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PHYSIOLOGICAL DISPOSITION AND METABOLISM OF H(3) COCAINE.(U)
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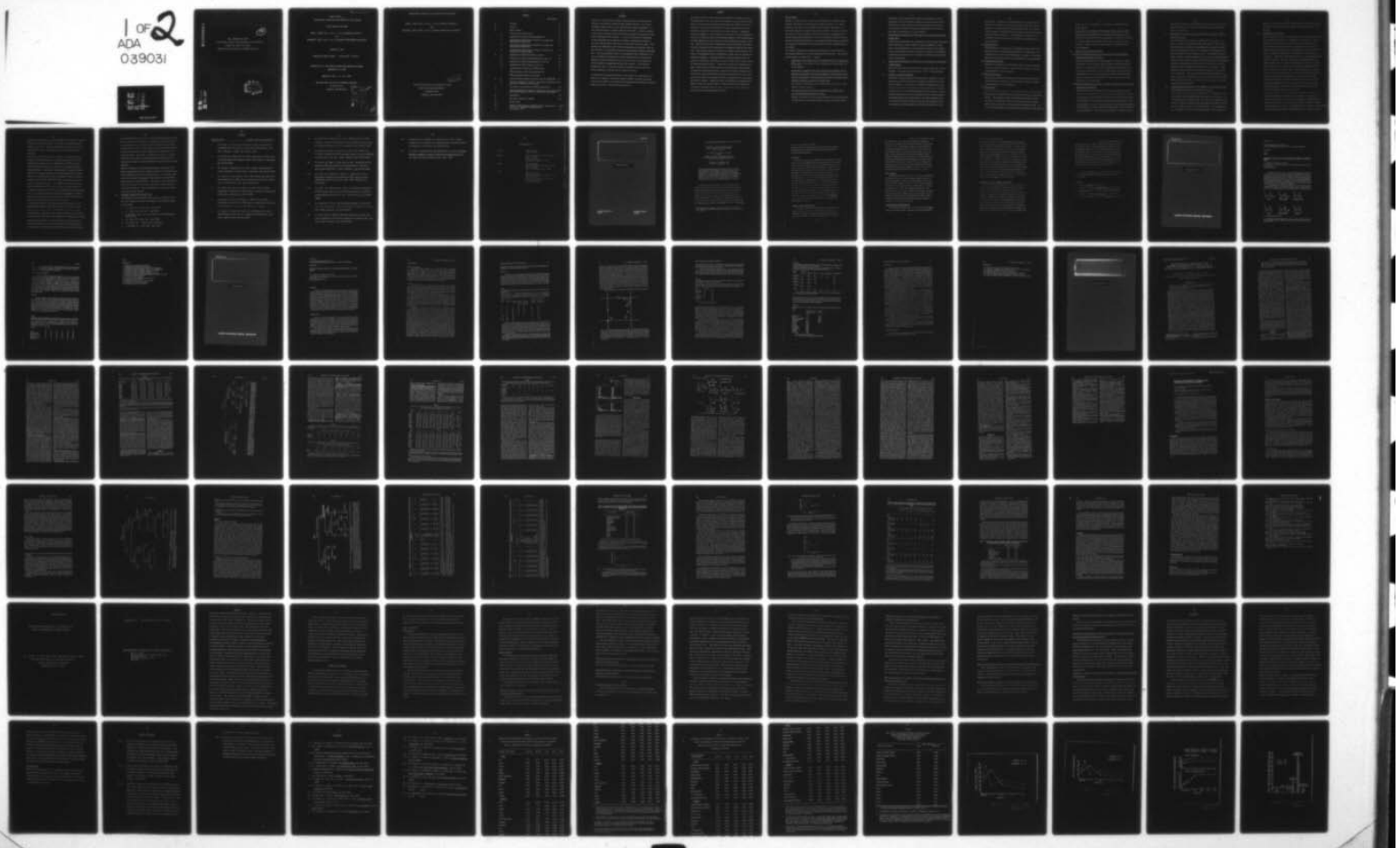

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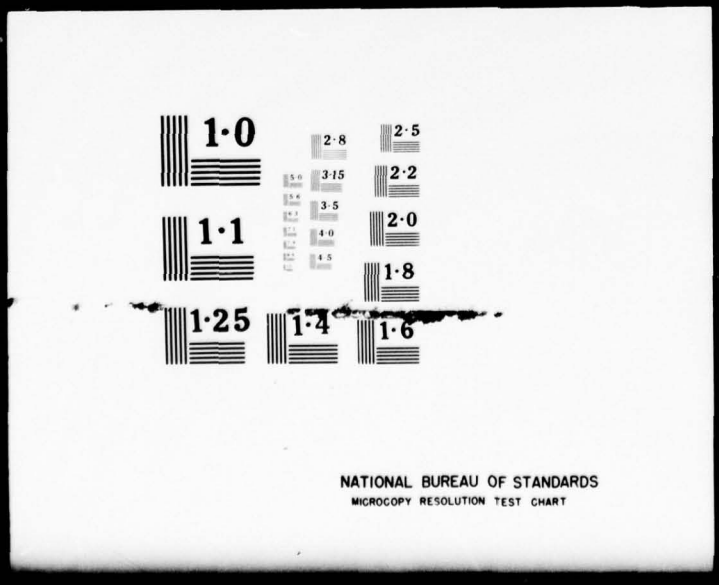

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FINAL COMPREHENSIVE REPORT

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PHYSIOLOGICAL DISPOSITION AND METABOLISM OF [^{3}H] COCAINE

FINAL COMPREHENSIVE REPORT

ANAND L. MISRA, Ph.D., F.R.I.C., F.A.I.C., RESEARCH SCIENTIST V

AND

SALVATORE J. MULE', Ph.D., F.A.I.C., ASSISTANT COMMISSIONER AND DIRECTOR

JANUARY 31, 1977

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PHYSIOLOGICAL DISPOSITION AND METABOLISM OF [^{3}H] COCAINE

Anand L. Misra, Ph.D., F.R.I.C., F.A.I.C., Research Scientist V

and

Salvatore J. Mule', Ph.D., F.A.I.C., Assistant Commissioner & Director

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New York State Office of Drug Abuse Services

Testing and Research Laboratory

80 Hanson Place

Brooklyn, New York 11217

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FOREWORD

Cocaine is a potent central nervous system stimulant of relatively short duration, low margin of safety and high systemic toxicity. Its intense euphoriant action leads to a very high degree of psychic dependence. It has little tolerance or physical dependence liability. Dramatic increases in the abuse of cocaine in the current drug subculture has greatly expanded public awareness of this problem. In view of the paucity of information on the disposition and metabolism of cocaine in experimental animals in acute and chronic states, there was an obvious need for such a study. This project was initiated in April 1973 by the authors and associates with support from the U.S. Army Medical Research and Development Command, Washington, D.C.. With the exception of work on the disposition and metabolism of cocaine in acutely and chronically treated monkeys(which has recently been submitted for publication),the major portion of work in this project has already appeared in appropriate professional journals. This study has provided new insights into the possible mechanism of action of cocaine, reverse tolerance to cocaine and its systemic toxicity.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

SUMMARY

This project deals in depth with the dispositional and metabolic aspects of [^{3}H] cocaine in the rat, dog and the monkey in acute and chronic states with a view to assess the possible relationship and significance of these parameters to the acquired sensitivity (reverse tolerance) and systemic toxicity to cocaine in these species on chronic treatment. Inter alia various metabolites of cocaine observed in the brain of these experimental animals e.g. [^{3}H] norcocaine, [^{3}H] benzoylnorecgonine, [^{3}H] benzoylecgonine and [^{3}H] ecgonine were also prepared and their disposition studied in the rat. Sensitive and specific methodologies for the estimation and identification of cocaine and its metabolites in biological materials were developed. This study has provided evidence that chronic administration of cocaine in subconvulsant doses to experimental animals does not produce dispositional tolerance. Benzoylnorecgonine and benzoylecgonine (but not cocaine or norcocaine) were shown to persist in the central nervous system of these experimental animals. Persistence of these polar metabolites in brain, their potent stimulant activity on intracisternal administration and possible mobilization by these metabolites of neuronal membrane-bound calcium from sites that control transmembrane permeability to sodium ions has been implicated by the authors to play a possible role in the reverse tolerance to cocaine. Comparative study of disposition and metabolism of [^{3}H] ψ-cocaine, the dextro isomer of cocaine in the rat has shown that differential pharmacokinetics of these 2 isomers may account in part for their differential pharmacological effects.

BODY OF REPORT

Cocaine is a potent central nervous system stimulant of relatively short duration, low margin of safety and high systemic toxicity. Its intense euphoric action leads to a very high degree of psychic dependence. It has little tolerance or physical dependence liability. The great potential for abuse, the lack of adequate sensitive and specific methodology to determine submicrogram quantities of cocaine in biological materials and the paucity of detailed information on its biological disposition and metabolism in acutely and chronically-treated experimental animals, prompted the present study.

The rational, objectives and background information on this project have been covered adequately in various publications listed below. The approach to this project was undertaken as follows:

1. Preparation of radiochemically pure randomly-labeled and ring-labeled [^{3}H] cocaine. For details on this subject refer to publication No.1 in the Appendix.

2. Development of suitable thin layer chromatographic methods for the separation of cocaine, some of its metabolites and congeners on Gelman silica gel sheets and development of methods for routine identification of cocaine metabolites in human urine.

   For details on methodology refer to publications No.2 and 3 in the Appendix on these subjects.

3. Physiological disposition and biotransformation of [^{3}H] cocaine in acutely and chronically-treated rats.

   Details on the sensitive and specific methodology for the estimation of [^{3}H] cocaine in biological materials, acute and chronic animal

experiments, tissue distribution, partition coefficients, binding in vitro of cocaine with plasma proteins of acutely and chronically-treated rats, biliary, urinary and fecal excretion of cocaine and its metabolites, metabolic studies and implications of results are described in publication No.4 in the Appendix.

4. Disposition and metabolism of [^{3}H] cocaine in acutely and chronically-treated dogs.

Details on the methodology for estimation of cocaine, acute and chronic animal experiments, identification of cocaine metabolites in dog brain, tissue distribution, urinary and fecal excretion and comparative urinary metabolic profile of cocaine in acutely and chronically-treated dogs and implications of results are described in publication No.5 in the Appendix.

5. Disposition and metabolism of [^{3}H] cocaine in acutely and chronically-treated monkeys.

Details of this study are covered in the enclosed report No.6 in the Appendix. This paper has recently been accepted for publication.

6. Studies on cocaine metabolites

Norcocaine, benzoylnorecgonine, benzoylecgonine, ecgonine have been shown by us to be the metabolites of cocaine in the brain of rats, dogs and monkeys. Norcocaine, the lipophilic metabolite was, like cocaine, a powerful CNS stimulant of short duration and contributes possibly in part to the pharmacological effects of cocaine. It was active by both systemic and intracisternal routes of administration. Benzoylnorecgonine and benzoylecgonine, the polar metabolites possessed potent stimulant activity only by the intracisternal route and ecgonine was pharmacologically inactive by both routes of ad-

ministration. Furthermore, benzoylnorecgonine and benzoylecgonine persisted in the CNS of rats, dogs and monkeys long after the disappearance of cocaine and its metabolite,norcocaine. These observations prompted a thorough study on the dispositional and metabolic profile of these metabolites. Identification of norcocaine as a metabolite of cocaine in rat brain is detailed in publication No.7 in the Appendix.

6.1 [^{3}H] Norcocaine

[^{3}H] Norcocaine was prepared by acid-catalysed exchange tritium labeling procedure and exhaustively purified in our laboratory. Its disposition in rat brain was studied after a 2 mg/kg i.v. injection. Details of these studies appear in publication No.8 in the Appendix.

6.2 [^{3}H] Benzoylnorecgonine

Preparation, estimation and disposition of this cocaine metabolite in the rat after a 10 mg/kg i.v. dose has been discussed in detail in publication No.9 in the Appendix.

6.3 [^{3}H] Benzoylecgonine

Preparation, estimation and disposition of this cocaine metabolite in the rat after a 10 mg/kg i.v. dose has been discussed in detail in publication No.10 in the Appendix.

6.4 [^{3}H] Ecgonine

Part of the tritiated ecgonine used for the synthesis of ring-labeled [^{3}H] cocaine was exhaustively purified by ITLC for use in disposition studies. The amphoteric nature and unfavourable partition characteristics of ecgonine have hampered the development of a suitable method for its estimation in the tissues and biological fluids. Details on the preparation and physiological disposition of [^{3}H] ecgonine

in the rat after a 10 mg/kg i.v. dose appear in publication No.11 in the Appendix.

7. Intracellular disposition of [^{3}H] cocaine, [^{3}H] norcocaine, [^{3}H] benzoylecgonine and [^{3}H] benzoylnorecgonine in the brain of rats.

Intracellular disposition of these compounds was studied in fractions of rat brain obtained by discontinuous sucrose density gradient procedure. Details of these studies appear in publication No.12 in the Appendix.

8. Other work on cocaine metabolites

8.1 Calcium binding property of cocaine and some of its metabolites - formation of molecular complexes.

Cocaine, benzoylnorecgonine, benzoylecgonine, norcocaine, ecgonine methyl ester but not ecgonine formed distinct molecular complexes with Ca^{2+}. Complexation with Ca^{2+} and possible mobilization of neuronal membrane-bound Ca^{2+} from sites that control the transmembrane permeability to Na+ may conceivably play a role in the excitatory action of cocaine and its metabolites. For details refer to publication No.13 in the Appendix.

8.2 Molecular complexes of cocaine, its active metabolites and some other stimulants with thiamine.

Thiamine and its phosphorylated esters appear to play an important role in nerve conduction and excitation at the molecular level, quite independently of their coenzymatic activity and have been implicated in transmembrane ion transport involving permeability changes at the sodium channel. Local anesthetics reportedly block nerve conduction by disorganizing the membrane around the sodium channel and blocking the sodium conductance. These observations prompted a study of possible

interaction of the active metabolites of cocaine and some otehr stimulants with thiamine. Details of this work appear in publication No.14 in the Appendix.

9. Disposition and metabolism of the dextro isomer of cocaine, [^{3}H] ψ-cocaine in the rat.

The toxicity and local anesthetic activity of the C_2-epimer of cocaine, ψ-cocaine is several-fold higher than cocaine. Both isomers have convulsant and paralysant properties, but ψ-cocaine is several times less potent than cocaine as an inhibitor of norepinephine uptake in ventricles and vas deferens slices. Differential effects of these 2 isomers on the EEG activities of the brain, cardiorespiratory functions, daily temporal pattern of self-administration and behavioral responses during drug-access sessions in the monkey have also been reported by the Neuropharmacology Group in our laboratory. Little information exists on the disposition and metabolism of the dextro isomer of cocaine. This investigation was therefore undertaken, as these parameters may possibly play a role in part in the differential effects of these 2 isomers. A report on this work appears in publication No.8 in the Appendix.

10. Neuropharmacological studies on the tolerance to the convulsant and cardiorespiratory effects of cocaine in the monkey.

These studies were principally conducted by Dr. M. Matsuzaki and his associates of the Neuropharmacology Laboratory in our Institute. It has been shown that repeated daily i.v. injections of cocaine in the monkey at a minimal convulsant dose (MCD) produced tolerance resulting in a marked increase in the MCD of cocaine. The repeated daily i.v. injections of cocaine at subconvulsant doses (2-4 mg/kg) also

produced a marked decrease in the cardiorespiratory stimulating effects. A detailed report on this subject appears in publication No.15 in the Appendix.

11. CONCLUSIONS OF THIS STUDY

Cocaine was shown to be very rapidly metabolised in all 3 species (rat, dog and monkey) studied by us. Norcocaine, benzoylecgonine, benzoylnorecgonine and ecgonine were observed to be the metabolites of cocaine in the brain of these species. Conversion of cocaine to norcocaine could be demonstrated in the CNS within a few minutes of the onset of convulsions following i.v. injection of a convulsant dose of cocaine. Norcocaine could contribute in part to the pharmacological effects of cocaine. The polar metabolites, benzoylnorecgonine and benzoylecgonine but not ecgonine,possessed potent stimulant activity only on intracisternal injection and were pharmacologically inactive when injected by the systemic route. These metabolites were shown to cross the blood-brain barrier only in small amounts but metabolism of cocaine and norcocaine in situ in the brain could lead to the formation of significant amounts of these metabolites. The longer $t_{\frac{1}{2}}$ of these metabolites in the CNS of experimental animals implies a slower clearance and accounts for their persistence long after the disappearance of cocaine and norcocaine.

The sequestration of cocaine in substantial amounts observed in the fat of the chronically treated rats for prolonged periods was not observed in the dog and the monkey. No significant differences were observed in the plasma-protein binding of cocaine in normal, acutely and chronically-treated rats. Norcocaine (in small amounts), benzoylecgonine (major), benzoylnorecgonine, ecgonine methyl ester, ecgonine and nor-

ecgonine were identified as urinary metabolites of cocaine in acutely and chronically-treated animals. The nature of unidentified metabolites is not known. Chronic administration of cocaine in subconvulsant doses to dogs and monkeys did not produce dispositional tolerance.

On the basis of this study, it was possible to speculate that persistence of benzoylnorecgonine and benzoylecgonine in the CNS and molecular complexation of these metabolites with Ca^{2+} may play a role in acquired sensitivity to cocaine in experimental animals on chronic treatment. Alteration or displacement of neuronal membrane-bound Ca^{2+} by benzoylnorecgonine and benzoylecgonine could conceivably cause a conformational change in membrane proteins, leading to changes in permeability to Na^{+} and K^{+} ions, depolarization of membrane and excitatory effects. Other alternative mechanisms (a) involving a reduction in Ca^{2+} binding sites on membrane protein and consequent interference with release of norepinephrine and acetylcholine, or (b) disorganization or expansion of hydrophobic regions of membrane, energy transfer changes by high affinity binding of lipophilic cocaine and norcocaine may also be involved.

An additional finding of this study was the demonstration of non-covalent intermolecular interaction of cocaine, its active metabolites and some other stimulants with thiamine, which has been reported to play a biophysical role in nerve conduction and excitation at the molecular level quite independently of its coenzymatic activity.

The question of tolerance or reverse tolerance to cocaine on repetitive administration in experimental animals remains unresolved. In contrast to the findings of our disposition and metabolism study,

the electrophysiological work by our Neuropharmacology Group has shown that cocaine given by constant i.v. infusion to the monkey produced a progressive increase in daily minimal convulsant dose on chronic administration and also a marked reduction in its stimulating effects on heart and respiratory rates following daily injections of sub-convulsant doses. Such studies underscore the need for a rigorous delineation of particular responses measured and limiting of conclusions on tolerance or reverse tolerance to cocaine solely to those responses.

Efforts to demonstrate the oxidation in vivo of norcocaine to its highly reactive nitroxyl free radical in rats after norcocaine or cocaine injection have not been successful so far. The failure could be due to the reduction in vivo of such radicals to corresponding norcompounds by membrane-SH group. Nevertheless, the involvement of such a free radical in the systemic toxicity of cocaine remains an attractive possibility. These conclusions are summarized in publication No.16 in the Appendix.

12. PERSONNEL PARTICIPATION IN THIS STUDY

In addition to the co-principal investigators, A.L. Misra and S.J. Mule', the following personnel participated in the different phases of this study.

1. P.K. Nayak, Ph.D. (April 1973 - April 1975)
2. V. V. Giri, Ph.D. (May 1974 - June 1976)
3. M. Matsuzaki, Ph.D., M.D. of our staff in the Neuropharmacological study
4. M.N. Patel, M.S. (April 1973 - June 1976)
5. V.R. Alluri, M.S. (August 1974 - June 1976)
6. V. Subbarow, M.S. (Nov. 1973 - Aug. 1974)

APPENDIX

Publication No.: Authors, Title and Journal Ref.

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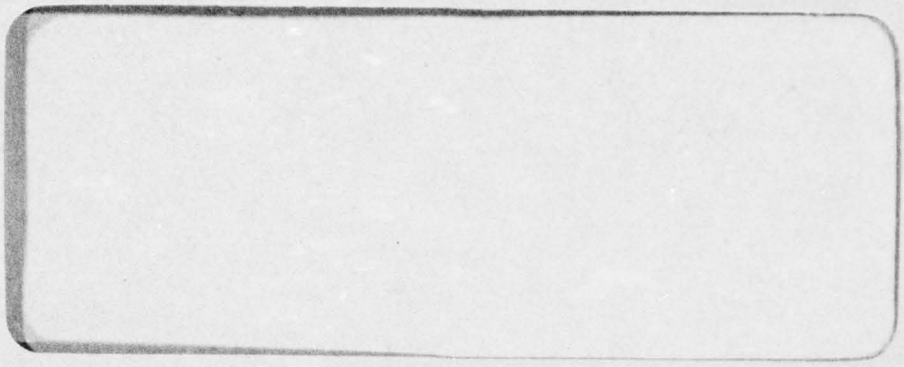

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PREPARATION OF RADIOCHEMICALLY PURE RANDOMLY LABELED AND RING LABELED [^{3}H] - COCAINE

P.K.Nayak, A.L.Misra*, M.N.Patel, S.J.Mule'

State of New York Drug Abuse Control Commission,Testing and Research Laboratory Brooklyn, N.Y. 11217, U.S.A.

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Accepted 6 December 1973

Acid catalysed-exchange tritium labeling of cocaine and subsequent purification furnished randomly labeled [^{3}H]-Cocaine of 98 % isotopic puritiy and specific activity 630 µCi/mg. Similar tritiation of ecgonine, esterification, benzoylation and exhaustive purification provided ring labeled [^{3}H]- Cocaine of 99 % isotopic purity and specific activity 48 µCi/mg. Both products were suitable for use in tracer studies on the biological disposition and biotransformation of cocaine.

In connection with the study on the physiological disposition and metabolism of cocaine in acute and chronically-treated experimental animals, need arose for tritium labeled cocaine of high specific activity. The preparation of ^{14}C-labeled cocaine with the label on the N-methyl, benzoyl and methyl ester groups[1,2] and ^{3}H-label on the benzoyl moiety[3] and in random position[4] has previously been reported. Radiolabeling in these positions however, has drawbacks in view of the *in vivo*

* Correspondence and request for reprints to: Dr.A.L.Misra DACC Testing and Research Laboratory 80 Hanson Place Brooklyn, N.Y. 11217

biologic lability of these positions.In this report we describe the preparation of radiochemically pure randomly labeled and ring labeled [^{3}H]-Cocaine.

EXPERIMENTAL

The melting points of the compounds were determined on Fisher-Johns melting point apparatus and are uncorrected. Baker analyzed silica gel /40-140 mesh/ was used for column chromatography and Gelman instant thin layer chromatography media, silica gel /ITLC/ was obtained from Gelman Instrument Company, Ann Arbor, Michigan. Radioscans of chromatograms were prepared by sectioning 1 cm planimetric strips of ITLC, transferring them to counting vials, adding methanol /0.5 ml/ and toluene phosphor solution /10 ml/ and determining the radioactivity in a liquid scintillation spectrometer. Cocaine base /50 mg, 1.7×10^{-4} mol/ and ecgonine /250 mg, 1.4×10^{-3} mol/ were tritiated /New England Nuclear Lab., Boston, Mass./ in solutions of glacial acetic acid in the presence of a platinum catalyst /25 mg/ and ^3H_2O /10 curies/ by stirring the reaction mixture overnight at 50°C. After filtration of the catalyst, labile tritium was removed **in vacuo** and the crude [^{3}H] -Cocaine residue taken up in methanol and [^{3}H]-ecgonine in water.

Randomly labeled [^{3}H] -Cocaine

The methanol solution of [^{3}H] -Cocaine was mixed with non-labeled cocaine /150 mg, 5.1×10^{-4} mol/ and the solution evaporated to dryness in a stream of nitrogen. The residue dissolved in chloroform /3 ml/ was chromatographed on a silica

gel column /15 x 1 cm/ and cocaine eluted with chloroform /100 ml/. The residue from the chloroform eluate was dissolved in 1N HCl /3 ml/, centrifuged to remove any insoluble matter and the clear solution basified to pH 9.5 with 1N NH_4OH. The precipitated cocaine was extracted using benzene /15 ml, four times/ and the benzene extract dried over anhydrous sodium sulfate. Residue obtained on evaporation of benzene extract was repeatedly recrystallized from isopropanol to a constant specific activity /630 μCi/mg/, yield, 176 mg. The chemical and isotopic purity /98 %/ of cocaine were checked by ITLC using benzene-ethyl acetate-conc. ammonia /85:15:0.1, v/v/, R_f 0.82 and n-butanol-acetic acid-water[5] /35:3:10, v/v/, R_f 0.92.

[^{3}H] -Ecgonine

Non-labeled ecgonine /75 mg, 4.1 x 10^{-4} mol/, was added to the aqueous solution of crude tritiated ecgonine and the solution lyophilized, yielding a yellowish colored residue /250 mg/. Crude [^{3}H] -ecgonine /125 mg, 7 x 10^{-4} mol/ was dissolved in methanol /3 ml/ and purified by chromatography on a silica gel column /20 x 2.5 cm/. The column was eluted first with methanol /100 ml, four times/ and then with methanol containing 2 % by volume of conc. ammonia /100 ml, three times/. The residues from these eluates were dissolved in a small volume of methanol to remove the non alkaloidal inorganic impurities /yield 85 mg, 4.6 x 10^{-4} mol, 68 %/.

Esterification of [^{3}H] -Ecgonine

[^{3}H] -Ecgonine /85 mg, 4.6 x 10^{-4} mol/ was dried *in vacuo* over P_2O_5 at 55°C overnight, dissolved in anhydrous methanol

/10 ml/ containing 10 % w/v of dry hydrochloric acid gas and the solution refluxed in a paraffin bath at 110°C for 2 hr. The residue obtained on evaporation of solvent in a stream of nitgen was triturated with acetone /10 ml/ and the precipitated [^{3}H] -Ecgonine methyl ester hydrochloride removed by centrifugation and dried *in vacuo* /79 mg, 3.4×10^{-4} mol, 73 %/. TLC using benzene-ethyl acetate-methanol-con. ammonia /70:10:1:0.1, v/v/ established the chemical purity of the compound /a single spot R_f 0.72, coincidental to authentic ecgonine methyl ester/. The hydrochloride dissolved in water /4 ml/ was adjusted to pH-9.5 with 1N NH_4OH, saturated with NaCl and the [^{3}H] -ecgonine methyl ester extracted using ethyl acetate-isopropanol /9:1, v/v/ /15 ml, four times/. Evaporation of organic phase furnished the yellowish oily methyl ester /61 mg, 3.1×10^{-4} mol/ which was dried *in vacuo* at 50°C over Drierite.

Benzoylation of [^{3}H] -Ecgonine methyl ester

[^{3}H] -Ecgonine methyl ester /61 mg., 3.1×10^{-4} mol/ dissolved in anhydrous benzene /3 ml/ was refluxed with three times its weight of benzoic anhydride at 110°C for 4 hr. A further reflux cycle for 2 hr. using same amount of benzoic anhydride was necessary for complete benzoylation. The residue obtained on evaporation of benzene in nitrogen was dissolved in 0.5N HCl / 3 ml/ and the precipitated benzoic acid removed by extraction with ether /15 ml, three times/. The aqueous phase was adjusted to pH 9.5 with 1N NH_4OH and [^{3}H] -cocaine extracted with benzene and recrystallized from isopropanol as previously described /84 mg, 2.9×10^{-4}, 90 %/. Although chem-

ically pure /m.p. 96 - 98°C/ by ITLC[5], [^{3}H] -Cocaine had only 75 % isotopic purity. Final purification was effected on preparative ITLC sheets /20 x 20 cm/ with benzene-ethyl acetate-conc. ammonia /85:15:0.1, v/v/ using 10 mg [^{3}H] -Cocaine per sheet as a streak. The cocaine band was eluted with methanol, the residue dissolved in dil. HCl and basified to pH 9.5 with dil. ammonia and cocaine extracted and recrystallized as described before. The isotopic purity of [^{3}H] -Cocaine thus obtained was 99 % and the specific activity 48 µCi/mg.

x

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Note

Separation of cocaine, some of its metabolites and congeners on glass fibre sheets

A. L. MISRA*, R. B. PONTANI and S. J. MULÉ

New York State Narcotic Addiction Control Commission, Testing and Research Laboratory, Brooklyn, N.Y. 11217 (U.S.A.)

(Received March 26th, 1973)

During the course of our study on the physiological disposition and metabolism of tritium ring labeled cocaine in experimental animals, need arose for the development of sensitive methods for the separation of labeled cocaine, some of its metabolites and congeners (Fig. 1). Paper[1–3], thin-layer[4,5] and gas chromatographic[6] methods have earlier been reported for the separation of cocaine from its metabolites, benzoyl ecgonine and ecgonine[7–11]. Pulmonary excretion of labeled carbon dioxide in experimental animals following administration of cocaine-N-$^{14}CH_3$ has provided suggestive evidence[12] for the formation of norecgonine.

This communication describes the application of glass fibre silica gel impregnated sheets (ITLC) to the separation of cocaine, some of its metabolites and congeners. This technique possesses the advantages of speed, convenience and ease

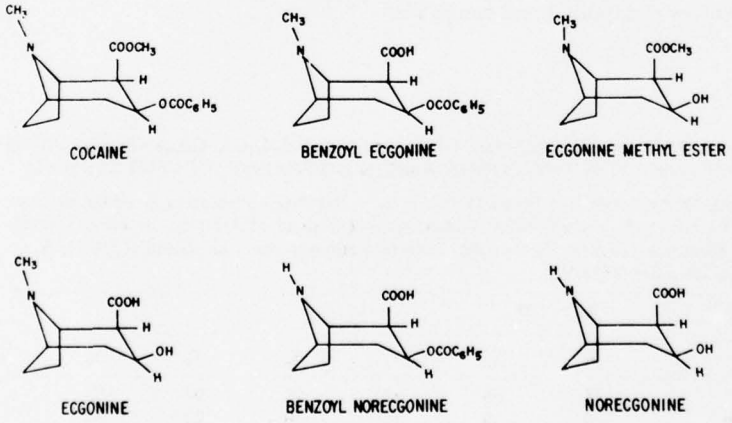

Fig. 1. Cocaine and related compounds.

* Correspondence should be addressed to: Dr. Anand L. Misra, New York State Narcotic Addiction Control Commission, Testing and Research Laboratory, 80 Hanson Place, Brooklyn, N.Y. 11217, U.S.A.

of radioscanning by direct transfer of sectioned planimetric strips of silica gel–glass fibre sheets to counting vials, addition of eluant and toluene–phosphor and assay of radioactivity in a liquid scintillation spectrometer.

MATERIALS AND METHODS

Cocaine was obtained commercially (Merck & Co., Inc., N.J.), ecgonine[13], ecgonine methyl ester[14] were prepared by methods previously described. Benzoyl ecgonine and benzoyl norecgonine were a kind gift from Dr. E. L. May, N.I.H., Bethesda, Md., and norecgonine was prepared from benzoyl norecgonine by modified acid hydrolysis procedure[13]. Gelman instant thin-layer chromatography media, silica gel (ITLC), obtained from Gelman Instrument Company, Ann Arbor, Mich., was used for TLC with application of standard techniques. The compounds were localized after development by spraying with (a) acidified iodoplatinate reagent and (b) Ludy Tenger's reagent[15] prepared fresh by dissolving 500 mg of bismuth carbonate in 1.5 ml of conc. HCl, adding 3 g of KI and making the volume to 50 ml with distilled water.

RESULTS

The results obtained with the mobilities of cocaine and related compounds on ITLC are given in Table I. Cocaine and ecgonine methyl ester could be separated from other compounds with solvent system S_1, and could be separated from each other with system S_2. Benzoyl norecgonine and norecgonine separated well from benzoyl ecgonine and ecgonine in systems S_3, S_4 and S_5. The compounds with higher mobility in system S_6, *e.g.* cocaine, benzoyl ecgonine and norcompounds, could be separated from slower moving polar conjugated *in vivo* metabolites in this system. Combinations of systems S_1, S_2, S_4, S_5, S_6, therefore could adequately separate cocaine, some of its metabolites and congeners.

TABLE I

CHROMATOGRAPHIC MOBILITIES ON GELMAN ITLC (SILICA GEL) OF COCAINE, SOME OF ITS METABOLITES AND CONGENERS IN DIFFERENT SOLVENT SYSTEMS

S_1 = Chloroform–acetone–conc. ammonia (5:94:1); S_2 = benzene–ethyl acetate–methanol–conc. ammonia (80:20:1.2:0.1); S_3 = chloroform–acetone–diethylamine (5:4:1); S_4 = ethyl acetate–methanol–conc. ammonia (17:2:1); S_5 = ethyl acetate–methanol–conc. ammonia (15:4:1); S_6 = *n*-butanol–acetic acid–water (35:3:10).

Compound	$R_F \times 100$					
	S_1	S_2	S_3	S_4	S_5	S_6
Cocaine	98	98	98	98	98	83
Benzoyl ecgonine	0	0	29	50	73	88
Ecgonine methyl ester	98	64	98	98	98	66
Ecgonine	0	0	33	11	28	48
Benzoyl norecgonine	0	0	12	30	46	95
Norecgonine	0	0	12	4	15	75

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ROUTINE IDENTIFICATION OF COCAINE METABOLITES IN HUMAN URINE

M. L. BASTOS, D. JUKOFSKY and S. J. MULÉ

New York State Narcotic Addiction Control Commission, Testing and Research Laboratory, 80 Hanson Place, Brooklyn, N.Y. 11217 (U.S.A.)

(Received September 11th, 1973)

SUMMARY

A method was developed whereby the major metabolites of cocaine (ecgonine and benzoylecgonine) were extracted from human urine, butylated and subsequently isolated and identified on Polygram silica gel sheets. A comparison of the butylated ecgonine thin-layer chromatographic technique (BETT) with the enzyme multiplied immunoassay technique (EMIT) for benzoylecgonine indicated complete agreement on the presence or absence of cocaine metabolites in 88% of the human urine samples analyzed. Disagreement on the positive urine samples was ascribed to the lower level of sensitivity of BETT (3–5 μg/ml of ecgonine or benzoylecgonine) in comparison to EMIT (1 μg/ml benzoylecgonine). Of the urine samples positive for the cocaine metabolites 18% were negative for other drugs of abuse and 71% were positive for methadone. Cross-reactivity studies with EMIT indicated a high degree of specificity for benzoylecgonine. The BETT and EMIT assays were found to be highly reliable, valid and sensitive tests for the presence of cocaine metabolites in human urine.

INTRODUCTION

Cocaine is extensively metabolized[1–3] and, therefore, little free cocaine is available for detection in urine. Apparently, the major metabolites[1] of this drug are benzoylecgonine and ecgonine. Unfortunately, these biotransformation products are water-soluble and thus not easily extracted by organic solvents, nor are they extracted readily by ion-exchange resins or absorbed on styrene-divinylbenzene copolymers (*i.e.*, XAD-2). For these reasons, cocaine, an apparently widely abused drug, is seldom detected in urinalysis screening programs for drugs subject to abuse.

This communication describes a method for extracting cocaine and its metabolites from human urine, the butylation of these metabolites, their thin-layer chromatographic detection, and subsequent comparison with the enzyme multiplied immunoassay technique for benzoylecgonine.

EXPERIMENTAL

Materials and methods

All chemicals were of reagent grade. Cocaine was obtained commercially from Merck and Co. (Rahway, N.J., U.S.A.). Ecgonine and benzoylecgonine were prepared in our laboratory from cocaine[4]. Benzoylecgonine and benzoylnorecgonine were also obtained as gifts from Dr. E. L. May, National Institute of Health, and Dr. R. J. Bastiani of the Syva Corporation. The urine samples were obtained from NACC treatment and rehabilitation centers as well as from the Syva Corporation. The assay for benzoylecgonine by the enzyme multiplied immunoassay technique (EMIT) was performed as suggested by the manufacturer[5].

Procedure

To 10 ml of urine in a 16 × 150 mm test tube about 250 mg of solid sodium bicarbonate is added to adjust the pH between 8–9. Five milliliters of a chloroform–ethanol (3:2) mixture are added, followed by mixing with a Genie Vortex for about 20 sec. After centrifuging the sample at about 1100 *g* for about 10 min, the upper aqueous phase is transferred to a clean test tube. The remaining organic layer contains unmetabolized cocaine and/or other extractable organic bases.

To the ethanolic aqueous layer containing the cocaine metabolites sufficient solid anhydrous potassium carbonate for saturation is added. The sample is mixed thoroughly using the Genie Vortex and allowed to stand for a few minutes. A dark brown suspension floats to the surface of the tube. It is then centrifuged at 1100 *g* for 10 min to separate the alcohol phase and subsequently transferred to a 40-ml centrifuge tube with a Pasteur pipette. One drop of 6 *N* HCl is added to the ethanolic extract and the pH is determined. If the pH is not below 7, additional 6 *N* HCl should be added.

To butylate the extracted cocaine metabolites 2 ml of 1-butanol and 0.1 ml of concentrated H_2SO_4 are added to the ethanolic extract and heated for 30 min in a glycerin bath maintained at 120–130°. The solution is allowed to cool to room temperature, 5 ml of toluene are added and the entire mixture is transferred to a clean test tube. The centrifuge tube is washed with 5 ml of water and the wash added to the toluene–butanol mixture. The mixture is shaken for 10 sec, centrifuged at 275 *g* for 5 min and the upper organic layer aspirated. The remaining aqueous layer is saturated with solid sodium bicarbonate and extracted with 10 ml of cyclohexane. The cyclohexane is separated from the aqueous layer by centrifugation at 275 *g* for 5 min. The organic phase is transferred to a clean tube and evaporated to dryness in a water-bath. The residue is dissolved in about 150 μl of chloroform and applied to a 0.25-mm Polygram silica gel sheet made by Macherey, Nagel & Co., Düren, G.F.R. (supplied by Brinkmann Instruments, Westbury, N.Y., U.S.A.). The sheets are developed in various solvent systems (see Table I), however, the 1 + 7 system is most commonly used. This solvent system combination (1 + 7) is also used for two-dimensional chromatography. Following development the chromatographic sheet is removed from the tank, air dried for 30 min, or placed in an oven at 90° for 5 min. The thin-layer sheet is sprayed with the iodoplatinate reagent[5], which reacts with butylated ecgonine to provide a bluish purple spot on a light background and with butylated benzoylecgonine or benzoyl-

norecgonine to provide a purple-brown spot. For optimum color development one should spray once and then respray 5 min later.

RESULTS

In Table I R_F values and sensitivity data are listed for the butylated cocaine derivatives and R_F values for morphine. Using a single solvent system it was difficult to separate morphine from butylated ecgonine, or the butylated compound was located close to the solvent front, associated with urinary extractable impurities, thus making detection difficult. A single solvent system was effective provided morphine was not present in the sample. Solvent systems No. 5 and No. 6 were acceptable, however, some interference from extractable substances was observed. All other

TABLE I

R_F VALUES AND SENSITIVITY LIMITS FOR DETECTION OF BUTYLATED COCAINE DERIVATIVES

Solvent systems: No. 1 = ethyl acetate–methanol–water (7:2:1), No. 2 = ethyl acetate–methanol–ammonia (15:4:1), No. 3 = chloroform–acetone–diethylamine (5:4:1), No. 4 = chloroform–acetone–ammonia (5:4, saturated), No. 5 = methanol–ammonia (100:1.5), No. 6 = benzene–ethyl acetate–methanol–ammonia (80:20:1.2:0.1), and No. 7 = chloroform–acetone–ammonia (5:94:1).

Solvent system No.	$R_F \times 100$*				*Sensitivity (μg/ml of urine)***	
	Morphine	*Butylated ecgonine*	*Butylated benzoyl-ecgonine*	*Butylated benzoyl-norecgonine*	*Butylated ecgonine*	*Butylated benzoylecgonine*
1	7	8	45	49	3	3–5
2	31	82	92	78	10	15
3	7	84	91	79	20	40
4	12	87	85	87	10	30
5	34	52	64	58	5	15
6	0	14	32	7	5	3
7	9	78	75	64	10	40
1+2	36	87	—	—	3	3–5
1+3	20	92	—	—	3	5
1+4	19	88	—	—	3	3–5
1+5	44	55	—	—	3	3–5
1+7	23	83	—	—	3	3

* Morphine was applied directly to the thin-layer chromatographic sheets (10–15 μg). The cocaine derivatives (ecgonine, benzoylecgonine and benzoylnorecgonine) were dissolved in either water or ethanol–water (95:5) in concentrations of 1 mg/ml and then butylated and extracted as described under Methods. The compounds were detected by spraying the sheets with the iodoplatinate reagent, which provided blue to purple color reactions. When two solvent systems were used the R_F was relative to the second solvent front and no R_F value was obtained when the derivatives were located above the second solvent front. Thus, the R_F value for these compounds would be the same as for the single solvent system No. 1, since the second solvent system was only allowed to travel a distance of 4–5 cm from the origin.

** Ecgonine and benzoylecgonine were added to urine in concentrations of 1–100 μg/ml and carried through the entire extraction and butylation procedure as described under Methods. Each value represents the minimum concentration detectable. A low level of detection (10–40 μg/ml) was due to extractable substances that interfered with the color reactions for the drugs in the various solvent systems.

single solvent systems were not considered effective for the detection of the cocaine derivatives. A double solvent system was used to separate morphine from butylated ecgonine. This separation is important since morphine glucuronide is extracted by the method described above and subsequently hydrolyzed to free morphine during the butylation procedure. However, little free morphine is finally extracted into cyclohexane. The solvent system combination 1 + 7 was found to be the most useful for the isolation and subsequent detection of butylated ecgonine. The R_F values for butylated benzoylecgonine and butylated benzoylnorecgonine with the double solvent system technique were the same as for the single solvent system No. 1, since these compounds were located above the second solvent system front. The sensitivity for detection of butylated ecgonine and benzoylecgonine varied from 3 to 40 μg/ml of urine. The greatest sensitivity was observed with solvent system No. 1 and the least with solvent systems No. 3 and No. 4. Using the double solvent system, of course, did not change sensitivity (3–5 μg/ml) since these values were related to the extraction and solvent system No. 1.

The butylated derivatives of ecgonine and benzoylecgonine may be effectively

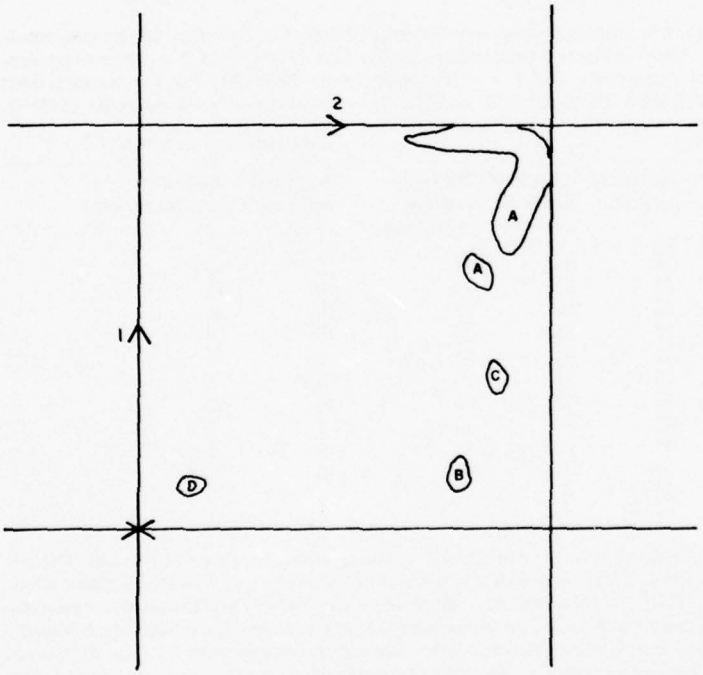

Fig. 1. Urine samples containing ecgonine, benzoylecgonine (10 μg/ml each) and control urines (no drug) were carried through the entire procedure as described under Methods. The butylated extracts as well as free morphine (10 μg) were applied at the origin and the chromatogram was developed in two different solvent systems (two-dimensional chromatography). The solvent system used for the first dimension was ethyl acetate–methanol–water (7:2:1) (Solvent system No. 1) and that used for the second dimension was chloroform–acetone–ammonia (5:94:1) (Solvent system No. 7). A = Urinary impurities; B = butylated ecgonine; C = butylated benzoylecgonine; D = morphine; X = origin.

separated from morphine and urinary impurities through the use of two-dimensional chromatography (Fig. 1). This technique, however, is limited and time consuming for the routine urinalysis of thousands of samples.

A total of 259 human urine samples analyzed for benzoylecgonine by EMIT were analyzed by the butylated ecgonine thin-layer chromatographic technique (BETT). The data (Table II) indicate that 88.0% of the samples that were either positive or negative by EMIT were confirmed by BETT and 11.9% were not. The largest

TABLE II

COMPARISON OF THE EMIT ASSAY FOR BENZOYLECGONINE WITH THE BETT ANALYSIS IN 259 HUMAN URINE SAMPLES

BETT is performed as described under Methods and EMIT as described by Syva Co., Palo Alto, Calif.[4]. About 3000 urine samples were screened by EMIT and those positive for benzoylecgonine as well as a random selection of negative samples were subsequently analyzed by BETT.

