Because of its simplicity the method is well suited for determining the inhibition of aromatic amino acid decarboxylase *in vivo*. It has the additional advantage of measuring the effect of decarboxylase inhibitors *in situ* rather than in homogenates prepared from a single organ. The method in its present form does not assay the inhibition of decarboxylase in the central nervous system.

Metabolism of Metaxalone

ROBERT B. BRUCE, LENNOX TURNBULL, JACK NEWMAN, AND JEFFERSON PITTS

Research Laboratories, A. H. Robins Company, Inc., Richmond, Virginia

Received August 10, 1965

The metabolism of metaxalone (I) has been studied in the dog and man. The major product is formed by oxidation of one of the methyl groups to yield 5-(3-methyl-5-carboxyphenoxymethyl)-2-oxazolidinone. This also occurs in the urine as the glucuronide. The ether linkage is also cleaved to give 3,5-xylenol and 5-hydroxymethyloxazolidinone. The oxazolidinone ring appears to be stable. In order to identify these metabolites the above acid and its triacetyl β-glucuronide methyl ester were synthesized.

Metaxalone, which is 5-(3,5-dimethylphenoxymethyl)-2-oxazolidinone (I), was first synthesized by Lunsford, *et al.*[1] This drug has been shown to be an effective muscle relaxant with low toxicity.[2] In an earlier attempt to study absorption and excretion of this compound in animals and man using the colorimetric method of Titus, *et al.*,[3] only extremely low concentrations in blood and urine could be found, in spite of the fact that recoveries of added amounts to biological fluids were satisfactory. The method described by Titus is based on the extraction of the drug from alkaline solution, and it was thus apparent that metaxalone was extensively metabolized and probably to an acidic compound. The following studies were carried out to investigate the metabolism of metaxalone in man and animals.

Experimental Section

Synthesis of Related Compounds.—A general review of the literature of the metabolism of structurally related compounds and the chemistry of metaxalone suggested a number of possible metabolites. The synthesis of these are described below.

5-(3-Methyl-5-carboxyphenoxymethyl)-2-oxazolidinone.—Three grams of metaxalone was suspended in 100 ml of water and stirred continuously at 75–80° for 3 hr while 2.14 g (0.5 equiv) of $KMnO_4$ in 25 ml of water was added. Removal of the MnO_2 and excess metaxalone by filtration and ether extraction of the filtrate gave a clear aqueous solution of pH 8–9. After concentrating to 25 ml, the solution was acidified; a gum (480 mg) separated. Thin layer chromatography (benzene–methanol–formic acid, 25:8:2) of this gum showed a major substance and a minor amount of a more polar acidic material, probably the phthalic acid derivative. Crystallization was induced, and, after several recrystallizations, the melting point was 157–192° (the material was poorly recrystallizable and the melting point did not improve; however, it was chromatographically pure).

Anal. Calcd for $C_{12}H_{13}NO_5$: C, 57.38; H, 5.22; N, 5.58. Found: C, 57.15; H, 5.16; N, 5.73.

5-(3-Methyl-5-carboxyphenoxymethyl)-2-oxazolidinone Triacetyl β-Glucuronide Methyl Ester.—A mixture of 1.5 g of 5-(3-methyl-5-carboxyphenoxymethyl)-2-oxazolidinone, 2.4 g of methyl bromacetylglucuronate, and 3.0 g of fresh silver oxide was stirred in 30 ml of pyridine with cooling in an ice bath for 15 min. The dark solution was filtered through Celite, and the filtrate was pumped to dryness. The residue was taken into $CHCl_3$ and the solution was extracted with 1 *N* HCl, dilute bicarbonate, and finally with water. Removal of the $CHCl_3$ gave 980 mg of dark gum, R_f 0.65 (tlc, benzene–acetone, 1:1). Florisil chromatography produced 591 mg of a clear glass, $[\alpha]^{25}D$ −30.6° (*c* 3, methanol). The material was chromatographically homogeneous.

Anal. Calcd for $C_{25}H_{29}NO_{14}$: C, 54.44; H, 5.30; N, 2.53. Found: C, 54.17; H, 5.31; N, 2.68.

