Urinary Metabolites of *dl*-Methadone in Maintenance Subjects

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A major pathway of metabolism of methadone (**1**) in maintenance subjects is N-demethylation which results in the urinary excretion of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (**2**), 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (**3**), and their ring-hydroxylated analogs **5** and **6**. Lesser amounts of 4-dimethylamino-2,2-diphenylvaleric acid (**8**) (formed by side-chain oxidation), 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (**4**) (resulting from subsequent N-demethylation and cyclization of **8**), ring-hydroxylated methadone (**9**), and normethadol (**7**) (formed by reduction and N-demethylation) were also found. The formation of **7** was of particular interest since it may represent an active metabolite of *d*-methadone. Contrary to earlier reports, methadone *N*-oxide was not found among the urinary metabolites but was shown to be formed from methadone in urine stored at 30°.

Over the past two decades there has been a continuing interest in the metabolism, chemistry, and pharmacology of methadone (**1**) and its cogeners in these laboratories as well as elsewhere. Because of the recent availability of new, more sensitive techniques, these studies are now proceeding more rapidly. The importance of these studies has also increased greatly because of the apparently successful use of methadone therapy for the maintenance of heroin addicts. Enzyme studies reported by Axelrod[1] in 1956 strongly suggested that methadone was metabolized by N-demethylation, a process that should have led to the formation of *N*-desmethylmethadone. However, when the synthesis of *N*-desmethylmethadone was attempted by Pohland, Sullivan, and Lee,[2] it was found that it was an unstable compound that spontaneously cyclized to the 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (**2**).† Indeed, this pyrrolidine was identical with the methadone metabolite obtained from rat liver slices or from dog bile. The compound was also the same as the metabolite isolated from rat bile in 1953 by Sung and Way[3] (see Way and Adler[4]). Later,[5] this metabolite was detected in human urine. Pohland, Boaz, and Sullivan[6] found that both the pyrrolidine metabolite and its *N*-desmethyl analog, 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (**3**), occur in the urine of humans receiving a typical 10-mg oral dose of *dl*-methadone. A quantitative study,[7] however, indicated that the amounts of these two metabolites and of unchanged methadone in the urine of heroin maintenance subjects accounted for a relatively small portion of an 80-mg dose. These results were in conflict with those of Basett and Casarett[8] who reported that almost all of the dose could be accounted for as methadone and the pyrrolidine metabolite **2**. We have now reinvestigated the metabolism of methadone in maintenance subjects using more sensitive techniques, principally combined gas chromatography–mass spectrometry (gcms). Two preliminary communications, which summarize early phases of our studies, have appeared.[9,10]

Methods

Subjects. Three male, methadone-tolerant subjects, ages 23–36 years, participated in the study. They had been participating in the methadone maintenance program at Man Alive Research, Inc., of Baltimore, Md., for a period of 4 months to 5 years. Each subject had a long history of multidrug abuse and was receiving a daily maintenance dosage of 80–100 mg of methadone.

Urine samples were collected under supervision for 24 hr following administration of the daily dose of methadone. Each urine collection was immediately frozen and was kept frozen at −10° until use because of the extreme sensitivity of the pyrrolidine metabolite to air oxidation and because methadone urines kept at room temperature tend to develop artifacts that mimic the behavior of methadone *N*-oxide.

