United States
Environmental Protection
Agency

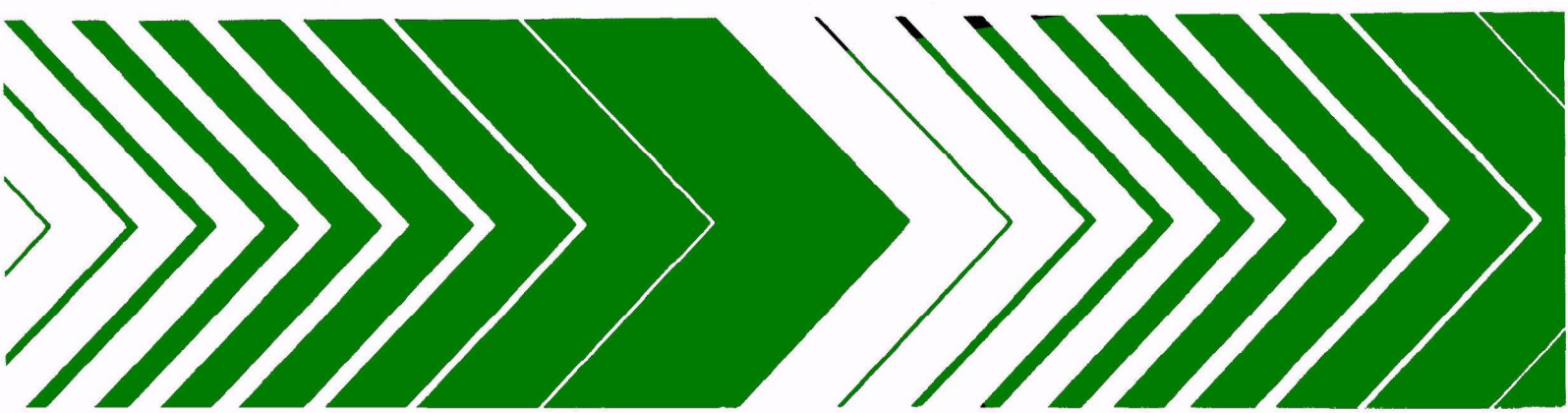
Health Effects Research
Laboratory
Research Triangle Park NC 27711

EPA-600/1-80-028
May 1980

Research and Development

EPA

Toxicity, Interactions, and Metabolism of Formamidine Pesticides in Mammals



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FOREWARD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report describes investigations of the mechanism(s) of acute toxicity of formamidine pesticides in mammals. Formamidine pesticides are a relatively new group of insecticide-acaricides which are particularly useful for the control of Lepidoptera, Hemiptera, phytophagous mites, and cattle ticks. Their widespread use for control of cotton insects and cattle ticks make the investigation of their toxicity extremely important.

F. Gordon Hueter
Director
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ABSTRACT

The overall goal of this research project was to investigate the mechanism(s) of acute toxicity of formamidine pesticides in mammals using chlordimeform (*N*'-(4-chloro-*o*-tolyl)-*N*,*N*-dimethylformamidine) and its metabolites as the primary model compounds. The role of biotransformations, particularly *N*-demethylation reactions, in generating potentially toxic metabolites was also studied.

By comparing the effects of hepatic microsomal mixed function oxidase inducers and inhibitors administered *in vivo* on the toxicity, metabolism, and distribution of metabolites in mouse tissues, it was concluded that although *N*-demethylation products are innately more toxic than chlordimeform, they are also less stable, and the best correlation of toxicity was obtained with the total level of formamidines in the brain, rather than with the level of any individual metabolite.

In a series of studies with dogs, rabbits, and cats, the cause of death was found to be cardiovascular collapse accompanied by respiratory arrest. Cardiovascular collapse resulted primarily from a peripheral local anesthetic-like effect of chlordimeform. Monoamine oxidase inhibition was not a major factor in lethality. Respiratory arrest was central in origin. Several other central effects of the formamidines were described, some of which may be local anesthetic actions, and a behavioral profile for chlordimeform poisoning in the rat was developed. The effectiveness of various drug treatments as potential therapeutic aids for formamidine intoxication were studied. Finally, the formamidines were found to possess a number of aspirin-like actions which resulted from an ability to inhibit prostaglandin synthesis.

This report was submitted in fulfillment of Research Grant No. R-803965 by Purdue Research Foundation under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period July 1st, 1975 to December 31st, 1978, and the work was completed as of July 1st, 1979.

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FIGURES

TABLES

LIST OF ABBREVIATIONS

ABBREVIATIONS

ACh	—	acetylcholine
CAA	—	5-chloroanthranilic acid
CDM	—	chlordimeform
CDU	—	1,1-dimethyl-3-(4-chloro-o-tolyl)urea
CNS	—	central nervous system
CT	—	4-chloro-o-toluidine
DCDM	—	N-demethyl chlordimeform
DCDU	—	1-methyl-3-(4-chloro-o-tolyl)urea
DDCDM	—	N,N-didemethyl chlordimeform
DDCDU	—	4-chloro-o-tolylurea
DFP	—	diisopropylphosphorofluoridate
ECG	—	electrocardiograph
EDTA	—	ethylenediamine tetracetate
EEG	—	electroencephalograph
G.C.	—	gas chromatography
GSH	—	glutathione
5-HT	—	5-hydroxytryptamine (serotonin)
ia	—	intraarterially
ip	—	intraperitoneally
iv	—	intravenously
ivc	—	intraventricularly
LSD	—	D-lysergic acid diethylamide
MAO	—	monoamine oxidase
MFO	—	mixed function oxygenase
NADPH	—	reduced nicotinamide-adenine dinucleotide phosphate
NE	—	norepinephrine (noradrenaline)
NFA	—	N-formyl-5-chloroanthranilic acid
NFT	—	N-formyl-4-chloro-o-toluidine
PCA	—	parachloroamphetamine
PG	—	prostaglandin
PGE_1	—	prostaglandin E_1
PGE_2	—	prostaglandin E_2
sc	—	subcutaneously
SD	—	standard deviation
SE(M)	—	standard error (of mean)
TCA	—	trichloroacetic acid
TLC	—	thin-layer chromatography

ACKNOWLEDGMENTS

The collaboration, advice, and technical assistance of the following people is gratefully recognized: Dr. D. E. Blake, Dr. C. Chinn, Mr. M. P. Holsapple, Mr. M. T. Lowy, Ms. V. Noland and Dr. W. R. Pfister and Mr. J. Rolley of the Department of Pharmacology and Toxicology, Purdue University; Dr. D. E. Nichols of the Department of Medicinal Chemistry, Purdue University; and Mrs. K. Dersch, Dr. G. Ghali, Mr. J. Leister, Dr. A. E. Lund and Dr. D. L. Shankland of the Department of Entomology, Purdue University.

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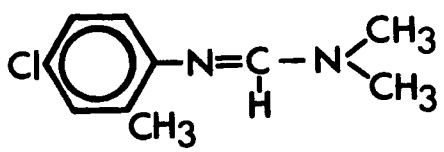
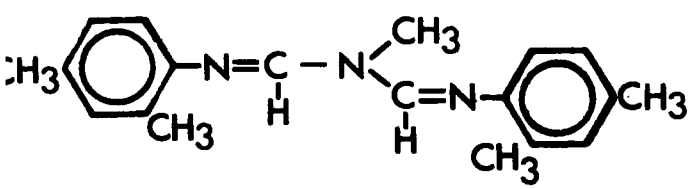
GENERAL INTRODUCTION

The formamidines are a relatively new group of insecticide-acaricides which are particularly useful for the control of Lepidoptera, Hemiptera, phytophagous mites, and cattle ticks (Hollingworth, 1976). In the U.S. two compounds are of primary interest, chlordimeform (I; N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine)) and amitraz (II; 1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene).



Chlordimeform
(Fundal : Nor-Am/Schering)
(Galecron : Ciba-Geigy)



Amitraz
(BTS-27419 : Boots)
(U-36059 : Upjohn)

During this grant period both compounds were reported to be carcinogenic in mice at high doses (Johnson, 1977). This is probably due to the metabolic release of carcinogenic anilines rather than because of any innate carcinogenicity of the parent formamidines (Ghali, 1980). Registration for amitraz has thus been limited to control of psylla on pears (Johnson, 1979), although it is used widely outside the U.S., e.g. for cattle tick control. By voluntary agreement, chlordimeform (CDM) is confined to use for control of cotton insects, in which situation it remains an important insecticide. It too has significant uses outside the U.S. A number of other compounds related to these formamidines are currently undergoing field testing.

The formamidines are structurally unlike other pesticides and their mechanism(s) of toxic action in vertebrates and invertebrates appear to be novel (Matsumura and Beeman, 1976; Lund et al., 1979b). At the start of this project it was known only that the symptoms of poisoning by

lethal doses of CDM in rodents were excitatory and included hyperexcitation, tremors, and convulsions preceding death (Beeman and Matsumura, 1973). The symptoms were described as sympathomimetic in nature (Aziz and Knowles, 1973). No cholinomimetic responses have been reported. The most likely site of action of formamidines was thought to be monoamine oxidase (MAO). Despite evidence that MAO was inhibited *in vitro* (Aziz and Knowles, 1973; Beeman and Matsumura, 1973), we felt that the rapid, excitatory poisoning symptoms were not congruent with the MAO theory, nor were the symptoms predominantly sympathomimetic, though this component was present. In particular it seemed important to determine the degree, duration, and reversibility of MAO inhibition *in vivo*. Not only is this crucial in order to interpret the role of MAO in poisoning, but also to evaluate the likelihood of dangerous interactions with dietary or medicinal amines (Sjoqvist, 1965) or of cumulative inhibition of MAO on repeated exposure to formamidines.

A number of other biochemical actions of formamidines have been described, such as mitochondrial uncoupling (Abo-Khatwa and Hollingworth, 1973) and inhibition of RNA synthesis (Murakami and Fukami, 1974), but these are not plausible as the cause of the symptoms observed (Lund *et al.*, 1979b). At the start of the project the actual cause of death from formamidines was unknown although Beeman and Matsumura (1974) noted briefly that CDM caused a depression of blood pressure in rabbits.

Before and during the work presented here a number of publications appeared describing the metabolism and fate of CDM in mammals (see Section 7 for references). A major metabolic route for CDM in all species studied is *N*-demethylation to yield *N*-demethyl chlordimeform (DCDM) (Knowles and Sen Gupta, 1970; Sen Gupta and Knowles, 1970; Morikawa *et al.*, 1975). Subsequently a second *N*-demethylation to give *N*,*N*-didemethyl chlordimeform (DDCDM) was reported (Benezet and Knowles, 1976b). Our preliminary results indicated that DCDM was more toxic than CDM to mice (Hollingworth, 1976) and more rapid in its actions. Also, piperonyl butoxide, an insecticide synergist which inhibits many *N*-demethylations, strongly antagonized some of the toxic effects of CDM, but not of DCDM, in cattle ticks, suggesting that conversion of CDM to DCDM is crucial for such toxicity (Knowles and Roulston, 1972). If this were true in mammals also, there are obvious implications regarding the appropriate compounds to use in mode of action studies, and for unexpected toxic interactions arising after exposure to mixed function oxidase (MFO) inducers or inhibitors.

The goals of this project were therefore:

(a). To define the symptoms and mechanism(s) of toxicity of CDM in mammals.

(b). To specifically examine the role of MAO in these processes and to evaluate the possibility of interactions or cumulative effects based on MAO inhibition.

(c). To develop information leading to potential means of therapy for formamidine overexposure.

(d). To study other pharmacological and behavioral actions of formamidines which might have toxicological implications, indicate potential sites of action, or have utility as an index of exposure to these pesticides.

(e). To investigate the metabolism of CDM and the accumulation of CDM and its metabolites *in vitro* and *in vivo*. Specific objectives in this respect were to determine whether *N*-demethylations are important activation reactions for formamidines, and to assess the possibility of significant interactions with MFO inducers and inhibitors.

CONCLUSIONS

1. Chlordimeform is a moderately potent, reversible inhibitor of monoamine oxidase. Inhibition of monoamine oxidase *in vivo* is ephemeral and never reaches high levels even at near lethal doses. Several other lines of evidence also indicate that monoamine oxidase inhibition is not a major mechanism underlying acute toxicity, and the possibility of dangerous interactions resulting from concurrent exposure to formamidines and natural or medicinal biogenic amines is slight. No evidence of such interactions was seen in cardiovascular studies performed with dogs. Inhibition of monoamine oxidase is unlikely to be cumulative with continued daily exposure to formamidines because of their rapid metabolism and the reversibility of inhibition.

2. Animals poisoned with chlordimeform suffer concurrent cardiovascular collapse and respiratory arrest. When given intravenously, chlordimeform has a biphasic action on blood pressure i.e. an intial depression followed (in survivors) by an elevation. Both actions are shared with lidocaine and are attributable to a local anesthetic-like effect of chlordimeform. The depressor effect results primarily from vasodilation and reduced cardiac contractility while the pressor response results centrally from enhanced sympathetic outflow. Respiratory arrest is a secondary effect arising centrally, probably through depression of the respiratory center following convulsions.

3. The effects of chlordimeform and lidocaine are also similar when administered intraventricularly in rats and when applied to the isolated frog sciatic nerve. Intraventricular chlordimeform induces EEG seizure discharges in the amygdala and blocks amygdalar inhibition by the raphe. Evidence for actions of formamidines on the nervous system other than as local anesthetics exists e.g. biogenic amine accumulation through partial inhibition of monoamine oxidase, a direct excitatory effect on some types of axons, and a partial -adrenergic agonist effect of *N*-demethylated formamidines. However, on the basis of present evidence the local anesthetic-like actions appear to explain well most of the symptoms and signs observed, including the primary cardiovascular depression, secondary increase in sympathetic outflow, and the convulsive and respiratory effects of chlordimeform. Like other local anesthetics, chlordimeform can induce "pharmacological kindling" i.e. precipitation of convulsions and other behavioral effects after repeated subliminal doses.

4. Therapeutic approaches to local anesthetic overdose are limited. Diazepam was clearly helpful in alleviating the central symptoms of

chlordimeform poisoning in rats and dogs, but only at low doses of diazepam. A combination of therapeutic steps is likely to be necessary and probably should include:

(a). Artificial resuscitation to combat respiratory depression.

(b). Low doses of diazepam.

(c). Vasoconstrictors and cardiac stimulants (e.g. epinephrine and calcium gluconate) for the cardiovascular depression.

5. In developing the "behavioral profile" for chlordimeform to serve as an aid in detecting and diagnosing overexposure, it was found to induce responses typical of the so-called "serotonergic behavioral syndrome". Most of these and the other behavioral effects of chlordimeform are not induced by known local anesthetics. However, further study casts doubt on the existence of this 'serotonergic syndrome' as presently defined and on any general role for serotonin in the behavioral effects of chlordimeform. The origins of these behavioral responses remains unclear in most cases although some possibilities other than local anesthetic action or presented in Conclusion 3.

6. Because of their ability to inhibit prostaglandin synthesis, the formamidines were found to have antipyretic, analgetic, and anti-inflammatory effects which are comparable to those of aspirin. However, they lack the potent gastric ulcerogenicity typical of aspirin-like agents.

7. The two successive metabolic N-demethylations of chlordimeform yield compounds of increased acute toxicity. These N-demethylation products are produced rapidly by mouse liver microsomes *in vitro* and *in vivo*. Metabolic reactions which result in the alteration of the formamidine nucleus are detoxications, and, in part, appear to be catalyzed by cytochrome P448 (3-methylcholanthrene inducible). Studies with several mixed function oxidase inhibitors and inducers indicate that N-demethylation *in vivo* tends to be a toxicologically 'neutral' process. Although the N-demethylation products are more toxic, they are also less stable. After dosing mice with chlordimeform, the best correlation of toxicity is found with the total level of all formamidines in the brain rather than with the level of any individual metabolite.

SECTION ONE

EVALUATION OF MONOAMINE OXIDASE

AS A TARGET FOR FORMAMIDINE PESTICIDES

INTRODUCTION

In considering the possible biochemical site of action of the formamidines, much attention has been directed to the actions of formamidines on monoamine-mediated neurotransmission in both mammals and arthropods (Matsumura and Beeman, 1976; Knowles and Aziz, 1974). In particular it has been reported that these compounds inhibit monoamine oxidase (MAO) activity in rat liver (Beeman and Matsumura, 1973; Aziz and Knowles, 1973) and in rat brain (Benezet and Knowles, 1976a). The symptoms of acute poisoning in the rat have been described as 'similar to those elicited by sympathomimetic agents including known MAO inhibitors' (Aziz and Knowles, 1973) and as sympathomimetic in nature and lacking normal cholinomimetic features (Beeman and Matsumura, 1973). Beeman and Matsumura (1973) also reported that treatment of rats with chlordimeform (CDM) caused an elevation in the serotonin and norepinephrine levels of the brain and also antagonized certain symptoms of reserpine intoxication which are generally believed to arise by release of biogenic amines from their presynaptic stores. These observations are stimulating since no other pesticides have been shown to exert their primary effect through aminergic mechanisms. Neumann and Voss (1977), recently have cast doubt on the role of MAO inhibition in the acute toxicity of CDM and some related toluidine derivatives on the basis of a lack of correlation of anti-MAO potency and lethality to rats among these compounds. Additionally, Robinson and Smith (1977), selectively depleted rat brain serotonin (5-HT) and norepinephrine (NE), or treated rats with the α-adrenergic agonist, phenylephrine. None of these pretreatments altered the acute toxicity of CDM, and the authors therefore concluded that MAO was probably not involved in its acute toxicity. Previously they had shown that pretreatments with reserpine, to deplete monoamines, or with α-adrenergic and serotonergic blockers also did not alter the LD_{50} of CDM (Robinson *et al.*, 1975). Meanwhile the view that MAO inhibition in particular, and interference with aminergic transmission in general, play a central role in generating the poisoning syndrome has been reiterated (Matsumura and Beeman, 1976; Knowles and Aziz, 1974; Knowles, 1976). Until now there has been no consideration of the potency of CDM and its metabolites as MAO inhibitors in other vertebrates than the rat, and no reports of their

effectiveness as MAO inhibitors in vivo. In the investigation presented here such studies were made and the results related to the hypothesis that MAO inhibition is an important feature of the toxicology of the formamidines in vertebrates.

MATERIALS AND METHODS

Chemicals

The radiolabelled MAO substrates were tryptamine bisuccinate (48.5 and 53 Ci/mol), 5-hydroxytryptamine (48.5 and 51 Ci/mol), and β-phenylethylamine (9.9 Ci/mol) from New England Nuclear, and tyramine hydrochloride (55 Ci/mol) from Amersham/Searle. All were labelled with ^{14}C in the side chain. MAO inhibitors, tranylcypromine hydrochloride and harmaline hydrochloride, were obtained from Sigma Chemical Co. Pheniprazine hydrochloride was the kind gift of Dr. C. Chinn. The formamidines N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine (CDM), N'-(4-chloro-o-tolyl)-N-methylformamidine (DCDM), and N-(4-chloro-o-tolyl)formamidine (DDCDM), and their metabolites N-formyl-4-chloro-o-toluidine (NFT) and 4-chloro-o-toluidine (CT) were synthesized and purified as described in Section 7. The identities and purity of these synthesized products were assessed by IR and NMR spectroscopy and by TLC on silica gel developed in benzene/diethylamine (19:1). Small amounts of NFT were sometimes present in the parent formamidines, particularly after storage. The amount of NFT did not exceed 2-3% and, when present, did not significantly affect the results obtained. Particular care was taken before the MAO assays to reduce the levels of NFT present in the formamidines.

Acute lethality

Male Swiss white mice (20-25 g) were obtained from Bellaire Acres, Danville, IN. The mice were starved for 4 hr before administration of the toxicants by gavage in a corn oil vehicle at 0.01 ml/g body wt. At least 5 doses with at least 10 mice/dose were utilized and the tests were replicated for each compound. Mortality was determined after 48 hr and the LD_{50} was determined by the method of Litchfield and Wilcoxon (1949).

Monoamine oxidase inhibition in vitro

MAO activity was assayed by the general radiometric method of Wurtman and Axelrod (1963). Mouse tissues were dissected, rinsed clean in saline, weighed after drying, and homogenized at 20 mg tissue/ml in 0.25 M sucrose containing 1 mM EDTA in a teflon-glass system. A mitochondrial fraction was isolated by differential centrifugation, initially at 600 g for 15 min and with further centrifugation of the supernatant at 12,000 g for 15 min. The resulting pellet was washed by resuspension in the same solution and recentrifugation. The washed mitochondria were then resuspended in the original volume of Tris buffer (50 mM, pH 7.5) containing 1 mM EDTA.

For the MAO assay, mitochondria (0.3 ml) were incubated with inhibitor (10 µl in ethanol) for 30 min at 25° in a stoppered centrifuge tube. The ^{14}C-labelled amine substrate (tryptamine, tyramine, β-phenylethylamine, or 5-HT at a final concentration of 10-30 µM) was added in 10-20 µl water and the MAO reaction allowed to proceed for exactly 2 min before addition of 0.2 ml 2N HCl. Toluene (5 ml) was added, the tube shaken, and 3 ml of the toluene layer was taken for scintillation counting of the amount of deaminated product present. Controls were run in which only 10 µl ethanol was added to the enzyme. Blank values obtained with 0.3 ml boiled enzyme were used to correct these data. In every MAO assay enzymatic rates were determined in triplicate and averaged. Under these conditions the assay was linear over several min with tissue concentrations up to 50 mg/ml. I_{50} values were calculated graphically from plots of percent inhibition of MAO against log of inhibitor concentration. The I_{50} values reported are the means of at least three such determinations.

The reversibility of the inhibition of MAO was examined by repeated washing of the mitochondria to remove the inhibitor. Mitochondria from mouse brain were incubated with 5×10^{-5} M CDM for 30 min. One portion was removed to assay for MAO activity using [^{14}C] tyramine, while the rest was centrifuged, and the mitochondrial pellet resuspended to its original volume in inhibitor-free buffer. After removing a further portion for enzyme assay the centrifugation and resuspension in fresh buffer was repeated several times more with samples removed for assay each time. An inhibitor-free MAO sample was treated similarly as a control. Protein was determined in each sample by a modified Lowry method (Schacterle and Pollack, 1973) and the activities of MAO were compared in the inhibited and control samples after correction for any loss of protein during the washing procedures.

In order to study the relation of MAO inhibition to time of exposure to the inhibitor, intestinal or brain mitochondria were incubated with CDM or DCDM at 5×10^{-5}M, or NFT at 5×10^{-6}M at 25°. Portions were removed at intervals from 20 sec-3 hr after addition of the inhibitor and immediately assayed for MAO using tryptamine. Activity was compared to a control sample incubated without inhibitor.

MAO inhibition in vivo

The effect of inhibitors on the activity of MAO in mouse liver and small intestine in vivo was assayed indirectly from the recovery of injected [^{14}C]-tryptamine. The method was based on that of Wang Lu and Domino (1976) for N,N-dimethyltryptamine. Potential MAO inhibitors were given orally as their hydrochlorides in water at sublethal doses except for NFT which was administered in corn oil. Two hr later, labelled tryptamine at 0.05 µCi/g body wt. was injected intraperitoneally. After a further 5 min the mice were killed and the liver and intestine were rapidly removed, rinsed with saline, and homogenized in 4 ml cold 1N HCl. The homogenate was centrifuged at 9000 g for 15 min, and the pellet was washed twice by resuspension in 1.5 ml portions of 0.1N HCl and centrifugation. The supernatants were combined and extracted with

10 ml ethyl acetate/toluene (1/1) and the aqueous phase was made alkaline by mixing with 2 ml 10 N NaOH. The residual tryptamine was then extracted with three 10 ml portions of the ethyl acetate/toluene. The organic phase was pooled and 5 ml samples taken for scintillation counting. Chromatography of such extracts from liver on silica gel plates developed with n-butanol/acetic acid/water (25/4/10) showed that the major peak of radioactivity cochromatographed with authentic tryptamine. When labelled tryptamine was added to the tissue during the initial homogenization, recovery of the added tryptamine ranged from 86-91% with a mean of 89%. The results were not corrected for recovery losses. Preliminary experiments showed that the tissue levels of tryptamine peaked at about 5 min after injection in untreated mice. Further initial experiments were conducted as described above but with various intervals between the administration of CDM.HCl and tryptamine from 15 min-24 hr to follow the change in MAO activity with time after exposure to the inhibitor.

RESULTS

Acute lethality of CDM and its metabolites to mice

The LD_{50} values for CDM, its two N-demethylation products (DCDM and DDCDM), and two hydrolysis products (NFT and CT) are presented in Table 1. It is notable that although CDM itself is only of medium potency (LD_{50} = 267 mg/kg) successive N-demethylations yielded increasingly more toxic metabolites with an LD_{50} for DDCDM of only 78 mg/kg. With CDM,

TABLE 1: ACUTE TOXICITY AND MAO INHIBITION IN VITRO BY CHLORDIMEFORM AND ITS MAJOR METABOLITES

		MAO[a], I_{50} (μM)		
	LD_{50}, mg/kg (mouse, oral)	Liver	Intestine	Brain
Chlordimeform (CDM)	267	47	121	122
N-Demethyl chlordimeform (DCDM)	163	21	61	69
N,N-Didemethyl chlordimeform (DDCDM	78[b]	133	73	104
N-Formyl-4-chloro-o-toluidine (NFT)	750	4.5	2.8	3.2
4-Chloro-o-toluidine (CT)	>1000	88	185	253

[a] substrate: tryptamine bisuccinate.
[b] solvent: propylene glycol.

DCDM, and DDCDM, all of which contain the formamidine nucleus, symptoms developed in less than 1 hr after dosage and were excitatory in nature.

Hyperexcitability, marked tremors in the head and limbs, gasping, and rapid death following one or more convulsive episodes were typical of all three compounds. The other two metabolites (NFT and CT) were less acutely toxic, slower-acting, and depressant in their effect. A gradual loss of responsiveness to external stimuli and hypothermia were noted in each case.

Innibition of MAO in vitro

The anti-MAO potency of CDM and these metabolites was compared in mitochondria from three tissues, the liver, brain, and small intestine. The I_{50} values are presented in Table 1. Although the tissues differed somewhat in their relative sensitivities to these agents, the differences were not extreme and no regular pattern of differential sensitivity was apparent. Most I_{50} values were in the range of 20-200 μM. Successive N-demethylations in this case did not greatly increase biological potency since although DCDM was about twice as effective as a MAO inhibitor compared to CDM, DDCDM was similar to CDM in its overall effectiveness. However, one metabolite, NFT, was notably better than any other compound as a MAO inhibitor with all three tissues, having I_{50} values in the 3-5 μM range. In contrast, the parent toluidine, CT, was somewhat less effective than CDM.

Reversibility of MAO inhibition

The recovery of mouse brain MAO from inhibition by CDM on repeated washing is shown in Fig. 1. The initial level of inhibition was 72%, this was reduced to 28% after one wash, 7% after the second, and activity returned to the control level with subsequent washes. This behavior was repeated with DCDM and the more potent inhibitor, NFT, as shown in Table 2. With these compounds the mitochondria were washed twice after exposure to the inhibitor for 30 min at 25°. In each case inhibition was reversed essentially to control levels. The high variability in the data for the washed mitochondria arises because of the substantial increase in MAO activity which occurred on washing the mitochondria in both the treatments and controls.

The time course of inhibition of MAO

When incubated with MAO from brain or intestine for periods from 20 sec-3 hr, all three compounds tested (CDM, DCDM, and NFT) gave essentially instantaneous inhibition. With NFT, inhibition after 20 sec was the same as after 3 hr and at all intermediate times. However, the two formamidines showed a more complex behavior. A large part of the final level of inhibition achieved at 3 hr was obtained 'instantaneously', but in each case a slow progressive component also was observed so that the degree of inhibition rose steadily about 7%/hr over the 3 hr observation period. This behavior is illustrated for the intestinal mitochondria in Fig. 2.

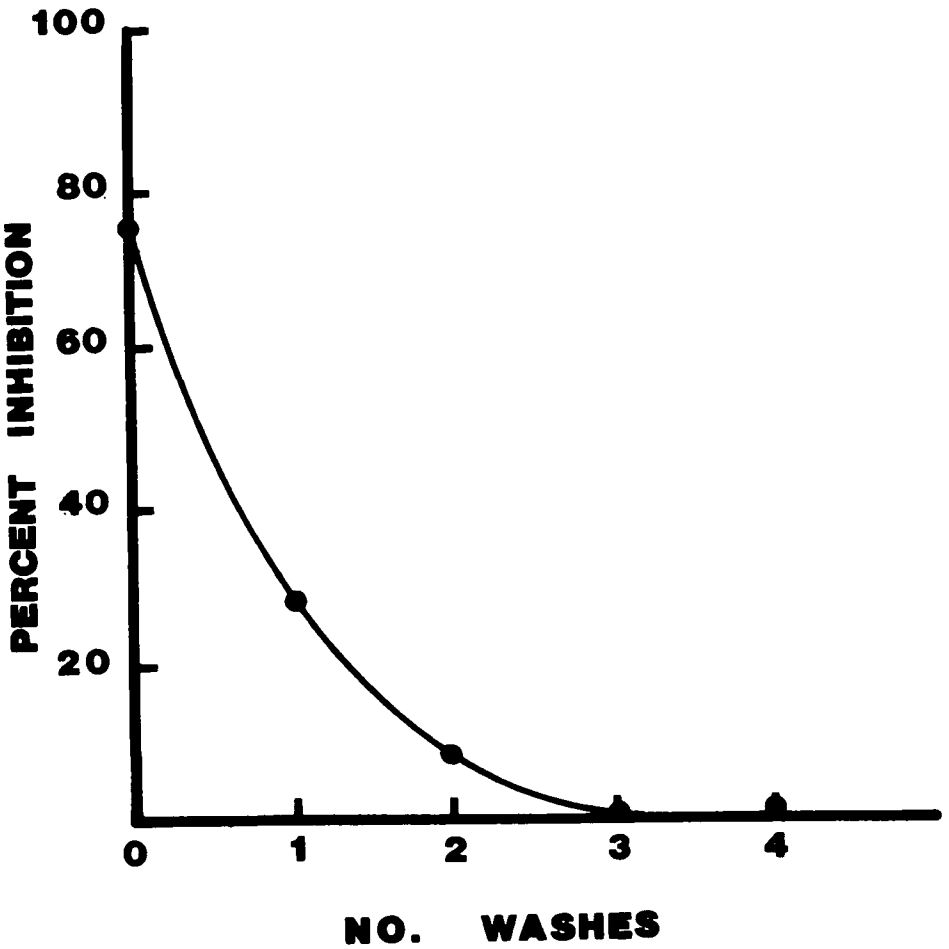


Figure 1. Recovery of mouse brain mitochondrial MAO from inhibition by CDM (50 μM) after successive washes to remove the inhibitor.

TABLE 2. REVERSIBILITY OF INHIBITION OF MITOCHONDRIAL MAO FROM MOUSE BRAIN BY CHLORDIMEFORM AND ITS METABOLITES ON WASHING

	Mean % Inhibition (±SD)[a]	
	Unwashed	Washed
Chlordimeform (50 μM)	68.5±4.9	2.4± 2.7
N-Demethyl chlordimeform (50μM)	86.1±0.7	8.9±14.2
N-Formyl-4-chloro-o-toluidine (5μM)	84.1±2.1	-1.4± 5.6

[a]Mean of three replicates. Tyramine hydrochloride as substrate.