3-Aminopropanediol was synthesized by the procedure of Bergmann, *et al.*[4]

Isolation of Metabolites. 5-(3-Methyl-5-carboxyphenoxymethyl)-2-oxazolidinone from Dog Urine.—A 14.5-kg, male, mongrel dog was given a daily oral dose of 5.73 g of metaxalone (395 mg/kg) for a 7-day period. The urine was collected during this period and preserved by the addition of NaF to the daily collection beaker. A 1.8-l. aliquot of the total collection (4.8 l.) was boiled and filtered through Celite to remove a flocculent precipitate. Saturation of the urine with $(NH_4)_2SO_4$ (about 1 kg) produced a gummy precipitate which was separated by decantation of the urine and which was extracted from excess $(NH_4)_2SO_4$ by treatment with warm ethanol. Removal of the ethanol produced 4.4 g of a glassy brown gum. A 500-mg sample of this gum was induced to crystallize from hot water and after three recrystallizations had a melting point of 145–170°; two further recrystallizations produced 120 mg, mp 153–173°.

Anal. Calcd for $C_{12}H_{13}NO_5$: C, 57.38; H, 5.22; N, 5.58. Found: C, 57.56; H, 5.27; N, 5.62.

This material cochromatographed on thin layer chromatography with the synthesized material. Their infrared and ultraviolet spectra were identical. The $[\alpha]^{26}D$ of the metabolite was −15° (*c* 3, methanol).

In order to confirm the structure of the metabolite, it was cleaved with HI. Treatment of 400 mg of the urinary acid with 10 ml of refluxing 57% HI for 4 hr followed by ether extraction gave 182 mg of extracted material. This was taken into bicarbonate solution which was extracted twice with ether to remove neutral material. Acidification of the aqueous bicarbonate solution and ether extraction gave 65 mg of crude *m*-hydroxytoluic acid. Recrystallization from methanol–water gave a pure sample, mp 208.5–210°. The material did not depress the melting point of authentic *m*-hydroxytoluic acid and the infrared curves were identical.

Anal. Calcd for $C_8H_8O_3$: C, 63.15; H, 5.30. Found: C, 63.48; H, 5.36.

5-(3-Methyl-5-carboxyphenoxymethyl)-2-oxazolidinone Triacetyl β-Glucuronide Methyl Esters.—A 75-kg male subject (J. E. P.) ingested orally 12.8 g of metaxalone (85 mg/kg per day) over a 2-day period, and the urine (1.93 l.) was collected

(1) C. D. Lunsford, R. P. Mays, J. A. Richman, Jr., and R. S. Murphey, *J. Am. Chem. Soc.*, **82**, 1166 (1960).

(2) M. N. Carrol, Jr., W. R. Luten, and R. W. Southward, *Arch. Intern. Pharmacodyn.*, **130**, 280 (1961); C. H. Carter, *Diseases Nervous System*, **23**, 98 (1962); J. F. Kurtzke and J. Gylfe, *Neurology*, **12**, 343 (1962); L. W. Morey and A. R. Crosby, *J. Am. Osteopath. Assoc.*, **62**, 517 (1963).

(3) E. Titus, S. Ulick, and A. P. Richardson, *J. Pharmacol. Exptl. Therap.*, **93**, 129 (1948).

(4) M. Bergmann, B. Erwin, and F. Dreyer, *Chem. Ber.*, **54B**, 936 (1921).

for 2.5 days. Saturation of a 1-l. aliquot of the urine with $(NH_4)_2SO_4$ produced 9.96 g of a gummy precipitate. Continuous ether extraction of a concentrated aqueous solution of this precipitate removed free 5-(3-methyl-5-carboxyphenoxymethyl)-2-oxazolidinone leaving 5.3 g of unextractable material. The unextractable material (1.1 g) was allowed to stand in 30 ml of 1:1 acetic anhydride–pyridine for 45 min. The reagents were removed *in vacuo*, and the crude product was esterified by treatment with 40 ml of 1.3% ethereal diazomethane. Thin layer chromatography (benzene–acetone, 1:1) of this acetylated, esterified fraction indicated (iodine vapor) at least seven components, the major component having R_f 0.65. This material was chromatographed on a Florisil column with elution by acetone–benzene mixtures. In the series of fractions obtained, 10% acetone–benzene produced a fraction containing 416 mg which was principally R_f 0.65 material. Hydrolysis of a small sample of this fraction with 1 *N* HCl produced free 5-(3-methyl-5-carboxyphenoxymethyl)-2-oxazolidinone. Rechromatography of the fraction gave 280 mg of a clear glass which was chromatographically homogeneous; $[\alpha]^{25}D$ −42.7° (*c* 3, methanol). Infrared spectra of the isolated and synthesized materials were identical.