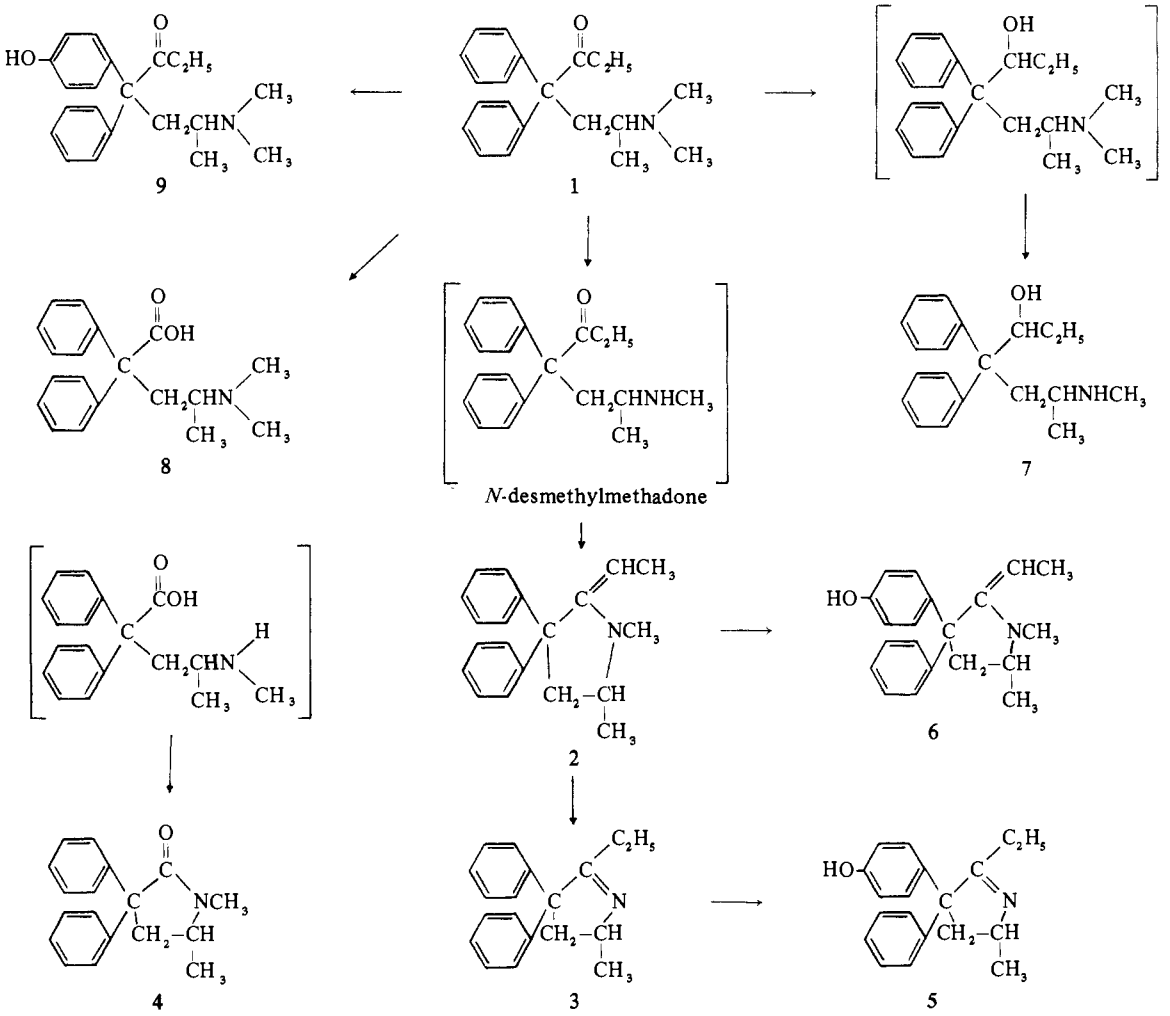
Samples Preparation. The urine samples were thawed and stirred to obtain homogeneity. A 500-ml aliquot of each urine sample was adjusted to pH 9.5 with 1 *N* NaOH and was extracted twice with equivalent volumes of CH_2Cl_2. The combined organic phase was evaporated to dryness *in vacuo*. The residual material was dissolved in 2.0 ml of a solution of 1 mg/ml of *n*-docosane in freshly distilled *n*-butyl chloride. Aliquots, 1 μl, of the resulting solution were introduced into the gc and gcms for analysis. Due to the extreme susceptibility of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (**2**) to air oxidation, it was imperative that gc analysis of the urine extracts be performed immediately following isolation. The development of a red color in solution of urine extracts and in solutions of authentic samples of this metabolite was indicative of the oxidation (*cf.* also ref 5).

