ation has been suggested to play an important role in aminoazo dye carcinogenesis,[11] and it seems that the same reaction may also be an essential metabolic reaction in producing MHb *in vivo*.

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REFERENCES

1. O. BODANSKY, *Pharmac. Rev.* **3,** 144 (1951).
2. W. KALOW, *Pharmacogenetics*, p. 162. Saunders, Philadelphia, Pa. (1962).
3. K. A. EVELYN and H. T. MALLOY, *J. biol. Chem.* **126,** 655 (1938).
4. P. B. HAWK, B. L. OSER and W. H. SUMMERSON, *Practical Physiological Chemistry*, 13th edn, p. 620. McGraw-Hill, New York (1954).
5. M. KIESE, *Ann. N.Y. Acad. Sci.* **123,** 141 (1965).
6. H. DANNENBERG and M. KIESE, *Naunyn–Schmiedebergs Arch. exp. Path. Pharmak.* **211,** 410 (1950).
7. J. A. MILLER and E. C. MILLER, *Adv. Cancer Res.* **1,** 339 (1953).
8. G. C. MUELLER and J. A. MILLER, *Cancer Res.* **11,** 271 (1951).
9. E. C. MILLER and C. A. BAUMANN, *Cancer Res.* **6,** 289 (1946).
10. A. H. M. KIRBY, *Cancer Res.* **7,** 333 (1947).
11. J. A. MILLER and E. C. MILLER, in *Physico-Chemical Mechanism of Carcinogenesis* (The Jerusalem Symposia on Quantum Chemistry and Biochemistry I), pp. 237–261. Israel Academy of Science and Humanities, Jerusalem (1969).

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Conversion of a substituted 3,4,5-trimethoxycinnamide to a 3,5-dihydroxy compound in the rat and inhibition of the conversion by neomycin

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IN OUR study of the metabolism of carbon-14 labeled *N*-(3,3-dimethylpropynyl)-3,4,5-trimethoxycinnamide* in rats, there was isolated from the urine a metabolite identified by nuclear magnetic resonance (n.m.r.) and mass spectrometry as α-(3,5-dihydroxycinnamido)isobutyric acid. This compound accounted for approximately 12 per cent of the radioactivity found in glucuronidase-sulfatase-treated rat urine, and could be extracted by diethyl ether at pH 7. It is not known that any mammalian system is capable of transforming a trimethoxyphenyl structure into a dihydroxyphenyl structure. Yet the dihydroxy compound was radioactive and therefore must have been derived from the radioactive parent drug, and some means had to exist for this conversion. We thus decided to investigate the source of this unique transformation.

* Abbott-25794.

The *p*-dehydroxylation of various phenolic compounds by intestinal microbes has been reviewed by Scheline.[1] Griffiths[2,3] has described the metabolism of sinapic acid and related compounds in the rat. Smith and Griffiths[4] have also demonstrated the conversion of 3,4,5-trihydroxyphenylacetic acid to 3,5-dihydroxyphenylacetic acid by intestinal microbes. This report describes what appears to be a microbial conversion *in vivo* of a 3,4,5-trimethoxycinnamic acid derivative to a 3,5-dihydroxycinnamic acid structure.

Experimental

Drug. The drug was prepared with carbon-14 label on the carbon atom alpha to the phenyl ring, and chemical and radiochemical purity was established to be 98 ± per cent by thin-layer chromatography on Silica gel plates developed in eight different solvent systems (solvents 4–11). The labeled drug had a specific activity of $1{\cdot}005 \times 10^7$ dpm/mg. This was diluted with unlabeled drug in order to obtain a 10 mg/kg dose which was administered to each rat. Rats in group I each received $2{\cdot}08 \times 10^7$ dis./min, and rats in groups II and III each received $2{\cdot}30 \times 10^7$ dis. min of ^{14}C-activity.

Animals. The rats used in this study were males of the Sprague–Dawley strain, obtained from the Charles River Laboratories, North Wilmington, Mass., and weighed approximately 200 g each. The rats had been kept on Purina Laboratory Chow and water *ad lib.*, and were maintained on the same diet throughout the duration of the experiment. Three groups of two rats each were used in this experiment. Each group was housed in a single stainless steel metabolism cage which was designed so as to separate liquid from solid excreta.

Neomycin was used to inhibit the bacteria in the gastrointestinal tract of the rats. Since repeated administration of neomycin can cause diarrhea in rats, we chose to employ a neomycin–kaolin–pectin* preparation in order to prevent diarrhea (with the resultant mixing of urine and feces) and yet to obtain effective sterilization of the gastrointestinal tract.

Dose schedule. Each rat was initially dosed (day 1) with 10 mg/kg of carbon-14 labeled drug in tragacanth suspension *per os.* Each rat was similarly dosed on day 2 with 10 mg/kg of unlabeled drug. Urines and feces were collected for 2 days following the radioactive dose. Starting on day 3 and continuing through day 8, the animals were dosed daily with 2·5 ml of the neomycin–kaolin–pectin preparation *per os.* In addition, on day 7, the rats were administered a second oral 10 mg/kg dose of carbon-14 labeled drug and this was followed with 10 mg/kg of unlabeled drug on day 8. Urines and feces were again collected for 2 days following the radioactive dose.