Classification of CDM and its metabolites as MAO inhibitors

In order to determine whether these compounds are Type A or Type B inhibitors, the inhibition of MAO from mouse intestine was assayed over a wide

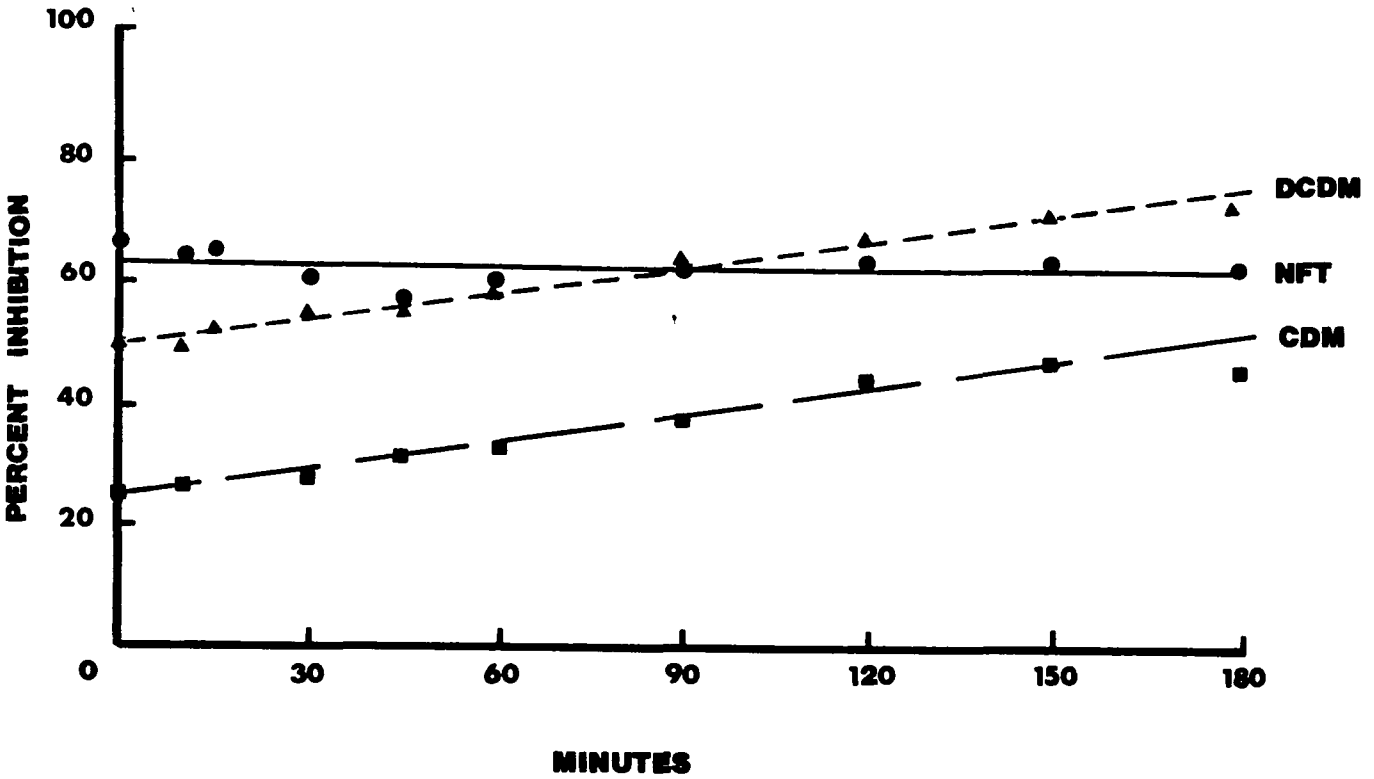


Figure 2. Time course of inhibition of MAO from mouse intestine by chlordimeform (CDM, 50μM), N-demethyl chlordimeform (DCDM, 50μM), and N-formyl-4-chloro-o-toluidine (NFT, 5μM).

range of inhibitor concentrations with three substrates, 5-HT (Type A), tryptamine (Mixed A and B), and β-phenylethylamine (Type B). The results are shown in Figs. 3 and 4, for CDM and NFT respectively, derived from three independent experiments in each case. DCDM behaved similarly to CDM. For each of the inhibitors, the MAO reaction with 5-HT is most sensitive to inhibition and β-phenylethylamine is the substrate least effective for inhibition, with tryptamine intermediate. The distinction between these three substances is most evident with NFT. The I_{50} values obtained for the three substrates, 5-HT, tryptamine, and β-phenylethylamine were 34,100, and 140 M respectively for CDM, 12, 33, and 39 μM for DCDM, and 1.2, 3.0, and 12 μM for NFT. In limited studies with tyramine, another mixed A and B type substrate, the results were very similar to those obtained with tryptamine.

Inhibition of MAO in vivo

The assumption that the inhibition of MAO would allow more injected tryptamine to survive intact in the tissues is confirmed by the results in Table 3. Although this method proved effective for assessing MAO activity in vivo in liver and small intestine, the amount of labelled tryptamine present

in the brain was too small to allow accurate determinations for this tissue. Tranylcypromine and pheniprazine are well-established and potent irreversible MAO inhibitors, and with these compounds 4-6 times as much tryptamine was recovered in the liver and intestine as in the untreated controls. Since tranylcypromine is an irreversible inhibitor, MAO inhibition was also assayed by homogenization of tissues from treated mice (15 mg/kg) followed by our normal MAO assays with isolated mitochondria using [^{14}C] tryptamine as substrate. Under these conditions tranylcypromine caused 80% inhibition of liver MAO and 92% inhibition of intestinal MAO *in vivo* 2 hr after dosage.

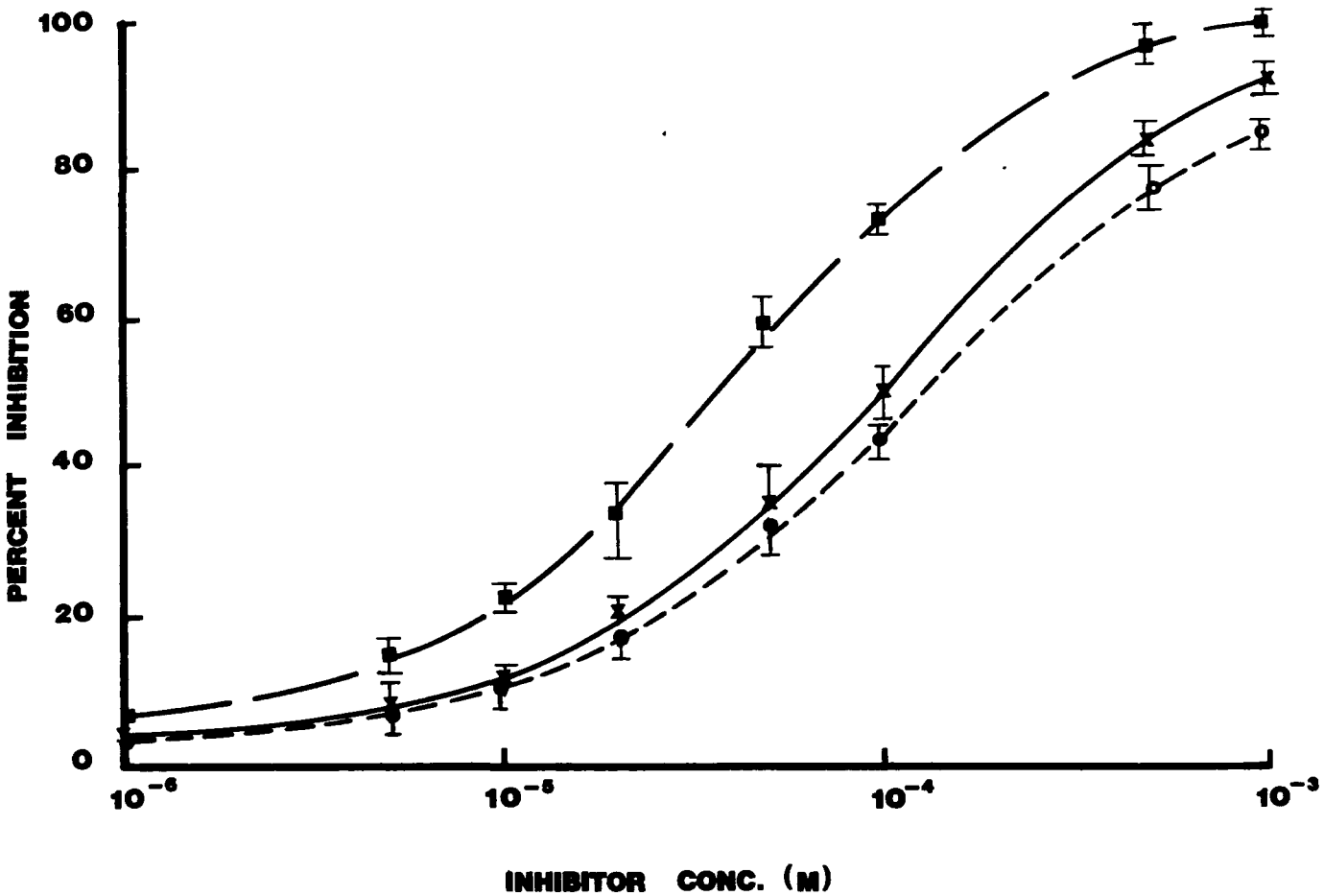


Figure 3. Inhibition of MAO from mouse intestine by CDM assessed with three MAO substrates: 5-Hydroxytryptamine, ■———■ ; Tryptamine, × ——— ×; β-Phenylethylamine, O ——— O.

Harmaline, a reasonably potent reversible inhibitor of MAO, did not protect tryptamine in the liver but did increase the amount of tryptamine surviving in the intestine. All of the standard MAO inhibitors were given at doses which caused no mortality and little if any external sign of toxicity.

CDM at 100 mg/kg also caused no strong poisoning symptoms and gave no significant increase in tryptamine recoveries. At 200 mg/kg poisoning symptoms were clear and an occasional death occurred. In this case a significant increase in tryptamine levels were seen, but this was much less than the increase caused by tranylcypromine and pheniprazine at their symptomless

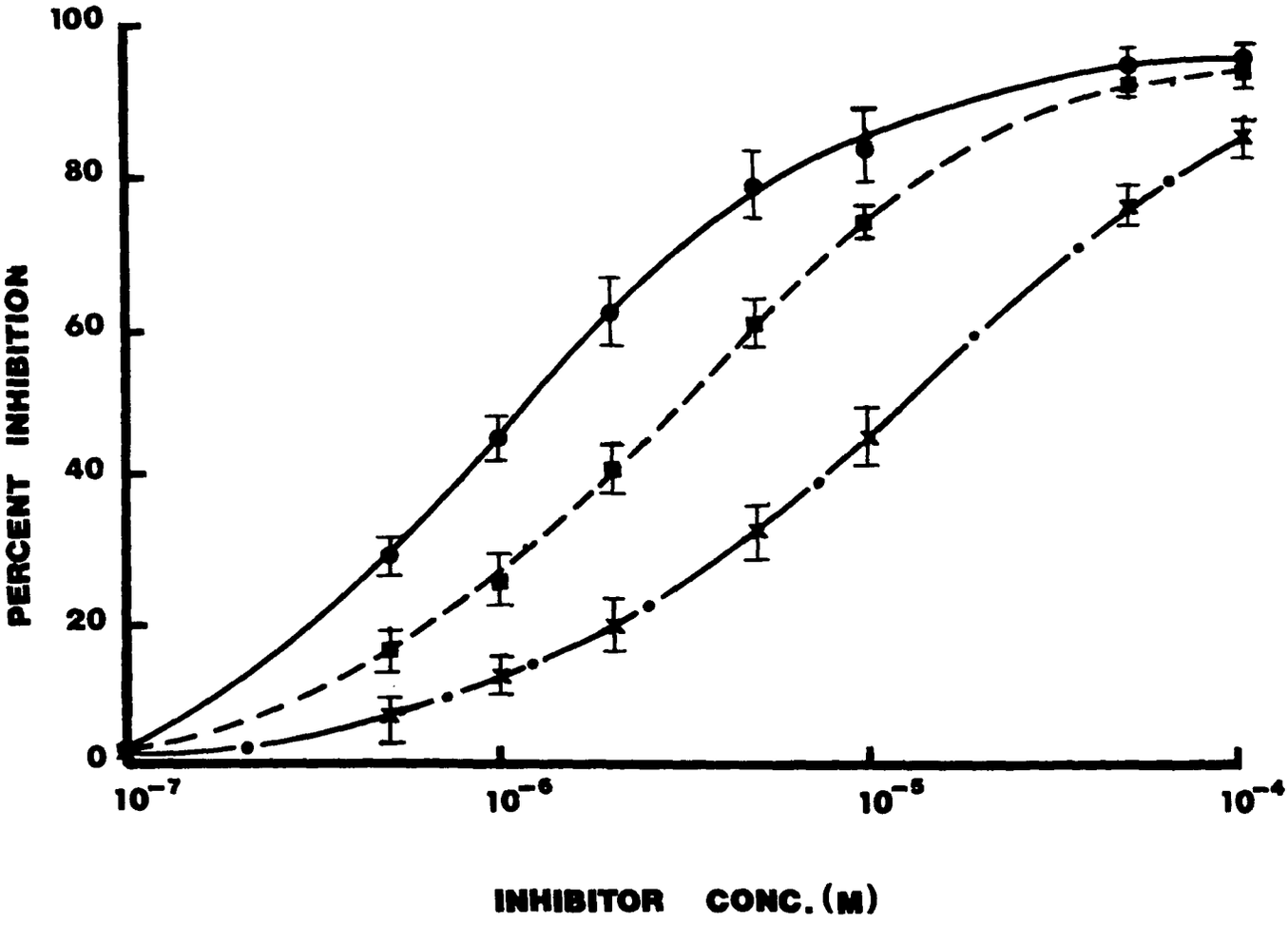


Figure 4. Inhibition of MAO from mouse intestine by NFT assessed with three MAO substrates: 5-Hydroxytryptamine, ●————●; Tryptamine,■————■; β-Phenylethylamine ×—·—·—×.

doses. NFT as a more potent MAO inhibitor than CDM caused a greater accumulation of tryptamine at 100 mg/kg than did CDM despite difficulties with getting the dose of NFT into solution in corn oil at this concentration.

The justification for the choice of 2 hr as the time delay after CDM dosage before administration of the tryptamine is illustrated by the data in Table 4. In this case varying times were left between the doses of chlordimeform (150 mg/kg) and tryptamine in order to evaluate the dynamics of MAO inhibition in vivo. The increase in tryptamine levels was not great at this dose but appeared to peak around the 2-4 hr period after dosage and to be declining by 6-12 hr with a return to normal levels within 24 hr.

TABLE 3. RECOVERY OF INJECTED [^{14}C] TRYPTAMINE AFTER PRETREATMENT OF MICE WITH MAO INHIBITORS

	Dose (mg/kg)	(pmol tryptamine recovered/g tissue) ±SD (n)[a] Liver	Intestine
Tranylcypromine	15	562 ± 183(8)[c]	632 ± 126(8)[c]
Pheniprazine	20	536 ± 90(8)[c]	482 ± 84(8)[c]
Chlordimeform	200	237 ± 59(10)[c]	233 ± 47(10)[b]
Chlordimeform	100	118 ±37(7)	152 ± 50(7)
N-Formyl-4-chloro-o-toluidine	100	198 ± 36(9)[c]	218 ± 84(9)
Harmaline	50	83 ± 24(9)	359 ± 155(9)[c]
Control	---	86 ± 27(10)	131 ± 39(10)

[a] S.D.(n) = standard deviation (number of observations).
[b] Significantly different from control at P = 0.05.
[c] Significantly different from control at P = 0.01.

DISCUSSION

The data presented in the initial section dealing with MAO inhibition *in vitro* establishes that with mitochondrial MAO from several mouse tissues, CDM is a readily reversible inhibitor of medium potency. *In vivo* it is rapidly metabolized by successive N-demethylations to yield DCDM and then DDCDM. These formamidines are susceptible to hydrolysis to NFT and then CT (see Section 7). These metabolites are in general rather similar to CDM in potency against MAO and, in the case of DCDM and NFT at least, are also reversible inhibitors. The only exception to these generalizations was the 10 to 40-fold greater potency of NFT compared to CDM as a MAO inhibitor *in vitro*. Thus the metabolism of CDM to NFT is potentially an activation reaction as far as MAO inhibition is concerned. The I_{50} values here are generally similar to those obtained by other investigators working with MAO from rat liver (Beeman and Matsumura, 1973; Aziz and Knowles, 1973) and rat brain (Benezet and Knowles, 1976). Benezet and Knowles (1976) in their more detailed study of the mechanism of inhibition of MAO by formamidines also noted the greater potency of NFT against rat brain, although in their earlier study with rat liver MAO, NFT was not found to be more effective than CDM. In our work with the mouse, NFT was clearly more potent than CDM against MAO from all tissues examined including liver. Otherwise MAO from the rat and the mouse responds to CDM and its metabolites similarly.

From the studies reported here it is evident that NFT inhibits MAO with no progressive component when enzyme and inhibitor are preincubated in the absence of substrate. However, superimposed on the 'instantaneous phase' with CDM and DCDM was a slow progressive increase in the degree of inhibition observed. This effect was also noted by Benezet and Knowles (1976a) for CDM and DCDM with their rat brain MAO. They suggested that this might be due to the slow generation of NFT by hydrolysis of the formamidines. Since NFT is a more potent inhibitor, the level of inhibition would thus slowly rise. This is a plausible explanation and in accord with the observed rates of hydrolysis of these formamidines (Section 7). Our observation that NFT itself lacks a progressive component of inhibition is compatible with this explanation.

TABLE 4. THE EFFECT OF TIME AFTER DOSING ON THE ABILITY OF CHLORDIMEFORM TO INCREASE THE RECOVERY OF INJECTED [^{14}C]TRYPTAMINE FROM MOUSE TISSUES

Hours after CDM dose[a]	Tryptamine recovered (pmol/g tissue) ± SD (n)[b] Liver	Intestine
0	65 ± 6(8)	105 ± 20(8)
0.25	95 ± 34(6)	121 ± 42(5)
0.50	81 ± 24(6)	97 ± 21(6)
1.0	85 ± 16(5)[d]	139 ± 57(6)
2.0	125 ± 32(5)[d]	136 ± 16(5)[c]
4.0	106 ± 28(5)[d]	163 ± 66(6)[c]
6.0	104 ± 28(4)[d]	142 ± 39(5)[c]
12.0	92 ± 8(4)	90 ± 15(8)
24.0	64 ± 6(5)	108 ± 11(5)

[a] Dose was 150 mg/kg, oral.
[b] SD (n) = standard deviation (number of observations).
[c] Significantly different from control at P = 0.05.
[d] Significantly different from control at P = 0.01.

It is known that mitochondrial MAO exists in several forms which may be distinguished by their differential responses to inhibitors, thermal inactivation, and differing substrate specificities (Squires, 1968 and 1972; Neff and Yang, 1974). Particular attention has been focused on the division of MAO enzymes into two classes, type A (with 5-HT and NE as preferred substrates) and type B (with β-phenylethylamine and benzylamine as preferred substrates). Several substrates are oxidized with similar efficiency by both types of MAO e.g. tryptamine, tyramine, dopamine, and kynuramine. Similarly some inhibitors such as tranylcypromine and pheniprazine are not particularly selective between the two forms of MAO while others are quite specific for one

of the types. Thus harmaline and clorgyline are considerably more potent as inhibitors of type A MAO while deprenyl is a relatively specific inhibitor of the B type. Different tissues vary widely in their relative contents of types A and B MAO and such variations are not constant among different species. The data in Figs. 3 and 4 show clearly that CDM, DCDM, and particularly NFT are more potent inhibitors when 5-HT is the substrate than when β-phenylethylamine is the substrate. Thus they appear to be selective inhibitors of type A MAO, although the degree of selectivity is quite low. As would be expected with tryptamine or tyramine as substrate, the potency is intermediate since these compounds act as efficient substrates for both types of MAO. However, if the situation were as simple as the assumptions above indicate, one would predict that a plateau in the inhibition curve should be observed using tryptamine, since the activity of the type A MAO against this substrate should be inhibited before that of the type B also present. Such plateaus are commonly seen with more highly selective inhibitors such as deprenyl, clorgyline, and harmine (Squires, 1972; Neff and Yang, 1974; Yang and Neff, 1974). However from these results it may be concluded that, like other selective inhibitors of type A MAO such as clorgyline (Yang and Neff, 1974), CDM and its metabolites *in vivo* should cause the preferential accumulation of the substrates for this form of MAO i.e. 5-HT, NE and dopamine if any high degree of inhibition were induced. Beeman and Matsumura (1973), report a 22% increase in NE and a 70% increase in 5-HT in the brains of rats 1 hr after an intraperitoneal dose of 200 mg/kg of CDM (LD_{90}). The results discussed above were obtained with mouse intestinal mitochondria. Recently Neumann and Voss (1977) obtained K_i values for CDM against rat liver MAO using the same substrates as in our study plus kynuramine. No trends in the K_i values with substrate were found which would allow classification on the type A/type B system. However, using rat brain MAO, Benezet and Knowles (1976) obtained results rather similar to ours with the same substrates.

The data presented in this section offer several lines of evidence regarding the probable contribution of MAO inhibition to the acute lethality of CDM. We believe that this evidence strongly disfavors the idea that MAO is the primary target. Despite the fact that CDM and its metabolites show some degree of potency as MAO inhibitors *in vitro*, there is no correlation between the ability to inhibit MAO and either the lethality or symptoms of poisoning. Sequential N-demethylations decrease the LD_{50} values in the formamidines (CDM, DCDM, DDCDM), but there is no corresponding trend in the I_{50} values for MAO from any of the tissues examined. On the other hand NFT is easily the best MAO inhibitor, but is not particularly toxic. Even the poorly toxic toluidine, CT, is an inhibitor with activity in about the same range as the formamidines. This suggests that MAO inhibition is more dependent on the presence of the substituted toluidine moiety than on the presence of an intact formamidine nucleus. A similar conclusion was reached by Neumann and Voss (1977) with a series of substituted chlorotoluidines including CDM and NFT. Furthermore, although CT is comparable to the formamidines in its potency against MAO, and NFT is more effective, their speed of action and symptoms are completely different from those of the formamidines. Rapid mortality accompanied by marked excitation was seen only for compounds with an intact formamidine nucleus. Since MAO is not an enzyme which is immediately essential to the integrity of nervous functions and for survival of the

organism, as compared for example to acetylcholinesterase, the rapid onset of symptoms and strong excitatory effects of CDM and the related formamidines is not in keeping with MAO as the site of action. Many potent MAO inhibitors such as tranylcypromine and the hydrazines exert their acute toxic actions through mechanisms other than MAO inhibition (Pletscher *et al.*, 1966) and CDM probably resembles them in this respect.

The results obtained here with MAO inhibition *in vivo* are entirely consistent with this conclusion. Since CDM is a readily reversible inhibitor of MAO, estimation of the degree of inhibition *in vivo* presents severe problems because *in vitro* assays of MAO which involve tissue homogenization and dilution before assay will partially reverse the inhibition. Thus an indirect but potentially more realistic method of assessing the status of MAO *in vivo* was adopted which measured the ability of injected [^{14}C]tryptamine to survive in the tissues. Judging by the observed actions of the known MAO inhibitors, tranylcypromine, pheniprazine, and harmaline, this method is an appropriate one for assessing the status of MAO *in vivo*. The first two compounds are potent, irreversible MAO inhibitors which are not selective between type A and type B MAO (Neff *et al.*, 1974). In both liver and intestine they cause a substantial increase in the amount of tryptamine recovered. Harmaline is a reversible inhibitor which is strongly selective for type A MAO (Neff *et al.*, 1974). It was found to preserve tryptamine in the intestine but not in the liver. This is reasonable since while the MAO of mouse intestine is about 70% of the A type, this enzyme class is virtually absent in mouse liver (Squires, 1968 and 1972). Thus, in the liver the tryptamine is destroyed by type B MAO which is insensitive to harmaline.

Assayed in this way, CDM causes only a moderate degree of MAO inhibition *in vivo* even at near-lethal doses. This is a degree of inhibition which other established MAO inhibitors, such as tranylcypromine and pheniprazine, easily exceed without any apparent signs of poisoning. It seems quite unlikely that the level of MAO inhibition after an oral dose of CDM as high as 200 mg/kg approaches the 85% or more necessary to cause significantly damaging accumulation of biogenic amines in the brain and other organs (Pletscher *et al.*, 1966. Tranylcypromine at 15 mg/kg does cause this degree of inhibition, but also leads to a far greater accumulation of tryptamine than does CDM at 200 mg/kg.

From the I_{50} data of Table 1, one would not expect CDM to be a more effective inhibitor of brain MAO *in vivo* compared to liver or intestinal MAO. However, this could not be assessed by the tryptamine recovery method and further data are needed regarding the status of the MAO in the brain during CDM poisoning.

The data of Table 4 reveal that the MAO inhibition which does result from dosage with CDM is not long-lasting, being essentially reversed within 12-24 hr after exposure. This is entirely what would be expected from a rapidly metabolized reversible inhibitor such as CDM. There thus seems to be little risk of the cumulative effects on MAO and possible interactions with natural or medicinal biogenic amines which are seen with many of the irreversible inhibitors (Sjoqvist, 1965). even with repeated exposure to rather large amounts of CDM.

Although our work does not exclude the possibility that under some circumstances, in some species, the MAO-inhibitory actions of CDM may present some hazard or contribute to the symptoms observed, it does indicate that this risk is probably not great and that the biochemical lesion underlying the acute lethality of the formamidines to mammals must be sought elsewhere. It has been suggested that other effects on the regulation of biogenic amines may be important in the lethality of CDM (Matsumura and Beeman, 1976; Knowles and Aziz, 1974; Knowles, 1976). Although this cannot be ruled out, there is little direct evidence to support this view, and other workers have shown that blockade of serotonergic and α-adrenergic receptors, and prior depletion of tissue stores of these amines did not reduce the lethality of CDM to rats (Robinson and Smith, 1977; Robinson et al., 1975). A more plausible hypothesis on the basis of current information relates the lethal actions of CDM and related formamidines to their local anesthetic-like actions (See Section 4) with a consequent depressor effect on the cardiovascular system (See Section 2) and marked central stimulation with enhanced sympathetic outflow which may account for the sympathomimetic symptomatology reported by other investigators (See Sections 3 and 4). A local anesthetic-like action could also account for the depression in motor end-plate sensitivity and inhibition of the depolarization-contracture process observed with the frog neuromuscular junction (Wang et al., 1975; Watanabe et al., 1976). In fact Watanabe et al., (1975) briefly noted some similarities between CDM and the local anesthetic, procaine, in their actions on the acetylcholine-stimulated contraction of the frog rectus abdominis muscle. However the formamidines are compounds with actions on multiple biochemical systems, and it is clearly premature to ascribe all observed effects of these compounds to any single type of biochemical action.

SECTION TWO

ACTIONS OF CHLORDIMEFORM ON THE CARDIOVASCULAR SYSTEM

INTRODUCTION

As a first step in attempting to define the cause of death and toxic mechanism with chlordimeform we started with the observation of Beeman and Matsumura (1974) that 200 mg/kg of CDM, ip, decreased the mean arterial bood pressure in the pentobarbital-anesthetized rabbit. The purpose of this investigation was therefore to analyze further the effects of CDM on the cardiovascular system. Parallel studies in these laboratories (Sections 3 and 4) showed that CDM shared many of the actions of local anesthetic agents, hence the cardiovascular actions of CDM were compared to those of lidocaine to determine if these agents had common sites and modes of action.

MATERIALS AND METHODS

Blood pressure, heart rate, and cardiac contractility

Fourteen dogs of both sexes, 7-12 kg each, were anesthetized with 30-35 mg/kg of pentobarbital sodium iv. The left femoral arterial bood pressure was monitored via a Statham pressure transducer with a Grass polygraph. Drugs were administered via a cannula in the cephalic vein. Lead II of the ECG and beat-to-beat heart rate were recorded on an E & M Physiograph. Artificial respiration was provided to the dogs using a Harvard Apparatus respirator, and right ventricular contractile force was monitored by means of a Walton-Brodie strain gauge sewn onto the right ventricle.

Blood pressure, heart rate, and peripheral resistance

Fifteen dogs of both sexes, 7-12 kg each, were anesthetized with 30-35 mg/kg of pentobarbital sodium iv. The right carotid artery and the left external jugular vein of each animal were cannulated and arterial and venous pressures were recorded. Lead II of the ECG was monitored along with the heart rate on a cardiotach. The dogs were given 600-700 units/kg of heparin. The right femoral artery was cannulated both toward and away from the heart. Blood was pumped from the femoral artery by a Harvard Apparatus constant-perfusion pump and back into the leg via the femoral artery. Perfusion pressure was monitored with a Statham pressure transducer distal to the pump and was displayed on the polygraph. The flow rate through the pump was adjusted (usually 27 ml/min) so that the perfusion pressure approximated the carotid pressure. The volume of the perfusion apparatus was 34 ml. Drugs were administered via a cannula in the cephalic vein or intraarterially (1 ml volume) into the perfusion apparatus distal to the pump.

Contractility of isolated rabbit hearts

Eighteen conventional Lagendorf rabbit heart preparations were perfused with 38°, oxygenated Locke-Ringer solution. Contractility was displayed on a Physiograph using a Type B, E & M myograph, and heart rate was monitored on another channel via a cardiotach. Drugs (1 ml volume) were injected into the perfusate as it entered the aorta.

Sympathetic outflow in the cat

Six cats, 2.6-3.4 kg, were anesthetized with 30 mg/kg of pentobarbital sodium. Both cervical sympathetic nerves were exposed and placed on bipolar electrodes for recording, and the cervical region was filled with warm mineral oil. Spike activity was amplified by a Tektronix 122 preamplifier and spike discharge frequency was recorded on the polygraph. Drugs were administered via a cannula in the cephalic vein.

RESULTS

Blood pressure

Chlordimeform or lidocaine at doses of 3-30 mg/kg iv, in dogs, caused dose-dependent decreases in mean arterial blood pressure within 1 min. A secondary increase was observed after CDM injection which returned to predrug values in less than 1 hr (Fig. 5, Table 5). Hyperventilation, tremors, particularly in the forelimbs, and occasionally clonic convulsions were associated with the transition from depressor to pressor responses. Animals under light anesthesia, as determined by the presence of active toe pinch and corneal reflexes, exhibited tremors and large secondary pressor responses after 10 or 30 mg/kg of CDM iv, while animals under deep anesthesia showed neither tremors nor pressor responses, indicating that these were of central

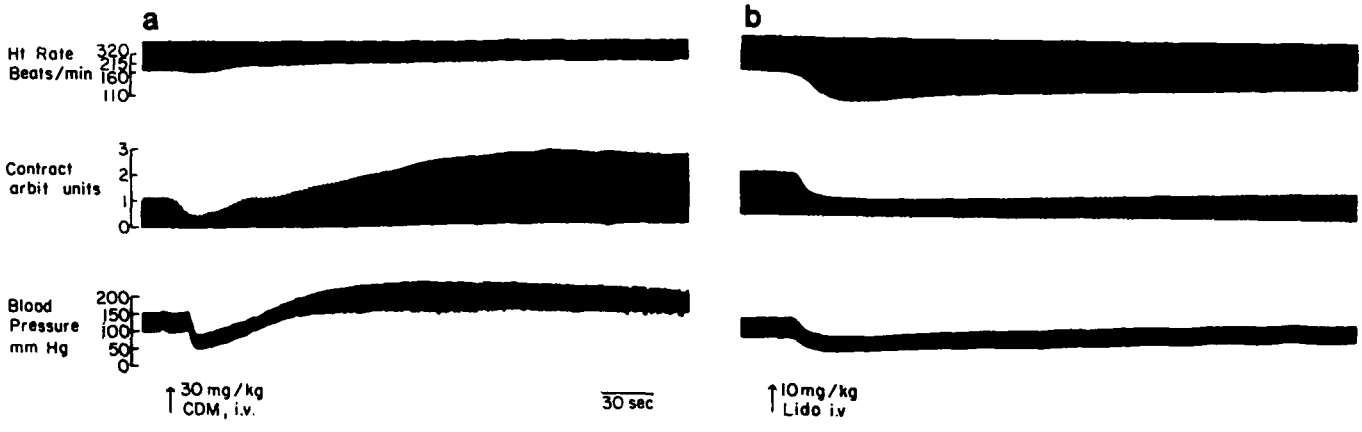


Figure 5. Typical records showing the effects of (a) CDM and (b) lidocaine on heart rate, cardiac contractility (in arbitrary units), and blood pressure in the pentobarbital-anesthetized dog.

origin. Secondary pressor responses were smaller and less frequently observed following lidocaine. Similarly, somatic manifestations of CNS stimulation by

TABLE 5. TIME COURSE OF THE PRIMARY (I^{o}) AND SECONDARY (II^{o}) BLOOD PRESSURE AND CARDIAC CONTRACTILITY RESPONSES IN CHLORDIMEFORM-TREATED DOGS

		Time (min) to:				
		I^{o}		II^{o}		
Dose (mg/kg iv)	n	I^{o} Depressor response	Decreased contractility response	II^{o} Pressor response	Increased contractility response	Recovery to control
3	9	0.7 ± 0.2[a]	0.9 ± 0.2	5.6 ± 1.3	3.8 ± 1.3	12.8 ± 0.7
10	9	0.8 ± 0.1	0.7 ± 0.1	10.5 ± 1.6	9.5 ± 1.6	29.5 ± 4.0
30	6	0.8 ± 0.1	0.7 ± 0.1	14.6 ± 0.5	14.2 ± 0.5	53.3 ± 4.3

[a]Mean ± SE.

lidocaine were less intense and less frequently observed than after CDM.

Dose-response data were collected on animals that were "light" by the above criteria. Level of anesthesia appeared to have no effect on the blood pressure depression caused by CDM or lidocaine. Blood pressure responses to CDM in four unanesthetized but restrained rabbits were qualitatively identical and quantitatively similar to those shown in Fig. 5.

Lethal doses of lidocaine (>30 mg/kg) or CDM (>50 mg/kg) caused an irreversible decrease in blood pressure. Although respiratory arrest occurred almost simultaneously, cardiovascular collapse was assumed to be the primary cause of death since artificial respiration did not protect the dogs against death. Lead II of the ECG showed that neither CDM nor lidocaine caused cardiac arhythmias at the doses used in these experiments.

Heart Rate and Cardiac Contractility

Figure 5 and Table 5 show that CDM caused a depression of cardiac contractility concurrent with the decrease in mean blood pressure, as well as a secondary increase concurrent with the increase in blood pressure. The dose-response curves for these events are shown in Fig. 6. The inotropic effects did not appear to result from changes in diastolic filling of the heart since vena cava pressure recordings at the level of the right atrium (six experiments) showed no decrease or increase in venous pressure associated with the decrease or increase in contractility, respectively. Therefore, CDM must have some effect either on the autonomic nervous system or on the myocardium itself.

