sodium bicarbonate solution. After drying over anhydrous sodium sulfate, the solvent was removed under reduced pressure. The resulting brown residue was taken up in ether containing a little acetone and the solution decolorized with Darco. Filtration and removal of the solvent yielded a crystalline residue. Recrystallization from heptane afforded VII (225 mg.) as needles, m.p. 175–178°. One additional recrystallization from heptane gave an analytical sample, m.p. 179–180°, $[\alpha]^{25}D$ +38.5°.

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.73; H, 8.58.

Reaction of VII with Hydrochloric Acid.—To a solution of VII (0.47 g.) in acetone (10 ml.) was added concd. hydrochloric acid (1 ml.). The solution was allowed to stand overnight at room temperature and then poured slowly into a mixture of ice and water. The precipitate was collected by filtration and washed with water. Recrystallization of the product (0.42 g.) from acetone–heptane gave IIIa, m.p. 223–226° which was identical with the material obtained previously from IIa by treatment with hydrochloric acid. Substitution of acetic acid for acetone in this experiment yielded the corresponding acetate (IIIb).

(16) R. E. Counsell and P. D. Klimstra, U. S. Patent 2,980,710 (1961).

The Metabolites of Ergometrine and Lysergic Acid Diethylamide in Rat Bile

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Received August 21, 1961

Administration of ergometrine (1 mg./kg.) to rats results in the biliary excretion, within six hours, of two metabolites which are more polar than ergometrine. It is suggested that these metabolites are the β-glucuronides of 12-hydroxyergometrine and 12-hydroxyergometrinine. Under the same conditions and at the same dose level lysergic acid diethylamide (LSD) is converted to two metabolites, which are β-glucuronides of a hydroxy-LSD and a hydroxy-*iso*LSD, hydroxylation occurring in the benzene ring. At 45 mg./kg. ergometrine is metabolized to give a number of additional metabolites two of which are the glucuronide ethers of ergometrine and ergometrinine, conjugation apparently having occurred on the hydroxyl group of the aminopropanol side chain.

Although the metabolism of simpler substituted indoles such as tryptophan[1] and skatole[2] has been studied in detail few examples of the metabolism of more complex indoles, such as are found in alkaloids, have been recorded. Because of their clinical importance and

(1) I. M. Dibner, A. Feldstein, H. Freeman, and H. Hoagland, *Fed. Proc.*, **19**, 3 (1960).
(2) E. C. Horning, C. C. Sweeley, C. E. Dalgliesh, and W. Kelly, *Biochem. Biophys. Acta*, **32**, 566 (1959).

the number of chemical investigations which have been carried out, the ergot alkaloids and substances derived from them present an interesting problem in metabolism studies.

The early work on this problem[3-5] was concerned with the distribution and excretion of various ergot alkaloids in different animals and was limited by the small amount of material which could be injected and the unspecific methods of chemical and biological estimation. The preparation of C^{14} side-chain labelled lysergic acid diethylamide (I) (LSD)[6] was soon followed by investigations of its metabolism in several laboratories. Axelrod, Brady, Witkop, and Evarts[7] studied the distribution of LSD in cats and monkeys, Stoll, *et al.*[8] used mice while Boyd, *et al.*[9] used rats. From this work it appeared that the liver was responsible for the metabolic transformation of LSD. Axelrod, *et al.*[7] using a system of guinea pig liver microsome and supernatant fraction, showed that, in the presence of TPNH and oxygen, LSD was oxidized to 2-oxy-LSD. The structure of this compound was confirmed by its synthesis.[10] That this *in vitro* oxidation differs from *in vivo* experiments can be seen from the results of Stoll *et al.*[8] and of Boyd.[11] Using rats with bile fistulae it was shown by paper chromatography that the bile contained, in addition to very small amounts of unchanged LSD, two main radioactive metabolites with R_f values in butanol : water of 0.13 and 0.18. Both compounds still showed the blue fluorescence in ultraviolet light characteristic of lysergic acid derivatives and gave a positive Ehrlich color reaction indicating that position 2 in the indole nucleus was unsubstituted. Boyd[11] also showed that the compounds with R_f values of 0.13 and 0.18 could be hydrolyzed by β-glucuronidase to give less polar compounds which were still radioactive and gave the same blue fluorescence in the ultraviolet.