Assay for metabolite. Previous work showed that the initial 48-hr urine sample following an oral dose of *N*-(3,3-dimethylpropynyl)-3,4,5-trimethoxycinnamide-^{14}C contained nearly all of the radioactivity which is excreted in the urine.[5]

The 0–48 hr urines were incubated with 0·5 per cent by volume of a commercial glucuronidase–sulfatase preparation† for 48 hr at pH 5 (acetate buffer, 37°) so as to hydrolyze glucuronide and sulfate conjugates. The hydrolyzed urines were then extracted three times at pH 7 by shaking in a separatory funnel with equal volumes of chloroform to remove the less polar metabolites, then extracted three times with equal volumes of diethyl ether. The ether layers were dried with anhydrous Na_2SO_4 and sampled for radioactivity. Thin-layer chromatography as well as isolation (below) showed that the ether extracts contained only α-(3,5-dihydroxycinnamido)isobutyric acid with a small amount of urinary impurities.

A final ether extraction was performed at pH 1. The constituents of this extract and the pH 7 $CHCl_3$ extract will be reported later.

The 0–48 hr urines from sixteen other rats which had received only a single dose of unlabeled *N*-(3,3-dimethylpropynyl)-3,4,5-trimethoxycinnamide were treated in like manner in order to obtain sufficient material for further workup.

Isolation and characterization of α-(3,5-*dihydroxycinnamido*)*isobutyric acid.* Following the above procedure for extraction of metabolite, the ether extracts were evaporated to dryness *in vacuo* at 37° or lower. The extracted metabolite was purified by column chromatography on a celite-acetic acid partition chromatographic column.[6] The radioactive peak obtained from this step was subjected to further purification by chromatography on a 20 mm × 12 cm column of Florisil packed in 2,2,4-trimethyl pentane. The column was eluted with 2,2,4-trimethyl pentane containing increasing concentrations of acetone. Evaporation of solvent from the radioactive peak which was eluted from this column yielded a white powder which was analyzed by n.m.r. and mass spectrometry.

* Kaomycin—a product of The Upjohn Company, Kalamazoo, Mich., U.S.A., containing 300 mg neomycin sulfate, 5·832 g kaolin, and 130 mg pectin per fluid ounce.

† Glusulase—a product of Endo Laboratories, Inc., Garden City, N.Y., U.S.A., containing both β-glucuronidase and sulfatase activity.

The n.m.r. was obtained at 100 MHz on a Varian Associates HA-100 spectrometer; n.m.r. (acetone-d_6): δ 1·39 (s, 6, gem-dimethyl), 6·38 (t, 1, aromatic, J = 2·0 Hz), 6·53 (d, 2, aromatic, J = 2·0 Hz), 6·58 (d, 1, vinyl, J = 16·0 Hz), 7·38 (d, 1, vinyl, J = 16·0 Hz).

The mass spectrum was obtained on an AEI-MS-902 mass spectrometer. Mass spectrum (70 eV) m/e (rel. intensity) 265 (0·5), 263 (1), 220 (28, $C_{12}H_{14}NO_3$), and 163 (100, $C_9H_7O_3$).

Thin-layer chromatography. Commercially prepared Brinkmann Silica gel F_{254} plates, 5 × 20 cm, 250 μ, were used with the following solvent systems: (1) benzene–methanol–glacial acetic acid (90:5:5, v/v); (2) ethyl acetate–methanol–28% NH_4OH (80:20:1, v/v); (3) chloroform–acetone (68:24, v/v); (4) toluene–methanol (70:30, v/v); (5) toluene–methanol (95:5, v/v); (6) toluene–methanol (99:1, v/v); (7) 100% benzene; (8) benzene–95% ethanol (95:5, v/v); (9) benzene–glacial acetic acid (95:5, v/v); (10) benzene–triethylamine–methanol (94:5:1, v/v); (11) 95% ethanol–28% NH_4OH (95:5, v/v; (12) toluene–methanol (7:1, v/v); and (13) dioxane–chloroform –glacial acetic acid (50:50:1, v/v).

The R_f values of parent drug were: 0·52 (solvent system 1); 0·86 (2); 0·72 (3); 0·73 (4); 0·40 (5); 0·06 (6); 0·00 (7); 0·44 (8); 0·10 (9); 0·46 (10); 0·80 (11); 0·54 (12); and 0·87 (13). The R_f values of α-(3,5-dihydroxycinnamido)isobutyric acid were: 0·12 (solvent system 1); 0·74 (2); 0·23 (3); 0·57 (4); 0·17 (12); and 0·62 (13).

Radiochemical assay. Aliquants of urine samples obtained from the rats were dissolved directly in diotol solution.* Extracts obtained from the urines were likewise dissolved in diotol solution. All samples were then counted in a liquid scintillation counter at conditions suitable for measurement of carbon-14 and corrected for quenching by the internal standard technique.