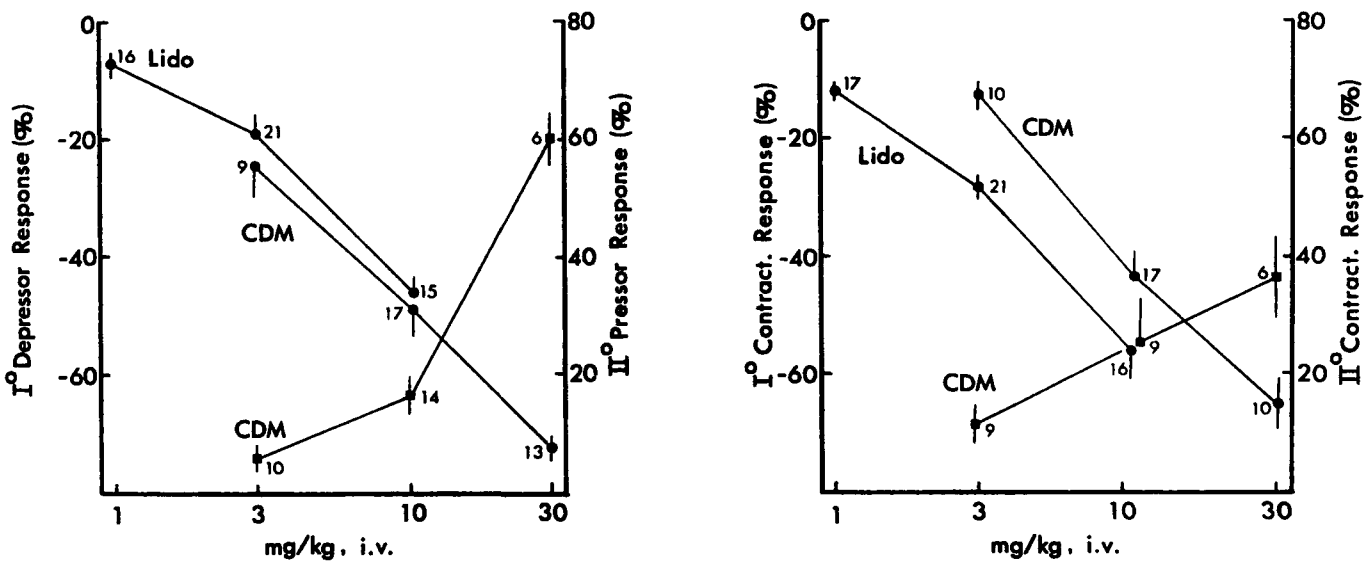


Figure 6. Initial I°(●——●), and secondary, II° (■————■), effects of CDM and lidocaine on blood pressure and cardiac contractility. Results are expressed as a percentage of predrug control (mean ± SE, number of observations).

Bilateral vagotomy (five experiments) or 1 mg/kg of atropine (three experiments) had no effect on the cardiac depression caused by 10 or 30 mg/kg of CDM. The same CDM doses had no effect on the positive chronotropic and inotropic effects of the ganglionic stimulant DMPP (1 µg/kg, two experiments) or the β-adrenergic agonist isoproterenol (1 µg/kg, three experiments). Furthermore, CDM caused a decrease in contractility very similar to that of lidocaine in the isolated rabbit heart (Fig. 7). Therefore, CDM depressed cardiac muscle independently of the autonomic nervous system in a way similar to that of lidocaine.

The secondary increase in cardiac contractility was observed following CDM in the dog but not in the isolated rabbit heart preparation and therefore it is not a direct cardiac effect. The observations in the dog that propranolol (1 mg/kg, three experiments), hexamethonium (10 mg/kg, three experiments), and diazepam (1 mg/kg, five experiments) all blocked the increase in contractility and blood pressure caused by 10 mg/kg of CDM further suggests that the positive inotropic effect of CDM results from sympathetic activity of central origin.

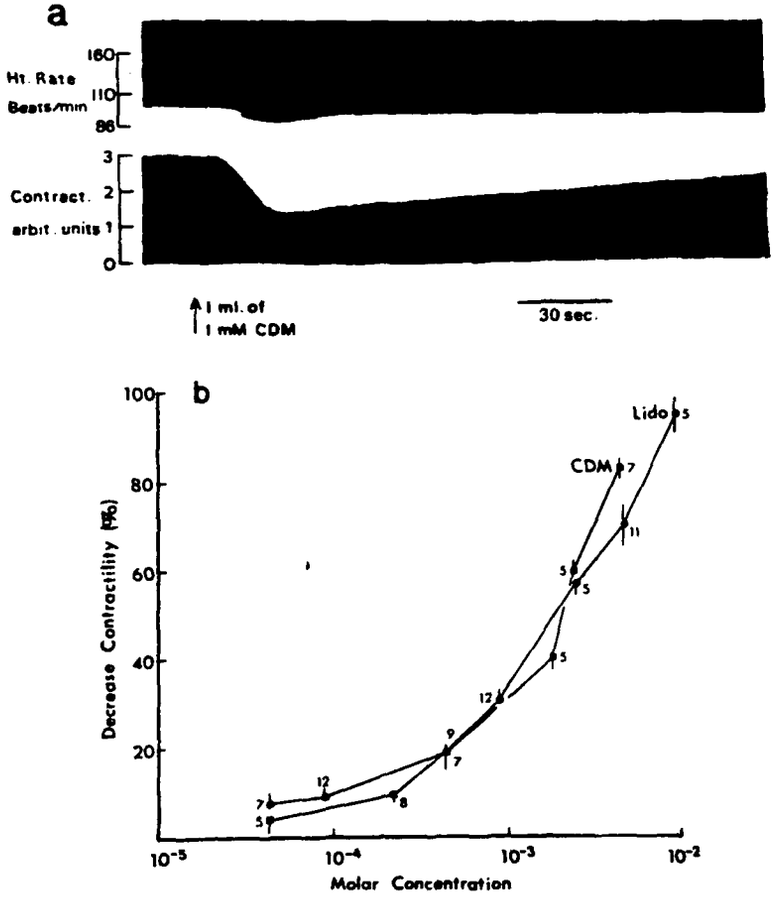


Figure 7. The effects of CDM (■ ——— ■) and lidocaine (● ——— ●) on heart rate and cardiac contractility (in arbitrary units) in the isolated rabbit heart preparation (7b). Doses are expressed as the concentration of 1 ml injected into the perfusate as it enters the aorta. Results are expressed as a percentage of predrug control (mean ± SE, number of observations). A typical record is shown in 7a.

Peripheral Resistance

Chlordimeform or lidocaine injected into the femoral artery caused a decrease in perfusion pressure in the perfused hind limb of the dog (Fig. 8a). Figure 8b shows that CDM was approximately 10 times more potent in this action than lidocaine. Propranolol at 2 mg ia (two experiments) or 5 mg of atropine ia (two experiments) blocked the decrease in perfusion pressure caused by 2 μg of acetylcholine (ACh) ia, respectively, but neither blocking drug attenuated the response to 10 mg of CDM ia. The increase in perfusion pressure caused by 2 μg of norepinephrine ia was not altered by 10 mg of CDM ia (three experiments). Tripelennamine at 2 mg ia blocked the decrease in resistance caused by 5 μg of histamine ia, but not that caused by CDM (two experiments).

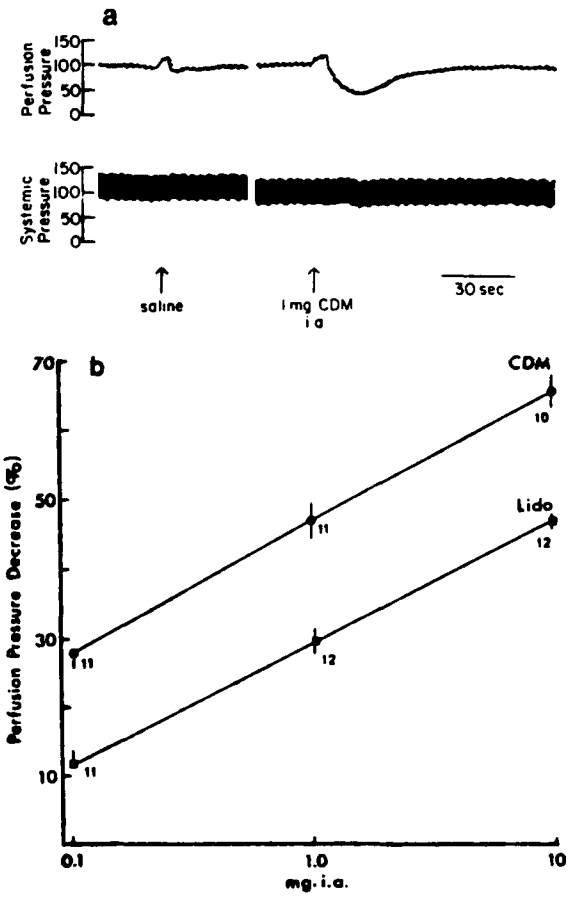


Figure 8. The effects of intraarterial CDM (●————●) and lidocaine (■————■) on the hind limb perfusion pressure in the pentobarbital-anesthetized dog (8b). Results are expressed as a percentage of the predrug control (mean ± SE, number of observations). A typical record is shown in 8a.

Finally, denervation of the perfused limb by the ligation of the femoral and sciatic nerves had no effect on the intraarterial CDM response. These findings suggest that the decrease in perfusion pressure caused by intraarterial CDM is caused by nonspecific relaxation of vascular smooth muscle and is not the result of adrenergic blockage or of cholinergic, β-adrenergic, or histaminergic stimulation.

When CDM or lidocaine was injected iv at 10-30 mg/kg, hind limb perfusion pressure decreased simultaneously with the systemic blood pressure. The secondary rise in systemic pressure caused by CDM was accompanied by a rise in perfusion pressure. This rise was abolished by ligation of the femoral and sciatic nerves (six experiments), by 10 mg/kg of hexamethonium iv (one experiment), and by 5 mg of phentolamine ia (one experiment). Therefore, it appears that CDM given iv in addition to its cardiac effects, caused a decrease in peripheral resistance that contributed to the depression of arterial blood pressure, and a secondary increase in resistance apparently mediated by the sympathetic nervous system which probably contributes to the secondary pressor response.

Central Sympathetic Involvement

CDM administered at doses of 1-30 mg/kg iv to cats produced an initial decrease in blood pressure followed by a secondary increase as in dogs. Recordings of spike discharge frequency from the cervical sympathetic nerves in six cats showed that 10 mg/kg of CDM caused a 46 $\pm$ 28% increase in mean discharge rate over the predrug control level (seven observations). This increase in rate was concurrent with the secondary blood pressure response, thereby providing further evidence that the secondary pressor response results from a central sympathetic discharge (Fig. 9). Surprisingly, the initial depressor response was accompanied by a decrease in cervical sympathetic discharge rate, suggesting that some central nervous system action of CDM might contribute to the initial blood pressure depression. Another observation lending support to this idea is that 10 mg/kg of CDM iv blocked the pressor response caused by bilateral carotid occlusion (five dogs).

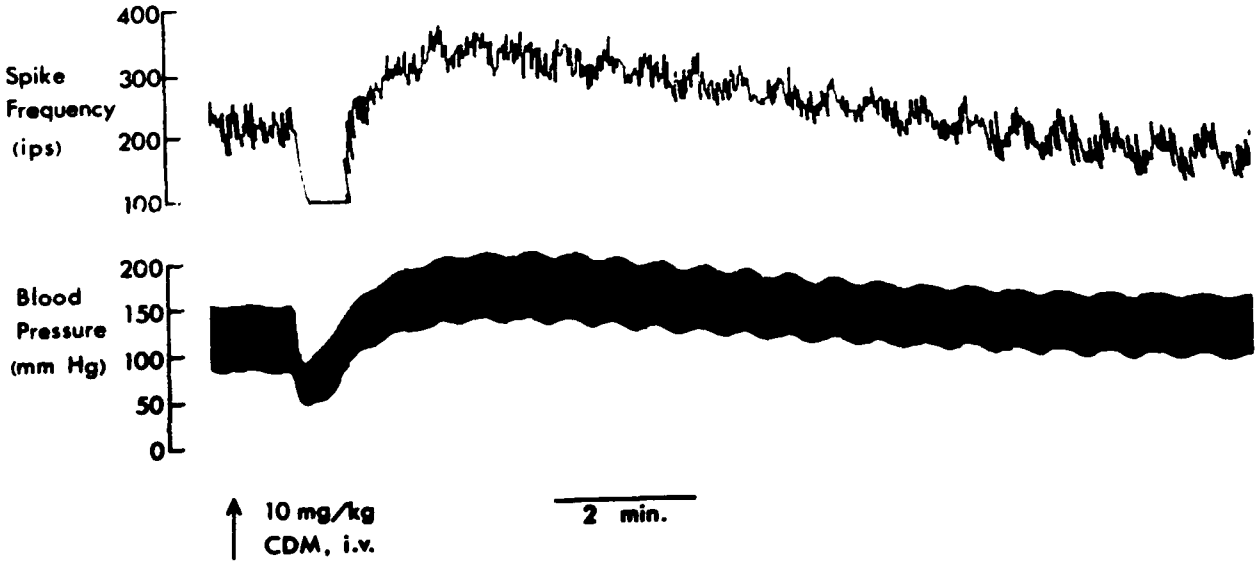


Figure 9. Typical record showing the effects of CDM on blood pressure and spike discharge frequency (in impulses per second) recorded preganglionically from the superior cervical nerve of the pentobarbital-anesthetized cat.

DISCUSSION

The experiments reported here show that cardiac depression and vasodilation resulting in hypotension can account for death in animals exposed to CDM. Respiration is depressed by CDM but artificial respiration does not alleviate or delay toxicity.

Both the cardiac and vascular depression involve direct effects of CDM on cardiac and vascular smooth muscle since neither denervation of these structures nor autonomic pharmacological manipulations reduced the depressor responses. Cardiovascular effects of toxic doses of local anesthetics have been well studied (for a review see Covino and Vassalo, 1976) and have been found to be identical to those described here for CDM. Although qualitatively identical effects are observed in this investigation with CDM and lidocaine, interesting quantitative differences are present. In concurrent studies (Section 4) we found that lidocaine is 10 times more potent than CDM in blocking the action potential of the frog sciatic nerve. Figures 6 and 7 show that CDM and lidocaine depress the dog heart, isolated rabbit heart, and blood pressure with similar potency while Fig. 8 shows that CDM is 10 times more potent than lidocaine in dilating peripheral blood vessels. The lack of any quantitative correlation between intrinsic anesthetic potency, as defined using nerve conduction blockade, and potency of effects on other excitable tissues is well documented for local anesthetics (Covino and Vassalo, 1976). It is therefore not particularly surprising that no such correlation is observed between CDM and lidocaine.

The increased contractility and vasoconstriction, which were responsible for the secondary pressor response, were blocked by sympatholytic agents and were absent in the isolated rabbit heart and denervated perfused hind limb preparations. Other observations also indicate that the secondary pressor response results from massive sympathetic discharge, which in turn is an autonomic component of CDM-induced seizures. Indeed, the blood pressure oscillations and discharge patterns of the superior cervical sympathetic nerve were similar to those observed during picrotoxin and strychnine seizures (Polosa et al., 1969). Further, the CDM-induced secondary pressor response was observed under conditions which favored the appearance of tremors and clonic limb movements. Both pressor and seizure responses were observed following the injection of 3 to 4 mg of CDM into the lateral ventricles of rats (Section 3). Finally, diazepam, which antagonizes local anesthetic seizures (de Jong, 1972), blocked both responses of intraventricular CDM (Section 3) as well as the secondary pressor response of intravenous CDM. Even the mydriasis induced by CDM in mice could be explained better by central sympathetic discharge than by monoamine oxidase inhibition, as suggested by Beeman and Matsumura (1973), since the present studies indicated that CDM had little or no direct sympathomimetic or parasympatholytic activity. Furthermore, CDM did not potentiate the action of the biogenic amines assayed on the cardiovascular system, an action which would be expected if CDM significantly inhibited MAO.

Centrally mediated pressor responses were also observed following intraventricularly administered lidocaine (Section 3). The smaller and less frequently observed secondary pressor response to lidocaine may be related to the weaker convulsive action of lidocaine as compared to CDM (Prince and Wagman, 1966; Pfister, W. F., Noland, V., and Yim, G. K. W., unpublished observations). The stronger convulsive action of CDM may also be due in part to direct neuronal excitation by CDM, which has been observed in cockroach nerve cord preparations (Beeman and Matsumura, 1973; Yamamoto and Fukami, 1976; Lund et al., 1979a).

Clearly these studies indicate that the cardiovascular toxicity of CDM is similar to that of local anesthetics. This implies that diazepam, vasoconstrictors, cardiac stumulants, and artifical respiration should be useful in treating cases of CDM poisoning. Observations in these laboratories with the dog suggest that this regimen is effective in reversing the hypotension caused by doses of CDM that otherwise would be marginally lethal, but is ineffective in antagonizing the severe cardiovascular depression caused by larger doses. Additional studies on this topic are presented in detail in Section 4.

SECTION THREE

THE CENTRAL ACTIONS OF CHLORDIMEFORM

INTRODUCTION

In rats and mice, chlordimeform produces hyperactivity, respiratory depression and convulsions. The mechanisms behind these central nervous system effects have not been elucidated. Since the chemical structure of CDM is similar to that of the local anaesthetic phenacaine, and since CDM exhibits a local anesthetic effect on the isolated frog sciatic nerve (Section 4) and cardiovasular system of several species (Section 2), the possibility was entertained that it may have actions on the central nervous system similar to that of local anaesthetics.

Lidocaine has been shown to produce an action on the amygdala which initiates seizures in the cat (Wagman et al., 1967). In dogs, lidocaine increases arterial blood pressure by a central mechanism (Kao and Jalar, 1959). This Section describes some central actions of chlordimeform in the urethane-anaesthetized rat and compares them to those of lidocaine. The arterial blood pressure and electrical activity in the amygdala were examined after intraventricular injections of chlordimeform or lidocaine. Since the raphe nucleus and serotonin exert inhibitory effects on seizures and on arterial blood pressure (Boggan and Seiden, 1973; Baum and Shropshire, 1975), the effects of raphe stimulation were also examined.

Local anesthetics and CDM (Wang and Narahashi, 1975) depress neuromuscular transmission although relatively high concentrations of CDM are needed (10^{-4}-10^{-3}M). Thus, studies were also initiated to identify whether the respiratory depression caused by CDM was of central or peripheral origin.

MATERIALS AND METHODS

Central responses to intraventricular drugs

Male Sprague-Dawley rats weighing 260-350g were used in all experiments. Rats were anaesthetized with pentobarbital sodium, 35 mg/kg, given intraperitoneally and fixed in a Kopf stereotaxic apparatus. Bipolar stainless-steel electrodes having diameters of 0.010 in. (303 series, Plastic Products Co.) were implanted in the dorsal raphe nucleus and left basal lateral part of the amygdaloid nucleus according to the stereotaxic atlas of Pelligrino and Cushman (1967). A right lateral ventricular cannula

was also implanted for drug injections. The lateral ventricular cannula was cut from a 23-gauge stainless-steel needle and fitted with an indwelling stylet. The electrodes and cannula were fixed to the calvarium by pouring cranioplastic cement around them and over two stainless-steel screws which had been inserted into the skull. After a minimum of 7 days recovery period, the animals were tested.

Rats prepared as described above were anaesthetized with urethane, 1.25 mg/kg, given intraperitoneally. The external jugular vein was cannulated with polyethylene tubing for intravenous injections, and the carotid artery cannulated for recording of blood pressure, using a Statham P 23 pressure transducer. Electrical activity from the basolateral nucleus of the amygdala was monitored on a Grass polygraph. Using a Grass S-88 stimulator, the raphe nucleus was stimulated, usually with a 10 sec train of square wave pulses (0.1-1.5 V, 0.5 msec duration, 10-50 Hz). Intraventricular drug injections were made from a needle fitted to polyethylene tubing connected to a microsyringe. Injection volumes were 10-20 µl. Body temperature was maintained at 37-38^{o} with a thermostatically controlled heating pad.

Drugs used in these experiments were chlordimeform, lidocaine HCl (Astra Pharmaceuticals, Inc.), and diazepam (commercially available Valium, Roche, Inc.)

Respiratory arrest

Rats were lightly anesthetized with urethane (1.2 g/kg, ip), and the test chemicals were infused over a period of 20-30 min via the femoral vein until respiratory arrest occurred. During the progression of symptoms, a Grass polygraph was used to monitor the following: Femoral arterial blood pressure (via a Statham P23A transducer); ECG via transthoracic electrodes; diaphragmatic contractions (via a Grass FT03 force displacement transducer); and end tidal pCO_2 levels (via a catheter in a tracheal tube and Beckman LB medical gas analyzer). The phrenic nerve was dissected free in the cervical region and an oil pool formed. Phrenic nerve discharges were detected via a pair of platinum electrodes, amplified (filter settings: 0.1-10 KHz), displayed on a Tektronix storage oscilloscope, and fed to a Grass AM7 audio monitor. The output was also fed to a Grass P7 Integrator and the integrated phrenic burst pattern was recorded on the polygraph. Displays of the phrenic discharges on the storage oscilloscope were also photographed using a Tektronix C5 oscilloscope camera.

When respiratory arrest occurred the status of the phrenic nerve-diaphragm system was assessed by delivering single and 2-4 sec long trains of square wave pulses (0.1 msec duration, 10 Hz, 0.2-5V) to the phrenic nerve via the pair of platinum electrodes, in order to monitor neuromuscular function.

RESULTS

Blood pressure responses to intraventricular injection of lidocaine or chlordimeform

In a total of 21 rats, the intraventricular injection of 300-1000 μg lidocaine produced variable responses ranging from pure pressor or depressor responses to biphasic ones. Of 21 rats, 7 exhibited pressor, 10 depressor and 4 biphasic responses. Because of the variable responses, it was not feasible to quantify the data. However, in any particular animal, the effect produced was dose related. A dose-dependent pressor response is illustrated in Figure 10. In 17 rats, the effects of CDM were also variable: 9 being pressor, 3 depressor and 5 biphasic. As with lidocaine, for a particular response, the effect was dose-dependent. Figure 10 illustrates the dose-dependent pressor response to CDM.

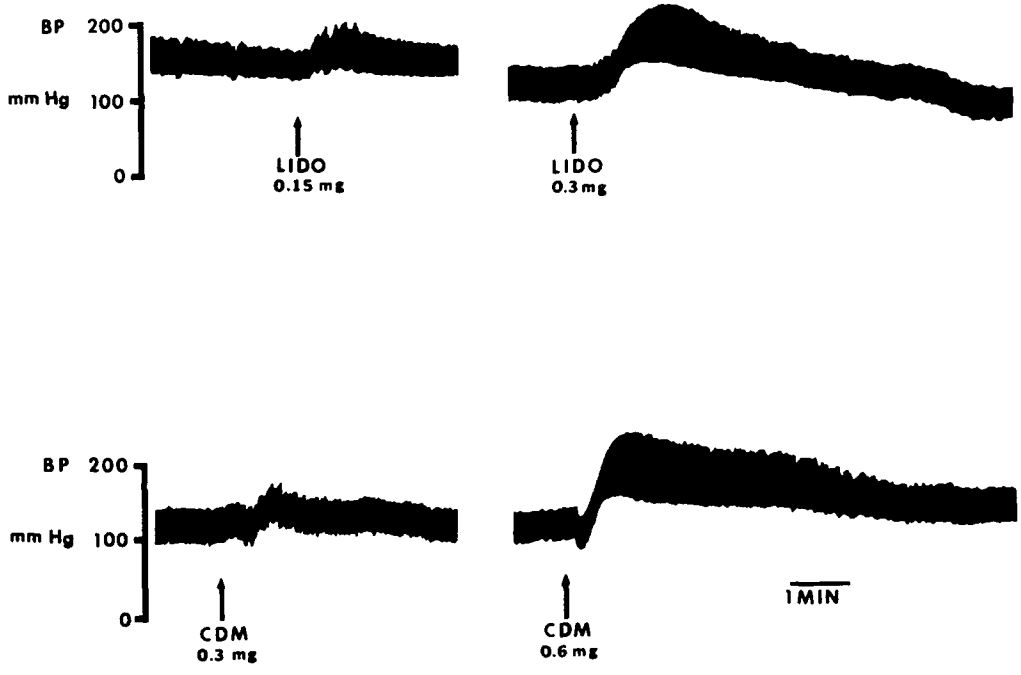


Figure 10. Pressor responses in the carotid artery after intraventricular injections of lidocaine or CDM in a rat anaesthetized with urethane.

Effects of lidocaine and CDM on changes in amygdaloid electrical activity induced by raphe simulation.

When the dorsal raphe was stimulated, the amplitude of the spontaneous electrical activity of the amygdala was suppressed. This suppression was antagonized by lidocaine or CDM, and the degree of antagonism was dose-dependent. These effects are illustrated in Figure 11. The duration of the EEG suppression was dependent on the frequency of raphe stimulation and was antagonized by lidocaine or CDM given intraventricularly. Such an effect is illustrated in Figure 12.

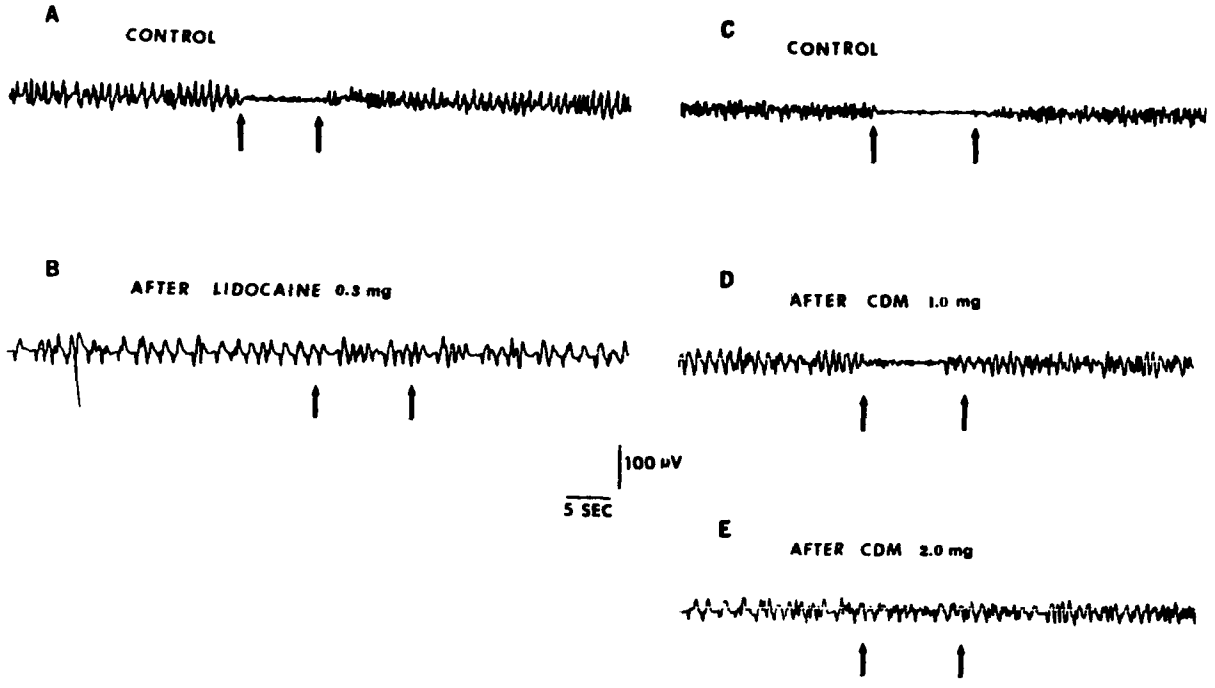


Figure 11. Suppression of amygdala EEG upon raphe stimulation and antagonism of this suppression by intraventricular lidocaine or CDM. Between arrows: 10 sec of dorsal raphe stimulation (1.5V. 0.5 msec. 20 Hz).

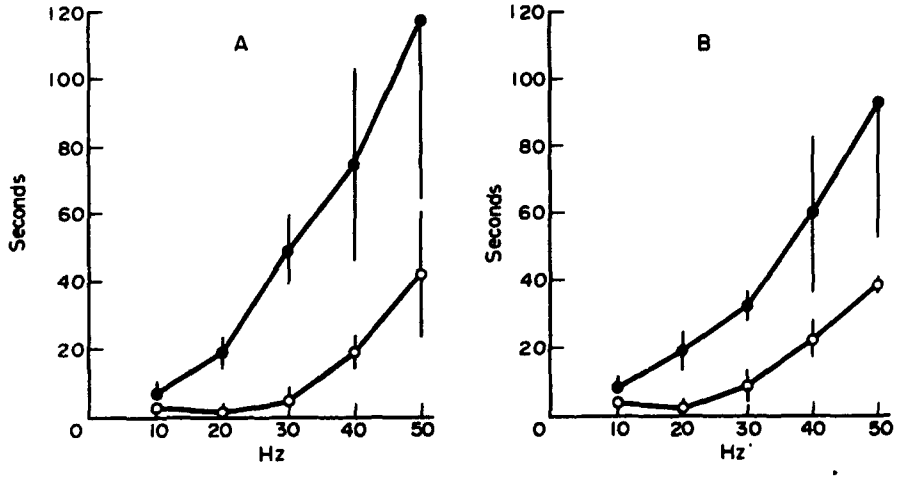


Figure 12. Duration of suppression of amygdala EEG activity and antagonism of this raphe-mediated EEG suppression by lidocaine and CDM. Panel A: duration of suppression at varying frequencies of raphe stimulation, control (●———●) and after 0.5 mg intraventricular lidocaine (○———○). Panel B; control (●———●) and after 1.0 mg intraventricular CDM (○———○). Vertical bars = SEM; n = 5.

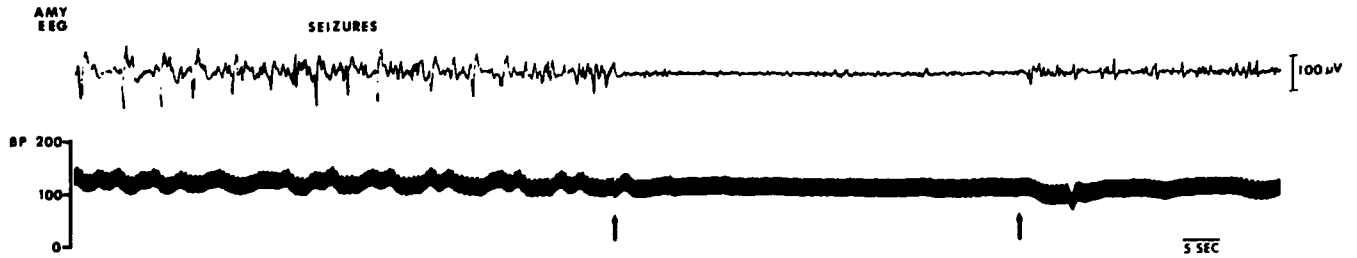


Figure 13. Amygdala EEG spikes and blood pressure oscillation recorded during overt CDM seizures. Dose: 3 successive 1 mg intraventricular doses. Between arrows, the dorsal raphe was stimulated electrically at 1.5V, 0.5 msec duration at 60 Hz.

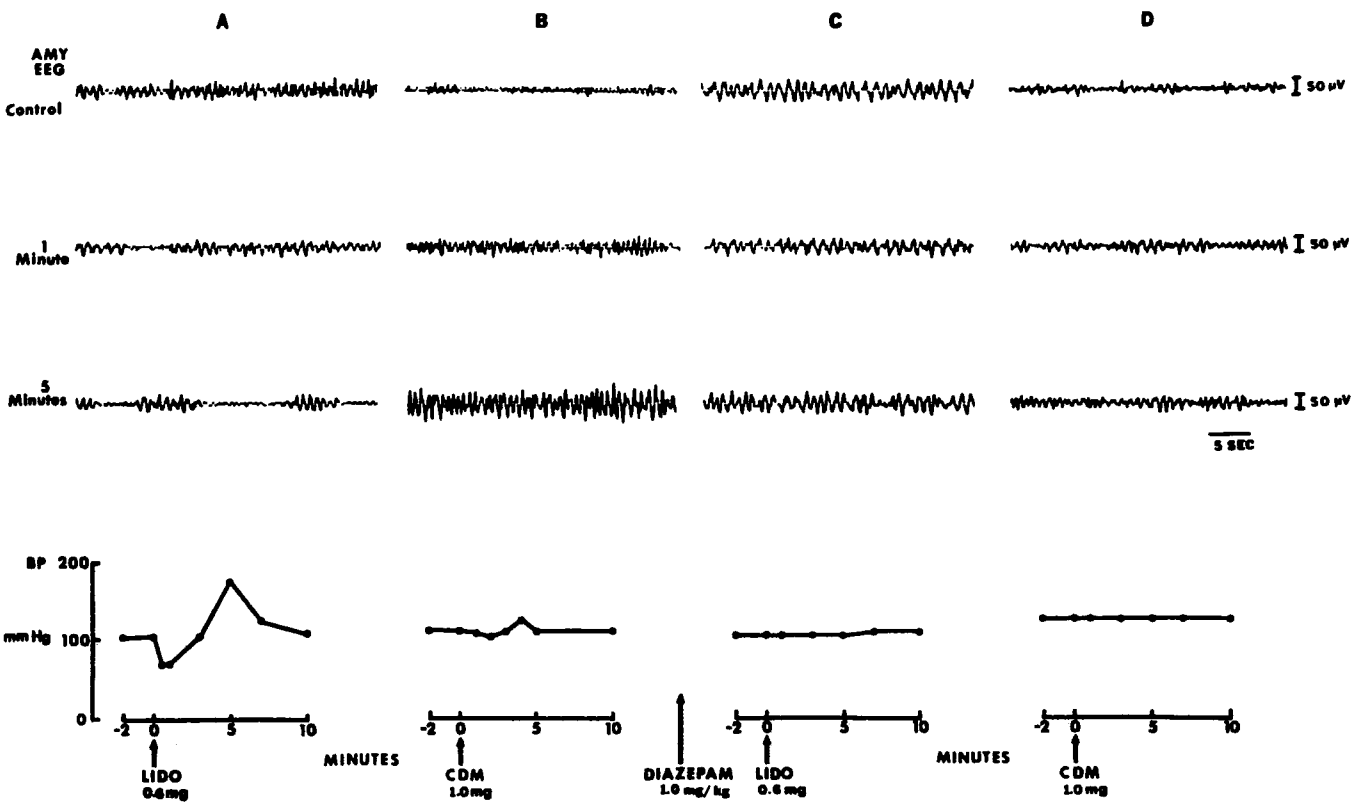


Figure 14. Antagonism by diazepam of the EEG and blood pressure effects of intraventricular lidocaine and CDM. Panels A and B: before; Panels C and D: after intravenous diazepam, 1.0 mg/kg.

