Pharmacology Biochemistry & Behavior, Vol. 38, pp. 505–512. © Pergamon Press plc, 1991. Printed in the U.S.A. 0091-3057/91 $3.00 + .00

3,4-Methylenedioxyamphetamine (MDA) Analogues Exhibit Differential Effects on Synaptosomal Release of ^{3}H-Dopamine and ^{3}H-5-Hydroxytryptamine

DENNIS J. McKENNA,*[1] X.-M. GUAN* AND A. T. SHULGIN†

*Department of Neurology & Neurological Sciences, Stanford University Medical Center, Stanford, CA 94305
†1483 Shulgin Road, Lafayette, CA 94549

Received 16 August 1990

McKENNA, D. J., X.-M. GUAN AND A. T. SHULGIN. *3,4-Methylenedioxyamphetamine (MDA) analogues exhibit differential effects on synaptosomal release of ^{3}H-dopamine and ^{3}H-5-hydroxytryptamine*. PHARMACOL BIOCHEM BEHAV **38**(3) 505–512, 1991.—The effect of various analogues of the neurotoxic amphetamine derivative, MDA (3,4-methylenedioxyamphetamine) on carrier-mediated, calcium-independent release of ^{3}H-5-HT and ^{3}H-DA from rat brain synaptosomes was investigated. Both enantiomers of the neurotoxic analogues MDA and MDMA (3,4-methylenedioxymethamphetamine) induce synaptosomal release of ^{3}H-5-HT and ^{3}H-DA in vitro. The release of ^{3}H-5-HT induced by MDMA is partially blocked by 10^{-6} M fluoxetine. The (+) enantiomers of both MDA and MDMA are more potent than the (−) enantiomers as releasers of both ^{3}H-5-HT and ^{3}H-DA. Eleven analogues, differing from MDA with respect to the nature and number of ring and/or side chain substituents, also show some activity in the release experiments, and are more potent as releasers of ^{3}H-5-HT than of ^{3}H-DA. The amphetamine derivatives (±)fenfluramine, (±)norfenfluramine, (±)MDE, (±)PCA, and d-methamphetamine are all potent releasers of ^{3}H-5-HT and show varying degrees of activity as ^{3}H-DA releasers. The hallucinogen DOM does not cause significant release of either ^{3}H-monoamine. Possible long-term serotonergic neurotoxicity was assessed by quantifying the density of 5-HT uptake sites in rats treated with multiple doses of selected analogues using ^{3}H-paroxetine to label 5-HT uptake sites. In the neurotoxicity study of the compounds investigated, only (+)MDA caused a significant loss of 5-HT uptake sites in comparison to saline-treated controls. These results are discussed in terms of the apparent structure-activity properties affecting ^{3}H-monoamine release and their possible relevance to neurotoxicity in this series of MDA congeners.

Methylenedioxyamphetamines Serotonin Dopamine Synaptosomes Neurotoxicity
Structure-activity relationships

MDA (3,4-methylenedioxyamphetamine) and its N-methyl analogue, MDMA (3,4-methylenedioxymethamphetamine; "Ecstasy") are ring-substituted phenylisopropylamine derivatives which bear structural and pharmacological similarities to both amphetamines and hallucinogens such as mescaline (15,38). Both behavioural (7, 18, 19) and pharmacological data indicate that MDMA and some of its derivatives may represent a new class of psychopharmacologic agents whose mechanisms of action are distinct from those of either classical hallucinogens or stimulants (15,17). MDA, MDMA, and some of their structural congeners are selectively neurotoxic to serotonergic nerve terminals in rats and primates (3, 14, 20, 24, 28, 31).

Although the exact mechanisms are incompletely understood, several investigators have proposed that the neurotoxicity induced by MDMA and its congeners is mediated by dopamine (DA) or its metabolites (9,44). These agents induce the presynaptic release of DA and serotonin (5-HT) both in vitro (12, 32, 42) and in vivo (32, 33, 46). The long-term serotonergic neurotoxicity can be significantly attenuated either by pretreatment with 5-HT uptake inhibitors such as citalopram (32), prior depletion of endogenous monoamines with reserpine, blockade of DA synthesis with alpha-methylparatyrosine (AMT), or prior lesioning of nigrostriatal dopamine neurons with 6-hydroxydopamine (44).

Structure-activity data, although limited, are also consistent with a role of DA in mediating the serotonergic neurotoxicity. For example, the enantiomers of MDA or MDMA are approximately equipotent as releasers of ^{3}H-5-HT from superfused brain slices, but the (+) enantiomers are more potent releasers of ^{3}H-DA than the (−) enantiomers (12) and are also significantly more potent in producing the long-term 5-HT depletion indicative of

[1]Requests for reprints should be addressed to Dennis J. McKenna at his present address: Shaman Pharmaceuticals, Inc., 887 Industrial Road, Suite G, San Carlos, CA 94070.

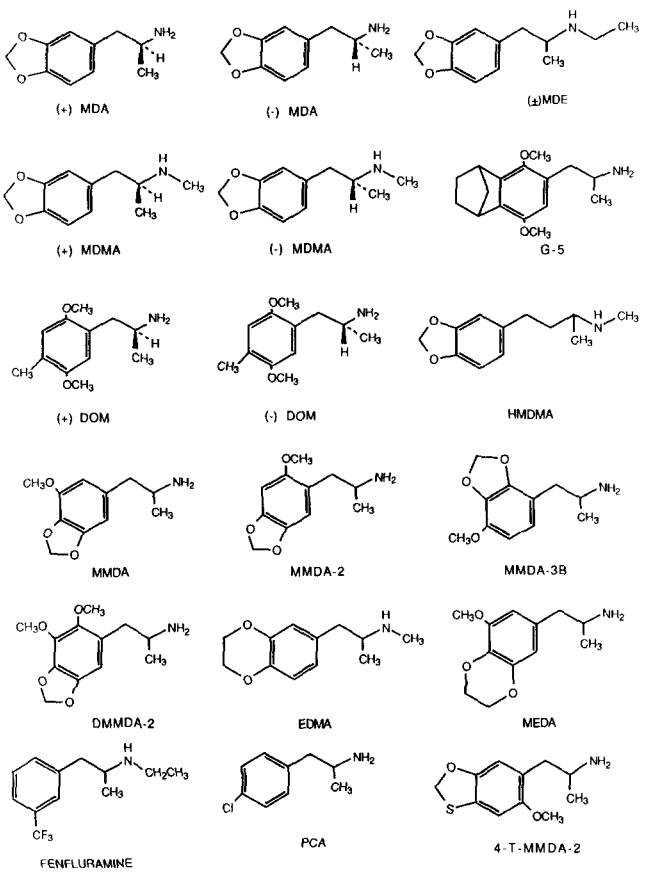

FIG. 1. Structures of amphetamine derivatives and MDA (3,4-methylenedioxyamphetamine) analogues investigated in the present study.

neurotoxicity (29,33). Moreover, N-ethylation of MDA to give the analogue MDE (3,4-methylenedioxyethylamphetamine) results in a compound which is less neurotoxic and less potent as a releaser of ^{3}H-DA in vitro than MDA or MDMA, but is approximately equipotent with these compounds as a releaser of ^{3}H-5-HT (12, 25, 29, 43).

