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β-HYDROXYLATION OF *N*-ACETYL-3,4-DIMETHOXYPHENETHYLAMINE AND THE INFLUENCE OF IPRONIAZID ON THE DEMETHYLATION OF THIS COMPOUND *IN VIVO* IN THE RAT

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Abstract—*N*-acetyl-β-hydroxy-3,4-dimethoxyphenethylamine was found in the urine of rats after intraperitoneal administration of either 3,4-dimethoxyphenethylamine or *N*-acetyl-3,4-dimethoxyphenethylamine. Identification was made from chromatographic, cocrystallization and mass spectral data. The monoamine oxidase inhibitor, iproniazid, was found to diminish the extent of demethylation of *N*-acetyl-3,4-dimethoxyphenethylamine *in vivo* by 17 per cent.

3,4-DIMETHOXYPHENETHYLAMINE (DMPEA), a compound found in human urine and of possible significance in the biochemistry of mental disorders,[1–5] is metabolized primarily to 3,4-dimethoxyphenylacetic acid (DMPAA).[6,7] While DMPEA produces hypokinesis in rats and has pharmacological effects in other species, DMPAA has no known pharmacological activity. It is of interest that *N*-acetylation, which occurs in rats both *in vitro* and *in vivo*, produces a metabolite of DMPEA, N-acetyl-3,4-dimethoxyphenethylamine (NADMPEA), which is ten times more potent than the amine in producing hypokinetic behavior.[7,8] Because a number of methoxylated phenethylamine derivatives have mescaline-like effects,[9–12] an investigation was undertaken in order to determine whether DMPEA was metabolized to any other derivatives that might be pharmacologically active.

As a first step, we administered DMPEA to rats, after blocking its deamination with iproniazid, and then analyzed the urine for *N*-acetylated metabolites. Subsequently, we administered NADMPEA with and without iproniazid pretreatment in order to determine whether iproniazid affected the degradation of NADMPEA, even though NADMPEA is not considered to be a substrate for amine oxidase.

MATERIALS AND METHODS

The reagents used in this study were obtained from commercial sources except for β-hydroxy-3,4-dimethoxy-phenethylamine(β-OH-DMPEA), which was donated by Dr. Albert A. Manian of the Psychopharmacology Service Center, National Institute of Mental Health.

Radioactive NADMPEA was prepared by acetylation of DMPEA-^{14}C with acetic

anhydride or by acetylation of non-radioactive DMPEA with acetic anhydride-^{14}C. Isotopes were purified and analyzed for impurities by extraction and chromatographic methods.

N-acetyl-β-hydroxy-DMPEA (NA-β-OH-DMPEA) was prepared by acetylating β-OH-DMPEA with acetic anhydride in saturated bicarbonate solution. Under these conditions, the β-hydroxy-group is not acetylated.[13] The product, after recrystallization from benzene, melted at 93°–95° (lit. 95·5°–96·5°).[14]

N,O - diacetyl - β - hydroxy - 3,4 - dimethoxyphenethylamine (*N,O* - diacetyl - β-OH-DMPEA) was prepared by allowing a mixture of β-OH-DMPEA·HCl, pyridine and acetic anhydride to stand at room temperature for 1·5 hr. After extraction of the product from dilute hydrochloric acid into chloroform, the chloroform extract was dried with anhydrous sodium sulfate and evaporated to remove the solvent. The diacetyl derivative was recrystallized from benzene. The melting point of the product varied from 77° to 103°. From experiments with acetic anhydride-^{3}H of known specific activity, it was shown that, under anhydrous conditions, β-OH-DMPEA incorporated two acetyl groups per mole, but the melting point varied with the solvent system used for crystallization. Mass spectra (Perkin–Elmer MS-270) of these products were identical regardless of melting point and were consistent with the structure of *N,O*-diacetyl-β-OH-DMPEA. These findings probably reflect the fact that *N,O*-diacetyl-β-OH-DMPEA exists in more than one crystalline form.

Male Wistar rats (100–175 g) were given water *ad lib.*, but were deprived of food for 7 hr prior to and during the urine collection period. Iproniazid (80 mg/kg) was injected i.p. in 0·5 ml saline at 5·5 and 1·5 hr prior to the injection of DMPEA or NADMPEA. Urine was collected for 16 hr. Acidic and neutral metabolites were isolated by multiple extractions of the urine at pH 1·5 with chloroform. When DMPEA was given to the rats, it was desirable to remove the acidic metabolites, DMPAA and homovanillic acid (HVA), from the chloroform by back extraction with 8% sodium bicarbonate. Basic metabolites were isolated by extraction of the urine at pH 10 with chloroform. Chloroform extracts were dried with anhydrous sodium sulfate, filtered, acidified with HCl and evaporated to a residue. Portions of the neutral fractions were chromatographed on Gelman I.T.L.C. fiberglass strips impregnated with silica gel in the solvent system benzene–heptane–diethylamine (5:4:1). Chromatograms were scanned for radioactive peaks. Samples of urine, chloroform extracts and sections of chromatograms were analyzed for radioactivity in a liquid scintillation counter.