Anal. Calcd for $C_{25}H_{29}NO_{14}$: C, 54.44; H, 5.30; N, 2.53. Found: C, 53.97; H, 5.46; N, 2.68.

In order to make an estimation of the quantities of these materials that were excreted by humans, the following studies were performed.

A male subject (F. P.) ingested 12.8 g of metaxalone over a 2-day period, and his urine was collected for a 2.5-day period giving a total volume of 2.5 l. A 100-ml aliquot of this was continuously extracted with ether for 24 hr, and the extract was esterified with ethereal diazomethane. Chromatography of this fraction on Florisil gave 324 mg of material which was eluted with 1:1 acetone–benzene. Rechromatography of this fraction gave 163 mg of pure ester of the urinary acid. This corresponds to 27% of the ingested dose of metaxalone.

Another 100-ml aliquot of the same urine was brought to 1 *N* by the addition of H_2SO_4, and refluxed for 1 hr. Formation and isolation of the methyl ester of the fraction was achieved just as in the preceding experiment. By this procedure, 267 mg of pure methyl ester was isolated which corresponds to 44% of the ingested dose of metaxalone. This value represents both free and conjugated acid.

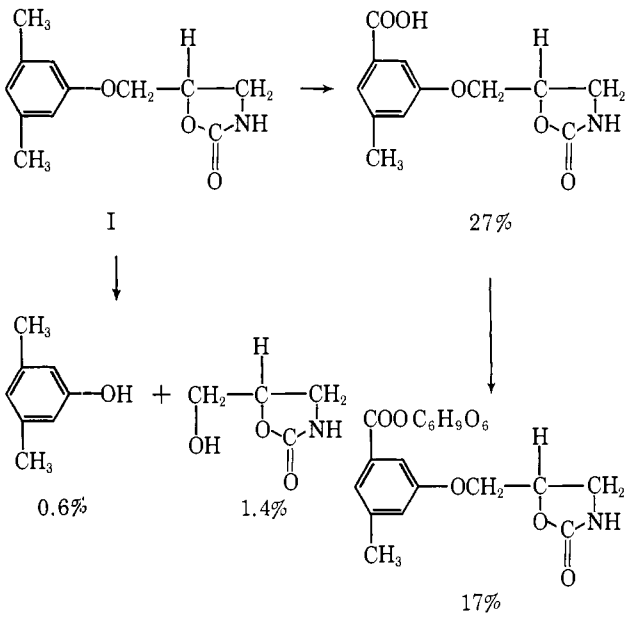
3-Aminopropanediol.—A 500-ml aliquot of human metaxalone urine (F. P.) was brought to 4 *N* by addition of solid NaOH and refluxed for 3 hr, after which time the cooled urine was extracted with three 500-ml portions of ether. The pH was adjusted to 2 with H_2SO_4, and the ether extraction was repeated. The urine was brought to pH 6 and concentrated to dryness, and the residual solids were extracted with boiling methanol. An aqueous solution of the methanol extract was passed onto a small Dowex-50(H^+) resin column, which, after a water wash, was eluted with 2 *N* NH_4OH. This eluate was then passed through a Dowex-21K(OH^-) column, and the column effluent was concentrated *in vacuo* to 90 mg of gum. Thin layer chromatography of this fraction (silica gel, methanol) gave a very polar minor spot (R_f 0.1) which was ninhydrin positive and which did not appear in control human urine. The material was treated with benzoyl chloride in pyridine and, after removal of the reagents, the product was chromatographed on Florisil. Elution with 5% acetone–benzene produced 65 mg of pure, crystalline N-(2,3-dibenzoyloxypropyl)benzamide, $[\alpha]^{25}D$ +3.0° (*c* 2, methanol). Recrystallization from absolute ethanol gave an analytical sample, mp 113–119.5°, which had an infrared spectrum identical with that of an authentic sample.[4] The melting point of the authentic sample was 113–114.5°, mmp 113–116.5°. The positive rotation of the metabolic sample may account for the melting point difference.

Anal. (metabolic sample) Calcd for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.47. Found: C, 71.53; H, 5.28; N, 3.73.

The 65 mg of isolated material accounts for 1.4% of the dose. However, it is likely that some aminopropanediol was not extracted during the methanol extraction of the urine solids.