Previous structure/activity studies of MDMA and its congeners have focused primarily on the differential potency of the enantiomers as neurotoxins or as ^{3}H-monoamine releasers, or on the influence of the side-chain nitrogen or alpha-carbon substituent on these parameters (12,16). In the present study, we investigated the effects of eleven ring-substituted analogues of MDA (Fig. 1) on synaptosomal release of ^{3}H-5-HT and ^{3}H-DA in vitro and evaluated selected derivatives for neurotoxicity in the rat. The results are compared with the effects of MDA and other amphetamine derivatives, some of which are known to exhibit serotonergic neurotoxicity in animals.

METHOD

Drugs and Chemicals

Drugs used in the in vitro release studies were obtained from the National Institute on Drug Abuse [(+)DOM, (−)DOM, (−)MDA, (+)MDA, (+)MDMA, (−)MDMA, (±)MDE], Sigma Chemical Co. (St. Louis, MO) [d-methamphetamine, (±)fenfluramine, (±)para-chloroamphetamine], or Research Biochemicals, Inc. (fluoxetine, bupropion). We are indebted to Dr. D. E. Nichols, Department of Medicinal Chemistry and Pharmacognosy, Purdue University, for the generous gift of (±)norfenfluramine. Other analogues used in the study were synthesized by A. T. Shulgin and P. Jacob, III. Synthesis and structural characterization of several of the analogues used in this study have been described elsewhere (35, 36, 39, 41) or will be published separately. All other chemicals used were of the highest quality commercially available.

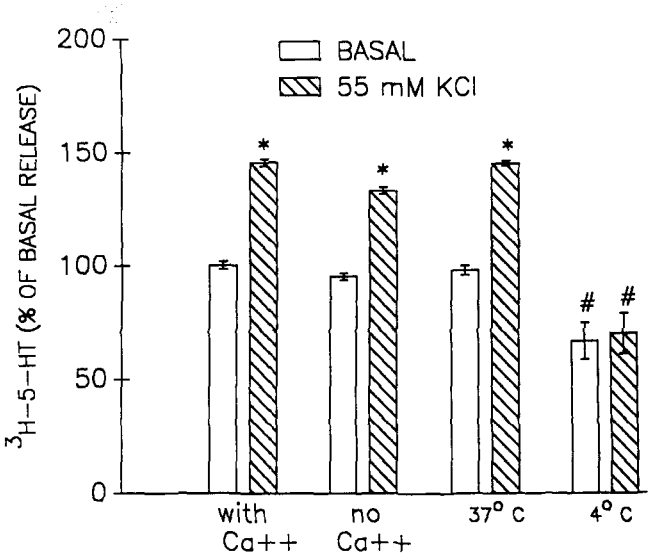

FIG. 2. Effect of calcium and temperature on the potassium-induced release of ^{3}H-5-HT from rat synaptosomes. *$p<0.05$ vs. basal; #$p<0.05$ vs. basal at 37°C.

Synaptosomal Release Preparations

In order to measure the in vitro synaptosomal release of ^{3}H-5-HT and ^{3}H-DA, we used a modification of a method described by Bender and co-workers (4). Male Sprague-Dawley rats (200–225 g; Simonsen Labs, Gilroy, CA) were lightly anaesthetized with halothane and decapitated. The brains were removed and cerebral cortex and hippocampus pooled and homogenized in 10 volumes of ice-cold 0.32 M sucrose with a Wheaton motor-driven Teflon-glass homogenizer (speed 6). The cortical-hippocampal tissue was used for the ^{3}H-5-HT release experiments, while pooled mesolimbic and striatal tissue, prepared in an identical manner, was used for the ^{3}H-DA release experiments. The homogenates were centrifuged at 3000 × g for 10 min and the supernatant, containing the crude synaptosomal fraction, was gently decanted and diluted 1:1 with Krebs-Hepes buffer [pH 7.4, composition (mM): NaCl, 117; KCl, 4.8; $CaCl_2$, 2.5; $MgCl_2$, 2.5; Hepes, 25; pargyline, 0.010]. The supernatant was recentrifuged at 10,000 × g for 20 min, and the final pellet was resuspended in 80 volumes of Krebs-Hepes buffer. The synaptosomal preparations were maintained on ice until used. The synaptosomal preparations (800 μl) were aliquoted into test tubes containing either ^{3}H-5-HT (specific activity: 12.5 Ci/mmol, NEN; final concentration, approx. 9 nM) or ^{3}H-DA (specific activity: 51.9 Ci/mmol, NEN; final concentration, approx. 2.3 nM) and incubated at 37°C for 15 min. Specific uptake was defined as the total minus blank taken in the presence of 10^{-4} M fluoxetine in the case of ^{3}H-5-HT, or 10^{-4} M bupropion in the case of ^{3}H-DA. After 15 minutes incubation, either buffer or varying concentrations of releasing drugs (0.01–10 μM) or KCl (final concentration: 50 mM) were added and the incubation at 37°C continued for an additional 15 min. Following