Results and discussion

Results from the extraction of rat urines obtained during this study appear in Table 1. It will be noted that whereas an average of 12 per cent of the radioactivity found in the urine goes to the neutral ether extract in the untreated (or normal) rat, this value is only 1 per cent when the neomycin preparation is administered to the same rats. Thin-layer chromatography of the neutral ether extracts in six different solvent systems (solvents 1–4, 12, 13) showed the presence of α-(3,5-dihydroxycinnamido) isobutyric acid, as shown by co-chromatography with authentic α-(3,5-dihydroxycinnamido)isobutyric acid, in the urines from all three groups before the administration of the neomycin preparation, but no dihydroxy metabolite could be detected when the neomycin preparation was administered along with parent drug. The 1 per cent radioactivity found in the neutral ether extract may be due to small amounts of contamination and/or to an amount of dihydroxy compound not detectable by the methods employed. The fact that there was essentially no difference in distribution of urinary radio-

TABLE 1. DISTRIBUTION OF RADIOACTIVITY FROM ABBOTT-25794-^{14}C IN VARIOUS EXTRACTS OF GLUCURONIDASE-SULFATASE-TREATED RAT URINE BEFORE AND AFTER THE ANIMALS WERE TREATED WITH NEOMYCIN

Extract	Urinary radioactivity % Group I*	Group II†	Group III‡
pH 7 $CHCl_3$	38·4	44·7	34·4
pH 7 $CHCl_3$ (neomycin)§	35·1	39·0	32·8
pH 7 EtOEt	10·4	8·7	17·0
pH 7 EtOEt (neomycin)	1·1	1·2	1·7
pH 1 EtOEt	17·2	17·6	15·6
pH 1 EtOEt (neomycin)	17·4	18·5	15·9
Aq. residue	34·1	29·0	33·0
Aq. residue (neomycin)	46·5	41·3	49·6

* Group I: av. wt. = 195 g; av. dose excreted in urine = 39·0 per cent.
† Group II: av. wt. = 215 g; av. dose excreted in urine = 39·7 per cent.
‡ Group III: av. wt. = 215 g; av. dose excreted in urine = 40·3 per cent.
§ Indicates sample was obtained from the urine of rats treated with Kaomycin.

* A mixture of 350 ml toluene, 350 ml dioxane, 210 ml methanol containing 73 g naphthalene, 4·6 g 2,5-diphenyloxazole and 80 mg 1,4-bis[2-(5-phenyloxazolyl)]benzene.

activity into the chloroform and pH 1 ether extracts between neomycin preparation-treated and untreated rats would seem to indicate that the other metabolites which are extracted under these conditions are unaffected by the neomycin treatment.

While it cannot be said that the absence of the dihydroxy metabolite from the urine following neomycin treatment is unequivocal proof that intestinal microbes are involved in the transformation of parent compound to α-(3,5-dihydroxycinnamido)isobutyric acid, there does not appear to be an alternative explanation for this observation if we bear in mind the fact that neomycin is not absorbed when orally administered. If we also take into account the earlier observations of Griffiths[2,3] and Smith and Griffiths,[4] and the similar structures which are involved in the transformations, it appears that intestinal microbes are involved in the conversion of *N*-(3,3-dimethylpropynyl)-3,4,5-trimethoxycinnamide to α-(3,5-dihydroxycinnamido)isobutyric acid.

These experiments again show that due care must be exercised in the interpretation of drug metabolism data when *in vivo* systems are used; it would be important that the investigator distinguish between mammalian and microbial metabolites of a given drug, especially since intestinal flora differ in various species and even among individual human beings.

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REFERENCES

1. R. R. SCHELINE, *J. pharm. Sci.* **57,** 2021 (1968).
2. L. A. GRIFFITHS, *Biochem. J.* **113,** 603 (1969).
3. L. A. GRIFFITHS, *Experientia* **26,** 723 (1970).
4. G. E. SMITH and L. A. GRIFFITHS, *Biochem. J.* **118,** 53P (1970).
5. G. J. IKEDA and C. B. ESTEP, *Fedn Proc.* **30,** 391 (1971).
6. J. T. MATSCHINER, T. A. MAHOWALD, W. H. ELLIOTT, E. A. DOISY, JR., S. L. HSIA and E. A. DOISY, *J. biol. Chem.* **225,** 771 (1957).

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Effect of morphine on esterified fatty acids in plasma and brain of the nontolerant, tolerant and abstinent rat

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IN A CONTINUING effort to ascertain the effect of morphine on lipid metabolism,[1,2] an attempt was made to determine the relationship, if any, between the action of morphine and the levels of esterified fatty acids in the plasma and brain of nontolerant, tolerant and abstinent rats. Such an effect might directly implicate fatty acid metabolism mechanistically in the agonistic as well as drug-dependent action of morphine either independently or possibly coupled with the phospholipid effects of this drug.[3–6] The results of this study are reported in this communication.

Nontolerant male Wistar rats weighing between 150 and 260 g were housed in cages in air-conditioned animal quarters and fed a Rockland mouse/rat diet and water *ad libitum*. Ten rats each were sacrificed at 1 and 2 hr by decapitation after the administration of 10 mg/kg (free base) of morphine subcutaneously. The blood was collected in heparinized beakers and the brain rapidly removed and immediately frozen.