RESULTS

Urinary metabolites of DMPEA

In the first experiments, saline solutions of DMPEA-^{14}C, 9·6 mc/m-mole (1–5 μmoles/kg), were injected i.p. into rats with or without pretreatment with iproniazid. The DMPEA-^{14}C was labeled on the α-position. In the NADMPEA-^{14}C studies (0·5 μmole/kg), either the α-carbon of DMPEA or the carbonyl-carbon of the acetyl group carried the label. The specific activities of these labeled compounds were 9·6 and 9·9 mc/m-mole respectively. Urine was analyzed for metabolites as described above. Acidic, basic and conjugated metabolites were identified by methods previously described.[7] Neutral fractions of all urine samples from rats given DMPEA-^{14}C or NADMPEA-^{14}C were found to contain two metabolites not previously reported. One, "a" (see Table 2) has not been identified. The other was identified as *N*-acetyl-β-

hydroxy-3,4-dimethoxyphenethylamine by analysis of chromatographic, crystallization and mass spectral data.

Chromatographic identification of a neutral metabolite as N-*acetyl-β-hydroxy*-3,4-*dimethoxyphenethylamine and verification by cocrystallization.*

The major metabolite of DMPEA was previously shown to be NADMPEA, which is rapidly degraded to *N*-acetyl-methoxytyramine (NAMT).[7] In the present study, another neutral metabolite was detected. This compound had the chromatographic characteristics of NA-β-OH-DMPEA. A portion of the neutral extract obtained from urine of rats given either DMPEA-^{14}C or NADMPEA-^{14}C was chromatographed in the benzene–heptane–diethylamine solvent system and the material was found to be isographic with authentic NA-β-OH-DMPEA. The radioactive material corresponding to this compound was eluted, acetylated and chromatographed with authentic *N,O*-diacetyl-β-OH-DMPEA. Radioactive material isographic with authentic *N,O*-diacetyl-β-OH-DMPEA was found. After elution of this area and rechromatography, using the same solvent system as before, a single radioactive peak (R_f 0·75 was obtained. The material eluted from this area cocrystallized perfectly with *N,O*-diacetyl-β-OH-DMPEA with benzene as the solvent.

Mass spectroscopic characterization of N-*acetyl-β-hydroxy*-3,4-*dimethoxyphenethylamine as a metabolite of DMPEA*

Nonradioactive DMPEA (0·2 m-moles/kg) was injected into a rat pretreated with iproniazid. Urine was collected and the neutral metabolites were isolated. Prior to chromatography, the neutral fraction, which contained the unknown metabolite, was mixed with a small amount (0·005 μg) of radioactive NA-β-OH-DMPEA obtained from urine of an experimental rat. The amount of added radioactive NA-β-OH DMPEA was so small that it was well below the detection limits of the mass spectrometer. After chromatography, a small radio-active peak was located by scanning and this area was eluted with methanol.

The eluate was concentrated, a portion was evaporated in a quartz capillary tube and a mass spectrum was obtained by direct probe at a temperature of 120°–130° when the total ion current was maximal. Then a mass spectrum of authentic, crystalline NA-β-OH-DMPEA was made. Agreement was found for mass number and relative abundances for the fragments seen in the two spectra. Furthermore, in both spectra the highest mass/charge ratio for which a peak was seen was 239 (discounting isotopic fragments), which corresponds to the mass of NA-β-OH-DMPEA. The five most abundant fragments in the spectra of both unknown and reference substances were at mass/charge ratios of 139, 167, 180, 124 and 108, given in the order of decreasing abundance.

The amount of NA-β-OH-DMPEA found in the urine of rats after giving DMPEA was approximately 0·7 per cent of total excreted metabolites and after giving NADMPEA was 4 per cent. In iproniazid-pretreated rats, these values were approximately doubled. The metabolites found in the urine of one rat given DMPEA are shown in Table 1. The recovery of injected ^{14}C in the urine was 69 per cent.

Except for NA-β-OH-DMPEA, the metabolites in Table 1 have been identified previously.[6,7,15,16] NAMT and HVA were excreted almost entirely as conjugates. The unknown basic metabolite was tentatively identified as a mixture of 3- and 4-methoxytyramine according to evidence from silica gel thin-layer chromatography.