Seizure activity induced by CDM and blockage by raphe stimulation

The successive injections of 3 to 4 intraventricular 1 mg doses of CDM resulted in clonic limb movements. This seizure activity was accompanied by oscillations in the arterial blood pressure, and the amygdala EEG was characterized by slow spikes (Fig. 13). spindle bursts (Fig. 14A) or high-amplitude rhythmic discharges (Fig. 14B). During seizure activity,

high frequency electrical stimulation of the dorsal raphe nucleus inhibited seizure activity along with its EEG and autonomic concommitants. This inhibition persisted during the entire period of electrical stimulation. Upon cessation of raphe stimulation, spiking of the EEG and oscillation of the blood pressure reappeared. Seizures did not return in the 5 rats observed. These effects are illustrated in Fig. 13.

Effects of diazepam

Intravenous diazepam, 1.0 mg/kg, blocked the effects of intraventricular lidocaine or CDM on the electrographic activity of the amygdala of the 5 rats. This dose of diazepam also antagonized the centrally mediated blood pressure responses produced by lidocaine or CDM. These results are illustrated in Figure 14.

Mechanism of respiratory arrest

The decreasing order of lethality and dose resulting in respiratory arrest were: cocaine (35.3 ± 11 mg/kg), lidocaine (35.4 ± 9.6 mg/kg), CDM (62.3 ± 6.0 mg/kg), and morphine (93.5 ± 11.0 mg/kg). In lidocaine-treated rats, the amplitude and rate of diaphragmatic movements and of phrenic nerve bursts gradually decreased until respiratory arrest (Figure 15).

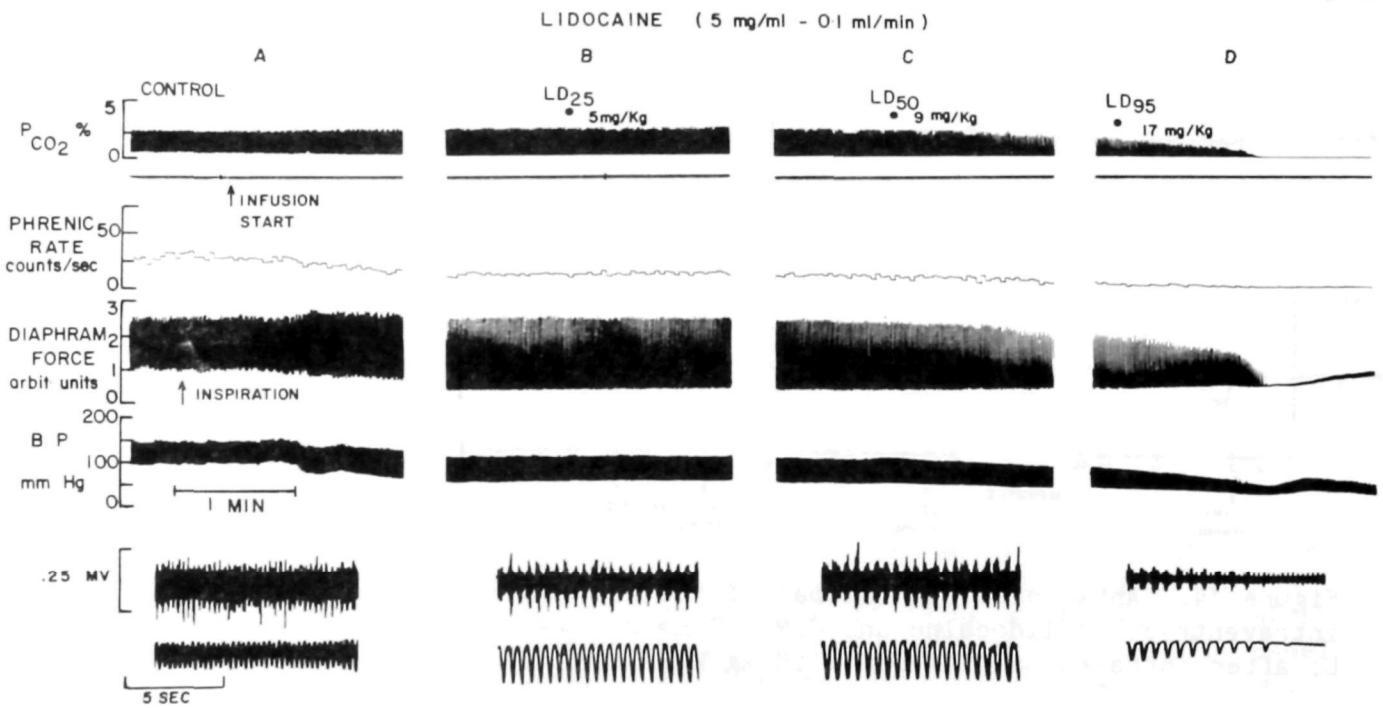


Figure 15. Respiratory and cardiovascular parameters of a urethane-anesthetized rat infused with a lethal iv dose of lidocaine. Measurements include: End-tidal pCO_2 levels; integrated discharge rate recorded from cut central end of phrenic nerve; diaphragmatic contractions; carotid blood pressure; oscilloscope tracings of phrenic bursts and diaphragmatic contractions.

In contrast, as shown in Figure 16, respiratory rate was initially increased by cocaine and CDM. Diaphragmatic contractile force and the amplitude of phrenic nerve bursts remained near control values, but disappeared abruptly upon respiratory arrest. The profile of morphine on the phrenic nerve activity was unique in that continous inter-burst discharge preceded the abrupt decrease in phrenic nerve amplitude and respiratory arrest. Naloxone reversed both actions of morphine but did not reverse the respiratory depression induced by the other agents. Following respiratory arrest produced by all of these agents, the diaphragm and gastrocnemius muscle still contracted following electrical stimulation of the phrenic and sciatic nerves, respectively. In contrast, central output in the phrenic nerve declined and disappeared at the time of respiratory arrest. These results indicate that respiratory arrest induced by these agents is central in origin, and not a result of neuromuscular blockade.

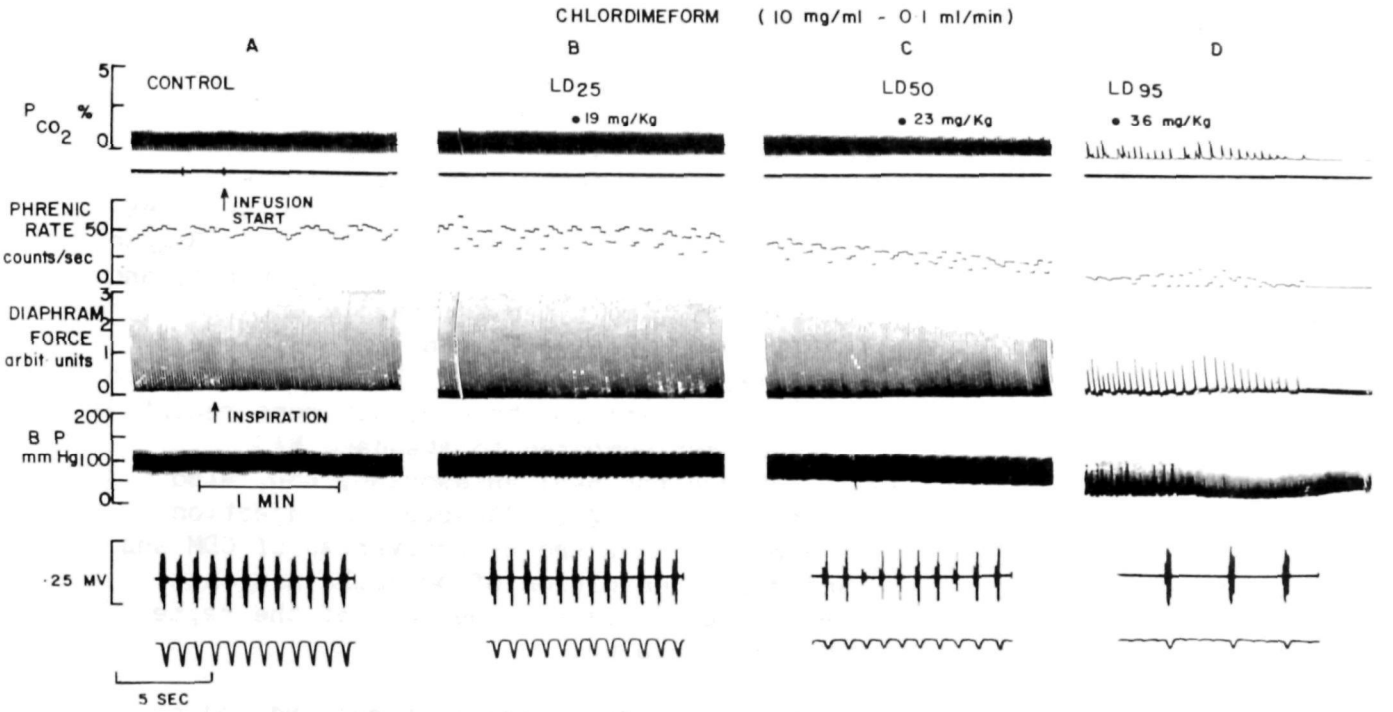


Figure 16. Same as Fig. 15 except drug infused was chlordimeform.

DISCUSSION

As previously found in the frog sciatic nerve (Section 4), the isolated rabbit heart, and dog blood pressure preparations (Section 2), the central actions of chlordimeform that were examined in the present study were qualitatively indistinguishable from those produced by the local anaesthetic lidocaine. Thus, when injected intraventricularly in the anaesthetized rat, both agents produced similar responses on blood pressure and electrical activity of the amygdala. The antagonism by intravenous diazepam of the effects of CDM and lidocaine were anticipated in view of diazepam's well established effectiveness in supressing local anaesthetic seizures (de Jong, 1970).

The studies of Wagman et al. (1967), and Eidelberg et al. (1963) have implicated the amygdala as the probable focus of seizure activity induced by the local anaesthetics, lidocaine and cocaine. The present study also points to the importance of the amygdala in the action of lidocaine as well as chlordimeform. The generally held scheme for explaining local anaesthetic seizures is that local anaesthetics selectively block tonic inhibitory mechanisms, thereby releasing excitatory mechanisms (de Jong, 1970). The observed block by lidocaine or chlordimeform of the suppression, induced by raphe stimulation, of the electrical activity in the amygdala raises the possibility that local anaesthetics may produce seizures by blockade of raphe inhibitory input to the amygdala.

Blockade by lidocaine and CDM of raphe inhibition of the amygdala is an attractive possibility in view of previous findings of antagonism of 5-hydroxytryptamine (5-HT) inhibition of cortical neurones by cocaine (Phillis, 1970) and antagonism of 5-HT-mediated rhythmic activity of cockroach malphighian tubules (Hollingworth, unpublished observations). Spontaneous firing of amygdala neurones is reduced following systemic 5-hydroxytryptophan (Eidelberg et al., 1967), iontophoretic 5-HT or dorsal raphe stimulation (Wang and Aghajanian, 1977). Moreover, electrical stimulation of the midbrain raphe inhibits seizures induced by electrical stimulation of the amygdala or by pentylenetetrazole (Kovacs and Zoll, 1974), and 5-hydroxytryptophan raises the threshold for audiogenic seizures and restores the reserpine-induced hypersensitivity to noise in mice (Boggan and Seiden, 1973). Although reduction of brain 5-HT levels facilitates, and elevation of brain 5-HT levels antagonizes, pentylenetretrazole or electrically induced seizures (Killian and Frey, 1973), brain 5-HT levels are elevated in CDM-treated rats (Beeman and Matsumura, 1973). The elevated 5-HT levels have been attributed to MAO inhibition by CDM, but more recent work suggests that the elevation is slight compared to standard MAO inhibitors (Benezet et al., 1978). Since the 5-HT antagonist, LSD, also causes an increase in [^{3}H]5-HT levels following [^{3}H]tryptophan injection (Diaz and Huttunen, 1971), it is apparent that the effectiveness of CDM and lidocaine in antagonizing 5-HT and raphe inhibition of amygdala neurones must be directly examined in order to better assess the role of the raphe system in seizures induced by CDM and lidocaine.

The blood pressure oscillations occurring during seizures induced by CDM are similar to those induced by convulsants such as picrotoxin and strychnine in the cat and described in detail by Polosa et al. (1969) and Polosa et al. (1972). This oscillatory behavior has been observed to be associated with synchronized periodic activity of sympathetic preganglionic neurones and appears to be generated in the central nervous system. Similar discharges have been recorded from peripheral sympathetic efferent nerves during the pressor response following intravenous CDM (Section 2). The pressor and convulsant effects of intraventricularly applied lidocaine and CDM might involve similar mechanisms, since both agents also blocked raphe inhibition of pressor responses evoked by stimulation of the amygdala. If the raphe system exerts a tonic inhibitory influence on amygdala and other nuclei responsible for vasomotor tone, then blockade of such inhibitory input could result in pressor responses such as those seen after

intraventricular lidocaine and chlordimeform. Since serotonergic neurones also exert an inhibitory influence upon spinal sympathetic neurones (Neurmayr et al., 1974), blockade of this descending inhibitory pathway may also contribute to the increase in blood pressure observed after CDM and lidocaine.

In regard to the mechanism underlying respiratory arrest induced by CDM, the results presented here clearly support the CNS rather than a blockade of neuromuscular junctions as the site of action. In CDM-poisoned rats, neuromuscular function appears to be relatively unimpaired, while CNS output via the phrenic nerve is diminished and disappears, indicating a probable block of the respiratory center in the brain stem.

SECTION FOUR

LOCAL ANESTHETIC PROPERTIES OF CHLORDIMEFORM AND POTENTIAL MEANS OF ALLEVIATING TOXICITY

INTRODUCTION

In the preceding two Sections, it was shown that CDM in both its peripheral and central actions is qualitatively indistinguishable from the local anesthetic, lidocaine. In addition, CDM, when injected into the lateral ventricle, caused a delayed respiratory arrest that was consistent with slow passage of CDM via the third ventricle and subsequent depression of the brain stem respiratory "center" by a local anesthetic-like action of CDM. CDM and local anesthetics have common toxic symptoms consisting of hyperexcitability, tremors, convulsions, and respiratory arrest (Beeman and Matsumura, 1974; deJong, 1970). Furthermore, the chemical structure of CDM is similar to that of the local anesthetics, phenacaine and guanicaine (Fig. 17).

The first objective of this study was to determine if CDM did have a local anesthetic action on the frog sciatic nerve preparation. Since diazepam is an effective antagonist of convulsions and lethality induced by local anesthetic agents (deJong, 1970; Richards et al., 1968), and was found to reverse the hypotensive effects of CDM in dogs (Section 2), a second objective of this study was to examine diazepam and other agents for their effects on CDM-induced convulsions and lethality.

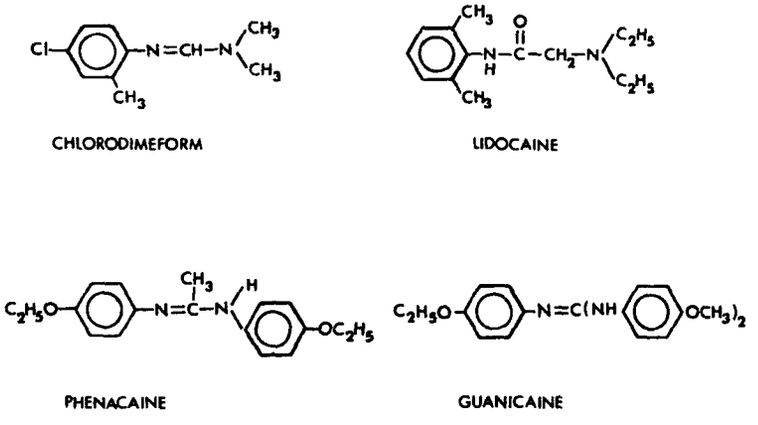


Figure 17. Chemical structure of chlordimeform and related local anesthetics.

MATERIALS AND METHODS

Frog sciatic nerve preparation

The hydrochloride salts of CDM, DCDM, and of the local anesthetic agents phenacaine (Holocaine, Winthrop-Stearns, Inc., New York), lidocaine (Xylocaine, Astra Pharmaceutical Products, Inc., Worcester, Massachusetts), and procaine (Mallinckrodt, Inc., St. Louis, Missouri) were dissolved in frog Ringer's solution (Wesseling et al., 1971) and the pH was adjusted to 7.0 ± 0.1 with 0.1 M NaOH. Sheathed and desheathed sciatic nerves dissected from frogs (Rana pipiens) measuring 3 to 3.5 in. in length were placed into Harvard nerve chambers with pairs of platinum wire stimulating and recording electrodes spaced at 1 cm intervals. Compound action potentials were evoked using supramaximal stimulation (0.6-4.0 V, 0.15 msec, 1 Hz) delivered from a Grass Model SD-5 stimulator. The action potential amplitude was monitored on a Tektronix 565 oscilloscope. Conduction rate was estimated as the ratio of the change in latency to the distance between two sets of recording electrodes. During twin pulse stimulation, the interval between pulses that resulted in 50% depression of the response to the second pulse was used as a measure of the relative refactory period. Drug solutions were applied to a 5 mm segment of the nerve between the stimulating and recording electrodes. Each nerve was used only once and six to eight nerves were run per drug concentration. Three or four concentrations of each agent enabled the calculation of the ED_{50} and standard error values by reverse regression analysis (Aldrete and Daniel, 1972).

CDM-drug interactions in vivo

White mice (25-30 g; Cox-Swiss, Laboratory Supply Co., Indianapolis, Indiana) were used in the studies of the convulsant and lethal actions of CDM. The mice were housed in groups under a 10 hr light-dark cycle for at least 1 week prior to testing. They were pretreated with the potential antagonists usually 30 min prior to being injected with approximate LD_{95} dose of CDM (100 mg/kg, ip). When assessing agents for possible enhancement of CDM toxicity, the pretreated mice were challenged with the approximate LD_5 dose of CDM (80 mg/kg CDM, ip). Comparisons were made using Fisher's method of calculating exact probabilities (Schwartz, 1974).

Diazepam (Valium: Hoffman-LaRoche Inc.) and the other pretreatments were injected intraperitoneally in a volume of 10 ml/kg. Saline was the solvent for diphenylhydantoin sodium, librium HCl (Hoffman-LaRoche Inc.), pentobarbital sodium (Nembutal, Abbott Laboratories), atropine sulfate (Nutritional Biochemicals Corp.), imipramine HCl (Geigy Pharmaceuticals), pyribenzamine HCl (Ciba Pharmaceutical Co.), phenoxybenzamine HCl (Smith, Kline & French), chlorphenoxamine HCl (Pitman-Moore Co.), neostigmine methylsulfate (Hoffman-LaRoche, Inc.), physostigmine (Eserine Salicylate, Mallinckrodt Chemical Works), cycloheximide (Calbiochem), and cinanserin (SQ 10,643: Squibb and Co.). Trimethadione (Tridione: Abbott Laboratories), mephenesin (K & K Laboratories, Inc.), and chlorpromazine HCl (Thorazine: Smith, Kline & French) were dissolved in 10% ethanol, 20% propylene glycol, 70% saline mixture.

RESULTS

Effects of CDM and DCDM on the frog sciatic nerve preparation

Figure 18 shows the effect of CDM, DCDM, phenacaine, procaine, and lidocaine on the amplitude of the compound action potential of the frog sciatic nerve. The slopes of the dose-response relationship for all compounds were similar: no significant departure from parallelism was observed between the regression lines for holocaine, procaine, DCDM, or lidocaine compared to that of CDM. The order of increasing potency for the compounds studied was found to be DCDM < CDM < procaine < phenacaine < lidocaine. The ED_{50}'s $\pm$ SE were 35 $\pm$ 6.2 mM (DCDM); 15.0 $\pm$ 3.7 mM (CDM); 8.7 $\pm$ 2.5 mM (procaine); 4.0 $\pm$ 1.5 mM (phenacaine), and 1.9 $\pm$ 0.3 mM (lidocaine). Hence, CDM and its demethylated metabolite, DCDM, were 0.6 and 0.3 times as potent as procaine respectively. The effects of CDM and DCDM on all other parameters studied were similar to those observed with procaine, phenacaine, and lidocaine. After 15 min of exposure to either CDM or procaine, threshold stimulus voltages and relative refractory periods were increased by approximately 30 and 20%, respectively. Conduction velocity was decreased by 15 and 20% after 15 min of exposure to CDM (30 mM, pH 6.0) and procaine (10 mM, pH 7.0).

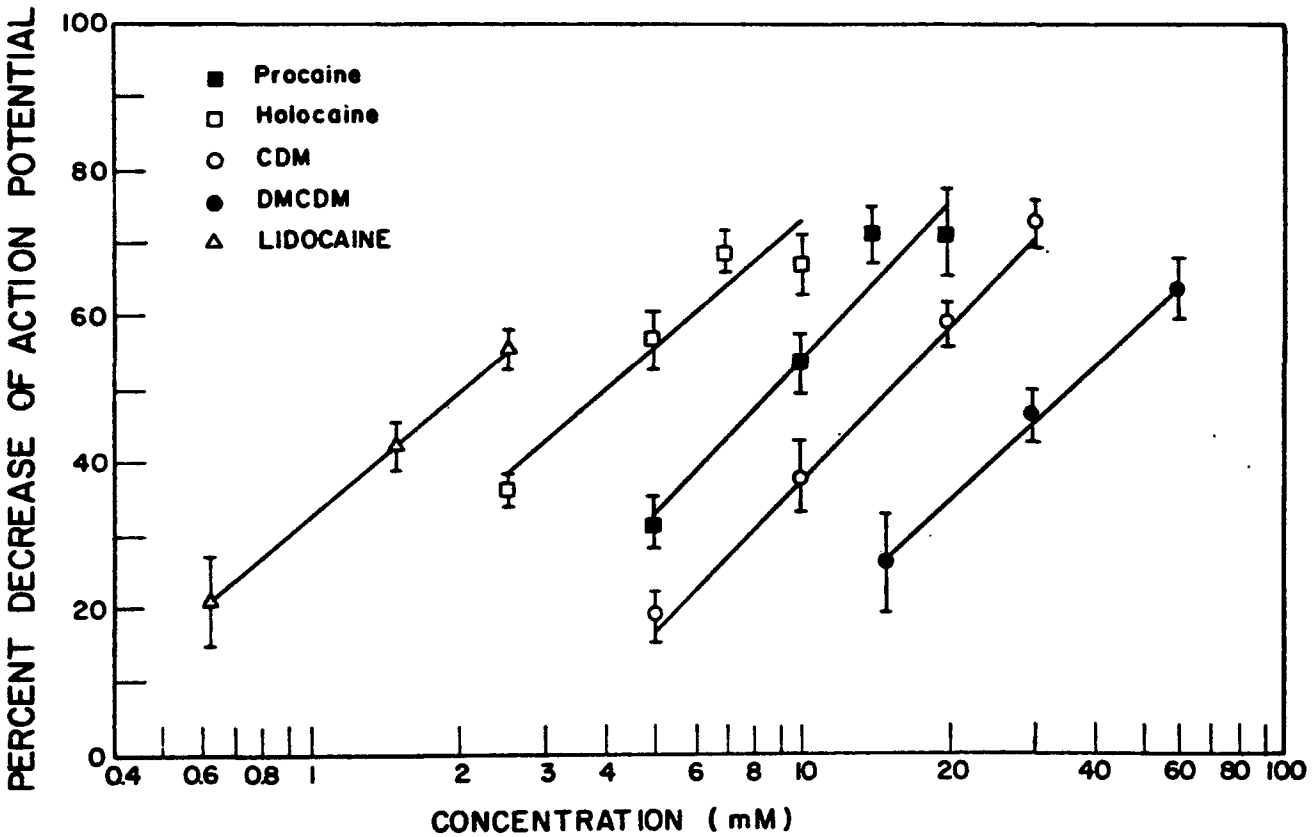


Figure 18. Effect of chlordimeform, N-demethylchlordimeform, procaine, holocaine, and lidocaine on the action potential in frog sciatic nerve. Abscissa; dosage (mM) on logarithmic scale; ordinate; percentage decrease of the compound action potential amplitude 15 min following drug application. Each point represents the mean $\pm$ SE (n = 6).

Repetitive or spontaneous firing was never observed. After complete blockage of the nerve by CDM, partial recovery was obtained following repeated washing of the nerve. When the degree of ionization of CDM was increased by lowering the pH from 7.0 to 6.0, the nerve-blocking effectiveness of 30 mM CDM was reduced by more than 50% (Fig. 19). With desheathed nerves, 1 mM CDM (pH 7.0) caused 20% depression (Fig. 19). Note that with intact nerves (Fig. 18) approximately 5 mM CDM was required to cause 20% depression. Thus CDM was about five times more active on the desheathed nerve.

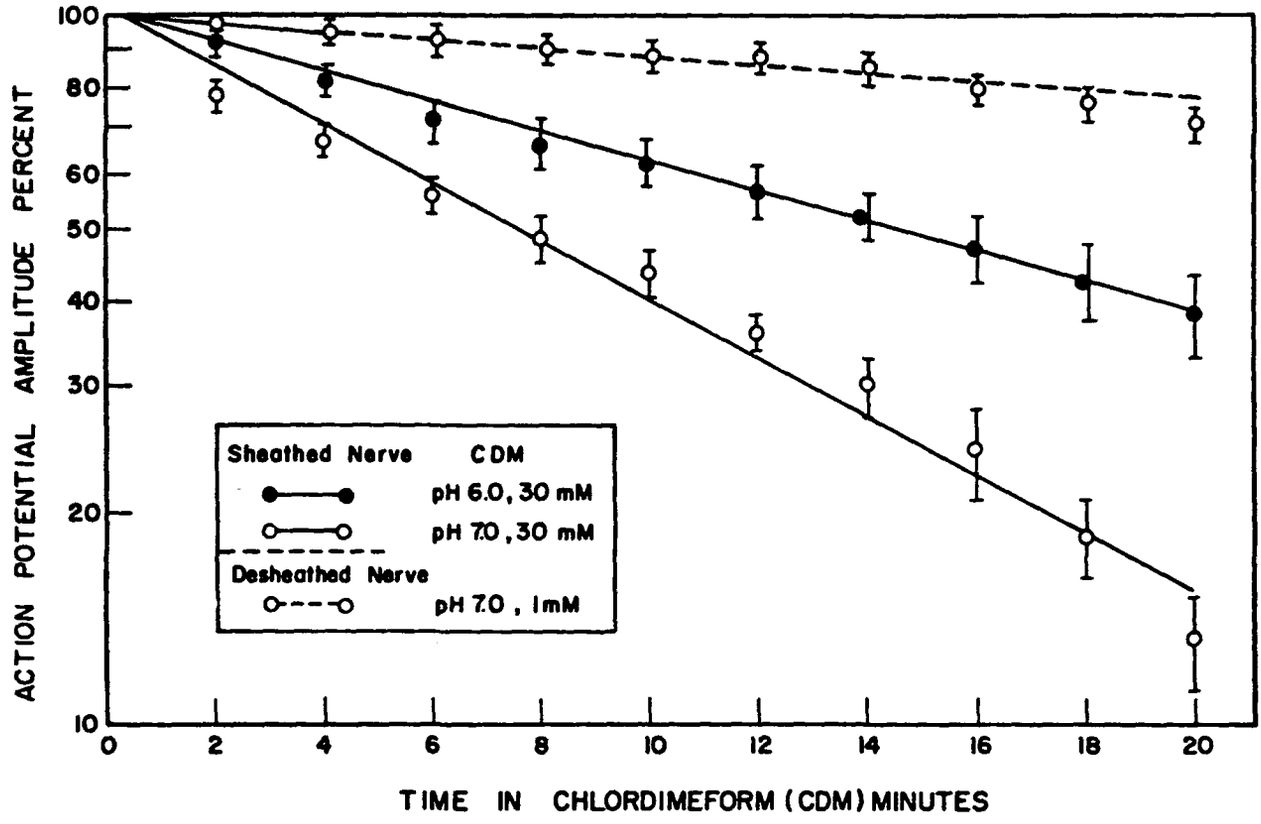


Figure 19. Effect of chlordimeform (30 mM) at pHs 6.0 and 7.0 in the sheathed and at pH 7.0 (1 mM) in the desheathed nerve on action potential amplitude at 2 min intervals following drug application. Abscissa: time (min) following application of chlordimeform to the nerve. Ordinate: action potential amplitude (percentage of control). Each point represents the mean $\pm$ SE (30 mM, pH 6.0; n = 7; 30 mM, pH 7.0; n = 6; 1 mM, pH 7.0; n = 6).

Diazepam and other agents on CDM convulsions and lethality

The approximate LD_{95} dose of CDM, 100 mg/kg, ip, resulted in periodic convulsive jerks (mean latency: 4 min--see Table 6). The frequency, severity, and duration of the convulsive episodes increased until a continuous seizure bout resulted in repiratory arrest and death (mean latency: 6-12 min for groups of 10 mice). The LD_{50} (and 95% confidence interval) for CDM was 85.5 (87.9-83.3) mg/kg). The threshold dose for inducing convulsions was well below 50 mg/kg, a dose at which no acute lethality was evident.

TABLE 6. EFFECTS OF ANTICONVULSANTS ON CDM CONVULSIONS AND LETHALITY IN MICE

Pretreatments[a]					
Compound	Dose (mg/kg)	No. convulsed/ No. injected	Mean latency (min)	No. deaths/ No. injected	Mean latency (min)
Saline controls	---	10/10	3.6	10/10	6.0
Saline controls	---	10/10	4.3	9/10	12.7
Saline controls	---	10/10	3.7	10/10	8.0
Saline controls[b]	---	10/10	2.3	10/10	6.2
Vehicle control	---	10/10	3.0	10/10	14.6
Diphenylhydantoin	40	10/10	2.2	10/10	6.2
Trimethadione	400	10/10	2.8	10/10	10.2
Mephenesin	100	8/10	---	7/10	8.7
Mephenesin	200	10/10	4.6	10/10	8.7
Diazepam	15	10/10	3.8	1/10*	15.0*
Diazepam	15[c]	10/10	4.7	3/10*	20.0*
Diazepam	30[c]	10/10	5.1	7/10	25.4*
Librium	15	10/10	3.2	5/10**	10.4
Librium	40	10/10	4.3	10/10	24.2*
Pentobarbital	15[c]	10/10	---	9/10	---
Pentobarbital	25[c]	4/10*	10.0*	6/10**	11.6

[a]Mice were pretreated 30 min prior to 100 mg/kg CDM, ip (LD_{95} dose). Exceptions: mephenesin (15 min), diphenylhydantoin (120 min), trimethadione (60 min).

[b]10% ethanol, 20% propylene glycol, 70% saline.

[c]Resulted in ataxia and decreased motor activity.

* $P \leq 0.001$

** $P \leq 0.01$

Pretreatment with the anticonvulsants diphenylhydantoin, trimethadione, or mephenesin did not result in prevention of either the convulsions or lethality that was observed following the LD_{95} dose of CDM (Table 6). At the 15 mg/kg dose, diazepam pretreatment reduced the severity and duration but did not appreciably reduce the incidence nor prolong the onset time of the CDM convulsions. However, lethality was reduced about 20%, and the onset time to death was lengthened to about 20 min. The 30 mg/kg dose of diazepam was also ineffective in preventing CDM convulsions, and it was less effective than the 15 mg/kg dose in preventing CDM lethality. Mice receiving this higher dose of diazepam exhibited motor impairment, ataxia, decreased motor activity, decreased responsiveness to sensory stimulation, and, in some cases, loss of righting reflex. Similar results were obtained with librium: the 15 mg/kg dose attenuated CDM lethality, whereas the 40 mg/kg dose prolonged the latency to death, but was ineffective in preventing CDM lethality. Pentobarbital reduced the incidence of convulsions and death, but only at the 25 mg/kg dose which was accompanied by marked sedation and depression.