*Results**	*Urine samples*	*%*
True positive	170	65.6
True negative	58	22.4
Total	228	88.0
False positive	26	10.0
False negative	5	1.9
Total	31	11.9

* All values are in reference to BETT.

percentage of false determinations were false positives (10.0%) *vs.* 1.9% for false negatives. Of the false positives 80.8% (21 samples) were at concentration levels in urine of 1–2 μg/ml of benzoylecgonine and 19.2% (5 samples) were between 2–3.5 μg/ml. The maximum sensitivity level for BETT was found to be 3–5 μg/ml (Table I). It is quite possible, therefore, that almost all the false positive samples were labeled so because of the limiting sensitivity of the confirmation technique (BETT). The false negatives may be ascribed to possible technician error in the application of either or both the EMIT and/or BETT analysis.

It was of interest to ascertain whether other drugs subject to abuse were present in the urine samples analyzed for cocaine. These data appear in Table III. Methadone was present in 71.0% of the urine samples truly positive for cocaine usage (confirmed by BETT) and in 76.0% of those samples unconfirmed by BETT (false positives). Morphine was present in 4.7% of the cocaine-positive urines, and quinine in 14.2%. Of the total samples (257) analyzed for cocaine metabolites, 21.4% were negative for other drugs subject to abuse, 69.6% contained methadone, 12.5% quinine, and 3.1% each were positive for morphine, barbiturates, and a miscellaneous group of drugs.

Many drugs chemically similar to derivatives of cocaine (Table IV) as well as those drugs commonly abused were tested for cross-reactivity in the EMIT assay for benzoylecgonine. Ecgonine was the only compound that provided a reasonable cross-reactivity, equivalent to 9.5 μg/ml of benzoylecgonine (relative reactivity of 0.105).

TABLE III

OTHER DRUGS OF ABUSE DETECTED IN 257 URINE SAMPLES ANALYZED FOR COCAINE METABOLITES BY EMIT AND BETT

The analysis for drugs other than cocaine was performed as described previously[6,7]. The data are presented both as whole numbers and percentages of the cocaine results.

Cocaine results	*Other drugs*					
	Negative	*Methadone*	*Quinine*	*Morphine*	*Barbiturates*	*Miscellaneous*[*]
True positive (169)	31 18.3%	120 71.0%	24 14.2%	8 4.7%	8 4.7%	5 3.0%
True negative (58)	16 27.6%	38 65.5%	6 10.3%	—	—	1 1.7%
False positive (25)	5 20.0%	19 76.0%	2 8.0%	—	—	2 8.0%
False negative (5)	3 60.0%	2 40.0%	—	—	—	—
Total (257)	55 21.4%	179 69.6%	32 12.5%	8 3.1%	8 3.1%	8 3.1%

[*] This includes the following: *d*-methorphan, chlorpromazine (Thorazine), phenylpropanolamine, *d*-amphetamine, and thioridazine (Mellaril).

Cocaine cross-reactivity in this assay was 460 μg/ml, a relative reactivity ratio of 0.002. The methyl ester of ecgonine and benzoylnorecgonine cross-reacted at concentrations of 380 and 335 μg/ml, respectively. All other compounds did not cross-react at concentrations ranging from 500 to 1000 μg/ml.

TABLE IV

CROSS-REACTIVITY OF VARIOUS DRUGS IN THE EMIT ASSAY FOR BENZOYLECGONINE

Drug	*Equivalent to 1.0 μg/ml of benzoylecgonine (μg/ml)*	*Relative reactivity*[*]
Benzoylecgonine	1.0	1.000
Ecgonine	9.5	0.105
Methyl ester of ecgonine	380	0.003
Benzoylnorecgonine	335	0.003
Norecgonine	NR_{1000}[**]	0.001
Cocaine	460	0.002
Atropine	NR_{500}	—
Nicotine	NR_{500}	—
Homatropine	NR_{500}	—
Scopolamine	NR_{500}	—
d-Amphetamine	NR_{500}	—
Morphine	NR_{500}	—
Methadone	NR_{500}	—
Secobarbital	NR_{500}	—

[*] All values relative to benzoylecgonine.

[**] NR = No cross-reactivity at the concentration indicated.

DISCUSSION

Cocaine apparently is a widely abused psychoactive drug that has been extremely difficult to detect on routine urine screening because of the extensive biotransformation of this drug. We therefore developed a method whereby ecgonine as well as benzoylecgonine could be extracted from biological material (urine) and butylated with subsequent isolation and detection on a thin-layer chromatogram. Because of extractable urinary substances and the possible presence of morphine, a double solvent system (1 + 7, Table I) proved to be most effective for the identification of butylated ecgonine. Two-dimensional chromatography, of course, may also be effectively used in detecting the butylated cocaine derivatives. Morphine present in the final extract was derived from morphine glucuronide, extractable with ecgonine and congeners and subsequently acid hydrolyzed to free morphine in the butylation procedure. Even though relatively little free morphine is extracted into cyclohexane, it is necessary to separate morphine (heroin metabolite) from the cocaine metabolites since heroin addicts quite commonly use cocaine.

A comparison of the EMIT assay for benzoylecgonine and the BETT assay on 259 urine samples indicated the reliability and validity of these tests. Although BETT confirmed only 88.0% of the EMIT tests, further inspection of the data revealed that all the false positive samples were in the concentration range of 3.5 μg/ml or less of benzoylecgonine. The sensitivity limits for the BETT assay were determined at 3–5 μg/ml, thus it appears that the false positives were simply due to the sensitivity limits of the confirmation assay (BETT). With the EMIT assay false positives may also be due to native lysozyme activity in the urine. This may be easily determined by analyzing the urine in the absence of antibody and the enzyme–drug complex (*i.e.* no reagent). The false negatives (1.9%) appear to be due primarily to technical error.

It is interesting to note that a high percentage of the samples positive for cocaine were also positive for methadone (71%) and that only 18% of the cocaine-positive samples were negative for other drugs. Quinine was present in 14% of the cocaine-positive samples and morphine was confirmed in 5%. Barbiturates and miscellaneous groups of drugs were present in 3 to 5% of the true positive cocaine samples. The data are indicative of the fact that cocaine is generally not used alone, but in conjunction with other drugs, especially heroin and methadone.

The EMIT assay for benzoylecgonine appears to be quite specific. All the drugs tested, with the exception of ecgonine, cross-reacted in the EMIT assay, at concentrations greater than 300 μg/ml benzoylecgonine equivalents.

It appears that the EMIT and BETT assays are highly reliable, valid and relatively sensitive tests for the determination of cocaine metabolites in human urine. These tests may be used independently to detect cocaine abuse, but preferably in combination as complimentary confirming tests.

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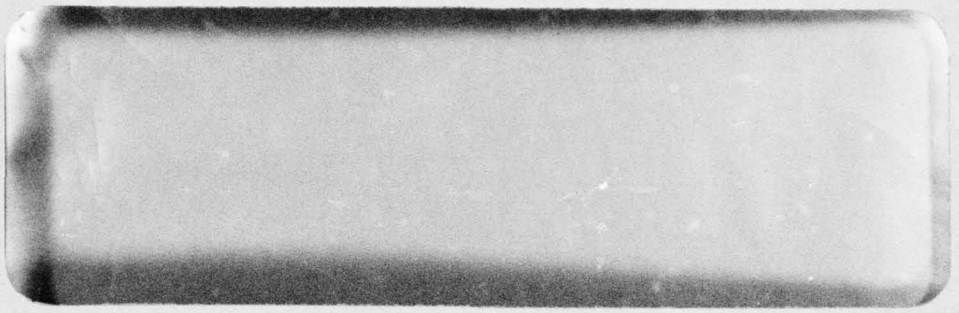

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PHYSIOLOGICAL DISPOSITION AND BIOTRANSFORMATION OF [^{3}H]COCAINE IN ACUTELY AND CHRONICALLY TREATED RATS[1, 2]

P. K. NAYAK, A. L. MISRA AND S. J. MULÉ

New York State Drug Abuse Control Commission, Testing and Research Laboratory, Brooklyn, New York

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ABSTRACT

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A sensitive method was developed for the estimation of [^{3}H]cocaine in biological materials. After an injection of 8 mg/kg i.v. in male Wistar rats, peak levels in brain, tissues and plasma occurred within 15 minutes and cocaine disappeared completely from brain and plasma 6 hours postinjection. The $T_{\frac{1}{2}}$ of cocaine in brain and plasma was 0.4 and 0.3 hour, respectively. No significant differences were observed in the rates of disappearance of cocaine from the subcutaneous site in acute and chronically treated rats after an injection of 20 mg/kg. After a 20 mg/kg s.c. dose, the peak levels of cocaine were attained gradually in 4 hours in brain, tissues and plasma with the exception of heart (0.5 hour) and fat (2 hours). These peak levels shifted from 4 to 2 hours in the chronically treated group. Consistently higher levels of cocaine were found to be sequestered in fat in the chronically treated animals. The $T_{\frac{1}{2}}$ of cocaine in brain and plasma of chronically treated rats was approximately 1.8 to 2 hours and that in the acutely treated animals, 0.8 to 1 hour. The brain/plasma ratios were also somewhat higher in chronically treated as compared to the acutely treated animals and were indicative of a high affinity of tissue for cocaine. Although cocaine did not persist in brains of acutely treated animals, measurable amounts were shown to persist in brain and other tissues of chronically treated animals long after the disappearance in plasma. Significantly high concentrations of metabolites of cocaine persisted in brain and plasma of acutely and chronically treated animals. No significant differences were observed in the plasma protein binding of cocaine in control, acutely and chronically treated rats. Unchanged cocaine was excreted in very small amounts in rat bile and approximately 36% of the dose (5 mg/kg i.v.) was excreted as metabolites 3.5 hours after injection. Excretion of free cocaine in urine and feces after a 20 mg/kg s.c. dose in acutely and chronically treated rats was 1.2 and 1.5%, respectively. Significantly higher excretion of total radioactivity occurred in feces in the chronic group (35.9%) as compared to the acute group (22.1%). Benzoylecgonine, benzoylnorecgonine, ecgonine methyl ester and ecgonine were identified as urinary metabolites in both acute and chronic animals. In addition, evidence was obtained for the presence of a phenolic

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Send reprint requests to: Dr. A. L. Misra, DACC Testing and Research Laboratory, 80 Hanson Place, Brooklyn, N.Y. 11217.

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metabolite and two other hydroxylated metabolites (with hydroxylation presumably in positions 6 and 7 of the pyrrolidine ring). Implications of these observations with respect to systemic toxicity, the absence of tolerance and physical dependence liability of cocaine are discussed.

The recent increase in the abuse of cocaine by the current drug subculture has prompted us to investigate the physiological disposition and metabolism of [^{3}H]cocaine in acutely and chronically treated rats. Cocaine (pK_a 8.70) is a potent central nervous system stimulant of relatively short duration, low margin of safety and high systemic toxicity (LD50 i.v. in rats, 17.5 mg/kg; fatal dose i.p. in rats 100 mg/kg). Its intense euphoric action leads to a high degree of psychic dependence (Tatum and Seevers, 1929; Downs and Eddy, 1932; Eddy *et al.*, 1965; Deneau *et al.*, 1969; Seevers, 1973) but physical dependence and tolerance to its euphoric and toxic effects have not been reported. Despite many reported studies (Oelkers and Raetz, 1933; Oelkers, 1933; Oelkers and Vincke, 1935; McIntyre, 1936; Langecker and Lewitt, 1938; Woods *et al.*, 1951; Sanchez, 1957; Ortiz, 1966; Werner, 1961; *Montesinos*, 1965; Fish and Wilson, 1969), there is still little knowledge of cocaine pharmacodynamics, and the basic mechanism of psychic dependence on cocaine remains unknown. Earlier methods used for the estimation of cocaine (Woods *et al.*, 1951; Gettler and Sunshine, 1951) lack adequate sensitivity and specificity to determine submicrogram quantities of this compound in biological materials, particularly in the central nervous system. Our recent studies (Misra *et al.*, 1974b) have provided evidence for the presence of norcocaine, benzoylecgonine, benzoylnorecgonine and ecgonine as metabolites of cocaine in rat brain.

In view of the great potential for the abuse of cocaine and the paucity of detailed information on its biological disposition, we describe in this communication a sensitive method for its estimation in biological materials and physiological disposition and metabolism of cocaine in acutely and chronically treated rats.

Methods

[^{3}H]cocaine (randomly labeled[3] and ring-labeled[3]) was prepared by a method described previously (Navak *et al.*, 1974). The radioactive material was diluted with nonradioactive cocaine to provide a specific activity of 12 μc/mg. Although cocaine was injected as its hydrochloride, all doses are expressed as the free base. Gelman instant thin-layer chromatography media (silica gel) (ITLC), was used for all thin-layer chromatography experiments. Radioscanning of chromatograms was performed by previous methods (Misra *et al.*, 1973a,1974c).

Standard samples of benzoylecgonine and ecgonine methyl ester were prepared by the method of Findlay (1954); benzoylnorecgonine and norcocaine by the method of Schmidt and Werner (1962); and ecgonine by the method of Bell and Archer (1960).

Estimation of [^{3}H]cocaine in biological materials. Two-milliliter aliquots of diluted urine or plasma (1:5) or tissue homogenate (10% in 0.5 N HCl, w/v) in duplicate were transferred to 40-ml centrifuge tubes containing 1 ml of nonradioactive cocaine hydrochloride as carrier (500 μg/ml as free base), the pH of the solution was adjusted to 9 by the addition of 1 ml of 1.5 N ammonia and the solution was buffered with 1 ml of 20% (w/v) K_2HPO_4 solution. Fifteen milliliters of cyclohexane were added and the mixture was shaken in an Eberbach shaker for 15 minutes. After centrifugation for 10 minutes, the aqueous layer was aspirated and the organic phase was vigorously shaken for 1 minute[4] with 4 ml of 0.1 N NaOH in a vibromixer (Vortex-Genie Scientific Industries, Springfield, Mass.). The solution was centrifuged for 10 minutes, the aqueous phase was aspirated and 10 ml of the organic phase were transferred to counting vials and evaporated at 45–50°C on a Fisher slide warmer. The residue was dissolved in 0.5 ml of isopropanol, 10 ml of toluene–phosphor were added and radioactivity was determined in a liquid scintillation spectrometer (Nuclear-Chicago Mark III). No quenching of radioactivity was observed in the cyclohexane extracts of urine, plasma and tissue extracts. Controls for background were run concurrently through the extraction procedure each time and these, along with sealed tritium standards, served as a check on the extraction technique and counting efficiency (approximately 60% for tritium). Background counts

[3] Distribution of [^{3}H]radioactivity in randomly labeled cocaine prepared by direct tritiation of cocaine has been shown (Werner and Schmidt, 1969) to be 28% in the ecgonine skeleton, 65% in the benzene ring and 7% in the OCH_3 group. Ring-labeled cocaine prepared by synthesis from [^{3}H]ecgonine has all the [^{3}H]radioactivity in the ring skeleton.

[4] Vigorous shaking for 1 minute with 0.1 N NaOH in the vibromixer was found to be sufficient for removal of small amounts of contaminating metabolites without affecting the recoveries of [^{3}H]cocaine.

for tritium were in the range of 18–20 cpm and the control plasma, urine and tissue extracts gave values in the same range. Consistent checks in counts for duplicate samples of extracted biofluids and tissues (each counted three times) and consistently higher absolute counts per minute (minus background counts per minute) for tissue extracts at later time periods showed that radioactivity could have cone only from [^{3}H]cocaine injected. Other details on the calibration curves for calculation of concentrations of cocaine in tissues and fluids, total radioactivity in urine and fecal samples have been reported previously (Misra *et al.*, 1973a, 1974a). *In vitro* recoveries of cocaine in the concentration range of 10 to 1000 ng/ml from diluted urine and plasma were 100 ± 1; from brain, liver and kidney homogenates 99 ± 0.5, 98 ± 0.5 and 99 ± 1 (S.E.M.), respectively.

Extraction of [^{3}H]cocaine (5–10 ng/ml) *in vitro* from diluted urine, plasma and brain homogenates in the presence of a concentration of 5 to 10 μg of nonlabeled cocaine as carrier (instead of 500 μg) as described above, evaporation of the organic solvent extract, dissolution of the residue in a small volume of methanol and ITLC in different solvent systems (Misra *et al.*, 1973b) demonstrated a single peak of radioactivity coincidental with the cocaine spot obtained by iodoplatinate spray. A further purification of the residue from the cyclohexane extract in the assay procedure was done for the identification of cocaine. The residue from the cyclohexane extract was dissolved in 0.1 N HCl, the aqueous acidic solution was extracted with cyclohexane and the organic phase discarded. The aqueous acid phase was adjusted to pH 8 to 9 with 1 N NH_4OH and re-extracted with cyclohexane. The residue from this cyclohexane extract was dissolved in a small volume of methanol and cochromatographed with 5 to 10 μg of nonlabeled cocaine to provide additional confirmation of the identity of extracted cocaine.

Specificity of the extraction procedure. The specificity of the above procedure was evaluated by extraction of urine, plasma or tissue homogenate of rats, injected subcutaneously with a 20 mg/kg dose of [^{3}H]cocaine, using thin-layer chromatography of the organic solvent extract and radioscanning of the chromatograms (Misra *et al.*, 1973a). The polar metabolites benzoylecgonine, benzoylnorecgonine and ecgonine were not extracted in significant quantities (less than 1%). Norcocaine, a highly lipophilic compound which has been shown (Misra *et al.*, 1974b, Misra *et al.*, 1975) to be a pharmacologically active metabolite of cocaine in rat brain, was quantitatively extracted by this procedure. Thin-layer chromatography of extracts of brains of rats injected with a 20 mg/kg subcutaneous dose of [^{3}H]cocaine and removed 0.5, 2, 4 and 6 hours after injection showed that norcocaine constituted 8 to 12% of the total values of cocaine in brain in the acute experiments and approximately 10 to 20% of those in brain in the chronically treated rats at these times (table 4). After an intravenous injection of 8 mg/kg of [^{3}H]cocaine in the rats, the contribution of norcocaine (from radioscans of ITLC of extracts of brains of rats removed 0.25, 1 and 2 hours after injection) to the total values of cocaine in brain (table 1) was approximately 15%. Norcocaine was not demonstrated in other tissues, possibly due to its further biotransformation to benzoylnorecgonine and norecgonine and was not observed to be a urinary metabolite in the rat. The solvent systems which adequately separated norcocaine and cocaine on ITLC (silica gel) were as follows: *n*-hexane–ethyl acetate–concentrated ammonia (60:40:0.1 v/v), R_f norcocaine and cocaine 0.72 and 0.96, respectively; *n*-hexane–dioxane–concentrated ammonia (90:10:0.3 v/v), R_f norcocaine and cocaine 0.43 and 0.86, respectively; *n*-heptane–dioxane–concentrated ammonia (90:10:0.3 v/v), R_f norcocaine and cocaine 0.69 and 0.92, respectively.

The amount of cocaine remaining at the site of injection after a subcutaneous administration was determined by cutting out a 1-inch square piece of skin and subcutaneous connective tissue surrounding the site of injection and homogenizing in 0.5 N HCl to give a 20% homogenate and extracting the sample as described previously.

Acute animals experiments. Male Wistar rats weighing 110 to 160 g were injected intravenously with an 8 mg/kg dose of [^{3}H]cocaine. The subcutaneous dose (20 mg/kg) was injected in the dorsal area above the right hind limb. After appropriate periods of time, the rats were lightly anesthetized with ether, and blood was drawn into heparin-treated Vacutainer tubes (Becton Dickinson & Co., Rutherford, N.J.) by cardiac punture. The plasma was immediately separated by centrifugation and quickly frozen. Brains were removed within 3 to 5 minutes after anesthetization, rinsed thoroughly with ice-cold 0.9% saline, blotted with Kimwipes tissue paper, weighed, wrapped in aluminum foil and stored frozen until analyzed. Known weights of tissue were homogenized in 0.5 N HCl to provide a 10% homogenate, and 2-ml aliquots in duplicate were analyzed as previously described. Plasma samples were diluted 1:5 with distilled water and similarly analyzed.

The urine and feces were collected in metabolism cages over a period of 1 week after injection. Diluted samples (1:5) of urine or fecal homogenates in 0.5 N HCl were analyzed in duplicate as described previously.

Chronic animal experiments. Rats were given s.c. injections of 20 mg/kg of nonlabeled cocaine twice daily for 3 weeks, then the same dose of [^{3}H]cocaine twice daily for 2 days before the terminal injection of same dose of labeled cocaine (five total labeled cocaine injections were administered). Blood and tissue samples were collected at various time intervals after the last injection.

Apparent partition coefficient [^{3}H]cocaine in

TABLE 1

Distribution of cocaine[a] in various tissues of male Wistar rats after a single intravenous injection of 8 mg/kg (free base) of [^{3}H]cocaine

Tissue or Fluid	0.25 Hr	0.5 Hr	1 Hr	2 Hr	4 Hr
Brain[b]	7.27 ± 0.18	2.74 ± 0.63	0.92 ± 0.13	0.21 ± 0.05	0.01 ± 0
Spleen	12.84 ± 0.86	7.34 ± 0.91	3.62 ± 0.43	0.52 ± 0.15	0.06 ± 0.01
Kidney	10.81 ± 0.67	3.46 ± 1.21	1.39 ± 0.34	0.37 ± 0.07	0.08 ± 0
Lungs	8.80 ± 0.37	3.77 ± 1.14	1.46 ± 0.25	0.51 ± 0.12	0.10 ± 0.01
Testes	6.75 ± 0.36	4.78 ± 0.66	3.64 ± 0.78	0.90 ± 0.17	0.05 ± 0.01
Intestine	6.71 ± 0.56	3.01 ± 0.61	1.31 ± 0.27	0.45 ± 0.18	0.03 ± 0.01
Liver	3.42 ± 0.64	1.07 ± 0.15	0.81 ± 0.05	0.29 ± 0.04	0.12 ± 0.01
Fat	3.48 ± 0.15	2.30 ± 0.59	1.63[c]	0.21 ± 0.05	0.01 ± 0
Muscle	2.98 ± 0.10	1.32 ± 0.25	0.59 ± 0.02	0.16 ± 0.03	0.01 ± 0
Heart	2.76 ± 0.23	1.22 ± 0.25	0.44 ± 0.08	0.12 ± 0.03	0.01 ± 0
Plasma	0.61 ± 0.06	0.30 ± 0.09	0.11 ± 0.01	0.03 ± 0.01	0

[a] Data represent the mean value ± S.E.M. (micrograms per gram of wet tissue weight or milliliters of fluid) of three animals at each time period.

[b] Norcocaine constituted approximately 15% of the total values expressed as cocaine in the rat brain at different times. As mentioned in the section on specificity of extraction procedure, norcocaine was not present in other tissues possibly due to its rapid biotransformation to the polar metabolites benzoylnorecgonine (and norecgonine).

[c] This value represents the mean from duplicate determinations from one animal only. Samples from the other two animals were lost in analysis.

1-octanol/0.1 M phosphate buffer, pH 7.4. The partition coefficient was determined at concentrations ranging between 0.1 and 1 μg/ml in buffer at ambient temperature by essentially the same procedure described previously (Misra *et al.*, 1974c).

Binding *in vitro* of [^{3}H]cocaine with plasma proteins from acute[5] and chronically treated[5] rats. Two-milliliter aliquots of plasma containing known concentrations of [^{3}H]cocaine in the range 0.1 to 1 μg/ml were transferred to cellophane bags and ultrafiltered by centrifugation using Amicon ultrafiltration supports (Amicon Corporation, Lexington, Mass.). Radioactivity was determined in 0.1-ml aliquots of solution before ultrafiltration and in filtrates using 10 ml of Aquasol solution (New England Nuclear Corporation, Boston, Mass.).

Biliary excretion of [^{3}H]cocaine and its metabolites in the rat. Male Wistar rats (180–350 g) were anesthetized by an intraperitoneal injection of a 50 mg/kg dose of sodium pentobarbital, the abdominal cavity was opened by midline incision and the biliary duct was cannulated using a 24-gauge hypodermic needle attached to a 10-inch length of polythene tubing (Dow Corning, Silastic brand, 0.03-inch internal diameter and 0.065-inch outer diameter). Prior to the intravenous injection of [^{3}H]cocaine (5 mg/kg), a blank sample of bile (0.3–0.5 ml) was collected over a period of 30 minutes. After the injection of drug, bile samples (0.3–0.5 ml) were collected every 30 minutes over a period of 2 to 3.5 hours. These samples were diluted with water to a measured volume and aliquots were analyzed for free drug and total radioactivity by methods described above.

Metabolic studies. Pooled urine collected in metabolism cages from five rats each injected with 20 mg/kg s.c. of [^{3}H]cocaine was filtered through a plug of glass wool and repeatedly passed through a column of Amberlite XAD-2 (300 × 25 mm). The column was washed with 100 ml of distilled water, unadsorbed radioactivity and waterwashings were combined and lyophilized to a residue and designated as fraction 1. The adsorbed metabolites of cocaine were eluted with methanol until the eluant showed little or no radioactivity. The eluate was concentrated to a residue under reduced pressure and designated as fraction 2. These two fractions were resolved into different metabolites by sequential thin-layer chromatography as shown in the flow chart (fig. 1). Cochromatography with authentic samples in different solvent systems (Misra *et al.*, 1973b) established the identity of cocaine, benzoylecgonine, benzoylnorecgonine, ecgonine methyl ester and ecgonine. Enzymic hydrolysis experiments with β-glucuronidase and peptidase on unidentified polar metabolites provided suggestive evidence of the absence of conjugation in these polar metabolites.

Results

Acute experiments. The results on the distribution of [^{3}H]cocaine after a single 8 mg/kg

[5] Blood was collected in heparin-treated vacutainer tubes by cardiac puncture 4 hours after s.c. injection of 20 mg/kg of nonlabeled cocaine in acutely treated animals and 2 hours after injection in chronically treated rats and was immediately centrifuged to separate the plasma.

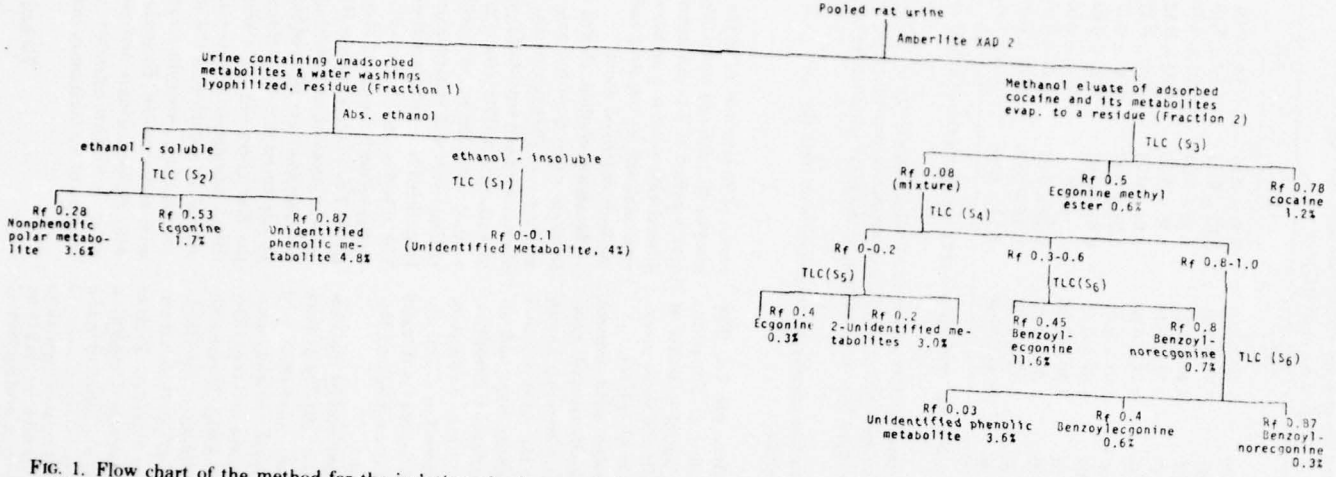

FIG. 1. Flow chart of the method for the isolation of urinary metabolites of cocaine after a single injection of a 20 mg/kg s.c. dose in male Wistar rats. The approximate excretion of radioactivity as a percentage of the dose in a 24-hour pooled urine sample from five rats was 36. The solvent systems used for the separation of different fractions by thin-layer chromatography were S_1, benzene–ethyl acetate–methanol–concentrated ammonia (20:40:40:2 v/v); S_2, benzene–ethyl acetate–methanol–ammonia (10:50:40:1 v/v); S_3, *n*-hexane–dioxane–ammonia (93:7:0.3 v/v); S_4, benzene–ethyl acetate–methanol–ammonia (40:45:15:1 v/v); S_5, ethyl acetate–methanol–dioxane–ammonia (40:40:20:1 v/v); S_6, *n*-butanol–ethyl acetate–dioxane–acetic acid–water (45:40:10:3:5 v/v).

intravenous injection in male Wistar rats appear in table 1. The peak levels of cocaine in brain, plasma and other tissues occurred within 0.25 hour. The highest concentrations of [^{3}H]cocaine were observed in the spleen followed by kidney, lungs, brain and testes. Concentrations lower than these were attained in liver and fat. Muscle, heart and plasma had still lower levels of cocaine. These levels declined sharply and the levels of cocaine 4 hours after injection were very low in brain and absent from plasma and completely disappeared from brain, heart, muscle, testes and fat 6 hours after administration. The values in spleen, kidney, lungs, intestine and liver 6 hours after i.v. injection were 0.05, 0.06, 0.09, 0.03 and 0.09 μg/g, respectively. No persistence of cocaine was observed in rat brain with this dose. The approximate half-lives of [^{3}H]cocaine in rat brain and plasma were found to be 0.4 and 0.3 hour, respectively.

After a convulsive dose of cocaine (10 mg/kg i.v.) in the rat, the levels of cocaine in brain and plasma were 35 and 5 μg/ml, respectively, within 1 to 2 minutes of injection. The formation of norcocaine as a metabolite of cocaine in rat brain within 1 to 2 minutes after a convulsive dose of cocaine (10 mg/kg i.v.) has been described (Misra *et al.*, 1974b).

Apparent partition coefficient of [^{3}H]cocaine in 1-octanol/0.1 M phosphate buffer, pH 7.4. The apparent partition coefficient of cocaine in the concentration range of 0.1 to 1 μg/ml was 7.6 ± 0.1 (S.E.M.).

Binding *in vitro* of [^{3}H]cocaine with plasma proteins from control, acutely and chronically treated rats. The values of mean percentage binding ± S.E.M. with plasma proteins from control, acutely and chronically treated rats at ambient temperature (25°C) were 33.4 ± 0.8, 38.6 ± 5.1 and 35.4 ± 0.3, respectively.

Biliary excretion of [^{3}H]cocaine in the rat. The biliary excretion of [^{3}H]cocaine and its metabolites after a 5 mg/kg dose by intravenous injection in male Wistar rats appears in table 2. Unchanged cocaine was excreted in the bile in very small amounts and only 0.04% of the dose was excreted in 3.5 hours. The excretion of the total amount of radioactivity (comprising metabolites of cocaine) was high at 0.5 and 0.5 to 1 hour collection and gradually declined thereafter. The mean percentage of the total amount of radioactivity excreted in bile within 3.5 hours was 36.0% of the administered dose.

Comparative rates of disappearance of [^{3}H]cocaine from s.c. site in acutely and chronically treated rats after a 20 mg/kg s.c. injection. Data on the comparative rates of disappearance of cocaine are given in table 3. No statistically significant differences were ob-

TABLE 2

Biliary excretion of [^{3}H]cocaine and its metabolites after a 5 mg/kg dose (i.v.) in male Wistar rats

Data represent mean values of determinations from two animals. The experiment was terminated 3.5 hours after injection.

	Biliary Excretion as a Percentage of Dose at Various Time Intervals							Total
	0–0.5 Hr	0.5–1 Hr	1–1.5 Hr	1.5–2 Hr	2–2.5 Hr	2.5–3 Hr	3–3.5 Hr	
Free cocaine	0.01	0.02	0.01	traces	traces	traces	traces	0.04
Total radioactivity	10.3	12.1	5.0	3.7	2.8	1.1	1.0	36.0

TABLE 3

Disappearance of cocaine[a] from the injection site after a 20 mg/kg (free base) subcutaneous injection of [^{3}H]cocaine in acutely and chronically treated[b] male Wistar rats

	Mean Percentage of Administered Dose Remaining at the Injection Site						
	0.5 Hr	1 Hr	2 Hr	4 Hr	6 Hr	12 Hr	24 Hr
Acute	31.8 ± 2.2	27.7 ± 8.2	13.3 ± 1.8	8.8 ± 1.8	0.6 ± 0.3	0	
Chronic	40.6 ± 5.7	20.8 ± 2.5	16.7 ± 1.6	6.5 ± 1.3	3.0 ± 1.0	0.2 ± 0.0	0

[a] Data represent the mean value ± S.E.M. of five animals at each time period.

[b] These animals were given a s.c. injection of 20 mg/kg of cocaine twice daily for 21 days, then with same dose of [^{3}H]cocaine twice daily for 2 days before the terminal injection of labeled drug.

served in the relative rates of disappearance of cocaine in the two groups.

Comparative distribution of [^{3}H]cocaine in acutely and chronically treated rats. Data on the comparative distribution of [^{3}H]cocaine in acutely and chronically treated rats after a 20 mg/kg s.c. injection appear in table 4. In acute animals, a characteristic pattern of a gradual increase in cocaine concentration to a peak at 4 hours was observed for plasma, brain and most tissues with the exception of heart. Peak levels in heart occurred within 0.5 hour and in fat 2 hours after injection. Metabolism of cocaine by liver resulted in lower levels in this tissue. An abrupt fall in the levels of cocaine occurred after 4 and 6 hours resulting in very low levels of cocaine in brain, testes and plasma at 12 hours and the drug was absent at 24 hours. High tissue and low plasma levels showed that cocaine possessed a marked affinity for the tissues examined.

The peak levels of cocaine in brain and plasma of chronically treated rats occurred between 1 to 2 hours after injection. A shift in peak tissue levels of cocaine from 4 to 2 hours and plasma levels from 4 to 1 hour occurred in

TABLE 4

Distribution of cocaine[a] in various tissues of acutely and chronically treated[b] male Wistar rats after subcutaneous injection of 20 mg/kg (free base) of [^{3}H]cocaine

Tissue or Fluid	Acute						
	0.5 Hr	1 Hr	2 Hr	4 Hr	6 Hr	12 Hr	24 Hr
Brain	2.24 ± 0.28	2.32 ± 0.21	2.96 ± 0.20	3.44 ± 0.36	0.49 ± 0.19	BD[c]	0
Kidney	1.88 ± 0.29	4.33 ± 0.25	5.20 ± 0.48	6.46 ± 0.38	0.77 ± 0.35	0.08 ± 0	0.05 ± 0
Lung	1.42 ± 0.30	4.53 ± 0.38	4.32 ± 0.27	6.35 ± 0.83	1.15 ± 0.44	0.09 ± 0	0.04 ± 0
Spleen	4.34 ± 0.84	2.72 ± 0.24	3.88 ± 0.44	4.32 ± 0.37	0.72 ± 0.27	0.13 ± 0.01	0.05 ± 0
Fat	1.56 ± 0.17	1.62 ± 0.23	3.23 ± 0.64	2.53 ± 0.19	0.43 ± 0.10	0.07 ± 0.01	0.03 ± 0.01
Testes	1.37 ± 0.25	1.98 ± 0.25	2.99 ± 0.21	3.85 ± 0.33	1.17 ± 0.28	BD	0
Intestine	2.09 ± 0.29	2.25 ± 0.52	2.87 ± 0.39	3.40 ± 0.38	0.56 ± 0.19	0.02 ± 0	0.01 ± 0
Muscle	0.68 ± 0.10	0.98 ± 0.06	1.34 ± 0.18	1.48 ± 0.19	0.26 ± 0.08	0.02 ± 0	BD
Liver	0.49 ± 0.23	0.73 ± 0.09	0.93 ± 0.19	1.35 ± 0.22	0.27 ± 0.07	0.11 ± 0.01	0.06 ± 0
Heart	3.67 ± 0.54	1.47 ± 0.18	1.25 ± 0.10	1.06 ± 0.12	0.28 ± 0.11	0.01 ± 0	0.01 ± 0
Plasma	0.25 ± 0.05	0.38 ± 0.03	0.45 ± 0.07	0.49 ± 0.06	0.08 ± 0.04	BD	0
Tissue or Fluid	Chronic						
	0.5 Hr	1 Hr	2 Hr	4 Hr	6 Hr	12 Hr	24 Hr
Brain[d]	1.31 ± 0.16	2.41 ± 0.27	3.05 ± 0.14	1.79 ± 0.22	0.49 ± 0.05	0.08 ± 0.03	0.03 ± 0
Kidney	2.21 ± 0.27	4.72 ± 0.66	5.59 ± 0.40	3.32 ± 0.42	1.30 ± 0.10	0.48 ± 0.03	0.42 ± 0.01
Lung	2.47 ± 0.45	6.85 ± 1.80	5.84 ± 0.68	3.29 ± 0.41	1.21 ± 0.05	0.43 ± 0.12	0.22 ± 0.01
Spleen	2.86 ± 0.34	5.22 ± 0.49	5.36 ± 0.37	4.02 ± 0.54	1.49 ± 0.12	0.56 ± 0.06	0.40 ± 0.02
Fat	2.37 ± 0.59	3.49 ± 0.50	6.10 ± 2.24	3.60 ± 0.30	3.21 ± 0.25	2.39 ± 0.13	1.24 ± 0.21
Testes	0.62 ± 0.08	1.87 ± 0.10	2.57 ± 0.18	1.95 ± 0.32	0.72 ± 0.09	0.04 ± 0.01	0.03 ± 0
Intestine	1.19 ± 0.09	2.38 ± 0.35	2.96 ± 0.30	1.17 ± 0.32	0.59 ± 0.07	0.12 ± 0.01	0.06 ± 0
Muscle	0.48 ± 0.06	0.88 ± 0.07	1.28 ± 0.10	0.79 ± 0.08	0.47 ± 0.05	0.22 ± 0.07	0.07 ± 0.02
Liver	0.54 ± 0.03	1.05 ± 0.18	1.25 ± 0.12	1.20 ± 0.17	0.72 ± 0.02	0.43 ± 0.03	0.28 ± 0.02
Heart	0.91 ± 0.17	1.56 ± 0.37	1.80 ± 0.14	1.04 ± 0.09	0.36 ± 0.04	0.08 ± 0	0.04 ± 0
Plasma	0.15 ± 0.03	0.50 ± 0.10	0.38 ± 0.08	0.21 ± 0.02	0.07 ± 0.01	0.01 ± 0	BD

[a] Data represent the mean value ± S.E.M. (micrograms per gram of wet tissue weight or milliliters of fluid) of five animals at each time period.

[b] The animals were given a 20 mg/kg dose of cocaine s.c. twice daily for 21 days, then the same dose of randomly labeled [^{3}H]cocaine twice daily for 2 days before the terminal injection of the same dose of labeled drug.

[c] BD denotes barely detectable levels of drug.

[d] Norcocaine constituted approximately 10% of the total values of cocaine in brain in the acute and approximately 10 to 20% of those in the chronic group (see section on specificity of extraction procedure). Its presence was not demonstrated in other tissues possibly due to its biotransformation to polar benzoylnorecgonine (and norecgonine).

TABLE 5

Brain/plasma ratios of the mean concentrations of [^{3}H]cocaine after a 20 mg/kg (free base) subcutaneous or 8 mg/kg (free base) intravenous injection in acutely and chronically treated male Wistar rats

Brain/Plasma Ratio	0.25 Hr	0.5 Hr	1 Hr	2 Hr	4 Hr	6 Hr	12 Hr	24 Hr	48 Hr
Subcutaneous (acute)[a]		9.0	6.1	6.6	6.9	6.2	2		
Subcutaneous (chronic)[b]		8.7	4.8	8.1	8.6	7.4	7.6	4.3	18
Intravenous (acute)	11.9	9.3	8.3	8.1					

[a] A single s.c. injection of 20 mg/kg (free base) of [^{3}H]cocaine was given in these acute experiments.

[b] The animals were given a 20 mg/kg dose of cocaine s.c. twice daily for 21 days, then the same dose of [^{3}H]cocaine twice daily for 2 days before the terminal injection of labeled drug. Blood and brain were collected at various time intervals after the last injection.

the chronic group. In chronically treated rats, cocaine was not detectable in plasma 24 hours after injection and barely detectable levels of extractable radioactivity were present in heart and testes at 48 and 96 hours. The mean values (micrograms per gram) of extractable radioactivity 48 and 96 hours after subcutaneous injection were as follows: brain 0.04, 0.02; kidney 0.28, 0.08; lung 0.15, 0.14; spleen 0.14, 0.13; fat 0.73, 0.66; intestine 0.05, 0.04; muscle 0.13, 0.04; and liver 0.13, 0.07, respectively. The comparatively higher levels of extractable radioactivity observed in fat declined very slowly over a period of 4 weeks to 0.18 μg/g. In spite of the high lipid solubility of cocaine, the relatively poor blood supply to the fat depots slows the transfer of cocaine from blood to fat. However, at 2 hours after injection, enough cocaine is finally transferred to bring the fat depots in both acutely and chronically treated animals to their equilibrium concentrations. The equilibration of cocaine in other tissues of the acute animals occurs only after the attainment of this equilibrium in heart and fat.

The prolonged course of absorption from the subcutaneous injection site (table 3) and the redistribution phenomena occurring during this phase complicates the pharmacokinetics of cocaine, and only an approximate estimate of its half-life in brain and plasma of acutely and chronically treated animals could be made on the basis of the present data. The half-lives of cocaine in brain and plasma of acute animals are 0.8 and 1.0 hour, respectively, that in brain and plasma of chronically treated animals 1.8 to 2.0 hours. The half-life of cocaine in fat of both acutely and chronically treated animals is 1.8 to 2.0 hours, a value similar to that for other tissues in the chronic group.

The data on brain/plasma ratios of cocaine in acutely and chronically treated groups after subcutaneous and i.v. injections are given in table 5. The values were, in general, somewhat higher in the chronic group compared to the acute group except at 1 hour when peak plasma levels of cocaine were attained in the chronic group. The brain/plasma ratio after a single intravenous injection (8 mg/kg) ranged between 8 and 12 (table 5).

Excretion. Comparative data on the excretion of free cocaine and total radioactivity in urine and feces, collected over a period of 1 week in acutely and chronically treated rats after a 20 mg/kg subcutaneous injection of ring-labeled [^{3}H]cocaine, is presented in figure 2. The percentages of the dose excreted free in urine and feces of acutely and chronically treated animals were 1.2 and 1.5, respectively, with the maximum excretion occurring in both groups within 24 hours after injection. Total radioactivity excreted in urine as a percentage of the dose administered in acutely (49.3%) and chronically treated rats (51.6%) was also similar, but significant differences were observed in the groups relative to the excretion of total radioactivity in feces. The values in the chronic group were consistently higher (35.9%) than that in the acute group (22.1%). Cumulative total radioactivity excreted in urine and feces of chronically treated rats was significantly higher (87.5%) compared to that in acutely treated group (71.4%).

Identification of urinary metabolites of [^{3}H]cocaine. Column and thin-layer chromatographic fractionation techniques and radioscan-

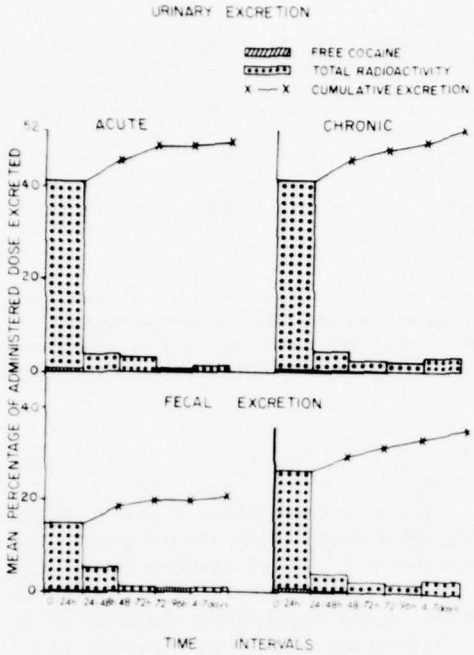

FIG. 2. Data represent the mean percentage of administered dose of ring-labeled [^{3}H]cocaine recovered as free cocaine and total radioactivity from the urine (top) and feces (bottom) of five male rats each in the acutely and chronically treated group. The animals were given a single subcutaneous injection of 20 mg/kg (free base) of cocaine in the acute group. In the chronically treated group, the animals were given subcutaneous injection of 20 mg/kg of cocaine twice daily for 21 days, then with same dose of ring-labeled [^{3}H]cocaine twice daily for 2 days before the final injection of labeled drug. Urine and feces were collected in metabolism cages throughout the 2 days on which [^{3}H]cocaine was administered in the chronic group. The 0 to 24 hour values of free cocaine and total radioactivity excreted as a percentage of the dose in urine and feces of chronic animals represent the mean values from all the five doses of labeled cocaine. Other excretion values after 24 hours in the chronic group represent time intervals after the last injection of labeled cocaine.

ning of chromatograms from pooled urine of rats injected subcutaneously with a 20 mg/kg dose of ring-labeled [^{3}H]cocaine furnished evidence for the presence of benzoylecgonine, benzoylnorecgonine, ecgonine methyl ester and ecgonine as urinary metabolites. Small amounts of unmetabolized cocaine were also detected. Norcocaine and norecgonine were not detected as urinary metabolites of cocaine. Suggestive evidence was obtained for the presence of a phenolic metabolite (by the aromatic hydroxylation) and, in addition, at least two other hydroxylated polar nonphenolic metabolites. Enzymic hydrolysis with β-glucuronidase, sulfatase and peptidase furnished indirect evidence that glucuronide, sulfate or amino acid conjugation of hydroxylated metabolites apparently did not occur. The possibility that some of the other unidentified metabolites may arise as artifacts of the fractionation procedure could not be completely ruled out. The known and hypothetical metabolic pathways of cocaine in the rat are given in figure 3.