Metaxalone and Acidic Metabolite in Feces.—A 2-day feces collection was taken from a 13.3-kg, male, mongrel dog following a single oral dose of 4.0 g of metaxalone. Vacuum-oven drying at 70° followed by blender treatment gave 36.4 g of dry powder. A 10-g portion was extracted with boiling absolute ethanol, 2 *N* in H_2SO_4. The filtered extract was neutralized with alkali and again filtered to remove Na_2SO_4. The solution was concentrated to dryness, and extraction of the solids with 280 ml of 50% aqueous methanol removed further insoluble material. This solution (50 ml) was passed through a small Dowex-50(H^+) column and the effluent was put onto a column of Dowex-21K(OH^-). The effluent from this column was concentrated to a residue of 41 mg of a clear gum. Thin layer chromatography showed that it consisted of a major substance, which cochromatographed with the administered drug, metaxalone. The spot was located by the hypochlorite–starch iodide reagent.

SCHEME I



The Dowex-21K(OH^-) column above was eluted with 2 *N* acetic acid to give after concentration, 49 mg. A major spot in this fraction cochromatographed (tlc, benzene–methanol–formic acid, 25:8:2) with 5-(3-methyl-5-carboxyphenoxymethyl)-2-oxazolidinone, the urinary metabolite. The spot was located by the hypochlorite–starch iodide method.

Based on these isolated fractions, the amount of metaxalone and its acidic metabolite in feces amounted to approximately 15–30% of the administered dose.

Discussion

The procedure described above for the isolation of 3-aminopropanediol would result in opening of the oxazolidinone ring if the latter were present in the urine, since alkali readily hydrolyzes oxazolidinone compounds to the amino alcohols.[1] That the unchanged oxazolidinone was the compound present in urine was shown by paper chromatography. Paper chromatography (butanol–acetic acid–H_2O, 50:11:25) of an amine extract of urine that had not been hydrolyzed did not show a spot corresponding to aminopropanediol; after alkaline hydrolysis this spot appeared. These results might indicate that a conjugated compound was present, but following acidic hydrolysis to which the ring is stable, no such spot was found. The stability of the oxazolidinone ring to metabolism was further confirmed by results obtained by Huf,[5] in which he showed that only traces of $^{14}CO_2$ were obtained from rats receiving metaxalone-^{14}C labeled on the carbonyl carbon.

Since the oxazolidinone ring was found, it was apparent that the other product of the cleavage of the ether linkage should also be present. This could have been either 3,5-dimethylphenol or 3-methyl-5-carboxy-

(5) E. G. Huf, personal communication.

phenol. The investigation for the presence of the xylenol in urine was carried out using gas chromatography. Phenols were separated from the urine of a dog that had received 18.7 g of metaxalone by continuous ether extraction of acidified urine. The acids were removed from the extract with $NaHCO_3$. Injection of an aliquot onto a 10% Apiezon on Chromosorb W column at 185° using a thermoconductivity detector showed the presence of 3,5-xylenol which was not present in control urine. Peak-height comparison with known amounts of the xylenol indicated that 0.6% of the dose was present in the urine. Enzymatic hydrolysis of a sample of the urine with glucuronidase and sulfatase did not increase the amount found. The urine was also investigated for the presence of 3-methyl-5-carboxyphenol by thin layer chromatography. The presence of this acid could not be definitely confirmed, although a spot corresponding to the known acid was found using toluene–ethyl formate–formic acid (5:4:1) and benzene–dioxane–acetic acid (18:5:0.8) on silica gel, from an extract of enzymatically hydrolyzed urine.

Since urinary excretion of metaxalone accounted for the major portion of the dose and only small amounts of unchanged drug appeared in the feces, the drug must be well absorbed in these species. These results indicate that in man and dog the metabolite changes shown in Scheme I take place.

The excretion of metaxalone-^{14}C was studied in the rat, rabbit, and dog. The rabbit appeared to excrete the major portion of the radioactivity in the urine very rapidly; 96% appeared within the first 48 hr. Within the same period, 11% appeared in the urine of the dog. The pattern of metabolites, by chromatography, appeared to be similar in these two species. The rat excreted 71% in the urine during a corresponding time. The pattern of metabolites in the rat, however, was different. At least five separate, radioactive spots could be distinguished on chromatography of the urine. These were not identified.