Extracted urine was acidified to pH 5.5 with dilute acetic acid and was hydrolyzed enzymatically by incubation with 0.50 ml of Glusulase (β-glucuronidase and aryl sulfatase, Endo Products, Inc.) at 37° for 24 hr. The hydrolyzed urine was neutralized to pH 7.6 with 1 *N* NaOH and was extracted twice with equivalent volumes of CH_2Cl_2. The combined organic phase was evaporated to dryness *in vacuo* and the residual mixture containing liberated metabolites of methadone was dissolved in 2.0 ml of CH_2Cl_2. A 0.5-ml aliquot of the resulting solution was evaporated to dryness *in vacuo* and the residue allowed to react with 1 ml of acetic anhydride containing 4 drops of pyridine for 4 hr. Another 0.5-ml aliquot was evaporated to dryness *in vacuo* and the residue allowed to react with an excess of CH_2N_2. Aliquots of the underivatized, acetylated, and methylated mixtures were introduced into the gc and gcms for the separation and identification of metabolites.

Gas Chromatography (gc). Gas chromatographic analyses were performed using an F & M (Hewlett-Packard) Model 402 gas chromatograph equipped with a hydrogen flame detector. The columns employed were 4-ft siliconized glass U tubes (2.5 mm i.d.) packed with 1% W-98 methylvinyl silicone gum rubber on 80–100 mesh Gas Chrom Q (Applied

†Attempts in this laboratory and elsewhere to prepare *N*-desmethylmethadone chemically have to date always led to the isolation of **2**. In unpublished studies we have carried out the *in vitro* enzymatic demethylation of methadone and attempted to "trap" *N*-desmethylmethadone with secondary amine trapping agents. Nevertheless, only **2** was isolated. It is our feeling that *N*-desmethylmethadone is sufficiently unstable so that it converts to **2** almost immediately following its *in vivo* formation.

Scheme I. Biotransformation of *dl*-Methadone in Human Maintenance Subjects[a]



[a]Bracketed compounds represent proposed metabolic intermediates that have not been isolated or identified.

Science Laboratories) or 10% OV-11 phenylmethyl (35% phenyl) silicone gum rubber on 100–120 mesh Supelcoport (Supelco, Inc.). The former column was maintained isothermally at 165° with the injector port temperature at 190° and the detector maintained at 180°. The latter column was heated at 200° with the injector port temperature at 220° and the detector at 210°. Helium at a flow rate of 60 ml/min was employed as the carrier gas in both columns. Hydrogen and oxygen were adjusted to give optimum response.

Conditioning of the W-98 column employed in the quantitative estimation of the known metabolites of methadone in the urine was obligatory. The column was heated to and maintained at 350° for 2 hr without carrier gas flow. It was then cooled to room temperature, carrier gas flow at 60 ml/min was initiated, and the temperature increased to and maintained at 250° for 24 hr. The oven temperature was lowered to 200° and a 10-μl aliquot of Silyl 8 (Pierce Chemical Co.) was injected onto the column. The temperature was maintained at 200° until a steady base line response was obtained (2–6 hr).

The quantitative estimation of the concentrations of methadone and of its two known basic metabolites was achieved by the use of the internal standard method of Sullivan and Blake.[7] The choice of *n*-docosane as internal standard was based upon its chemical inertness and its gc retention time, 6.0 min, relative to that of methadone, 4 min; 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (**2**), 2.4 min; under the conditions used for gas chromatographic analysis. After gc analysis of the urine extracts, the ratios of the individual peak heights of methadone and metabolites to that of *n*-docosane were determined. Quantitation was achieved utilizing these experimental ratios and standard curves constructed from ratios obtained by gc assay of *n*-butyl chloride solutions containing known concentrations of methadone (0.25–3.0 mg/ml) and its two previously known metabolites (25–300 μg/ml) and 1 mg/ml of *n*-docosane. The results thus obtained were corrected for extraction losses using data obtained by addition of a known quantity of [2-^{14}C]methadone (New England Nuclear, Inc., 5.2 μCi/mmol) to human urine prior to the extraction process and the determination of recovery in the final extract by liquid scintillation counting. Data obtained by gc analysis of extracts of human urine containing known concentrations of metabolites **2**, **3**, and **4** revealed extraction losses comparable to that of methadone. Quantitation of metabolites **5** and **6** was not possible due to the lack of pure authentic samples.