The effects of other agents that were tested as possible antagonists of CDM are summarized in Table 7. Chlorpromazine (20 mg/kg) increased the latency to convulsions and the latency to death, but provided little protection against CDM lethality. Agents that did not reduce either the severity of CDM convulsions or the incidence of CDM convulsions and lethality included haloperidol, chlorphenoxamine, cycloheximide, neostigmine, and physostigmine. Physostigmine increased the latency to death. Cinanserin increased the onset time and decreased the severity and incidence of convulsions as well as increased the latency to death and decreased the incidence of lethality in mice treated with CDM. Animals treated with the 50 mg/kg cinanserin were ataxic and exhibited ptosis and decreased muscle tone. During preliminary cardiovascular studies with CDM, it appeared that atropine and pyribenzamine intensified the hypotensive action of CDM. Hence, possible enhancement of CDM lethality by atropine and other agents that possess "local anesthetic" activity was tested by pretreating mice with these agents, and then challenging them with 80 mg/kg CDM (approximate LD_5 dose). This dose of CDM caused convulsions in 100% of the saline-pretreated mice (mean latency: 5.2 $\pm$ 0.2 min - see Table 8). As expected, no pretreatment reduced the incidence of convulsions. The onset time for CDM convulsions was reduced in the animals treated with imipramine, phenoxybenzamine, and atropine but not with the mice dosed with pyribenzamine. CDM lethality was enhanced following pretreatment with pyribenzamine, imipramine, and phenoxybenzamine, but not after atropine or lidocaine pretreatment.

DISCUSSION

The acute toxic symptoms of CDM are similar to those observed following local anesthetic overdose, i.e., clonic convulsions, respiratory depression, and cardiovascular collapse followed by death (deJong, 1970). The effectiveness of diazepam and pentobarbitol in reducing lethality by toxic doses of CDM also parallels the effectiveness of these agents in decreasing lethality from local anesthetic agents such as lidocaine (Richards *et al.*, 1968; Wesseling *et al.*, 1971; Aldrete *et al.*, 1972). The ineffectiveness of

TABLE 7. EFFECT OF OTHER PHARMACOLOGICAL AGENTS ON CDM CONVULSIONS AND LETHALITY IN MICE

Pretreatments[a]					
Compound	Dose (mg/kg)	No. convulsed/ No. injected	Mean latency (min)	No. deaths/ No. injected	Mean latency (min)
Saline controls	----	10/10	2.8	10/10	6.4
Saline controls	----	10/10	2.3	9/10	6.2
Saline controls	----	10/10	3.6	10/10	6.0
Chlorpromazine	5	9/9	3.5	9/9	----
Chlorpromazine	20	5/10	6.0*	7/10	15.3*
Haloperidol	1	9/10	3.1	9/10	9.8
Haloperidol	5	10/10	3.6	10/10	8.1
Chlorphenoxamine	5	10/10	2.4	9/10	9.5
Cyloheximide	0.7	8/10	3.8	8/10	9.5
Neostigmine	0.1	10/10	2.0	10/10	5.9
Physostigmine	0.25	10/10	2.9	10/10	18.1*
Physostigmine	0.50	8/8	3.6	6/8	14.0*
Cinanserin	25[b]	8/10	6.0*	9/10	9.0*
Cinanserin	50[b]	6/10**	7.2*	6/10**	13.8*

[a]Mice were pretreated 30 min prior to 120 mg/kg CDM, ip (LD_{95} dose). Exceptions: cycloheximide (2 hr), chlorpromazine (3 hr).

[b]Resulted in ataxia and decreased motor activity.

* $P \leq 0.001$

** $P \leq 0.01$

TABLE 8. TOXICITY IN MICE OF CDM COMBINED WITH AGENTS POSSESSING LOCAL ANESTHETIC ACTIVITY

Pretreatment	Dose (mg/kg)	Latency to convulse (min)	No. deaths/ No. injected
Saline	—	5.2±0.2	7/70
Pyribenzamine	10	4.4±0.3	5/10*
Pyribenzamine	30	4.2±0.6	8/10*
Atropine	1	3.6±0.2*	2/20
Atropine	10	3.0±0.2*	3/20
Imipramine	10	4.5±0.2	4/10**
Imipramine	30	3.2±0.5*	4/10**
Phenoxybenzamine	1	3.1±0.3*	4/10**
Phenoxybenzamine	10	3.6±0.4**	4/10**
Lidocaine	10	5.2±0.4	4/30
Lidocaine	20	4.0±0.2	3/20

[a]Mice were pretreated 15 min prior to 80 mg/kg CDM ip ($LD_{5\%}$ dose) except lidocaine (3 min); pyribenzamine 30 mg/kg (30 min).

* $P \leq 0.001$

** $P \leq 0.01$

diphenylhydantoin, trimethadione, and mephenesin in reducing CDM lethality was anticipated in view of the previously reported inability of these agents in antagonizing local anesthetic toxicity (deJong, 1970). The observed enhancement of CDM toxicity by the antihistaminic, pyribenzamine, the α-adrenergic blocker, phenoxybenzamine, and the tricyclic antidepressant, imipramine, could have been due to the local anesthetic actions of this diverse group of pharmacologic agents (Crescitelli and Geissman, 1951; Guerrero and Molgo, 1974). However, CDM toxicity was not enhanced by atropine, which also has comparable local anesthetic activity (Sheu et al., 1969), or by lidocaine.

In the frog sciatic nerve preparation, CDM and its metabolite DCDM produced effects similar to those of the local anesthetic agents studied: amplitudes and conduction velocities of the compound action potential were depressed, and threshold voltages and refractory periods were increased. Reversibility of the nerve block, increased effectiveness in desheathed preparations, and decreased potency at pHs favoring ionization of the compounds (pKa of CDM is near 7.0) are additional characteristic actions shared by CDM and the local anesthetics (deJong, 1970; Ritchie and Greengard, 1966).

In our comparisons of the actions of CDM and local anesthetics in vivo (Sections 2 and 3), we found that both CDM and lidocaine caused similar biphasic cardiovascular responses when injected intravenously in dogs, and similar amygdala EEG spike discharges, overt seizures, and blood pressure responses when injected into the lateral ventricle of rats. Further, like lidocaine and cocaine (Post et al., 1975), CDM induced "pharmacological kindling" in rats i.e. a type of reverse tolerance in which repeated daily subconvulsant doses eventually precipitated generalized convulsions (Yim, et al., 1977). These results indicate that some of the pharmacological and toxic actions of CDM are due to actions that are shared by local anesthetic agents.

Unlike the situation with local anesthetic seizures, which are readily antagonized by diazepam (deJong, 1970), CDM seizures could not be prevented even at doses higher than that which reduced CDM lethality. This was especially surprising since we found that diazepam did antagonize seizures and amygdala EEG spike discharges that resulted from intraventricular CDM (Section 3). We also observed that diazepam blocked the secondary pressor response of CDM, an autonomic consequence of CDM-induced seizures (Section 2). The latter studies were carried out in anesthetized preparations and perhaps an additive anticonvulsant effect of the anesthetics might explain the lack of protection observed in this study with unanesthetized mice. The general CNS depressant actions of pentobarbital and cinanserin might likewise explain their effectiveness against the CDM seizures and lethality.

The resistance of CDM convulsions to diazepam might also reflect manifestations of direct excitatory actions of CDM that are not shared by local anesthetics. Indeed, Beeman and Matsumura (1974), reported repetitive firing from the cockroach ventral nerve cord treated with CDM. Moreover, repetitive firing in cockroach giant axons (Lund et al., 1979a) and increased

firing of caudate neurons (Pfister, W. and Yim, G. K. W., unpublished) have been noted following the application of CDM but not lidocaine. The greater intensity of the CDM convulsions, as compared to lidocaine seizures, is also consistent with this possibility. These indications of a direct excitatory action of CDM on neuronal membranes suggest that CDM may have effects on sodium and potassium conductances beyond the depression characteristic of local anesthetics such as lidocaine and procaine. The acute symptoms of CDM toxicity are qualitatively similar to those produced by DDT, which also elicits repetitive firing in the cockroach giant axon (Narahashi and Yamasaki, 1960). Although cycloheximide is effective in preventing acute lethality and decreasing the severity of convulsions induced by DDT (Hrdina and Singhal, 1972). cycloheximide failed to protect the mice from convulsions and lethality induced by CDM.

An understanding of the mechanisms of CDM-induced lethality must precede the analysis of the diazepam-CDM interactions observed in this study. In the lightly anesthetized dog intravenous CDM can result in simultaneous respiratory arrest and cardiovascular collapse, and the irreversible hypotension is mediated primarily by direct depressant effects of CDM on the heart and blood vessels (Section 2). It seems unlikely that diazepam would prevent these direct cardiovascular depressant actions. The report by Wang et al., (1975) of CDM block of acetylcholine depolarization at the frog neuromuscular junction raises the question of whether CDM-induced respiratory arrest involves peripheral blockade of neuromuscular transmission. The observed inability of neostigmine and physostigmine to reduce CDM lethality does not support the possibility of a peripheral site of action, and is in keeping with the conclusion already reached in Section 3. It is more likely that CDM-treated mice succumbed from postconvulsive depression of the respiratory "centers." Diazepam at 15 mg/kg appeared to have sufficiently attenuated the severity of the CDM convulsions to prevent the secondary postical respiratory arrest and consequent death. The ineffectiveness of 30 mg/kg of diazepam in reducing CDM lethality could possibly be due to additive central respiratory depressant actions of CDM and the higher dose of diazepam. Block by diazepam of the secondary pressor response of CDM could also unmask an irreversible hypotensive action by CDM. The interactions of diazepam with CDM and local anesthetic on central respiratory mechanisms deserve further study. An unequivocal interpretation of these results is not presently possible.

SECTION FIVE

BEHAVIORAL EFFECTS OF CHLORDIMEFORM

INTRODUCTION

Recently, Jacobs described a behavioral model in the rat consisting of a spectrum of symptoms termed the "serotonergic syndrome" which is purported specifically to reflect "increased central serotonergic transmission". Regimens that induced the syndrome include release of serotonin (5-HT) by parachloroamphetamine (PCA), or direct stimulation of postsynaptic 5-HT receptors by L-tryptophan plus monoamine oxidase (MAO) inhibition (Jacobs, 1976).

Interestingly, sub-toxic doses of CDM elicit some of the component symptoms characteristic of the 5-HT syndrome in rats. The symptoms of CDM behavioral toxicity have previously been likened to serotonin poisoning in the presence of a MAO inhibitor (Beeman and Matusumura, 1973). In addition, CDM possesses moderate MAO inhibitory potency (Section 1) and brain levels of both 5-HT and norepinephrine are elevated in rats receiving toxic doses of CDM (Beeman and Matsumura, 1973), although recent work suggests that the elevations are low compared to those induced by other MAO inhibitors (Beeman et al., 1978).Since CDM inhibits MAO and elevates brain 5-HT levels, while PCA (Jacobs, 1976) indirectly, and quipazine (Hong et al., 1969) directly, stimulate postsynaptic 5-HT receptors, each should be capable of eliciting the 5-HT syndrome.

The first aim of this study was to determine if the serotonergic syndrome could be produced by CDM, quipazine and by intraventricular 5-HT. The second objective was to determine if the serotonergic syndrome produced by these agents could be blocked by 5-HT antagonists (cinanserin and methysergide). An additional objective was to develop a "behavioral profile" for the effects of lethal and sublethal doses of CDM in the rat.

MATERIALS AND METHODS

Adult, male Sprague-Dawley derived albino rats (350-450 g) (Laboratory Supply Co., Indianapolis, IN) were housed in groups (8-10/cage) except animals with lateral ventricular cannulas which were housed individually in air-conditioned rooms (24) in a quiet environment, with lighting between 07:00 and 18:00 hours. Animals were supplied with food (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL) and water ad libitum for approximately one week prior to use.

For the intraventricular injection of 5-HT, stainless steel cannulas were implanted 0.5mm above the lateral ventricles. The stereotaxic coordinates were: L = 1.5 mm; AP = 1.0 mm: H = 3.0 below dura. 5-HT, dissolved in saline, was injected in a volume of 10 µl over a period of 30 sec through a stainless steel needle which protruded 1 mm below the tip of the cannula. Doses are expressed as the total salt.

The presence (or absence) of the following six components of the "Serotonergic syndrome" was assessed: rhythmic reciprocal forepaw treading; slow lateral head weaving; hindlimb abduction (splayed out posture); rigidity (manifested by increased resistance of the hindlimbs to passive extension and flexion); Straub tail, and resting tremor (especially of the head and forelimbs). Positive control regimens for inducing the 5-HT syndrome included PCA (5 mg/kg), and L-tryptophan (200 mg/kg, ip) 1 hr following pargyline (50 mg/kg, ip). Animals were injected with appropriate drugs and observed at 30, 60, and 90 min, except animals injected with 5-HT which were observed at 4 x 15 min intervals following injection. The animals were also observed for the presence of slow periodic backing movements; periodic circling; hyperactivity (periodic movement about the cages); hyperreactivity (startle reaction and/or vocalization accompanying handling); hyperreflexia (periodic clonic spasms) and salivation.

All drugs were prepared immediately prior to use and administered intraperitoneally to rats in volumes of 1 ml/kg of body weight. Chlordimeform HCl (CDM); parachloroamphetamine HCl (PCA) (Regis Chemical Co., Chicago, IL); quipazine, 2-(1-piperazinyl)quinoline maleate (Miles Laboratories, Elkhart, IN); cinanserin HCl (E. R. Squibb and Sons, New Brunswick, NJ); and pargyline (Eutonyl®, Abbott Laboratories, North Chicago, IL) were dissolved in physiological saline. L-Tryptophan (Calbiochem, Los Angeles, CA) and methysergide (Sandoz Inc., Hanover, NJ) were made up in a 1.5% suspension of methocellulose (USP) and acidified with a few drops of 0.1 N HCl. 5-Hydroxytryptamine creatine sulfate complex (5-HT, Sigma Chemical Co., St. Louis, MO) was dissolved in physiological saline and administered intraventricularly (ivc).

Where applicable, data were analyzed for significance between treatment groups by using Fisher's method for calculating exact probabilities (Fisher, 1970), calculating X^2 for 2 x 2 contingency tables, or using Student's t test. Three doses of each agent enabled the calculation, by reverse regression analysis (Brownlee, 1965), of threshold doses and standard errors for eliciting 4 out of 6 serotonergic or additional symptoms.

RESULTS

Rats treated with both PCA and CDM exhibited the serotonergic syndrome in a dose-related manner (Fig. 20). The calculated threshold PCA and CDM doses ($\pm$ SE) for eliciting 4 of the 6 symptoms were 4.7 $\pm$ 0.8 and 94.8 $\pm$ 6.7 mg/kg, respectively. In addition, the slopes of the dose response lines of PCA and CDM were not significantly different ($F_{1,2} = 0.72$; $p > 0.5$). Since pargyline plus L-tryptophan elicited behaviors indistinguishable from those of PCA, all further comparisons were made between non-lethal sub-threshold doses of PCA (5 mg/kg). CDM (80 mg/kg), and quipazine (20 mg/kg).

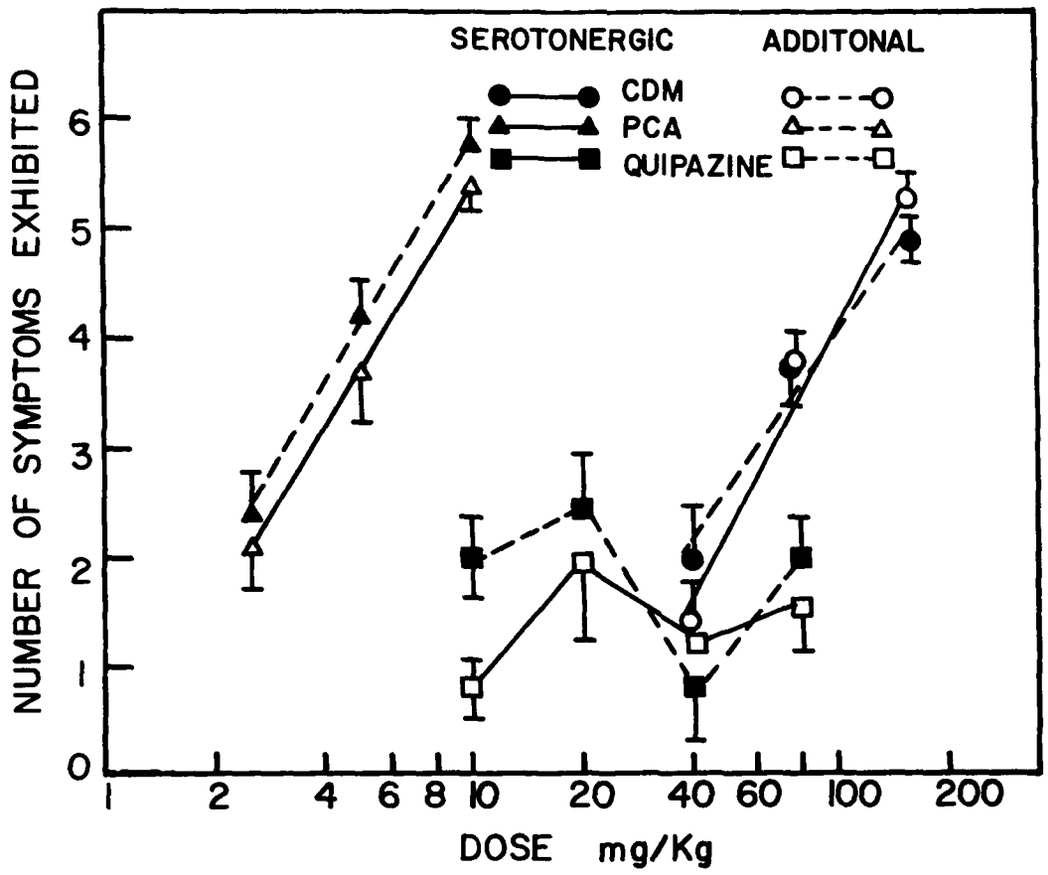


Figure 20. Dose response effect of PCA, CDM, and quipazine in eliciting serotonergic syndrome (closed symbols) and additional symptoms (open symbols). Each point represents the mean ± SE number of symptoms observed (n = 6-12/dose).

The mean number of symptoms exhibited per rat and the frequencies with which each of the 6 different component symptoms of the 5-HT syndrome were exhibited by the groups of rats treated with 5 mg/kg PCA, 80 mg/kg CDM and 20 mg/kg quipazine are given in the first three columns of Table 9. Thus the eleven animals treated with 5 mg/kg of PCA exhibited an average of 3.7 ± 0.5 of the 6 possible "5-HT-like" responses. CDM at 80 mg/kg also effectively elicited the 5-HT syndrome, as the 12 animals averaged 4.3 ± 0.3 responses. The onset of PCA and CDM-induced behavioral alteration was rapid (5-10 min), and of long duration (up to 4 hours at the highest doses tested). Forepaw treading, head weaving and tremor were the most frequently observed symptoms, whereas Straub tail responses were infrequently observed.

Since the quipazine response changed from an excitatory to a depressant phase after about 30 min, the quipazine-treated rats were observed several times during the first 30 min. In contrast to PCA and CDM, quipazine did not effectively elicit the serotonergic syndrome in rats; no dose response relationship was evident (Fig. 20). The mean number of symptoms exhibited by the 12 rats that received 20 mg/kg quipazine was only 0.8 ± 0.3 of the 6

possible (Table 9). The three responses seen most often with PCA and CDM (forepaw treading, head weaving, and tremor) were absent in the 12 quipazine-dosed rats. Forepaw treading and tremor could be elicited only with doses of quipazine producing acute lethality (40 and 80 mg/kg). Even with the 40 and 80 mg/kg dose (Fig. 20), the overall incidence of the 6 serotonergic symptoms was far below the 4 of 6 criteria of Jacobs (1976).

Cinanserin (20 mg/kg) effectively antagonized the 5-HT syndrome induced by PCA in rats (Fig. 21), with the total incidence of the components of the syndrome halved (3.7 $\pm$ 0.5 vs 2.0 $\pm$ 0.6, $t_{20} = 2.19$, $p < 0.05$). The dose of cinanserin employed (20 mg/kg) produced no overt behavioral symptoms of its own, except a decrease in muscle tone. Cinanserin also completely prevented quipazine from inducing any signs of the 5-HT syndrome (Table 9 and Fig. 21). In contrast, the incidence of the components of the 5-HT syndrome elicited by CDM was unchanged by pretreatment with cinanserin (20 mg/kg) (4.3 $\pm$ 0.5 vs 3.8 $\pm$ 0.4, $t_{18} = 1.05$, $p < 0.05$). Higher doses of cinanserin (40-60 mg/kg) could not be tested for their effectiveness in antagonizing the CDM-induced 5-HT symptoms, since these higher doses of cinanserin alone induced hindlimb abduction as well as marked signs of depression (decreased motor activity, diminished responsiveness to external stimuli and respiratory depression).

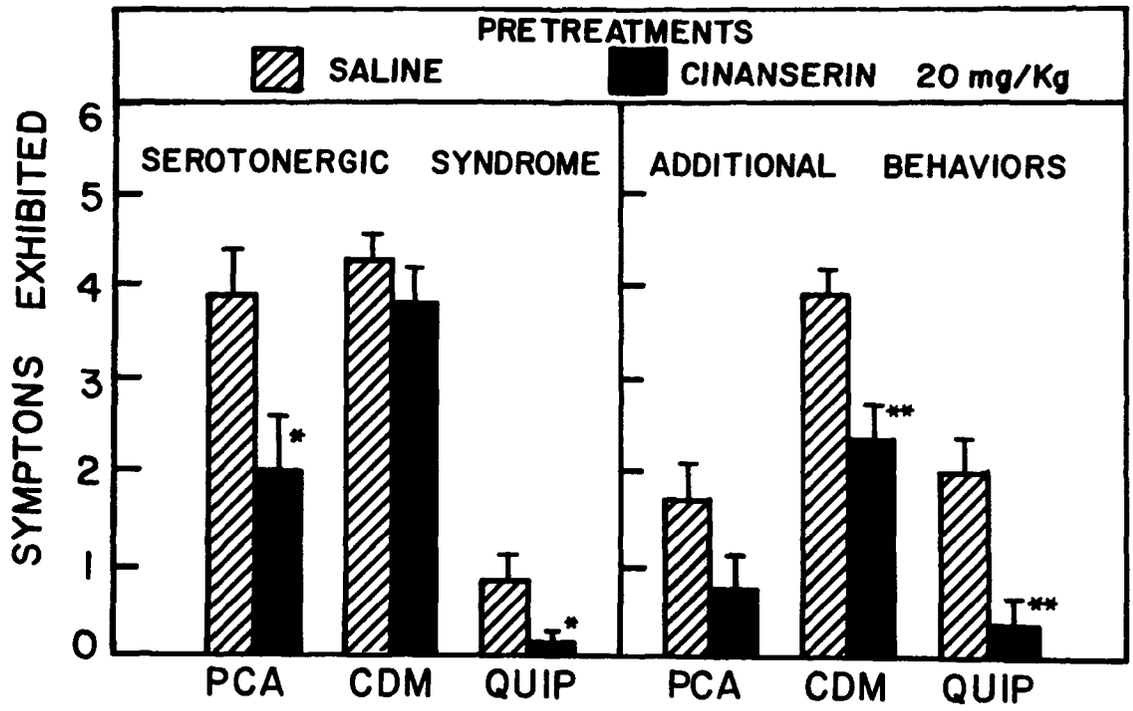


Figure 21. Effect of cinanserin (20 mg/kg) pretreatment (30 min) on the serotonergic syndrome and additional symptoms elicited by PCA (5 mg/kg), CDM (80 mg/kg), and quipazine (10 mg/kg). Values = $\bar{X} \pm$ SE; (n = 6-12). * $p < 0.05$, ** $p < 0.01$.

TABLE 9. EFFECT OF CINANSERIN ON SEROTONERGIC SYNDROME PRODUCED BY PCA, CDM AND QUIPAZINE

Treatments	Dose	Controls			After Cinanserin (20 mg/kg)			After Methysergide (10 mg/kg)	
		PCA	CDM	Quip-azine	PCA	CDM	Quip-azine	PCA	CDM
	mg/kg	5	80	20	5	80	20	5	80
#Symptoms Exhibited/Rat	$\bar{X}$ ±SE	3.7 ±0.5	4.3 ±0.3	0.8 ±0.3	2.0* ±0.6	3.8 ±0.4	0.0* ±0.0	3.0 ±0.6	3.6* ±0.2
Symptoms									
Forepaw Treading		7/11	12/12	0/12	5/11	8/8	0/8	0/5*	0/10*
Head Weaving		10/11	12/12	0/12	5/11*	8/8	0/8	1/5*	2/10*
Hindlimb Abduction		6/11	8/12	2/12	1/11*	4/8	0/8	4/5	10/10
Rigidity		6/11	6/12	6/12	2/11	3/8	0/8*	5/5	10/10
Tremor		10/11	12/12	0/12	7/11	8/8	0/8	4/5	4/10*
Straub Tail		2/11	1/12	2/12	1/11	1/8	0/8	1/5	10/10
Totals		41/66	51/72	10/72	22/66	32/48	0/48	15/30	36/60
Percent Incidence		62%	71%	14%	33%	67%	0%	50%	60%

* $P < 0.05$ when compared to corresponding controls (Fisher, 1970).

TABLE 10. EFFECT OF CINANSERIN ON ADDITIONAL SYMPTOMS PRODUCED BY PCA, CDM AND QUIPAZINE.

Treatments	Dose	Controls			After Cinanserin (20 mg/kg)			After Methysergide (10 mg/kg)	
		PCA	CDM	Quip-azine	PCA	CDM	Quip-azine	PCA	CDM
	mg/kg	5	80	20	5	80	20	5	80
No. Symptoms Exhibited/Rat	$\bar{X}$ ±SE	4.3 ±0.4	3.8 ±0.3	2.0 ±0.4	0.7** ±0.3	2.3* ±0.4	0.3** ±0.3	2.8* ±0.4	2.7 ±0.2
Symptoms									
Backing		4/11	4/8	0/8	0/11	2/8	0/8	0/5	2/10
Circling		6/11	6/8	8/8	1/11*	1/8*	1/8	3/5	0/10*
Hyperactivity		11/11	8/8	8/8	1/11*	7/8	1/8*	5/5	6/10
Hyperreactivity		9/11	8/8	1/8	2/11*	7/8	0/8	2/5	10/10
Hyperreflexia		7/11	3/8	2/8	0/11*	2/8	0/8	0/5*	9/10
Salivation		11/11	0/8	1/8	2/11*	0/8	0/8	4/5	0/10
Totals		48/66	29/48	20/48	6/66	19/48	2/48	14/30	27/60
Percent Incidence		72%	60%	42%	9%	40%	4%	47%	45%

* $p < 0.05$ when compared to corresponding controls (Fisher, 1970).
** $p < 0.01$ when compared to corresponding controls.

Methysergide (10 mg/kg) pretreatment (30 min) significantly reduced the incidence of the components of the serotonergic syndrome in CDM (80 mg/kg) treated rats (4.3 ± 0.3 vs 3.6 ± 0.2, $t_{20} = 2.31$, $p < 0.05$) but not following treatment with PCA (Table 9). Methysergide alone did not elicit any overt behavioral effects.

In addition to the 5-HT syndrome, PCA, CDM, and quipazine elicited other effects which are categorized in Table 10. Only PCA and CDM exhibited these effects in a dose related manner (Fig. 20). The calculated doses ($\bar{X}$ ± SE) for eliciting 4 of these 6 additional effects were 4.7 ± 1.0 and 91.5 ± 6.9 mg/kg. The fact that these doses are not significantly different from doses that elicit the serotonergic syndrome raises the possibility that these symptoms have common underlying mechanisms.

As illustrated in Figure 21 and detailed in Table 10, cinanserin pretreatment also reduced the overall incidence of the 6 additional behaviors elicited by PCA (4.3 vs 0.7, $t_{20} = 10.2$, $p < 0.01$); by CDM (3.8 vs 2.3, $t_{14} = 3.23$, $p < 0.01$); and by quipazine (2.0 vs 0.3, $t_{14} = 3.5$, $p < 0.01$). CDM-induced hyperactivity was noticeably resistant to antagonism by cinanserin. In contrast, hyperactivity and circling induced by PCA or quipazine were almost completely prevented by cinanserin. Methysergide (10 mg/kg) pretreatment did attenuate the total incidence of the additional symptoms following PCA (4.3 ± 0.4 vs 2.8 ± 0.4, $t_{14} = 2.48$, $p < 0.05$) but not following CDM.

When administered into the lateral ventricles, 5-HT depressed the spontaneous activity of the rat but did not elicit the serotonergic syndrome (Table 11). In particular, Straub tail, tremor and rigidity were not observed. Of the additional symptoms, circling and head twitches (pinnial reflex) were present.

TABLE 11. EFFECT OF 5-HT[a] ON SEROTONERGIC SYNDROME AND ON ADDITIONAL BEHAVIORS IN THE RAT

Dose (g)	Mean (± SE) number of symptoms and percent incidence	
	Serotonergic Syndrome	Additional Symptoms
0	0.3 ± 0.2 (4%)	1.0 ± 0.4 (17%)
50	0.8 ± 0.5 (13%)	0.5 ± 0.3 (18%)
100	2.0 ± 0.4** (25%)	0.8 ± 0.4 (17%)
200	1.5 ± 0.5* (25%)	0.3 ± 0.3 (4%)
400	1.5 ± 0.3***(37%)	1.0 ± 0.4 (17%)

[a]See Methods for classification of Serotonergic Syndrome and Additional Symptoms. Significant differences from controls: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

DISCUSSION

The demonstrated ability of CDM to induce the "5-HT syndrome" and the cinanserin block of the PCA-induced "5-HT syndrome" seem compatible with Jacobs' view that "the 5-HT syndrome" is a model of increased 5-HT transmission, since CDM is an MAO inhibitor and elevates brain 5-HT levels (Beeman and Matsumura, 1973; Benezet et al., 1978) while cinanserin is a 5-HT antagonist (Salas et al., 1966).

Clearly in our study central injections of 5-HT did not produce hyperactivity but rather depressed motor activity, and this is consistent with previous reports that both intraventricular (Green et al., 1976a) and systemic (Jacobs and Eubanks, 1974) administration of 5-HT depresses motor activity and produces sedation.

Even though 5 µl injections into the lateral ventricle rapidly reach the fourth ventricle, brain stem nuclei distant to the ventricular surfaces and the spinal area would be inaccessible (Myers and Yaksh, 1968). Hence, the ineffectiveness of intraventricular 5-HT in inducing the complete serotonergic syndrome could well be due to 5-HT not reaching all the appropriate sites of action. In the case of quipazine, however, no explanation is apparent for the inability of this direct 5-HT agonist to induce the 5-HT syndrome. Although Green et al. (1976b) reported that quipazine (25 and 50 mg/kg) exhibited a motor syndrome similar to that induced by L-tryptophan following tranylcypromine, they did not provide data detailing the frequencies with which the component symptoms were observed.

The ineffectiveness of the two 5-HT antagonists in blocking the 5-HT syndrome induced by CDM likewise cannot be easily explained. Since PCA was about 20 times as potent as CDM in its ability to induce the syndrome, it is unlikely that these results are due to greater affinity of CDM (as compared to PCA) for the 5-HT receptors involved. CPP, 6-chloro-2-(piperazinyl)-2-pyrazine, is a new agent which also induces the 5-HT syndrome (Clineschmidt et al., 1977). In this case also the 5-HT syndrome was not antagonized by cinanserin. One other confounding finding was that the higher doses of cinanserin alone elicited hindlimb abduction, one of the components of the "5-HT syndrome"! Clineschmidt et al. suggested that the 5-HT motor syndrome could be due to central tryptaminergic activation to explain the ineffectiveness of the specific 5-HT antagonist cinanserin. However, this possibility was not supported by the effective antagonism by cinanserin of the PCA-induced motor syndrome observed in this study. Thus, these latter findings are difficult to explain by the simple concept that the serotonergic syndrome specifically reflects increased serotonergic transmission.

The behavorial symptoms other than the components of the 5-HT syndrome (Table 11) deserve further comment. Although the stereotypic behavior observed with PCA, CDM and quipazine is usually associated with dopaminergic agonists, this stereotypic activity was readily antagonized by cinanserin. Quipazine-induced stereotypy had previously been shown to be abolished by antiserotonergic agents as well as by dopamine receptor antagonists, leading Grabowska et al. (1974) to suggest that serotonergic as well as dopaminergic

pathways are essential for the full expression of the stereotypy. Our preliminary observation of a block of CDM-induced stereotypy by both cinanserin and by haloperidol is consistent with Grabowska's suggestion.

In view of these contradictions and uncertainties, the concept of a specific 5-HT syndrome is questionable and needs further validation. CDM certainly has a number of behavioral effects in rats at sublethal doses, as described above and summarized in Table 13, but whether they are wholly due to serotonergic actions is dubious in view of the ineffectiveness of 5-HT blockers in preventing their expression in most instances. In those cases where methysergide was effective i.e., forepaw treading, head weaving, and tremor, even if 5-HT is involved, it remains to be shown whether this response is due to an increase in brain 5-HT through CDM's MAO inhibitory action or whether other direct or indirect serotonimimetic actions are occurring.