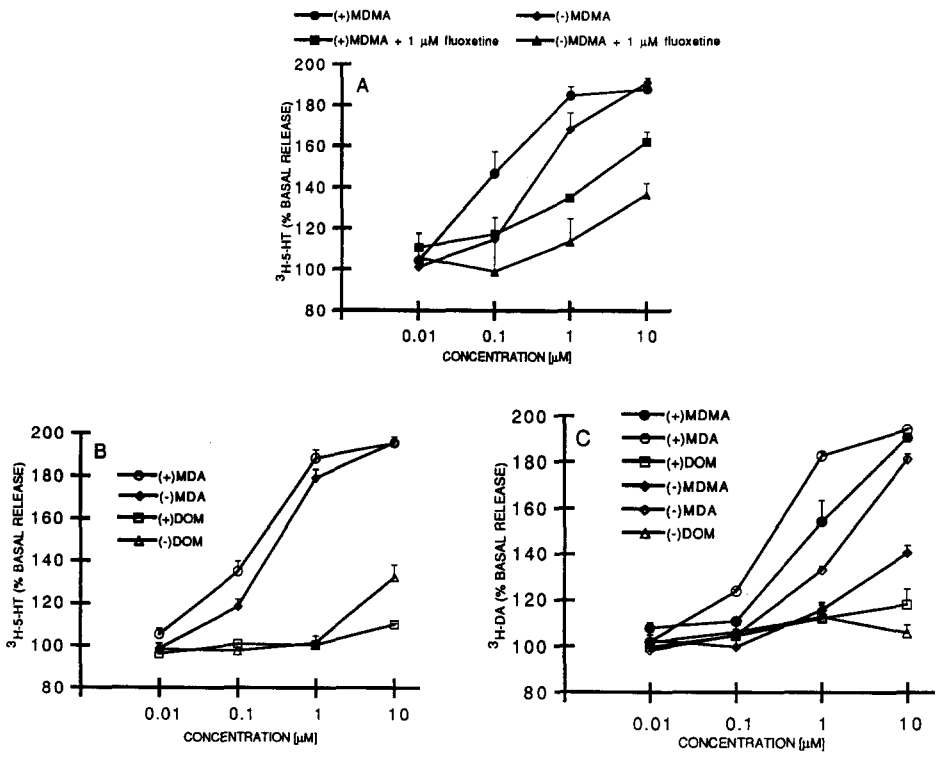

FIG. 3. (A) Synaptosomal release of ^{3}H-5-HT induced by MDMA enantiomers and their inhibition by 10^{-6} M fluoxetine. (B) Synaptosomal release of ^{3}H-5-HT induced by the enantiomers of MDA and DOM. (C) Synaptosomal release of ^{3}H-DA induced by (+) and (−) enantiomers of MDMA, MDA and DOM.

incubation, the assays were terminated by rapid filtration through No. 32 glass fiber filters (Schleicher & Schuell, Keene, NH) presoaked in Krebs-Hepes buffer. The filters were rinsed twice with 5 ml Krebs-Hepes buffer and counted in 2 ml Biosafe™ scintillation fluid (Research Products Inc.) in a Packard 1900CA scintillation counter at 41% efficiency. Effect of releasing agents was expressed as a percent of basal release, as follows: % of basal release = (100 − % retained) + 100. By this calculation, controls (basal release) equal 100%, and assays showing release equal 100 + % released. Assays were performed in triplicate, and results are reported as the means ± standard error of the mean, of 3 or more independent experiments.

Characterization of in vitro synaptosomal release. We conducted initial experiments aimed at characterizing the nature of the synaptosomal release of tritiated monoamines observed in our system; specifically, we investigated the effect of calcium and potassium, temperature, and 5-HT uptake blockers on release, in addition to examining the effects of MDA and various analogues. These experiments were conducted using synaptosomes prepared from pooled cortical and hippocampal tissues, incubated in the presence of ^{3}H-5-HT.

Release of ^{3}H-5-HT from synaptosomes by potassium was measured by incubating synaptosomes for 15 min at 37°C in the presence of ^{3}H-5-HT, then adding 1.0 ml 105 mM KCl dissolved in buffer, to give a final KCl concentration of 55 mM. Tubes were incubated an additional 15 min at 37°C, then filtered and counted as described above. To test for calcium independence of the potassium-induced release, the experiment was conducted using calcium-free buffer containing 2.5 mM ethyleneglycoltetraacetate (EGTA), a calcium chelator.

To test the effect of temperature on potassium-induced release, controls were incubated at 37°C prior to and after the addition of KCl; a duplicate set of tubes was incubated at 37°C for 15 min in the presence of ^{3}H-5-HT to load the synaptosomes, then cooled on ice to 4°C prior to the addition of KCl.

The effect of MDA and the other analogues on synaptosomal release was investigated by loading the synaptosomes with either ^{3}H-5-HT or ^{3}H-DA for 15 min at 37°C, then adding varying concentrations of releasing drug (0.01–10 μM) and incubating for a further 15 min. The effect of fluoxetine, a selective 5-HT uptake blocker, on synaptosomal release induced by MDMA was investigated by first loading the synaptosomes with ^{3}H-5-HT for 15 min at 37°C as described above; following synaptosomal loading, fluoxetine (10^{-6} M, final concentration) was added and the preparation incubated for an additional 5 min; then various concentrations (0.01–10 μM) of (+)MDMA or (−)MDMA were added and the incubation was continued for an additional 10 minutes before filtering. Preparations were then filtered and counted as described above.

Neurotoxicity Studies

Animals and drug administration. Neurotoxicity studies utilized male Sprague-Dawley rats (300–325 g; Simonsen Labs, Gilroy, CA). Animals were administered repeated doses IP (2 × 5 mg/kg/day, for 4 consecutive days) of various MDA analogues, dissolved in saline. Analogues screened for neurotoxicity were selected on the basis of structural features and their action as ^{3}H-5-HT or ^{3}H-DA releasers in vitro. Control animals were injected with saline (1.0 ml/kg). As a positive control, another group of animals received MDA, but a slightly lower dose was used (2 × 3 mg/kg/day, 4 consecutive days) due to a high rate of mortality