In the present work the nature of these two metabolites of LSD has been further investigated and the metabolites present in rat bile after the administration of low level and high level doses of ergometrine (II) have been studied by paper chromatographic and chemical methods.

(3) J. Rosenbloom and C. B. Shildecker, *J. Am. Med. Assoc.*, **63**, 1203 (1914).
(4) J. C. Kopet and J. M. Dille, *J. Am. Pharm. Assoc.*, **31**, 109 (1942).
(5) E. Rothlin, *Helv. Chim. Acta*, **29**, 1290 (1946).
(6) A. Stoll, J. Rutschmann, and A. Hofmann, *ibid.*, **37**, 820 (1954).
(7) J. Axelrod, R. O. Brady, B. Witkop and E. N. Evarts, *Ann. N. Y. Acad. Sci.*, **66**, 435 (1957).
(8) A. Stoll, E. Rothlin, J. Rutschmann, and W. R. Schalch, *Experientia*, **11**, 396 (1955).
(9) E. S. Boyd, E. Rothlin, J. F. Bonner, I. H. Slater, and H. C. Hodge, *J. Pharmacol. Exptl. Therap.*, **113**, 6 (1955).
(10) K. Freter, J. Axelrod, and B. Witkop, *J. Am. Chem. Soc.*, **79**, 3191 (1957).
(11) E. S. Boyd, *Arch. int. pharmacodyn.*, **120**, 292 (1959).

I	R = $N(C_2H_5)_2$	lysergic acid diethylamide
II	R = $NHCH(CH_3)CH_2OH$	ergometrine
III	R′ = $N(C_2H_5)_2$	isolysergic acid diethylamide
IV	R′ = $NHCH(CH_3)CH_2OH$	ergometrinine

Methods

Male and female albino rats (200–300 g.) were anesthetized with urethan and the bile duct was cannulated with the shaft of a hypodermic needle attached to a length of polythene tubing. Doses of LSD and of ergometrine maleate at levels of 3 mg./kg. were injected into a femoral vein. Ergometrine maleate was also administered at a dose level of 45 mg./kg. The bile was collected for 6 hr., diluted with ethanol and evaporated under reduced pressure in a stream of nitrogen at 60°. The residue was exhaustively extracted with hot methanol and the methanolic extracts were combined and concentrated for paper chromatography.

Paper Chromatography.—*System A*, butanol: acetic acid: water (4:1:5); *System B*, butanol: water (satd.); *System C*, butanol: ammonia: water (satd.).

Whatman No. 1 or No. 3 paper was used depending upon the quantity of material to be chromatographed. The chromatograms were impregnated by dipping in a 30% acetone solution of the aqueous phase and allowing the acetone to evaporate. The R_f values and solvent systems used are given in Table I. The chromatograms were visualized under an ultraviolet lamp or by spraying with a modified Ehrlich reagent[12] to detect the indole nucleus or with diazotized sulfanil-

TABLE I

PAPER CHROMATOGRAPHY OF ERGOMETRINE AND LSD METABOLITES AND THEIR HYDRAULIC PRODUCTS

Compound	R_f values for system A	B	C
Ergometrine	0.68	0.85	0.94
Ergometrine Metabolite A	.19	.04	.13
Ergometrine Metabolite B	.33	.06	.38
Ergometrine Metabolite C	.43	.17	.44
Ergometrine Metabolite D	.60	.27	.56
Ergometrine Metabolite E	.72	.18	.41
Ergometrine Metabolite F	.72	.32	.63
Ergometrine Metabolite G	.73	.50	.73
Ergometrine Metabolite H	.78	.65	.73
Hydrolyzed Metabolite A	.48	.21	—
12-Hydroxyergometrine	.48	.21	—
LSD	.86	.74	.95
LSD Metabolite A	.28	.07	.38
LSD Metabolite B	.34	.1	.53
Hydrolyzed Metabolite A	.67	.57	—
Hydrolyzed Metabolite B	.78	.60	—

(12) N. P. Kefford, *J. Exptl. Bot.*, **6**, 129 (1955).

amide and sodium carbonate[13] to detect phenols. For preliminary separation of metabolites from bile pigments the methanolic extracts were streaked on to sheets of Whatman No. 3 paper (9″ × 22″) and chromatographed using system A. (The methanolic extract of approximately 60 ml. of bile required 24 sheets of paper). The fluorescent bands were cut out and eluted with hot methanol. The methanol eluates were concentrated under nitrogen and rechromatographed on system A. The process was repeated using system B, again on system A and finally on system B.