Discussion

In view of the low levels of cocaine appearing in plasma and brain, a sensitive method for the estimation of [^{3}H]cocaine from biological materials was developed which provided quantitative recoveries in the 10 to 1000-ng range. In preliminary experiments, no significant differences were observed in values of free cocaine in tissues using either randomly labeled or ring-labeled [^{3}H]cocaine. Randomly labeled [^{3}H]cocaine, which was isotopically stable *in vivo* and had a high specific activity, was therefore used in comparative tissue distribution experiments. Synthesized ring-labeled [^{3}H]cocaine of lower specific activity was chosen for comparative excretion studies in acutely and chronically treated rats, because of the *in vivo* metabolic lability of benzoyl and methyl ester groups which in randomly labeled [^{3}H]cocaine have been shown (Werner and Schmidt, 1969) to have 65 and 7% of the total [^{3}H] radioactivity of cocaine molecule.

After injection of 8 mg/kg i.v. of cocaine in the rat, the initial peak levels in plasma declined quickly as a result of tissue distribution, metabolism and some excretion. High lipid solubility of cocaine played an important role in its rapid entry into the brain. Cocaine possessed a marked affinity for the tissues as borne out by the high brain/plasma ratio (table 5). The erythrocyte/plasma ratio of cocaine (0.9–1.8:1), determined with 0.5 to 5 μg/ml concentrations of cocaine in our laboratory, agreed well with that (1.2–1.4:1) reported earlier by Woods *et al.* (1951). No significant differences were observed in plasma-protein binding of cocaine in control, acutely and chronically treated rats. In view of the relatively small plasma-protein binding values for cocaine and elimination of cocaine primarily by biotransformation, it is apparent

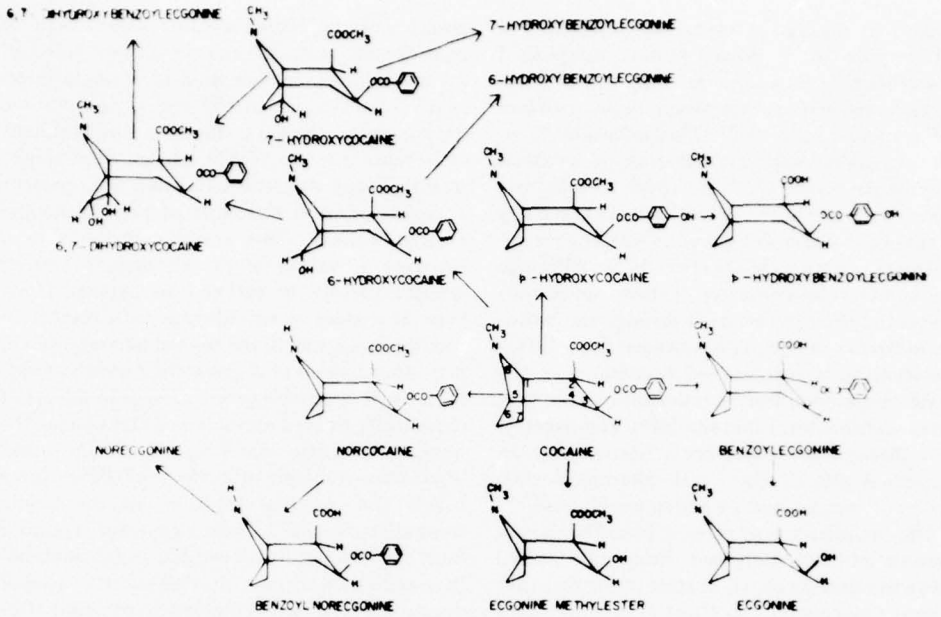

FIG. 3. The known and hypothetical metabolic pathways of cocaine in the rat.

that binding of lipophilic cocaine to tissues may play a more important role in determining its overall pharmacokinetics than the plasma-protein binding. Previous studies on binding of cocaine to guinea-pig vas deferens *in vitro* (Marks *et al.*, 1967) showed complete uptake within 8 minutes, with 90% of the radioactivity being washed out after 4 minutes with little additional washout occurring after 16 minutes, indicating a specifically bound cocaine fraction.

Biliary excretion studies showed that cocaine was very rapidly metabolized and unchanged cocaine was excreted in very small amounts in bile. Comparative urinary and fecal excretion studies in acutely and chronically treated rats also showed that only 1 to 1.5% of the dose was excreted as unmetabolized cocaine.

Norcocaine was observed as a metabolite of cocaine in rat brain during the onset of convulsions after a 10 mg/kg i.v. dose of cocaine (Misra *et al.*, 1974b). As mentioned earlier in the section on specificity of extraction procedure, the highly lipophilic norcocaine constituted approximately 15% of the values expressed as cocaine in brain of rats injected intravenously with an 8 mg/kg dose of [^{3}H]cocaine and approximately 10% of those in brain of rats injected subcutaneously with 20 mg/kg of cocaine. Norcocaine has further been shown to be a pharmacologically active metabolite of cocaine in the brain of dogs injected intravenously with a 5 mg/kg dose of [^{3}H]cocaine (A. L. Misra, M. N. Patel, V. R. Alluri, S. J. Mulé and P. K. Nayak, unpublished results) and also in the brain of the monkeys (Hawks *et al.* 1974). Evidence for the formation of norcocaine, benzoylecgonine, benzoylnorecgonine and ecgonine as metabolites of cocaine in rat brain after an injection of 20 mg/kg s.c. has previously been reported (Misra *et al.*, 1974b).

In vitro (liver) microsomal enzymes reportedly (Ramos-Aliaga and Chiriboga, 1970; Leighty and Fentiman, 1974) convert cocaine to norcocaine and benzoylecgonine. Norcocaine, benzoylecgonine and benzoylnorecgonine were shown (Misra *et al.*, 1975) to have potent stimulant activity after intracisternal injection in the rat. Benzoylecgonine and benzoylnorecgonine, however, did not produce observable pharmacological effects even after high doses (200 mg/kg) when administered systemically. These two polar metabolites persisted in selected anatomic areas of the CNS of the dog, long after the disappearance of cocaine after a

single 5 mg/kg intravenous injection of [^{3}H]cocaine (A. L. Misra *et al.*, unpublished observations). Furthermore, these metabolites formed molecular complexes with calcium (Misra and Mulé, 1975). This molecular complexing, along with mobilization of neuronal membrane-bound Ca^{++} by these metabolites, may play a role in the potent stimulant activity of these compounds observed in rats after intracisternal injection (Misra *et al.*, 1975). Although the highly polar character of these compounds prevented their penetration through the blood-brain barrier in adequate concentrations, direct metabolism of cocaine and norcocaine in the brain tissue could lead to significant amounts of these metabolites. Lipid solubility and penetration through the blood-brain barrier play an important role in the overall pharmacological action of cocaine and its active metabolites.

The mobilization of cocaine from the subcutaneous site in acutely and chronically treated rats was rapid (table 1), in spite of the fact that a local vasoconstriction effect of cocaine could limit the rate of its absorption. No significant differences were observed in relative rates of disappearance of cocaine from subcutaneous site in acutely and chronically treated rats. In acute animals injected subcutaneously the peak plasma levels, as in the intravenous experiment, were lower than those in other tissues and were sustained for a 1 to 4 hour period. This may represent a state of relative equilibrium between absorption from the subcutaneous site and detoxication mechanisms. A similar profile was also observed earlier for the dog (Woods *et al.*, 1951). No marked selective deposition or persistence of cocaine was observed in brain or other tissues, and the compound disappeared from brain and plasma fairly rapidly after i.v. or s.c. injections. This observation is similar to that earlier observed by us for thebaine, an opioid stimulant with little or no physical dependence liability (Misra *et al.*, 1973c, 1974c). By contrast, it is interesting to note that opioid depressant drugs such as morphine and methadone persisted in brain in small concentrations for prolonged periods (Misra *et al.*, 1971, 1973a, 1974a; Misra and Mulé, 1972); even after a single subcutaneous injection.

In comparative drug uptake studies in acutely and chronically treated rats as described here, an important factor merits consideration. Chronic dosing of rats over a period of 3 weeks with unlabeled cocaine would lead to considerable tissue saturation with cocaine and its metabolites. Introduction of a single injection of [^{3}H]cocaine at the end of the chronic dosing period presents, therefore, the problems of isotopic dilution of the labeled drug subsequently taken up, with unlabeled drug present at many sites in the body of the chronically treated animal. This could contribute to a lowering of values in the chronically treated group especially at earlier time periods. However, if a state of equilibrium with respect to cocaine levels within the central nervous system and other tissues at a given time were to exist, then the free cocaine levels present in tissues of chronically treated animals would influence the quantity of drug taken up after subsequent injections. Multiple injections of [^{3}H]cocaine before the terminal injection in chronically treated rats would, in such a case, satisfactorily label the tissue stores of cocaine and its metabolites radioactively and provide a true value of drug concentrations in biological materials after chronic drug administration. Peak levels of free cocaine in brain and other tissues and plasma of acutely treated animals did not differ significantly from those in chronically treated animals. Peak concentrations in tissues, however, were shifted from 4 to 2 hours in the chronically treated group. The half-lives of cocaine in brain and plasma of chronically treated rats (1.8–2 hours) were higher than those in the acutely treated group (0.8–1 hour). The brain/plasma ratios in chronically treated rats were also, in general, somewhat higher than those in acutely treated animals with the exception of the 1-hour period at which peak levels of cocaine occurred in the plasma in the chronic group. The data indicated an altered distribution of cocaine in the chronic group. The rise and fall of CNS levels of cocaine coincided roughly with the change of levels in plasma and suggested three alternative possibilities: 1) that cocaine was probably present in the CNS in the interstitial compartment; 2) its penetration into and exit out of the cell must be rapid and 3) any significant intracellular or extracellular binding in the CNS must be freely reversible.

A significant and important finding of this study was sequestration of cocaine in substantial amounts in fat, observed in the chronic group in contrast to the acute animals given s.c. or i.v. doses of cocaine. Thus, values of cocaine

in fat, 6 hours to 4 weeks after injection, were comparatively much higher than those in other tissues of the chronically treated animals. The sequestration of cocaine in fat depots of chronically treated animals may produce a slow and prolonged release of cocaine long after its disappearance from the plasma and may also be responsible for prolonging its persistence in brain and other tissues in this group. The total radioactivity of brains removed 1 week after injection was several-fold higher than the extractable free cocaine. We have observed a similar pattern (A. L. Misra *et al.*, unpublished observations) in the selected anatomical areas of the CNS of the dog injected intravenously with a single 5 mg/kg dose of [^{3}H]cocaine, where the levels of total radioactivity in anatomic areas of the CNS 1 week after injection ranged between 343 and 441 ng/g and that in plasma 493 ng/ml; the values of free cocaine in these areas were barely detectable 24 hours after injection of [^{3}H]cocaine. Thin-layer chromatographic experiments on brains from chronically treated rats removed 1 week after injection of 20 mg/kg s.c. of cocaine revealed the presence of benzoylecgonine, benzoylnorecgonine, ecgonine, small amounts of norcocaine and cocaine. The half-lives of benzoylecgonine and ecgonine in rat brain after an intravenous injection of 10 mg/kg have been shown (Misra *et al.*, 1974d, 1975) to be 1.3 and 7.8 hours, respectively, and in plasma, 0.8 and 3.8 hours, respectively. This may account for the comparatively slower clearance of these compounds from the rat brain.

Liver is known to be a primary site for the detoxication of cocaine. Our study has provided evidence that cocaine is metabolized fairly rapidly, and only small quantities (1–1.5%) are excreted free in urine and feces. The excretion of metabolites, as reflected by the total radioactivity values, continues for several days. There were only minor differences in the excretion of free cocaine or total radioactivity in urine from acutely and chronically treated groups. Significant differences, however, were observed in the fecal excretion of total radioactivity in chronic (35.9%) as compared to that in the acute group (22.1%) (fig. 2), and it is conceivable that this difference may be due to the use of multiple injections of labeled drug in the chronic group. Benzoylecgonine, benzoylnorecgonine, ecgonine methyl ester and ecgonine were identified as urinary metabolites of cocaine. Norcocaine, the pharmacologically active metabolite detected in rat brain, was not excreted in the urine possibly due to its further biotransformation to benzoylnorecgonine. In addition to these metabolites, evidence for the presence of phenolic (hydroxylation in the *para* position of the aromatic ring) and ring-hydroxylated metabolites has also been obtained. The positions where hydroxylation presumably occurs are 6 and 7, respectively, in the pyrrolidine ring. Evidence for the presence of these hydroxylated metabolites, however, is only suggestive at the present time. Our previous studies have shown that ecgonine (Misra *et al.*, 1974d, 1975) is not metabolized in the rat and the metabolism of benzoylecgonine is primarily to ecgonine and two minor metabolites, one of which has phenolic characteristics. It is conceivable, therefore, that hydroxylation in the pyrrolidine ring and aromatic ring occurs before the de-esterification to polar benzoylecgonine and ecgonine. Similarly *in vitro* incubation of norcocaine with rat brain homogenates showed its biotransformation to benzoylnorecgonine and norecgonine as metabolites. Benzoylecgonine was not converted to benzoylnorecgonine or norecgonine *in vivo* or in the brain (fig. 3). Evidence for glucuronide conjugation of hydroxylated metabolites was not obtained in this study, but the possibility of formation of such conjugates exists and could not be completely ruled out. The pattern of metabolites in chronically treated rats was qualitatively similar to that in the acutely treated group with somewhat higher excretion of benzoylecgonine and ecgonine methyl ester.

Interaction of cocaine with neural membrane phospholipids through electrostatic and nonpolar forces has been reported to produce alterations in the ordering and fluidity of nonpolar regions (Hauser *et al.*, 1969; Seeman, 1972; Singer, 1973), marked inhibition in functioning of the membrane as a cation-exchange site and displacement of calcium from membrane sites at which it participates in the generation of action potential (Feinstein and Paimre, 1966). Changes in brain tissue respiration, oxidative phosphorylation (Kuzhman, 1969) and cerebral metabolism (Rogers and Nahorski, 1973) have also been reported with cocaine. It is conceivable, therefore, that increased partitioning of lipophilic cocaine and norcocaine into the hydrocarbon domains of the neural membranes, a

high affinity binding coupled with hyperthermia which enhances its toxicity (Peterson and Hardinge, 1967), may modify the membrane function, its structural integrity and consequently alter the energy metabolism of nerve tissue. Furthermore, repeated administration of cocaine could also lead to the oxidation *in vivo* of norcocaine to its highly reactive nitroxide free radical. This and the prolonged sequestration of cocaine in fat in chronic animals may play an important role in the systemic toxicity of cocaine. The following observations support this postulate: 1) oxidation of cocaine at ambient temperature with hydrogen peroxide reportedly (Rozantsev and Sholle, 1969) leads to a fairly stable nitroxide radical; 2) preliminary work in our laboratory on the oxidation of norcocaine with *m*-chloroperbenzoic acid has shown the formation of N-oxyl compound; 3) electron spin resonance spectroscopy (Dwek *et al.*, 1973; Keith *et al.*, 1973) has provided evidence for conformational alterations in membranes as a result of binding of a wide variety of similar spin label probes to membrane proteins; 4) nitroxide free radicals *in vivo* are known to be reduced to norcompounds with membrane SH groups (Giotta and Wang, 1972); and 5) toxicity of cocaine does not depend exclusively on catecholamine release (Hatch and Fischer, 1972).

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Disposition and Metabolism of [^{3}H]Cocaine in Acutely and Chronically treated Dogs

A. L. MISRA, M. N. PATEL, V. R. ALLURI, S. J. MULÉ and P. K. NAYAK

New York State Office of Drug Abuse Services, Testing and Research Laboratory, Brooklyn, New York 11217, U.S.A.

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1. Beagle dogs were chronically treated with cocaine, 5 mg/kg subcutaneously twice daily for 6 weeks, followed by same dose of [^{3}H]cocaine given intravenously.

2. The $t_{1/2}$ values of cocaine in plasma, liver, spleen and heart, in acutely and chronically treated dogs, were: 1·2, 1·1; 2·2, 1·8; 1·8, 1·3; 2·0, 1·2 h, respectively. In both groups, cocaine disappeared from all areas of the central nervous system 12–24 h after injection but significant amounts of radioactivity due to benzoylnorecgonine and benzoylecgonine persisted in the CNS even 1 week after administration of cocaine. Brain-to-plasma ratios of cocaine were lower in chronically-treated than in acutely-treated dogs 2 and 4 h after injection.

3. Norcocaine, benzoylnorecgonine, benzoylecgonine and ecgonine were metabolites of cocaine in dog brain in both groups. Norcocaine and benzoylnorecgonine were present in higher amounts in brains of chronically treated dogs. Rate of disappearance of norcocaine was similar to cocaine in both groups.

4. The amounts of cocaine excreted in urine and faeces as percentage of dose were 0·9–5·0, 1–1·6 in the acute and 2·2–3·3 and 0·2–0·3 in the chronically treated dogs. Major excretion of radioactivity occurred in urine within 24 h in both groups. Total radioactivity (65% of dose) in urine plus faeces was similar in both groups.

5. Norcocaine, benzoylnorecgonine, benzoylecgonine, ecgonine, norecgonine, ecgonine methyl ester and unidentified compounds were urinary metabolites of cocaine in both groups. Benzoylnorecgonine and ecgonine were excreted in higher amounts and benzoylecgonine and norecgonine in lower amounts in the acute than in the chronically treated dogs.

6. The possible role of persistence of benzoylnorecgonine and benzoylecgonine (which possessed potent stimulant activity intracisternally) in the CNS is discussed.

Introduction

In view of the emergence of cocaine as a major problem of drug abuse, it has become essential to determine the physiological disposition of this compound following acute and chronic administration to species which exhibit the psychological effects characteristic of chronic cocaine dependence in man. Self-administration studies in the monkey (Deneau, Yanagita & Seevers, 1969) have established the strong psychic dependence potential of cocaine but little or no tolerance and physical dependence liability. Previous work (Grode, 1912; Tatum & Seevers, 1929; Downs & Eddy, 1932; Gutierrez-Noriega & Zapata-Ortiz, 1944; Kosman & Unna, 1968; Hatch & Fischer, 1972; Simon *et al.*, 1972) has shown that dogs (*a*) exhibit true cocaine psychosis with marked craving for cocaine, (*b*) become hypersensitive rather than tolerant to cocaine on

repeated dosing and (*c*) this increase in sensitivity persisted unabated for a couple of weeks after complete withdrawal of cocaine. Apart from an earlier acute study in the dog by Woods, McMahon & Seevers (1951), no information is available on the physiological disposition of cocaine in acute and chronic states in this species.

We have extended our previous work on cocaine in the rat (Misra *et al.*, 1974 b, d; 1975 b; Nayak *et al.*, 1976 and other references cited therein) to investigate the disposition, metabolism of [^{3}H]cocaine in the acutely and chronically treated dogs with a view to assess the possible relationship and significance of these parameters to the hypersensitivity observed with cocaine on chronic treatment.

Materials and methods

The method for the preparation of ring-labelled and randomly labelled [^{3}H]cocaine has been described (Nayak *et al.*, 1974). Other details on radiochemical purity, specific activity (20 μCi/mg), method of estimation in biological materials, recoveries etc., have previously been described (Nayak *et al.*, 1976). Although cocaine was injected as its hydrochloride, all doses of the drug are expressed as the free base. Gelman instant thin-layer chromatography media (silica gel, ITLC) from Gelman Instrument Company, Ann Arbor, Michigan, was used for all t.l.c. experiments. The technique for radioscanning thin-layer chromatograms, counting radioactivity, efficiency of counting in Nuclear Chicago Mark III liquid scintillation counter, etc., have been described (Misra *et al.*, 1973 a; Misra, Pontani & Mulé, 1974 c).

Randomly labelled [^{3}H]cocaine (specific activity 630 μCi/mg) which was isotopically stable *in vivo* was used in comparative tissue distribution experiments for the estimation of free cocaine. Ring-labelled [^{3}H]cocaine (specific activity 48 μCi/mg) was chosen for comparative excretion and metabolic studies in acutely and chronically treated dogs because of the metabolic lability *in vivo* of methyl ester and benzoyl groups (Nayak *et al.*, 1976).

Specificity of the extraction procedure for [^{3}H]cocaine

The specificity of the extraction procedure (Nayak *et al.*, 1976) was evaluated by extraction of urine, plasma or tissue homogenates of dogs injected intravenously with 5 mg/kg dose of [^{3}H]cocaine, t.l.c. of the cyclohexane extract and radioscanning of the chromatograms (Misra, Pontani & Mulé, 1973 b). The polar metabolites benzoylecgonine, benzoylnorecgonine, norecgonine, ecgonine and ecgonine methyl ester, were not extracted in significant quantities (less than 1%). Norcocaine, a highly lipophilic compound which has been shown (Misra *et al.*, 1975 b) to be a pharmacologically active metabolite of cocaine in the brain of rats and dogs was quantitatively extracted by this procedure.

Animal experiments

Acute: Male and female beagle dogs (approximately 3–5 years of age) were housed in air-conditioned quarters and allowed 3–4 weeks to get acclimatized to the animal quarters. Purina Dog Chow and water were supplied *ad libitum*. Food was withdrawn from cages approximately 16 h before administration of cocaine. Beagle dogs of either sex weighing 6–10 kg were given intravenous

injection of 5 mg/kg (free base) of [^{3}H]cocaine. After appropriate periods of time, the animals were sacrificed by a rapid intravenous injection (40–80 mg/kg) of thiopental sodium, followed by exsanguination. Blood samples were collected in heparinized Vacutainer tubes (Becton-Dickinson Co., Rutherford, N.J.) approximately 1–2 min before the selected time interval (prior to the injection of thiopental sodium) by venipuncture of either the cephalic or lateral saphenous vein. Plasma immediately obtained by centrifugation was quickly frozen ($-20°$).

Brain, heart, lung, liver, kidney, intestine, muscle, fat etc., were quickly removed, washed with ice-cold 0·9% saline, wiped dry in tissue paper, wrapped in aluminium foils and stored frozen ($-20°$) until analysed. A sample of bile and cerebrospinal fluid was also obtained at the time of sacrifice and stored frozen. Before analysis, brain was carefully separated into various selected anatomical areas and these and other tissue samples (approximately 1 g wherever possible) were homogenized in 0·5 M HCl with a teflon pestle to provide a 10 or 20% homogenate. Duplicate samples of 2 ml of these homogenates were analysed for free cocaine. Plasma samples were diluted (1:5) with distilled water and analysed similarly. Total radioactivity due to metabolites and free drug in plasma and tissue homogenates was concurrently measured by dissolving appropriate aliquots (0·2 ml) in 10 ml Aquasol solution (New England Nuclear, Boston, Mass.) and counting the radioactivity in liquid scintillation counter.

Chronic studies

Beagle dogs (6–9 kg) were subcutaneously injected with a 5 mg/kg (free base) dose of non-radioactive cocaine twice daily (9–10 a.m. and 3–4 p.m.) for 6 weeks, then with a 5 mg/kg subcutaneous dose of randomly-labelled [^{3}H]cocaine twice daily for 2 days followed by a terminal intravenous injection of the same dose of labelled cocaine. Samples of plasma, bile, tissues were obtained at various time intervals and analysed for free cocaine and total radioactivity by the previous procedure.

Urinary and faecal excretion of ring-labelled [^{3}H]cocaine in acutely and chronically treated dogs

Following a 5 mg/kg intravenous dose of ring-labelled [^{3}H]cocaine to two female dogs, urine was collected at hourly intervals from an indwelling Bardex–Foley Catheter (Fr. 8) for the first 6 h after injection, followed by 24 h collections of urine and faeces in metabolism cages every day for a period of 7 days. After collection of urine samples, cages were washed with a small volume of water and the washings were added to the sample. All samples were kept frozen until analysed. Faeces were homogenized in a known volume of 0·5 M HCl, filtered through a coarse cloth and aliquots of filtrate analysed for free cocaine and total radioactivity as previously described.

The same dogs used in the acute experiments were treated chronically with non-labelled cocaine for 6 weeks and then given a single intravenous dose (5 mg/kg) of ring-labelled [^{3}H]cocaine. Urine and faeces were collected as described above.

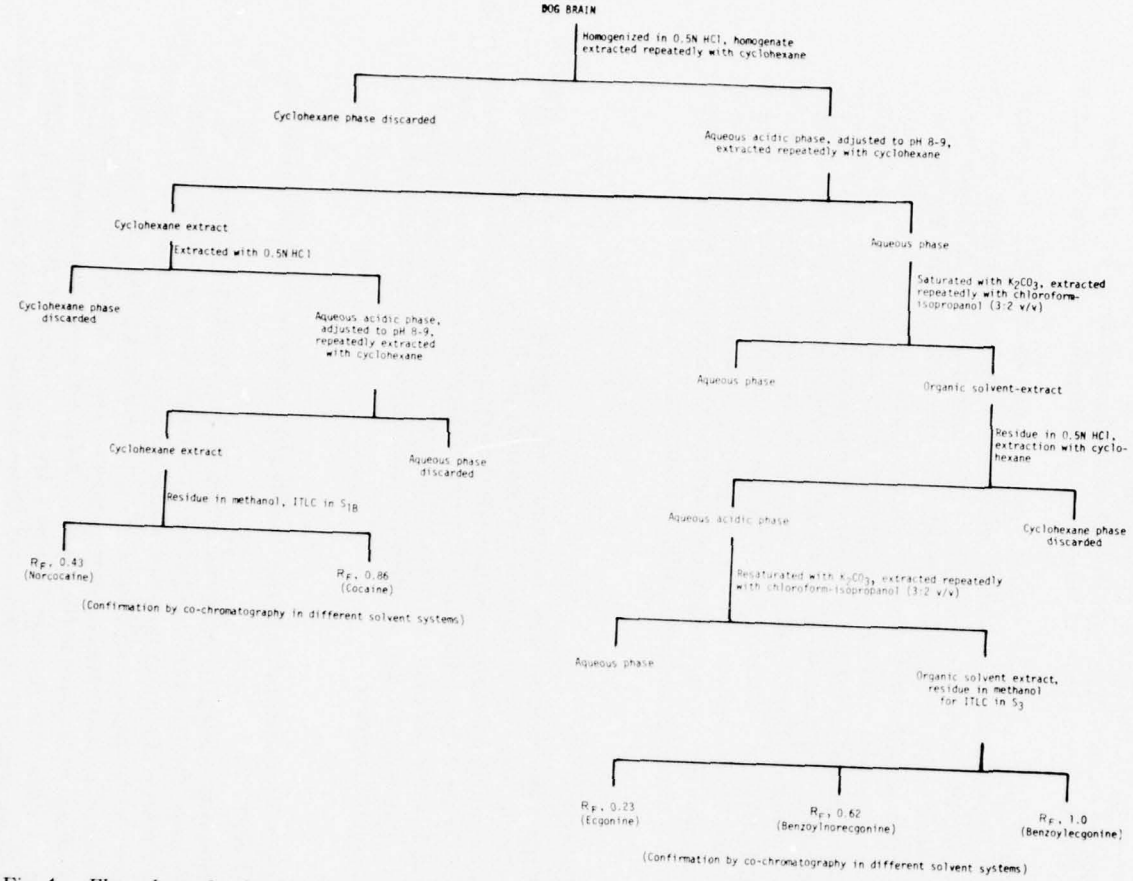

Fig. 1. *Flow chart showing the presence of metabolites of cocaine in brains of acutely and chronically treated dogs injected intravenously with [^{3}H]cocaine.*

The solvent systems used for the separation of different metabolites were $S_{1\text{-}B}$, n-hexane–dioxane–ammonia soln. (sp. gr. 0·88) (90:10:0·3, by vol.) and S_3, ethyl acetate–methanol–ammonia soln. (sp. gr. 0·88) (15:4:1). The dose of [^{3}H]cocaine was 5 mg/kg.

Identification of cocaine metabolites in the brains of acutely and chronically treated dogs

Brain tissue (5 g) from dogs sacrificed at various times after 5 mg/kg intravenous injection of [^{3}H]cocaine was homogenized in 0·5 M HCl to provide a 10% homogenate and processed as shown in the flow chart (Fig. 1).

Identification of urinary metabolites of [^{3}H]cocaine in the acutely and chronically treated dogs

The method used for the identification of urinary metabolites of cocaine in acutely and chronically treated dogs is outlined in Fig. 2.

Results

Pharmacological and behavioural effects

A 5 mg/kg intravenous injection of cocaine to the dogs produced mild excitation, restlessness, anxious behaviour, rapid respiration, rise of body temperature, muscle tremors for a few minutes, some salivation, and pupillary dilatation but no convulsions. The duration of responses to this injection generally ranged between 40–60 min. An intravenous dose of 10 mg/kg produced very severe convulsions and hyperexcitation. During the course of chronic administration of cocaine to the dogs, a marked desire for drug was clearly evident. No impairment of general physical condition, no significant change in food intake, body weight or mortality occurred in response to cocaine. All the female dogs exhibited convulsions, extensor rigidity, ataxia lasting for approximately 45 min during the first week of chronic treatment with cocaine. Male dogs did not show these effects. During the fifth week of chronic treatment, two male dogs however went into severe convulsions, mydriasis and extreme restlessness twice during the week. A female dog exhibited these effects once during the sixth week of chronic treatment. A male dog chronically treated with cocaine for 10 weeks with 2 daily 5 mg/kg subcutaneous doses, suddenly withdrawn for 2 days did not show any withdrawal symptoms or after-depression. Resumption of the treatment in this dog however, produced severe clonic-tonic convulsions, catatonic behaviour, prostration, incoordination, ataxia, extensor rigidity, hyperthermia and death within 1 h of injection.

Comparative results in acutely and chronically treated dogs

Distribution of [^{3}H]cocaine in biological fluids and tissues: Data on the distribution of free cocaine in tissues of 2 acutely and 2 chronically treated dogs following 5 mg/kg intravenous dose of [^{3}H]cocaine are given in Table 1. The mean values of cocaine in plasma, muscle, heart, fat, duodenum, small intestine, spleen, lungs and kidney were higher in chronic as compared to the acute group. The mean values of cocaine in liver were lower in chronic dogs up to 2 h after injection and somewhat higher than those in the acute group at later times. The mean values of cocaine in bile were lower initially (0·5, 1 h) in the chronic group, higher at 2 and 4 h and again dropped lower than those in the acute group at later times. Comparatively faster disappearance of cocaine occurred from bile of chronically treated dogs 4 h after injection as compared to the acute. Cocaine disappeared from plasma in both acutely and chronically treated dogs by 12 h.

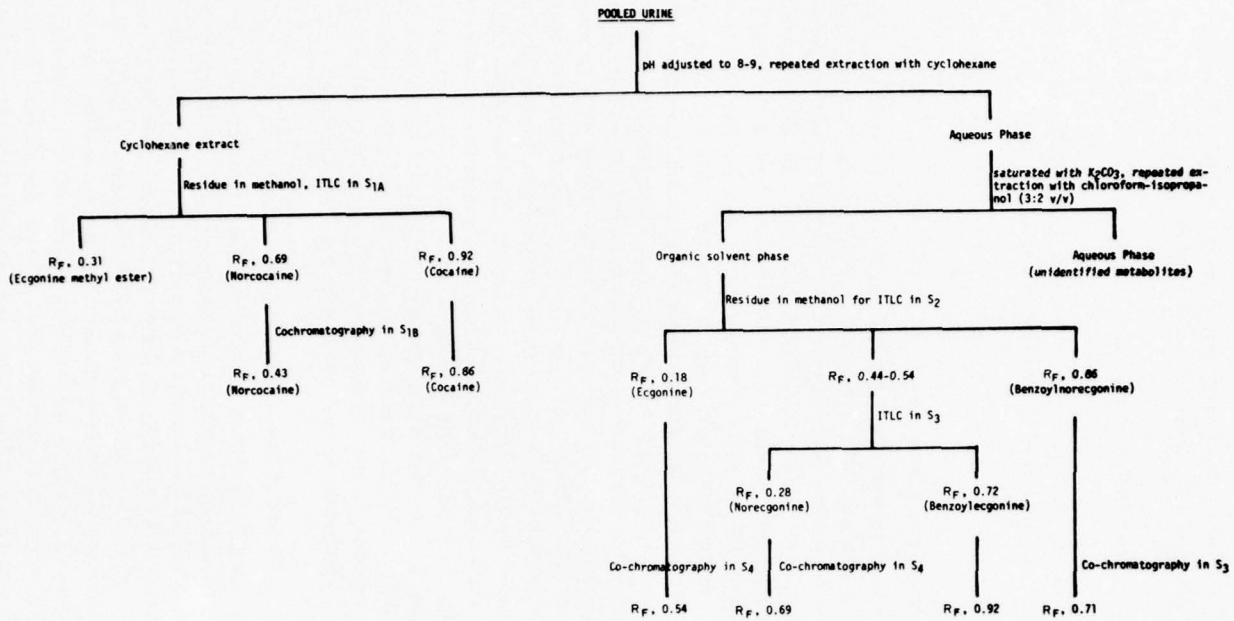

Fig. 2. *Flow chart outlining the method used for the identification of urinary metabolites of cocaine in acutely and chronically treated dogs injected intravenously with [ring-^3H]cocaine.*

The dose of [^{3}H]cocaine was 5 mg/kg. Pooled urine was filtered through glass wool and processed as shown. The excretion of radioactivity as a percentage of dose in acute dogs (in 48 h urine) and chronically treated dogs (in 24 h urine) was 58 and 56%, respectively. The solvent systems used for the separation of different metabolites were: S_{1-A}, n-heptane–dioxane–ammonia soln. (sp. gr. 0·88) (90:10:0·3, by vol.); S_{1-B}, n-hexane–dioxane–ammonia soln. (sp. gr. 0·88) (90:10:0·3); S_2, n-butanol–ethyl acetate–dioxane–acetic acid–water (45:40:10:3:5); S_3, ethyl acetate–methanol–ammonia soln. (sp. gr. 0·88) (15:4:1); S_4, n-butanol–acetic acid–water (35:3:10).

Table. 1. Distribution of cocaine in various tissues and fluids of acutely and chronically treated dogs at intervals after intravenous [^{3}H]cocaine

Acute									Chronic						
0·5 h	1 h	2 h	4 h	6 h	12 h	24 h	1 week	Tissues and fluids*	0·5 h	1 h	2 h	4 h	6 h	24 h	1 week
9·87	15·44	17·86	21·21	24·03	15·34	9·98	0·08	Bile	8·12	8·67	39·11	38·24	11·00	5·78	0·02
9·25	8·60	3·30	0·87	0·54	0·25	0·23	0·05	Liver	7·16	6·17	2·06	1·46	0·87	0·32	0·08
6·18	4·21	1·41	0·68	0·25	0·06	0·04	0	Kidney	9·27	5·20	2·22	1·14	0·31	0·09	0·01
3·51	3·48	1·46	0·49	0·23	0·08	0·04	0	Lungs	6·81	4·89	1·42	1·14	0·32	0·13	0·02
3·47	3·92	1·50	0·50	0·39	0·09	0·04	0	Spleen	10·78	6·90	2·60	1·22	0·45	0·19	0
2·91	1·63	0·65	0·25	0·23	0·08	0·01	0	Small intestine	3·85	2·16	1·01	0·74	0·14	0·02	0
2·63	1·98	0·87	0·63	0·27	0·04	0·01	0	Duo-denum	4·31	2·06	1·40	0·75	0·17	0·02	0
2·50	2·06	1·54	0·40	0·50	0·03	0·01	0	Fat	4·66	2·20	1·81	0·69	0·22	0·04	0·04
2·23	1·57	0·53	0·18	0·14	0·03	0·02	0	Heart	3·78	2·75	0·84	0·35	0·16	0·06	0
2·19	1·46	0·48	0·15	0·07	0·01	0	0	Muscle	2·69	1·64	0·74	0·27	0·08	0·01	0
0·64	0·46	0·12	0·04	0·03	0	0	0	Plasma	0·82	0·56	0·31	0·09	0·02	0	—

Beagle dogs were subcutaneously injected with a 5 mg/kg dose of non-radioactive cocaine twice daily for six weeks, then with a 5 mg/kg subcutaneous dose of randomly-labelled [^{3}H]cocaine twice daily for two days, followed by a terminal injection of the same dose by intravenous route in the chronic experiments.

Values are means (μg/g wet tissue or ml) from two dogs sacrificed at the stated times. Duplicate determinations were performed on the individual tissues of each animal and results averaged. Two dogs were sacrificed at each time for the acute study. For the chronic study, 1 dog each was used at 6 and 24 h. The values of the individual animals did not deviate more than ± 15% from the mean for lung, small intestine, duodenum, fat, heart, muscle and plasma at 1 h and thereafter. The values in bile, liver, kidney, spleen showed deviations ± 25% from the mean at 0·5 and 1 h after injection; subsequently these deviations fell in the range for other tissues.

*Norcocaine was present only in traces in tissues possibly due to further biotransformation to benzoylnorecgonine and norecgonine.

Table 2. Distribution of cocaine in selected tissues of the CNS of acutely and chronically treated dogs at intervals after intravenous [^{3}H]cocaine

Acute								Chronic						
0·5 h	1 h	2 h	4 h	6 h	12 h	24 h	Selected tissues of the CNS*	0·5 h	1 h	2 h	4 h	6 h	24 h	1 week
4·51	2·71	1·03	0·31	0·17	0·02	0	Temporal cortex (grey)	5·94	3·17	1·26	0·48	0·21	0·01	0
4·92	2·69	1·15	0·30	0·18	0·02	0	Temporal cortex (White)	5·70	3·06	1·35	0·49	0·20	0·01	0
3·52	2·18	0·81	0·24	0·14	0·02	0	Cerebellum	4·92	2·45	1·04	0·42	0·16	0·01	0
4·16	2·44	1·23	0·39	0·17	0·02	0	Spinal Cord	4·34	2·74	1·54	0·61	0·31	0·01	0
3·80	2·10	0·83	0·28	0·15	0·01	0·01	Hypothalamus	4·65	2·65	1·04	0·43	0·16	0·01	0
4·21	2·46	0·91	0·28	0·16	0·02	0	Thalamus	5·36	2·95	1·22	0·46	0·19	0·01	0
3·75	2·20	0·90	0·27	0·16	0·02	0	Medulla	4·55	2·59	1·15	0·43	0·21	0·01	0
3·99	2·26	0·95	0·27	0·14	0·02	0	Pons	4·73	2·56	1·35	0·48	0·19	0·01	0
3·67	2·11	0·83	0·27	0·15	0·02	0	Mesencephalon	4·49	2·55	1·09	0·43	0·19	0·01	0
4·29	2·44	1·18	0·36	0·20	0·02	0	Caudate nucleus	5·11	3·47	1·37	0·52	0·25	0·01	0
1·07	0·57	0·24	0·03	0·03	0	0	Cerebrospinal fluid	0·93	0·43	0·23	0·08	0·02	0	—

Each figure represents the mean value (μg/g wet tissue or ml) obtained from 2 dogs sacrificed at various time intervals. Duplicate determinations were performed on the individual tissues of each animal and the results averaged. Two dogs were sacrificed at each time interval for acute study. For the chronic study 1 dog each was used at 6 and 24 h. The values of the individual animals did not deviate more than $\pm$ 12% from the mean for the areas of the CNS and CSF. Other details as in Table 1. Dose of [^{3}H]cocaine was 5 mg/kg (free base).

*Norcocaine constituted approximately 10–18% of the total values expressed as cocaine in brain of acutely treated dogs and 21–30% of those in the chronically treated dogs (see Fig. 5).

With the exception of bile and liver, other tissues had very low levels of free cocaine 24 h after injection and only bile and liver had detectable amounts (50–80 ng/ml or g) of free cocaine 1 week after injection.

Table 3. Half-lives of cocaine in biological fluids, selected tissues and anatomical areas of the CNS of the acutely and chronically treated dogs after intravenous [^{3}H]cocaine

Tissues and fluids	Half-life (h)	
	Acute	Chronic
Temporal cortex (grey)	1·5	1·2
Temporal cortex (White)	1·5	1·2
Cerebellum	1·5	1·2
Spinal cord	1·5	1·5
Hypothalamus	1·5	1·2
Thalamus	1·5	1·2
Medulla	1·5	1·3
Pons	1·5	1·2
Mesencephalon	1·5	1·2
Caudate nucleus	1·6	1·3
Cerebrospinal fluid	1·0	1·1
Plasma	1·2	1·1
Liver	2·2	1·8
Kidney	1·8	1·2
Spleen	1·8	1·3
Heart	2·0	1·2

Details as given in Table 1. Dose of [^{3}H]cocaine was 5 mg/kg (free base)

The half-lives were calculated from semi-log linear regression plots of values on the distribution of cocaine in tissues and fluids of the acutely (0·5–12 h) and the chronically treated (0·5–6 h) dogs given in Tables 1 and 2.

Comparison of the slopes of the regression lines for different tissues and biological fluids in 2 groups by t test showed that the $t_{1/2}$ values in the chronically treated dogs were not significantly different from those in the acute group.

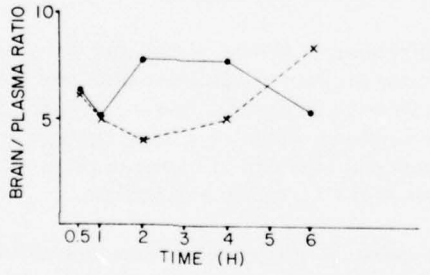

Fig. 3. *Brain-to-plasma ratios of mean concentrations of cocaine in acutely and chronically treated dogs following intravenous injection of* [^{3}*H*]*cocaine.*

Data represent mean values obtained from the ratio of concentration of cocaine in 10 selected anatomical areas of brain to plasma at different times post-injection of [^{3}H]cocaine (5 mg/kg free base). Acute expt. are shown as solid line, chronic expt. as broken line.

The approximate estimates of peak values of total radioactivity comprising free cocaine plus metabolites (μg/g or ml expressed as cocaine equivalents) and those 1 week after injection of 5 mg/kg dose of [^{3}H]cocaine in biofluids and tissues of acutely and chronically treated dogs respectively were as follows: bile 812, 5·4 and 748, 1·5; plasma 4·2, 0·6 and 4·5, 0·6; liver 51·4, 2·4 and 23·6, 2·0; kidney 27·3, 0·6 and 15·9, 0·7; lung 8·7, 0·5 and 8·1, 0·4; spleen 8·2, 0·6 and 13·4, 0·5, small intestine 5·8, 0·4 and 6·4, 0·5; duodenum 6·0, 0·5 and 8·7, 0·6; fat 3·7, 0·1 and 5·5, 0·4; heart 4·6, 0·5 and 5·6, 0·4; muscle 4·5, 0·5 and 4·3, 0·5.

Distribution of [^{3}*H*]*cocaine in the CNS:* Data on the comparative distribution of free cocaine in selected areas of the CNS of the acutely and chronically treated dogs following 5 mg/kg intravenous dose of [^{3}H]cocaine are given in Table 2. In general, the values of free cocaine were higher in chronic in all CNS areas as compared to the acute. The values of cocaine in the cerebrospinal fluid, however, were somewhat higher in the acute as compared to the chronic group. Complete disappearance of free cocaine was observed from all the selected anatomical areas of the CNS of acutely and chronically treated dogs 12–24 h after injection of [^{3}H]cocaine and no persistence of cocaine was observed in areas of CNS in two groups.

The approximate estimates of peak values of total radioactivity comprising free cocaine plus metabolites (μg/g or ml expressed as cocaine equivalents) and those 1 week after injection of 5 mg/kg dose of [^{3}H]cocaine in selected anatomical areas of the CNS of acutely and chronically treated dogs respectively were as follows: temporal cortex (grey) 6·2, 0·4 and 8·0, 0·5; temporal cortex (white) 6·9, 0·4 and 8·0, 0·5; cerebellum 5·1, 0·4 and 6·9, 0·5; spinal cord 5·9, 0·3 and 6·3, 0·4; hypothalamus 5·7, 0·4 and 7·3, 0·5; thalamus 6·2, 0·4 and 8·2, 0·5; medulla 5·6, 0·4 and 7·0, 0·4; pons 5·8, 0·4 and 7·0, 0·4; mesencephalon 5·6, 0·4 and 7·1, 0·5; caudate nucleus 6·1, 0·4 and 7·6, 0·5; cerebrospinal fluid 1·9, 0·5 and 2·5, 0·6. The values of total radioactivity in different areas of the CNS and CSF of chronically treated dogs were comparatively higher than those in the acute group up to 24 h and these tended to reach roughly similar levels thereafter in the two groups. At later time (1 week after injection) significant amounts of radioactivity due to metabolites persisted in the areas of the CNS, long after the disappearance of free cocaine.