TABLE 1

EFFECT OF MDA AND MDA ANALOGUES ON RELEASE OF ^{3}H-5-HT AND ^{3}H-DA FROM RAT BRAIN SYNAPTOSOMES*

Drug (1 μM)	^{3}H-5-HT	^{3}H-DA
(+)MDA	190 ± 4†	183 ± 2†‡
(±)PCA	190 ± 1†	183 ± 1†
(+)Methamphetamine	165 ± 4†	180 ± 6†
(+)MDMA	185 ± 4†	155 ± 9†‡
(−)MDA	180 ± 4†	133 ± 2†‡
(±)Norfenfluramine	186 ± 1†	131 ± 5†
(±)Fenfluramine	180 ± 1†	120 ± 3†
(±)EDMA	175 ± 1†	120 ± 7†
(−)MDMA	170 ± 8†	115 ± 1†‡
(±)MMDA	172 ± 2†	115 ± 6
(−)DOM	100 ± 4	113 ± 6
(±)MEDA	145 ± 23†	113 ± 5†
(±)MMDA-3B	185 ± 4†	112 ± 2†
(+)DOM	100 ± 0	112 ± 6
(±)MDE	170 ± 1†	110 ± 2
(±)MMDA-2	103 ± 3	106 ± 6
(±)HMDMA	117 ± 1†	105 ± 5
(±)G-5	100 ± 2	100 ± 16
(±)4-T-MMDA-2	115 ± 4†	92 ± 4†
(±)DMMDA-2	130 ± 7†	90 ± 6

*Values are percent of basal ^{3}H-monoamine released at a drug concentration of 1 μM, ± standard error of the mean (see the Method section). Results are given in rank order of potency for ^{3}H-DA release (column 3). †$p<0.05$ vs. control (Student's t-test). ‡$p<0.05$ vs. opposite enantiomer (Student's t-test).

among the MDA-treated animals given the higher dose. Five to seven animals were used for each treatment. Fourteen days after the last drug treatment, the animals were lightly anaesthetized with halothane, decapitated, and the cortex and midbrains removed and rapidly frozen in isopentane over dry ice. Frozen tissue samples were stored at −70°C until used.

^{3}H-Paroxetine binding. The density of 5-HT uptake sites labeled by the selective 5-HT uptake blocker, ^{3}H-paroxetine, was determined by performing saturation binding experiments (11). Significant reduction in the B_{max} value, which is a measure of the density of 5-HT uptake sites, relative to saline-treated controls was interpreted as evidence of ablation of serotonergic terminals resulting from neurotoxicity (3).

RESULTS

Effects of Experimental Conditions on In Vitro Synaptosomal Release

In our experimental system, the synaptosomal release induced by 55 mM KCl was Ca^{++}-independent (Fig. 2). The potassium-induced release was temperature-dependent; the percent of controls induced by 55 mM KCl at 37°C was 145%, while at 4°C, the amount of release in the presence of potassium was equivalent to basal levels. The basal levels at the lower temperature were significantly less than basal levels at 37°C (Fig. 2). These results show that basal release, as well as potassium-stimulated release, is temperature-dependent.

Effects of MDA and MDA Analogues on In Vitro Synaptosomal Release

The effects of the enantiomers of MDMA, MDA and DOM on synaptosomal release of ^{3}H-5-HT are shown in Fig. 3A and B; the effects of these enantiomers on synaptosomal release of ^{3}H-DA are shown in Fig. 3C. The (+) enantiomers of MDA and MDMA are more potent releasers of both tritiated monoamines than the corresponding (−) enantiomers (Fig. 3). The enantiomers of MDMA and MDA are approximately equipotent releasers of ^{3}H-5-HT at a concentration of 1 μM (Fig. 3A and B, Table 1) but the (+) enantiomers of both compounds are significantly more potent releasers of ^{3}H-DA than the corresponding (−) enantiomers (Fig. 3C, Table 1). These results are consistent with previously reported findings (12, 28, 32). The release of ^{3}H-5-HT by both enantiomers of MDMA was significantly reduced in the presence of 10^{-6} M fluoxetine (Fig. 3A). These results provided evidence that the release being measured was at least partially carrier-mediated. Neither enantiomer of DOM induced a significant release of ^{3}H-DA at any concentration tested (Fig. 3C);

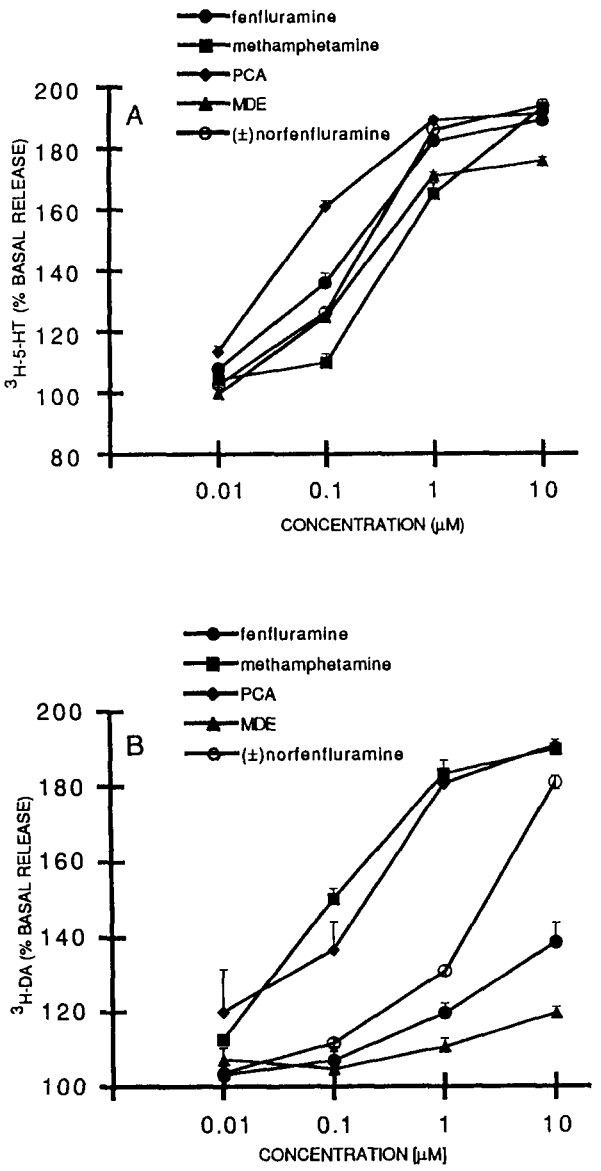

FIG. 4. (A) Synaptosomal release of ^{3}H-5-HT induced by (±) fenfluramine, (±)norfenfluramine, d-methamphetamine, (±)PCA, and (±)MDE. (B) Synaptosomal release of ^{3}H-DA induced by (±)fenfluramine, (±)norfenfluramine, d-methamphetamine, (±)PCA, and (±)MDE.