Hydrolysis of Metabolites with β-Glucuronidase.—This was carried out in acetate buffer (5 ml., pH 4.5, 0.1 *M*) with a β-glucuronidase preparation[14] (1 mg = 500,000 units/g.) at 36°. After incubation the solution was evaporated to dryness under nitrogen (reduced pressure) and the methanol soluble material chromatographed as described previously. For inhibition of glucoronidase 10% excess of the calculated amount of 0.003 *M* solution of saccharic acid was added.

Pharmacological Testing of LSD Metabolites

(a) **Estimation of Metabolites.**—This was done spectrophotometrically on the assumption that the metabolites had a molecular weight of 517, and that they had the same extinction coefficient, at the maximum absorption of 315 mμ, as LSD. The optical densities of the metabolites in aqueous solution was measured at 315 mμ, and the concentration was determined.

(b) **Pharmacological Procedure.**—Uteri were taken from virgin female rats (150–180 g.) which had received stilbestrol (100 μg./rat) 24–48 hr. before use. One horn of the uterus was suspended in an organ bath maintained at 29° containing the oxygenated solution.[15] Contractions were recorded by an isotonic lever writing on a smoked drum. Doses of 5-hydroxytryptamine and acetylcholine were chosen to produce equistimulant actions and were alternated at 5 min. intervals. It was first established that extracts made from the bile of normal rats did not interfere with responses to 5-hydroxytryptamine, nor with the inhibitory action of LSD in the test. The substance to be tested was then added to the bath and maintained there throughout successive applications of the stimulant drugs. The method of studying anti-5-hydroxytryptamine activity is substantially that used by Cerletti and Doepfner.[16]

(a) **Ergometrine.**—The bile collected after low dose levels of ergometrine maleate (3 mg./kg.) showed two metabolites on paper chromatograms each giving a blue color with Ehrlich's reagent and fluorescing blue in ultraviolet light. These metabolites have been named ergometrine *metabolites A* (more polar) and *B* (less polar). Repeated chromatography of both metabolites on systems A and B produced only amorphous brown powders each of which showed the same ultraviolet spectra: $\lambda_{max.}$ 315 mμ; $\lambda_{min.}$ 270 mμ. At high dose levels (45 mg./kg.) paper chromatograms of bile extracts showed six further metabolites (in addition to ergometrine metabolites A and B) together with some unchanged ergometrine and its isomer ergometrinine. All substances gave a blue fluorescence in ultraviolet light and gave a blue color with Ehrlich reagent. The new metabolites were named ergometrine *metabolites C, D, E, F, G, and H* in order of decreasing polarity (Table I).

(13) D. Bolling, H. A. Sober, and R. J. Block, *Fed. Proc.*, **8**, 185 (1949).
(14) R. I. Cox, *Aust. J. Sci.*, **19**, 202 (1957).
(15) J. H. Gaddum, W. S. Peart, and M. Vogt, *J. Physiol.*, **108**, 467 (1949).
(16) A. Cerletti and W. Doepfner, *J. Pharmacol. Exptl. Therap.*, **122**, 124 (1958).

(1) **Isomerism of Ergometrine Metabolites.**—Ergometrine metabolites A and B purified by repeated paper chromatography were incubated in phosphate buffer (pH 5.5) at 36° as described for LSD metabolites. Under these conditions metabolite A was converted into a mixture of metabolites A and B; similarly ergometrine metabolite B was converted into a mixture of both metabolites A and B.