Combined Gas Chromatograph-Mass Spectroscopy (gcms). Mass fragmentation patterns of components present in the urinary extracts were obtained using an LKB-9000 combined gas chromatograph-mass spectrometer (gcms). A 4-ft siliconized glass column (2.5 mm i.d.) packed with 1% W-98 methylvinyl silicone gum rubber on 80–100 mesh Gas Chrom

Table I. Methadone and Unconjugated Metabolites in Human Urine after Oral Administration of Maintenance Doses of Methadone Hydrochloride

Subject	M. K.	V. L.	R. L.
Age	23	28	36
Dose, mg/day	100	90	50
24-hr urine vol, ml	1050	750	1050
Metabolite	Total 24-hr recovery, mg		
Methadone	26.2	3.8	6.3
2	10.5	2.9	14.2
3	0.25	0.25	1.3
4	*a*	*a*	1.1

[a] Concentrations of metabolite **4**, 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone, were not determined in these samples.

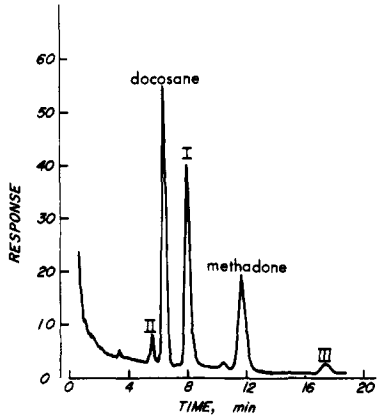


Figure 1. Gas chromatogram of the separation of methadone and unconjugated metabolites in a urine extract employing a 10% OV-11 column at 200°. Docosane is the internal standard. I is 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (**2**), II is 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (**3**), and III is 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (**4**).

Q was employed as the gc column. The column temperature was 180° and the carrier gas (helium) flow was 40 ml/min. An electron energy of 70 eV was used for ionization.

Authentic samples of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (**2**), 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (**3**), 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (**4**), 4-dimethylamino-2,2-diphenylvaleric acid (**8**), and normethadol (**7**) used for gcms structure confirmation and gc standards were obtained from Mr. Richard Booher of the Lilly Research Laboratories. Methadone *N*-oxide was prepared by the published procedure.[11]

Results

In our earlier studies[6] we identified metabolites **2** and **3** in the urine of a volunteer receiving 10 mg of *dl*-methadone orally. In a later study we found that a subject receiving 80 mg of *dl*-methadone daily excreted 12% of the dose per day as a mixture of methadone and metabolites **2** and **3**. In the present study it seemed desirable to substantiate these results. Table I summarizes the recoveries of methadone and metabolites **2** and **3** in three maintenance subjects.

Gcms analysis of the urine extract from subject R. L. revealed that the gc peak of methadone contained a small amount of a second component that appeared to be drug related. Utilization of a more polar gc column, OV-11, under appropriate conditions permitted the separation of this unknown metabolite **4** from the parent drug as well as an excellent separation of metabolites **2** and **3** (Figure 1). The