Design and Synthesis of Thiolesters for the Histochemical Demonstration of Esterase and Lipase *via* the Formation of Osmiophilic Diazo Thioethers[1]

JACOB S. HANKER, LIONEL KATZOFF, HOWARD R. ROSEN, MYRON L. SELIGMAN, HIROMI UENO, AND ARNOLD M. SELIGMAN

Departments of Surgery, Sinai Hospital of Baltimore, and The Johns Hopkins University School of Medicine, Baltimore, Maryland

Received November 17, 1965

The design, preparation, and properties of thiolesters as substrates for the cytochemical demonstration of esterolytic enzymes is described. The relative reactivity with osmium tetroxide of several diazo thioethers formed by S coupling with diazonium salts of the thiophenols resulting from enzymatic hydrolysis of the thiolesters was studied. This information has resulted in the development of a new technique for the demonstration of esterases and lipase by the selective deposition at the enzyme sites of osmium black, an ideal end product for light and electron microscopy. Examples of results with light and electron microscopy are included.

This investigation was prompted by the need for developing methods for esterase and lipase in electron microscopy that, in addition to utilizing well-established histochemical reactions, would yield electron-opaque end products.[2a-d] Thiolesters appeared to be worthy of synthesis as substrates to fulfill this need for several reasons. Fatty acid esterases have been known for some time to hydrolyze thiolesters.[3a-c] The thiophenols and thionaphthols produced on hydrolysis of aryl thiolesters are unable to undergo nuclear coupling with diazonium salts[4a] but do couple very readily on the sulfur (Figure 1) at both acid and alkaline pH to form insoluble yellow diazo thioethers (S-azo compounds[4a-c]). This would permit a rapid capture reaction, upon enzymatic hydrolysis of the thiolester, which would greatly improve localization. Moreover, aryl diazo thioethers are quite unstable and are readily decomposed to yield thiophenols.[5]

Since thiophenols as well as thiols readily reduce OsO_4 and react with it to form mercaptides, it was expected that conditions would be found where diazo thioethers would react with OsO_4 to yield osmium black, an ideal end product for light and electron microscopy.[2a-c] A formulation for the over-all reaction is shown in Figure 1.

Once it was shown[2a,b] that phenyl thiolacetate (I) gave satisfactory histochemical localization of esterase in thin, formalin-fixed sections of rat kidney and rat liver, other thiolesters were prepared in three categories. 2-Naphthyl thiolacetate (II) was prepared to study the effects of increasing the size of the molecule, and two large aliphatic thiolesters were prepared to compare esterase localization with that of the aromatic esters. These two substrates were the octadecyl thiolacetate (III) and the triphenylmethyl (trityl) thiolacetate (IV).

(1) This investigation was supported by a research grant (CA-02478) from the National Cancer Institute, U. S. Public Health Service, Washington, D. C.

(2) (a) J. S. Hanker, A. R. Seaman, L. P. Weiss, H. Ueno, R. A. Bergman, and A. M. Seligman, *Science*, **146**, 1039 (1964); (b) A. M. Seligman in Proceedings of the Second International Congress of Histo- and Cytochemistry, Springer-Verlag, 1964, p 9; (c) J. Hanker, A. Seaman, L. Weiss, H. Ueno, H. Dmochowski, L. Katzoff, and A. M. Seligman, *J. Histochem. Cytochem.*, **13**, 3 (1965); (d) L. A. Sternberger, E. J. Donati, J. S. Hanker, and A. M. Seligman, *Exptl. Mol. Pathol.*, in press.

(3) (a) J. Suzuoki and T. Suzuoki, *J. Biochem.* (Tokyo), **40**, 599 (1953); (b) J. Suzuoki and T. Suzuoki, *Nature*, **173**, 83 (1954); (c) G. B. Koelle and J. S. Friedenwald, *Proc. Soc. Exptl. Biol. Med.*, **70**, 617 (1949).

(4) (a) A. Hantzsch and H. Freese, *Ber.*, **28**, 3237 (1895); (b) P. Friedlaender and A. Chwala, *Monatsh.*, **28**, 247 (1907); (c) J. S. Hanker, L. Katzoff, L. D. Aronson, M. L. Seligman, H. R. Rosen, and A. M. Seligman, *J. Org. Chem.*, **30**, 1779 (1965).

(5) K. Saunders, "The Aromatic Diazo Compounds," Edward Arnold and Co., London, 1949.