Ergometrine metabolite C (0.02 mg.) purified by paper chromatography using systems A, B, C was refluxed under nitrogen with 3 ml. of 2% aqueous potassium hydroxide for 2 hr. After neutralization with acetic acid, the solution was evaporated under reduced pressure in a stream of nitrogen and the methanol soluble material of the residue chromatographed on system A. Two bands which fluoresced blue in ultraviolet light and gave a positive Ehrlich test were present. These were eluted and were shown to be ergometrine metabolites C and D by comparative paper chromatography on systems A and B. Ergometrine metabolite D when similarly treated was also converted to a mixture of metabolites C and D.

Similarly the four ergometrine metabolites E, F, G and H present in trace amounts only after high dose levels were shown to consist of isomeric pairs E, F and G, H.

(2) **Hydrolysis of Ergometrine Metabolites with β-Glucuronidase.**—Ergometrine metabolites A and B were both hydrolyzed to give products which were less polar on paper chromatograms and which fluoresced blue in ultraviolet light, gave a blue color with Ehrlich reagent and a distinct purple color with diazotized sulfanilamide. The hydrolysis was inhibited by saccharic acid.

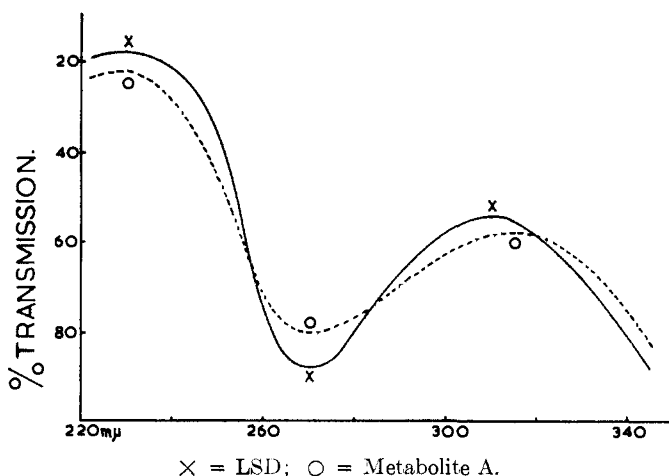
The hydrolytic product obtained from metabolite A and purified by paper chromatography using systems A and B was applied to chromatograms together with a sample of 12-hydroxyergometrine (kindly supplied by Dr. A. Hofmann and purified by rechromatography on paper just prior to the experiment). The hydroxy compound produced from the hydrolysis of metabolite A had the same R_f values as 12-hydroxyergometrine on both systems of paper chromatography (Table I) and gave a similar purple color with diazotized sulfanilamide.

Ergometrine metabolites C and D when incubated with β-glucuronidase were hydrolyzed to less polar compounds which fluoresced blue in ultraviolet light, gave a blue color with Ehrlich's reagent but did not give a color with diazotized sulfanilamide. When eluted from chromatograms and compared with ergometrine and ergometrinine on systems A, B, and C the hydrolytic products of metabolite C could not be separated from ergometrine, and that formed from ergometrine metabolite D could not be separated from ergometrinine (IV).

(b) **Lysergic Acid Diethylamide.**—Two metabolites which fluoresced blue in ultraviolet light and which gave a blue color with Ehrlich reagent could be detected on chromatograms of the bile extracts. These have been called LSD metabolite A and LSD metabolite B and their R_f values are shown in Table I. From 12 mg. of LSD injected into 12 rats a small amount of crystalline material was isolated from the eluates of each of the metabolite bands of the chromatograms. After only one recrystallization LSD metabolite A had m.p. 322–325° (yield 0.05 mg.) and LSD metabolite B had m.p. 317–320° (yield 0.06 mg.). Ultraviolet spectra of both metabolites showed $\lambda_{max.}$ 315 mμ, $\lambda_{min.}$ 270 mμ (Figure 1).

When tested pharmacologically LSD metabolite A showed 5% of the activity of lysergic acid diethylamide while LSD metabolite B was less than 0.5% as active as lysergic acid diethylamide after 10 min. contact with the uterus.

(1) **Isomerism of LSD Metabolites.**—A solution of LSD metabolite A pre-



× = LSD; ○ = Metabolite A.

Fig. 1.—Ultraviolet absorption spectra of lysergic acid diethylamide (LSD) and LSD *metabolite A*.