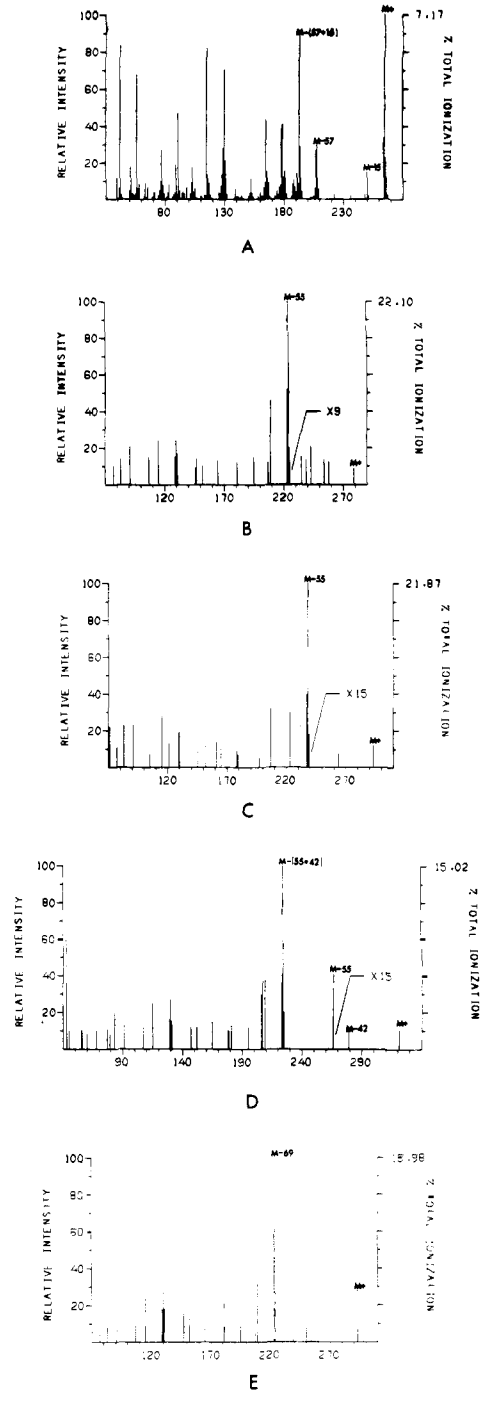


Figure 2. Relative intensity plots obtained from the mass fragmentation patterns of A, 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (**4**); B, 2-ethyl-5-methyl-3-(hydroxyphenyl)-3-phenyl-1-pyrroline (**5**); C, methylated derivative of metabolite **5**; D, acetylated derivative of metabolite **5**; and E, 2-ethylidene-1,5-dimethyl-3-(hydroxyphenyl)-3-phenylpyrrolidine (**6**) which were separated from urine extracts by gcms.

mass fragmentation pattern of metabolite **4** (Figure 2A), obtained by gcms analysis, showed its structure to be that of 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (**4**). The most intense peak in the pattern was that of the molecular ion at 265 amu. The M − 15 peak represented the loss of the *C*-methyl group while the loss of the $-CONCH_3$ moiety was responsible for the M − 57 peak. The major peak at 193 amu is due to the combined loss of both of these groups, M − (57 + 15). The remaining peaks were all consistent with those obtained from compounds in the methadone series.

Confirming evidence for the structure of metabolite **4** was obtained by comparison of its fragmentation pattern to that of an authentic sample of 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone. The urines of subjects M. K. and V. L. were not assayed for metabolite **4**.

In an attempt to account for a greater proportion of the daily dose of methadone, these studies were extended to an investigation of the conjugated metabolites remaining in urine after extraction of methadone and **2**, **3**, and **4**. Extracted urine samples were incubated with a mixture of β-glucuronidase and aryl sulfatase and then were extracted with methylene chloride. Initial gcms analysis of these extracts revealed the presence of three hydroxylated metabolites. The more abundant of these had a retention time of 9 min and was tentatively identified by its mass fragmentation pattern as 2-ethyl-5-methyl-3-(hydroxyphenyl)-3-phenyl-1-pyrroline (**5**). The mass fragmentation pattern of **5** (Figure 2B) showed a molecular ion at 279 amu and a most abundant ion at M − 55 due to the loss of the $C_2H_5C{=}N$ group. This fragmentation has been shown to be consistent with the pyrroline ring system.[6] Conversion of **5** to the *O*-methyl derivative by reaction with diazomethane confirmed the presence of a phenolic hydroxyl group. The mass fragmentation pattern (Figure 2C) of the *O*-methyl derivative of **5** showed a molecular ion of 293 amu and the most abundant ion was again found to be the M − 55 peak at 238, indicating the loss of the $C_2H_5C{=}N$ moiety. The mass fragmentation pattern of **5** acetate (Figure 2D) showed an M^+ at 321 amu, a major peak again at M − 55, and a peak at M − 42 representing the loss of ketene. This latter fragmentation is characteristic of a phenolic acetate thus confirming the presence of an aromatic hydroxyl group in the metabolite. The most abundant ion was that at 224 amu representing the combined loss of 55 and 42.

The second hydroxylated metabolite isolated from the hydrolyzed urine samples had a gc retention time of 11 min and was identified by mass spectrographic analysis as 2-ethylidene-1,5-dimethyl-3-(hydroxyphenyl)-3-phenylpyrrolidine (**6**). Its mass fragmentation pattern (Figure 2E) showed an M^+ ion at 293 amu. The most abundant ion occurred at 224 amu representing the loss of the $-C(=CHCH_3)NCH_3$ moiety (M − 69), a fragmentation that is unique for the pyrrolidine ring. The remaining fragments were identical with those found for metabolite **5**. The ms of the methoxy ether and of the acetate of **6** confirmed the proposed structure. A third phenolic metabolite was found in this fraction. A careful comparison of the mass spectrum of the minor metabolite with that of methadone and its analogs strongly suggested that this metabolite was hydroxymethadone (**9**). Although metabolites **5** and **6** could have been formed from **9**, studies in rats (to be published) suggested that these two metabolites were formed sequentially from **2**. The assignment of the hydroxy group to the para position is based solely on analogy with the metabolism of other compounds.