TABLE 2

EFFECT OF MDA AND MDA ANALOGUES ON ^{3}H-PAROXETINE BINDING

Drug	K_d (nM)	B_{max} (pmol/g tissue)
Saline	0.23 ± 0.03	38 ± 2.1
(±)MDA	0.27 ± 0.09	19 ± 1.7*
(±)MMDA	0.26 ± 0.03	40 ± 3.4
(±)DMMDA	0.24 ± 0.05	33 ± 2.6
(±)EDMA	0.23 ± 0.04	32 ± 4.1

*$p<0.05$, one-way ANOVA.

similarly, neither DOM enantiomer induced a significant release of ^{3}H-5-HT, except at the highest concentration used (10 μM) (Fig. 3B).

In addition to the enantiomers of MDA, MDMA, and DOM, the effect of five other amphetamine derivatives on synaptosomal release of ^{3}H-5-HT and ^{3}H-DA was investigated. All of these derivatives [(±)fenfluramine, (±)norfenfluramine, (±)para-chloroamphetamine, (±)MDE, and d-methamphetamine] have been shown by previous investigators to exhibit varying degrees of serotonergic neurotoxicity (8, 21, 25, 26, 29, 30, 34, 43, 45). All five of the neurotoxic derivatives showed a similar degree of potency as releasers of ^{3}H-5-HT (Fig. 4A, Table 1) with d-methamphetamine showing the weakest activity. With respect to the induction of ^{3}H-DA release, however, differential potencies were observed (Fig. 4B). (±)para-Chloroamphetamine (PCA) and d-methamphetamine were approximately equipotent releasers of ^{3}H-DA, while (±)fenfluramine and (±)MDE were approximately equipotent to each other, but both derivatives showed much weaker activity compared to methamphetamine and PCA. However, these derivatives still induced significant levels of ^{3}H-DA release, relative to basal controls, at concentrations of 1 μM and 10 μM. (±)Norfenfluramine was more potent than (±)fenfluramine at inducing ^{3}H-DA release at 1 and 10 μM, but the difference was significant only at 10 μM.

Of the nine additional MDA analogues tested, nearly all were more active as releasers of ^{3}H-5-HT than of ^{3}H-DA (Fig. 5A and B; Table 1). The most active of the ^{3}H-5-HT releasing drugs were (±)MMDA-3B, (±)MMDA, and (±)EDMA; the others all showed significantly less potency at a concentration of 1 μM (Table 1). All of the analogues tested were much less potent as inducers of ^{3}H-DA release, but the three compounds named above showed the most activity as ^{3}H-DA releasers as well, even though this activity was markedly reduced in comparison to their effect on ^{3}H-5-HT release (Fig. 5, Table 1).

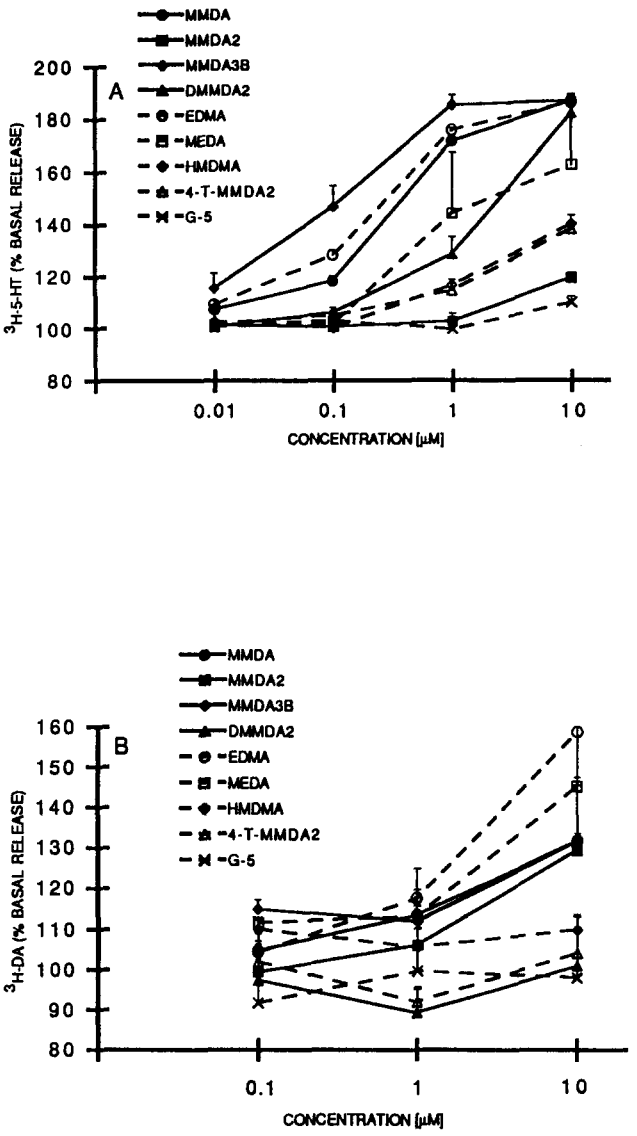

FIG. 5. (A) Synaptosomal release of ^{3}H-5-HT induced by structural analogues of MDA and MDMA. (B) Synaptosomal release of ^{3}H-DA induced by structural analogues of MDA and MDMA. All compounds listed are racemic.

Effect of Selected Analogues on Density of ^{3}H-Paroxetine Binding Sites

The K_d and B_{max} values obtained from Scatchard analyses of saturation-binding assays with ^{3}H-paroxetine in animals treated with (±)MDA or various analogues [(±)EDMA, (±)MMDA-3B, or (±)DMMDA-2] were compared with saline controls and analyzed by one-way ANOVA. The K_d provides a measure of the affinity of the labelled ligand for its receptor, while the B_{max} measures the density of the uptake sites. No significant differences in K_d values were found between treated animals and saline controls. The B_{max} value of rats treated with (±)MDA (2 × 3 mg/kg, 4 consecutive days) was significantly reduced with respect to saline controls, but the B_{max} values of animals treated with the analogues were not significantly different than controls (Table 2). Animals treated with the analogues (±)EDMA and (±)DMMDA-2 had somewhat reduced B_{max} values compared to controls, but this difference did not reach statistical significance.