TABLE 1. URINARY METABOLITES IN ONE RAT 16 hr AFTER ADMINISTRATION OF 3,4-DIMETHOXYPHENETHYLAMINE-α-^{14}C (5 μmoles/kg, I.P.)

Metabolite	Per cent of excreted ^{14}C
3,4-Dimethoxyphenylacetic acid (DMPAA)	61·1
Homovanillic acid (HVA)	1·9
3,4-Dimethoxyphenethylamine (DMPEA)	11·4
Unidentified basic metabolite	1·4
N-acetyl-3,4-dimethoxyphenethylamine (NADMPEA)	0·1
N-acetyl-3-methoxytyramine (*n*-NAMT)	21·8*
N-acetyl-β-hydroxy-3,4-dimethoxyphenethylamine (NA-β-OH-DMPEA)	0·7
Unidentified neutral metabolite (a)	0·1

*This includes 1% which was excreted as the isomer, *N*-acetyl-4-methoxytyramine (iso-NAMT).

As further evidence for this, an aliquot of the basic fraction was acetylated and chromatographed. Three peaks were found: these were isographic with iso-NAMT, *n*-NAMT and NADMPEA respectively.

The neutral fractions of urine after administration of DMPEA-^{14}C contained four metabolites. These were NADMPEA, NAMT, NA-β-OH-DMPEA and an unidentified neutral metabolite 'a'. The amounts of these four compounds present in urine are indicated in Table 1. When NADMPEA was given to rats, these four metabolites were excreted in approximately the same relative proportions. After giving DMPEA, some of the unidentified basic metabolite, presumed to be methoxytyramine, may be acetylated and contribute to the amount of total NAMT. However, NADMPEA is much more rapidly demethylated than DMPEA. Therefore, most of the NAMT formed is probably via demethylation of NADMPEA. Since the relative amounts of neutral metabolites from DMPEA and NADMPEA are the same, the major pathway for formation of these compounds from DMPEA appears to be via acetylation and subsequent demethylation or β-hydroxylation. Moreover, when either DMPEA or NADMPEA was given after iproniazid treatment, the same change in the pattern of urinary metabolites was observed. There was an increased excretion of dimethoxy metabolites and a decreased excretion of demethylated metabolites.

Effect of iproniazid on NADMPEA metabolism

Acetyl-^{14}C- or α-^{14}C-NADMPEA (0·5 μmole/kg) was administered to rats pretreated with iproniazid (80–100 mg/kg) or given no drug; the urine was collected for 16 hr and analyzed for metabolites. After extraction at acidic and basic pH, urine samples were adjusted to pH 1·5 and placed in a boiling water bath for 15 min in order to hydrolyze conjugates. Each sample was adjusted to pH 5·0 and the mixture was evaporated with nitrogen at 50°. To the residue was added 5 ml of 1 M acetate buffer, pH 5·0, and 10 mg each of sulfatase and β-glucuronidase. This mixture was incubated at 37° for 20 hr and extracted for neutral compounds. While this procedure failed to release all the conjugated ^{14}C, the same pattern of metabolites was obtained after further hydrolysis of the residue, It can be assumed, therefore, that hydrolyzed metabolites were representative of the remaining unhydrolyzed material. Portions of the neutral fractions were chromatographed in the benzene–heptane–diethylamine

solvent system. All chromatograms of neutral metabolites were scanned and then divided into sections for counting the ^{14}C associated with each metabolite. The results are shown in Table 2.

The amount of total NAMT excreted was diminished by 17 per cent after iproniazid pretreatment. This reduced excretion of NAMT could occur via one or more of several possible mechanisms. For example, if iproniazid pretreatment resulted in the deacetylation of NADMPEA, less NADMPEA would be available for demethylation to NAMT. In order to determine whether NADMPEA was being deacetylated *in vivo*, several animals with and without the iproniazid pretreatment were injected with

TABLE 2. URINARY METABOLITES OF NADMPEA-^{14}C IN THE RAT EXPRESSED AS PER CENT OF EXCRETED ^{14}C*

Metabolite	Pretreatment	
	None	Iproniazid (80–100 mg/kg†)
Unknown neutral metabolite	0·5 (0·4–0·6)	1·6 (1·1–2·0)
NA-β-OH-DMPEA	4·2 (2·4–5·7)	10·9 (10·3–11·7)
Unconjugated NAMT	7·0 (3·2–13·1)	1·1 (0·2–3·2)
NADMPEA	0·5 (0·3–1·0)	4·5 (3·7–5·4)
Conjugated NAMT	86·5 (81·4–89·9)	75·3 (73·2–77·9)
Total NAMT	93·5 (92·0–94·6)	76·4 (74·9–78·4)

*Each percentage represents the mean of four experiments. The ranges are given in parentheses.
†In one experiment of four, the iproniazid dose was 100 mg/kg rather than 80 mg/kg. Results from this experiment were in the range of the other results and are included.