A fourth acetylated metabolite was found in the acetylated extract of hydrolyzed urine. As described elsewhere[10] this material was *O,N*-diacetyl-*N*-demethylmethadol. This finding demonstrated that normethadol (**7**) was excreted as a conjugated metabolite by maintenance subjects. Approximately 3% of the methadone dose was excreted as **7**.

Hydrolyzed urine samples, following extraction at pH 9.5, were neutralized to pH 7.0 and freeze-dried. The residual material was extracted with ethanol. The ethanol extract was evaporated to dryness *in vacuo* and the residue treated with diazomethane. Gcms analysis of derivatized extract showed it to contain a methadone metabolite that was identified by its mass fragmentation pattern as the methyl ester of 4-dimethylamino-2,2-diphenylvaleric acid (**8**). Proof of structure was obtained by comparison of its fragmentation pattern to that of an authentic sample of methyl 4-dimethylamino-2,2-diphenylvalerate. Metabolite 8 was also found in unhydrolyzed urine indicating that it is excreted in unconjugated form.

In a recent communication, Beckett, *et al.*,[12] have reported that methadone *N*-oxide is a urinary metabolite of methadone in man. In the present study comparison tlc and gcms analysis of urine extracts obtained using the extraction procedure described by Beckett, *et al.*, failed to show demonstrable levels of methadone *N*-oxide in the urine of any of six subjects receiving maintenance doses of *dl*-methadone. Authentic methadone *N*-oxide, added to human control urine, was quantitatively recovered and identified using this extraction procedure.

Analysis of extracts obtained from urine samples aged at 30° for 5-10 days prior to extraction did, however, reveal appreciable quantities of methadone *N*-oxide. In addition, urine extracts, originally devoid of methadone *N*-oxide, were found upon reanalysis after storage at 30° to contain detectable amounts of the *N*-oxide. These results suggested that methadone *N*-oxide was not a metabolite of methadone but rather was a result of chemical oxidation of unchanged methadone eliminated in the urine. This supposition was confirmed by tlc and gcms analysis of organic solutions of authentic methadone free base. Fresh solutions of methadone were free of any contaminant including the *N*-oxide. After storage at 30°, these solutions were found by tlc and gcms analysis to have developed a new component that was identified as methadone *N*-oxide. The relative quantity of the *N*-oxide present increased with time of storage.

Discussion

Scheme I summarizes our current understanding of the biotransformation of methadone in humans. From the present studies it is clear that N-demethylation to metabolite **2** is quantitatively the most important route of metabolism of methadone in man. The secondary metabolites **3**, **5**, and **6** all arise by further N-demethylation and by hydroxylation of the primary metabolite **2**. Of these four metabolites **2** and **5** are quantitatively the more important. Apparently **3** is a more active substrate for hydroxylation than is **2** thus accounting for the relatively minor amount of **3** found in urine.

Of the two minor pathways, the formation of normethadol (**7**) is the most intriguing. The presence of this metabolite in urine implies that methadol is formed as a primary metabolite by enzymatic reduction of the keto group of methadone. Since *in vitro* studies show that methadol is an excellent substrate for microsomal *N*-demethylase,[13] it is not surprising that the reduced metabolite appearing in urine is normethadol rather than methadol. In the rat both methadol and normethadol appear as metabolites.[10] The interest in this pathway of metabolism is enhanced by the observation that α-*l*-normethadol possesses analgesic activity comparable to that of *l*-methadone.[10] Therefore, since the *in vivo* reduction of *d*-methadone, the least active optical isomer of methadone,[14-16] leads to α-*l*-methadol and to α-*l*-normethadol,[10,17] it is not unreasonable to suppose that some of the reported activity of *d*-methadone[16] may be due to its partial conversion to α-*l*-methadol and α-*l*-normethadol. The relevance of these observations to the pharmacological response

produced in man by administration of *dl*-methadone has yet to be assessed.