DISCUSSION

The major finding of the present study is that those compounds which other investigators have shown are the most potent serotonergic neurotoxins in vivo show the greatest potency as releasers of ^{3}H-DA rather than of ^{3}H-5-HT. This can be seen in Table 1, where the compounds investigated are ranked according to their potency as in vitro ^{3}H-DA releasers. For example, the S(+) enantiomers of MDA and MDMA are both more potent neurotoxins than the R(−) enantiomers (12) and this relative potency is reflected by their relative ranking as ^{3}H-DA releasers. Similarly, MDE displays a relatively low potency for inducing ^{3}H-DA release, and requires an approximately 4-fold higher dose to induce

an equivalent long-term reduction in 5-HT, compared to MDMA (25). However, the compounds (±)fenfluramine and its N-deethyl congener, (±)norfenfluramine, provide striking exceptions to this apparent correlation. Our study shows that fenfluramine is less potent than (+)MDMA as a ^{3}H-DA releaser, but according to one study (45) fenfluramine is approximately 3 times more toxic than MDMA. In an effort to explain this discrepancy, we also tested (±)norfenfluramine, the primary metabolite of fenfluramine (6). Although our results indicate that (±)norfenfluramine is more potent than (±)fenfluramine at inducing ^{3}H-DA release from synaptosomes, this difference is significant only at the highest concentration used (Fig. 4B, Table 1). The fact that fenfluramine and its metabolite, norfenfluramine, are neurotoxic yet lack potency as ^{3}H-DA releasers is evidence that the serotonergic neurotoxicity of these amphetamine derivatives may involve mechanisms unrelated to (or in addition to) endogenous DA release. An alternative possibility that has yet to receive extensive experimental investigation is that the endogenous release of norepinephrine (NE), rather than DA, is the primary mechanism mediating the neurotoxicity of these agents. If NE or its metabolites mediates the neurotoxicity of these drugs, this would also explain the apparent discrepancy between the anatomical distribution of the serotonergic lesions (cortex, hippocampus) and the relatively low abundance of DA-containing neurons in these regions.

A secondary finding of this study is that relatively narrow structural determinants can result in large differential effects on the ability of MDA and its congeners to release ^{3}H-5-HT and ^{3}H-DA from synaptosomes. Centrally active phenylisopropylamines can be separated into two classes, differing with respect to their probable mechanisms of action and behavioral and psychological effects. The hallucinogen DOM, along with its halogenated congeners DOB (2,5-dimethoxy-4-bromo-phenylisopropylamine) and DOI (2,5-dimethoxy-4-iodo-phenylisopropylamine) represent a class of hallucinogenic phenylisopropylamines which exhibit high affinity for 5-HT$_2$ receptors (10,13) and act at these postsynaptic sites primarily as agonists or partial agonists (22,27). Compounds in this class also generalize to LSD or other hallucinogens in the drug discrimination test (1) and exhibit a human psychopharmacology typical of LSD-like hallucinogens (37). Although investigations are limited, compounds in this class are apparently *not* serotonergic neurotoxins. One study used immunocytochemistry to compare the neurotoxicity of PCA (parachloroamphetamine) and MDA with the phenylisopropylamine hallucinogen, DOM (2,5-dimethoxy-4-methyl-phenylisopropylamine) and found no evidence for serotonergic neurodegeneration in animals treated with the latter compound (5).

The second class is typified by MDMA and its congeners; these compounds typically display a low affinity for 5-HT$_2$ receptors (2,23), induce the presynaptic release of 5-HT and DA from nerve terminals, and frequently do display serotonergic neurotoxicity, at least in some animals under some dose regimens. Drugs in this class exhibit similarities to both hallucinogens and stimulants in the drug discrimination assay (7, 18, 19) and their human psychopharmacology has been characterized as "entactogenic" rather than hallucinogenic (15,17). Structurally, compounds in the first (DOM-like) class are 2,5-dimethoxy-4-substituted phenylisopropylamines [the nature and polarity of the 4-substituent can vary greatly without loss of central activity; cf. (37)], while compounds in the second (MDMA-like) class typically have a methylenedioxy ring substitution.

Some of the analogues investigated in the present study belong to yet a third class of centrally active phenylisopropylamine derivatives, typified by the compounds MMDA (3,4-methylenedioxy-5-methoxy-phenylisopropylamine) and DMMDA (2,5-dimethoxy-3,4-methylenedioxy-phenylisopropylamine) (35, 36, 39). Structurally, these compounds are intermediates between the DOM-like compounds and the MDA-like compounds, in that they incorporate the methylenedioxy ring, together with one or two additional methoxy ring substituents (cf. Fig. 1). The human psychopharmacology of these compounds is also apparently intermediate between that of the "entactogenic" MDMA-like drugs, and the "hallucinogenic" DOM-like drugs (40). There is little or no information available on their behavioral effects in the drug discrimination assay. The present study provides the first information on the possible neurotoxicity and synaptosomal releasing ability of compounds in this intermediate class. While the results from the in vivo neurotoxicity studies presented here must be regarded as preliminary, they indicate that the presence of one or two methoxy substituents on the ring, in addition to the methylenedioxy substituent, abolishes the serotonergic neurotoxicity and dramatically attenuates the capacity of these agents to release ^{3}H-DA from synaptosomes (Table 2, Fig. 5B); these derivatives still retain considerable potency as releasers of ^{3}H-5-HT, however (Table 1, Fig. 5A). If the synaptosomal release of ^{3}H-DA were a reliable index of serotonergic neurotoxicity, then the analogues in this intermediate class would be expected to lack neurotoxicity; however, some of these agents display similar potencies to (±)fenfluramine, (±)norfenfluramine, and (±)MDE as ^{3}H-DA releasers and these compounds are known to display serotonergic neurotoxicity. Although none of the analogues tested showed evidence of neurotoxicity at the comparatively low doses used in this preliminary study, it is possible that at higher doses they may exhibit activity as serotonergic neurotoxins.