SECTION SIX

INHIBITION OF PROSTAGLANDIN SYNTHESIS BY FORMAMIDINES

INTRODUCTION

As presented in the previous Sections, CDM possesses a number of actions (i.e. blockade of nerve conduction, inhibition of MAO, psychomotor stimulation, and induction of stereotypic behavior) which are known to be shared by cocaine. This encouraged us to make a further comparison between these compounds in regard to their relative antinociceptive (analgesic) activity in rats. CDM (40 mg/kg, ip) and cocaine (20 mg/kg, ip) both increased tail flick latencies and raised the threshold for vocalization induced by electrical stimulation of the tail. The narcotic antagonist, nalorphine (5 mg/kg, sc), did not block CDM- or cocaine-induced antinociception. Lidocaine produced analgesic responses only at doses which resulted in loss of the righting reflex (i.e. 60-80 mg/kg). Because of this non-narcotic analgesic effect of CDM, which is probably not related to its local anesthetic actions, further studies of the mechanism of analgesia were conducted to detect any aspirin-like analgesic properties of CDM using the isolated guinea pig ileum. Aspirin, a prostaglandin synthesis inhibitor, cause depression of the electrically-induced ileum twitch which is reversed by prostaglandin E_1 (PGE_1) but not by the morphine antagonist, naloxone. PGE_1 is believed to stimulate the release of the actual transmitter, acetylcholine, in this preparation (Ehrenpreis *et al.*, 1976). When CDM was found to block the ileum twitch, further studies were performed to define other aspirin-like actions of CDM (antipyretic, anti-inflammatory) and its activity as a PG synthesis inhibitor.

MATERIALS AND METHODS

Male Sprague-Dawley rats (300-400 g) were obtained from Laboratory Supply Co., Indianapolis, Indiana. Chlordimeform and amitraz were kindly provided by Ciba-Geigy Corporation and the Upjohn Co. respectively. They were recrystallized before use to a final purity of over 98%.

The drugs included: acetycholine iodide (Calbiochem); clonidine HCl (Catapres, Boehringer Ingelheim, Ltd. Dist.); indomethacin (Indocin, Sigma Chemical Co.); morphine sulfate (Mallenkrodt); naloxone HCl (Narcan, Endo Laboratories); l-norepinephrine bitartrate (Sigma Chemical Co.); phentolamine mesylate, USP (Regitine, Ciba Pharmaceutical Co.); tolazoline HCl (Priscoline, Ciba Pharmaceutical Co.); and prostaglandin E_1 (PGE_1, Sigma Chemical Co.). Indomethacin and PGE_1 were dissolved in 95% ethanol and stored in the freezer until needed. All other compounds were prepared fresh in distilled water.

Guinea pig ileum preparation

Strips of longitudinal muscle were obtained from the guinea pig ileum as described by Rang (1964). The strips were equilibrated for one hour in 37° Krebs solution aerated with 95% O_2-5% CO_2, and were "field" stimulated as described by Paton (1957). A Grass S88 stimulator was used to deliver square wave pulses (60 V, 2-4 msec, 0.1 Hz) and the submaximal (approximately 80% maximum) contractions were recorded on a Grass Model 5 polygraph via Grass FT-03 transducers. Postsynaptic effects were assessed using cumulative ACh concentration responses according to the method of Ariens et al., (1964).

Antipyretic and anti-inflammatory assays

Fever was induced in 24-hour fasted rats by the subcutaneous injection of a 15% suspension of dried brewer's yeast (10 mg/kg). Fifteen hours later the test drugs, suspended in 1.5% methylcellulose, were injected intraperitoneally and body temperature was monitored rectally over a further 7 hours. Edema was induced by injection of 0.05 ml of 1% suspension of carrageenin (Viscareen, Marine Colloids) into the plantar tissue of the left hind paw (Winter et al., 1962). The formamidines were administered as in the antipyretic assay, 30 min before the carrageenin. The resultant edema was expressed as the percent difference in paw volume, measured by mercury displacement, compared to the volume of the untreated right hind paw.

Assay of prostaglandin synthesis

PG synthesis was assayed using bovine seminal vesicle microsomes (Flower et al., 1973). Lyophilized microsomes (1.5 mg) were incubated with ^{3}H-arachidonic acid (5 Ci per mmole) at pH 8.2 in the presence of glutathione (5 mM) and epinephrine (5 mM) for 5 min. The reaction (0.51 ml) was terminated with 0.25 ml 1 N HCl, and extracted with 2 ml ethyl acetate. Authentic PGE_2 was added to a 1 ml portion of the extract and the ethyl acetate was removed. The residue was taken up in 0.1 ml ethanol and chromatographed on silica gel plates in ethyl acetate:acetone:acetic acid (90:10:1). The PGE_2 spot was visualized by exposure to iodine vapor, scraped off, and counted in a scintillation counter. Control reactions with a boiled enzyme sample were run similarly, and the PGE_2 counts from the other incubations were corrected accordingly. Corrected rates of synthesis of PGE_2 (arachidonate at 0.4 μM final concentration) in the absence of inhibitors ranged from 15-20 pmoles per 5 min per mg protein. At least five concentrations of each inhibitor were used, without preincubation with the enzyme before addition of 0.4 or 50 μM arachidonic acid. From two to five replicates of each assay were run. I_{50} (50% inhibitory) concentrations were determined graphically from plots of % inhibition of PGE_2 synthesis against log of inhibitor concentration.

RESULTS

CDM produced a dose-related inhibition of the twitch responses of the guinea-pig longitudinal muscle preparation induced by electrical stimulation (Figures 22 and 23). The ED_{50} as determined by reverse regression analysis

was 2.9 x 10^{-5} M (7 trials, Figure 22A), with a 95% confidence interval of 2.4 - 3.6 x 10^{-5} M. At this concentration, ACh-induced contractions were reduced by approximately 15%.

As illustrated in Figure 22B, ACh-induced contractions were reduced by approximately 30% in the presence of 10^{-4} M CDM (8 trials), a concentration that depressed twitch responses by approximately 70% (Figure 22A). ACh contractions were enhanced by the presence of 10^{-8} M PGE_1 (8 trials, Figure 22B). ACh responses, in the presence of both 10^{-4} M CDM and 10^{-8} M PGE_1, were not appreciably different from control ACh responses (8 trials, Figure 22B).

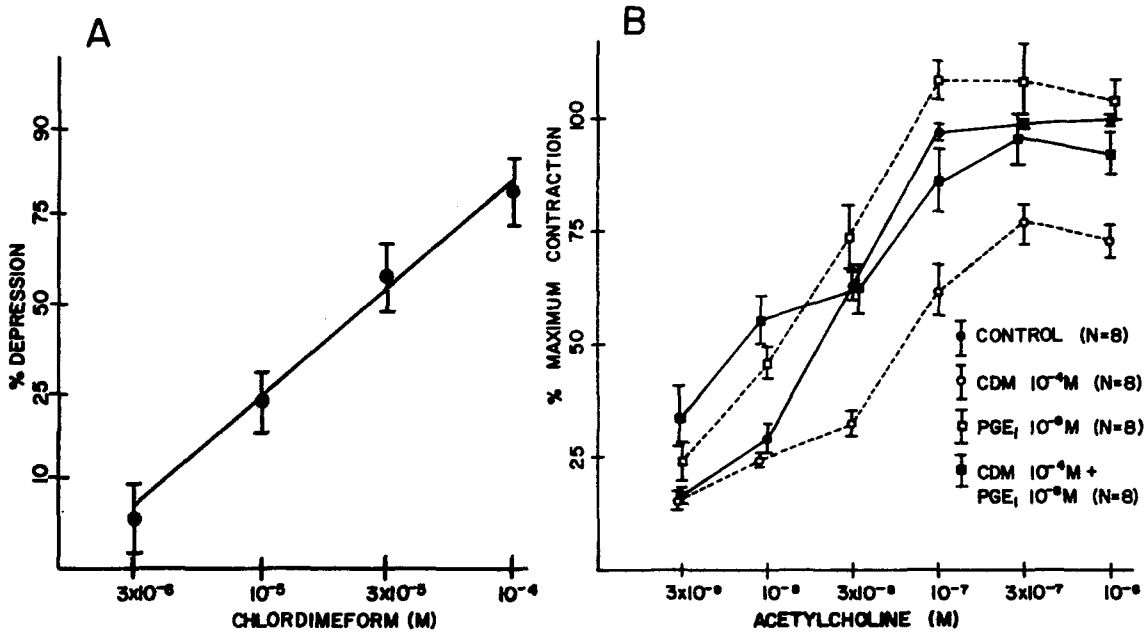


Figure 22. A: CDM-induced depression of the electrically stimulated twitch height. Ordinate: mean ± SE (n = 7) percent depression from control height spaced on a probit scale. Abscissa: log cumulative concentration of CDM. B: ACh responses in the presence of CDM and PGE_1. Ordinate: mean ± SE (n = 8) percent of maximum control ACh contraction. Abscissa: cumulative ACh concentration.

Twitch depression induced by CDM was not antagonized by naloxone (3 x 10^{-7} M to 10^{-4} M, 3 trials, Figure 23A). In contrast, twitch depression produced by morphine (ED = 3 x 10^{-7} M), was readily reversed by naloxone (3 x 10^{-7} M, 2 trials). This concentration of naloxone did not affect control twitch height (2 trials). The depression induced by 3 x 10^{-5} M CDM (45.6 ± 4.7% of control) was reversed by 3 x 10^{-5} M tolazoline (117 ± 5.3% of control, n = 5, Figure 23B), but not by phentolamine (3 x 10^{-5} M, 3 trials, Figure 23C). Twitch depression produced by norepinephrine or by clonidine was readily reversed by either tolazoline (2 trials) or by phentolamine (3 trials). At 10^{-4} M (3 trials), phentolamine alone had only a slight depressant effect on the twitch response. In contrast, tolazoline alone (3 x 10^{-5} M) increased the twitch response to 143 ± 4.5% of control (n = 6).

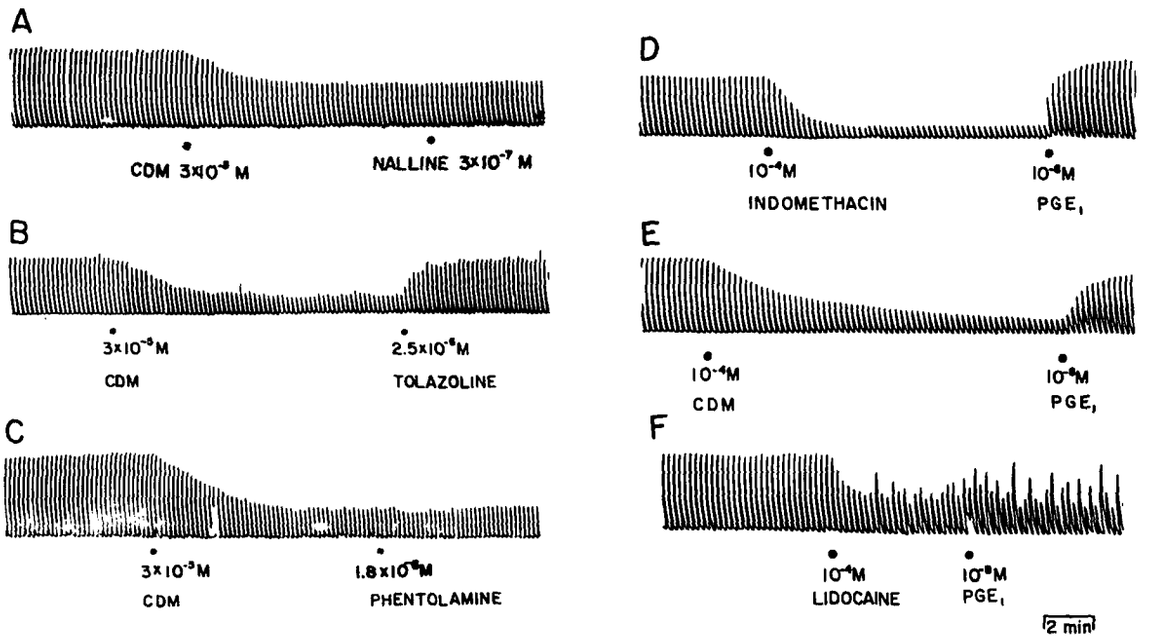


Figure 23. Effects of (A) naloxone; (B) tolazoline; and (C) phentolamine on twitch depression by 3 x 10^{-5} M CDM. Effects of PGE_1 on twitch depression induced by 10^{-4} M (D) indomethacin; (E) CDM; or (F) lidocaine.

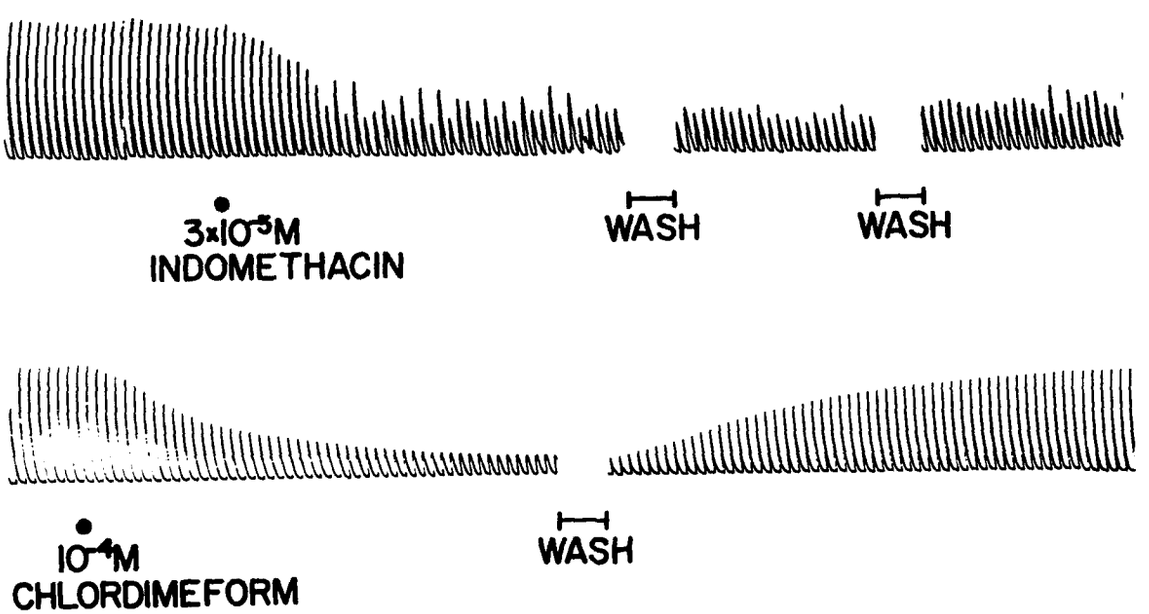


Figure 24. Effects of drug free wash on twitch depression induced by 3 x 10^{-5} M indomethacin (top polygraph tracing); or by 10^{-4} M CDM (lower tracings).

PGE_1 (10^{-8} M) readily reversed twitch depression induced by 10^{-4} M indomethacin (2 trials, Figure 23D), and by 10^{-4} M CDM (27.7 ± 2.3% of control with CDM: 86.9 ± 3.6% of control with CDM plus PGE_1, n = 9, Figure 23E). The depression by 3 x 10^{-5} M CDM (49.4 ± 1.8% of control) was readily reversed by PGE_1 (10^{-8} M, 102.4 ± 2.1% of control, n = 6). Washing the strip with fresh drug-free Kreb's buffer readily reversed twitch depression induced by CDM, but not that induced by the irreversible PG synthesis inhibitor, indomethacin (Figure 24). Depression of the twitch by lidocaine (50% at 10^{-4} M) was only partially and erratically reversed by PGE_1 (10^{-8} M, 5 trials, Figure 23F).

The antipyretic activities of the formamidines, CDM and amitraz, were assessed against yeast-induced fever in rats and compared to the effects of two known antipyretic inhibitors of PG synthesis, indomethacin and aspirin. Examination of the resulting dose responses presented in Fig. 25 yields the following rank-order of the compounds according to their antipyretic activity: indomethacin > chlordimeform > amitraz > aspirin. The reduction by chlordimeform of the rectal temperatures to 37° in the yeast-treated rats is consistent with the hypothermic effects of chlordimeform which we previously observed in normal rats (Pfister and Yim, 1977).

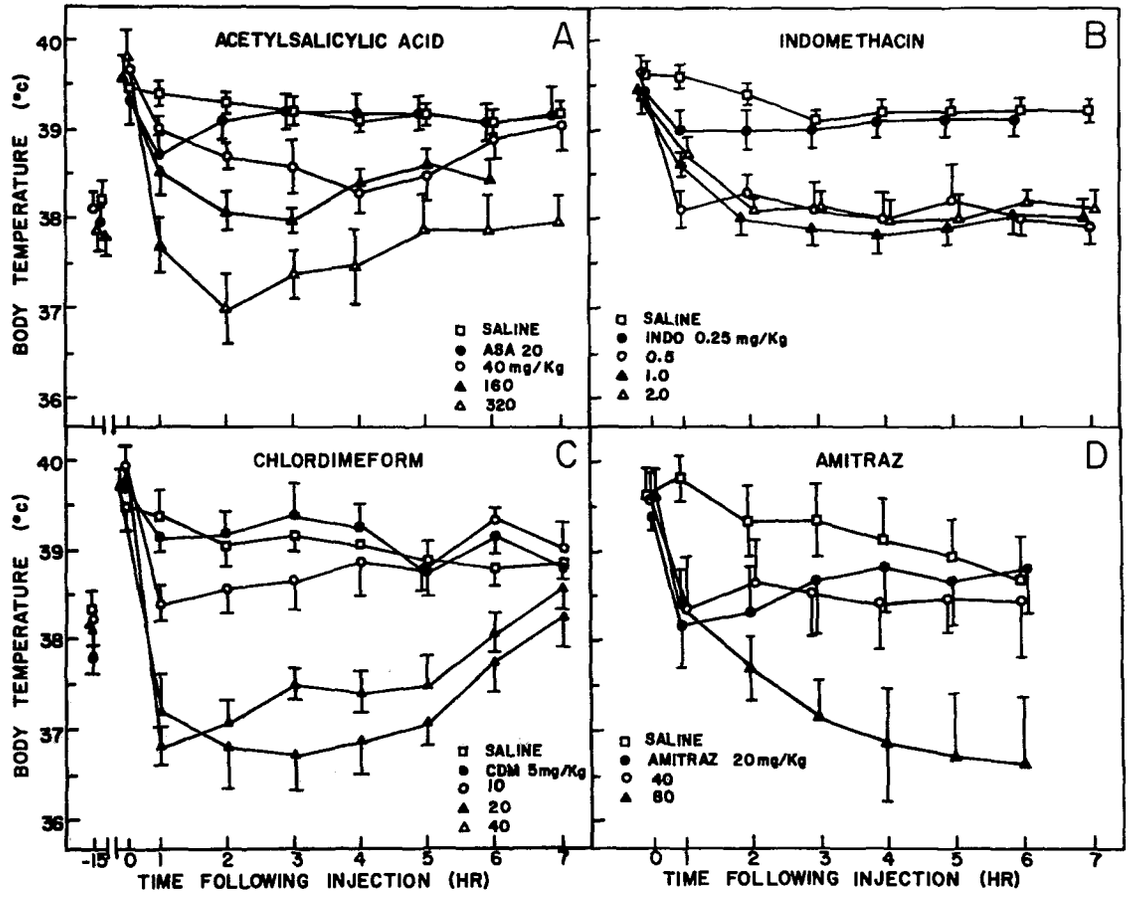


Figure 25. Antagonism of yeast-induced fever in rats by acetylsalicylic acid (aspirin), indomethacin, and two formamidine pesticides, chlordimeform and amitraz. Normal body temperature at time of administration of yeast (-15 hr) is shown in A and C. Values = $\bar{X}$ ± ; n = 6.

TABLE 12. INHIBITION OF PGE_2 SYNTHESIS BY BOVINE SEMINAL VESICLE MICROSOMES WITH TWO FORMAMIDINES AND TWO COMMON NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

Inhibitor	I_{50} (μM ± SD) at arachidonic acid concentration of:	
	0.4 μM	50 μM
Chlordimeform	34 ± 19	145 ± 36
Amitraz	880 ± 170	>1500
Indomethacin	0.4 ± 0.08	1.0 ± 0.2
Aspirin	790	3900

To assay the inhibition of PG synthetase we used a standard method based on the conversion of ^3H-arachidonic acid to PGE_2 by bovine seminal vesicle microsomes. The values in Table 12 reveal that chlordimeform is an inhibitor of PG synthesis of some potency while amitraz is less active, approximating aspirin in its activity under these conditions. Figure 26 demonstrates that chlordimeform and amitraz are anti-inflammatory agents. The control curves show the time course of the edema produced by the injection of carrageenin into the plantar tissue of the hind paw. Paw swelling was reduced by approximately 50% in the chlordimeform-treated rats (40 mg/kg). and by 25% in those treated with amitraz (80 mg/kg). Aspirin at 140 mg/kg reduced paw swelling by 60-65%. The ability of aspirin and indomethacin to reduce the edema induced by carrageenin and other irritants is well documented (Winter, 1965).

DISCUSSION

CDM produced a dose-related depression of the electrically-stimulated twitch responses of the guinea-pig longitudinal muscle preparation. In this study, the concentration of CDM which depressed the twitches by 50% (3×10^{-5} M) and 70% (10^{-4} M) reduced ACh-induced contractions by 15% and 30% respectively. In comparison, twitch depression by morphine (Paton, 1957), by NE (Paton and Vizi, 1969), by clonidine (Kroneberg and Oberdorf, 1974), and by indomethacin (Ehrenpreis *et al.*, 1974) primarily involve decreased ACh release, with minimal or no postsynaptic depression of ACh responses.

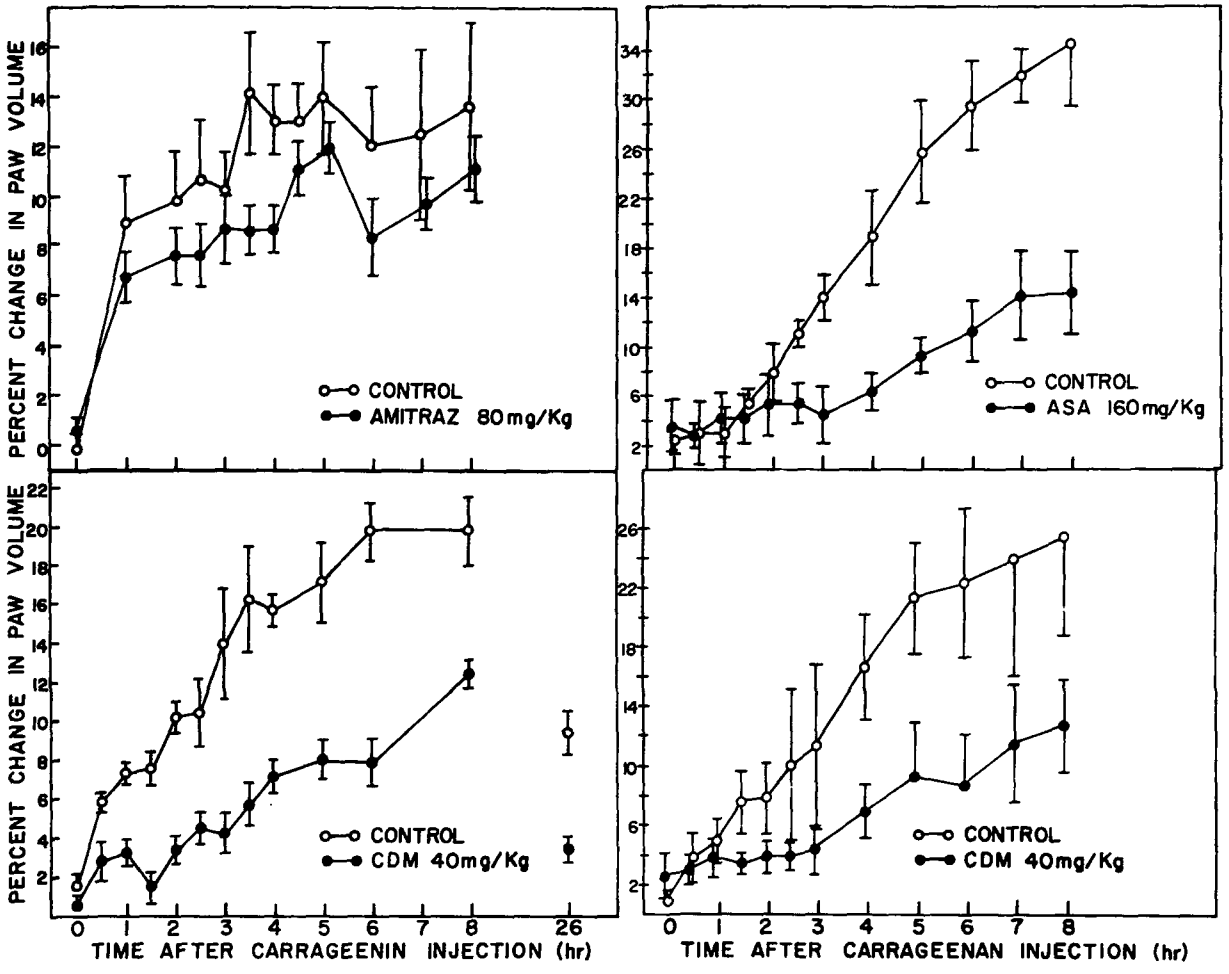


Figure 26. Antagonism of the carrageenin-induced edema of rat paw by the formamidines amitraz and chlordimeform and aspirin (ASA). Compounds were administered 30 minutes before the carrageenin. Values = $\bar{X} \pm SE$; n = 6.

The CDM-induced twitch depression was not like that produced by narcotic-type analgetics, because it was not antagonized by naloxone, which readily reversed the twitch depression by morphine (Paton, 1957). In the rat tail flick preparation, we had already found that CDM-induced antinociception is likewise not antagonized by naloxone.

The CDM-induced twitch depression was also not like the depression produced by appetite stimulants, clonidine, or another alpha-adrenergic agonist, norepinephrine, since phentolamine, which is an alpha antagonist, reversed the depression induced by either clonidine or norepinephrine, but did not antagonize the CDM-induced effect. The ability of tolazoline, another alpha-adrenergic antagonist, to readily reverse the depression of the twitch height by CDM may possibly involve the previously reported anticholinesterase action of tolazoline (Dzoljic, 1967). It is also unlikely that the local anesthetic action of CDM is primarily responsible for the depression of the

ileal twitch responses since PGE was much more effective in antagonizing CDM-induced than lidocaine-induced depression.

Thus, the present studies indicate that the twitch depression produced by CDM was most similar to that produced by the nonsteroidal anti-inflammatory agent, indomethacin; both drugs were consistently reversed by exogenously added PGE_1. Indomethacin is a well-known irreversible inhibitor of prostaglandin synthesis (Gryglewski, 1974). The results above show that chlordimeform is also an effective inhibitor of prostaglandin synthesis. The I_{50} values reported here for aspirin and indomethacin with the bovine seminal vesicle synthetase fall within the range of existing values in the literature (Gryglewski, 1974). The mechanism and kinetics of inhibition by the formamidines remain to be established, but preliminary results indicate that chlordimeform is a reversible, non-progressive inhibitor. In each case in Table 12 the inhibitory potency decreased at the higher substrate concentration. Normal concentrations of arachidonate at the active site of the synthetase *in vivo* are hard to estimate since it is believed that the fatty acid precursors for PG are stored as phospholipids and are released as needed by the action of phospholipases. Tissue levels of the free acid are very low (Hinman, 1972). It is significant that the potencies of the four compounds assayed as synthetase inhibitors are directly related to their antipyretic activities as shown in Fig. 25.

The washing studies with the guinea pig ileum uncovered an important difference between the actions of CDM and indomethacin. The persistence, after washing with fresh Kreb's buffer, of the twitch depressant action of indomethacin, has been attributed by Ehrenpreis *et al.*, (1976), to irreversible inhibition of PG synthesis (Ku and Wasvary, 1975). The reversibility of the twitch depression induced by CDM is in keeping with the conclusion that it is a reversible PG synthesis inhibitor. Thus, the guinea pig ileal preparation may be a convenient screen to distinguish reversible from irreversible inhibitors of PG synthesis.

We have shown that CDM has several aspirin-like actions (antinociception, anti-inflammation, antipyretic, and block of guinea pig ileum twitch). It is likely that aspirin achieves most or all of these actions by inhibition of prostaglandin synthesis (Vane, 1971; Flower, 1974; Robinson and Vane, 1974), an inhibition which we have found also to be shared by the formamidines. Since aspirin and indomethacin are progressive, irreversible inhibitors of PG synthetase (Gryglewski, 1974), and chlordimeform appears to be reversible and non-progressive, a direct comparison of I_{50} values can be misleading. However, we feel that inhibition of PG synthesis can provide a reasonable explanation of the several aspirin-like pharmacological actions of the formamidines.

Our present results are of interest from a number of aspects: (i) PG synthetase inhibitors with the general structural characteristics of the formamidines have not been previously reported. Most known inhibitors are acids rather than bases (Robinson and Vane, 1974). Further, the ability of pesticides to interfere with prostaglandin-dependent processes has not apparently been the subject of previous study. (ii) Early death from single

high doses of the formamidines has been attributed to direct depression myocardial and vascular muscle and accompanying respiratory depression, which are local anesthetic-like actions. It is intriguing to note in this regard the recent report that PG can antagonize the block of nerve conduction induced with local anesthetics, and that the PG synthetase inhibitor indomethacin also caused a conduction block which could be reversed by the addition of PGE_1 (Horrobin et al., 1977). Thus there may be a connection between the PG synthetase inhibiting and local anesthetic actions of the formamidines. (iii) Finally, the gastric ulcers, intestinal hemmorhaging, and death in 2-5 days observed in animals treated with indomethacin and other non-steroidal

TABLE 13. BEHAVIORAL AND PHARMACOLOGICAL PROFILE OF CDM IN THE RAT

	Dose (mg/kg, ip)					
Effects	1-20	21-40	41-60	61-80	81-100	200
HYPERPHAGIA	+++	+	-	-	-	-
HYPERGLYCEMIA	+	++	+++			
ANTIPYRESIS	+	++	+++			
ANALGESIA	+	++	+++	+++		
ANTIINFLAMMATORY	+	++	+++			
ANOREXIA	-	-	+	++	+++	
STRAUB TAIL	-	-	-	+	++	+++
HYPERTONICITY	-	+	++	+++	+++	+++
HIND LIMB ABDUCTION	-	+	++	+++	+++	+++
HYPERREACTIVITY	+	+	++	+++	+++	+++
INCREASED MOTOR ACTIVITY	+	+	++	+++	+++	+++
CIRCLING	-	-	+	++	+++	
BACKING	-	-	+	++	+++	
TREMOR	-	-	-	+	++	+++
HEAD WEAVING	-	-	-	+	++	+++
SPASTICITY-HYPERREFLEXIA	-	-	+	++	+++	+++
CONVULSIONS	-	-	-	+	++	+++
RESPIRATORY ARREST	-	-	-	-	-	+++
CARDIAC ARREST	-	-	-	-	-	+++
ELEVATED BRAIN 5-HT						++
ELEVATED BRAIN NE						+
DEATH	-	-	-	-	-	+++

Effects are rated subjectively according to intensity: + = present (low intensity); ++ = moderate intensity; +++ = marked intensity; - = not present; empty space = not known. The effects produced by CDM are arranged in decending rank order of appearance over the dose range indicated.

anti-inflammatory agents has been attributed to the inhibition of PG synthesis (Robert, 1976). This raises the question of whether the formamidines also may initiate these toxic consequences of the inhibition of PG synthesis.

Preliminary studies have shown that CDM in fact is much less potent than aspirin in inducing gastric ulcers. This may be attributable to the reversibility of the inhibition of PG synthesis by CDM (Yanagi and Komatsu, 1976) and the fact that it is a base rather than an acid like aspirin (Brune et al., 1976). Thus, CDM and related formamidines comprise a new and interesting class of nonsteroidal anti-inflammatory agents which lack some of the undesirable side-effects of current compounds.

A summary of the behavioral and pharmacological actions of CDM in the rat as observed in the studies reported in Sections 2 through 6, and in other studies in our laboratories, is provided in Table 13.

SECTION SEVEN

BIOTRANSFORMATION OF CHLORDIMEFORM AND ITS RELATION TO TOXICITY

INTRODUCTION

Clearance and metabolism of chlordimeform in mammals.

The metabolism of CDM and some of its intermediate metabolites has been investigated in dogs and goats (Sen Gupta and Knowles, 1970), rats (Knowles and Sen Gupta, 1970; Ahmad and Knowles, 1971a, 1971b; Morikawa et al., 1975; Benezet and Knowles, 1976b) and mice (Knowles and Benezet, 1977). In each case, when CDM labelled with ^{14}C in the ring methyl was orally administered, it was readily metabolized and rapidly eliminated in the first 24 hours, primarily in the urine. About 85% of the ^{14}C-CDM equivalents were eliminated in the rat urine (Knowles and Sen Gupta, 1970), while 70-80% was eliminated in the dog urine in the first 24 hours. Slightly less than 5% of the administered dose was accounted for in the bile of dogs with biliary cannulas in 72 hours (Sen Gupta and Knowles, 1970). The clearance of CDM was also rapid in goats. Excretion was faster in males, since 80% of the administered dose was eliminated in the urine of male goats compared to only 65% eliminated in the urine of lactating female goats in the same experimental period of 24 hours. The elimination of chlordimeform in feces after an oral dose is considered a minor route, since it accounted for only 7.5%, 0.6% and 1.8% of the administered dose in 72 hours in rats, dogs and goats respectively.

The pathway of excretion seems to be dependant on the route of administration since, after ip administration to mice of ^{14}C-CDM, 39% of the administered radiocarbon was eliminated in the urine and 45% in the feces in 24 hours (Knowles and Benezet, 1977), compared to about 81% recovered in the urine of mice in the same experimental period when ^{14}C-CDM was given orally (Ghali and Hollingworth, unpublished).