A relatively minor pathway involved the oxidation of methadone to 4-dimethylamino-2,2-diphenylvaleric acid (**8**). This acidic metabolite appeared to be subsequently N-demethylated, in part, to an intermediate metabolite (not isolated), 4-methylamino-2,2-diphenylvaleric acid, which by ring closure yielded metabolite **4**, 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone. The conversion of methadone to a carboxylic acid has some precedent in the known enzymatic oxidation of acylbenzenes to benzoic acid.[18]

In the studies reported here, no methadone *N*-oxide was found in freshly obtained or frozen samples. Upon standing at room temperature, however, the urine samples appeared to develop a component which had characteristics similar to methadone *N*-oxide. These results are at variance with those of Beckett, Vaughan, and Essien[12] who report methadone *N*-oxide to be an important metabolite of methadone.

Despite the relatively large number of methadone metabolites found in human urine, they probably do not account for the entire daily maintenance dose. Further studies, probably involving labeled drug, will be required to answer this question.

Acknowledgment. My interest in methadone research began 20 years ago in the laboratory of Dr. Albert Pohland, to whom I express my appreciation for his continued interest and support. I also wish to acknowledge the advise and encouragement I have received from Dr. Robert E. McMahon and Dr. Irwin H. Slater. The authors are indebted to Dr. David Blake, Man Alive Research, Inc., and University of Maryland School of Pharmacy for selecting the subjects and for supervision of the urine collection and shipment.

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Structural Requirements for Tumor-Inhibitory Activity among Benzylisoquinoline Alkaloids and Related Synthetic Compounds†

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The results of a study directed toward determination of the structural requirements for tumor-inhibitory activity among benzylisoquinoline alkaloids and related synthetic compounds are reported. Monomeric benzylisoquinolines and aporphines appear to have no inhibitory activity against the W-256 tumor system. In contrast, significant inhibitory activity is manifested by a wide range of bis(benzylisoquinoline) derivatives. The comparable activity of *dl*-*O*-methyldauricine (**11a**) and of **14** with those of tetrandrine (**2a**) and thalidasine (**6**) indicates that a macrocyclic ring is not required. Methylation of the nitrogen atoms does not appear to be necessary, for **9**, **11b**, and **12** each show inhibitory activity. The significant activity of **9** and **12** indicates the apparent absence of stereospecificity requirements for biological activity in the series. One possible rationalization, that bis(benzylisoquinolines) may exert their tumor-inhibitory activity by a mechanism involving initial metabolic dehydrogenation to bis(dihydroisoquinolinium) derivatives, is rendered unlikely by the observed inactivity of **10** and the significant activity of **11c**.

Interest in the bis(benzylisoquinoline) alkaloids was stimulated by our report in 1966 that the alkaloids thalicarpine[2] and *dl*-tetrandrine[3] have significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 in the rat over a wide dosage range.[4,5] Subsequent studies revealed that the dextrorotatory enantiomer, tetrandrine, shows activity of the same order as the racemate. Thalicarpine (**1**)[6] and tetrandrine (**2a**)[7] have undergone extensive preclinical toxicological studies and have been selected for clinical evaluation now in progress under the auspices of the National Cancer Institute.

In view of the increasing significance of the biological properties of the benzylisoquinoline alkaloids, we deemed it important that studies be undertaken which are directed toward elucidation of the mode of action of the active alkaloids. We report herein some recent findings concerning the first step, *viz.*, the definition of structural and stereochemical requirements for tumor-inhibitory activity.

The monomeric benzylisoquinolines and aporphines appear to have no tumor-inhibitory activity against the W-256 tumor system. For example, *dl*-1-benzyl-2-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (**3**),[8] in the form of its pamoate [*i.e.*, salt of 4,4′-methylenebis(3-hydroxy-2-naphthoic acid)], was found to be toxic at doses equivalent to 50 mg/kg of free base and higher and inactive at lower doses. None of the additional dozen or more benzyliso-

† Tumor Inhibitors. 89. For paper 88, see ref 1.