Half-lives of [^{3}*H*]*cocaine in plasma, tissues and CNS:* Data on the apparent half-lives of [^{3}H]cocaine in plasma, selected tissues and the areas of the CNS of acutely and chronically treated dogs are given in Table 3. Statistical comparison of the slopes of the semi-log. linear regression plots of cocaine concentrations versus time for tissues and biofluids in 2 groups using t test did not show any significant differences in the $t_{1/2}$ values in 2 groups.

Brain-to-plasma ratios of [^{3}*H*]*cocaine:* The brain-to-plasma ratios of the mean concentrations of cocaine in the acutely and chronically treated dogs following 5 mg/kg intravenous dose of [^{3}H]cocaine are given in Fig. 3. These values, not different in two groups at 0·5, 1 h, were lower in the chronic group at 2, 4 h and higher at 6 h as compared to the acute group. The CSF to plasma ratios in the two groups (Fig. 4) were higher in acute than in the chronic dogs up to 2 h and not different at 4 and 6 h after injection.

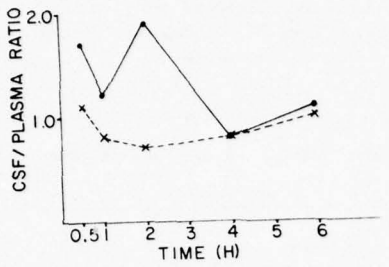

Fig. 4. *Ratio of mean concentration of cocaine in cerebrospinal fluid (CSF) to plasma (P) in acutely and chronically treated dogs following intravenous injection of [^{3}H]cocaine.*

Dose of [^{3}H]cocaine was 5 mg/kg (free base). Acute studies are shown by the solid line, chronic studies by the broken line.

Metabolites of cocaine in brain: Norcocaine, benzoylecgonine, benzoylnorecgonine and ecgonine were found to be the metabolites of cocaine in the brain of acutely and chronically treated dogs. Processing of brains of chronically treated dogs 1 week after the 5 mg/kg intravenous injections however, showed the presence of benzoylecgonine and benzoylnorecgonine only. The ratios of the amount of norcocaine to that of cocaine in brain of dogs in acutely and chronically treated groups at different times are given in Fig. 5. Norcocaine constituted

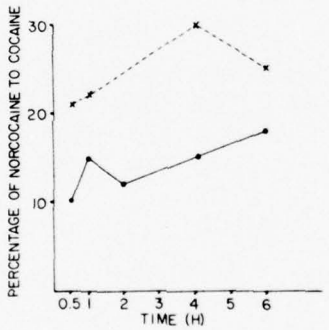

Fig. 5. *Ratio of mean concentrations of norcocaine to cocaine in brain of acutely and chronically treated dogs after intravenous [^{3}H]cocaine.*

The ratios were determined from the radioscans of t.l.c. of brain extracts at different times. Weighed samples of brain were homogenized in 0·5 M HCl to give a 10 or 20% homogenate which was adjusted to pH 9 with 1M NH_4OH and extracted with cyclohexane as described for cocaine. The residue from the organic solvent extract was subjected to t.l.c. (Nayak *et al.*, 1976) and relative proportions of norcocaine and cocaine determined from the radioscans of chromatograms.

Dose of [^{3}H]cocaine was 5 mg/kg. Acute studies are shown as solid line, chronic studies by the broken line.

10–18% of the total values of cocaine in brain of acutely treated animals and approximately 21–30% of those in the chronically treated dogs at these times (Table 2). The apparent half-life of norcocaine in dog brain was calculated to be approximately 1 h for the fast phase and 3·3 h for the slower phase following

Table 4. Urinary and faecal excretion of free cocaine and total radioactivity after intravenous injection of [*ring*-^{3}H]cocaine in acutely and chronically treated dogs

	Mean percentage of dose excreted at different time intervals								
	0–1 h	1–2 h	2–4 h	4–6 h	6–24 h	24–48 h	48–72 h	3–7 days	Total*
Acute									
Free cocaine (A)	0·2	0·2	0·3	0·1	0·1	0	0	—	0·9
in urine (B)	0·9	1·7	1·8	—	0·6	0	0	—	5·0
Free cocaine (A)	—	—	—	—	1·1	0·3	0·1	0·1	1·6
in faeces (B)	—	—	—	—	—	0·8	0·1	0·2	1·1
Total radio-activity (A)	7·4	9·5	6·2	6·6	14·5	2·2	1·1	1·6	49·1
in urine (B)	3·0	6·9	14·4	—	17·3	4·4	1·3	1·0	48·3
Total radio-activity (A)	—	—	—	—	13·7	2·4	1·6	2·7	20·4
in faeces (B)	—	—	—	—	—	10·5	0·8	1·7	13·0
Chronic									
Free cocaine (A)	0	1·2	0·7	0·1	0·2	0	0	—	2·2
in urine (B)	1·5	0·3	1·0	0·4	0·1	0	0	—	3·3
Free cocaine (A)	—	—	—	—	0·2	0	0	—	0·2
in faeces (B)	—	—	—	—	0·3	0	0	—	0·3
Total radio-activity (A)	0·1	19·6	13·8	5·9	16·4	1·6	0·7	1·0	59·1
in urine (B)	2·1	7·1	18·9	7·9	19·9	2·5	0·8	0·8	60·0
Total radio-activity (A)	—	—	—	—	4·8	0·5	0·2	0·2	5·7
in faeces (B)	—	—	—	—	3·5	1·3	0·2	0·4	5·4

Beagle dogs were subcutaneously injected with a 5 mg/kg dose of non-radioactive cocaine twice daily for 6 weeks, then with 5 mg/kg intravenous dose of [*ring*-^{3}H]cocaine in the chronic experiments.

Data represent mean values obtained from 2 female beagle dogs A and B. Urine was collected through an indwelling catheter every hour up to 6 h;. thereafter urine and faeces were collected in metabolism cages. Traces of free cocaine continued to be excreted in urine and faeces for several days.

*Statistical comparison using non-correlated 't' test showed no significant differences in values of free cocaine in urine, faeces and total radioactivity in faeces in the acutely and chronically treated dogs. Total radioactivity values in urine however, were significantly higher ($P < 0{\cdot}05$) in the chronically treated as compared to the acutely treated dogs.

5 mg/kg intravenous injection of [^{3}H]cocaine. The rate of disappearance of norcocaine from the brain of chronically treated dogs was similar to that in the acute group. Like cocaine, no persistence or accumulation of norcocaine was observed in the CNS of the acute or chronic dogs. In brains of chronically treated dogs, the amount of benzoylnorecgonine was approximately 4 times that in the acute dogs (460 ng/g) at 0·5 h and twice that in the acute (730 and 650 ng/g) in the 4 and 24 h period. The amount of benzoylecgonine in brains of chronically treated dogs however, was approximately one-third of that in the acute group (1040 ng/g) at 0·5 h and approximately twice that in acute (280 and 25 ng/g) at 4 and 24 h after injection. Ecgonine was present in several-fold higher concentration in brains of chronically treated dogs as compared to the acute 0·5 h post-injection.

Urinary and faecal excretion of cocaine and its metabolites: Data on the comparative urinary and faecal excretion of free cocaine and total radioactivity following 5 mg/kg intravenous dose of ring-labelled [^{3}H]cocaine in 2 dogs in both groups are given in Table 4. The amount of cocaine appearing in the urine of acutely and chronically treated dogs represented only a small percentage of the injected dose and varied in 2 animals (0·9, 5·0% in the acute, 2·2, 3·3% of dose in the chronic group). Similarly, the excretion of cocaine as the percentage of dose in faeces in the acute dogs was 1·1, 1·6; and 0·2, 0·3 in the chronic group. Major excretion of total radioactivity occurred in the urine within 24 h after injection in both acute and chronic groups. Excretion of metabolites as reflected in values of total radioactivity however, continued for

Table 5. Comparative urinary metabolic profile of cocaine in acutely and chronically treated dogs after intravenous [^{3}H]cocaine

	Mean % of dose excreted*	
	Acute	Chronic†
Cocaine	2·9	2·8
Norcocaine	0·8	0·7
Ecgonine methyl ester	0·1	0·1
Benzoylecgonine	2·5	3·8
Benzoylnorecgonine	9·1	6·9
Norecgonine	0·9	2·2
Ecgonine	37·1	33·0
Unidentified metabolites	5·6	6·7

Beagle dogs were subcutaneously injected with a 5 mg/kg dose of non-radioactive cocaine twice daily for 6 weeks, then with a 5 mg/kg intravenous dose of [*ring*-^{3}H]cocaine in the chronic experiments.

Data represent mean values from 2 female beagle dogs for each group. Urine was collected in metabolism cages.

*The mean percentage of dose excreted in 48 h after injection of labelled cocaine was 58% in the acute group and 56% in the chronic group.

†The values of free cocaine in individual animals in acutely and chronically treated dogs deviated approximately ±25 and ±20% from the mean respectively. The estimates of metabolites in individual animals obtained from the radioscans of the t.l.c. deviated approximately ±20 and ±15% from the means in the acutely and chronically treated dogs respectively. Statistical comparison of these means and deviations using standard non-correlated '*t*' test showed that the values in the 2 groups were not significantly different.

several days. There was a comparatively higher excretion of total radioactivity in urine as a percentage of dose in chronic (59–60%) as compared to the acute (48–49%), but total radioactivity values in faeces were higher in the acute (13–20%) than in the chronic group (5–6%). Excretion of total radioactivity as percentage of dose in urine and faeces in the two groups was, however, very similar.

Urinary metabolites of cocaine: Norcocaine, benzoylecgonine, benzoynorecgonine, norecgonine, ecgonine methyl ester, and ecgonine were identified as urinary metabolites of cocaine in both acutely and chronically treated dogs. Approximately 5–7% of the radioactivity was present as unidentified urinary metabolites in 48 h urine of dogs in both groups. The comparative urinary metabolic profile in acutely and chronically treated dogs is given in Table 5. There were no significant differences in the mean percentage of dose excreted as norcocaine and ecgonine methyl ester or unidentified metabolites. Benzoylnorecgonine and ecgonine were excreted in higher amounts in acute as compared to the chronic group and the amounts of benzoylecgonine and norecgonine were lower in the acute as compared to the chronic group.

Discussion

The rationale for the multiple injections of labelled cocaine in dogs chronically treated with non-labelled cocaine has been discussed earlier (Misra *et al.*, 1975 a; Nayak *et al.*, 1976). Rapid metabolism of cocaine in dogs was indicated by the high levels of radioactivity due to metabolites in bile at early times after injection and excretion of only a very small fraction of the dose as unmetabolized cocaine in urine and faeces. The faster mobilization of cocaine from the fat tissue of the chronically treated dogs was in sharp contrast to its prolonged sequestration in this tissue in chronically treated rats (Nayak *et al.*, 1976). Although comparatively higher values of urinary benzoylecgonine and norecgonine, rapid urinary elimination of cocaine metabolites, and lack of accumulation of radioactivity in tissues of the chronically treated dogs may suggest a faster metabolism of cocaine in this group, no significant differences in $t_{1/2}$ values of cocaine in tissues and biological fluids were observed in two groups.

The exact nature of the unidentified urinary metabolites of cocaine is not known at the present time but partition characteristics of these in different solvents indicated the highly polar nature of these metabolites.

The lower CSF-to-plasma ratio of cocaine in the chronically treated dogs indicated a rapid resorption of cocaine into plasma from the CSF in this group as compared to the acute. The lower brain to plasma ratios (Fig. 3) in the chronically-treated dogs 2 and 4 h after injection as compared to the acute indicated an altered distribution of cocaine in the two groups 1 h after injection of the drug. The rise and fall of the levels of cocaine in the CNS of dogs in the two groups coincided roughly with the change of levels in plasma and suggested that penetration of cocaine into and exit out of the cell in the CNS must be fairly rapid and any significant intra- or extra-cellular binding in the the CNS must be freely reversible.

The lack of persistence of cocaine in the CNS of acutely and chronically treated dogs is similar to that previously observed by us with thebaine, another

opiate stimulant (Misra *et al.*, 1974 c), but not with opiate depressants (Misra, Mitchell & Woods, 1971; Misra & Mulé, 1972; Misra *et al.*, 1973 a; Misra *et al.*, 1974 a). Norcocaine, an active metabolite of cocaine (Misra *et al.*, 1974 b; Hawks *et al.*, 1974), also did not persist in the CNS of the dog or the rat (Misra *et al.*, 1976 b). Metabolism of cocaine and norcocaine *in situ* in the CNS however, could lead to significant amounts of polar metabolites benzoylecgonine and benzoylnorecgonine, which possessed potent stimulant activity intracisternally in the rat (Misra *et al.*, 1975 b). The longer $t_{1/2}$ of benzoylnorecgonine, benzoylecgonine and ecgonine in rat brain and plasma as compared to cocaine (Misra *et al.*, 1974 d, 1975 b, 1976 a) may imply a comparatively slower clearance of these polar metabolites from the dog brain.

Persistence of polar metabolites, benzoylnorecgonine and benzoylecgonine, in the CNS of the chronically treated dogs, long after the disappearance of cocaine and norcocaine and molecular complexation of these metabolites with Ca^{2+} ions (Misra & Mulé, 1975), could conceivably play a role in the acquired sensitivity to cocaine observed in dogs on chronic treatment. The role of Ca^{2+} in the electrical stability of the neuronal membrane (Narahashi, Anderson & Moore, 1966), in inward current of action potential (Baker, Hodgkin & Ridgway, 1971), in coupling the electrical events of the membrane to the physiological response of the cell (Bianchi, 1968) and regulation of permeability of cell membrane for ions and neutral molecules (Whittam, 1968; Romero & Whittam, 1971; Holloszy & Narahara, 1967) is well documented. Alterations or displacement of neuronal membrane-bound Ca^{2+} (which normally keeps the membrane in a condensed state) by benzoylnorecgonine and benzoylecgonine could conceivably cause a conformational change in membrane proteins leading to changes in permeability to Na^{+} and K^{+}, depolarization of membrane and excitatory effects. Concurrently, a reduction in the Ca^{2+}-binding sites on membrane protein could also interfere with the release of neurotransmitters, e.g. norepinephrine and acetylcholine. As the adsorption of local anesthetics on the membrane induces a disordering and expansion of the hydrophobic regions of the membrane, an alteration in the interfacial tension at membrane–water interface and modification of energy transfer (Seeman, 1972), cocaine and its lipophilic metabolite norcocaine could also contribute in part to the sensitization observed in dogs on chronic treatment.

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COMMUNICATION NR. 6

DISPOSITION AND METABOLISM OF [^{3}H] COCAINE IN ACUTELY AND CHRONICALLY-TREATED MONKEYS

A.L. Misra, V.V. Giri, M.N. Patel, V.R. Alluri and S.J. Mule'

New York State Office of Drug Abuse Services

Testing and Research Laboratory

Brooklyn, New York 11217

Running Title: Cocaine disposition in monkeys

Correspondence, galley-proof & reprint requests to:

Dr.A.L. Misra
ODAS Testing and Research Laboratory
80 Hanson Place
Brooklyn, New York 11217
(U.S.A.)

ABSTRACT

Monkeys were chronically-treated with cocaine, 1 mg/kg s.c. twice daily for the first week and four times daily for the subsequent 21 weeks followed by the same dose of [^{3}H] cocaine intravenously. The $t_{\frac{1}{2}}$ values (hr) of cocaine in plasma, CSF, liver, kidney and heart of acutely and chronically-treated monkeys were 1.32, 0.85; 0.91, 0.75; 1.60, 1.29; 0.90, 0.91; 1.02, 0.91 respectively. In areas of the CNS the values in the acute and chronic group ranged between 0.75-0.90 and 0.62-0.84 hr respectively. With the exception of temporal cortex, cerebellum and caudate nucleus, the $t_{\frac{1}{2}}$ were not significantly different in 2 groups. Norcocaine, benzoylnorecgonine, benzoylecgonine and minor amounts of ecgonine were the metabolites of cocaine in brain of monkeys. Significant amounts of total radioactivity due to benzoylnorecgonine and benzoylecgonine persisted in the CNS of chronically-treated monkeys. Norcocaine constituted approximately 3,21, 8, 6% of the values of cocaine in the CNS areas in the acute and 14, 13, 14, 16% in the chronically-treated monkeys 0.25, 0.5, 1 and 2hr after cocaine injection. Biphasic pattern of peak levels in plasma and bile of chronically-treated monkeys indicated entero hepatic circulation of cocaine. Brain to plasma and CSF to plasma ratios of cocaine were higher in the chronic as compared to the acute group. The amounts of cocaine excreted in urine and feces as percentage of dose were 0.23-5.1; 0.1-0.23 in the acute and 0.54-7.0;0.1-0.14 in the chronic group. Major excretion of radioactivity occurred in urine within 24hr and the mean values of the total radioactivity in urine and feces (96hr) in the acute and chronic group were 60.4, 63.2% of dose respectively. Norcocaine, benzoylnorecgonine, benzoylecgonine, ecgonine methyl ester, ecgonine and unidentified compounds were the urinary metabolites of cocaine in the 2 groups. The percentage of benzoylecgonine was higher, that of benzoylnorecgonine lower in the chronic as compared to the acute group and the values of other metabolites were not markedly different in 2 groups. Data support the postulate that chronic treatment of monkeys with cocaine does not produce dispositional tolerance.

Recent increases in the abuse of cocaine in the current drug subculture have led to an increasing concern over its effects on prolonged administration. Although few systematic studies on the pharmacology of cocaine in man have been reported (1), studies in the monkey (2-4) have established the strong psychic dependence potential of cocaine but little or no tolerance and physical dependence liability. Considerable evidence exists for a reverse tolerance or increase in sensitivity to cocaine on repeated dosing (5-10). Apart from a recent study in the monkey (11), no information is available on the biological disposition of cocaine in this species. We have extended our previous work in the rat (8) and the dog (9) to investigate the disposition and metabolism of [^{3}H] cocaine in acutely and chronically-treated monkeys, a species which exhibits psychological effects so characteristic of chronic cocaine dependence in man.

MATERIALS AND METHODS

The preparation of [^{3}H] cocaine has previously been described (12) Randomly-labeled [^{3}H] cocaine (sp. act. 40 μCi/mg) was used in comparative tissue distribution experiments and ring-labeled [^{3}H] cocaine (sp. act. 48 μCi/mg) for excretion and metabolic studies because of the *in vivo* metabolic lability of methyl ester and benzoyl groups (8). Other details on radiochemical purity, method of estimation in biological materials, recoveries, specificity of extraction procedures, technique for radioscanning of thin layer chromatograms (Gelman ITLC, silica gel), counting

of radioactivity in Nuclear Chicago Mark III liquid scintillation counter etc. have been described (8, 13, 14). The methods of preparation of samples of different metabolites of cocaine have also been described (8).

Animal Experiments

Acute Studies

Male and female monkeys (M. Mulatta, approximately 3 years of age), received from the supplier were tuberculin-negative and carefully observed over a quarantine period of 4 weeks. Subsequently, they were transferred to large community cages accommodating 5 monkeys where they were permitted complete freedom of movement. The housing quarters were air-conditioned and the floors were covered with wood shavings as litter to maintain the animals in a dry and relatively clean condition. The animals were maintained on a diet of Purina Monkey Chow and continuous running water for drinking purposes was supplied to minimize any danger arising from stagnant water source. Food was withdrawn from cages approximately 16 hr prior to the administration of cocaine.

Male and female monkeys (3.5-4.5 Kg) were transferred to a restraining chair and given intravenous injections of 1 mg/kg (free base) dose of [^{3}H] cocaine. After appropriate periods of time, the animals were sacrificed by a rapid i.v. injection of thiopental sodium, followed by ex-sanguination. Blood samples were collected in heparinised vacutainer tubes (Becton-Dickinson Co, Rutherford, N.J.) approximately 1-2 min before the selected time (prior to the injection of thiopental sodium) by vein-puncture. Plasma immediately obtained by centrifugation was quickly frozen (-20^{o}).

Brain, heart, lung, liver, kidney, intestine, muscle, fat etc. were quickly removed, washed with ice-cold 0.9% saline, wiped dry in tissue paper, wrapped in aluminum foils and these tissues and samples of bile and cerebrospinal fluid were stored frozen until analysed. Prior to analysis, brain was carefully separated into various selected anatomical areas and these and other tissue samples (approximately 1g. wherever possible) were homogenized in 0.5 M HCl with a teflon pestle to provide a 10 or 20% homogenate. Duplicate samples (2 ml) of these homogenates and diluted plasma (1:5 with water) were analysed for free cocaine and total radioactivity as previously described (8,9).

Chronic studies

Five monkeys were injected subcutaneously each with 1 mg/kg (free base) dose of nonlabeled cocaine twice daily(at 10 AM and 3PM)for the first week and four times daily between (8:30AM and 5:30PM) for the subsequent 21 weeks. A 1 mg/kg s.c. dose of [^{3}H] cocaine was then administered four times during the day followed by a terminal dose of labeled cocaine by i.v. injection the next day. Samples of plasma, bile, CSF and tissues were obtained at various time intervals and analysed for free cocaine and total radioactivity by the previous procedure (8,9).

In the course of chronic studies, periodic weight check, blood and urine analysis were carried out.

Urinary and fecal excretion of ring-labeled [^{3}H] cocaine in acutely and chronically-treated monkeys:

Following a 1 mg/kg i.v. dose of ring-labeled [^{3}H] cocaine in 2 female monkeys, urine was collected at hourly intervals from an indwelling Bardex-Foley catheter (Fr.8) for the first six hours of injection.

The monkeys were given i.m. injections of gentamicin (5mg/kg) 6, 24, 48 and 72 hr after the injection of cocaine to prevent infection Urine and feces were subsequently collected in metabolism cages every 24 hr for 4 days. After collection of urine samples, cages were washed with a small volume of water and the washings were added to the sample. All samples were kept frozen until analysed. Feces were homogenized in a known volume of 0.5 M HCl, filtered through a coarse cloth and aliquots of filtrate analysed for free cocaine and total radioactivity as previously described (8). Aliquots of urine diluted with water were similarly analysed.

The same monkeys used in the acute experiments were treated chronically with nonlabeled cocaine (1 mg/kg s.c.) twice daily during the first week, then 4 times daily with same dose for a period of 3 weeks, followed by a single 1 mg/kg i.v. injection of ring-labeled [^{3}H] cocaine. Urine and feces were collected and analysed as above.

Identification of cocaine metabolites in the brain and urine of acutely and chronically-treated monkeys.

The method used was essentially similar to that described earlier (9).

Polyacrylamide gel electrophoresis of plasma proteins in the acutely and chronically-treated monkeys.

Electrophoresis of plasma samples was done as described earlier (15).

RESULTS

Pharmacological and behavioral effects

The 1 mg/kg (free base) dose of cocaine by i.v. injection produced restlessness and excitement in the monkeys immediately after injection but no convulsions. . . . . .

With higher doses (5 mg/kg i.v.) convulsions occurred intermittently and started within 5 min of injection, along with the spasmodic lateral movements of the head. These effects lasted for 1 hr and in some monkeys, this excitatory phase was followed by depression in which the animals went into shock and died of respiratory and cardiac arrest 2.5 hr after injection. A 3 mg/kg i.v. dose of cocaine produced rapid breathing, restlessness, tremors, spasmodic movements of the head and intermittent convulsions immediately after injection and these effects lasted for approximately 45 min. Some animals went into post-stimulatory depression phase 2 hr after injection even with this dose and died 2 hr later. With these doses, hyperthermia (approximately 43°C) was observed in all monkeys. The exact cause of death in these monkeys is not clear. A sensitivity to cocaine or lack of adaptation to the primate chair and consequent stress during the onset of convulsions could possibly be involved. Clinical analysis of blood obtained by cardiac puncture immediately after death showed low levels of blood sugar. Significantly high levels of metabolites e.g. benzoylnorecgonine and benzoylecgonine were observed in the areas of the CNS of the post-mortem samples.

During the course of chronic treatment with cocaine, a marked craving for drug was observed and the animals would come out of the community cage in an orderly fashion to offer their backs for s.c. injections. During the course of chronic treatment, no significant change in food intake, physical condition or body weight was observed in response to cocaine. Repeated administration (1 mg/kg s.c. four times a day) of cocaine induced very rapid psychological dependence in the monkeys.

Comparative distribution of [^{3}H] cocaine in biological fluids and tissues of acutely and chronically-treated monkeys.

Data on the distribution of free cocaine in biological fluids and tissues of acutely and chronically-treated monkeys after 1 mg/kg (free base) dose of [^{3}H] cocaine by i.v. injection are given in Table 1. The values of free cocaine in bile of chronically-treated monkeys were initially higher at 0.25, 0.5 and 4 hr and comparatively lower at 1 and 2 hr as compared to those in the acutely-treated monkeys. The values in liver were higher in the chronically-treated group as compared to the acute at all times. The values in kidney, lungs, spleen, pancreas, small intestines, fat and heart were initially higher at 0.25, 0.5, 1 hr in the chronically-treated group but fell somewhat lower than those in the acutely-treated monkeys at later times. No marked differences in values of cocaine were observed in duodenum and muscle in the 2 groups. A biphasic pattern of rise and fall was observed for free cocaine in plasma of the chronically-treated monkeys and these values were slightly higher in chronically-treated monkeys as compared to the acute up to 1 hr. The $t_{1/2}$ values of cocaine in some tissues and fluids of monkeys (Table 3) although comparatively lower in the chronically-treated animals, were not significantly different from those in the acutely-treated monkeys.

The values of total radioactivity (as cocaine equivalents) comprising free cocaine plus metabolites were comparatively higher in bile, spleen, intestine, pancreas, fat, heart and muscle in the chronically-treated monkeys as compared to the acute. The values in liver, kidney and duodenum were comparatively lower in the chronic as compared to the acute. The values in lungs initially higher up to 60 min, declined to values lower than those in the acute group at later times.

Distribution of cocaine in selected anatomical areas of the CNS of acutely and chronically-treated monkeys

Data on the distribution of free cocaine in selected areas of the CNS and in CSF of acutely and chronically-treated monkeys after 1 mg/kg (free base) dose of [^{3}H] cocaine by i.v. injection are given in Table 2. The values of free cocaine in various areas of the CNS and the CSF of the chronically-treated monkeys were higher at 0.25 and 0.5 hr as compared to those in the acutely-treated monkeys. These values in the chronic group were either similar or lower than those in the acute group at later times. With the exception of cortex, cerebellum and caudate nucleus, the $t_{\frac{1}{2}}$ values of cocaine in selected areas of the CNS although comparatively lower in the chronically-treated animals, were not significantly different from those in the acutely-treated monkeys (Table 3).

In general, the values of total radioactivity (as cocaine equivalents) comprising free cocaine plus metabolites were higher in all areas of the CNS of the chronically-treated monkeys at all times after administration of [^{3}H] cocaine. The values in CSF in the chronically-treated monkeys were approximately twice those in the acute group.

Comparative profile of metabolites of cocaine in the brain of acutely and chronically-treated monkeys.

Norcocaine, benzoylnorecgonine, benzoylecgonine and minor amounts of ecgonine were observed as metabolites of cocaine in the CNS of acutely and chronically-treated monkeys. Norcocaine, determined from the radioscans of tlc of brain extracts at different times after 1 mg/kg i.v. injection of [^{3}H] cocaine by a previous procedure (9), constituted approximately 3, 21, 8, 6% of the total values expressed as cocaine 0.25, 0.5, 1 and 2 hr after

cocaine injection in the acute experiments and 14, 13, 14,16% respectively in the chronic experiments. Benzoylnorecgonine values in chronic group were approximately 15, 7, 5, 2.5, 10 times those in the CNS of the acutely-treated monkeys at 0.25, 0.5, 1, 2, 4 hr respectively. The content of benzoylecgonine in the CNS of chronically-treated monkeys although similar in the 2 groups at 0.5 hr was approximately 1.5 to 2.5 times higher in the chronic group as compared to the acute at subsequent times. With higher i.v. doses (3 and 5 mg/kg) of cocaine, the same metabolites were observed in the CNS of monkeys. In all cases of death with these higher doses of cocaine, the content of benzoylnorecgonine in the CNS was found to be markedly higher than that of norcocaine. In 4 monkeys which died in the post-stimulatory depression phase after cocaine administration, the values of benzoylnorecgonine in brain were 19, 4, 2,6 times those of norcocaine respectively.

Brain to plasma ratios of cocaine in acutely and chronically-treated monkeys

With the exception of values at 1 hr after cocaine injection, the values of brain to plasma ratios of cocaine 0.25, 0.5, 2 and 4 hr after injection were comparatively higher in chronically-treated monkeys as compared to the acute (Fig.1).

CSF to plasma ratios of cocaine in acutely and chronically-treated monkeys

With the exception of values at 1 hr, the CSF to plasma ratios 0.25, 0.5, 2 and 4 hr after cocaine injection were comparatively higher in the chronically-treated monkeys as compared to the acute (Fig.2)

Electrophoretic plasma-protein profile in acutely and chronically-treated monkeys.

Polyacrylamide gel electrophoresis of proteins from the freshly prepared plasma of the control, acutely and chronically-treated monkeys did not show any observable differences.

Urinary and fecal excretion of free cocaine and total radioactivity in the acutely and chronically-treated monkeys.

The percentage of dose excreted as free cocaine in urine in 2 animals each in acutely (0.23, 5.1) and chronically-treated (0.54, 7.02) monkeys differed considerably (Fig. 3). Fecal excretion of free cocaine in both groups ranged between 0.1 - 0.2% of the dose. There were no significant differences in the mean values of total radioactivity in urine in acutely (55.4%) and chronically-treated (56.2%) monkeys. The mean fecal excretion of total radioactivity was 4.95 and 7.04% of the dose in the acutely and chronically-treated monkeys respectively. Major excretion of total radioactivity and of free drug in urine occurred within 0 to 6 and 6 to 24 hr. in both groups.

Comparative urinary metabolic profile of cocaine in acutely and chronically-treated monkeys.

Norcocaine, benzoylnorecgonine, benzoylecgonine, ecgonine methyl ester, ecgonine were the urinary metabolites of cocaine in monkeys in the 2 groups (Fig. 4). The percentage of dose excreted as benzoylecgonine was higher and of benzoylnorecgonine lower in the chronic as compared to the acute group. The values of ecgonine methyl ester, norcocaine and unidentified metabolites were not markedly different in 2 groups. No attempt was made to characterize the unidentified metabolites of cocaine which were highly polar in nature.

DISCUSSION

The rationale for the multiple injections of labeled cocaine in experimental animals chronically-treated with non-labeled cocaine has previously been discussed (8,9). As in the case of the rat and the dog (8,9), high levels of cocaine metabolites in bile and excretion of only a very small fraction of the dose as unmetabolised cocaine in urine and feces of monkeys indicated very rapid metabolism of cocaine. The use of one animal each at different time periods in the acutely and chronically-treated groups precludes a statistical comparison of the individual values of cocaine in the 2 groups. However, a valid statistical comparison of the rates of disappearance of cocaine in 2 groups could be made from the slopes of the semi-log. regression plots of values on the distribution of cocaine in tissues and fluids of animals in the 2 groups. With the exception of temporal cortex, cerebellum and caudate nucleus (Table 2), the $t_{\frac{1}{2}}$ of cocaine in other areas of the CNS and most of the tissues of the chronically-treated monkeys were not significantly different from those in the acutely-treated group. This would suggest that chronic treatment of monkeys with cocaine does not produce dispositional tolerance, an observation in accord with the recent work of Kibbe and Wilson (11) in the monkey and our previous work in the dog (9)

Biphasic pattern of peak levels of cocaine in plasma and bile may suggest enterohepatic circulation of cocaine in the chronically-treated monkey. Higher CSF to plasma ratio's at different times in chronically-treated monkeys indicated a slower resorption of cocaine into plasma from CSF in this group as compared to the acute. Differences in brain to plasma ratios at different times after injection indicated an altered distribution of cocaine

in 2 groups. The lack of persistence of cocaine and its lipophilic metabolite norcocaine (16-18) in the CNS of the monkeys in both groups was similar to that observed earlier in the rat and the dog (8,9) and suggested that the penetration of cocaine into and the exit from the cell in the CNS of the monkey must be fairly rapid and any significant intra or extracellular binding must be freely reversible. The longer $t_{\frac{1}{2}}$ of the polar metabolites, benzoylnorecgonine, benzoylecgonine and ecgonine in brain and plasma as compared to cocaine and norcocaine (19-21), which probably accounts for their persistence in significant amounts in the CNS of the monkeys and the ability of these polar metabolites to form complexes with Ca^{2+} (22) have been implicated by us (8,9) to play a possible role in the reverse tolerance or increase in sensitization to cocaine on repeated dosing reported by several workers (5-10). Other authors (23) have however suggested that progressively increasing effects of cocaine on pathological behavior and seizures rather than tolerance in experimental animals may be related to electrical kindling, a phenomenon in which repetitive subthreshold stimulation of the limbic system is eventually associated with major motor seizures.

Recent electrophysiological studies in our laboratory (24) with cocaine given by constant i.v. infusion to the monkey, have however, shown a progressive increase in daily minimal convulsant dose of cocaine upon chronic administration and a marked reduction in stimulatory effects of cocaine on heart and respiratory rates following daily injection of the same dose of cocaine. In our disposition studies and those of others (5-7,10) in which reverse tolerance or increase in sensitization on

chronic cocaine treatment has been observed, the compound has been generally administered systemically to the animals in subconvulsant doses. In view of the very rapid metabolism of cocaine, its effects varied considerably with the dose and route of administration. It is conceivable that the neuronal mechanism underlying tolerance to the convulsant effects of cocaine in the monkey (24) may differ from those for other excitatory effects. In regard to the concepts of tolerance or reverse tolerance to cocaine, recent electrophysiological studies (24,25) have underscored the need for a rigorous delineation of the particular response measured and limiting of conclusions solely to those responses.

Acknowledgment

This work was supported by U. S. Army Medical Research and Development Command Contract No. DADA.-17-73-C-3080. The authors express their grateful thanks for excellent assistance provided by Dr. A. Goldberg in blood chemistry work, Dr. M. Matsuzaki, Mrs. J. Vardy and Mr. A. Kramer in other phases of this work.

LEGENDS FOR FIGURES

Fig. 1 Brain to plasma ratios of mean concentrations of cocaine in acutely and chronically-treated monkeys following intravenous injection of 1 mg/kg (free base) dose of [^{3}H] cocaine. Data represent mean values obtained from the ratio of concentrations of cocaine in 10 selected anatomical areas of brain to plasma at different times after injection. Acute studies are shown as solid lines and chronic studies as dotted lines.

Fig. 2 Ratio of mean concentrations of cocaine in cerebrospinal fluid to plasma in acutely and chronically-treated monkeys following intravenous injection of 1 mg/kg (free base) dose of [^{3}H] cocaine. Acute studies are shown as solid lines and chronic studies as dotted lines.

Fig. 3 Cumulative urinary and fecal excretion of free [^{3}H] cocaine and total radioactivity after intravenous injection of 1 mg/kg (free base) dose of [^{3}H] cocaine in 2 acutely and 2 chronically-treated female monkeys. Data represent mean percentages of dose excreted at different times from 2 female monkeys. For the chronic study, the same monkeys used in the acute study were given s.c. injection of 1 mg/kg dose of non-labeled cocaine twice daily for 1 week and 4 times daily for subsequent 3 weeks before the final i.v. injection of [ring-^{3}H] cocaine. Urine was collected through an indwelling catheter every hour up to 6 hr and thereafter urine

and feces were collected in metabolism cages.

Fig. 4 Comparative urinary profile of cocaine and its metabolites in acutely and chronically-treated monkeys after a 1 mg/kg (free base) dose by i.v. injection. Data represent mean values from 2 female monkeys for each group. The schedule of chronic experiments is stated in footnote of Fig 3. The mean percentage of radioactivity excreted in urine over 96 hr was 55.4 and 56.2% of the dose in the acute and chronic group respectively.

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TABLE 1

Comparative distribution[a] of [^{3}H] cocaine in various tissues and fluids of the acutely and chronically-treated[b] rhesus monkeys at intervals after a 1 mg/kg (free base) dose by intravenous injection

Tissues[c] and fluids	0.25 hr	0.5 hr	1 hr	2 hr	4 hr
ACUTE					
Bile	1.63	10.51	25.07	24.64	0.84
Liver	0.62	0.68	0.64	0.35	0.14
Kidney	1.57	1.18	0.96	0.32	0.09
Lungs	1.34	1.43	0.79	0.48	0.09
Spleen	1.21	1.27	0.82	0.37	0.08
Small Intestine	0.20	0.46	0.19	0.10	0.04
Duodenum	0.91	0.96	0.61	0.26	0.10
Pancreas	1.66	1.36	0.79	0.32	0.09
Fat	0.17	0.37	0.10	0.33	0.04
Heart	0.30	0.42	0.26	0.12	0.03
Muscle	0.65	0.53	0.27	0.08	0.02
Plasma	0.05	0.04	0.02	0.02	0.006
CHRONIC					
Bile	3.92	15.26	12.48	11.03	11.70
Liver	1.47	1.17	0.85	0.64	0.18
Kidney	2.29	1.10	0.64	0.20	0.11
Lungs	2.74	2.08	1.09	0.28	0.15
Spleen	2.76	1.86	1.17	0.32	0.11
Small Intestine	0.59	0.57	0.38	0.13	0.03
Duodenum	0.97	0.72	0.44	0.20	0.19
Pancreas	1.66	1.94	1.12	0.26	0.11
Fat	0.17	0.46	0.33	0.08	0.04
Heart	0.65	0.51	0.31	0.08	0.04
Muscle	0.54	0.49	0.37	0.06	0.02

Bile	1.63	10.51	25.07	24.64	0.84
Liver	0.62	0.68	0.64	0.35	0.14
Kidney	1.57	1.18	0.96	0.32	0.09
Lungs	1.34	1.43	0.79	0.48	0.09
Spleen	1.21	1.27	0.82	0.37	0.08
Small Intestine	0.20	0.46	0.19	0.10	0.04
Duodenum	0.91	0.96	0.61	0.26	0.10
Pancreas	1.66	1.36	0.79	0.32	0.09
Fat	0.17	0.37	0.10	0.33	0.04
Heart	0.30	0.42	0.26	0.12	0.03
Muscle	0.65	0.53	0.27	0.08	0.02
Plasma	0.05	0.04	0.02	0.02	0.006
CHRONIC					
Bile	3.92	15.26	12.48	11.03	11.70
Liver	1.47	1.17	0.85	0.64	0.18
Kidney	2.29	1.10	0.64	0.20	0.11
Lungs	2.74	2.08	1.09	0.28	0.15
Spleen	2.76	1.86	1.17	0.32	0.11
Small Intestine	0.59	0.57	0.38	0.13	0.03
Duodenum	0.97	0.72	0.44	0.20	0.19
Pancreas	1.66	1.94	1.12	0.26	0.11
Fat	0.17	0.46	0.33	0.08	0.04
Heart	0.65	0.51	0.31	0.08	0.04
Muscle	0.54	0.49	0.37	0.06	0.02
Plasma	0.07	0.03	0.06	0.01	0.003

a. Each figure represents mean value (micrograms per g. wet tissue weight or ml. of fluid) obtained from the monkeys sacrificed at various time intervals. Duplicate determinations were performed on the individual tissues of each animal and the results averaged. One monkey was used for each time period in acute and chronic experiments.

b. The monkeys were injected s.c. with 1 mg/kg (free base) dose of nonlabeled cocaine twice daily for the first week and four times daily for the subsequent 21 weeks. A 1 mg/kg s.c. dose of randomly-labeled [^{3}H] cocaine was then administered four times during the day, followed by a terminal injection of the same dose by i.v. injection the next day.

c. Norcocaine constituted not more than 5% of the total values expressed as cocaine in different tissues possibly due to its rapid biotransformation to benzoylnorecgonine.

TABLE 2

Comparative distribution[a] of [^{3}H] cocaine in selected tissues of the CNS of the acutely and chronically-treated[b] rhesus monkeys at various intervals after a 1 mg/kg (free base) dose by intravenous injection

Selected tissues of the CNS[c]	0.25 hr	0.5 hr	1 hr	2 hr	4 hr
ACUTE					
Temporal Cortex (gray)	0.82	0.98	0.62	0.20	0.04
Temporal Cortex (white)	0.84	0.91	0.63	0.20	0.04
Cerebellum	0.70	0.79	0.61	0.18	0.03
Spinal Cord	0.69	0.67	0.53	0.33	0.04
Hypothalamus	0.77	0.82	0.57	0.20	0.03
Thalamus	0.78	0.85	0.57	0.18	0.03
Medulla	0.66	0.67	0.41	0.23	0.03
Pons	0.79	0.73	0.56	0.22	0.03
Mesencephalon	0.71	0.75	0.46	0.20	0.03
Caudate Nucleus	0.93	1.05	0.71	0.29	0.06
Cerebrospinal fluid	0.13	0.18	0.12	0.04	0.01
CHRONIC					
Temporal Cortex (gray)	1.62	1.20	0.68	0.16	0.03
Temporal Cortex (white)	1.37	1.10	0.69	0.16	0.03
Cerebellum	1.47	0.90	0.56	0.22	0.02
Spinal Cord	0.97	0.75	0.64	0.13	0.05
Hypothalamus	1.20	0.90	0.52	0.16	0.03
Thalamus	1.33	0.99	0.52	0.13	0.03
Medulla	1.12	0.89	0.54	0.15	0.03
Pons	1.26	0.98	0.59	0.17	0.03
Mesencephalon	1.32	0.90	0.55	0.13	0.03
Caudate Nucleus	1.60	1.20	0.69	0.18	0.04
Cerebrospinal fluid	0.29	0.24	0.14	0.04	0.01

ACUTE					
Temporal Cortex (gray)	0.82	0.98	0.62	0.20	0.04
Temporal Cortex (white)	0.84	0.91	0.63	0.20	0.04
Cerebellum	0.70	0.79	0.61	0.18	0.03
Spinal Cord	0.69	0.67	0.53	0.33	0.04
Hypothalamus	0.77	0.82	0.57	0.20	0.03
Thalamus	0.78	0.85	0.57	0.18	0.03
Medulla	0.66	0.67	0.41	0.23	0.03
Pons	0.79	0.73	0.56	0.22	0.03
Mesencephalon	0.71	0.75	0.46	0.20	0.03
Caudate Nucleus	0.93	1.05	0.71	0.29	0.06
Cerebrospinal fluid	0.13	0.18	0.12	0.04	0.01
CHRONIC					
Temporal Cortex (gray)	1.62	1.20	0.68	0.16	0.03
Temporal Cortex (white)	1.37	1.10	0.69	0.16	0.03
Cerebellum	1.47	0.90	0.56	0.22	0.02
Spinal Cord	0.97	0.75	0.64	0.13	0.05
Hypothalamus	1.20	0.90	0.52	0.16	0.03
Thalamus	1.33	0.99	0.52	0.13	0.03
Medulla	1.12	0.89	0.54	0.15	0.03
Pons	1.26	0.98	0.59	0.17	0.03
Mesencephalon	1.32	0.90	0.55	0.13	0.03
Caudate Nucleus	1.60	1.20	0.69	0.18	0.04
Cerebrospinal Fluid	0.29	0.24	0.14	0.04	0.01

a. Each figure represents mean value (micrograms per g. wet tissue weight or ml. fluid) obtained from the monkeys sacrificed at various time intervals. Duplicate determinations were performed on the individual tissues of each animal and the results averaged. One monkey was used for each time period in acute and chronic experiments.

b. The monkeys were injected s.c. with 1 mg/kg (free base) dose of nonlabeled cocaine twice daily from the first week and four times daily for the subsequent 21 weeks. A 1 mg/kg s.c. dose of randomly-labeled [^{3}H] cocaine was then administered four times during the day, followed by a terminal injection of the same dose by i.v. injection the next day.

c. Norcocaine constituted approximately 3, 21, 8, 6 % of the total values expressed as cocaine 0.25, 0.5, 1 and 2 hr after injection of [^{3}H] cocaine in the acute experiments and 14, 13, 14, 16 % respectively in the chronic experiments.

TABLE 3

Half-lives[a] of cocaine in biological fluids, selected tissues and anatomical areas of the acutely and chronically-treated monkeys[b] after 1 mg/kg (free base) intravenous injection of [^{3}H] cocaine

Tissues and Fluids	Half-life (hr) Acute	Chronic[c]
Temporal Cortex (gray)	0.80	0.64
Temporal Cortex (white)	0.80	0.66
Cerebellum	0.77	0.62
Spinal Cord	0.90	0.84
Hypothalamus	0.76	0.70
Thalamus	0.75	0.68
Medulla	0.83	0.71
Pons	0.78	0.69
Mesencephalon	0.79	0.68
Caudate nucleus	0.89	0.70
Cerebrospinal fluid	0.91	0.75
Liver	1.60	1.29
Kidney	0.90	0.91
Lungs	0.94	0.88
Heart	1.02	0.91
Muscle	0.73	0.74
Plasma	1.32	0.85

a. The half-lives were calculated from semi-log regression plots of values of cocaine in tissues and fluids of monkeys given in Tables 1 and 2.

b. The schedule of chronic experiments is stated in footnotes of Tables 1, 2.

c. Comparison of the slopes of the regression lines for different tissues and biological fluids in 2 groups by "t" test showed that the t 1/2 values in the chronically-treated monkeys were significantly different($p < 0.05$) from those in acutely-treated animals only for temporal cortex (gray and white), cerebellum and caudate nucleus. The values for other tissues were not significantly different in 2 groups.