The elimination of two chlordimeform metabolites after oral administration was also rapid, but the relative importance of the routes of excretion were changed compared to CDM. When ^{14}C-labeled DCDM or CT were orally administered to rats, 35% and 71% of the radiocarbon from the two compounds were eliminated in the urine and 64% and 24% were eliminated in the feces respectively in 24 hours (Knowles and Sen Gupta, 1970; Ahmad and Knowles, 1971a).

In all of these studies, the majority of the radioactive materials in urine were unidentified water soluble materials, most likely glucuronide and sulfate conjugates, which yielded to arylsulfatase/β-glucuronidase cleavage.

No individual conjugates have been identified, but some of the radiocarbon-containing aglycones were identified after deconjugation as CDM, DCDM, NFT, and CT, in addition to other unidentified materials (Sen Gupta and Knowles, 1970). The organosoluble metabolites in the urine account only for 10-25% of the total radiocarbon. This relatively wide range of variation in the amount of organosoluble metabolites indicates a difference in the rates of degradation of CDM by different mammalian species. The major organosoluble metabolites recovered were NFT, CT and N-formyl-5-chloroanthranilic acid (NFA). In addition to these major metabolites, others such as DCDM, DDCDM, and 5-chloroanthranilic acid (CAA) were also present in all mammalian species studied. Recently, Knowles and Benezet (1977) added three urea derivatives to this list of metabolites, i.e. 1,1-dimethyl-3-(4-chloro-o-tolyl)urea (CDU), 1-methyl-3-(4-chloro-o-tolyl)urea (DCDU) and 4-chloro-o-tolylurea (DDCDU).

In vitro studies using various hepatic cellular subfractions have indicated that the maximum activity in degrading CDM in rats was associated with the microsomal subfraction (Ahmad and Knowles, 1971a; Morikawa *et al.*, 1975). The metabolites detected *in vitro* were well correlated qualitatively with those found *in vivo*. With the elucidation of the molecular structure of these metabolites, the sites of enzymatic attack on the formamidine molecule can be tentatively established and a metabolic pathway (Fig. 27) has been postulated (Ahmad and Knowles, 1971a; Morikawa *et al.*, 1975; Knowles and Benezet, 1977).

The initial metabolism of CDM is thought to be catalyzed exclusively by mixed function oxidases (MFO). One major pathway involves successive N-demethylations to yield DCDM and DDCDM. The initial N-demethylation reaction is NADPH and O_2 dependent, inhibited by SKF 525-A, and therefore is probably catalyzed by microsomal MFO (Ahmad and Kowles, 1971a). However, it is not known whether the second N-demethylation reaction is catalyzed by the same enzymes. Oxidative N-demethylation usually proceeds through an N-hydroxymethyl intermediate (McMahon, 1966). This intermediate, however, was not isolated with CDM *in vitro* (Ahmad and Knowles, 1971a; Morikawa *et al.*, 1975).

Both of the N-demethylated metabolites (DCDM and DDCDM), as well as CDM itself, can be cleaved to NFT in a reaction which overall appears to be a hydrolysis. The mechanism of this cleavage is an unresolved issue. Ahmed and Knowles (1971a) suggested a nonenzymatic hydrolytic mechanism for formamidine cleavage. Morikawa *et al.* (1975), although not entirely excluding this possibility, provided evidence that this reaction is probably mediated by an oxidative mechanism. This conclusion was based on the fact that production of the N-formyl derivative in hepatic microsomal incubation was significantly dependent on the concentration of NADPH, and was inhibited by SKF 525-A, a known inhibitor of microsomal drug oxidation. However, since the N-formyl derivative can be produced non-enzymatically even more readily from the two N-demethylated derivatives than from CDM itself, and since the N-demethylation reaction is also impaired by microsomal inhibition, then the decrease in the rate of production of the N-formyl derivative might also be an indirect result of the decrease in the rate of N-demethylation.

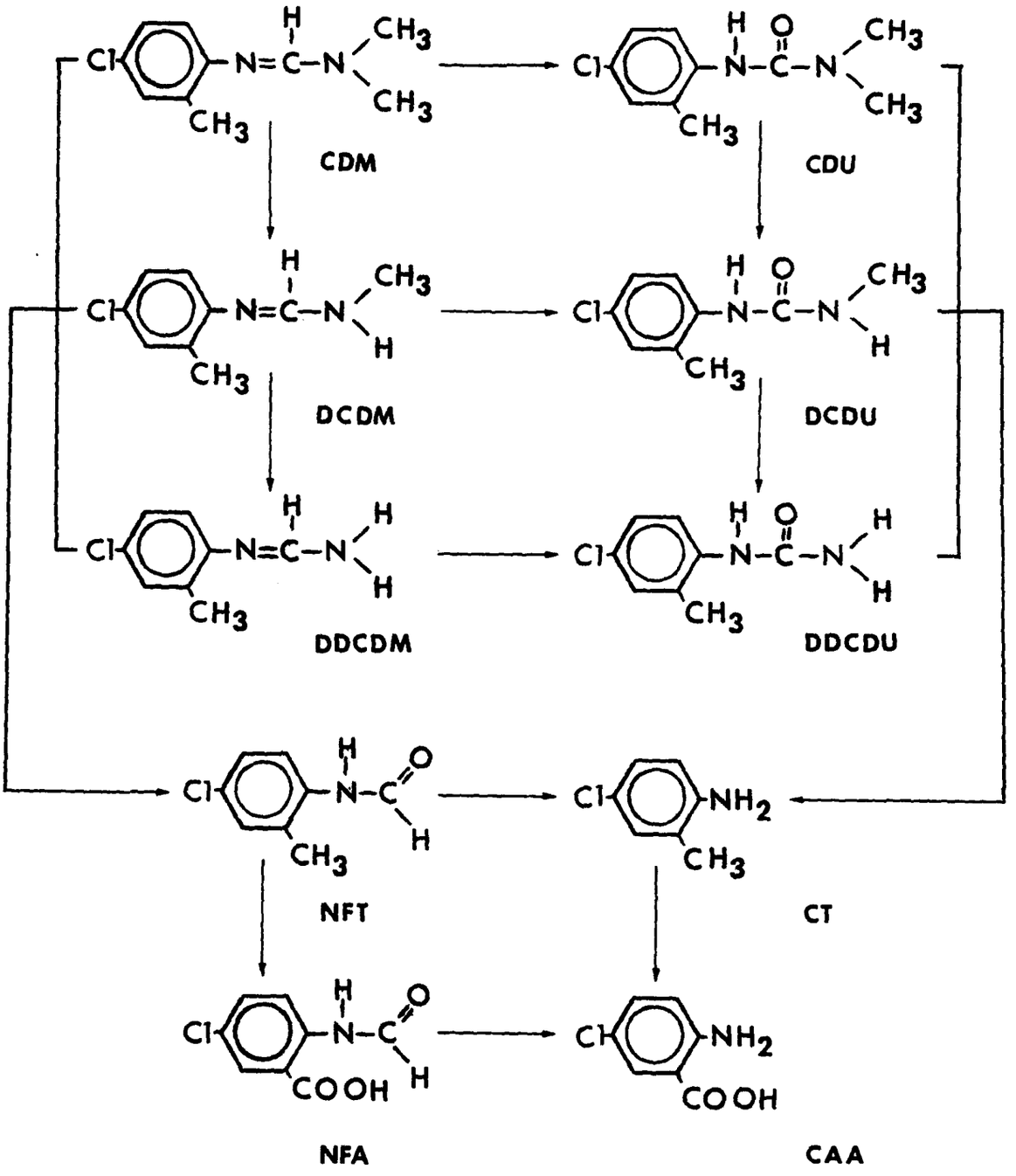


Figure 27. Metabolic map showing fate of chlordimeform in mammals.

NFT can be deformylated to CT. This reaction is inhibited by the organophosphate DFP, but not by SKF 525-A, and is probably carried out by the soluble liver formamidase which normally catalyses the deformylation of N-formylkynurenine, and is inhibited by DFP (Mehler and Knox, 1950; Shinohara and Ishiguro, 1970; Ahmad and Knowles, 1971b). This enzyme was also capable of deformylating the corresponding anthranilic acid (NFA) to CAA, a common metabolite from CDM.

CDU, one of the three urea metabolites recently reported by Knowles and Benezet (1977), may be formed by hydroxylation of the central (amidine) carbon of CDM followed by an enol-keto rearrangement. The other two urea metabolites could be formed by a similar mechanism from their respective formamidines or by sequential oxidative N-demethylation of CDU. Oxidative N-dealkylation is known to be an important pathway in the mammalian metabolism of substituted phenylurea herbicides (Geissbuhler et al., 1975). CT could also be produced by a direct cleavage of the ureas. Direct cleavage of substituted phenylureas in mammals to yield substituted anilines has been postulated but apparently is not a major pathway (Geissbuhler et al., 1975). Therefore, it seems likely that most of the 4-chloro-o-toluidine is derived by deformylation of NFT. In addition to the production of CT and NFT, which with their conjugates, are major terminal metabolites, an additional reaction has been observed. This involves oxidation of the ring methyl moiety yielding various anthranilic acids (Knowles and Benezet, 1977). It is not known at what stage of metabolism the ring-methyl group is oxidized, but ring-methyl oxidation products (alcohol, aldehyde, or acid) with an intact formamidine group have not been reported.

CDM is not a substrate for mammalian hepatic glutathione transferases (Ghali, G. and Hollingworth, R. M., unpublished). No in vitro studies of the conjugation of CDM or its metabolites by glucuronyl transferases, sulfotransferases, or other conjugating enzymes have been reported, although from the results described on the aglycones present after urinary deconjugation, direct conjugation is a possible reaction with formamidines (Sen Gupta and Knowles, 1970).

MATERIALS AND METHODS

Synthesis of chlordimeform and its major metabolites

Chlordimeform (CDM) and its N-demethylated metabolites, DCDM and DDCDM, were synthesized by coupling 4-chloro-o-toluidine with N,N-dimethylformamide, N-methylformamide, or formamide in the presence of phosphorous oxychloride (Hollingworth, 1976).

In a typical synthesis of chlordimeform, N,N-dimethylformamide (19 g, 0.260 moles) in 25 ml of dry benzene was added to phosphorous oxychloride (12 g, 0.078 moles) in 20 ml of dry benzene in a 250 ml three-neck flask attached to a water condenser. The mixture was stirred at room temperature for 30 min, then 4-chloro-o-toluidine (7.4 g, 0.052 moles) in 30 ml of dry benzene was added at room temperature with stirring for a further 3 hr.

In the case of DCDM and DDCDM, the order of addition of reactants was changed in order to avoid the accumulation of a sticky intermediate complex i.e., the formamide and the toluidine were added simultaneously to the phosphorous oxychloride in the flask. Furthermore, the formamide was dissolved in acetonitrile and the stirring continued for only one hr at room temperature.

In the case of CDM and DCDM, the precipitate from this reaction (crude hydrochloride salt) was collected by filtration and treated with 250 ml of ice-cold 1 N sodium hydroxide under cooling and continuous stirring for about 10 min. The oily layer was then extracted with 100 ml of benzene. The benzene layer was washed with water and dried over anhydrous sodium sulfate. The compound was either redistilled (CDM; $b_{0.3}$ 103-108°, n_D^{24} 1.5918), or recrystallized (CDM, DCDM, and DDCDM) several times until purity was evident by thin layer chromatography (Whatman K5F silica gel, developed with benzene:diethylamine, 95:5) and melting point (Fisher-Johns hot plate). CDM and DCDM were recrystallized from benzene while DDCDM was recrystallized from ether by the gradual addition of hexane.

In the case of DDCDM, which is more easily hydrolyzed under basic conditions, the precipitate was taken into a separatory funnel which contained prechilled ether and an equal volume of ice-cold 0.5 N sodium hydroxide, and shaken continuously for 2-3 min. The ether phase was then separated, washed several times with water, and dried over anhydrous sodium sulfate. The compound was recrystallized several times until purity was evident as before. All three of these formamidines as the free base gave colorless cystals with melting points of 30-31°, 91-92°. and 88° respectively. The hydrochloride salts of these formamidines were made by passing HCl gas into their ethereal solutions. The salts were recrystallized from ethanol-acetone (1:1) giving water soluble white crystals with melting points of 221-223°, 192-194° and 220-221° for CDM, DCDM and DDCDM respectively.

The formamidine metabolite, N-formyl-4-chloro-o-toluidine (NFT), was synthesized by refluxing 4-chloro-o-toluidine with an excess of formic acid in benzene with concomitant removal of the water produced (Fieser and Jones, 1955). After removing the solvent, the product was recrystallized from 1:1 ethanol-acetone mixture producing colorless crystals with a sharp melting point at 120°C.

The urea metabolite, 4-chloro-o-tolylurea (DDCDU). was synthesized by the reaction of equimolar potassium cyanate and 4-chloro-o-toluidine in acetonitrile (Geissbuhler et al. 1975) at room temperature. The urea produced in this reaction precipitated as a highly insoluble product in good yield. The product was purified by washing the precipitate several times with acetonitrile and recrystallization from dimethylsulfoxide. The material so obtained was a white powder (m. 204°).

1-Methyl-3-(4-chloro-o-tolyl)urea (DCDU) was synthesized by the reaction of equimolar amounts of 4-chloro-o-toluidine and methyl isocyanate in benzene. The isocyanate was added to the toluidine with stirring at 40°.

The precipitate was filtered off and washed with water and then with acetonitrile and recrystallized from dimethylsulfoxide (m. 213°).

The structures of these compounds were confirmed by elemental analysis, and infrared, nuclear magnetic resonance, and g.c.- mass spectroscopy. 4-Chloro-o-toluidine was purified from the commercial material (Aldrich Chemical Co.) by vacuum distillation. A further metabolite, 5-chloroanthranilic acid, was obtained from Ciba-Geigy Agrochemicals, Greensboro, NC.

Chlordimeform labeled with ^{14}C in the ring methyl group with specific activity of 20 μCi per mg (4 mCi per mmole) and a radiochemical purity of 96-99% (major impurity NFT, formed on storage) was obtained from Ciba-Geigy Agrochemicals. Other ^{14}C-labeled metabolites were biosynthesized by incubating 20 μCi of ^{14}C-chlordimeform for 20 min with the microsomal fraction of mouse liver and NADPH (1.7 mM) in Tris buffer (pH 7.6) at 37°, and subsequent extraction of the metabolites with chloroform, and purification by preparative two-dimensional TLC (Knowles and Benezet, 1977). Compounds were eluted from the TLC plates with acetone.

Effect of mixed function oxidase inducers and inhibitors on the acute toxicity of CDM and its N-demethylated metabolites

The mice were pretreated with either of two hepatic mixed function oxidase (MFO) inhibitors or with one of three MFO inducers. The inhibitors SKF 525-A (in 0.9% NaCl) and piperonyl butoxide (in corn oil) were administered ip at the rate of 50 and 400 mg/kg respectively two hours prior to subsequent treatment with graded oral doses of CDM, DCDM, or DDCDM as described before. In the case of the inducers, phenobarbital (in saline solution) was administered ip at four successive daily doses of 50 mg/kg prior to assay on day five. Aroclor 1254 (in corn oil), a polychlorinated biphenyl mixture, was given once at 500 mg/kg ip five days prior to treatment, and 3-methylcholanthrene (in corn oil) was administered ip in two successive daily doses of 40 mg/kg with the second dose two days prior to the toxicity tests. Control animals were pretreated with the vehicle alone. LD_{50}'s were determined as described in Section 1.

Subcellular fractionations

Animals were killed by a blow to the head. The desired tissues such as livers, brains, and lungs were rapidly removed and washed with cold 0.25 M sucrose solution. The tissues were then weighed, minced, and homogenized in fresh prechilled sucrose at the rate of 5 ml/g tissue using a Potter-Elvehjem type glass homogenizer fitted with a teflon pestle. The system was kept ice cold throughout. The microsomal and mitochondrial fractions were isolated by differential centrifugation, initially 600 x g for 10 min, then the supernatant was spun down at 12,000 x g for another 10 min to isolate the mitochondrial fraction, and then by further centrifugation of the supernatant at 105,000 x g for one hr using a Beckman L2-65 ultracentrifuge to separate the microsomal fraction. The resulting microsomal pellet was kept frozen at -20° for a maximum of four days before determination of the MFO activities.

The supernatant from the 105,000 g spin was used as the source of soluble enzymes. It and the mitochondrial fraction was used within two hours of preparation. Hepatic microsomes were also prepared from mice pretreated with MFO inducers or inhibitors as already described.

Metabolism of chlordimeform in vitro

Microsomal pellets (or mitochondrial fractions) of hepatic, brain, or lung tissues were resuspended in ice-cold 0.05 M Tris buffer (pH 7.6 at 37°) to a final concentration corresponding to 5 g of liver and 20 g of brain or lung tissues per 100 ml. Soluble fraction of liver was diluted 5 times with buffer. Rates of metabolism were determined at 37° in an incubating shaker in open 50 x 5 mm tubes containing 4.2×10^{-4} M ^{14}C-labeled substrate (CDM, DCDM, or DDCDM), 10 µl 10^{-2} M NADPH in the Tris buffer, and 50 µl of microsomal (or mitochondrial) suspension or liver soluble fraction. The final incubation volume was 60 µl. The substrate was added as a solution in hexane and the hexane was carefully evaporated before addition of the other components. Five µl samples were taken from the incubating mixture at several different times over 10 min after the addition of enzyme, spotted on silica gel TLC plates (Whatman K5F, 250 µ) along with the chromatographic standards in some runs, and developed in a two dimensional system modified from Knowles and Benezet (1977). The first system consisted of benzene:acetone:diethylamine (95:5:5), while the second system consisted of benzene:dioxane:acetic acid (90:10:4). Individual metabolite zones were located by autoradiography on X-ray film and scraped for scintillation counting in a toluene base scintillation cocktail containing 0.4% 2,5-diphenyloxazole and 4% Cab-O-Sil powder. The nature of the metabolites was confirmed by cochromatography in the system above and by mass spectrometry in some cases.

To study the effect of the absence of molecular oxygen on the microsomal metabolism of CDM, a modified Warburg vessel with two gas valves was used. The enzyme and NADPH were placed in one compartment while the substrate was placed in the other. A source of nitrogen was connected to the gas inlet while both valves were open. The nitrogen was allowed to pass for 10 min before the valves were closed. This method gave equivalent results to a second one in which the flask was alternately evacuated and then flushed with N_2. However, the latter method resulted in foaming and possible enzyme denaturation. The vessel was warmed to 37° and the enzyme and NADPH were admitted to the substrate compartment and mixed well before incubation for 10 min in a shaking water bath at 37°. The reaction was stopped by dropping the vessel in a cooling mixture (-20°).

The effect of carbon monoxide on the microsomal metabolism of CDM was also studied by bubbling CO gas for 5 to 10 min at a rate which did not cause foaming into a mixture of the enzyme and NADPH before adding them to the substrate in the usual incubation tubes.

Metabolism of chlordimeform in vivo

Male Swiss white mice (23-25 g) were orally dosed with ^{14}C-chlordimeform after pretreatment with piperonyl butoxide, phenobarbital,

3-methylcholanthrene, or vehicle as previously described. Four mice were used for each treatment and each mouse was given 10 μCi of the toxicant (1:1 mixture of labelled and unabelled CDM) in 0.25 ml corn oil. This dose of CDM amounted to 40 mg/kg. After 40 min, the animals were sacrificed. Blood was collected in heparinized vials. Livers and brains were rapidly removed, placed in preweighed scintillation vials, and immediately frozen in an acetone/dry ice bath and stored at -20° for analysis. Total tissue radioactivity was assayed using a 50 mg sample of well-macerated tissue in a scintillation vial. The tissue was digested overnight with 0.5 ml of Soluene-100 (Packard Instrument Co., Downers Grove, IL) at room temperature, bleached with 0.5 ml 30% hydrogen peroxide, and then treated with 0.2 ml glacial acetic acid to overcome chemiluminescence. Scintillation cocktail (10 ml, Aquascint 1, ICN) was added to the vials which were counted in a scintillation spectrometer (Packard Tri-Carb 3310). Efficiency of counting was determined with ^{14}C-toluene internal standard.

To study the nature and magnitude of the individual metabolites, about 200-400 mg of each tissue was weighed and homogenized in 2 ml 2% HCl, 0.5 ml 10% trichloroacetic acid (TCA) was added, and the mixture was centrifuged for 30 min at 3,000 rpm. The supernatant was transferred to another tube and the pellet re-extracted with another 2 ml of 2% HCl followed by TCA and centrifugation as before. The two supernatants were combined, treated with another 0.5 ml of 10% TCA and spun down for a further 30 min. The clear acidic extract was then transferred to a larger tube, the pH was carefully adjusted to 9.0 using 1 N NaOH with rapid mixing, and the solution was extracted three times with prechilled ether. The combined ether extractswere dried over anhydrous sodium sulfate, and evaporated to near dryness using a gentle stream of nitrogen under cooling conditions to minimize evaporative losses of volatile components. The residual tissue material from the acidic extractions was further extracted with two portions of ether to remove any residual NFT. These two ether extracts were combined, dried over anhydrous sodium sulfate, and evaporated to near dryness as before. The ethereal extracts were analyzed by two-dimensional TLC/scintillation counting as already described. The nature of the metabolites was determined initially by cochromatography on TLC.

Rates of hydrolysis of chlordimeform and its N-demethylated metabolites

The rates of hydrolysis of CDM, DCDM, and DDCDM were investigated at pH 7.4 and pH 8.7. A 10 mM stock solution of each compound was made in methanol. One ml of each of these solutions was placed in a 12 ml graduated tube with glass stopper and made to 10 ml using 0.01 M Tris buffer which was prewarmed to 37°. A 5 μl sample (zero time) was taken right after mixing and before placing the tube in a shaking water bath at 37°. Samples (5 μl) were taken at 5 min intervals over the first 30 min then less frequently for 3 hr and every 24 hr until the reaction was completed.

The amount of the hydrolysis product, N-formyl-4-chloro-o-toluidine (NFT), was monitored by injecting the samples immediately into a Water Associates Liquid Chromatograph equipped with a 30 cm x 4 mm i.d. Micron Bondapak C-18 column, UV absorbance detector (Model 440) of a fixed wavelength (245 nm), and a Water Associates Pump Model M-6000A. An

attenuation unit full scale of 0.05 to 0.1, a flow rate of 2 ml per minute, and a pressure of 2000 psi were used. The mobile phase was a mixture of acetonitrile:water:methanol (45:45:10). All solvents were "L.C. Distilled in Glass" grade. Retention time for the hydrolysis product under these conditions was 3.5 min. Retention times for the parent formamidines were much longer (> 20 min). The experiment was duplicated for each compound at each pH and the results averaged. The pH of the reactions was monitored and was steady throughout the experiment. In order to ensure that the reaction had reached completion, the tubes were boiled for 30 min at the end of the reaction and the amount of the hydrolysis product was checked before and after boiling. However, no change in the amount of the hydrolysis product was observed.

The NFT peak heights were measured at each time interval and a first order plot relating time to $\log[(h_\alpha - h_o)/(h_\alpha - h_t)]$ was made, where h_α is the final peak height of NFT at t_α and h_o is the peak height at t_o, and h_t is the peak height at a given time, t. The pseudo first order rate constant (k) was calculated from the equation, k = slope x 2.303, and the half life ($t_{1/2}$) of each compound was calculated from the equation, $t_{1/2} = 0.693/k$.

RESULTS

Toxicity and symptoms in mice after oral exposure to CDM and its metabolites

Data regarding the acute toxicity of CDM and its major metabolites have already been shown in Table 1. Additional studies with two of the more minor urea metabolites (DCDU and DDCDU) indicated LD_{50} values over 500 mg/kg and an absence of the rapid excitatory symptoms typical of the three formamidines.

The symptoms elicited by lethal doses of CDM and the two N-demethylated metabolities, although not identical, are very similar. These compounds are rapidly acting and symptoms are manifested as restlessness, hyperreflexia, tremors, particularly of the head and forelimbs, developing to one or more episodes of clonic convulsions. Death occurs generally within the first hour during one of these convulsive episodes and is marked by labored breathing and gasping. The tremors are not seen in the case of animals poisoned with the N-demethylated derivatives and the convulsive stage is significantly longer. The CDM-treated animals usually show signs of locomotor difficulty partly due to frequent hyperextension of the hind legs a later stages of poisoning, but this was not seen with DCDM and DDCDM.

Since N-demethylations appear to be the only metabolic reactions leading to more acutely toxic products, and these are most likely catalyzed by microsomal mixed function oxidases (MFO), the effect of ip pretreatment with inhibitors or inducers of hepatic MFO systems on the toxicity of CDM was studied. The results are shown in Table 14. Surprisingly, the MFO inhibitors (SKF 525-A and piperonyl butoxide) had no effect on the toxicity of CDM. Neither did the MFO inducer, phenobarbital. The other two inducers, 3-methylcholanthrene and Aroclor 1254 had the even more paradoxical effect of decreasing the toxicity of CDM about 2-fold. The same trends were also seen in the effect of some of these pretreatments on the toxicity of DCDM and DDCDM. 3-Methylcholanthrene had a clear protective effect, approximately

doubling the LD_{50} for both DCDM and DDCDM. Aroclor 1254 was tested only with DCDM but had a comparable protective action. Piperonyl butoxide pretreatment again did not have any effect on the toxicity of DCDM or DDCDM.

TABLE 14. THE EFFECT OF SEVERAL PRETREATMENTS AFFECTING MICROSOMAL OXIDATIONS ON THE TOXICITY OF FORMAMIDINES TO MICE

		LD_{50} (±95% Conf. Intervals). mg/kg	
Pretreatment	Formamidine	Control	Pretreated
Piperonyl butoxide	CDM	233(183-295)	260(206-327)
SKF 525-A	CDM	290(223-377)	280(215-364)
Phenobarbital	CDM	250(225-279)	266(229-309)
Aroclor 1254	CDM	295(268-324)	510(425-612)
3-Methylcholanthrene	CDM	250(223-280)	410(360-462)
Piperonyl butoxide	DCDM	185(162-209)	190(168-214)
Aroclor 1254	DCDM	167(145-199)	350(273-447)
3-Methylcholanthrene	DCDM	167(145-199)	310(258-371)
Piperonyl butoxide	DDCDM	76(61-95)	84(67-105)
3-Methylcholanthrene	DDCDM	100(81-123)	170(132-210)

Non-enzymatic hydrolysis of chlordimeform and its N-demethylated metabolites.

Since it has been suggested that much of the NFT produced from these formamidines arises by non-enzymatic hydrolysis, and because this hydrolysis is a clear detoxication reaction (although potentially leading to mutagenic/carcinogenic anilines), we measured the rates of hydrolysis of CDM, DCDM, and DDCDM. As shown in Fig. 28 plots of log $[(h_\alpha - h_o)/(h_\alpha - h_t)]$ against time were linear, indicating a pseudo first order rate of hydrolysis for each compound. First order kinetics were observed over at least 60% of the total reaction. The rate constants (k) and half lives ($t_{1/2}$) calculated from these plots at 37° and either pH 7.4 or 8.7 are given in Table 15.

Under 'physiological conditions' i.e. pH 7.4 and 37°, the formamidines are relatively stable with half lives ranging from 161 min for DDCDM to 538 min for CDM. In each case, raising the pH to 8.7 approximately triples the rate of hydrolysis. The hydrolysis product in each case, NFT, could also be hydrolyzed further to CT. However, under the reaction conditions and times employed here, NFT was stable i.e. no hydrolysis was detected in 12 hr at pH 8.7.

Metabolism of formamidines by mouse enzymes in vitro

In order to determine which tissue(s) and subcellular fraction(s) were most active in degrading CDM, a preliminary study was conducted with mouse liver, brain, kidney, heart, skeletal muscle, and lung homogenates fortified

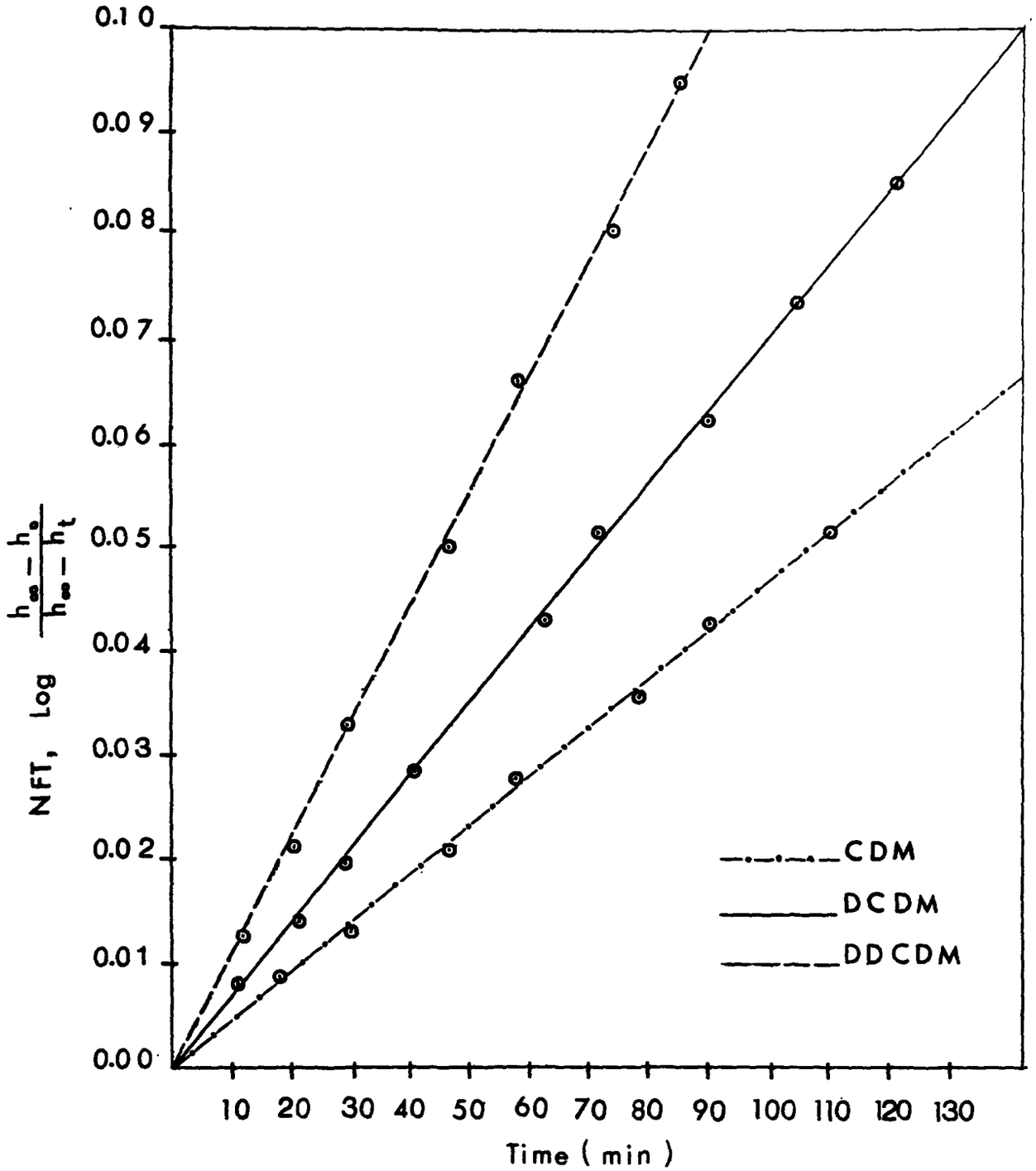


Figure 28. Rates of hydrolysis of chlordimeform and its N-demethylated metabolites at pH 7.4 and 37°.

TABLE 15. RATE CONSTANTS AND HALF LIVES FOR THE HYDROLYSIS OF CHLORDIMEFORM AND ITS N-DEMETHYLATED METABOLITES.

	CDM		DCDM		DDCDM	
	pH 7.4	pH 8.7	pH 7.4	pH 8.7	pH 7.4	pH 8.7
Rate constant (k, min^{-1})	1.29×10^{-3}	3.10×10^{-3}	2.78×10^{-3}	7.80×10^{-3}	4.33×10^{-3}	1.25×10^{-2}
Half life ($t_{1/2}$, min)	538	224	250	88.8	161	55.2

with NADPH (1 mM) and GSH (5 mM). These, and all other data for metabolism *in vitro* were corrected for the slight spontaneous breakdown of formamidines to NFT, as measured by blanks run concurrently with the appropriate enzyme preparation denatured by boiling. Significant metabolism (loss of CDM greater than 5% in 20 min) was seen only with the liver homogenate. Further studies with subfractions from these tissues were conducted and the results are shown in Table 16. Of the liver subfractions, CDM-metabolizing activity was confined almost exclusively to the microsomal fraction. It is significant that no detectable metabolism of CDM was seen with brain microsomes. The lung and liver microsomal fraction was virtually inactive in degrading CDM even when prepared from mice pretreated with the MFO inducers, phenobarbital and 3-methylcholanthrene. Since the liver microsomal system is so predominant in the biotransformation of CDM, further metabolic studies *in vitro* were confined to this fraction.

TABLE 16. RELATIVE ACTIVITIES OF MOUSE TISSUE MICROSOMES AND OTHER CELLULAR SUBFRACTIONS IN DEGRADING CHLORDIMEFORM.