NADMPEA-^{14}C, prepared by acetylating DMPEA-^{14}C with acetic anhydride. No radioactive acidic or basic metabolites were detected. Furthermore, there was no significant difference in the quantitative output of metabolites in these animals compared with that of animals injected with NADMPEA-^{14}C prepared by acetylating nonradioactive DMPEA with acetic anhydride-^{14}C. NADMPEA is, therefore, not deacetylated in measurable amounts.

The decreased formation of NAMT from NADMPEA in animals pretreated with iproniazid was associated with an increased output of NA-β-OH-DMPEA, unidentified neutral metabolite (a), and NADMPEA. Thus, there is an increased excretion of dimethoxy metabolites in iproniazid-pretreated animals and a decreased excretion of demethylated products. It appears that iproniazid interferes with the demethylation of NADMPEA, resulting in increased availability of NADMPEA for β-hydroxylation. An alternative possibility is that iproniazid increases the rate of β-hydroxylation of NADMPEA directly. If this were the case, the amount of NADMPEA excreted after iproniazid pretreatment would be expected to be less than the amount excreted when no iproniazid was given. Since the proportion of unmetabolized NADMPEA excreted is increased in the presence of iproniazid, this latter possibility appears to be ruled out. It has been pointed out that most of the NAMT is excreted in conjugated form. However, considerable variation among individual animals was found in the amounts of unconjugated and conjugated NAMT. Variation in conjugation mechanisms may be related to a number of genetic and physiological factors.[17]

DISCUSSION

The pathways of DMPEA metabolism, obtained in the main from studies with rats, are shown in Fig. 1. As can be seen, DMPEA and dopamine have several metabolites in common. It has been shown previously that DMPEA can be β-hydroxylated *in vitro* with partially purified dopamine-β-hydroxylase.[18] It is not surprising, therefore,