The results found with some of the analogues which bear an even closer structural relationship to MDMA also deserve comment. The addition of a single methylene to the ring alkylidenedioxy substituent to give the analogue (±)EDMA (3,4-ethylenedioxymethamphetamine, Fig. 1) results in a compound which lacks neurotoxicity and shows markedly attenuated ^{3}H-DA releasing ability, but is comparable to (−)MDMA as a releaser of ^{3}H-5-HT. Interestingly, this compound lacks detectable psychotropic effects in man at doses up to 200 mg orally. Addition of a ring-methoxy group to this compound to give the compound MEDA (Fig. 1) attenuates its potency as a ^{3}H-5-HT releaser, but does not further diminish its already low potency as a ^{3}H-DA releaser. Extension of the side-chain of MDMA by one carbon to give the homologue HMDMA (41), or replacement of one methylenedioxy oxygen with sulfur to give the compound 4-T-MMDA-2 (Fig. 1) essentially abolishes the ^{3}H-5-HT and ^{3}H-DA releasing capability, as well as the central psychotropic effect in man (Shulgin and Jacob, unpublished). The possible neurotoxicity of these analogues is not addressed in the present study.

The results reported here indicate that synaptosomal release of ^{3}H-DA shows some relationship to serotonergic neurotoxicity *in vivo* of MDA and its congeners; however, some derivatives, notably fenfluramine and norfenfluramine, are conspicuous exceptions to this apparent relationship, indicating that additional mechanisms must play a role in mediating the serotonergic neurotoxicity of these agents.

ACKNOWLEDGEMENTS

We thank Ms. Mary Keller for assistance in the preparation of the manuscript. Two of us (D.J.M., X-M.G.) thank Dr. S. J. Peroutka for constructive discussion and for financial support in the form of postdoctoral fellowships. This work was supported in part by the Stanley Foundation and National Institutes of Health grants NS 12151-14 and NS 23560-03.