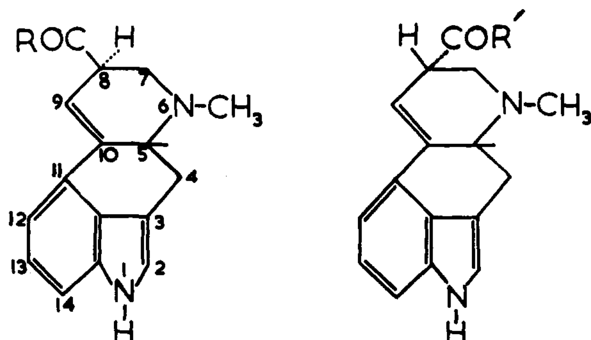
pared by chromatography was dissolved in 5 ml. of phosphate buffer (pH 5.5) and incubated at 36° for 72 hr. After evaporation under nitrogen (reduced pressure) the methanol soluble material was chromatographed on system A and two bands which fluoresced blue in ultraviolet and which gave a blue color with Ehrlich's reagent were observed. On elution these metabolites could not be separated from LSD metabolites A and B using chromatography systems A and B. Using the same method LSD metabolite B was also converted to a mixture of metabolites A and B.

(2) **Hydrolysis of LSD Metabolites with β-Glucuronidase.**—LSD metabolites A and B after treatment with β-glucuronidase were both converted to less polar substances which fluoresced blue in the ultraviolet, gave a blue color with Ehrlich reagent and a distinct purple color with diazotized sulfanilamide. No hydrolysis occurred when the LSD metabolites A and B were incubated with β-glucuronidase in the presence of saccharic acid. The R_f values of the hydrolytic products of the metabolites are shown in Table I.

Discussion

Ergometrine.—The metabolites of ergometrine present in rat bile after doses of 3 mg./kg. (ergometrine metabolites A and B) possess chemical properties which are consistent with hydroxylation of the benzene ring of the indole nucleus having occurred prior to conjugation with glucuronic acid. The inability to separate the hydrolytic products of ergometrine metabolite A from a sample of synthetic 12-hydroxyergometrine by paper chromatography indicates that hydroxylation of the alkaloid has most probably occurred in position 12 which is regarded as a position of high electron density in the indole

nucleus.[17] This conclusion, resting as it does almost entirely on paper chromatographic evidence, will require confirmation from further work. As ergometrine metabolites A and B may each be converted to a mixture of both by treatment with acid or alkali, they are most probably related to one another as are ergometrine and ergometrinine (derivatives of D-lysergic acid and D-isolysergic acid, respectively). Ergometrine metabolite B would therefore be the glucuronide of 12-hydroxyergometrinine.



The substances present in the bile after high doses of ergometrine (45 mg./kg.) present a complex picture. In addition to ergometrine metabolites A and B eight other substances have been revealed on paper chromatograms. Two of these could not be separated from ergometrine and ergometrinine on paper chromatograms, and the remaining six substances could also be divided into three isomeric pairs by their behavior with acid and alkali, suggesting that the members of each pair are derivatives of ergometrine and ergometrinine. Of these only metabolites C and D (Table I) have been examined in detail. These are more polar than ergometrine and since they may be hydrolyzed with β-glucuronidase to give non-phenolic products which could not be separated from ergometrine and ergometrinine it would appear that metabolites C and D are glucuronides of ergometrine and ergometrinine, respectively. The glucuronic acid residues are presumably attached to the propanolamide side chain. Such a conjugation of glucuronic acid with a primary alcohol group is not entirely unexpected as Williams[18] mentions several examples of primary alcohols forming glucuronide ethers particularly at high dose levels. Chloramphenicol is excreted as a glucuronide of the primary al-

(17) R. D. Brown, *Australian J. Chem.*, **12**, 152 (1959).