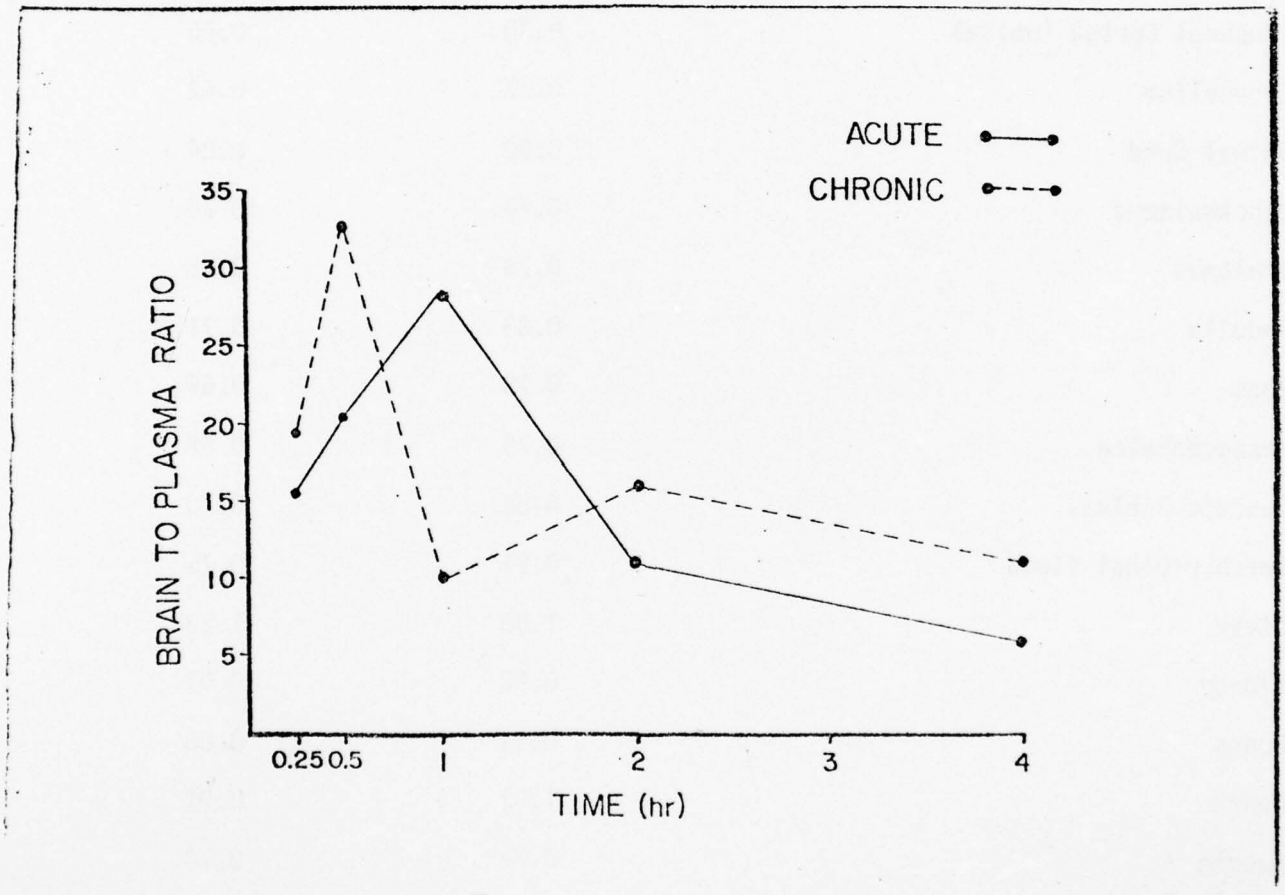

FIG. 1 -- MISRA ET AL

[^{3}H] Cocaine

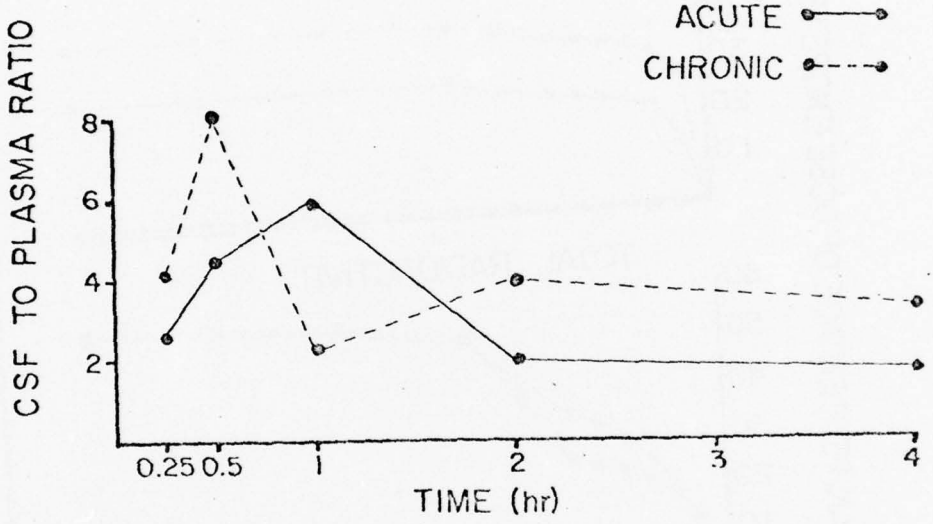

FIG 2 - MISRA ET AL

[^{3}H] Cocaine

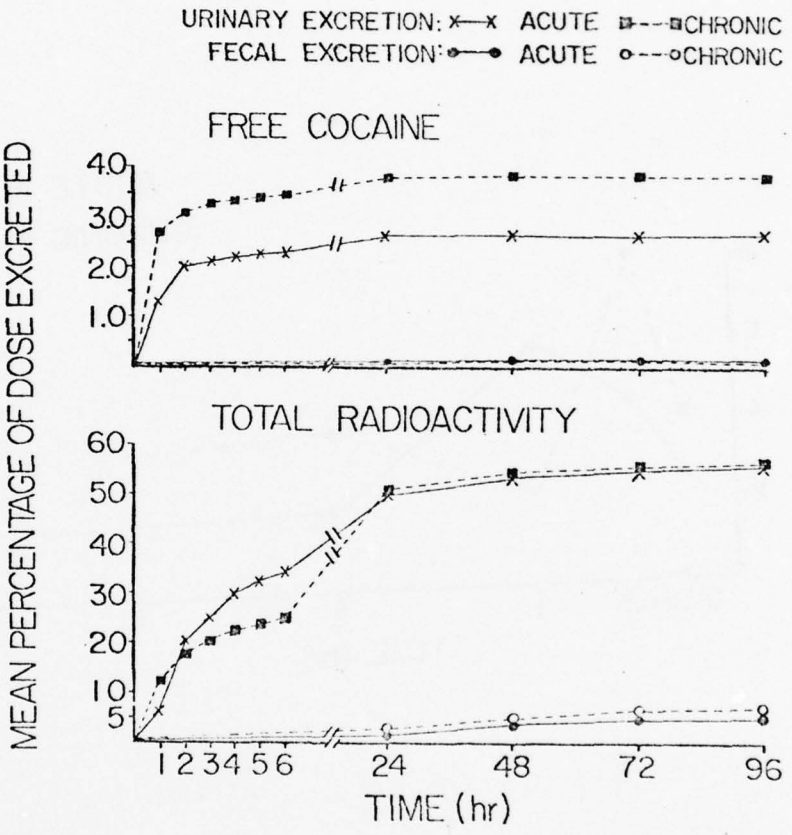

FIG 3 — MISRA ET AL

[^{3}H] Cocaine

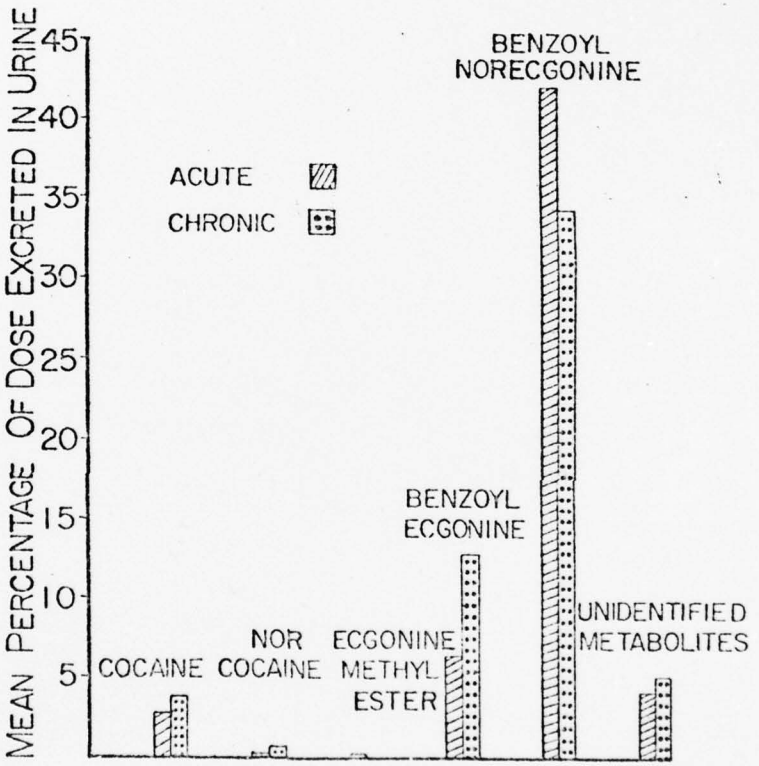

FIG 4 — MISRA ET AL

(^{3}H) Cocaine

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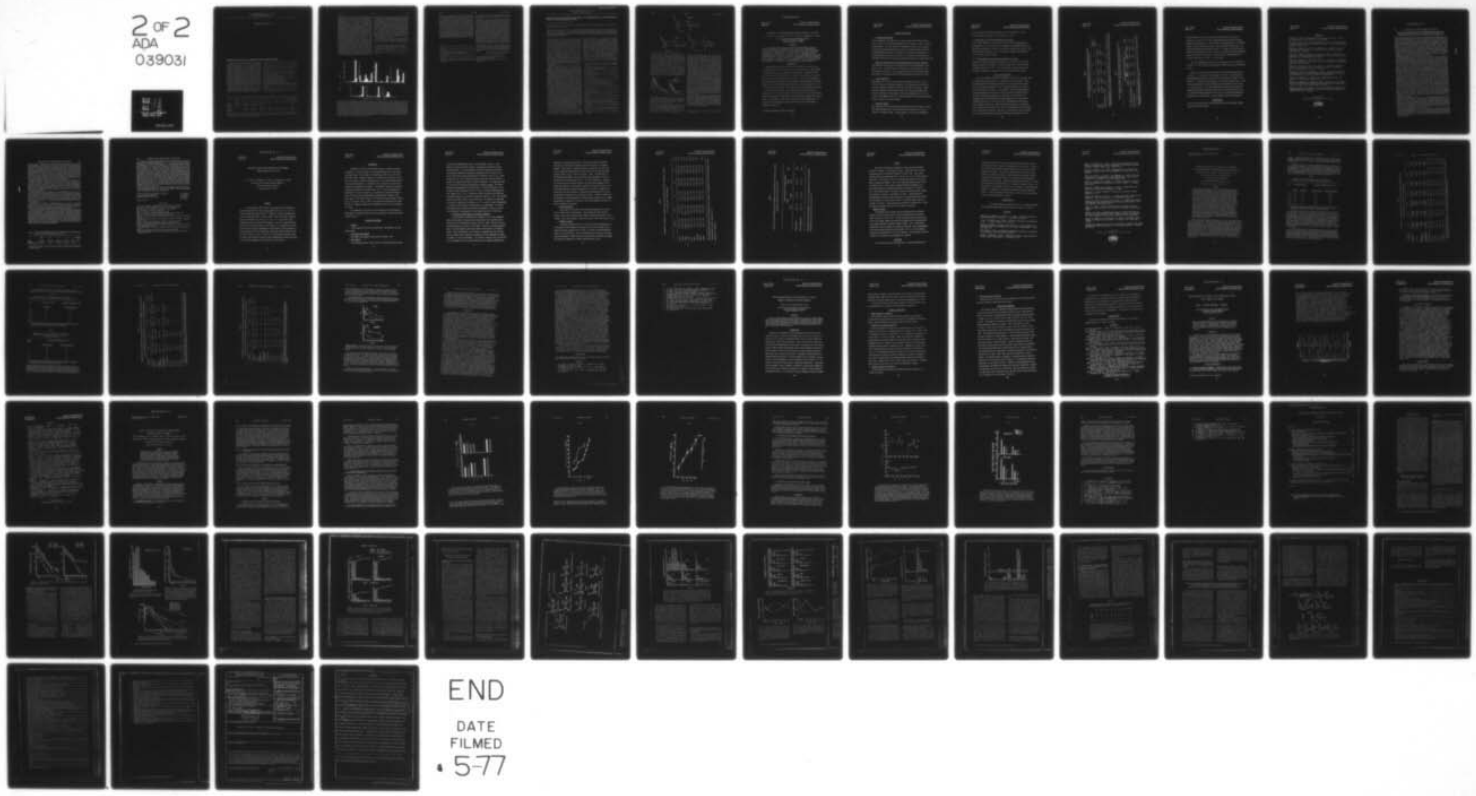

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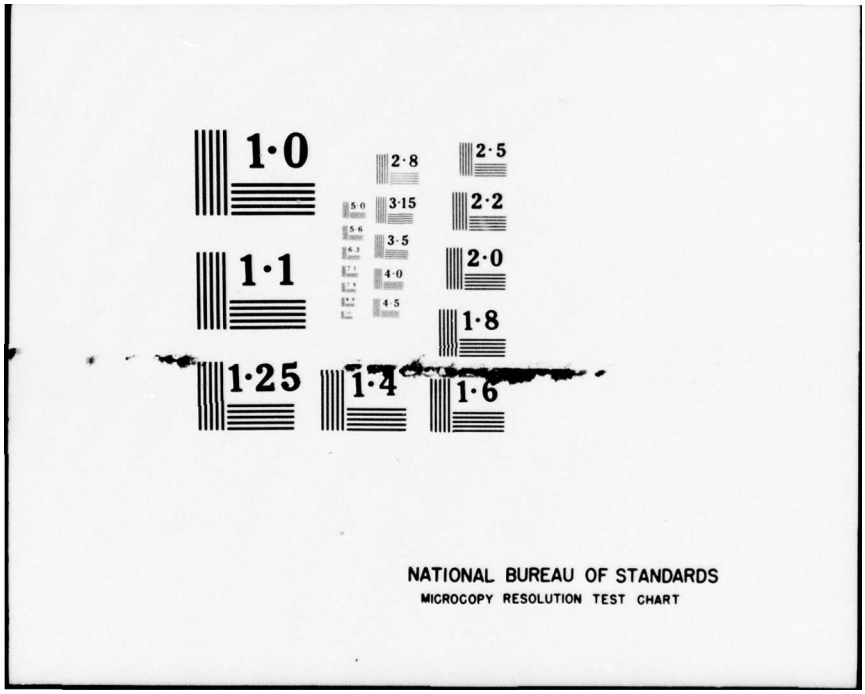
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Separatum EXPERIENTIA *30*, 1312 (1974)
Birkhäuser Verlag, Basel (Schweiz)

PUBLICATION NR 7

Identification of Norcocaine as a Metabolite of [^{3}H]-Cocaine in Rat Brain

Cocaine is a powerful central nervous system stimulant of relatively short duration, low margin of safety, high systemic toxicity (LD_{50} i.v. in rats 17.5 mg/kg) and little recognized medical use. Its intense euphoriant action leads to a very high degree of psychic dependence[1,2] and a profound and dangerous type of drug abuse. Physical dependence and tolerance to its euphoria and toxic effects has, however, not been reported to develop. In spite of much work[3–14], the dispositional and metabolic profile of cocaine in the central nervous system remains unknown. This study deals with these parameters and demonstrates that: a) cocaine disappeared fairly rapidly from the rat brain after s.c. and i.v. injections; b) in addition to the formation of benzoylecgonine, benzoylnorecgonine and ecgonine as metabolites, N-demethylation occurs rapidly in the brain to form norcocaine, a lipophilic compound with convulsant properties similar to those of cocaine.

Materials and methods. Male Wistar rats (120–150 g) were injected s.c. with 20 mg kg^{-1} (free base) dose or i.v. with 8 mg kg^{-1} (free base) dose of [^{3}H] cocaine prepared as described previously[15] (radiochemical purity 98%,

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Uptake of [^{3}H] cocaine in brain and plasma of male Wistar rats after a single 8 mg kg^{-1} (free base) i.v. or 20 mg kg^{-1} (free base) s.c. injection

	0.25 h	0.5 h	1 h	2 h	4 h	6 h	12 h	24 h	Half-life (h)
Brain (i.v.)	7269 ± 177	2738 ± 634	924 ± 131	212 ± 48	8 ± 3	0	—	—	0.4
Plasma (i.v.)	612 ± 62	296 ± 90	111 ± 14	26 ± 8	0	—	—	—	0.3
B/P[a] (i.v.)	11.9	9.3	8.3	8.1	—	—	—	—	
Brain (s.c.)	—	2237 ± 282	2320 ± 205	2963 ± 195	3439 ± 362	485 ± 194	4 ± 1	0	4.8
Plasma (s.c.)	—	249 ± 51	378 ± 31	448 ± 74	494 ± 59	78 ± 35	2 ± 1	0	5.0
B/P[a] (s.c.)	—	9.0	6.1	6.6	6.9	6.2	2	—	

Data represent the mean value ± S.E.M. (ng/g of tissue or ml of fluid) of 3 animals for i.v. and 5 animals for s.c. group at each time period.
[a] B/P represents the ratio of mean brain to plasma concentrations.

specific activity 12 μCi/mg). The brains and plasma at different periods of time were obtained as previously described[16–19]. Free [^{3}H] cocaine was determined by homogenization of brain in 0.5 *M* HCl (10% homogenate), adjusting the pH of a 2 ml aliquot of homogenate or diluted plasma (1:5) containing 1 ml nonradioactive cocaine hydrochloride carrier (500 μg/ml) with dilute ammonia to pH 8–9; this was buffered with 1 ml of 20% K_2HPO_4 solution and extracted with 15 ml cyclohexane by shaking for 15 min and the organic phase was centrifuged for 10 min, washed with 4 ml 0.1 *M* NaOH solution. The residue obtained by evaporating 10 ml aliquots of organic phase in counting vials was dissolved in 0.5 ml isopropanol and radioactivity counted with 10 ml toluene-phosphor solution. In vitro recoveries of [^{3}H] cocaine from tissue homogenates and fluids by this method were quantitative in the concentration range 1–1000 ng and the minimal amount of cocaine detectable was 1 ng. The method did not extract benzoylecgonine, benzoylnorecgonine and ecgonine, but norcocaine, a minor metabolite was extracted. A rough estimate of the amount of norcocaine present in rat brain 3.5 h following a 20 mg kg^{-1} s.c. dose was 10% of the extracted value.

The qualitative identification of [^{3}H] cocaine and its metabolites was done on groups of 5 brains removed 3.5 h after a single s.c. injection of 20 mg kg^{-1} dose. The brains were homogenized in 0.5 *M* HCl (20% homogenate), centrifuged to remove debris, the pH of the supernatant adjusted to 7.5 and chromatographed on Amberlite XAD-2 The adsorbed cocaine and its metabolites were eluted with methanol and the residue from the eluate was chromatographed[20] on ITLC (silica gel) with 4 solvent systems and chromatograms radioscanned. The brain homogenate supernatant containing unadsorbed radioactivity from Amberlite XAD-2 column was adjusted to pH 10–12 with dilute NaOH and extracted repeatedly with 12 times its volume of chloroform-methanol (68:32, v/v). The filtered organic extract was evaporated to dryness in vacuo and the residue was chromatographed on ITLC with solvent systems: S_1 (Figure g), S_2 and S_4, respectively and paper chromatographed with *n*-butanol-acetic acid-water (4:1:5, v/v).

Results and discussion. The mean values on the uptake of [^{3}H] cocaine by rat brain and plasma at different time intervals after a single s.c. injection of 20 mg kg^{-1} dose or

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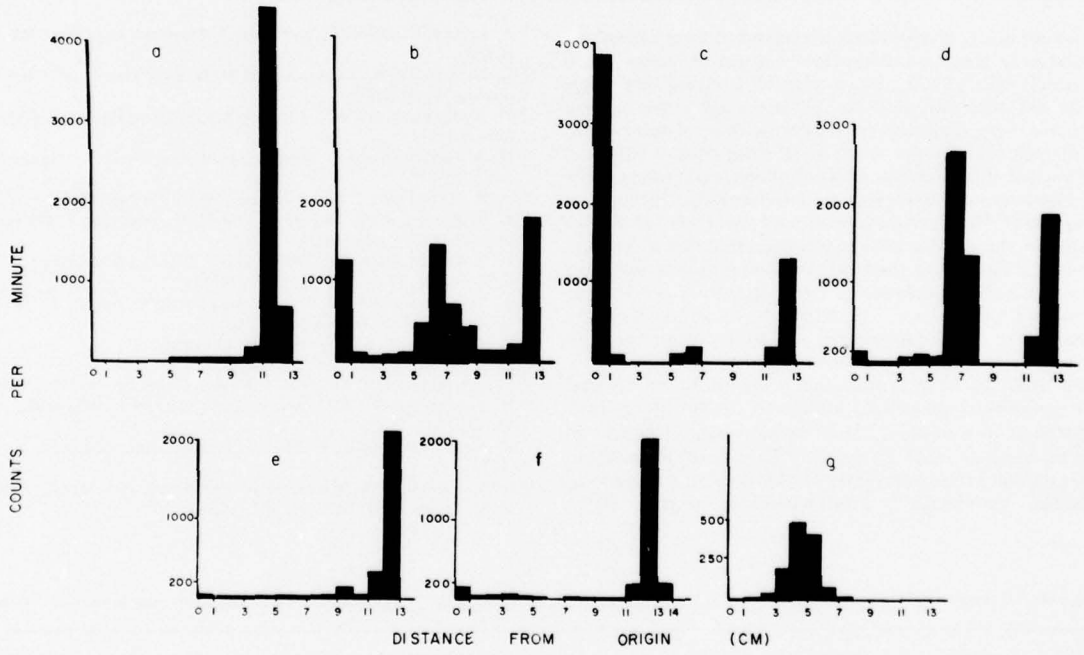

Radioscans of thin-layer chromatograms (silica gel) of brains of rats injected s.c. with 20 mg kg^{-1} dose of [^{3}H] cocaine, removed 3.5 h after injection, processed on Amberlite XAD-2 resin, methanol-soluble residue from Amberlite XAD-2 resin in a) solvent system S_1: *n*-butanol-acetic acid-water (35:3:10, v/v) a single peak of radioactivity Rf 0.88; b) in solvent system S_2: chloroform-acetone-diethylamine (5:4:1, v/v), 3 peaks of radioactivity Rf 0.05 (benzoylnorecgonine), 0.5 (benzoylecgonine), 0.96 (cocaine and norcocaine) with distribution of radioactivity as 20, 49, 31%, respectively; c) in solvent system S_3: *n*-hexane-ethyl acetate-concentrated ammonia (60:40:0.1, v/v), 3 peaks of radioactivity Rf 0.05 (benzoylnorecgonine, benzoylecgonine), 0.55 (norcocaine), 0.96 (cocaine) with distribution of radioactivity as 66, 8, 26%, respectively; d) in solvent system S_4: ethyl acetate-methanol-concentrated ammonia (17:2:1, v/v) 4 peaks of radioactivity Rf 0.05 (unknown), 0.35 (benzoylnorecgonine), 0.55 (some benzoylnorecgonine and benzoylecgonine), 0.96 (cocaine and norcocaine) with distribution of radioactivity as 3, 4, 61 and 32%, respectively; e) separation of norcocaine and cocaine on ITLC (silica gel), solvent system S_3, Rf norcocaine and cocaine 0.72, 0.96, respectively; f) ITLC (alumina) in solvent system benzene-ether (8:2, v/v), Rf norcocaine, cocaine 0.05–0.3 and 0.9 respectively; g) ITLC of the organic solvent extract of brain homogenate from Amberlite XAD-2 column containing unadsorbed radioactivity, in solvent system S_1, a single peak of radioactivity due to ecgonine Rf 0.43.

8 mg kg^{-1} i.v. injection dose are given in the Table. The radioscans (details given in Figure a–d) provided evidence for 4 metabolites which were separately eluted by previous techniques[16–19] and characterized by co-chromatography with standard samples[21], as benzoylnorecgonine, benzoylecgonine, norcocaine and an unidentified metabolite. Details of separation of norcocaine from cocaine on ITLC (silica gel) and ITLC (alumina) are shown in Figure e–f. Norcocaine was also detected as a metabolite of cocaine in rat brains within 1 min of onset of convulsions following a 10 mg kg^{-1} i.v. dose of [^{3}H] cocaine. A single peak of radioactivity in solvent systems S_1 (Figure g), S_2, S_4 and paper chromatogram and co-chromatography with standard ecgonine confirmed the identify of Amberlite XAD-2-unadsorbed metabolite as ecgonine. The brain debris remaining after the extraction with 0.5 *M* HCl after washing with water, recentrifugation and counting by earlier procedure[22] showed significant bound radioactivity.

Oxidation of cocaine at ambient temperature with hydrogen peroxide reportedly[23] leads to the formation of fairly stable nitroxide free radical and such radicals in vivo have been reported[24] to be reduced to norcompounds by membrane sulfhydryl groups. The formation of norcocaine as a metabolite of cocaine in rat brain conceivably may play an important role in the systemic toxicity of cocaine. The lack of persistence of cocaine in rat brain for a prolonged period is similar to that observed previously[19,25] with thebaine, an opioid stimulant which also possesses little or no physical dependence liability. In contrast, depressant narcotic analgesic drugs such as morphine[22] and methadone[16,18] with very high tolerance and physical dependence liability, persisted in the rat brain for prolonged periods even after a single s.c. injection. These observations may have some relevance to the absence of tolerance and physical dependence liability with cocaine[26].

Zusammenfassung. Nach s.c. und i.v. Injektionen von Cocain in Ratten wurde Cocain im Hirn rasch metabolisiert. Ausser Benzoylecgonin, Benzoylnorecgonin und Ecgonin wurde Norcocain als wichtiger Metabolit identifiziert.

A. L. Misra, P. K Nayak, M. N. Patel, N. L. Vadlamani and S. J. Mulé

New York State Drug Abuse Control Commission, Testing and Research Laboratory, 80 Hanson Place, Brooklyn (New York 11217, USA),
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[^{3}H]-Norcocaine and [^{3}H]-Pseudococaine: Effect of N-Demethylation and C_2-Epimerization of Cocaine on its Pharmacokinetics in the Rat[1]

A. L. Misra, R. B. Pontani and S. J. Mule'

New York State Office of Drug Abuse Services, Testing and Research Laboratory, 80 Hanson Place, Brooklyn (New York 11217, USA), 12 February 1976.

Summary. After i.v. injections of cocaine, norcocaine, pseudococaine to the rat, the $T_{1/2}$ in brain were 0.4, 0.6, 0.2 h respectively and in plasma 0.4, 0.5, 0.2 h respectively. Benzoylnorecgonine and norecgonine were the metabolites of norcocaine in brain. Pseudonorcocaine, pseudobenzoylnorecgonine, pseudobenzoylecgonine and pseudoecgonine were the metabolites of pseudococaine in rat brain. Benzoylnorecgonine and pseudobenzoylecgonine had potent stimulant activity intracisternally in the rats.

Norcocaine (Figure 1) has been reported to be an important metabolite of cocaine in the rat[2,3] dog[4] and monkey[5]. Like cocaine, it is a powerful central nervous system stimulant[6,7] of short duration and it contributes possibly in part to the pharmacological effects of cocaine. Pseudococaine (dextro isomer and C_2-epimer of cocaine) has the C_2-methyl ester group in equatorial configuration and *trans* to the nitrogen and C_3-benzoyloxy side chain[8] (Figure 1). Its toxicity and local anesthetic activity is several-fold higher than cocaine[9–11]. Both isomers have convulsant and paralysant properties[10–14], but pseudococaine is several times less potent than cocaine as an inhibitor of norepinephrine uptake in ventricles and vas deferens slices[13,15]. It has been reported to be more extensively degraded than cocaine[16,17] in cats. No information exists on the dispositional and metabolic profile of norcocaine and pseudococaine in the central nervous system. This study was undertaken to obtain information on these parameters.

Materials and methods. Norcocaine was prepared by a procedure previously described[18]. Pseudococaine was prepared by conversion of cocaine to pseudo ecgonine methyl ester[8] and subsequent benzoylation with benzoylchloride in benzene using standard procedures. Samples (50 mg) of these non-labelled compounds were tritiated (New England Nuclear, Boston, Mass.) by acid-catalyzed exchange tritium labelling using 0.3 ml acetic acid, 25 mg platinum catalyst and 10 curies of tritiated water. The crude products were exhaustively purified in our laboratory by repeated solvent extractions and silica gel column chromatography (eluant, benzene-methanol mixtures of increasing polarity). The specific activities and radiochemical purities of [^{3}H]-norcocaine and [^{3}H]-pseudococaine were 2.56 mCi/mg, 96% and 1.97 mCi/mg, 98% respectively. The hydrochlorides of these compounds were suitably diluted with nonradioactive compounds in 0.9% saline to specific activities of 64 and 40 μCi/mg respectively for the preparation of injection solutions.

Male Wistar rats (120–150 g) were injected i.v. with 2 mg kg^{-1} (free base) dose of [^{3}H]-cocaine, [^{3}H]-norcocaine or [^{3}H]-pseudococaine. The brain and plasma at different times were obtained as previously described[19,20]. Free [^{3}H]-norcocaine and [^{3}H]-pseudococaine were determined in brain and plasma by the procedure used for cocaine[2,3]. In vitro recoveries of [^{3}H]-norcocaine and [^{3}H]-pseudococaine were in the range described for cocaine[2,3]. Other details on the specificity of extraction procedure, counting technique, etc., have been previously described[3].

The qualitative identification of metabolites of norcocaine and pseudococaine was done in groups of 5 brains of rats injected with 2 mg kg^{-1} i.v. dose of these compounds. The brains were homogenized in 0.5 *M* HCl (20% homogenate) centrifuged, supernatant removed and its pH adjusted to 7.5 with dilute ammonia. The resultant solution was chromatographed on Amberlite XAD-2 (2 × 15 cm) and the adsorbed compounds and their metabolites eluted with methanol. The residue from this methanol eluate was chromatographed on Gelman instant thin layer chromatography media silica gel (ITLC) with different solvent systems[21] for characterization of metabolites. The solution containing unadsorbed radioactivity from the Amberlite XAD-2 column was saturated with K_2CO_3 and extracted repeatedly with chloroform-isopropanol (2:1, v/v), the organic phase evaporated to dryness in vacuo and the residue therefrom submitted to ITLC for characterization of the metabolites.

Results and discussion. Comparative distribution of [^{3}H]-cocaine, [^{3}H]-norcocaine and [^{3}H]-pseudococaine in rat brain and plasma after a 2 mg kg^{-1} i.v. injection

[1] Acknowledgment. This work was supported by U.S. Army Medical Research and Development Command Contract No. DADA-17-73-C-3080.

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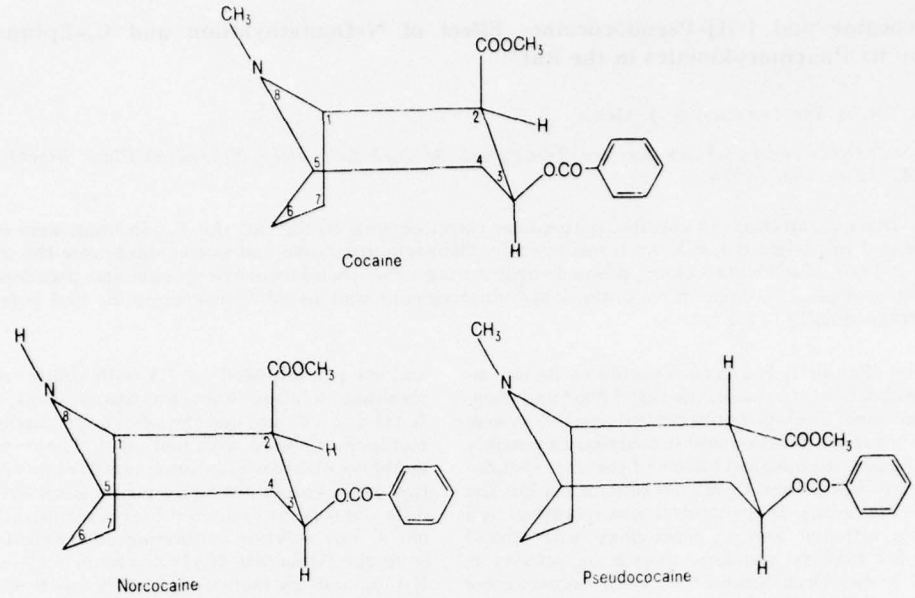

Fig. 1. Structures of cocaine, norcocaine and pseudococaine.

is shown in Figure. 2. The peak concentrations of norcocaine in brain and plasma were approximately 2.3 times those of cocaine. The apparent partition coefficient of [^{3}H]-norcocaine in 1-octanol-M/15 phosphate buffer, pH 7.4 in the concentration range 1–10 µg/ml at 25°C was 18.5 ± 0.2 (SEM). Inspite of the high lipid solubility of pseudococaine (apparent partition coefficient in 1-octanol-M/15 phosphate buffer, pH 7.4, 77.2 ± 2.2 (SEM), its peak concentrations in brain were approximately half those of cocaine, while the plasma levels were approximately 2.5 times those of cocaine. Rapid metabolism of pseudococaine led to comparatively lower brain levels. Like cocaine, norcocaine and pseudococaine did not persist in the CNS and the levels in brain and plasma were barely detectable 6 h post-injection. The half-lives of

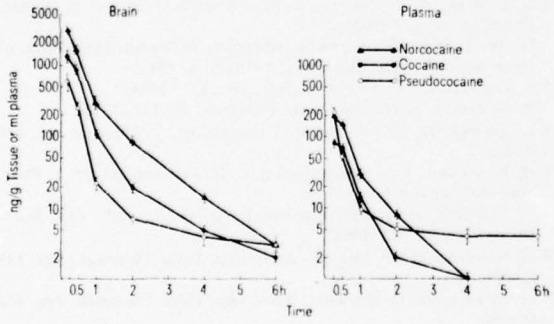

Fig. 2. Comparative distribution of [^{3}H]-cocaine, [^{3}H]-norcocaine and [^{3}H]-pseudococaine in brain and plasma of male Wistar rats after a single 2 mg kg^{-1} (free base) dose by i.v. injection. Data represent mean value ± SEM (ng per g wet tissue weight or ml fluid) of 6 determinations from 3 animals at each time.

cocaine, norcocaine and pseudococaine in rat brain were 0.4, 0.6 and 0.2 h respectively; that in plasma 0.4, 0.5 and 0.2 h respectively. The brain to plasma ratios 0.25, 0.5, 1, 2, 4 h post-injection were as follows: cocaine 15.9, 11.5, 7.9, 9.5, 5; norcocaine 15.7, 10.6, 9.7, 10.9, 14; pseudococaine 2.8, 4.7, 2.4, 1.4, 1.0 respectively.

Thin layer chromatographic experiments on brain extracts (30 min) of rats injected i.v. with norcocaine provided evidence for the presence of major amounts of norcocaine (72%), benzolnorecgonine (24%) and minor amounts of norecgonine (4%). Benzoylnorecgonine has earlier been shown[6] to possess potent stimulant activity intracisternally (i.c.) in the rat and formed molecular complex[22] with Ca^{2+}. Similar experiments with pseudococaine provided evidence for the presence in brain of pseudonorcocaine (12%), pseudobenzoylnorecgonine (12%), pseudobenzoylecgonine (8%) and pseudoecgonine (minor amounts) as metabolites of pseudococaine (66%).

Our preliminary studies on the pharmacological activity of these metabolites showed that pseudoecgonine and pseudoecgonine methyl ester (1 mg kg^{-1} i.c.) showed no activity in the rat. Pseudobenzoylecgonine at this dose produced intermittent convulsions lasting approximately 15 sec and death within 10 min; lower doses 0.5 mg kg^{-1} (i.c.) produced rapid heart beat, laboured breathing, running activity, shivering, disorientation, jerking and convulsions 4 min post-injection lasting until death 30 min later. A 0.25 mg kg^{-1} dose (i.c.) produced similar effects without mortality. Pseudococaine (0.5–1 mg kg^{-1} i.c. rats) produced rapid heart beat, laboured breathing, gasping and death within 1 min and lower doses produced similar effects without mortality. Norcocaine (i.v.) was approximately 2 to 3 times more potent a stimulant as compared to cocaine.

This study demonstrates that N-demethylation and C_2-epimerization of cocaine lead to compounds which have significantly different dispositional profile in the CNS, and have more potent stimulant activity as compared to cocaine. In addition, some of the polar metabolites of norcocaine and pseudococaine, e.g. benzoylnorecgonine and pseudobenzoylecgonine, possessed potent stimulant activity intracisternally in the rat.

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DISPOSITION OF [^{3}H] BENZOYLNORECGONINE (COCAINE METABOLITE) IN THE RAT

A.L. MISRA*, V.V. GIRI, M.N. PATEL, V.R. ALLURI, R.B. PONTANI AND S.J. MULE'

NEW YORK STATE OFFICE OF DRUG ABUSE SERVICES
TESTING AND RESEARCH LABORATORY
BROOKLYN, NEW YORK, 11217

ABSTRACT

The preparation and disposition of [^{3}H] benzoylnorecgonine, which has potent stimulant activity intracisternally in the rat, has been described. The T1/2 of [^{3}H] benzoylnorecgonine in brain and plasma of rats injected with a 10 mg kg^{-1} i.v. dose were 3.0, 1.2 h respectively. The ratio of mean peak concentration in brain to that in plasma was 0.03. No metabolites of benzoylnorecgonine were observed in rat brain. The mean percentage of dose excreted in urine and feces in 96 h were 85 and 2.2, respectively, with major excretion (82.5%) occurring within 24 h in urine. Approximately 90% of the radioactivity in urine was due to unmetabolised benzoylnorecgonine and 10% due to an unidentified metabolite. Norecgonine was not detected as a urinary metabolite.

INTRODUCTION

Benzoylnorecgonine has been shown to be a metabolite of cocaine in the rat (Misra et al., 1974a; Nayak et al., 1976), dog (Misra et al., 1976a; 1976b) and the monkey (Misra et al., unpublished observations). Although not active by systemic routes of administration, it possessed potent stimulant activity in the rat on intracisternal injection (Misra et al., 1975) and was approximately ten times more potent compared to benzoylecgonine, another metabolite of cocaine. Benzoylecgonine and benzoylnorecgonine persisted in selected anatomic areas of the CNS of the dog, long after the disappearance of cocaine following a 5 mg kg^{-1} i.v. injection of [^{3}H] cocaine. These polar metabolites formed molecular complexes with Ca^{2+} (Misra and Mule', 1975) and may play a role in the acquired sensitivity to cocaine in experimental animals on chronic treatment (Misra et al., 1976b). No information exists on the disposition of benzoylnorecgonine. We report here the preparation and physiological disposition of [^{3}H] benzoylnorecgonine in the rat.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

1. [^{3}H] Benzoylnorecgonine

This was prepared by the hydrolysis of carrier-diluted [^{3}H] norcocaine (Misra et al., 1976c) by a microscale adaptation of the procedure of Findlay (1954) for benzoylecgonine. The final product was repeatedly recrystallized from ethanol to furnish [^{3}H] benzoylnorecgonine, specific activity approximately 188 uci/mg and radiochemical purity 95%. This material was diluted with nonradioactive benzoylnorecgonine to a specific activity of 15 uci/mg for the preparation of injection solution in 0.9% saline, using appropriate amount of 1N hydrochloric acid.

2. Method of estimation of [^{3}H] benzoylnorecgonine in biological materials

The method used was essentially that described for [^{3}H] benzoylecgonine (Misra et al., 1975). The concentration of nonlabeled carrier solution used in the extractions was 250 ug/ml. The in vitro recoveries of [^{3}H] benzoylnorecgonine were similar to those described for [^{3}H] benzoylecgonine (Misra et al., 1975).

3. Animal experiments

Male Wistar rats (110-160g.) were injected intravenously (tail vein) with a 10 mg kg^{-1} dose of [^{3}H] benzoylnorecgonine. After appropriate time intervals, brain and blood were collected as described previously (Misra et al., 1974b). Blood was immediately centrifuged to obtain the plasma and the samples kept frozen till analysed. Known weights of brain were homogenised in 0.9% saline to give a 20% homogenate and 2 ml. aliquots were taken in duplicate and analysed. Plasma, diluted, was similarly treated.

4. Metabolic studies

Four male Wistar rats were injected intravenously each with a 10 mg kg^{-1} dose of [^{3}H] benzoylnorecgonine and urine and feces were collected at 24, 48, 96 h intervals in metabolism cages. Diluted samples of urine or fecal homogenate

were analysed for [^{3}H] benzoylnorecgonine and total radioactivity by the previous method (Misra et al., 1975; Nayak et al., 1976).

5. Metabolites of [^{3}H] benzoylnorecgonine in rat brain

Brain of rats injected i.v. with 10 mg kg^{-1} dose of [^{3}H] benzoylnorecgonine and removed 30 min. after injection were homogenised and extracted without the addition of nonradioactive carrier by the previous procedure (Misra et al., 1975). The residue from the organic phase was dissolved in methanol and subjected to ITLC in different solvent systems.

6. Urinary metabolites of [^{3}H] benzoylnorecgonine in the rat

Pooled urine from rats injected intravenously with a 10 mg kg^{-1} dose of [^{3}H] benzoylnorecgonine was repeatedly extracted (without carrier) by the previous procedure (Misra et al., 1975). The residue from the organic solvent extract dissolved in methanol was subjected to ITLC in different solvent systems.

RESULTS AND DISCUSSION

Data on the uptake of [^{3}H] benzoylnorecgonine in rat brain and plasma, brain to plasma ratios after 10 mg kg^{-1} i.v. injection are given in Table 1. Mean peak concentrations of [^{3}H] benzoylnorecgonine in brain were attained within 15 min. and were sustained upto 30 min. The levels declined to 0.10 ug/g in brain and 0.44 ug/ml in plasma 6 h post-injection. Barely detectable amounts of benzoylnorecgonine were present in brain and plasma 48 h post-injection. The ratio of mean peak concentration in brain to that in plasma was 0.03 (apparent partition coefficient of [^{3}H] benzoylnorecgonine in 1-octanol-M/15 phosphate buffer, pH 7.4, 0.21+0.01 (S.E.M). Benzoylecgonine (Misra et al., 1975), ecgonine (Misra et al., 1974c) and cocaine (Misra et al., 1974a) gave brain to plasma ratios of 0.05-0.12, 0.03, 11.9 respectively with a similar dose by the same route of injection in the rats. The low values of brain to plasma ratio of benzoylnorecgonine showed that the highly polar character of this

TABLE 1

Uptake[a] of [^{3}H] benzoylnorecgonine in brain and plasma of male Wistar rats after a 10 mg kg^{-1} dose by intravenous injection.

	0.25 h	0.5 h	1 h	3 h	6 h	Half-life (h)
Brain	0.41±0.01	0.39±0.02	0.26±0.03	0.17±0.02	0.10±0.01	3.0
Plasma	15.27±0.61	11.02±0.70	5.11±0.54	1.29±0.01	0.44±0.01	1.2
Brain/plasma ratio	0.027	0.035	0.051	0.13	0.23	

a. Data are mean values ± S.E.M. (ug g^{-1} wet tissue weight or ml fluid) from 3 animals at each time. Duplicate determinations were performed on tissue and fluid of each animal and the results averaged.

TABLE 2

Urinary and fecal excretion[a] of [^{3}H] benzoylnorecgonine and total radioactivity after a 10 mg kg^{-1} intravenous injection in male Wistar rats.

	Mean percentage of dose excreted					
	URINE			FECES		TOTAL
	0-24 h	24-48 h	48-96 h	0-24 h	24-96 h	
Free [^{3}H] benzoylnorecgonine	82.5±3.8	1.6±0.5	0.9±0.3	1.8±0.3	0.4±0.1	87.2
Total radioactivity	85.0±4.6	1.8±0.6	1.0±0.3	2.4±0.3	0.5±0.1	90.7

a. Data represent the mean percentage ± S.E.M. of the administered dose of [^{3}H] benzoylnorecgonine recovered from the urine and feces of 4 male rats.

compound prevented its entry through the blood-brain barrier in high concentrations. In spite of this, small concentrations entered the CNS and were gradually cleared within 48 h. The presence of benzoylnorecgonine in significant amounts in brains of dogs (Misra et al., 1976a) and monkeys (Misra et al., unpublished observations) administered 5 mg kg^{-1} i.v. doses of [^{3}H] cocaine would therefore imply metabolism in part of cocaine to norcocaine and benzoylnorecgonine in situ in the CNS of these animals. The half-life of benzoylnorecgonine in rat brain and plasma was 3.0 and 1.2 h respectively.

Thin layer chromatographic studies on brain extracts of rats injected with 10 mg kg^{-1} i.v. dose of [^{3}H] benzoylnorecgonine showed absence of any metabolites in brain.