Cellular subfraction	% of added CDM recovered unchanged[a]
Liver	
Microsomes	62.9
Mitochondria	98.2
Soluble	98.7
Brain	
Microsomes	99.1
Lung	
Microsomes	98.2
Control	
Buffer	99.4

[a]Means of two independent experiments with two replicates each. Incubation time: 10 min.

The nature of the microsomal enzymes attacking CDM was investigated, with the results given in Table 17. In the absence of NADPH, the rate of CDM degradation was reduced virtually to zero. Replacement of O_2 by N_2 also greatly decreased the rate of metabolism (80% inhibition). Even in the presence of atmospheric O_2 pretreatment of the microsomal suspension with CO gas inhibited the degradation of CDM by about 75%.

Comparative metabolism of formamidines by mouse liver microsomes

The rates and pathways of microsomal metabolism of CDM, DCDM, and DDCDM were compared at several times over 20 min. The data presented in Table 18 for 10 min incubations are typical. In addition to the parent compound (CDM), five other types of metabolites were assayed; DCDM, DDCDM, NFT, CT and a 'Polar' fraction. This last fraction stayed on or near the origin in the

TABLE 17. METABOLITES FORMED IN REACTION OF ^{14}C-CHLORDIMEFORM WITH MOUSE LIVER MICROSOMES.

Reaction Conditions			% of Substrate recovered as[a]:			
Enzyme	NADPH	Gas	CDM	DCDM	DDCDM	NFT
-	+	Air	62.3	26.8	1.1	2.4
+	-	Air	98.4	0.0	0.0	0.4
+	+	N_2	91.5	3.2	0.1	0.2
+	+	CO	89.3	7.6	0.2	0.6

[a]Means of two independent experiments with two replicates each. Incubation Ortime: 10 min.

2-dimensional TLC system employed. The nature of the constituents was not determined, but rechromatography with a more polar developing system showed at least 7 components. The nature of the major metabolities (CDM, DCDM, DDCDM and NFT) was confirmed by GC-MS Analysis. Rather small amounts of the urea derivatives (CDU, DCDU, and DDCDU) were detected (less than 5%). Since these compounds are neither highly toxic, nor major metabolites they were not quantified further.

Liver microsomes degraded the three formamidines in the order DDCDM>DCDM>CDM. DDCDM was very readily metabolized since only 15% of the added DDCDM was recovered intact after 10 min, and 60% was hydrolyzed to NFT, whereas 63% of the CDM added was recovered unchanged. Only small amounts of DDCDM were recovered starting from CDM and DCDM, although this may reflect the instability of DDCDM as much as a slow rate of synthesis. When CDM was the substrate, the major metabolite (27%) was DCDM with relatively small amounts of the other metabolites. Thus the first N-demethylation (CDM to DCDM) occurs readily. The rate of the second N-demethylation (DCDM to DDCDM) is hard to evaluate because of rapid further degradation of DDCDM, but it is toxicologically significant that DDCDM does not accumulate to any high level.

Effect of manipulations of MFO activity on the in vitro metabolism of chlordimeform.

Intraperitoneal pretreatments of mice with inducers and inhibitors of MFO considerably affected the rate and type of microsomal metabolism of CDM in vitro as shown in Table 19. Despite their lack of effect on toxicity, the MFO inhibitors, SKF 525-A and piperonyl butoxide, decreased both the rate of destruction of CDM and the rate of accumulation of the apparently more toxic metabolites DCDM and DDCDM. Phenobarbital, as expected, showed the reverse effect, causing a much more rapid loss of CDM and a considerably greater accumulation of the two N-demethylation products.

3-Methylcholanthrene and Aroclor 1254 were even more effective inducers as judged by the overall loss of CDM. However, in contrast to the other compounds, these two inducers also considerably increased the rate of production of NFT as well as the rate of N-demethylation. Compared to the controls, more of both DCDM and DDCDM accumulated after 3-methylcholanthrene

TABLE 18. *IN VITRO* METABOLISM OF CHLORDIMEFORM AND ITS *N*-DEMETHYLATED METABOLITES BY MOUSE LIVER MICROSOMES.

	Percentage of substrate recovered as[a]:					
Substrate	CDM	DCDM	DDCDM	NFT	CT	Polar
CDM	62.72 ± 1.58	26.88 ± 1.10	1.02 ± 0.30	4.22 ± 0.70	1.10 ± 0.30	2.33 ± 0.70
DCDM	---	49.92 ± 2.20	1.83 ± 1.50	15.26 ± 1.36	7.43 ± 0.40	12.90 ± 1.35
DDCDM	---	---	15.17 ± 0.15	60.04 ± 1.40	9.23 ± 0.60	10.66 ± 0.90

[a]Mean (±SD) of two independent experiments with two replicates each. Incubation time: 10 min.

TABLE 19. EFFECT OF SEVERAL PRETREATMENTS AFFECTING MICROSOMAL OXIDATIONS ON THE IN VITRO METABOLISM OF CHLORDIMEFORM BY MOUSE LIVER MICROSOMES.

Pretreatment	Metabolites, percent as[a]: CDM	DCDM	DDCDM	NFT	CT	Polar
Control	62.72 ± 1.53	26.88 ± 1.10	1.02 ± 0.30	4.22 ± 0.70	1.10 ± 0.29	2.33 ± 0.69
Phenobarbital	40.04 ± 1.06	44.86 ± 0.12	4.09 ± 0.37	5.85 ± 0.07	1.06 ± 0.33	3.18 ± 0.33
3-Methylcholanthrene	32.78 ± 2.18	37.37 ± 4.00	1.40 ± 0.17	16.18 ± 1.33	1.56 ± 0.04	9.59 ± 0.33
Aroclor 1254	19.99 ± 0.46	54.26 ± 0.50	7.69 ± 0.30	8.54 ± 0.38	1.62 ± 0.18	5.35 ± 0.21
Piperonyl butoxide	75.03 ± 6.20	16.86 ± 4.71	0.61 ± 0.14	3.93 ± 0.40	0.93 ± 0.28	1.50 ± 0.57
SKF 525-A	70.81 ± 2.05	19.18 ± 0.16	0.67 ± 0.03	3.76 ± 0.01	1.60 ± 0.01	1.54 ± 1.10

[a]Means of two independent experiment with two replicates in each. Control is the average of ten independent experiments with two replicates in each. Incubation time: 10 min.

and Aroclor pretreatments. However, 3-methylcholanthrene caused only a small rise in the level of DDCDM compared to phenobarbital and Aroclor 1254. 3-Methylcholanthrene was also the most effective compound stimulating the pathways to NFT and polar metabolites.

Effects of manipulations of MFO activity on in vivo metabolism of chlordimeform.

The effect of pretreatment with the inducers phenobarbital and 3-methylcholanthrene and the inhibitor piperonyl butoxide on the levels of CDM metabolites in the liver, blood and brain was determined 40 min after dosage with CDM. The 40 min interval was chosen since this was the typical time for the development of severe toxicity symptoms and death at lethal doses of CDM. Recoveries of added CDM, DCDM, DDCDM, and NFT by this method of extraction ranged from 80-85%. The data in Tables 21 and 22 are corrected for this level of recovery. The recovery of CT was not checked and no recovery correction of the data for this compound was made.

The percentage of the dose present in the tissues after each treatment is shown in Table 20. For the purposes of calculation the total blood volume of the mice was taken as 6.0% of their body weight. Liver weight averaged 1.44 g and brain weight 0.42 g. Relatively large amounts of radioactivity were present in the liver with much less in the blood and brain.

TABLE 20. TOTAL RADIOACTIVITY IN TISSUES AFTER AN ORAL DOSE OF CHLORDIMEFORM.

	Total radioactivity as percent of the dose[a]		
Pretreatment	Liver	Brain	Blood
Control	11.15 ± 0.26	0.717 ± 0.071	1.23 ± 0.072
Phenobarbital	12.53 ± 0.55	0.694 ± 0.051	1.33 ± 0.081
3-Methylcholanthrene	11.16 ± 0.52	0.693 ± 0.037	1.37 ± 0.110
Piperonyl butoxide	6.36 ± 0.34	0.352 ± 0.051	0.65 ± 0.052

[a]Mean ± SD (n = 3). Time after dosage, 40 min.

Pretreatment with phenobarbital or 3-methylcholanthrene had no effect on the levels of radioactivity in the tissues. However, piperonyl butoxide unexpectedly reduced the amount of radiolabel by about 50% in all the tissues. This effect proved to be reproducible in a replicate run. The levels of the individual metabolites in these tissues are presented in terms of the relative percentage they represent of the total radioactivity recovered (Table 21) and in terms of their absolute levels (Table 22).

The data for the liver in Table 21 show that pretreatment with phenobarbital or 3-methylcholanthrene hardly affects the percentage of the radioactivity present as the parent compound, CDM, which averages about 10%. However, the MFO inhibitor, piperonyl butoxide radically changes this picture, reducing metabolism so that 56% of the radioactivity is present as CDM. Piperonyl butoxide also increases the proportion of DCDM present in the liver

but the other treatments have little effect in this regard. However, the proportion of DDCDM was much reduced by piperonyl butoxide, and much increased by the inducer pretreatments. NFT and polar metabolites were greatly reduced by piperonyl butoxide. The most notable change resulting from inducer pretreatment was the 140% increase in NFT after 3-methylcholanthrene which was not seen with the other MFO inducer, phenobarbital.

A rather similar picture is presented in the data for the blood and, to a lesser extent, for brain also. The few notable differences are the low percentage of DCDM in the blood and brain after 3-methylcholanthrene treatment, and the high percentage of NFT in the brain compared to the other tissues. In fact, 45% of the radioactivity in the brain is NFT in the 3-methylcholanthrene-treated animals compared to 18% in the controls. Clearly 3-methylcholanthrene increases NFT levels in the brain at the expense of DCDM. The same conclusion holds, to a lesser extent, in the blood also. As in the liver, so in the blood and brain, piperonyl butoxide greatly increases the proportion of label present as CDM, and, to a lesser extend, DCDM, while radically decreasing the other metabolites. Polar metabolites are less important in the brain, perhaps because of the blood-brain barrier.

Since the total levels of radioactivity in these tissues were virtually identical in the control, phenobarbital, and 3-methylcholanthrene pretreatments (Table 20), the above conclusions also apply when the data are presented as the percent of the total dose recovered in each form in Table 22. However, because piperonyl butoxide pretreatment reduced the total radioactivity in all the tissues, the data presented in Table 22 are significantly different for this pretreatment. It is these data which should be related to the observed toxicity in Table 1. Although CDM levels are still quite elevated after piperonyl butoxide, the DCDM concentration is lower than the control in the blood and brain, and the amount of DDCDM in all tissues is extremely low. This is true for the other metabolites (NFT, CT and polar fraction) also.

On the basis of the data in Tables 1 and 22, an attempt was made to correlate the effects of the several pretreatments on the toxicity of CDM with the levels of formamidines singly or in combination in liver, blood, or brain. In this comparison, the levels of CDM, DCDM, and DDCDM singly or in the combinations DCDM plus DDCDM, and CDM plus DCDM plus DDCDM (total formamidines) were examined. The only plausible correlations obtained are shown in Table 23. The effects on the toxicity of CDM are presented as a ratio of LD_{50} (control)/LD_{50}(pretreatment). A ratio of greater than 1.0 indicates that the pretreatment increased the toxicity of CDM while a value of less than 1.0 reveals a decrease in toxicity. Thus a direct correlation of this toxicity ratio with tissue levels of any 'active' compound(s) would be expected.

TABLE 21. EFFECT OF SEVERAL PRETREATMENTS AFFECTING MICROSOMAL OXIDATIONS ON THE IN VIVO METABOLISM AND DISTRIBUTION OF CHLORDIMEFORM IN MICE.

	—Metabolites as percent of total radioactivity in the liver[a]					
Pretreatments	CDM	DCDM	DDCDM	NFT	CT	Polar
Control	10.11±2.79	14.30±1.86	6.92±0.99	7.70±1.20	3.26±0.19	30.67±3.97
Phenobarbital	9.39±1.03	17.88±0.62	15.66±1.34	8.58±1.44	6.32±0.87	22.17±1.89
3-Methylcholanthrene	10.79±0.50	14.85±4.11	13.47±0.47	18.72±1.58	2.89±0.51	26.72±2.24
Piperonyl butoxide	56.47±1.47	26.11±1.55	3.88±0.25	3.49±1.33	3.09±0.84	4.52±1.34
	Metabolites as percent of total radioactivity in the blood[a]					
Pretreatments	CDM	DCDM	DDCDM	NFT	CT	Polar
Control	7.27±1.03	11.34±2.90	4.58±0.91	11.13±0.39	3.73±0.88	36.11±4.50
Phenobarbital	10.06±0.91	8.16±1.69	5.71±0.79	10.82±3.16	9.92±1.32	21.84±2.38
3-Methylcholanthrene	10.25±2.79	4.93±0.13	12.36±3.30	19.78±0.47	3.07±0.81	27.27±3.55
Piperonyl butoxide	61.59±1.98	13.15±0.88	1.01±0.07	3.74±1.20	2.54±0.37	4.04±0.35
	Metabolites as percent of total radioactivity in the brain[a]					
Pretreatments	CDM	DCDM	DDCDM	NFT	CT	Polar
Control	6.78±2.03	27.99±4.65	4.37±1.17	18.23±0.89	6.74±1.19	13.86±4.20
Phenobarbital	4.89±0.26	30.18±0.37	5.28±0.44	30.33±1.76	3.05±1.10	12.06±2.91
3-Methylcholanthrene	6.92±1.46	8.84±1.03	5.36±0.34	45.06±6.32	1.72±0.64	15.78±5.26
Piperonyl butoxide	33.05±2.70	32.80±1.95	1.16±0.11	4.27±1.20	3.90±0.79	4.62±1.29

[a]Mean (± SD). Three animals, duplicate assays on each extract.

TABLE 22. EFFECT OF SEVERAL PRETREATMENTS AFFECTING MICROSOMAL OXIDATIONS IN THE MOUSE ON THE IN VIVO METABOLISM OF CHLORDIMEFORM.

	Metabolites found in the liver as percent of total dose[a]					
Pretreatments	CDM	DCDM	DDCDM	NFT	CT	Polar
Control	1.12±0.28	1.59±0.17	0.77±0.10	0.86±0.12	0.36±0.02	3.42±0.52
Phenobarbital	1.16±0.14	2.21±0.11	1.93±0.83	1.06±0.19	0.78±0.11	2.74±0.24
3-Methylcholanthrene	1.20±0.11	1.64±0.04	1.50±0.04	1.86±0.02	0.32±0.07	2.99±0.04
Piperonyl butoxide	3.59±0.11	1.66±0.17	0.25±0.22	0.23±0.10	0.20±0.05	0.29±0.10
	Metabolites found in the blood as percent of total dose					
Pretreatements	CDM	DCDM	DDCDM	NFT	CT	Polar
Control	0.089±0.015	0.137±0.030	0.056±0.008	0.136±0.010	0.046±0.012	0.410±0.036
Phenobarbital	0.134±0.019	0.109±0.027	0.075±0.012	0.140±0.034	0.132±0.020	0.228±0.220
3-Methylcholanthrene	0.141±0.045	0.066±0.006	0.167±0.032	0.269±0.026	0.045±0.009	0.406±0.037
Piperonyl butoxide	0.398±0.022	0.086±0.011	0.006±0.001	0.024±0.008	0.016±0.003	0.025±0.003
	Metabolites found in the brain as percent of total dose[a]					
Pretreatments	CDM	DCDM	DDCDM	NFT	CT	Polar
Control	0.049±0.016	0.202±0.046	0.031±0.011	0.130±0.016	0.048±0.008	0.098±0.028
Phenobarbital	0.034±0.002	0.210±0.016	0.037±0.005	0.210±0.004	0.021±0.009	0.086±0.019
3-Methylcholanthrene	0.048±0.009	0.062±0.003	0.037±0.003	0.314±0.060	0.012±0.004	0.108±0.030
Piperonyl butoxide	0.118±0.022	0.116±0.024	0.004±0.001	0.015±0.006	0.014±0.005	0.016±0.004

[a]Mean (± SD). Three animals, duplicate assays on each extract.

TABLE 23. COMPARISON OF THE EFFECT OF PRETREATMENTS ON THE TOXICITY OF CDM TO MICE AND ON THE LEVELS OF SELECTED FORMAMIDINES IN THEIR TISSUE.

		% of dose as[a]:		
		DCDM in:		
Pretreatment	Toxicity Ratio[a]	Brain	Blood	Total Formamidines in Brain
Control	1.00	0.202	0.137	0.282
Phenobarbital	0.94	0.210	0.109	0.281
3-Methylcholanthrene	0.61	0.062	0.066	0.147
Piperonyl butoxide	0.89	0.116	0.086	0.238

[a]Calculated as LD_{50}(control)/LD_{50}(pretreatment). Data from Table 1.

Table 23 shows that only three correlations relate toxicity to metabolite levels in all the treatments with any degree of fidelity. The levels of DCDM in brain and blood (which might reasonably be expected to be correlated with each other) show the same general trend as the toxicity ratios but are not well correlated quantitatively with toxicity. However, by combining the levels of all formamidines present in the brain, a correlation is achieved which matches the pattern of toxicity changes quite well quantitatively.

DISCUSSION

Toxicity tests with CDM and its metabolites establish that only those compounds which retain the formamidine nucleus (CDM, DCDM, DDCDM) have a high acute toxicity and induce strong excitatory symptoms. Only after the initial excitatory phase (and most mortality) is complete do the mice show the prolonged depressed behavior typical of the non-formamidine metabolites. Because of the clear increase in toxicity and more rapid toxic action in the order CDM<DCDM<DDCDM (Table 1), the possibility that much, or all, of the toxicity of CDM is due to its N-demethylation products is plausible. This view is supported by the results of Benezet et al. (1978), who found that the N-demethylated analogs of CDM are more toxic than CDM to the rat and also act more rapidly to produce excitation when injected ivc. In each effect, DDCDM was the most active compound.

The data of Tables 16 and 17 demonstrate that the biotransformation of CDM in mice, including the N-demethylations, is largely attributable to the hepatic microsomal MFO system. This agrees with previous studies in rats (Ahmad and Knowles, 1971a; Morikawa et al., 1975). Little metabolism of CDM is accomplished by lung or brain tissues even after pretreatment of the mice with MFO inducers. From these results, one might predict that hepatic MFO inducers, by increasing the rate of N-demethylation, should increase the toxicity of CDM, while MFO inhibitors conversely should decrease its toxicity. But our data show that none of these predictions are correct. Neither of the MFO inhibitors (SKF 525-A and piperonyl butoxide) significantly changed the toxicity of CDM. Of the MFO inducers, phenobarbital pretreatment also did not alter the LD_{50} of CDM, while Arocloron or near the origin in the 1254 and

3-methylcholanthrene actually decreased the toxicity of CDM to about half its normal value.

Despite the unexpected results obtained in the toxicity studies, the MFO inducers and inhibitors do have their predicted effects on hepatic microsomal metabolism when administered *in vivo*. The data in Table 19 show that inhibitor pretreatments of the mice reduce the amount of CDM destroyed by the isolated microsomes. Even in the control microsomes the degradation of CDM was quite rapid (39% in 10 min), but this was greatly increased in microsomes from inducer-pretreated mice, with 60%, 67%, and 80% of the CDM destroyed after the phenobarbital, 3-methylcholanthrene, and Aroclor treatments respectively. The two MFO inhibitors had similar effects on the pattern of metabolites produced from CDM. All metabolites were reduced, although the effect was rather small with NFT compared to the decrease in DCDM, DDCDM, and polar metabolites. The three inducers, however, produced rather different changes in the spectrum of metabolites. Phenobarbital and Aroclor considerably increased the amounts of DCDM and DDCDM, with less effect on the amount of NFT. 3-Methylcholanthrene on the other hand increased the level of NFT by 260% but increased DCDM only by 35% and there was no increase at all in the level of DDCDM.

Two further conclusions can be drawn from these results. First, phenobarbital in known to increase the activity of those MFO reactions catalyzed by cytochrome P-450 while 3-methylcholanthrene induces predominantly the related cytochrome P-448 (Conney, 1967; Alvares *et al.*, 1973). Aroclor 1254, a mixture of polychlorinated biphenyls, induces both types of cytochromes (Alvares *et al.*, 1973). All three inducers increase the rate of *N*-demethylation of CDM, though the P-450 inducers are more effective, but the hydrolysis of the formamidines to NFT appears to be rather selectively increased by the P-448 inducers, particularly 3-methylcholanthrene. The same relationship is true for the 'polar' fraction. Thus much of the cleavage of the formamidines to NFT is probably catalyzed by cytochrome P-448.

It has been suggested (Ahmad and Knowles, 1971a), that the hydrolytic production of NFT is largely non-enzymatic, although Morikawa *et al.* (1975) concluded that the MFO system also might be involved. As pointed out earlier, this does not prove that the MFO system produces NFT directly. However, the data in Table 19 cannot be interpreted except in terms of a direct MFO-catalyzed hydrolysis of one or more of the formamidines. The rates of spontaneous hydrolysis of CDM, DCDM, and DDCDM are shown in Table 15. Although hydrolysis to NFT does occur, it is relatively slow e.g. at pH 7.4 and 37^{o}, as used in the MFO studies above, in the 10 min incubation period employed, the percentage hydrolysis of CDM, DCDM, and DDCDM to NFT would be 1.3, 2.7, and 4.2 respectively. This compares to the presence of 16.2% of the added CDM as NFT in the MFO assay after the 3-methylcholanthrene pretreatment, and this value has already been corrected for spontaneous production of NFT (boiled enzyme blank). The mechanism of this formamidine cleavage reaction is unknown. One possibility is that the primary MFO attack in this case is by hydroxylation of the amidine carbon which may lead either to formamidine cleavage and release of NFT or, by tautomerization, to the corresponding urea derivatives.

The second conclusion in comparing Tables 1 and 19 is that it is difficult to relate these *in vitro* metabolic results to the effects of the same pretreatments on toxicity. The two pretreatments which decrease the toxicity of CDM i.e. Aroclor and 3-methylcholanthrene do not reduce the level of any metabolite, and Aroclor greatly *increases* the level of the more toxic metabolites, DCDM and DDCDM. These pretreatments do reduce the amount of CDM remaining, but the LD_{50}'s of the other four treatment were not greatly different, yet the amount of CDM unmetabolized ranges from 40% to 75%.

A partial answer to this lack of evident correlation of toxicity and metabolism probably lies in Table 18 where the metabolism of CDM, DCDM, and DDCDM is compared in hepatic microsomes from untreated mice. Clearly the *N*-demethylated products are more rapidly degraded than CDM. DDCDM, the most toxic of these formamidines is also the least stable with only 15% of the parent compound recoverable after 10 min and 60% conversion to NFT. Toxicity and metabolic stability are therefore negatively correlated. Thus the more potent metabolites (DCDM, DDCDM) are also the ones likely to have the shortest survival times in the body.

Because of the confusing metabolism-toxicity relations so far developed, a study was performed to analyze the levels of each of the critical metabolites in liver, blood, and brain at a time chosen to coincide with the peak of symptoms in mice given lethal doses of CDM. Because of time and radioisotope limitations this study was confined to phenobarbital, 3-methylcholanthrene, and piperonyl butoxide as the pretreatments. The data for recovery of total radioactivity in the three tissues again gave an unexpected, but important, result. Phenobarbital and 3-methylcholanthrene pretreatments have no significant effect on the total ^{14}C present in any tissue compared to the control. Piperonyl butoxide reduces this to half the control value in each case. Thus the piperonyl butoxide must reduce uptake of the oral dose of CDM. Why this decreased uptake should occur is not obvious, especially since the piperonyl butoxide was given ip and the CDM orally. One possibility is that the uptake of CDM from the gastrointestinal tract is speeded by rapid metabolic destruction of CDM in the liver. When this metabolism is decreased by piperonyl butoxide, the rate of uptake is slowed. This interesting effect deserves to be studied further.

Examination of the distribution of CDM and its metabolites in the three tissues when expressed as the % of the total metabolites recovered (Table 21) reveals a picture qualitatively similar to that seen in Table 19 with metabolism by the hepatic microsomes *in vitro*, but there are some notable quantitative differences. With the control and inducer treatments *in vivo* relatively little unchanged CDM was present in the liver (about 10% of the quantitative differences. With the control and inducer treatments *in vivo* relatively little unchanged CDM was present in the liver (about 10% of the recovered radioactivity) indicating a very rapid biodegradation for this compound. DDCDM was found to be a much more prominent metabolite *in vivo* than *in vitro*.

Further, major differences in the tissue levels of CDM between the inducer pretreatments and control are not evident in most cases *in vivo*. A further difference between the *in vivo* and *in vitro* results is the high

effectiveness of piperonyl butoxide in preventing the degradation of CDM (e.g. in the brain; 56% as CDM versus 10% as CDM in the control). In particular the proportion of metabolites present as DDCDM was greatly reduced with this pretreatment. Thus it is again evident that *in vitro* metabolic studies have only a limited ability to predict *rates* of metabolism *in vivo* although *pathways* of metabolism can often be deduced *in vitro*.

The crucial comparison in relating the toxicity of CDM to its metabolism lies with the data in Table 22, and the further correlations in Table 23. The concentration of neither CDM nor DDCDM in any tissue correlates well with toxicity after the different pretreatments e.g. if CDM itself were the major toxicant, the data in Table 22 suggest that the piperonyl butoxide pretreatment should clearly increase the toxicity of CDM since the levels of this compound are elevated 2-3 fold in all tissues compared to the control. On the other hand, if DDCDM were the major toxicant, piperonyl butoxide should greatly decrease the toxicity of CDM because the tissue levels of DDCDM are considerably lowered. In fact, neither of these results is seen; piperonyl butoxide has only a minor effect on the toxicity of CDM. Similar comparisons with the other pretreatments lead to the same general conclusion. Of the individual formamidines, the trend in DCDM levels can be related to the toxicity quite closely (Table 23) and a reasonable quantitative correlation is obtained between the toxicity ratio and DCDM in brain (correlation coefficient, $r = 0.897$) and blood ($r = 0.884$) for the four treatments. However, in most cases all three of the formamidines are present in the tissues at roughly comparable concentrations. The most reasonable assumption, based on our knowledge of their pharmacological actions, is that all three have some innate toxicity and the overall effect on the animal is the sum of the effects of the three compounds acting in concert rather than the action of only one compound such as DCDM. This hypothesis receives support from the data in Table 23 where an excellent quantitative correlation is obtained between the toxicity ratio after the various pretreatments and the total level of formamidines in the brain. The correlation coefficient for this relationship is 0.985. Further support for this view comes from preliminary experiments where we have observed that the toxicities of CDM, DCDM and DDCDM are additive in mice - each can replace an equivalent toxic fraction of another without a change in lethality or symptoms.

In this relationship, the major factor underlying the lowered toxicity of CDM after 3-methycholanthrene pretreatment is the strongly increased activity in cleaving the toxic formamidines to the poorly toxic metabolite, NFT, probably through the induction of cytochrome P-448. The same reason may underly the lower toxicity after Aroclor treatment which also induces P-448 and increases NFT production. Piperonyl butoxide, as one might expect, tends to preserve the formamidines from further degradation and should, therefore, increase the toxicity of CDM by our hypothesis. However, this effect is balanced by the reduced rate of penetration of CDM after piperonyl butoxide pretreatment so that, overall, the tissue levels of total formamidines differ little from those of the control, and toxicity of CDM is not changed greatly. Phenobarbital pretreatment does not alter the pattern of metabolites found in the brain very significantly, and thus has no effect on the toxicity of CDM. Our results offer no support for the idea that *N*-demethylation is an

obligatory activation reaction for CDM, or that the most toxic compound, DDCDM, is the sole or even major toxic species *in vivo*.

These conclusions must be tentative since only the determination of a time-concentration profile for each of the major metabolites in each tissue containing important sites of action would be definitive in determining the role of the individual formamidines in such a complex toxicological relationship.

The reason why DCDM and DDCDM are more toxic than CDM when given orally (Table 1) is unclear. Comparisons of the relative potencies of CDM and DCDM on most biochemical and physiological systems which plausibly might be involved in their toxicity show no striking differences between the compounds. DDCDM, as a relatively newly discovered toxic metabolite, has, unfortunately, been less widely studied. As shown in Section 1, DCDM is only slightly better than CDM as an inhibitor of MAO. In Section 4, DCDM was found to be somewhat less effective than CDM as a local anesthetic, at least on the frog sciatic nerve. Although only preliminary experiments were performed to assess the effects of DCDM on the cardiovascular system of the dog, using the methods described for CDM in Section 2, DCDM was quantitatively and qualitatively rather similar to CDM although some differences in action were noted. Further, the two compounds acted very rapidly in this system (Table 5), suggesting that *N*-demethylation of CDM (or DCDM) is not obligatory for the cardiovascular effects which arise through peripheral actions. For this reason we did not include further work with DCDM in our cardiovascular studies.

The only major difference in pharmacological potency between CDM and its *N*-demethylation products so far known is in their α-adrenergic and octopaminergic actions. DCDM has a much stronger effect than CDM as a partial agonist at α-adrenergic sites (rabbit ear artery) as shown by Robinson and Bittle (1979). The same distinction is also found in their action as octopaminergic agonists in invertebrates, a type of system which is analogous to the α-adrenergic system of vertebrates. In this case DDCDM is also active, but somewhat less so than DCDM, while CDM itself is relatively ineffective (Hollingworth and Murdock, 1980).

However, although it is attractive to try to relate the higher toxicity of DCDM and DDCDM to their greater potency in stimulating α-adrenergic neurotransmission, there are problems with this hypothesis. α-Adrenergic agonists (e.g. NE itself) are central depressants not central stimulants, as seen with formamidines at lethal levels (Feldberg, 1963). Further, we found that phentolamine, an α-adrenergic blocker, did not prevent the peripheral cardiovascular depression caused by CDM (Section 2). Robinson *et al.* (1975) reported that phentolamine given ip to rats did not reduce the lethality of CDM, and concluded that "it seems improbable that death following chlordimeform poisoning in rats results from stimulation of α-adrenergic . . .receptors." This view was strengthened by the subsequent observation (Robinson and Smith, 1977) that phenylephrine, itself an α-agonist, did not increase the toxicity of CDM as might be expected if CDM, or a metabolite, had important actions at the same site.

The exact α-adrenergic effects of DCDM (and probably DDCDM) are difficult to predict since partial agonists are, by definition, partial antagonists also and thus may stimulate α-adrenergic transmission at one dose and partially block it at a higher dose. This action of the formamidines deserves further study, particularly with regard to the central effects of CDM, but as pointed out above, the evidence presently available does not strongly support this type of action as a major factor in the acute toxicity of CDM, whereas local anesthetic-like effects can explain most of the symptoms observed. The greater toxicity of DCDM and DDCDM may therefore lie as much in the area of pharmacokinetics (more rapid accumulation at the site(s) of action) as in the area of pharmacodynamics (greater potency at, or a different, site of action).

Overall, the toxicological actions of CDM, and probably related formamidines, is rather complex, with several active compounds present simultaneously, each of which may have several significant actions on the nervous system. Much is now known concerning the neurotoxicology of formamidines and its relationship to their toxicity, but numerous questions remain to be answered before a satisfactory and complete picture of their pharmacological and toxicological effects can emerge.

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/1-80-028		

4. TITLE AND SUBTITLE	5. REPORT DATE
Toxicity, Interactions, and Metabolism of Formamidine Pesticides in Mammals	May 1980
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
R. M. Hollingworth and G.K.W. Yim	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Purdue University Department of Entomology West LaFayette, Indiana 47907	1EA615
	11. CONTRACT/GRANT NO.
	Grant No. R-803965
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
US Environmental Protection Agency Health Effects Research Laboratory Research Triangle Park, NC 27711	14. SPONSORING AGENCY CODE EPA 600/11

15. SUPPLEMENTARY NOTES

16. ABSTRACT

The overall goal of this research project was to investigate the mechanism(s) of acute toxicity of formamidine pesticides in mammals using chlordimeform (N'-(4-chloro-o-tolyl)-N,N-dimethylformamidine) and its metabolites as the primary model compounds. The role of biotransformations, particularly N-demethylation reactions, in generating potentially toxic metabolites was also studied.

By comparing the effects of hepatic microsomal mixed function oxidase inducers and inhibitors administered in vivo on the toxicity, metabolism, and distribution of metabolites in mouse tissues, it was concluded that although N-demethylation products are innately more toxic than chlordimeform, they are also less stable, and the best correlation of toxicity was obtained with the total level of formamidines in the brain, rather than with the level of any individual metabolite.

In a series of studies with dogs, rabbits, and cats, the cause of death was found to be cardiovascular collapse accompanied by respiratory arrest. Cardiovascular collapse resulted primarily from a peripheral local anesthetic-like effect of chlordimeform. Monoamine oxidase inhibition was not a major factor in lethality. Respiratory arrest was central in origin. Several other central effects of the formamidines were described, some of which may be local anesthetic actions, and a behavioral profile for chlordimeform poisoning in the rat was developed. The effectiveness of various drug treatments as potential therapeutic aids for formamidine intoxication were studied. Formamidines also have aspirin-like actions due to an inability to inhibit prostaglandin synthesis.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Formamidine pesticides Toxicity in mammals Administered In Vivo		06,F,T,

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	114
	20. SECURITY CLASS *(This page)*	22. PRICE
	UNCLASSIFIED	

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE