real difference in the antibacterial activity of the two; Fraction I possesses very good activity against *E. coli* but Fraction II has little, if any, activity against this organism. Fraction III, on the other hand, demonstrated about the same amount of antibacterial activity as Fraction I.

The nitro groups are responsible to a large degree for the antimicrobial activity of ORF-1744, as with other nitroheterocycles. Information on the metabolic fate of nitroheterocyclic compounds is meager and is confined principally to a few nitrofurans and a nitroimidazole. Among the nitrofuran studies, two[1, 2] describe a reduction of the nitro group. On the other hand, no reduction of the nitro group was found in the case of the nitroimidazole.[3, 4]

The experiments with ORF-1744 herein described were designed only to detect congeners of 2,4-dinitropyrrole; reduction of the nitro group would not have been detected because such a transformation would probably have caused a cleavage of the pyrrole ring, resulting in the loss of the characteristic u.v. absorption. Detection of

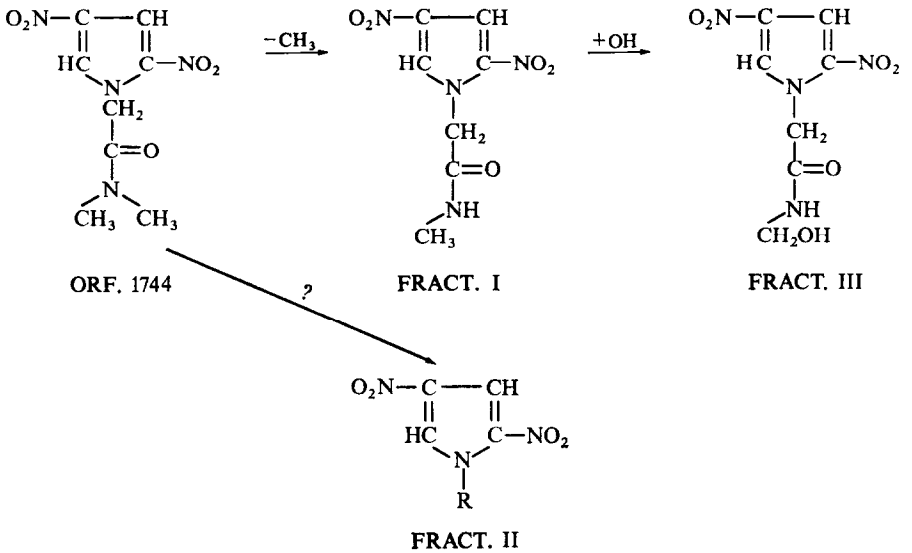

FIG. 6. A proposed metabolic route for ORF-1744 in mice.

the parent compound or its metabolites in urine extracts was dependent on this u.v. absorption. We may conclude that the primary objective of the investigation was successful, i.e. it resulted in the isolation and identification of the active antimicrobial metabolites. However, it is apparent that the complete pattern of metabolic degradation of dinitropyrroles in mice remain to be established.

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REFERENCES

1. A. H. BECKETT and A. E. ROBINSON, *J. mednl pharm. Chem.* **1**, 135 (1959).
2. J. OLIVARD, S. VALENTI and J. A. BUZARD, *J. mednl pharm. Chem.* **5**, 524 (1962).
3. R. M. J. INGS, G. L. LAW and E. W. PARNELL, *Biochem. Pharmac.* **15**, 515 (1966).
4. R. W. MANTHEI and L. G. FEO, *Wiad. parazyt.* T.X., 177 (1964).