Data on the urinary and fecal excretion of [^{3}H] benzoylnorecgonine after a 10 mg kg^{-1} i.v. injection in the rat appear in Table 2. Renal excretion of benzoylnorecgonine began very fast and 82.5% of the dose was excreted in urine within 24 h. Excretion of approximately 91% of the total radioactivity in urine and feces showed that this polar compound was not sequestered in rat tissues on systemic injection. Thin layer chromatographic studies on the extracts of pooled urine provided evidence that 90% of the urinary radioactivity was due to unmetabolised benzoylnorecgonine and 10% due to an unidentified metabolite. Norecgonine was not detected as a urinary metabolite of benzoylnorecgonine.

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Estimation and disposition of [^{3}H]benzoylecgonine and pharmacological activity of some cocaine metabolites

Benzoylecgonine (pKa 8·65, 8·80, 11·80) is known to be the major metabolite of cocaine (ref. cit. Misra, Nayak & others, 1974a). No information exists on the physiological disposition and metabolism of benzoylecgonine. Its amphoteric nature and unfavourable partition characteristics have hampered the development of a sensitive method for its estimation in biological materials. Previous methods (Misra, Pontani & Mulé, 1973a; Valanju, Baden & others, 1973; Koontz, Besemer & others, 1973; Bastos, Jukofsky & Mulé, 1974) lack adequate sensitivity for its estimation in sub-microgram range in tissues. This communication describes an improved method of assaying [^{3}H]benzoylecgonine in biological materials, its distribution and metabolism in the rat and its gross pharmacological activity in the rat and that of some other metabolites of cocaine (benzoylnorecogonine, ecgonine, norcocaine and ecgonine methylester) after intravenous and intracisternal injections.

[^{3}H]Benzoylecgonine (sp. act. 31 μCi mg^{-1}) was prepared by the hydrolysis of carrier-diluted [^{3}H]cocaine (Nayak, Misra & others, 1974) by a micro-scale adaptation of the procedure of Findlay (1954). Benzoylecgonine, ecgonine methyl ester were prepared according to Findlay (1954), benzoylnorecgonine and norcocaine by the method of Schmidt & Werner (1962) and ecgonine by the method of Bell & Archer (1960).

In vitro *recoveries of [^{3}H]benzoylecgonine from biological materials.* Two ml aliquots of diluted urine, plasma (1 : 5) or tissue homogenates of rats (20% *in* 0·9% saline) containing known concentrations of [^{3}H]benzoylecgonine in the 5–1000 ng range were transferred to 40 ml centrifuge tubes containing 1 ml non-radioactive benzoylecgonine (500 μg ml^{-1}) as carrier. *To this solution* 2 g solid K_2CO_3 was added (pH, 11–12) followed by 15 ml chloroform–isopropanol (2 : 1, v/v) and the mixture shaken for 20 min. After centrifugation for 10 min, the aqueous phase was aspirated and 10 ml organic phase evaporated to dryness at 45–50° in counting vials. The residue was dissolved in 0·5 ml methanol, 10 ml toluene phosphor added and radioactivity determined in a liquid scintillation counter. Other details on counting technique, calibration curve for calculation of benzoylecgonine concentrations in tissues and fluids have been described by Misra & Mulé (1972), Misra, Mulé & others (1973b) and Misra, Pontani & Mulé (1974b). *In vitro* recoveries of [^{3}H]benzoylecgonine from aqueous solutions in the concentration range 5–1000 ng were: 95·4 $\pm$ 0·3% (s.e.); from urine and plasma 91·0 $\pm$ 1·7% (s.e.); brain 90·0 $\pm$ 0·6% (s.e.); and liver 81·3 $\pm$ 0·9% (s.e.). Extractions at lower pH with or without saturation of aqueous phase with sodium chloride or washing of organic phase with dilute 4% K_2HPO_4 buffer lowered the recoveries. Ecgonine was also quantitatively extracted by this procedure. For the specific extraction of cocaine from biological materials see Misra & others (1974a).

Distribution of [^{3}H]benzoylecgonine in the rat. Male Wistar rats (130–160 g) were injected in the tail vein with a 10 mg kg^{-1} dose of [^{3}H]benzoylecgonine (sp. act. 6·2 μCi mg^{-1}). At chosen times, animals were lightly anaesthetized with ether and blood drawn into heparinized vacutainer tubes by cardiac puncture. Plasma was obtained immediately by centrifugation. Known weights of brain were homogenized in 0·9% saline to give a 20% homogenate and 2 ml aliquots were taken in duplicate and analysed. Plasma, diluted, was similarly treated.

For intracisternal injections, rats were lightly anaesthetized with ether and injected with [^{3}H]benzoylecgonine 1 mg kg^{-1} in 10 μl. Brains were removed 0·5 h later and analysed for benzoylecgonine. Cocaine and other metabolites were similarly injected intracisternally in volumes of 10 μl, the pH of solutions being adjusted to 6·8–7·0 with 0·1 N NaOH.

Metabolic studies. Male Wistar rats were injected intraperitoneally with 200 mg kg^{-1} benzoylecgonine and the urine was collected for 48 h, pooled, evaporated under vacuum, and the residue repeatedly extracted with methanol. The methanol-soluble portion, on preparative thin-layer chromatography (Gelman instant thin-layer chromatography media [ITLC] silica gel, 20 × 20 cm sheets) with solvent system: n-butanol–acetic acid–water (35:3:10, v/v), provided 2 major bands R_F, 0·15–0·50 (A) and 0·8–1·0 (B). These were eluted with methanol and separately rechromatographed on the same medium with ethyl acetate–methanol–conc. ammonia (15:4:1, v/v). This procedure resolved A into two compounds R_F 0·05 (minor) and 0·24 (major) and B into R_F 0·3 (minor) and 0·8 (major), respectively.

[^{3}H]Benzoylecgonine concentrations in rat brain and plasma after a 10 mg kg, i.v. injection are in Table 1. Mean peak concentrations in brain were attained 0·5 h after injection and declined to 20–28 ng g^{-1} or ml by 6 h. The ratio of mean peak concentration in brain to plasma was 0·12 (apparent partition coefficient of [^{3}H]benzoylecgonine in 1-octanol–phosphate buffer pH 7·4, 0·15 ± 0·01 s.e.). The brain to plasma ratio with [^{3}H]ecgonine (Misra, Vadlamani & others, 1974c) after a similar intravenous dose was 0·03.

Cocaine (apparent partition coefficient in 1-octanol–buffer pH 7·4, 7·6 ± 0·1 s.e.), however provided a brain to plasma ratio of 11·9, 15 min after injection of a subconvulsive 8 mg kg^{-1} intravenous dose (Misra & others, 1974a) and the peak concentrations of [^{3}H] cocaine in brain and plasma at this time were 7269 ± 177 ng g^{-1} and 612 ± 62 ng ml^{-1}, respectively. The half-lives of benzoylecgonine, ecgonine and cocaine in rat brain were [illegible]3, [illegible] and 0·4 h, respectively, that in plasma 0·8, 3·8 and 0·3 h, respectively.

After intracisternal injection of [^{3}H]benzoylecgonine (1 mg kg^{-1}) to the rats, approximately 5–11% of the dose g^{-1} of brain was observed 0·5 h post-injection. Extraction of rat brains and thin-layer chromatography of the extract provided no evidence for *N*-dealkylation of benzoylecgonine to benzoylnorecgonine.

The compounds of R_F 0·24 and 0·8 were shown to be ecgonine and benzoylecgonine by co-chromatography. A minor compound R_F 0·3 showed the presence of a phenolic group by Folin-Ciocalteau and $FeCl_3$-$K_3Fe(CN)_6$ reagents. A compound of R_F 0·05 gave a positive test for glucuronide (Dische, 1947) and on hydrolysis with β-glucuronidase at pH 6·8 generated the phenolic compound. It was also intensely positive to cobalt nitrate reagent (Azouz, Parke & Williams, 1953). These experiments provided evidence for the existence of benzoylecgonine and the formation of ecgonine as a major metabolite and two minor metabolites, i.e. a phenolic compound (*p*-aromatic hydroxylation) and its conjugate as glucuronide.

Table 1. *Distribution[a] of [^{3}H]benzoylecgonine in brain and plasma of male Wistar rats after a 10 mg kg^{-1} dose by intravenous injection.*

	0·25 h	0·5 h	1 h	3 h	6 h	Half-life (h)
Brain	208 ± 14	366 ± 114	283 ± 52	111 ± 7	20 ± 2	1·3
Plasma	4150 ± 282	3088 ± 175	2008 ± 284	338 ± 115	28 ± 4	0·8
Brain/plasma ratio	0·05	0·12	0·14	0·32	0·71	

(a) Data are mean values ± s.e.m. (ng g^{-1} wet tissue weight or ml fluid) of 6 determinations from 3 animals at each time.

No observable pharmacological effects were noted with ecgonine and ecgonine methyl ester after high doses (200 mg kg^{-1}, i.v. or 10 mg kg^{-1}, i.c.) and also with benzoylecgonine after doses of 250 mg kg^{-1} (i.v.). Cocaine and norcocaine, the lipophilic compounds, were active by both intravenous and cisternal routes. A 20 mg kg^{-1} (i.v.) injection or 1–2 mg kg^{-1} (i.c.) injection of cocaine and norcocaine to the rats caused excessively rapid heart beat, convulsions and death within 3–5 min. Lower doses (5–10 mg kg^{-1}, i.v. or 0·5–1·0 mg kg^{-1}, i.c.) produced similar results without mortality, and the effects lasted for 5–20 min. At 1 mg kg^{-1} (i.c.) benzoylecgonine, piloerection, running and jumping activity, jerking, rapid breathing and squeaking appeared within 5 min and these effects lasted for approximately 4 h; with higher doses (2 mg kg^{-1}) these effects became more violent without ensuing mortality. Benzoylnorecgonine (100 μg kg^{-1}, i.c.) produced effects similar to benzoylecgonine within 1 min of administration and death within 30 min. The potent stimulant effects observed with these two compounds on intracisternal injections were dose-dependent and their nature were distinctly different from those observed after the injections of cocaine or norcocaine.

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New York State Drug Abuse Control Commission,
Testing and Research Laboratory,
Brooklyn, New York 11217, U.S.A.

A. L. MISRA
P. K. NAYAK
R. BLOCH
S. J. MULÉ

January 27, 1975

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PHYSIOLOGIC DISPOSITION AND METABOLISM OF [^{3}H] ECGONINE (COCAINE METABOLITE) IN THE RAT

A.L. Misra, N.L. Vadlamani, R. Bloch, P.K. Nayak and S.J. Mule'

New York State Drug Abuse Control Commission

Testing and Research Laboratory

Brooklyn, New York 11217

ABSTRACT

A simple method is described for the estimation of [^{3}H] ecgonine in biological materials which gave *in vitro* recoveries of 47.2 ± 1.7 (S.D.) % in the concentration range 5-1000 ng. Following a 10 mg/kg intravenous dose in the rat, mean peak levels in brain, kidney, liver, lung, heart, intestine, muscle and spleen were 0.7, 145.6, 13.9, 13.5, 7.7, 7.6, 5.8 and 5.6 µg/g respectively within 15 minutes after injection. The ratio of mean concentration in brain to that in plasma at this time was 0.03. Ecgonine was not metabolized in the rat and the percentages of dose excreted in urine and feces in 96 hours were 90.6 and 8.8, respectively, with major excretion (86.2%) occurring within 24 hours in urine. In spite of its highly polar character, ecgonine crosses the blood-brain barrier in small concentrations and has been found to be an inactive metabolite of [^{3}H] cocaine in the rat brain.

INTRODUCTION

Metabolism of cocaine in experimental animals is thought to proceed by hydrolysis of two ester groups to yield benzoylecgonine and finally ecgonine (Woods, et al., 1951; Sanchez, 1957; Montesinos, 1965; Ortiz, 1966; Fish and Wilson, 1969). However, evidence for the formation of ecgonine as a metabolite of cocaine in tissues is tentative and it is not known whether ecgonine is further biotransformed. No information exists concerning the physiologic disposition and metabolism of ecgonine. The amphoteric nature and unfavorable partition characteristics of ecgonine (pk_a, 10.91, 11.15) have hampered the development of a sensitive method for its estimation in biological materials. Paper, thin layer and gas chromatographic methods (Majlát and Bayer, 1965; Misra, et al., 1973; Valanju, et al., 1973) and derivatisation methods (Koontz, et al., 1973; Bastos, et al., 1974) for the separation and detection of ecgonine from its congeners, generally lack adequate sensitivity for its estimation in tissues.

This communication describes a sensitive method for the estimation of [^{3}H] ecgonine in biological materials and its physiologic disposition and metabolism in the rat.

MATERIALS AND METHODS

1. Ecgonine

It was prepared from cocaine hydrochloride by the method of Bell and Archer (1960).

2. Ludy Tenger Spray Reagent

The reagent was prepared as described (Ludy Tenger, 1951).

3. [^{3}H] Ecgonine

Acid-catalysed exchange tritium labeling of ecgonine hydrochloride was

carried out at Amersham Searle Corp., Arlinton Heights, Illinois. After removal of labile tritium and catalyst, the product was dissolved in water and freeze-dried. The crude residue was dissolved in methanol diluted with nonradioactive ecgonine and subjected to preparative ITLC, silica gel (20 X 20 cm) with solvent system, n-butanol-acetic acid-water (35:3:10, v/v). Ecgonine band (R_f 0.3 - 0.5) located by spraying a guide strip with Ludy Tenger's reagent, was exhaustively eluted with 500 ml 95% ethanol. The eluate was freed of silica gel by filtration and evaporated to dryness in vacuo. The residue obtained had approximately 75% isotopic purity. Further purification of this residue was done by preparative ITLC (20 X 20 cm) with ethyl acetate-methanol-conc. ammonia (17:2:1, v/v). Ecgonine was eluted as before and recrystallized from ethanol-acetone to a colourless crystalline product m.p. 198 - 199° (decomp.), isotopic purity 95%, specific activity 33 μCi/mg. The chemical and radiochemical purity of ecgonine was confirmed by m.p., I.R. and thin layer chromatography in different solvent systems and radioscanning of chromatograms. The radioactive ecgonine was diluted with nonradioactive compound to a specific activity of 3.3 μCi/mg for the preparation of the injection solution in 0.9% saline.

4. Estimation of [^{3}H] Ecgonine in Biological Materials

One ml aliquots of diluted urine or plasma (1:5) or tissue homogenates (20% in 0.9% saline) in duplicate were transferred to 40 ml centrifuge tubes containing 0.5 ml nonradioactive ecgonine carrier (1 mg/ml), the pH of the solution was brought to 10-12 with 1.0 ml 1N NaOH. Thirty ml of chloroform-methanol (68:32, v/v) were added and the mixture shaken for 20 minutes in an Eberbach shaker. After centrifugation for 15 minutes, top aqueous phase was aspirated and 15 ml organic phase transferred to counting vials and evaporated to dryness at 45-50°C on a Fisher Slide Warmer. The residue in counting vials was dissolved in 0.5 ml methanol and 10 ml toluene-phosphor solution added and radioactivity

determined in a Nuclear Chicago Mark II Liquid Scintillation Spectrometer. The counts were corrected for quenching using [^{3}H] toluene as an internal standard. Other details on counting technique, calibration curve for calculation of ecgonine concentrations in tissues and fluids have been described previously (Misra, et al., 1973; Misra, et al., 1974). The recoveries of [^{3}H] ecgonine from diluted urine, plasma and tissue homogenates in the concentration range 5-1000 ng were 47.2 ± 1.7 (S.D.)%, respectively. Saturation of solutions with solid sodium chloride in the extraction procedure did not improve the recoveries. Extraction of ecgonine with chloroform alone or with chloroform containing different concentrations of lecithin or sodium lauryl sulfate gave very poor recoveries. Cocaine and benzoylecgonine will also be quantitatively extracted by this procedure at pH 8-9. As specific methods for the estimation of these compounds are already available (Nayak, et al., 1974a,b) determination of ecgonine in a mixture of these three compounds can easily be done by differential estimation.

5. Animal Experiments

Male Wistar rats (120-150 g) were injected intravenously (tail vein) with a 10 mg/kg dose of [^{3}H] ecgonine. After appropriate time intervals, tissues, blood, urine and feces were collected as described previously (Misra and Mule', 1972; Misra and Mule', 1973; Misra, et al., 1973; Misra, et al., 1974).

6. Metabolic Studies

An aliquot of pooled urine of 10 rats each injected intravenously with 10 mg/kg dose of [^{3}H] ecgonine was brought to pH 10-12 with 1N NaOH and the solution extracted repeatedly with 12 times its volume of chloroform-methanol (68:32, v/v) for 30 minutes. After centrifugation for 10 minutes, the organic phase was separated, filtered and the residue obtained on evaporation in vacuo was dissolved in methanol for further characterization by ITLC.

TABLE 1

Distribution of ecgonine in various tissues of male Wistar rats after intravenous injection of a 10 mg/kg dose of [^{3}H] ecgonine

	0.25 h	0.5 h	1 h	3 h	6 h	16 h
Brain	0.70 ± 0.21	0.43 ± 0.08	0.31 ± 0.06	0.26 ± 0.06	0.20 ± 0.07	0.09
Plasma	23.02 ± 10.29	18.91 ± 3.59	11.56 ± 1.85	1.03 ± 0.26	0.53 ± 0.14	0.09
Kidney	145.63 ± 51.44	64.63 ± 5.19	54.23 ± 10.98	6.52 ± 1.39	1.89 ± 0.86	0.75
Liver	13.89 ± 3.67	15.31 ± 2.86	12.84 ± 4.61	3.86 ± 0.57	1.06 ± 0.31	0.42
Lung	13.49 ± 3.29	9.54 ± 0.45	6.64 ± 1.38	2.11 ± 0.36	1.03 ± 0.13	0.26
Heart	7.71 ± 2.35	4.18 ± 0.49	3.49 ± 0.78	1.59 ± 0.25	0.92 ± 0.03	0.33
Intestine	7.55 ± 1.42	6.52 ± 0.78	2.56 ± 1.21	3.83 ± 1.00	3.15 ± 1.85	0.46
Muscle	5.78 ± 1.83	2.67 ± 0.64	2.42 ± 0.47	1.37 ± 0.17	1.07 ± 0.37	a
Spleen	5.55 ± 1.64	4.36 ± 0.96	4.03 ± 1.26	2.19 ± 0.45	1.09 ± 0.15	0.25

Data represent mean value ± S.E.M. (microgram/g tissue weight or microgram/ml fluid) from three animals at each time period upto 6 h and 2 animals at 16 h.

a. Sample lost in analysis.

TABLE 2

Urinary and fecal excretion of [^{3}H] ecgonine and total radioactivity following a 10 mg/kg intravenous injection in male Wistar rats

	Mean Percentage of Excreted Ecgonine			
	URINE		FECES	TOTAL
	0-24 h	24-96 h	0-96 h	
Free ecgonine	86.2 ± 7.1	4.4 ± 0.4	8.8 ± 1.7	99.4
Total radioactivity	87.4 ± 7.3	4.3 ± 0.4	11.9 ± 1.7	103.6

Data represent the mean percentage ± S.E.M. of the administered dose of [^{3}H] ecgonine recovered from the urine and feces of 10 male rats.

RESULTS

Data on the distribution of ecgonine in various tissues and plasma following a 10 mg/kg i.v. dose appear in Table 1. Mean peak levels of ecgonine attained within 15 minutes of injection in kidney, liver, lung, heart, intestine, muscle and spleen were 145.6, 13.9, 13.5, 7.7, 7.6, 5.8 and 5.6 μg/g, respectively. The values at 15 min. in brain were the lowest (0.7 μg/g) and those in plasma were 23 μg/ml. The ratio of mean peak concentrations in brain to plasma was 0.03. Tissue levels of ecgonine declined considerably by 16 hours after injection but comparatively higher levels were still present in liver, intestine and kidney. The data on urinary and fecal excretion of ecgonine following a 10 mg/kg i.v. dose appear in Table 2. Renal excretion of ecgonine began very fast and the major amount of the administered dose (86.2%) was eliminated in the urine within 24 hours. Significant amounts were also excreted in the feces. Almost quantitative excretion of radioactivity in urine and feces occurred within 96 hours after injection of ecgonine.

Metabolic Studies

The methanol-soluble residue from the extracted urine was chromatographed on ITLC silica-gel sheets (20 X 20) cm and developed with n-butanol-acetic acid-water (35:3:10, v/v). A single radioactive band R_f 0.45-0.55 positive to Ludy Tenger reagent, was eluted with methanol and co-chromatographed with standard ecgonine in different solvent systems. Only a single peak of radioactivity with R_f identical to ecgonine was observed. Paper chromatography with solvent system n-butanol-acetic acid-water (4:1:5, v/v) also produced a single peak of radioactivity R_f 0.4 due to ecgonine. These radioscans provided evidence for the absence of any metabolites of ecgonine.

DISCUSSION

The low value of the ratio of peak brain to plasma concentrations 15

minutes after injection of ecgonine indicates that the blood-brain barrier can prevent entry of a highly polar compound into the rat brain. In spite of this, small concentrations of ecgonine entered the brain and were cleared gradually in 16 hours. The decline of ecgonine levels with time in other tissues was comparatively faster. Almost quantitative clearance of radioactivity in urine and feces suggests that ecgonine was not sequestered or retained in the rat tissues. Ecgonine was not metabolized in the rat. Definitive evidence has been obtained for the formation of ecgonine as one of the metabolites of [^{3}H] cocaine in the rat brain (Misra et al., unpublished observations). The absence of any observable pharmacological effect even with higher i.v. doses of ecgonine (100-200 mg/kg) in the rat would indicate that it was an inactive metabolite of cocaine.

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INTRACELLULAR DISPOSITION OF [^{3}H]-COCAINE, [^{3}H]-NORCOCAINE, [^{3}H]-BENZOYLECGONINE AND [^{3}H]-BENZOYLNORECGONINE IN THE BRAIN OF RATS

Salvatore J. Mule'. George A. Casella and Anand L. Misra

New York State Office of Drug Abuse Services

Testing and Research Laboratory

80 Hanson Place, Brooklyn, New York 11217

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Summary

[^{3}H]-cocaine, [^{3}H]-norcocaine, [^{3}H]-benzoylecgonine and [^{3}H]-benzoylnorecgonine were administered i.c. in equi-potent pharmacologic doses and the intracellular disposition and metabolism of each drug determined. Norcocaine and cocaine rapidly entered and egressed from the brain so that 4.8-6.1% of the radioactivity present in brain at one minute was observed at 30 minutes. The highest levels of subcellular radioactivity were generally found in the microsomal plus supernatant, followed by the nuclear and shocked mitochondrial fractions. No apparent localization of the radioactivity occured in synaptic membranes. The brain/plasma (B/P) ratio curves for cocaine and norcocaine were similar; however, the norcocaine values were considerably higher at each time interval. Benzoylecgonine and benzoylnorecgonine had higher comparative B/P ratios than cocaine or norcocaine and persisted in brain for a longer period of time so that 0.6-2.1% of the radioactivity present in brain at 1 hour was detected at 24 hours. Cocaine and norcocaine were extensively metabolized to the benzoylmetabolites. Benzoylecgonine was metabolized to benzoylnorecgonine and benzoylnorecgonine was unmetabolized. The brain disposition data and B/P ratios agreed quite well with the overall pharmacologic action of cocaine and its metabolites.

Following the administration of [^{3}H]-cocaine to rats, the metabolites norcocaine, benzoylecgonine, benzoylnorecgonine and ecgonine were shown to be present in whole brain (1). Cocaine and norcocaine were pharmacologically active when administered by either intravenous or intracisternal route (2). Benzoylecgonine and benzoylnorecgonine exhibited potent stimulant activity only after intracisternal administration whereas ecgonine and its methyl ester were inactive by either route (2, 3). In the dog and monkey, benzoylecgonine and benzoylnorecgonine persisted in the CNS for a much longer period of time than cocaine or norcocaine (4, 5, 6). These observations on cocaine and its metabolites prompted us to study *in vivo* the distribution of these compounds in intracellular and synaptic membrane fractions of rat brain in order to gain insight into their mechanisms of action. The results of these studies are reported herein.

fraction. There did not appear to be a localization of drug in the synaptic membrane fractions (M_1 0.9, 1.0, 1.2). Twenty minutes after administration of cocaine, no radioactivity was detected in either the synaptic membrane or pellet fractions.

A metabolic analysis of the brain homogenate at various time intervals indicated extensive metabolism of cocaine (Table 2) that increased with time. The metabolites of cocaine identified were norcocaine, benzoylecgonine and benzoylnorecgonine. The percentage of these metabolites increased with time as more of cocaine and the metabolite, norcocaine, were converted to benzoylecgonine and benzoylnorecgonine. At 30 minutes there was almost equivalent quantities of benzyolnorecgonine and cocaine in the brain (31.6-35.6%).

TABLE 2

Metabolic Profile in Brain at Various Time Intervals
Following a 0.5 mg/kg Intracisternal Injection of [3H]-Cocaine.a

TIME	CYCLOHEXANE EXTRACT		CHLOROFORM:ISOPROPANOL EXTRACT	
	%		%	
	Cocaine	Norcocaine	Benzoylecgonine	Benzoylnorecgonine
3 min.	57.3	20.7	7.4	14.6
5 min.	55.6	16.4	9.4	18.6
10 min.	46.1	13.9	13.1	26.9
20 min.	40.5	13.5	15.9	30.1
30 min.	35.6	13.3	19.4	31.6

a. All values represent the percentage of the dose present in the brain homogenate of two pooled rat brains at each time interval.

Significantly high levels of radioactivity were observed at one minute in brain (Table 3) after the intracisternal administration of 0.125 mg/kg [3H]-norcocaine. The level of radioactivity in brain declined rapidly so that only 4.8% present at one minute remained after 30 minutes. The microsomal plus supernatant fraction, followed by the nuclear and shocked mitochondrial fractions, contained the major quantity of the radioactivity at each time interval. Again, as with cocaine, the M_1 (0.8) subfraction contained considerably higher levels of drug(s) however, there appeared to be a tendency to localize in the M_1 (1.0) synaptic membrane fraction during the first five minutes after drug administration. Radioactivity was still present in all the fractions through one hour indicating a greater degree of binding in comparison to cocaine.

At three minutes after the intracisternal injection of [3H]-norcocaine, about 8% of the dose present in the brain homogenate was benzoylnorecgonine and 92% as the administred norcocaine (Table 4). As the time after drug administration increased, a greater percentage of norcocaine was metabolized to benzoylnorecgonine so that at 30 minutes 72% norcocaine and 28% benzoylnorecgonine was observed in brain. No other metabolites of norcocaine were identified in brain during this 30 minute time interval.

TABLE 3

DISTRIBUTION OF TOTAL RADIOACTIVITY IN SUBCELLULAR AND SYNAPTIC MEMBRANE FRACTIONS OF RAT BRAIN AFTER 0.125 mg/kg OF [^{3}H]-NORCOCAINE.[a]

FRACTION	1 min.	3 min.	5 min.	10 min.	20 min.	30 min.	1 hr.
Homogenate	2,508,325 ± 245,056	1,764,294 ± 151,064	1,584,719 ± 193,868	631,962 ± 27,916	231,835 ± 27,502	120,174 ±14,026	57,302 ±3,465
Nuclear	1,094,476 ± 98,188	698,379 ± 67,873	652,401 ± 80,467	262,654 ± 11,350	96,162 ± 16,297	47.782 ± 4,209	23,459 ±2,118
Shocked Mitochondria	62,006 ± 4,507	42,801 ± 3,093	37,812 ± 2,577	19,129 ± 745	10,261 ± 1,333	5,774 ± 610	3,997 ± 200
Microsomes and Supernatant	1,267,191 ± 208,612	1,105,506 ± 90,729	915,361 ± 89,898	379,278 ± 18,127	131,133 ± 14,855	59.762 ± 4,627	25,832 ±2,363
M_1 (Suspension)	17,653 ± 1,684	15,074 ± 967	12,845 ± 842	7,339 ± 343	5,286 ± 339	3,772 ± 527	2,837 ± 27
M_1 (0.8)	5,251 ± 205	4,883 ± 420	4,043 ± 444	2,203 ± 24	1,397 ± 315	715 ± 83	732 ± 120
M_1 (0.9)	769 ± 76	761 ± 57	627 ± 32	450 ± 36	382 ± 72	379 ± 39	154 ± 14
M_1 (1.0)	1,138 ± 152	1,089 ± 85	994 ± 19	432 ± 11	504 ± 83	276 ± 25	152 ± 32
M_1 (1.2)	728 ± 115	968 ± 135	805 ± 50	528 ± 56	491 ± 36	698 ± 65	436 ± 76
M_1 (Pellet)	805 ± 118	768 ± 83	868 ± 37	826 ± 165	1,294 ± 22	596 ± 126	392 ± 15

a. The data represents the mean value ± S.E. (Disintegrations Per Minute/gm wet weight of brain) of three experiments of two rats each at each time period. Specific activity of 720 DPM/ng [^{3}H]-Norcocaine.

The intracellular distribution of radioactivity following the intracisternal administration of 0.75 mg/kg of [^{3}H]-benzoylecgonine appears in Table 5.

TABLE 4

Metabolic Profile in Brain at Various Time Intervals Following a 0.125 mg/kg Intracisternal Injection of [^{3}H]-Norcocaine.[a]

TIME	CYCLOHEXANE EXTRACT	CHLOROFORM:ISOPROPANOL EXTRACT
	% Norcocaine	% Benzoylnorecgonine
3 min.	92	8
5 min.	88	12
10 min.	89	11
20 min.	77	23
30 min.	72	28

a. All values represent the percentage of the dose present in the brain homogenate of two pooled rat brains at each interval.

TABLE 6

Metabolic Profile in Brain at Various Time Intervals Following a 0.75 mg/kg Intracisternal Injection of [^{3}H]-Benzoylecgonine. [a]

TIME (hrs)	CHLOROFORM:ISOPROPANOL EXTRACT	
	% Benzoylecgonine	Benzoylnorecgonine
1	96	4
4	94	6
6	95	5
8	91	9
16	74	26

a. All values represent the percentage of the dose present in the brain of two pooled rat brains at each time interval.

Considerable radioactivity was present per g brain after one hour and this slowly declined to a level of 1.2% after 16 hours. The radioactivity was predominantly localized in the microsomal plus supernatant fraction and declined through eight hours at about the same rate as observed for the brain homogenate. Nuclear and shocked mitochondrial fractions contained considerably less

TABLE 5

DISTRIBUTION OF TOTAL RADIOACTIVITY IN SUBCELLULAR AND SYNAPTIC MEMBRANE FRACTIONS OF RAT BRAIN AFTER THE INTRACISTERNAL ADMINISTRATION OF 0.75 mg/kg OF [^{3}H]-BENZOYLECGONINE.[a]

FRACTION	1 hr.	4 hrs.	6 hrs.	8 hrs.	16 hrs.
Homogenate	371,081 ± 16,116	139,881 ± 4,056	44,799 ± 3,937	29,486 ± 2,428	4,375 ± 158
Nuclear	70,062 ± 2,831	25,315 ± 1,696	9,342 ± 1,130	6,756 ± 599	
Shocked Mitochondria	2,683 ± 30	1,455 ± 51	528 ± 64	139 ± 37	
Microsomes and Supernatant	321,196 ± 8,481	111,709 ± 4,416	35,133 ± 2,456	22,153 ± 4,510	
M_1 (Suspension)	910 ± 15	573 ± 13	223 ± 27	104 ± 26	
M_1 (0.8)	401 ± 9	275 ± 71	117 ± 2	---	
M_1 (0.9)	61 ± 7	29 ± 4	---	---	
M_1 (1.0)	41 ± 3	30 ± 3	---	---	
M_1 (1.2)	90 ± 4	67 ± 7	---	---	
M_1 (Pellet)	210 ± 32	109 ± 17	---	---	

a. The data represents the mean value ± S.E. (Disintegrations Per Minute/gm wet weight of brain) of three experiments of two rats each at each time period. Specific activity 20.8 DPM/ng [^{3}H]-Benzoylecgonine.

TABLE 7

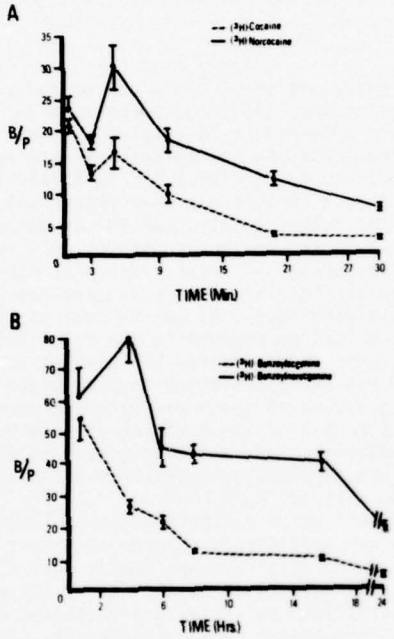
DISTRIBUTION OF [^{3}H]-BENZOYLNORECGONINE IN SUBCELLULAR AND SYNAPTIC MEMBRANE FRACTIONS OF RAT BRAIN AFTER THE INTRACISTERNAL ADMINISTRATION OF 0.05 mg/kg OF THE DRUG.[a]

FRACTION	1 hr.	4 hrs.	6 hrs.	8 hrs.	16 hrs.
Homogenate	1,114.0 ± 96.5	519.2 ± 11.3	411.6 ± 8.8	264.7 ± 32.3	72.0 ± 1.3
Nuclear	357.1 ± 39.2	160.8 ± 12.9	123.3 ± 5.3	51.4 ± 6.5	
Shocked Mitochondria	18.2 ± 2.7	8.5 ± 1.4	6.5 ± 0.5	6.3 ± 1.5	
Microsomes and Supernatant	815.5 ± 60.5	372.9 ± 19.0	288.2 ± 4.1	230.1 ± 25.3	
M_1 (Suspension)	5.6 ± 1.2	3.0 ± 0.3	2.4 ± 0.1	1.7 ± 0.4	
M_1 (0.8)	3.2 ± 0.1	1.6 ± 0.3	1.2 ± 0.1	----	
M_1 (0.9)	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	----	
M_1 (1.0)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	----	
M_1 (1.2)	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	----	
M_1 (Pellet)	0.3 ± 0.0	----	----	----	

a. The data represent the mean value ± S.E. (ng/g wet weight of brain) of three experiments of two rats each at each time period. Specific activity 543.7 DPM/ng [^{3}H]-Benzoylnorecgonine.

of the radioactivity at each time internal. There was no apparent localization of the radioactivity in the synaptic membrane fractions and no radioactivity could be detected in these fractions or the pellet at 6 and 8 hours. The M_1 (0.8) fraction contained the highest levels of drug(s) of the M_1 subfractions through 6 hours

[^{3}H]-Benzoylecgonine was metabolized to benzoylnorecgonine but only to the extent of 9% during the first eight hours after drug administration (Table 6). At 16 hours, the percentage of benzoylecgonine and benzoylnorecgonine in brain was 74 and 26, respectively.



Figures 1A and B. Brain/plasma (B/P) ratios (Disintegrations per minute per gram brain/Disintegrations per minute per ml plasma). The data represent the mean value ± S.E. of three experiments of two rats each at each time period.

The distribution of [^{3}H]-benzoylnorecgonine in subcellular and synaptic membrane fractions after 0.05 mg/kg intracisternal administration of the drug is summarized in Table 7. Six percent of the drug administered was observed per g of brain at one hour and 0.4% remained at 16 hours (72 ng/g). The drug distributed in high concentrations to the microsomal plus supernatant fraction followed by the nuclear and shocked mitochondrial fractions. An apparently similar distribution of the drug occured in the M_1 synaptic membrane fractions (9, 1.0, 1.2) at 1, 4, and 6 hours. No drug was present in these fractions at 8 hours. Benzoylnorecgonine was also absent in the pellet fraction at 4, 6 and 8 hours.

Analysis of the brain homogenate at 1, 4, 6, 8, and 16 hours revealed that only benzoylnorecgonine was present indicating the absence of any metabolism for this drug at time intervals described.

Figures 1A and B show the brain/plasma ratios for cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine at various time intervals. The B/P ratios were higher for norcocaine in comparison to cocaine at each time interval, and the overall pattern of decay was identical. The values were statistically significant ($P < .05$) at each time interval except for the one minute value. The B/P ratios for benzoylnorecgonine and benzoylecgonine were considerably higher than those observed for cocaine and norcocaine. Benzoylnorecgonine values were higher than benzoylecgonine at each time interval (significant at $P < .05$ at all times except one hour) and especially at four hours where the values were 81.04 and 27.03, respectively. At 24 hours the B/P ratios were still elevated and were 20.18 for benzoylnorecgonine and 3.82 for benzoylecgonine.

Dissussion

The intracellular distribution of [^{3}H]-cocaine and related surrogates and metabolites [^{3}H]-norcocaine, [^{3}H]-benzoylecgonine and [^{3}H]-benzoylnorecgonine in the brain of rats appeared to be related to the lipophilic nature and polarity of each compound. Cocaine and norcocaine with apparent partition coefficients of 7.6 and 18.5, respectively (3, 8) gained rapid entry into the brain matrix and although these drugs were extensively metabolized, they appeared to leave the brain rapidly. At 30 minutes only 4.8-6.1% of the radioactivity present in the brain homogenates at 1 minute after norcocaine or cocaine administration was still present in brain. The half-lives for cocaine in brain and plasma after i.v. adminstration 0.3-0.4 (3) and for norcocaine in brain and plasma (0.5-0.6) (8) as well as the B/P ratios reported in this study lend further support to the relatively rapid clearance of these drugs from the CNS. Furthermore, the binding of these drugs to intracellular fractions was easily reversed and the levels of radioactivity decreased rapidly over a period of one hour following drug administration. Following [^{3}H]-cocaine i.c. 51% of the radioactivity in brain at 30 minutes was provided by [^{3}H]-benzoylecgonine and [^{3}H]-benzoylnorecgonine. Following [^{3}H]-norcocaine 28% of the radioactivity in brain at 30 minutes was due to [^{3}H]-benzoylnorecgonine.

The penetration, distribution and metabolism of [^{3}H]-benzoylecgonine and [^{3}H]-benzoylnorecgonine was considerably slower than that observed for either cocaine or norcocaine. Obviously, this was due in part at least to the highly polar character and lower lipid solubility of these compounds as indicated by the apparent partition coefficients of 0.15 and 0.21 for benzoylecgonine (2) and benzoylnorecgonine (9), respectively. The half-lives of these drugs in brain and plasma after i.v. administration were 1.3 - 3.0 hours in brain and 0.2 - 1.2 hours in plasma for benzoylecgonine and benzoylnorecgonine, respectively (2, 9). These data provide additional evidence for the persistence of the polar metabolites in brain and blood. At 24 hours after the administration of benzoylecgonine 2,385 ± 211 DMP/g wet weight brain was observed in the brain homogenate (0.6% of the radioactivity present at 1 hour in the brain homogenate). The value for benzoylnorecgonine at 24 hours was 23.2 ng/g wet weight of brain (2.1% of the drug present at 1 hour in the brain homogenate). There was no apparent localization of these compounds in the synaptic membranes and the intracellular disposition appeared to parallel that observed in the brain homogenate over the time period of analysis. Benzoylecgonine was slowly metabolized to benzoylnorecgonine (26% present in brain at 16 hours) and no evidence was obtained for the metabolism of benzoylnorecgonine after i.c. administration. Neither ecgonine nor norecgonine were identified as metabolites of cocaine, norcocaine, benzoylecgonine or benzoylnorecgonine after intracisternal administration of the drug. Earlier studies at different time intervals following systemic administration of these drugs indicated the presence of both norecgonine and ecgonine in brain (1, 3, 8). It appears after cocaine or norcocaine administraiton i.c. that metabolism

occurs almost immediately indicating it occurs directly within brain cells. In the case of benzoylecgonine no metabolism of this drug was observed in brain at 0.5 hr (2) and only 4% of the drug was converted to benzoylnorecgonine at one hour (this study) which of course in more than sufficient time for the drug to recycle through the liver and thus be converted to the metabolite.

The brain to plasma ratios (B/P) after administration of cocaine or norcocaine were considerably lower than those obtained following the administration of benzoylecgonine or benzoylnorecgonine. The norcocaine B/P values were higher than cocaine indicating a greater tissuue affinity, but the curves and their slopes were almost identical, indicating rapid penetration into, and egression from the brain and plasma. The B/P ratios at 30 minutes ranged from 2.7 - 7.0 for cocaine and norcocaine, respectively. The B/P ratios for the polar metabolites (benzoylecgonine and benzoylnorecgonine) indicate a much greater tissue affinity than observed for either cocaine or norcocaine with the values for benzoylnorecgonine considerably higher than those for benzoylecgonine throughout the 24 hours. At 24 hours the B/P ratio for benzoylecgonine and benzoylnorecgonine were 3.8 and 20.2, respectively. The data obtained concerning the disposition of these drugs within brain and the B/P ratios after intracisternal administration of equi-potent pharmacologic doses coincides quite nicely with the overall pharmacologic effects of cocaine and its metabolites. The comparative potency ratios (mg/kg) in comparison with cocaine were: 10, 4, and 0.67 for benzoylnorecgonine, norcocaine, and benzoylecgonine, respectively. The lipophilic drugs cocaine and norcocaine penetrated the brain and the intracellular structure immediately and egressed rapidly from the brain and plasma as shown by the B/P ratios and brain disposition data. The pharamcologic effect in the rat (rapid heart rate, running, rapid breathing) lasted about 5-10 minutes for cocaine and 15-20 minutes for norcocaine. At least in part the cocaine and norcocaine effects my be ascribed to the metabolites of these drugs. Benzoylecgonine and benzoylnorecgonine provided a much longer duration of action (2-4 hours) and in addition caused a greater stimulatory effect including jumping, squeaking, circling, twitching, and piloerection. The disposition in brain, the B/P ratios, and the half-lives of these drugs (benzoylecgonine, benzoylnorecgonine) clearly reflect the longer duration of action. Previous studies (2) indicated the potent stimulant and dose dependent nature of their action as well as the lack of effect observed with very high doses of benzoylecgonine and benzoylnorecgonine (250 mg/kg, i.v.) following systemic administration, indicating a blood-brain barrier effect and the importance of the metabolism of cocaine or norcocaine to the benzoyl metabolites within brain cells.

Cocaine, norcocaine, benzoylecgonine and benzoylnorecgonine form molecular complexes with calcium chloride (12). The molecular complexing along with mobilization of neuronal membrane bound calcium may play a significant role in the potent stimulant activity,(pharmacologic action) and duration of action observed in rats after intracisternal administration of these drugs.

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CALCIUM-BINDING PROPERTY OF COCAINE AND SOME OF ITS ACTIVE METABOLITES-FORMATION OF MOLECULAR COMPLEXES

Anand L. Misra and Salvatore J. Mule'

New York State Drug Abuse Control Commission
Testing and Research Laboratory
Brooklyn, New York 11217

ABSTRACT

Cocaine, benzoylecgonine, benzoylnorecgonine, norcocaine, ecgonine methyl ester but not ecgonine formed distinct molecular complexes with calcium chloride of the general formula [B. $CaCl_2 \cdot 2H_2O$]. The particularly strong interaction observed with benzoylecgonine, benzoylnorecgonine and calcium may play a role in the potent stimulant activity of these 2 compounds observed in rats after intracisternal administration.

INTRODUCTION

Norcocaine, benzoylecgonine, benzoylnorecgonine and ecgonine have been shown (Misra et al., 1974a) to be the metabolites of cocaine in rat brain. In the course of our study on the pharmacological activity of these metabolites (Misra et al., 1974b; 1975; Nayak et al., 1975) we observed that cocaine and norcocaine were pharmacologically active both by intravenous and intracisternal routes of administration; benzoylecgonine and benzoylnorecgonine possessed potent stimulant activity only by intracisternal route; ecgonine and ecgonine methyl ester were inactive by both routes. Furthermore, benzoylecgonine and benzoylnorecgonine persisted in selected anatomic areas of the CNS of the dog, long after the disappearance of cocaine following a single 5 mg kg^{-1} i.v. injection of [^{3}H] cocaine (Misra et al., unpublished observations). These observations prompted a study of possible interaction of these cocaine metabolites with calcium, which is known to participate in a number of nerve functions at the membrane level e.g. in excitation of neuronal membranes (Singer and Tasaki,

1968), potassium transport (Blum and Hoffman, 1972), release of neurotransmitter substances (Katz, 1966; Heuser and Miledi, 1971) and intracellular mediation of catecholamine response through the cyclic AMP system (Greengard et al., 1972).

This report describes calcium-binding property of cocaine and some of its active metabolites and formation of distinct molecular complexes.

MATERIALS AND METHODS

1. Standard samples of compounds

Benzoylecgonine, ecgonine methyl ester were prepared by the method of Findlay (1954); benzoylnorecgonine and norcocaine by the method of Schmidt and Werner (1962) and ecgonine by the method of Bell and Archer (1960).

2. Preparation of molecular complexes

Molecular complexes of cocaine and its metabolites with calcium were prepared by mixing solutions of compounds and calcium chloride in a 1:1 molar ratio in isopropanol, heating on a water bath for 1 h. at 60-80°, and evaporation to a small volume in a stream of nitrogen. Under these conditions, complexes of benzoylecgonine and benzoylnorecgonine precipitated, were centrifuged, washed repeatedly with small volumes of hot isopropanol, dried at 55°C in vacuo and recrystallized from ethanol-isopropanol mixture. Complexes of cocaine, norcocaine, ecgonine methyl ester with calcium were obtained by the addition of ether to the concentrated isopropanol solutions of their mixture, removing the precipitate by centrifugation, washing repeatedly with ether and chloroform. The yields of benzoylecgonine and benzoylnorecgonine complexes were approximately 45%. Ecgonine did not form a complex under these conditions. Complexes gave a positive test with silver nitrate and iodoplatinate reagent.