REFERENCES

1. Appel, J. B.; Cunningham, K. A. The use of drug discrimination procedures to characterize hallucinogenic drug action. Psychopharmacol. Bull. 22:959–969; 1986.
2. Battaglia, G.; Brooks, B. P.; Kulsakdinun, C.; DeSouza, E. B. Pharmacologic profile of MDMA (3,4-methylenedioxymethamphetamine) at various brain recognition sites. Eur. J. Pharamcol. 149:159–163; 1988.
3. Battaglia, G.; Zaczek, R.; DeSouza, E. B. MDMA effects in brain: Pharmacologic profile and evidence of neurotoxicity from neurochemical and autoradiographic studies. In: Peroutka, S. J., ed. Ecstasy: The clinical, pharmacological, and neurotoxicological effects of the drug MDMA. Boston: Kluwer Academic Publishers; 1990:171–199.
4. Bender, A. S.; Wu, P. H.; Phillis, J. W. The rapid uptake and release of [^{3}H]adenosine by rat cerebral cortical synaptosomes. J. Neurochem. 36:651–660; 1981.
5. Berger, W.; Hung, G.; Molliver, M. E.; Grzanna, R. Psychotropic amphetamines have different sites of action at serotonergic (5-HT) synapses: a comparison of p-chloroamphetamine (PCA) and 3,4-methylenedioxyamphetamine (MDA) with 2,5-dimethoxy-4-methylamphetamine (DOM). Soc. Neurosci. Abstr. 13:906; 1987.
6. Bruce, R. B.; Maynard, W. R., Jr. Fenfluramine metabolism. J. Pharm. Sci. 57:1173–1176; 1968.
7. Callahan, P. M.; Appel, J. B. Differences in the stimulus properties of 3,4-methylenedioxymethamphetamine and 3,4-methylenedioxymethamphetamine in animals trained to discriminate hallucinogens from saline. J. Pharmacol. Exp. Ther. 246:866–70; 1988.
8. Fuller, R. W.; Perry, K. W.; Molloy, B. B. Reversible and irreversible phases of serotonin depletion by 4-chloroamphetamine. Eur. J. Pharmacol. 33:119–124; 1975.
9. Gibb, J. W.; Stone, D. M.; Johnson, M.; Hanson, J. R. Neurochemical effects of MDMA. In: Peroutka, S. J., ed. Ecstasy: The clinical, pharmacological, and neurotoxicological effects of the drug MDMA. Boston: Kluwer Academic Publishers; 1990:133–150.
10. Glennon, R. A.; McKenney, J. D.; Lyon, R. A.; Titeler, M. 5HT$_1$ and 5-HT$_2$ binding characteristics of 1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane analogues. J. Med. Chem. 29:194–199; 1986.
11. Habert, E.; Graham, D.; Tahraoui, L.; Claustre, Y.; Langer, S. Z. Characterization of [^{3}H]paroxetine binding to rat cortical membranes. Eur. J. Pharmacol. 118:107–114; 1986.
12. Johnson, M. P.; Hoffman, A. J.; Nichols, D. E. Effects of the enantiomers of MDA, MDMA, and related analogues on [^{3}H]serotonin and [^{3}H]dopamine release from superfused rat brain slices. Eur. J. Pharmacol. 132:269–276; 1986.
13. McKenna, D. J.; Peroutka, S. J. Differentiation of 5-hydroxytryptamine$_2$ receptor subtypes using ^{125}I-R-(−)2,5-dimethoxy-4-iodophenylisopropylamine and ^{3}H-ketanserin. J. Neurosci. 9:3482–3490; 1989.
14. McKenna, D. J.; Peroutka, S. J. Neurochemistry and neurotoxicity of 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy"). J. Neurochem. 54:14–22; 1990.
15. Nichols, D. E. Differences between the mechanism of action of MDMA, MBDB, and the classic hallucinogens. Identification of a novel therapeutic class: entactogens. J. Psychoactive Drugs 18:305–313; 1986.
16. Nichols, D. E.; Lloyd, D. H.; Hoffman, A. J.; Nichols, M. B.; Yim, G. K. Effects of certain hallucinogenic amphetamine analogues on the release of [^{3}H]serotonin from rat brain synaptosomes. J. Med. Chem. 25:530–535; 1982.
17. Nichols, D. E.; Hoffman, A. J.; Oberlender, R. A.; Jacob, P., III; Shulgin, A. T. Derivatives of 1-(1,3-benzo-dioxo-5-yl)-2-butanamine: Representatives of a novel therapeutic class. J. Med. Chem. 29:2009–2015; 1986.
18. Oberlender, R.; Nichols, D. E. Drug discrimination studies with MDMA and amphetamine. Psychopharmacology (Berlin) 95:71–76; 1988.
19. Oberlender, R.; Nichols, D. E. Studies of the discriminative cue of S-(+)-MBDB, the alpha-ethyl homologue of MDMA. Soc. Neurosci. Abstr. 15:249; 1989.
20. O'Hearn, E.; Battaglia, G.; De Souza, E. B.; Kuhar, M. J.; Molliver, M. E. Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain: Immunocytochemical evidence for neurotoxicity, J. Neurosci. 8:2788–2803; 1988.
21. Peat, M. A.; Warren, P. F.; Bakhit, C.; Gibb, J. W. The acute effects of amphetamine, methamphetamine, and p-chloroamphetamine on the cortical serotonergic system of the rat brain: evidence for differences in the effects of methamphetamine and amphetamine. Eur. J. Pharmacol. 116:11–16; 1985.
22. Pierce, P. A.; Peroutka, S. J. Antagonism of 5-hydroxytryptamine$_2$ receptor-mediated phosphatidylinositol turnover by d-lysergic acid diethylamide. J. Pharmacol. Exp. Ther. 247:918–925; 1988.
23. Pierce, P. A.; Peroutka, S. J. Ring-substituted amphetamine interactions with neurotransmitter receptor binding sites in human cortex. Neurosci. Lett. 95:208–212; 1988.
24. Ricaurte, G.; Bryan, G.; Strauss, L.; Seiden, L.; Schuster, C. Hallucinogenic amphetamine selectively destroys brain serotonin nerve terminals. Science 229:986–988; 1985.
25. Ricaurte, G. A.; Finnigan, K. F.; Nichols, D. E.; DeLanney, L. E.; Irwin, I.; Langston, J. W. 3,4-methylenedioxyethylamphetamine (MDE) a novel analogue of MDMA, produces long-lasting depletion of serotonin in the rat brain. Eur. J. Pharmacol. 137:265–268; 1987.
26. Sanders-Bush, E.; Sterenka, L. R. Immediate and long-term effects of p-chloroamphetamine on brain amines. Ann. NY Acad. Sci. 305: 208–221; 1978.
27. Sanders-Bush, E.; Burris, K. D.; Knoth, K. Lysergic acid diethylamide and 2,5-dimethoxy-4-methylamphetamine are partial agonists at serotonin receptors linked to phosphoinositol hydrolysis. J. Pharmacol. Exp. Ther. 246:924–928; 1988.
28. Schmidt, C. J. Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine. J. Pharmacol. Exp. Ther. 240:1–7; 1987.
29. Schmidt, C. J. Acute administration of methylenedioxymethamphetamine: comparison with the neurochemical effects of its N-desmethyl and N-ethyl analogues. Eur. J. Pharmacol. 136:1–88; 1987.
30. Schmidt, C. J.; Ritter, J. K.; Sonsalla, P. K.; Hanson, G. R.; Gibb, J. W. Role of dopamine in the neurotoxic effects of methamphetamine. J. Pharmacol. Exp. Ther. 233:539–544; 1985.
31. Schmidt, C. J.; Wu, L.; Lovenberg, W. Methylenedioxymethamphetamine: A potentially neurotoxic amphetamine analogue. Eur. J. Pharmacol. 124:175–178; 1986.
32. Schmidt, C. J.; Levin, J. A.; Lovenberg, W. *In vitro* and *in vivo* neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain. Biochem. Pharmacol. 36:747–755; 1987.
33. Schmidt, C. J.; Taylor, V. L. Neurochemical effects of methylenedioxymethamphetamine in the rat: acute versus long-term changes. In: Peroutka, S. J., ed. Ecstasy: The clinical, pharmacological, and neurotoxicological effects of the drug MDMA. Boston: Kluwer Academic Publishers; 1990:151–169.
34. Schuster, C. R.; Lewis, M.; Seiden, L. S. Fenfluramine neurotoxicity. Psychopharmacol. Bull. 22:148–151; 1986.
35. Shulgin, A. T. Psychotomimetic amphetamines: Methoxy-3,4-dialkoxyamphetamines. Experientia 20:366–367; 1964.
36. Shulgin, A. T. 3-methoxy-4,5-methylenedioxyamphetamine a new psychotomimetic agent. Nature 201:1120–1121; 1964.
37. Shulgin, A. T. Psychotomimetic drugs: Structure-activity relationships. In: Iversen, L. L.; Iversen, S. D.; Snyder, S. H., eds. Handbook of psychopharmacology, vol. 11. New York: Plenum Press; 1978:243–333.
38. Shulgin, A. T. The background and chemistry of MDMA. J. Psychoactive Drugs 18:291–304; 1986.
39. Shulgin, A. T.; Sargent, T. Psychotropic phenylisopropylamines derived from apiole and dillapiole. Nature 215:1494–1495; 1967.
40. Shulgin, A. T.; Sargent, T.; Naranjo, C. Animal pharmacology and human psychopharmacology of 3-methoxy-4,5-methylenedioxyphenylisopropylamine (MMDA). Pharmacology 10:12–18; 1973.
41. Shulgin, A. T.; Jacob, P., III. Potential misrepresentation of 3,4-methylenedioxyamphetamine (MDA). A toxicological warning. J. Anal. Toxicol. 6:71–75; 1982.
42. Steele, T. D.; Nichols, D. E.; Yim, G. K. W. Stereochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related am-

phetamine derivatives on inhibition uptake of [3H]-monoamines into synaptosomes from different regions of rat brain. Biochem. Pharmacol. 36:2297–2303; 1987.
43. Stone, D. M.; Johnson, M.; Hanson, G. R.; Gibb, J. W. A comparison of the neurotoxic potential of methylenedioxyamphetamine (MDA) and its N-methylated and N-ethylated derivatives. Eur. J. Pharmacol. 134:245–248; 1987.
44. Stone, D. M.; Johnson, M.; Hanson, G. R.; Gibb, J. W. Role of endogenous dopamine in the central serotonergic deficits induced by 3,4-methylenedioxymethamphetamine. J. Pharmacol. Exp. Ther. 249: 79–87; 1988.
45. Wagner, J. A.; Peroutka, S. J. Comparative neurotoxicity of fenfluramine and 3,4-methylenedioxymethamphetamine (MDMA). Soc. Neurosci. Abstr. 14:327; 1988.
46. Yamamoto, B. K.; Spanos, I. J. The acute effects of methylenedioxymethamphetamine on dopamine release in the awake-behaving rat. Eur. J. Pharmacol. 148:195–203; 1988.