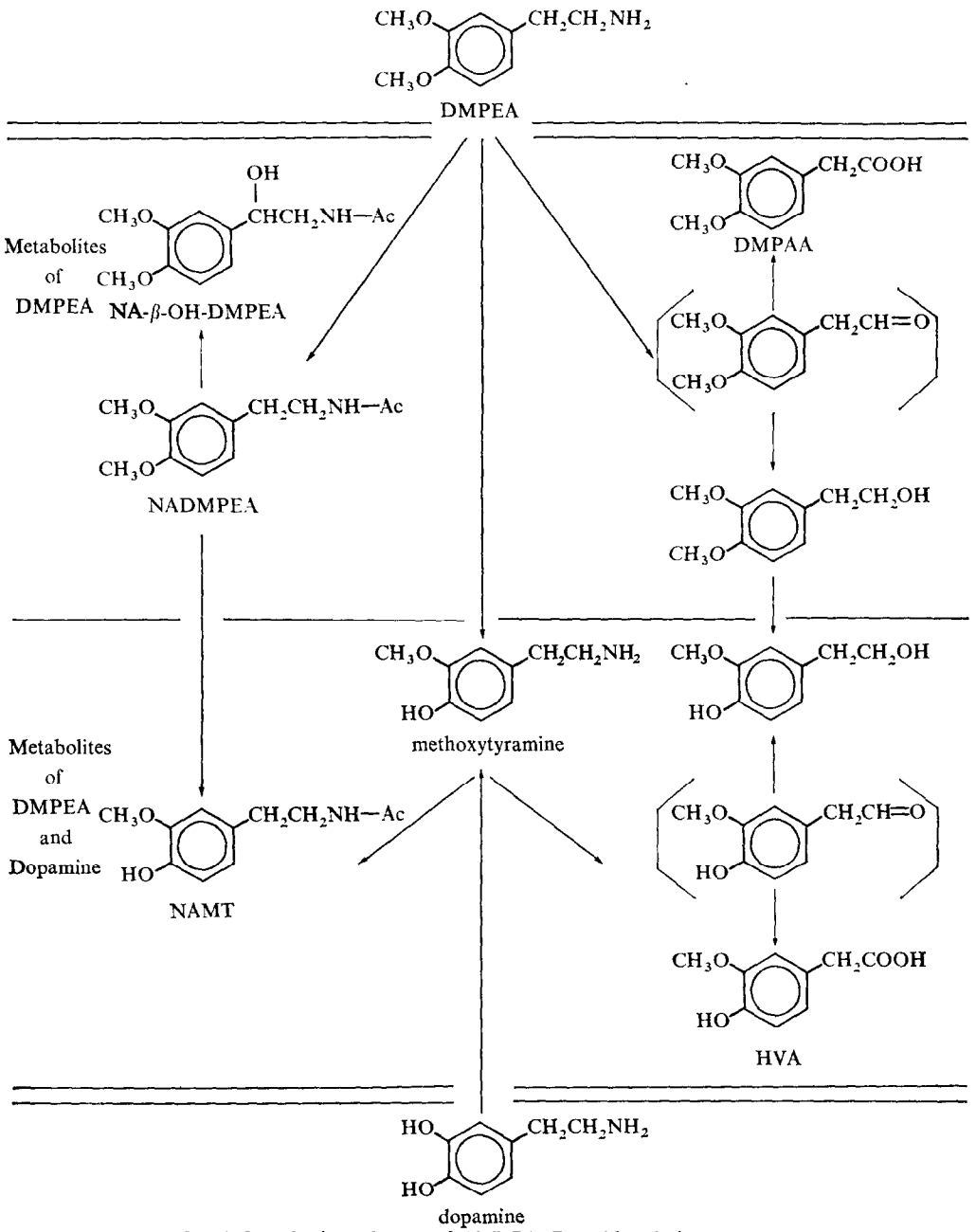

FIG. 1. Metabolic pathways of DMPEA. For abbreviations, see text.

to find a β-hydroxylated derivative as a metabolite *in vivo* of DMPEA. Moreover, it has been demonstrated that many phenethylamines,[19] as well as certain phenyl derivatives with neutral side chains such as *o*-acetaminoethyl benzene,[20] are enzymatically hydroxylated to form derivatives of benzyl alcohol.

While DMPEA may also be β-hydroxylated, we have not detected β-OH-DMPEA in these experiments, although this compound might be a precursor of NA-β-OH-DMPEA. It has been suggested that β-OH-DMPEA may represent one of a number of possible psychoactive toxins.[21] NA-β-OH-DMPEA represents the further metabolism of DMPEA to a compound that retains the 3,4-dimethoxy substituent. A study of substituted phenethylamines has shown that a para-methoxy substitutent is essential for compounds having mescaline-like effects in experimental animals.[8–12] However, NA-β-OH-DMPEA, administered to a small number of rats, did not appear to be a potent compound and had only a slight hypokinetic effect.

It is not clear why iproniazid interferes with the demethylation of NADMPEA. Monoamine oxidase inhibitors of the hydrazine type do affect enzymes other than monoamine oxidases, such as decarboxylases and nictinamide-adenine dinucleotidases.[22] Iproniazid has been shown to complex with NAD in rat tissues and may cause a nicotinamide deficiency *in vivo*.[23,24]

The nature of microsomal demethylation reactions is not completely understood. It has been shown that NADPH, a necessary cofactor in microsomal incubations, releases electrons to oxygen by means of the heme-like pigment, cytochrome P-450.[25] It is not known whether one or many enzymes are involved. Inhibition of demethylation by iproniazid may occur in a number of ways. Iproniazid may act on enzymes which alter the concentrations of coenzymes such as NADPH or other components necessary for metabolism. The concentration of available oxygen may be changed, or inactivation of cytochrome P-450 or its reductase might result. For example, SKF 525-A is known to convert cytochrome P-450 *in vitro* to an inactive metabolite,[25] and may inhibit oxidative drug metabolism, including demethylation reactions, in this manner. If iproniazid interfered with the formation of NADPH or with the NADP⁻NADPH oxidation system, this might explain its effect on metabolism of NADMPEA, inasmuch as the NADP⁻NADPH oxidation system is involved in the displacement of the methoxy group by a hydroxy group in the demethylation reaction. In previous unreported studies, we have found that SKF 525-A has an effect similar to that of iproniazid on DMPEA metabolism. Both drugs block hexobarbital metabolism, another NADP⁻NADPH-dependent hydroxylation system.[26] It is possible also that iproniazid, by inhibiting monoamine oxidase, produces increased concentrations of endogeneous amines or *N*-acetylated amines, which may carry methoxy groups and act as competitive inhibitors in the demethylation of administered NADMPEA.[27]

In a large number of experimental studies, methylated metabolites have been investigated after amine oxidase inhibition with iproniazid.[28] The results may have been influenced appreciably and in an unpredictable fashion by the action of this inhibitor. An inhibitory action of iproniazid on demethylation should be explored further in relation to the relapses produced in schizophrenics by administration of this compound.

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