3. Elemental analyses of complexes

All microchemical analyses were performed by Childers Laboratory, Inc., Milford, New Jersey.

4. Infrared spectra of complexes

These spectra were run as KBr pellets of complexes (1%) to check any differences with respect to band intensities or shape.

RESULTS AND DISCUSSION

The colourless molecular complexes of benzoylecgonine and benzoylnorecgonine with calcium chloride had the general formual (B. $CaCl_2$. $2H_2O$) where B stands for the ligand. Benzoylecgonine complex, $C_{16}H_{19}NO_4$. $CaCl_2 2H_2O$ requires C, 44.00; H, 5.27; Cl, 16.27; Ca, 9.16: Found C, 45.63; H, 5.54; Cl, 15.67; Ca, 8.86. This complex turned brown at 275-280°C and did not melt up to 300°C. Benzoylnorecgonine complex, $C_{15}H_{17}NO_4$. $CaCl_2$. $2H_2O$ requires C, 42.62; H, 4.97; Cl, 16.81; Ca, 9.47: Found C, 41.04; H, 5.39; Cl, 16.88; Ca, 10.22. This complex turned brown at 255°C and gradually melted with charring at 275-295°C. Both complexes were hygroscopic. A distinct change in crystallinity occurred on formation of these complexes. These complexes dissociated to generate free ligands on tlc in weakly acidic or alkaline solvent systems. Complexes were stable in absence of water. Yields of complexes with cocaine, norcocaine, ecgonine methyl ester were slightly lower compared to benzoylecgonine and benzoylnorecgonine due to their dissociation on recrystallization. These complexes were also hygroscopic, and gradually decomposed on heating without melting up to 300°C. Under these conditions ecgonine did not form a complex. The infrared spectra of complexes of benzoylecgonine and benzoylnorecgonine with Ca^{2+} displayed slight differences in the fingerprint region and appearance of a new band at 1420 cm^{-1}. Complexes of benzoylecgonine and benzoylnorecgonine with $MgCl_2$ were comparatively much more soluble both in ethanol and isopropanol and difficult to crystallize. Reaction of $MgCl_2$ with cocaine in ethanol gave cocaine hydrochloride mp. 190-192°C possibly due to the liberation of hydrochloric acid from $MgCl_2$ on heating.

An increase in the concentration of Ca^{2+} is known to raise markedly the threshold of nerve to stimulation, whereas a decrease increases excitability. Complexation of active metabolites of cocaine with Ca^{2+} and the possible mobilization of neuronal-membrane bound Ca^{2+} from sites that control transmembrane permeability to Na^{+} may conceivably play some role in the excitatory actions of cocaine and some of its metabolites. Further work in this direction is in progress in our laboratory.

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MOLECULAR COMPLEXES OF COCAINE, ITS ACTIVE METABOLITES AND SOME OTHER STIMULANTS WITH THIAMINE

ANAND L. MISRA* AND NARASIMHAN L. VADLAMANI

NEW YORK STATE OFFICE OF DRUG ABUSE SERVICES
TESTING AND RESEARCH LABORATORY
BROOKLYN, NEW YORK 11217

ABSTRACT

Cocaine, its pharmacologically active metabolites, norcocaine benzoylnorecgonine, benzoylecgonine and other central nervous system stimulants e.g. dextrococaine, nicotine, caffeine and p-hydroxy norephedrine formed molecular complexes with thiamine. The possible implications of such an interaction are discussed.

INTRODUCTION

Thiamine and its phosphorylated esters appear to play an important role in nerve conduction and excitation at the molecular level, quite independently of their coenzymatic activity and have been implicated in transmembrane ion transport involving permeability changes at the sodium channel (Von Muralt, 1962; Itokawa and Cooper, 1970). Local anesthetics reportedly block nerve conduction by disorganizing the membrane around the sodium channel and blocking the sodium conductance (Seeman, 1972). Some synthetic local anesthetics including cocaine (Eckert, 1962; Cuisinand et al., 1969; Thyrum et al., 1969), indoles (Biaglow et al., 1969) and some neurotransmitters (Galzigna, 1969) have previously been reported to form charge-transfer complexes with thiamine. These observations led us to examine the possible interaction of active metabolites of cocaine, and some other stimulants, with thiamine.

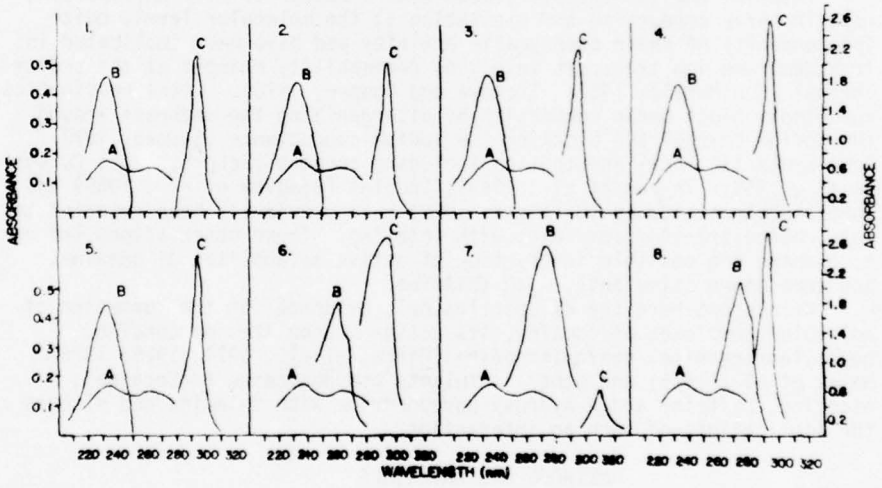
We present here the UV spectroscopic evidence for the formation of molecular complexes of cocaine, its active metabolites norcocaine, benzoylnorecgonine, benzoylecgonine (Misra, et al., 1974; 1975; 1976a; Nayak et al., 1976) and other stimulants pseudococaine (d-Cocaine), nicotine, caffeine and p-hydroxy norephedrine with thiamine and discuss the implications of such an interaction.

METHODS AND MATERIALS

1. Samples of standard compounds: Benzoylecgonine and ecgonine methyl ester were prepared by the method of Findlay (1954): benzoylnorecgonine and norcocaine by the method of Schmidt and Werner (1962); ecgonine

*To whom correspondence should be addressed.

Fig. 1. The UV-spectra of thiamine hydrochloride (A), the ligand (B) and the molecular complexes (C) with thiamine of (1) cocaine (2) norcocaine (3) benzoylnorecgonine (5) pseudo cocaine (6) nicotine (7) caffeine and (8) p-hydroxy norephedrine. The left ordinate represents absorbances of the spectra of the individual components, thiamine hydrochloride (1.86×10^{-5} Mol/l.) and the ligands cocaine hydrochloride (3.53×10^{-5} Mol/l.), norcocaine hydrochloride (3.69×10^{-5} Mol/l.), benzoylnorecgonine (3.86×10^{-5} Mol/l.), benzoylecgonine (3.68×10^{-5} Mol/l.), pseudo cocaine hydrochloride (3.53×10^{-5} Mol/l.), nicotine sulfate (4.73×10^{-5} Mol/l.), caffeine hydrochloride (6.17×10^{-5} Mol/l.), and p-hydroxy norephedrine hydrochloride (3.92×10^{-4} Mol/l.). The right ordinate represents absorbances of the spectra of all molecular complexes (C). The concentrations of individual components in these complexes were as follows: thiamine hydrochloride (0.88×10^{-2} Mol/l.) and (1) cocaine hydrochloride (1.76×10^{-2} Mol/l.); (2) norcocaine hydrochloride (1.84×10^{-2} Mol/l.); (3) benzoylnorecgonine (1.93×10^{-2} Mol/l.); (4) benzoylecgonine (1.84×10^{-2} Mol/l.); (5) pseudo cocaine hydrochloride (1.76×10^{-2} Mol/l.); (6) nicotine sulfate (0.71×10^{-2} Mol/l.); (7) thiamine hydrochloride (1.07×10^{-1} Mol/l.) and caffeine hydrochloride (2.70×10^{-1} Mol/l.); (8) thiamine hydrochloride (5.9×10^{-3} Mol/l.) and p-hydroxy norephedrine hydrochloride (1.96×10^{-2} Mol/l.).



by the method of Bell and Archer (1960) and Ψ-cocaine (dextrococaine) by the method of Findlay (1954); Misra et al., (1976b). Nicotine, caffeine and p-hydroxy norephedrine were obtained commercially.

2. UV-Spectra of compounds and complexes: All the spectra were measured in aqueous solutions at ambient temperature 25°C in a Carl-Zeiss PMQ II spectrophotometer. The same concentration of drugs present in the complex was used in the reference cuvette in the spectral determinations of the complexes.

RESULTS AND DISCUSSION

The details on the formation of molecular complexes of these stimulants and thiamine are given in Fig. 1. Cocaine, norcocaine, benzoylnorecgonine, benzoylecgonine, and pseudococaine formed distinct molecular complexes with well-defined absorption bands at wavelength (max. 294 nm) longer than those of the individual components and their intensities depended on the concentrations of these components. Higher temperatures (37-50°C) slightly reduced the absorbance of complexes of cocaine, its derivatives and thiamine. Ecgonine and ecgonine methyl ester, the pharmacologically inactive metabolites of cocaine did not form such complexes. The molecular complex of nicotine and thiamine (max. 293 nm) formed very slowly upon keeping the mixture of aqueous solutions of these compounds at ambient temperature (25°C) for 48-72 h. Caffeine formed such a complex (max. 310 nm) at comparatively much higher concentrations of the individual components and the p-hydroxy norephedrine complex showed absorption maxima at 292 nm. Cocaine and benzoylecgonine also formed molecular complexes (max. 290 nm) with thiamine disulfide.

Thiazolium ring in thiamine is known for its moderate electron acceptor properties and it is conceivable that the stimulants described above may act as electron-donors towards thiamine in the formation of molecular complexes. The electron donation from these compounds may occur either through the nonbonding lone pair of electrons on the nitrogen atom or through the electron donating groups or through the aromatic pi-electron system in some of these molecules. The change in electron structure of the thiamine acceptor induced by the formation of such complexes will result in a basic change in its reactivity and may inhibit the biochemical function of thiamine in nerve structures. The imposition of such a molecular complex upon a semi-conducting biopolymer matrix of protein-lipid could be expected to produce localized changes in charge-density on the surface of the matrix, leading to alterations in the electrical character of the axonal membrane and resultant stimulatory response. Previous work on charge-transfer processes involving xeno-biotics and substrates or functioning biological units has provided possibilities for the understanding of noncovalent intermolecular interactions in biological systems and furnished evidence for the pharmacological activity of several drugs (Szent-György, 1960; Pullman, 1968; Doukas, 1975).

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COCAINE: TOLERANCE TO ITS CONVULSANT AND CARDIORESPIRATORY STIMULATING EFFECTS IN THE MONKEY

Masaji Matsuzaki, Philip J. Spingler, Anand L. Misra and Salvatore J. Mule'

New York State Office of Drug Abuse Services, Testing and Research Laboratory

80 Hanson Place, Brooklyn, New York 11217

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Summary

Repeated daily I.V. injections of cocaine in the monkey at a minimal convulsant dosage (MCD) produced tolerance resulting in a marked increase in the MCD of cocaine. The repeated daily I.V. injections of cocaine at subconvulsant doses (2.0-4.0 mg/kg) produced a marked decrease in the cardiorespiratory stimulating effect and apparent plasma half-life of cocaine in the monkey.

Previous studies (1-11) have shown that chronic cocaine administration does not produce tolerance or physiological dependence. Reverse tolerance or increasing sensitivity to cocaine with increasing use has been reported (2-14). Other studies (15-17) have shown the development of tolerance to cocaine in man. In the rat, tolerance was demonstrated to a water-reinforced task after chronic injections of cocaine in high doses (18). In these reports, however, it is difficult to find causal relations between the dose of cocaine and the magnitude of responses to this drug. There were also no adequate systemic parameters to quantify alterations in the dose-related responses following repeated administrations of the drug. This communication provides evidence for the development of tolerance to the convulsant and cardiorespiratory stimulating effect (19,20) of cocaine in the monkey.

Methods

Subjects. Rhesus monkeys with permanently implanted electrodes in the brain were used as subjects (Ss). Bipolar stainless steel electrodes were implanted stereotaxically in the subcortical structures, and bipolar silver-ball electrodes were placed on the dura in various cortical areas, under pentobarbital anesthesia (45 mg/kg, IV). Electrodes for recording eye movements were implanted in the orbital plate. An indifferent electrode was located in the bone over the frontal sinus. The needle electrodes and a chest-movement transducer were attached to the chest of the Ss for recordings of heart rate (H.R.) and respiratory rate (R.R.) respectively. For the injection of the drug, a silastic catheter was chronically inserted in the femoral or jugular vein under appropriate anesthesia.

Experimental procedures. The study was carried out in four main parts; these experimental procedures were as follows.

Part 1. Study on the alteration in convulsant effect during short-term session

Nine Ss used in this study were restrained in a primate chair and placed in an observation chamber (90 x 88 x 75 cm.) and the polygraphs of EEGs, eye movement, H.R. and R.R. were recorded daily (5 to 12 days). Cocaine solution (5.0 mg/ml as free base in 0.9% saline) was injected once daily at a minimal convulsant dosage (MCD) that produced a minimal grade of convulsions lasting 20-30 sec. The MCD was established by injecting cocaine at a constant rate of 1.0 ml/10 sec. through a venous catheter using a remote control technique. The injection was terminated as the onset of the convulsive seizures were manifested by the EEGs, and at this point, the daily MCD was calculated. One to two days before beginning the drug session, Ss were habituated to the chair, and the control experiments were conducted. Following the completion of each experimental session of daily drug administrations, the Ss were returned to their home cages and no drugs were administered to these animals for a period of 3 to 4 weeks.

Part 2. Study on the alteration in MCD and behavioral responses during long-term session

Two Ss used in this study were permanently housed in open-face cubicle (74 x 71 x 86 cm) and fitted with a monkey jacket (nylon net) connected to a restraining joint-arm which attached to the rear wall of the cubicle in order to allow relative free movement to the Ss. Cocaine was injected once daily at a MCD using the same procedures as described in Part 1. A session consisted of daily cocaine injections and polygraphic recordings for 5 consecutive days every week over a 5 to 6 month period.

Part 3. Study on the alterations in cardiorespiratory stimulating effect

Six Ss used in this study were restrained in the chair, and the polygraphs of EEGs, eye movement, H.R. and R.R. were recorded daily for 6 to 12 days. Following the control experiments for 1-2 days, cocaine was injected once daily at subconvulsant dosage (2-4 mg/kg, IV) for 5 to 10 days using the remote control technique. Mean H.R. and R.R. per min. were determined by 10 randomly derived observations from polygraphic recordings at various times before and after a daily injection of cocaine (5, 10, 30, 60 min. and every 0.5 hour thereafter up to 5-6 hours after injection). The Ss were returned to their home cages and no drugs were administered to these Ss for a period of 3 to 4 weeks.

Part 4. Study on the alteration in plasma half-life of cocaine

Two Ss used in this study were restrained in the chair and the blood samples were drawn from the Ss at various times after I.V. injection of [^{3}H] cocaine (4.0 mg/kg as free base) during recording of the polygraphs. [^{3}H] Cocaine concentration in plasma were determined by the method previously described (21). Mean half-lives ($T_{\frac{1}{2}}$) of [^{3}H] cocaine were determined following the [^{3}H] cocaine injection on the first and fourth day, respectively, of the experimental session. The daily injection on the two intervening days (second and third day) was the same dose of non-labeled cocaine (4.0 mg/kg).

Results

1. Alteration in MCD of cocaine in the short-term session

Intravenous injection of cocaine in doses of 3.1 to 6.0 mg/kg produced clonic convulsions with seizures lasting 20 sec. to 5 min. Severity and duration of the convulsions varied depending upon the dosage of cocaine. With

repeated daily injections of cocaine, the convulsant effect markedly decreased. Typical changes in the MCD for cocaine following repeated daily injections are given in Figure 1. An injection of cocaine at a 3.1 mg/kg produced convulsions that lasted for 30 sec. on the first day but no convulsions on the subsequent three days (Fig. 1A). Following three days without a cocaine injection, an increased dose of 4.7 mg/kg was required to produce the convulsion. The same dose on the subsequent day did not produce a convulsion.

In another session (Fig. 1B) the same dose was given daily over a two-day span. An injection of 5.4 mg/kg produced a convulsion on the first day but no convulsion on the next day. A 7.0 mg/kg dose produced a convulsion on the third day but no convulsion on the fourth day. Following two days without cocaine, the MCD increased to 8.6 mg/kg on the seventh day but this same dose produced no convulsion on the eighth day.

Figure 2 is representative of the data on the MCD of cocaine following repeated daily injections. In the first session (S1) the MCD on the first day was 5.2 mg/kg and it rapidly increased to 5.6, 6.0 and 7.0 mg/kg on the second, third and fourth day, respectively. Following two days without a drug injection, the MCD on the seventh day was 7.6 mg/kg and increased continuously to 12.0 mg/kg by the eleventh day of drug administration. In the second session (S2) which started 35 days after the first session (no drug administered during this period) the MCD on the first day was 6.9 mg/kg which increased to 10.7 mg/kg within 8 days.

Figure 3 represents the mean MCD of cocaine and the mean percent increase in the MCD from the first dose for the first five days from eight experimental sessions using six different monkeys. The mean MCD on the first day was 5.3 mg/kg and it increased to 6.4, 7.6, 8.1 and 9.4 mg/kg, respectively, on the second, third, fourth and fifth day. The differences in the mean MCD for each day in comparison to the first MCD and to each other were statistically significant (see Fig. 3).

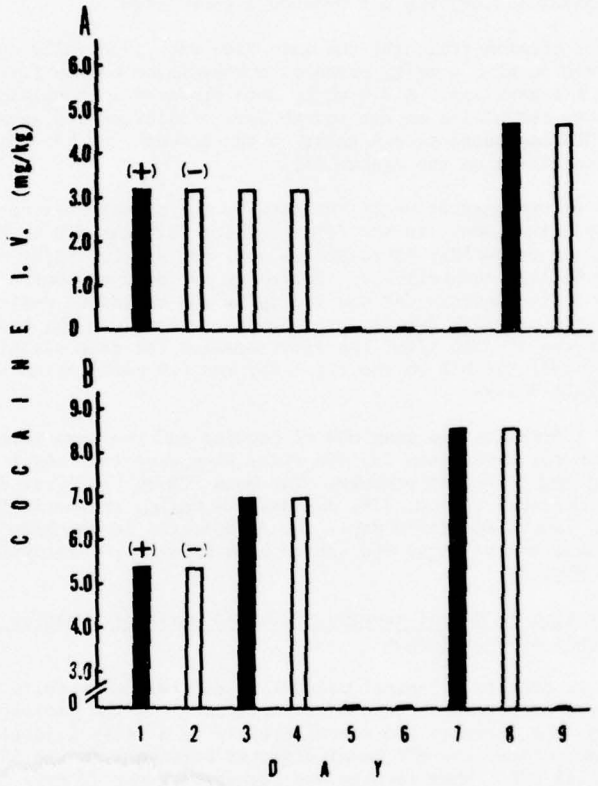
2. Alteration in MCD of cocaine, EEGs and animals' behavior following long-term chronic administration

Figure 4 represents a typical pattern of alteration in daily MCD of cocaine during a long-term chronic administration. In the prolonged experimental session, the elevated MCD was maintained by a daily injection of cocaine and during this period, the MCD level migrated between a 50 and 100% increase above the initial MCD. When cocaine was withdrawn after 39 days of chronic administration, the MCD returned to the initial control level (4.1 mg/kg) at 41 days after the discontinuance of cocaine. Following chronic administration and subsequent withdrawal of the drug, the Ss became gentle and inactive and were persistently depressed in their overall behavior. Upon withdrawal, the Ss exhibited persistent high-amplitude rhythmic slow-waves (5-6 Hz) predominantly in the neocortical EEGs associated with their behavioral depression.

On resumption of chronic administration of cocaine to the Ss, which did not receive any drug for 40 days (see S2-1 and S2-2 of Fig. 4) the MCD rapidly increased by 80% above the first dose within a two-week period of daily injections. Thereafter, the MCD gradually decreased from the peak of 80% level to a level of 25-40% above the control and was maintained at this level for five weeks. Subsequently, the MCD elevation stabilized at a 20-33% level, and the animals began to have a constant intensity and magnitude of convulsions lasting 20-25 sec. as manifested in the EEGs' and behavioral responses.

The animals displayed post-convulsive hyperexcitation which was also stabilized simultaneously with the convulsions and usually lasted for 2-2.5

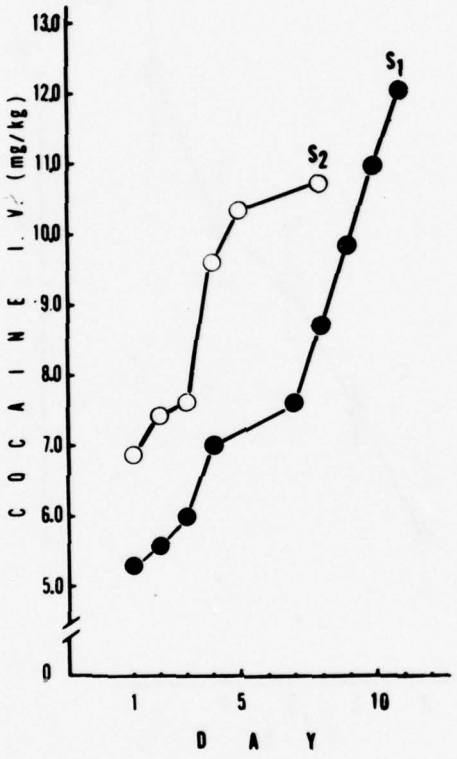
FIG. 1



Typical pattern of changes in the minimal convulsant dosage (MCD) of cocaine following repeated daily injections in the monkeys. In Graphs A and B, black bar (+) indicates the MCD and open bar (-) the dose which did not produce convulsions. No drug was given on the fifth, sixth and seventh day in Graph A and on the fifth, sixth and ninth day in Graph B. Different monkeys were used for the data in each of two graphs.

hours. The animals displayed self-mutilation (biting and chewing of digits of extremities, hands, arms and legs) during the post-convulsive hyperexcitation. Except during the post-convulsive excitatory period, the Ss were gentle, inactive and behaviorally depressed most of the time. At this stage of chronic

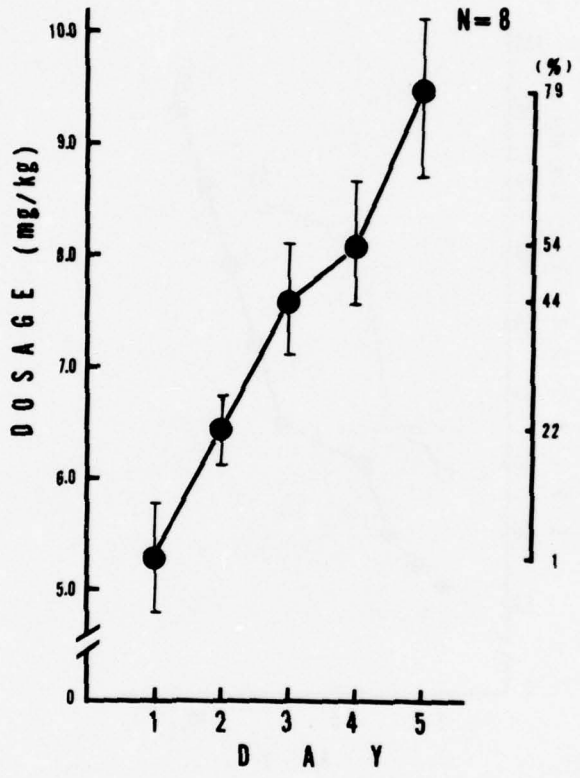
FIG. 2



Typical pattern of changes in the minimal convulsant dosage (MCD) of cocaine following repeated daily injections in the monkeys. S1 (● - ●) represents daily MCD for the first 11 days out of 30 days' session (first session) and S2 (o-o) the daily MCD for the first 8 days of the second session which started 35 days after the end of the first session (S1). No drug was given on the fifth and sixth day in S1 and on the sixth and seventh day of the second session (S2).

treatment, the Ss showed hypersensitive excitatory response to the subconvulsant low dose, suggesting an increase in sensitivity to the excitatory effect of cocaine. These results indicated that chronic treatment with daily

FIG. 3



The mean minimal convulsant dosage (MCD) of cocaine and the mean percent increases of the MCD above the first MCD for the first 5 days of 8 experimental sessions using 6 different monkeys. Each session consisted of daily injections of cocaine (once daily) for 5 to 10 days. The left ordinate represents I.V. dosage of cocaine and the right ordinate percent increases of the MCD. The abscissa represents days of the experimental sessions. Each value represents the mean ± S.E.M. (vertical lines). The mean MCD for the second to fifth day differ significantly from the first MCD ($P<0.01$) and from each other ($P<0.05$) using Student's T-test.

MCD of cocaine produced a constant intensity of convulsions and post-convulsive excitatory response, and upon the withdrawal, the Ss showed chronic alterations in the EEGs' and behavioral responses.

These results indicated that the repeated daily administration of cocaine in the monkeys resulted in tolerance to the convulsant effect of cocaine and this tolerance was maintained by a long-term chronic administration of this drug. Following withdrawal of the drug, the animals showed chronic alterations in the EEGs and their behavioral responses.

3. Alterations in cardiorespiratory stimulating effects

An intravenous injection of cocaine at a subconvulsant dose (2-4 mg/kg) as well as a convulsant dose produced marked increases in heart rate (H.R.) and respiratory rate (R.R.). However, these effects were reduced by repeated daily injections of cocaine (Fig. 5). In the control experiments (no drug injections) the mean H.R./min. ranged from 121 ± 3.5 (S.E.M.) to 181 ± 4.0, and the mean R.R./min. ranged from 21.7 ± 0.60 (S.E.M.) to 30.3 ± 0.60, in six different Ss.

An injection of cocaine at 4.0 mg/kg, I.V. (a subconvulsant dose) produced an average of 46.1 and 24.0% increases above the pre-drug control in H.R. at 10 min. and 30 min., respectively, after the injection on the first day (Fig. 5A). However, the same dose on the second day produced an average of only 23.3 and 10.9% increases in H.R. at 10 min. and 30 min. after injection. An average of 18.4 and 10.5% increases at 10 min. and 30 min., respectively, were observed on the third day.

Simultaneously, the R.R. (Fig. 5B) increased an average of 65.3 and 46.4% above the pre-drug control at 10 min. and 30 min., respectively after injection on the first day. However, the average increase of R.R. was 40.0 and 24.1% at 10 min. and 30 min., respectively, on the second day, and 28.2 and 17.8% on the third day. The differences in the mean percent increases in H.R. and R.R. observed on the second and third day as compared to those on the first day were statistically significant ($P<0.01$). In the control experiments, injections of saline (1-2 ml/kg) produced no alteration in H.R. and R.R. under the same experimental conditions.

These results indicated that the repeated daily injections of cocaine at the same doses produced a significant reduction in the effect of this drug in producing increases in the heart and respiratory rates.

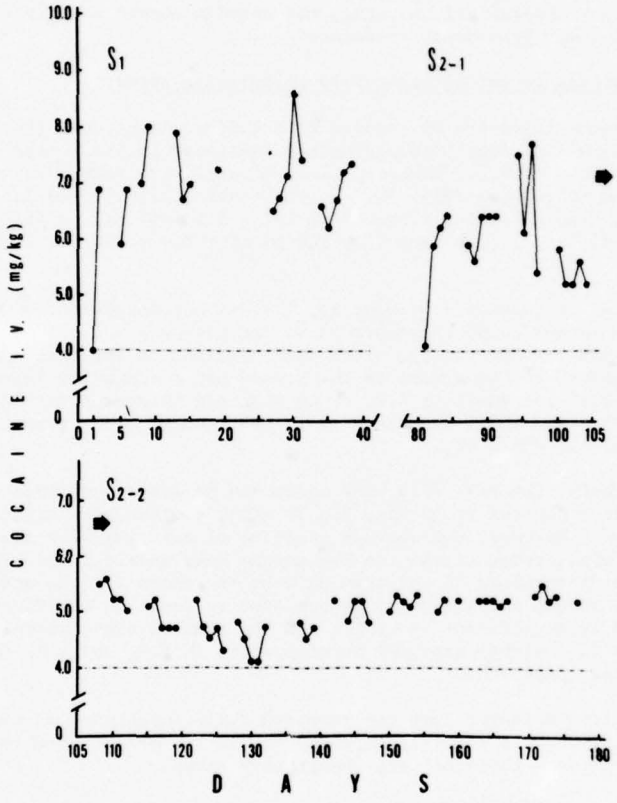
4. Alteration in plasma half-life of [^{3}H] cocaine

The repeated daily injections of cocaine (4.0 mg/kg, I.V.) also reduced the apparent $T_{\frac{1}{2}}$ of cocaine in the plasma of two monkeys. The mean plasma $T_{\frac{1}{2}}$ of [^{3}H] cocaine on the first day was 93 min., and decreased to 48 min. on the fourth day after two daily injections of the same dose of non-labeled cocaine. The statistical significance of this difference in $T_{\frac{1}{2}}$ values, however, was not evaluated.

Discussion

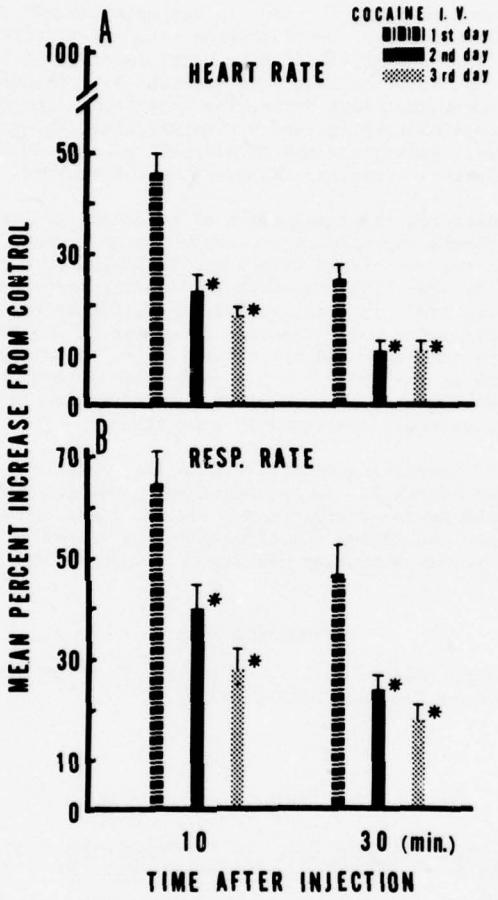
Previous studies (1-11) on the chronic administration of cocaine in experimental animals have demonstrated no tolerance to the excitatory effects of cocaine and no abstinence syndrome on abrupt withdrawal (9,11). A pronounced increase occurred in severity of behavioral and toxic effects of cocaine on repeated daily administration (2-4,7,12,14). In most of these studies, however, the drug was administered in subconvulsant doses either

FIG. 4



Typical pattern of changes in the daily minimal convulsant dosage (MCD) of cocaine during chronic injections in the monkey. In the upper graph, S1 represents the daily MCD for the first session (39 days) and S2-1 represents the MCD for the second session which started after 41 days without drug injection following the first session (S1). S2-2 in the lower graph is a continuation of the S2-1 (shown by black arrows). The ordinate represents I.V. dosage of cocaine, and the abscissa the days of the experimental session. Black dots indicate the daily MCD and the connecting lines between dots indicate that the daily injections were given on the consecutive days. Usually, a daily injection was given for 4 to 5 consecutive days per week (no drug was given for 2 days over the weekend). The horizontal broken lines in the graph indicate the control MCD level (the first MCD).

FIG. 5



Mean percent increase above control in heart rate/min. (A) and in respiratory rate/min. (B) at 10 min. and 30 min., respectively, after the daily I.V. injection of cocaine (4.0 mg/kg) for the first, second and third days of eight experimental sessions using four different monkeys. Each value represents the mean ± S.E.M. (vertical lines). *Values differ significantly from appropriate values observed on the first day ($P<0.01$). The differences in values observed on the third, fourth and fifth day as compared to those on the second day were not statistically significant.

orally, intraperitoneally or intramuscularly and the effect of the drug evaluated by gross visual observations of the responses of the animals.

Because of the very rapid metabolism of cocaine (22) its effect varied considerably with the route of its administration. In our study, the drug was given intravenously at high doses and high rate of infusion (5 mg/ml/10 sec.) and the MCD was determined daily in order to evaluate the effects of drug on EEGs' and behavioral responses. The injection rate was a critical factor in producing convulsions during or immediately after intravenous injections. In order to determine the MCD of cocaine, the constant I.V. injection rate proved adequate for producing convulsions during the injection. The measurement of MCD and the electrophysiological parameters on the heart and respiratory rates enabled us to precisely quantitate the development of tolerance to the convulsant and cardiorespiratory stimulant responses in the monkeys.

Our study demonstrated the development of tolerance to the convulsant and cardiorespiratory stimulating effects of cocaine in the monkey. The development of tolerance to the convulsant effect was indicated by a decrease in the effect of this drug in producing convulsions following repeated daily injections at the same dose and a progressive increase in daily MCD of cocaine following chronic administration. A marked reduction in the stimulating effects of cocaine on the heart and respiratory rates following repeated daily injections of cocaine at the same dose provided evidence for the development of tolerance to these effects. The recovery from tolerance to the convulsant effects of cocaine required a considerably long time.

The mechanism of increasing sensitivity to the excitatory effect of cocaine is still controversial. It is conceivable that the neuronal mechanism of convulsions by cocaine may differ from those for other excitatory effects. The central mechanisms underlying the development of tolerance to the convulsant and cardiorespiratory stimulant effects of cocaine also remain to be explored.

Acknowledgments

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DISPOSITION AND BIOTRANSFORMATION OF COCAINE

Anand L. Misra

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INTRODUCTION

Cocaine (methyl-3β-hydroxy-1-αH, 5αH-tropane-2β carboxylate-3-benzoate), pk_a 8.70, is a potent central nervous system stimulant of relatively short duration, low margin of safety, and high systemic toxicity ($^{LD}50$ i.v. in rats and rabbits 17.5 and 17 mg/kg respectively; fatal dose i.p. in rats 100 mg/kg). It is much less toxic by oral ingestion than by other routes of administration. Its intense euphoriant action leads to a very high degree of psychic dependence.[1-8] Craving for cocaine is more apparent by intravenous and not by oral route of administration. Cocaine has little or no tolerance and physical dependence liability, and considerable evidence has been presented[1-9] for a reverse tolerance or increase in sensitivity to cocaine on repeated dosing. This sensitization persisted unabated for a couple of weeks after complete withdrawal of cocaine. In spite of several studies,[10-20] there is a paucity of detailed information on the biological disposition and metabolism of cocaine in acute and chronic states in experimental animals, particularly in species (e.g., dogs and monkeys) which exhibit psychological effects so characteristic of chronic cocainism in man.

This chapter deals with dispositional and metabolic aspects of cocaine in rat, dog, and monkey in acute and chronic states, with a view to assess the possible relationship and significance of these parameters to the acquired sensitivity and systemic toxicity to cocaine in these species on chronic treatment.

DISPOSITION AND METABOLISM OF [^{3}H] COCAINE IN RATS

Materials and Methods

The method for the preparation of randomly labeled and ring-labeled [^{3}H] cocaine has been described earlier.[21] In our studies, randomly labeled [^{3}H] cocaine, which was isotopically stable in vivo and had high specific activity, was used for the tissue distribution studies, and ring-labeled [^{3}H] cocaine with lower specific activity was used for the excretion studies in view of the metabolic lability of methyl ester and benzoyl groups of cocaine. Other details on method of estimation of cocaine in biofluids and tissues, recoveries, specificity of extraction procedure,[22] radioscanning of thin-layer radiochromatograms (Gelman® ITLC, silica gel sheets), and counting procedures, etc. have previously been described.[23,24]

Distribution of [^{3}H] cocaine in Tissues and Biofluids of Rats After Intravenous Injection

The data on the distribution of [^{3}H] cocaine after a single 8 mg/kg intravenous injection in male Wistar rats appear in Figure 1. The peak levels of cocaine in brain, plasma, and other tissues occurred within 15 min. Highest concentrations of cocaine were observed in spleen followed by kidney, lung, brain, and testes. Liver and fat had lower concentrations as compared to these tissues. Muscle, heart, and plasma had still lower levels of [^{3}H] cocaine. These levels declined sharply and 4 hr after injection were very low in brain and absent from plasma. After 6 hr cocaine disappeared completely from brain, heart, muscle, testes, and fat. The values in spleen, kidney, lung, intestine, and liver 6 hr post-injection ranged between 0.03 to 0.09 μg/g. No persistence of cocaine in rat brain was observed with this dose. The half-life of cocaine in rat brain and plasma with this dose was 0.4 and 0.3 hr respectively. After a 10 mg/kg convulsive dose of [^{3}H] cocaine by intravenous injection in the rat, the levels of cocaine in rat brain and plasma were 35 μg/g and 5 μg/ml respectively within 2 min of injection. N-Dealkylation of cocaine to norcocaine was observed in rat brain within 1 to 2 min after 10 mg/kg i.v. dose of cocaine. High lipid solubility of cocaine (apparent partition coefficient in the concentration range 0.1 to 1 μg/ml in 1-octanol-0.1 M phosphate buffer, pH 7.4, 7.6 ± 0.1 [S.E.M.]) played an important role in rapid entry of cocaine into rat brain. The high brain to plasma ratio (12 to 8) between 0.25 to 2 hr post-injection of 8 mg/kg i.v. dose indicated a marked affinity of cocaine for the tissues.

Biliary Excretion Studies in the Rat

These studies[22] after 5 mg/kg i.v. injection in the rat showed that cocaine was very rapidly metabolized, and unchanged cocaine was excreted in very small amounts in bile (Figure 2) up to 3.5 hr post-injection. The excretion of total radioactivity comprising metabolites of cocaine was high at 0.5 and 0.5 to 1 hr and gradually declined thereafter. Total radioactivity as mean percentage of dose excreted in rat bile was approximately 36 in 3.5 hr post-injection.

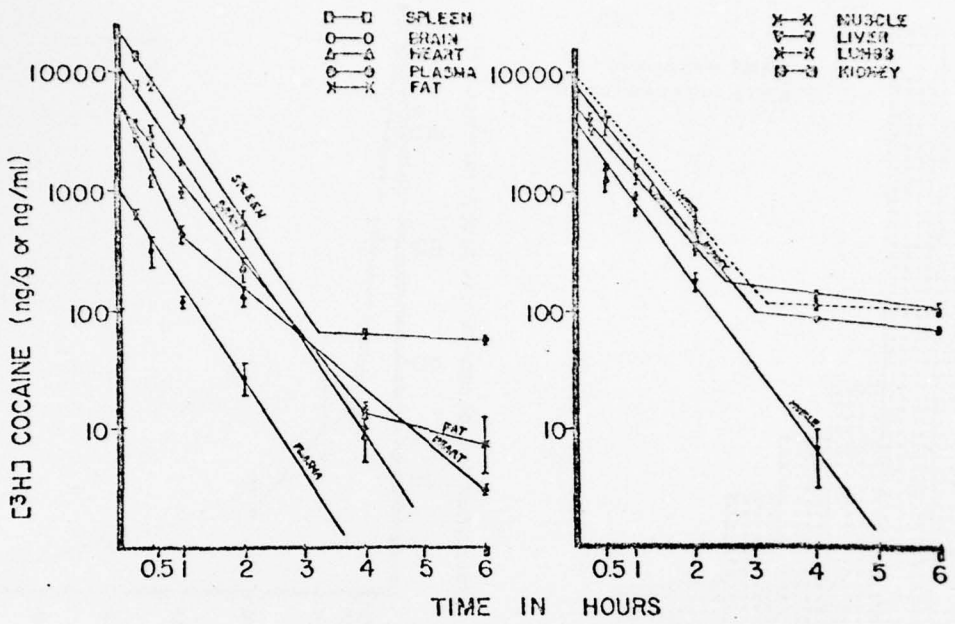

FIGURE 1. Distribution of [^{3}H] cocaine in tissues and biofluids of male Wistar rats after a single 8 mg/kg intravenous injection (n = 3).

Distribution of [^{3}H] Cocaine in Tissues and Biofluids of Acutely and Chronically Treated Rats After Subcutaneous Injection

The mobilization of cocaine from the injection site after a 20 mg/kg subcutaneous injection in acutely and chronically treated male Wistar rats (Figure 3) was rapid, in spite of the fact that local vasoconstriction effect of cocaine could limit its absorption. No significant differences were observed in relative rates of disappearance of cocaine from the subcutaneous site in the two groups.[22]

No significant differences were observed in plasma-protein binding of cocaine (mean value, 33.4 to 38.6%) in vitro at ambient temperature (25°C) in control, acutely, and chronically treated rats using an earlier procedure.[24] In view of the relatively small degree of plasma-protein binding of cocaine and its elimination primarily by biotransformation, binding of lipophilic cocaine to tissues may play a more important role in determining its overall pharmacokinetics than the plasma-protein binding.

Data on the comparative distribution of [^{3}H] cocaine in acutely and chronically treated rats following a 20 mg/kg subcutaneous injection are given in Figure 4. In acute animals, a characteristic pattern of gradual increase in cocaine concentration to a peak at 4 hr was observed for plasma, brain, and most tissues, with the exception of heart and fat, where peak levels occurred at 0.5 and 2 hr respectively. Metabolism of cocaine by liver resulted in much lower levels in this tissue. The sustained peak plasma levels between 1 to 4 hr, which were lower than those in other tissues, represented a state of relative equilibrium between absorption from the subcutaneous site and detoxication mechanisms. An abrupt fall in levels of cocaine occurred from plasma and most tissues at 4 and 6 hr, resulting in very low levels in brain, testes, and plasma at 12 hr and the absence of drug at 24 hr. The fairly rapid disappearance of cocaine from rat brain and plasma is similar to that observed earlier by us with thebaine,[24,25] a morphine congener and stimulant with little or no tolerance and physical dependence liability. In contrast, the opioid depressant drugs[23,26-28] (e.g., morphine and methadone) with high tolerance and physical dependence liability persisted in the CNS in small concentrations for prolonged periods, even after a single subcutaneous injection.

Peak levels of cocaine in brain and plasma of chronically treated rats occurred between 1 to 2 hr post-injection (Figure 4), and a shift in cocaine levels from 4 to 2 hr in brain and 4 to 1 hr in plasma occurred in chronically treated rats. However, no significant differences were observed in

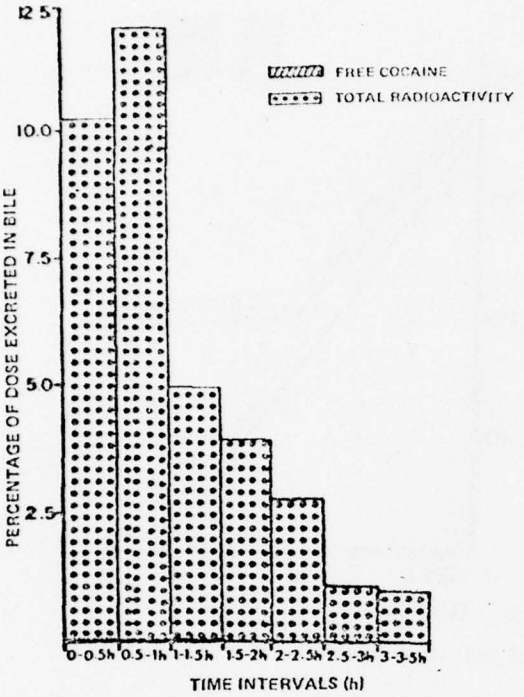

FIGURE 2. Biliary excretion of [^{3}H] cocaine and total radioactivity after a 5 mg/kg intravenous injection in male Wistar rats.

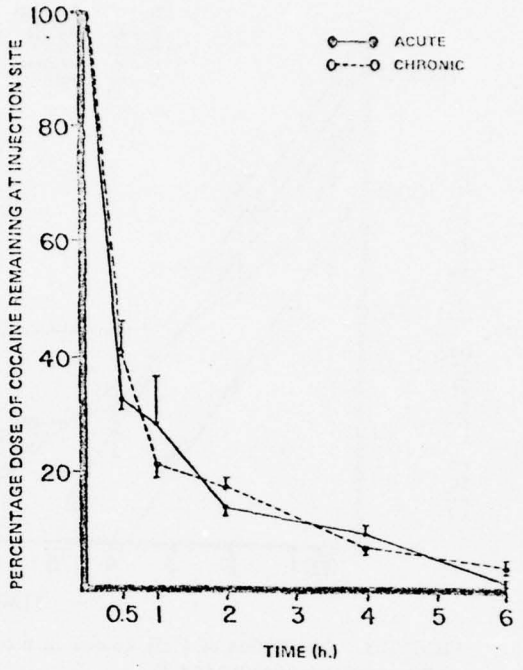

FIGURE 3. Comparative rates of disappearance of [^{3}H] cocaine from subcutaneous site in acutely and chronically treated rats after 20 mg/kg subcutaneous injection ($n = 5$ in each group). For chronic experiments, the animals were given s.c. injection of 20 mg/kg dose of nonradioactive cocaine twice daily for 3 weeks, then with same dose of labeled cocaine twice daily for 2 days before the terminal injection of same dose of labeled cocaine.