(18) R. T. Williams, "Detoxification Mechanisms," 2nd Ed., Chapman and Hall, London, 1959, p. 46 *et seq.*

cohol group rather than undergoing hydroxylation in the benzene ring prior to conjugation.[19,20] The remaining ergometrine metabolites E, F, G, and H (Table I) could not be examined further as they were present in very small amounts. They are however less polar than ergometrine and this would indicate that they have probably not undergone hydroxylation. It is suggested without the support of any experimental evidence that these compounds may have been formed by N-demethylation such as occurs in the metabolism of dimethyltryptamine.[21]

Lysergic Acid Diethylamide.—The similarity of the behavior of LSD metabolites A and B on paper chromatography and their interconversion by acids and alkalies suggest that they are derivatives of D-lysergic and D-isolysergic acids. As LSD metabolite A (more polar) blocks the action of 5-hydroxytryptamine on the isolated rat uterus it may be concluded that it is derived from D-lysergic acid whereas the inactive metabolite B (less polar) is a derivative of D-isolysergic acid (III). It should be noted that on the same systems of paper chromatography D-lysergic acid has a higher polarity than D-isolysergic acid. The fact of the hydrolysis of both metabolites by saccharic acid, a specific inhibitor of this enzyme,[22] gives further support to the findings of Boyd[11] that the two important metabolites of LSD are glucuronides. The color reaction by these metabolites with Ehrlich reagent indicates that position 2 in the indole nucleus is unsubstituted and as the hydrolyzed metabolite gives a phenolic test with diazotized sulfanilamide, hydroxylation of the benzene ring of the indole nucleus would appear to have occurred in both LSD metabolites A and B. The position of the hydroxyl group has not been fixed but the ultraviolet spectra of the metabolites indicates that a derivative of lumilysergic acid has not been formed as the spectra of this substance would resemble that of a simple indole. The fact that one metabolite is pharmacologically active also makes this unlikely. It is tentatively suggested that, by analogy with ergometrine, hydroxylation probably has occurred in position 12 of the indole nucleus.

Acknowledgments.—We wish to thank Burroughs Wellcome (Aust.) Ltd., and Merck Sharp & Dohme (Aust.) for grants (M.B.S.) and Dr. H. Hofmann (Sandoz AG, Basle) for making available to us a sample of 12-hydroxyergometrine. We are also grateful to Sandoz

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(20) A. J. Glazko, L. M. Wolf, W. A. Dill and A. C, Bratton, *J. Pharmacol. Exptl. Therap.*, **96**, 445 (1949).

(21) S. Szara and J. Axelrod, *Experientia*, **15**, 216 (1959).

(22) G. A. Levvy, *Biochem. J.*, **52**, 464 (1952).

AG., Basle, and Burroughs Wellcome (Aust.) Ltd., for supplying lysergic acid diethylamide and ergometrine. The pharmacological testing of the LSD metabolites was carried out by Miss J. N. Pennefather, Pharmacology Department, University of Sydney.

The Fate and Excretion of Polythiazide[1] in the Dog

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Received January 4, 1962

Studies on the fate and excretion of polythiazide in the dog using doses in the range of 0.1 mg./kg. indicate that the compound is well absorbed. After either oral or intravenous administration, the drug is completely excreted within 5 days; 80–85% in the urine and 15–20% in the feces. The bulk (60–90%) is excreted in the first 24 hr. The time course of excretion of high doses (100 mg./kg.) is similar, a total of 63% being excreted over 5 days—35–45% in the urine and 20–30% in the feces. The polythiazide is excreted as a mixture of the unchanged drug with up to about 30% of a degradation product, 3-(methylsulfamyl)-4-amino-6-chlorobenzenesulfonamide (II). Another product of the formation of II, S-trifluoroethylthioglycolic acid, has been detected in the urine but the quantitative aspects of its formation and excretion have not been determined.

Several thiazide diuretics are currently in clinical use. Metabolic studies, however, have been reported only on the two simplest compounds, chlorothiazide and hydrochlorothiazide. In the species studied, dogs[2] and humans[3] in the case of chlorothiazide, and rats[4] and humans[5] for hydrochlorothiazide, both drugs proved to be metabolically stable and were completely excreted unchanged within 24 hr. following intravenous administration. Recoveries were somewhat less when the compounds were administered orally. Excretion

(1) Renese®.

(2) J. E. Baer, H. L. Leidy, A. V. Brooks and K. H. Beyer, *J. Pharmacol. Exptl. Therap.*, **125**, 295 (1959).

(3) H. R. Brettell, J. K. Aikawa and G. S. Gordon, *A.M.A. Arch. Internal Med.*, **106**, 57 (1960).

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