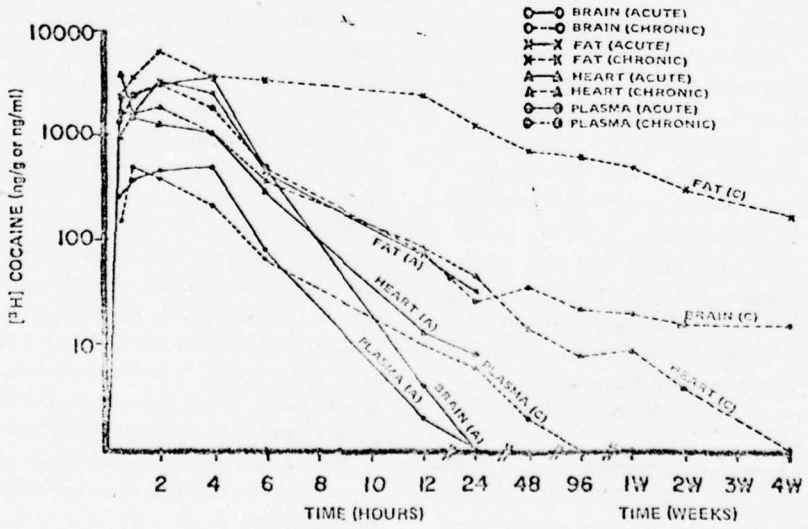

FIGURE 4. Comparative distribution of [^{3}H] cocaine in tissues and biofluids of acutely and chronically treated rats after 20 mg/kg subcutaneous injection (n = 5 in each group). Other details on chronic treatment as given in footnote of Figure 3.

peak levels of cocaine in brain and plasma in two groups. Cocaine was not detectable in plasma of chronically treated rats 24 hr post-injection and barely detectable in heart and testes at 48 and 96 hr. Comparatively high levels of cocaine were sequestered in fat of chronically treated rats, and these declined gradually over a period of 4 weeks to 0.18 μg/g. Although the relatively poor blood supply to fat depots slows the transfer of cocaine from blood to fat, enough cocaine is finally transferred in 2 hr to bring the fat depots in both groups to their equilibrium concentrations. The half-life of cocaine in fat in acutely and chronically treated rats was 1.8 to 2.0 hr. The sequestration of cocaine in substantial amounts in fat depots of chronically treated rats, in contrast to the acute, provided a slow and prolonged release and may account for its persistence in brain in small amounts in this group. The equilibrium of cocaine in other tissues of the acute animals occurred only after the attainment of equilibrium in heart and fat. The pharmacokinetics of cocaine after subcutaneous injection in the rat is complicated by the prolonged course of its absorption from the subcutaneous site and the redistribution phenomena occurring during this phase. The approximate half-life of cocaine in brain and plasma of acute animals was 0.8 and 1 hr respectively, and that in brain, plasma, and tissues of chronically treated rats was 1.8, to 2.0 hr respectively.

The brain to plasma ratios of cocaine in chronically treated rats after 20 mg/kg subcutaneous injection were in general higher than those in the acute animals, with the exception at 1 hr when peak levels of cocaine occurred in plasma in the chronic group. The data indicated an altered distribution of cocaine in the chronic group.

The rise and fall of cocaine levels in rat brain coincided roughly with the change of levels in plasma and suggested that (a) cocaine was probably present in the CNS in the interstitial compartment, (b) its penetration into and exit out of the cell in the CNS must be fairly rapid, and (c) any significant intracellular or extracellular binding in the CNS must be freely reverisble.

Metabolites of Cocaine in Rat Brain

Norcocaine, benzoylecgonine, benzoylnorecgonine, and ecgonine were shown[29] to be the metabolites of cocaine in brains of both acutely and chronically treated rats. Norcocaine represented approximately 15% of the values expressed as cocaine in brain in Figure 1 and approximately 10% of those expressed as cocaine in brain in Figure 4. In chronic experiments, the content of norcocaine[22] in brain of rats injected with a 20 mg/kg s.c. dose of [^{3}H] cocaine was approximately 20% of that expressed as cocaine (Figure 3). Rat liver microsomal enzymes in vitro have been reported[30,31] to convert cocaine to norcocaine and benzoylecgonine. The half-life of benzoylecgonine and ecgonine in rat brain following a 10 mg/kg intravenous injection of these compounds was 1.3 and 7.8 hr respectively; that in plasma was 0.8 and 3.8 hr respectively.[32,33] This may imply a slower clearance of these polar metabolites from rat brain. Our studies[32] on the pharmacological activity of these metabolites after intravenous and intracisternal injection in the rat showed that norcocaine, like cocaine, possessed potent stimulant activity by both routes of administration, while benzoylecgonine and benzoylnorecgonine were active only by the intracisternal route, and ecgonine and ecgonine methyl ester were inactive by both routes of administration. Benzoylnorecgonine was approximately ten times more potent intracisternally as compared to benzoylecgonine in the rat.[32]

Urinary and Fecal Excretion of Cocaine and its Metabolites in Acutely and Chronically Treated Rats

Comparative data on the urinary and fecal excretion of cocaine and total radioactivity in the two groups are given in Figure 5. Cocaine was metabolized very rapidly in the rat and only 1 to 1.5% of the dose was excreted unchanged in the rat urine and feces, with maximum excretion occurring within 24 hr in the two groups. The excretion of metabolites continued for several days as reflected by the values of total radioactivity which were similar in the acute (49.3% of the dose) and the chronic group (51.6%). Significant differences, however, were observed in the two groups relative to the excretion of total radioactivity in feces, the values in the chronic group being consistently higher (35.6% of the dose) than those in the acute group (22.1%). Cumulative total radioactivity in urine and feces in the chronic group was significantly higher (87.5% of the dose) as compared to the acute (71.4%).

Urinary Metabolites of Cocaine in Acutely and Chronically Treated Rats

Column and thin-layer chromatographic

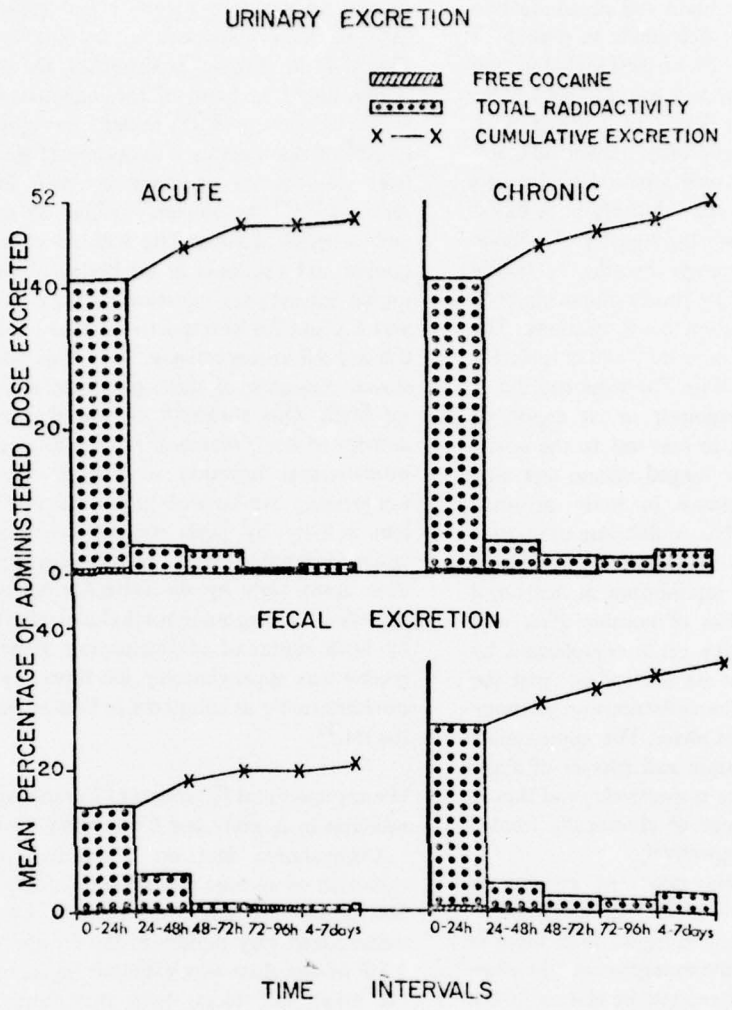

FIGURE 5. Comparative urinary and fecal excretion of free cocaine and total radioactivity in acutely and chronically treated rats after 20 mg/kg s.c. injection of [^{3}H] cocaine (n = 5 in each group). (From Nayak, P. K., Misra, A. L., and Mulé, S. J., *J. Pharmacol. Exp. Ther.*, 196, 556, 1976. With permission.)

studies[3,4] on pooled urine of rats injected subcutaneously with a 20 mg/kg dose of [^{3}H] cocaine provided evidence for the presence of benzoylecgonine, benzoylnorecgonine, ecgonine, and ecgonine methyl ester as urinary metabolites in addition to unmetabolized cocaine. Norcocaine and norecgonine were not detected as urinary metabolites. Tentative evidence was also obtained for a phenolic metabolite (*p*-aromatic hydroxylation) and ring-hydroxylated metabolites (hydroxy groups presumably in the 6,7 position of the pyrrolidine ring). Such hydroxylation probably occurs before the hydrolysis of cocaine or norcocaine to polar benzoylecgonine or benzoylnorecgonine. In our study,[22] evidence for glucuronide conjugation of these metabolites was not obtained. The pattern of urinary metabolites in acutely and chronically treated rats was qualitatively similar, with somewhat higher excretion of benzoylecgonine and ecgonine methyl ester in the chronic

group as compared to the acute. The known and hypothetical metabolic pathways of cocaine in the rat are outlined in Figure 6.

DISPOSITION AND METABOLISM OF COCAINE IN THE DOG

Distribution of Cocaine in Acutely and Chronically Treated Dogs[35]

Beagle dogs of either sex (6 to 10 kg) were given intravenous injection of 5 mg/kg (free base) dose of [^{3}H] cocaine. This dose produced mild excitation, restlessness, anxious behavior, rapid respiration, some rise of body temperature, muscle tremors for a few minutes, salivation and pupillary dilatation, but no convulsions. An intravenous dose of 10 mg/kg produced very severe convulsions and hyperexcitation. For the chronic study, beagle dogs were administered a 5 mg/kg dose of nonradioactive cocaine subcutaneously twice daily for 6 weeks, followed by the radioactive dose (5 mg/kg) by intravenous injection. Comparative data on the distribution of cocaine in tissues and body fluids of acutely and chronically treated dogs after 5 mg/kg i.v. injection of [^{3}H] cocaine appear in Figure 7. The values of cocaine in plasma, muscle, heart, fat, duodenum, spleen, lung, and kidney were higher in the chronic as compared to the acute group. The values of cocaine in liver were lower in the chronic group up to 2 hr post-injection and somewhat higher than those in the acute at later times. In the chronic group, the values of cocaine in bile were lower initially (0.5 and 1 hr), higher at 2 and 4 hr, and again dropped lower than those in the acute group at later times. Comparatively faster disappearance of cocaine occurred from bile 4 hr post-injection in the chronic as compared to the acute group. Cocaine disappeared from plasma in the two groups by 12 hr, and with the exception of bile and liver, other tissues had very low levels of cocaine 24 hr post-injection. The levels of cocaine in bile and liver 1 week post-injection were in the range of 50 to 80 ng/ml or g respectively. The half-lives of cocaine in plasma, liver, kidney, spleen, and heart in the acute dogs were 1.2, 2.2, 1.8, 1.8, and 2.0 hr respectively, and in the chronic group, 1.1, 1.8, 1.2, 1.3, and 1.2 hr respectively.

The values of total radioactivity in plasma and fat were higher in the chronic group, but those in liver, kidney, lung, and spleen were lower in the chronic as compared to the acute study. No changes in the levels of total radioactivity occurred in muscle in the two groups at earlier times.[35]

Data on the distribution of cocaine in selected anatomic areas of the CNS of the acutely and chronically treated dogs [35] appear in Figure 8. With the exception of cerebrospinal fluid, the levels of free cocaine in general were higher in the chronic group in all areas of the CNS as compared to the acute. Cocaine did not persist in the CNS of dog in the two groups, and by 12 to 24 hr post-injection, complete disappearance of cocaine occurred from all areas of the CNS in both groups. The half-lives of cocaine in selected areas of the CNS of the acute dogs were in the range 1.5 to 1.6 hr, and in the chronic group, 1.2 to 1.5 hr. There was no change in the half-life of cocaine in the cerebrospinal fluid and the spinal cord. The lack of persistence of cocaine in the CNS and plasma of the dog in two groups is similar to that observed for the rat.[22] The rise and fall of cocaine levels in the CNS of the dog coincided roughly with changes in plasma levels of cocaine. This suggested that penetration of cocaine into and exit out of the cell in the CNS must be fairly rapid and any extra- or intracellular binding in the CNS must be freely reversible.

The brain to plasma ratios of cocaine (Figure 9) showed no significant differences at 0.5 and 1 hr, were lower in the chronic group at 2 and 4 hr post-injection as compared to the acute, and indicated an altered distribution of cocaine in the chronic group.[35] The CSF to plasma ratios of cocaine (Figure 10) were significantly higher in the acute as compared to the chronic group up to 2 hr, but showed no significant differences thereafter.[35]

The total radioactivity values were significantly higher in the areas of the CNS of chronically treated dogs as compared to the acute up to 24 hr, and they tended to reach similar levels thereafter due to rapid metabolism and elimination of cocaine and its metabolites in chronically treated dogs. Significant amounts of radioactivity persisted in selected areas of the CNS of the chronically treated dogs 1 week post-injection, long after the disappearance of cocaine and norcocaine from these areas.

Metabolism of Cocaine in Brain of Acutely and Chronically Treated Dogs[35]

As previously observed in the rat,[22] norcocaine, benzoylecgonine, benzoylnorecgonine,

FIGURE 6. The known and hypothetical metabolic pathways of cocaine in the rat. (From Nayak, P. K., Misra, A. L., and Mule, S. J., *J. Pharmacol. Exp. Ther.*, 196, 556, 1976. With permission.)

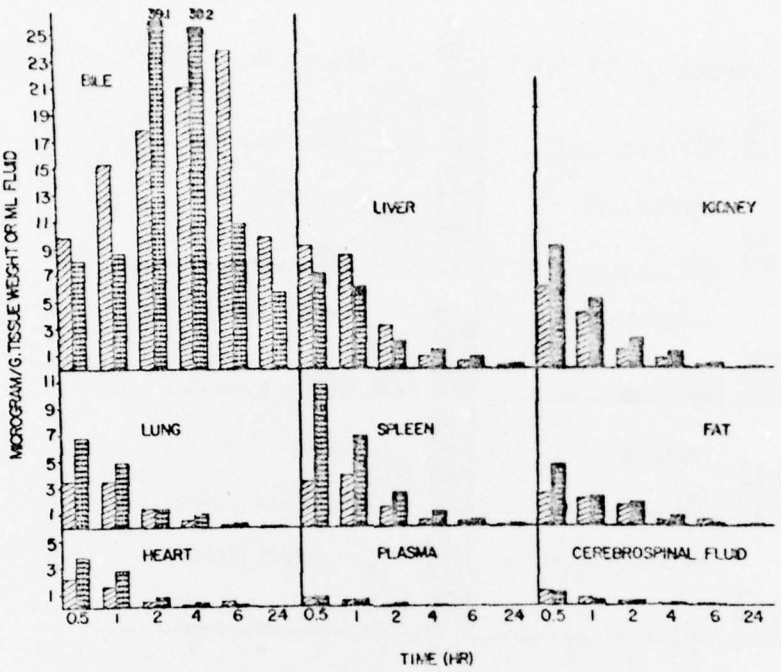

FIGURE 7. Comparative distribution of free cocaine in tissues and biofluids of acutely (slanted-line bar) and chronically treated (dotted bar) dogs after 5 mg/kg intravenous injection of [^{3}H] cocaine. Each figure represents the mean value from two dogs sacrificed at various times, and duplicate determinations were performed on the individual tissues of each animal and the results averaged. For chronic experiments, beagle dogs were injected s.c. with a 5 mg/kg dose of nonradioactive cocaine twice daily for 6 weeks, then with a 5 mg/kg s.c. dose of randomly labeled [^{3}H] cocaine twice daily for 2 days, followed by a terminal injection of the same dose by i.v. route.

and ecgonine were identified as metabolites of cocaine in the dog brain in the two groups. At later times, however, only benzoylecgonine and benzoylnorecgonine were present in the dog brain. The amounts of norcocaine as percentage of cocaine values in brain (Figure 11) were higher in the chronic as compared to the acute group. The half-life of norcocaine in the brain of dogs injected with 5 mg/kg i.v. dose of [^{3}H] cocaine was 1 hr for the fast phase and 3.3 hr for the slower phase. Norcocaine did not persist in the CNS of the dog, and its rate of disappearance in brain was similar to that of cocaine. In brains of chronically treated dogs, benzoylnorecgonine was approximately four times that in the acute at 0.5 hr and twice that at the 4- and 24-hr periods, while benzoylecgonine was approximately one-third the amount in the acute group at 0.5 hr and approximately twice that at the 4- and 24-hr periods. Ecgonine concentration was several times higher in brains of chronically treated dogs as compared to the acute 0.5 hr post-injection. Although the lower lipid solubility of polar benzoylecgonine[32] and benzoylnorecgonine (apparent partition coefficients at 25°C in 1-octanol-M/15 phosphate buffer, pH 7.4, 0.15, and 0.21 respectively) prevented their penetration through the blood-brain barrier in adequate concentration, direct metabolism of cocaine and norcocaine in brain could lead to significant amounts of these metabolites in the selected anatomic areas of the CNS of the dog.

Urinary and Fecal Excretion of [^{3}H] Cocaine and Total Radioactivity in Acutely and Chronically Treated Dogs[35]

The excretion profile of cocaine and total radioactivity in acutely and chronically treated dogs is given in Figure 12. As previously observed,[15] cocaine was rapidly metabolized in the dog and a comparatively small fraction of the dose was

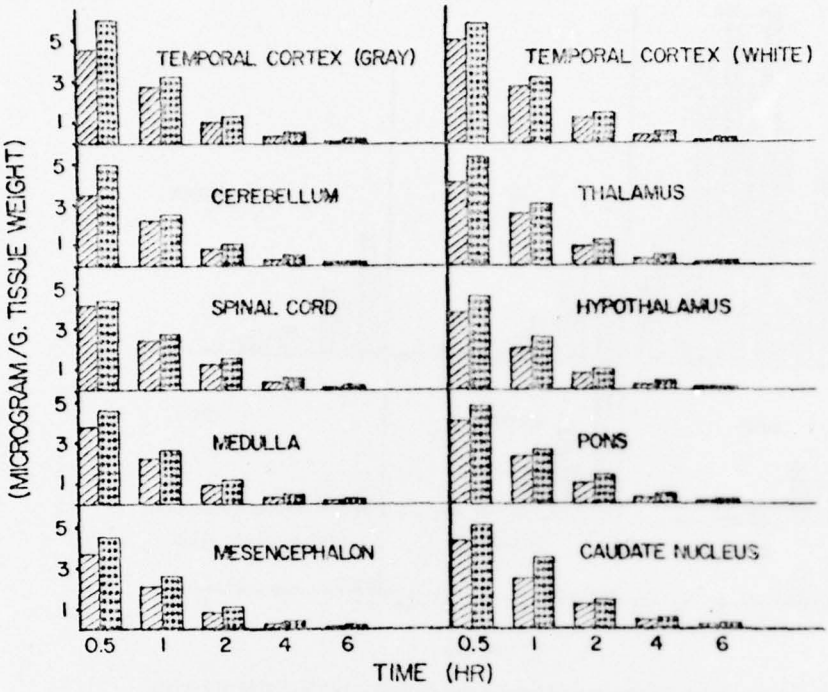

FIGURE 8. Comparative distribution of free cocaine in selected areas of the CNS of the acutely (slanted-line bar) and chronically treated (dotted bar) dogs after 5 mg/kg intravenous injection of [^{3}H] cocaine. Other details as in footnote of Figure 7.

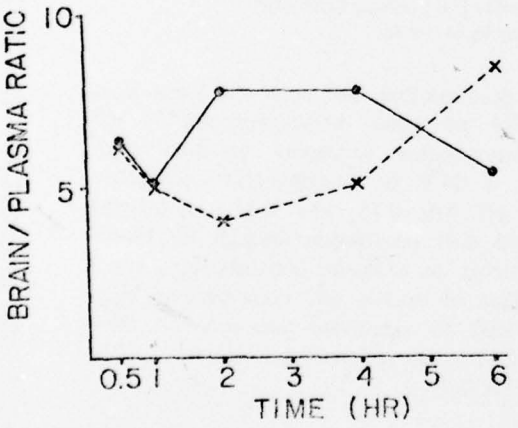

FIGURE 9. The brain to plasma ratio of mean concentration of cocaine in acutely (solid line) and chronically treated (dotted line) dogs after 5 mg/kg intravenous injection of [^{3}H] cocaine. Data represent mean values obtained from the ratio of concentration of cocaine in ten selected anatomic areas of brain to plasma at different times post-injection. Other details as in footnote of Figure 7. (From Misra, A. L., Patel, M. N., Alluri, V. R., Mulé, S. J., and Nayak, P. K., *Xenobiotica*, in press, 1976. With permission.)

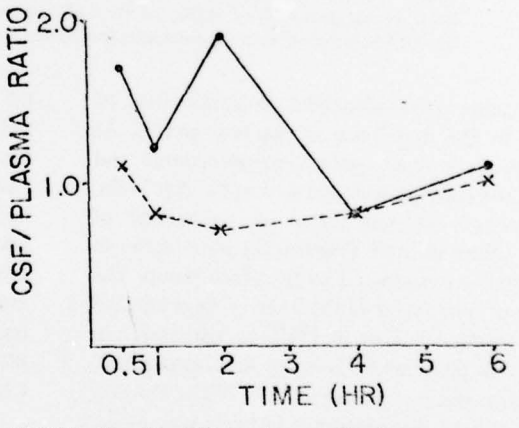

FIGURE 10. The CSF to plasma ratio of mean concentrations of cocaine in acutely (solid line) and chronically treated (dotted line) dogs after 5 mg/kg intravenous injection of [^{3}H] cocaine. Other details as in footnote of Figure 7. (From Misra, A. L., Patel, M. N., Alluri, V. R., Mulé, S. J., and Nayak, P. K., *Xenobiotica*, in press, 1976. With permission.)

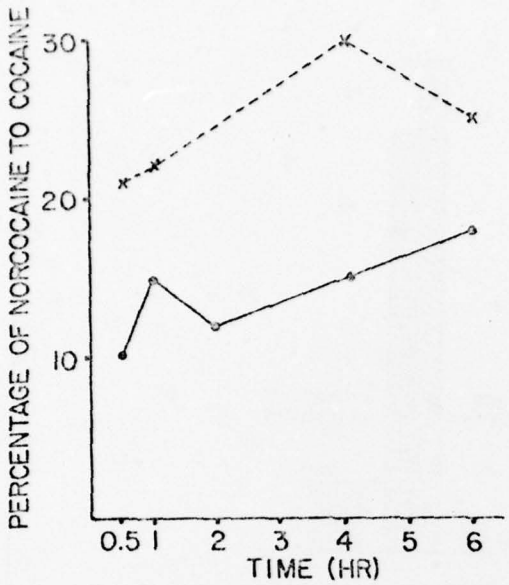

FIGURE 11. The ratio of amounts of norcocaine to cocaine in brain of acutely (solid line) and chronically treated (dotted line) dogs after 5 mg/kg intravenous injection of [^{3}H] cocaine. Ratios were determined from radioscans of the brain extracts processed by the method of extracting cocaine from tissues and biofluids. Other details as in footnote of Figure 7. (*From Misra, A. L., Patel, M. N., Alluri, V. R., Mulé, S. J., and Nayak, P. K., Xenobiotica*, in press, 1976. With permission.)

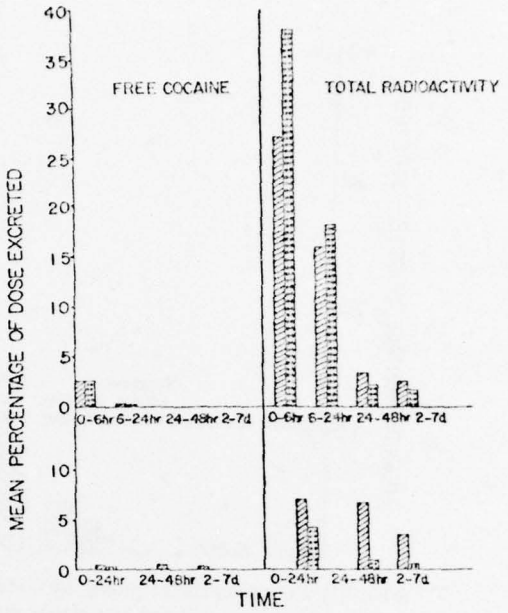

FIGURE 12. Comparative urinary (top) and fecal (bottom) excretion of cocaine and total radioactivity in two acutely (slanted-line bar) and two chronically treated (dotted bar) dogs after 5 mg/kg intravenous injection of ring-labeled [^{3}H] cocaine. The values represent mean percentage of dose excreted at different times in two dogs. The same dogs used in acute experiments were subcutaneously injected with a 5 mg/kg dose of nonradioactive cocaine twice daily for 6 weeks, then with a 5 mg/kg i.v. dose of ring-labeled [^{3}H] cocaine for the chronic experiments.

excreted unchanged in urine and feces. The excretion of cocaine as mean percentage of dose in urine and feces of two acute dogs was 7.0 and 1.4, and in the chronically treated dogs, 2.8 and 0.3 respectively. Major excretion of total radioactivity occurred in urine within 24 hr in both groups, but excretion of total radioactivity continued for several days. There was comparatively higher excretion of total radioactivity in urine as percentage of dose in the chronic (60%) as compared to the acute group (49%). Cumulative percentages of total radioactivity in urine and feces in the two groups, however, were very similar.

Comparative Profile of Urinary Metabolites of Cocaine in Acutely and Chronically Treated Dogs[3,5]

Data on the comparative metabolic profile of cocaine are given in Figure 13. Norcocaine, benzoylnorecgonine, benzoylecgonine, norecgonine, ecgonine methyl ester, and ecgonine were identified as urinary metabolites of cocaine in both groups. Approximately 6 to 7% of the dose was present as unidentified metabolites in 48-hr urine in both groups. There were no significant differences in mean percentages of norcocaine, ecgonine methyl ester, and unidentified metabolites. Benzoylnorecgonine and ecgonine were present in higher amounts, and benzoylecgonine and norecgonine in lower amounts, in the acute as compared to the values in the chronically treated dogs.

DISPOSITION AND METABOLISM OF [^{3}H] COCAINE IN THE MONKEY

Pharmacokinetic Studies on [^{3}H] Cocaine in Monkeys after Intravenous Injections

Although several studies have been reported on the self-administration of cocaine in the monkey,[7,8] detailed information on the pharmacokinetics and metabolism of cocaine in this species in the acute and chronic states is lacking. In our laboratory, studies were carried out

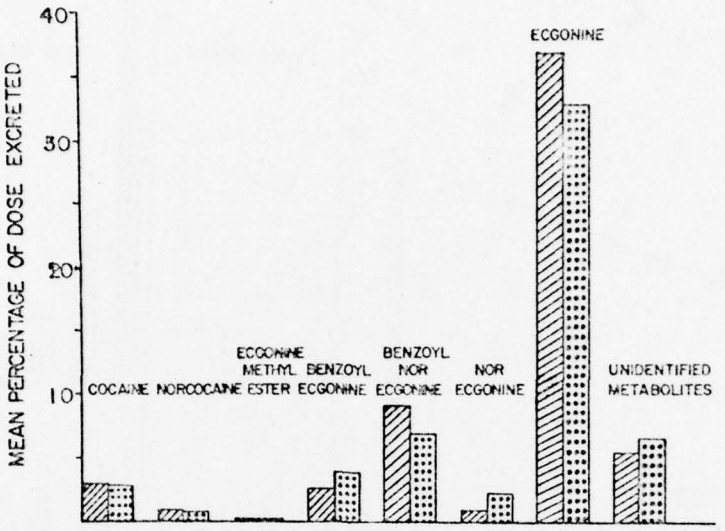

FIGURE 13. Comparative profile of urinary metabolites of cocaine in two acutely (slanted-line bar) and two chronically treated (dotted bar) dogs after 5 mg/kg intravenous injection of ring-labeled [^{3}H] cocaine (the details on chronic treatment of dogs as given in the footnote of Figure 12). Data represent mean values from two dogs in each group. The mean percentages of dose excreted in 48 hr after injection of labeled cocaine in acute and chronic dogs were 58 and 56.2, respectively.

using different intravenous doses (5,3, and 1 mg/kg) of [^{3}H] cocaine in the monkey. Female monkeys were catheterized with Bardex-Foley® indwelling catheters (size 8, 12 Fr.) and transferred to a restraining chair. With a 5 mg/kg intravenous injection of [^{3}H] cocaine to the monkey, severe convulsions were observed within 5 min of administration of drug, along with the spasmodic lateral movements of the head. These effects and hyperthermia lasted for 1 hr, and this was followed by a depression phase in which the monkey went into shock and died of respiratory arrest approximately 2.5 hr after the injection of cocaine. Analysis of tissues and biofluids removed immediately after the death gave the following values (μg/g or ml) of free cocaine: liver 0.42, spleen 0.67, kidney 0.25, pancreas 2.78, heart 0.30, lung 1.13, bile 10.88, cerebrospinal fluid 0.27, and plasma 0.03. The values (μg/g) of free cocaine in selected areas of the CNS of the monkey were as follows: temporal cortex (gray) 0.60, temporal cortex (white) 0.58, cerebellum 0.50, spinal cord 0.53, hypothalamus 0.52, thalamus 0.51, medulla 0.59, pons 0.61, mesencephalon 0.53, and caudate nucleus 0.49. The approximate half-life of cocaine in plasma obtained up to the time of death was approximately 1 hr.

With a 3 mg/kg intravenous injection of [^{3}H] cocaine to the monkey, convulsions were observed within 15 min of administration and the effects lasted for about 30 min. After 50 min, the hyperthermic monkey (43°C) went into severe depression and died of respiratory arrest 1.5 hr after administration of cocaine. Attempts to revive the animal by lowering the body temperature were unsuccessful. The levels of free cocaine (μg/g or ml) in tissues and biofluids of this monkey removed immediately after death were as follows: liver 0.41, spleen 0.80, kidney 0.48, pancreas 1.71, heart 0.33, lung 0.87, bile 13.67, cerebrospinal fluid 0.20, and plasma 0.01. The values of free cocaine (μg/g) in selected anatomic areas of the CNS of the monkey were as follows: Temporal cortex (gray) 0.56, temporal cortex (white) 0.57, cerebellum 0.57, spinal cord 0.56, hypothalamus 0.51, thalamus 0.51, medulla 0.51, pons 0.66, mesencephalon 0.54, and caudate nucleus 0.55.

Metabolites of Cocaine in the Brain of Monkeys

Analysis of brains of monkeys administered a 3 or 5 mg/kg intravenous injection of [^{3}H] cocaine

removed immediately after death showed the presence of norcocaine, benzoylecgonine, and benzoylnorecgonine as metabolites. The formation of norcocaine as a metabolite of cocaine in the brain of monkeys has been reported recently.[3,6] The ratio of the amounts of benzoylnorecgonine to benzoylecgonine in brain with 5 mg/kg i.v. injection was 3:2 and that with 3 mg/kg i.v. injection 1:3 respectively.

Pharmacokinetic Studies in Acutely and Chronically Treated Monkeys Injected Intravenously with 1 mg/kg Dose of [^{3}H] Cocaine

In chronic experiments, two female monkeys were administered 1 mg/kg s.c. dose of cocaine twice daily for one week, 2 mg/kg s.c. dose twice daily for the following week, and 3 mg/kg s.c. dose twice daily for the third week followed by an intravenous injection of 1 mg/kg dose of [^{3}H] cocaine. Comparative data on the distribution of [^{3}H] cocaine in acute and chronically treated monkeys are given in Table 1. In both groups, the plasma values showed a biphasic pattern between 5 min to 1 hr. The approximate half-life of cocaine in two acute experiments was 1.3 and 1.2 hr respectively; that in the chronically treated monkeys was 1.0 and 0.8 hr respectively. The peak plasma levels of cocaine and total radioactivity values were also much lower in the chronically treated monkeys as compared to the acute, and significant amounts (38 to 85 ng/ml) of total radioactivity persisted in the plasma of chronically treated monkeys 48 hr after injection. Cocaine was either absent or present in barely detectable concentrations in plasma 6 hr after injection in both groups.

Administration of 1 mg/kg dose of [^{3}H] cocaine by intravenous injection in another monkey produced restlessness and excitement immediately after injection, and these effects lasted for approximately 45 min as previously observed in other monkeys with a similar dose. However, this monkey went into a severe post-stimulatory depression 1 hr after injection and died of respiratory arrest 1 hr and 45 min after injection. Intravenous injection of 5% glucose or lowering of body temperature, etc. during the depression phase did not revive the animal. The exact cause of death in these monkeys is not known. A sensitivity to cocaine or lack of adaptation to the primate chair and consequent stress during the onset of convulsions could possibly be involved. The levels of free cocaine (μg/g or ml) in tissues and biofluids of monkey removed immediately after death were as follows: liver 0.17, spleen 0.08, kidney 0.13, pancreas 0.68, heart 0.14, lung 0.27, bile 5.54, cerebrospinal fluid 0.07, and plasma 0.008. The values (μg/g) in selected

TABLE I

Distribution[a] of [^{3}H] Cocaine and Half-lives in Plasma of Acutely and Chronically Treated[b] Monkeys After 1 mg/kg (Free Base) Dose by i.v. Injection[c]

	5 min	10 min	20 min	0.5 hr	1 hr	2 hr	4 hr	6 hr	Half-lives[d] (hr)
Acute									
(A)	45	16	20	37	32	15	5	2	1.3
(B)	97	84	67	90	49	9	5	3	1.2
Chronic									
(A)	26	15	23	11	17	6	2	0	1.0
(B)	19	17	20	15	21	16	2	0	0.8

[a]Each figure represents the average of duplicate determinations in ng per ml plasma from two female monkeys (A) and (B).
[b]For chronic experiments, the same monkeys were administered 1 mg/kg dose of nonradioactive cocaine subcutaneously twice daily for one week, 2 mg/kg dose twice daily for the following week, 3 mg/kg dose twice daily for the third week, followed by 1 mg/kg dose of [^{3}H] cocaine by i.v. injection.
[c]This dose produced restlessness and excitement but no convulsions in the monkeys immediately after injection.
[d]Correlation coefficient $r = -0.997, -0.919$ for data in the acute experiments, $P < 0.05$. Correlation coefficient $r = -0.985, -0.974$ for data in the chronic experiments, $P < 0.05$.

areas of the CNS of the monkey were as follows: temporal cortex (gray) 0.25, temporal cortex (white) 0.27; cerebellum 0.26, spinal cord 0.33, hypothalamus and thalamus 0.23, medulla 0.25, pons 0.32, mesencephalon 0.26, and caudate nucleus 0.26.

Urinary and Fecal Excretion of Free Cocaine and Total Radioactivity After 1 mg/kg Intravenous Injection in Acutely and Chronically Treated Monkeys

In acute experiments, the percentage of dose excreted in two female monkeys as free cocaine in urine and feces collected over a period of 96 hr post-injection was (a) 0.2 and 0.2, and (b) 5.1 and 0.1, respectively. The total radioactivity values in urine and feces for these monkeys were (a) 43.2 and 6.1, and (b) 67.6 and 3.8%, respectively. The same monkeys on chronic treatment with cocaine excreted (a) 0.5 and 0.1, and (b) 7.0 and 0.1%, respectively as free cocaine in urine and feces. The total radioactivity values as percentage of dose in urine and feces in the chronic group were (a) 42.7 and 9.5, and (b) 69.6 and 4.6, respectively. Major excretion of free cocaine and total radioactivity occurred within 24 hr.

Urinary Metabolites of Cocaine in the Monkey

In addition to small amounts of unchanged cocaine, minor amounts ($< 0.1\%$) of norcocaine, ecgonine methyl ester, benzoylnorecgonine (33%), and benzoylecgonine (5%) were identified as urinary metabolites in pooled urine of monkeys collected over a period of 96 hr post-injection of a 1 mg/kg i.v. dose of [^{3}H] cocaine.

THE IMPLICATIONS OF DISPOSITION AND BIOTRANSFORMATION ON THE ACQUIRED SENSITIVITY TO COCAINE AND ITS SYSTEMIC TOXICITY IN EXPERIMENTAL ANIMALS ON CHRONIC TREATMENT

Chronic treatment of dogs and monkeys with cocaine has been reported to produce increased sensitization (instead of tolerance) which persisted unabated for 2 weeks or more after the discontinuance of cocaine.[1,3,4] Tolerance to and physical dependence on cocaine have not been reported to develop on repeated dosing.[1-9] In our study, dogs chronically treated with 5 mg/kg s.c. doses of cocaine twice daily for 20 weeks and suddenly withdrawn for 2 days did not show any withdrawal symptoms or after-depression. Resumption of 5 mg/kg s.c. dose produced severe convulsions, prostration, incoordination, extensor rigidity, and death within 1 hr of injection. Recent studies have shown that toxic and behavioral effects of cocaine did not depend exclusively on cathecholamine release.[37,38]

In all three species (rats, dogs, and monkeys) studied by us, cocaine was shown to be very rapidly metabolized, and the metabolites in brain were identified as norcocaine, benzoylecgonine, benzoylnorecgonine, and ecgonine. Cocaine and norcocaine did not persist in the CNS of these animals in acute or chronic states 12 to 24 hr post-injection, and the decay of norcocaine in the CNS was similar to that of cocaine. Benzoylecgonine and benzoylnorecgonine persisted in the CNS long after the disappearance of cocaine or norcocaine. These polar metabolites also formed distinct molecular complexes with calcium ions[39] and possessed potent stimulant activity after intracisternal injection in the rat. Benzoylnorecgonine was approximately ten times more potent as a stimulant compared to benzoylecgonine.[32] Alteration or displacement of neuronal membrane-bound Ca^{2+} (which normally keeps the membrane in a condensed state) by benzoylnorecgonine and benzoylecgonine could conceivably cause a conformational change in membrane proteins, leading to changes in permeability to Na^+ and K^+, depolarization of membrane, and excitatory effects. Concurrently, a reduction in the Ca^{2+}-binding sites on membrane proteins could also interfere with release or uptake of neurotransmitters.

Previous work[40-43] has shown that the interaction of lipophilic cocaine with neural membrane phospholipids through electrostatic and nonpolar forces produces alteration in ordering and fluidity of nonpolar regions, marked inhibition in the functioning of membrane as a cation exchange site, and displacement of Ca^{2+} from membrane sites at which it participates in the generation of action potential. Changes in brain tissue respiration, oxidative phosphorylation,[44] and cerebral metabolism[45] have also been reported with cocaine. Increased partitioning, therefore, of lipophilic cocaine and its metabolite norcocaine into the hydrocarbon domains of neural membrane and

high affinity binding coupled with hyperthermia (which enhances its toxicity[46]) may modify the membrane function and its structural integrity, and consequently alter the energy metabolism of the nerve tissue. Furthermore, higher doses or repeated administration of cocaine could conceivably lead to the oxidation in vivo of cocaine to the fairly stable and highly reactive nitroxide free radical. Such an oxidation reportedly[47] occurs at ambient temperature with hydrogen peroxide, and nitroxide free radicals are known[48] to be reduced to the corresponding norcompounds with membrane-SH groups. The mechanistic scheme for the formation of such a free radical, its binding with neural membrane proteins and phospholipids, and its dissociation as a result of reduction with membrane-SH groups are outlined in Figure 14.

A most interesting property of cocaine is the potentiation of adrenergic responses and lack of potentiation in denervated organs. The "unitary" hypothesis[49-58] of cocaine action postulates that cocaine blocks a specialized nerve membrane transport system for sympathomimetic amines, prevents their inactivation by uptake and storage, and thus diverts larger amounts of amines to appropriate tissue receptor, therefore potentiating the response. Although the inhibition of uptake of norepinephrine by cocaine has been confirmed for the peripheral tissues, brain norepinephrine uptake is unaffected.[59] Other workers[60-62] have suggested that the block of norepinephrine uptake may not be an important factor in the supersensitivity produced by cocaine, and a direct action on the effector cells leads to hyperresponsiveness. The changes in effector cells following denervation may be of the same type as those produced by cocaine, and they are maximal by the time sensitization is fully developed.[62] Most of these experiments on supersensitivity to norepinephrine have been performed on isolated tissue preparations in vitro, and it is not certain to what extent these tissues metabolize cocaine to benzoylecgonine and benzoylnorecgonine. These metabolites form distinct molecular complexes with Ca^{2+}, which is known to participate in a

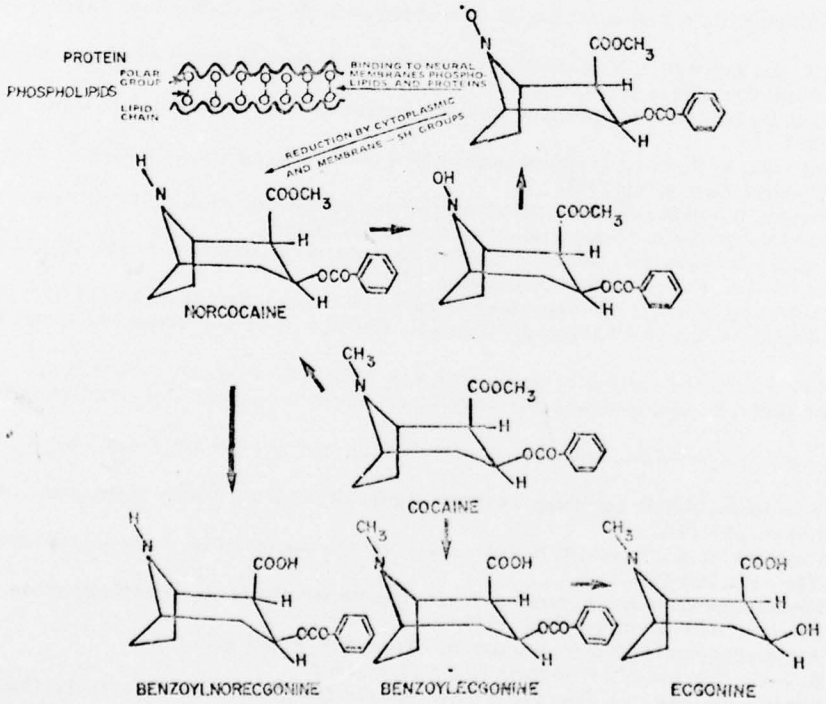

FIGURE 14. Metabolic pathways of cocaine in brain of rats, dogs, and monkeys and the mechanistic scheme for the formation of nitroxide free radical, its binding with neural membrane proteins and phospholipids, and its dissociation as a result of reduction with membrane-SH groups.

number of nerve functions at the membrane level, e.g., in excitation of neuronal membranes,[63] potassium transport,[64] release of neurotransmitter substances,[65,66] and intracellular mediation of catecholamine response through the cyclic AMP system.[67] Studies[68] on binding of [^{3}H] cocaine to guinea pig vas deferens in vitro showed complete uptake within 8 min, with 90% radioactivity being washed out after 4 min and little additional washout occurring after 16 min, indicating a specifically bound radioactive cocaine or its metabolite.

ACKNOWLEDGMENT

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ABSTRACT

This project deals in depth with the dispositional and metabolic aspects of [^{3}H] cocaine in the rat, dog and the monkey in acute and chronic states with a view to assess the possible relationship and significance of these parameters to the acquired sensitivity (reverse tolerance) and systemic toxicity to cocaine in these species on chronic treatment. Inter alia various metabolites of cocaine observed in the brain of these experimental animals e.g. [^{3}H] norcocaine, [^{3}H] benzoylnorecgonine, [^{3}H] benzoylecgonine and [^{3}H] ecgonine were also prepared and their disposition studied in the rat. Sensitive and specific methodologies for the estimation and identification of cocaine and its metabolites in biological materials were developed. This study has provided evidence that chronic administration of cocaine in subconvulsant doses to experimental animals does not produce dispositional tolerance. Benzoylnorecgonine and benzoylecgonine (but not cocaine or norcocaine) were shown to persist in the central nervous system of these experimental animals. Persistence of these polar metabolites in brain, their potent stimulant activity on intracisternal administration and possible mobilization by these metabolites of neuronal membrane-bound calcium from sites that control transmembrane permeability to sodium ions has been implicated by the authors to play a possible role in the reverse tolerance to cocaine. Comparative study of disposition and metabolism of [^{3}H] ϕ-cocaine, the dextro isomer of cocaine in the rat has shown that differential pharmacokinetics of these 2 isomers may account in part for their differential pharmacological effects.