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Chick Microsomal Oxidases. Isolation, Properties, and Stimulation by Embryonic Exposure to 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane*

M. B. Abou-Donia† and D. B. Menzel‡

ABSTRACT: The optimum conditions for the activity of the chick liver microsomal oxidative *N,N*-dimethylaniline demethylase have been established. Reduced nicotinamide–adenine dinucleotide phosphate was required and was partially replaced by reduced nicotinamide–adenine dinucleotide. The enzyme was competitively inhibited by β-diethylaminoethyl diphenylpropylacetate. Michaelis constants in the absence and in the presence of β-diethylaminoethyl diphenylpropylacetate were determined for *N,N*-dimethylaniline and *N,N*-dimethylaniline *N*-oxide. The data presented in this work demonstrate that the rate of *N,N*-dimethylaniline *N*-oxide demethylation by chick liver microsomal fraction is sufficiently high for the *N*-oxide to be considered as an intermediate in the oxidative demethylation of *N,N*-dimethylaniline. Ferrous ions activated the microsomal demethylase when incubated with nicotinamide–adenine dinucleotide phosphate and gave a greater oxidative reaction in the absence of nicotinamide–adenine dinucleotide phosphate. The microsomal fraction oxidized *N,N*-dimethylaniline when incubated with hydrogen peroxide generating system. The activity of this enzyme system in chicks hatched from eggs which had been injected with 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane, and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene prior to the incubation has been determined. 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane and its major metabolites in the chick stimulated microsomal oxidative activity. However, there were no changes in the enzyme activity requirements or in the Michaelis constants both in the presence and absence of β-diethylaminoethyl diphenylpropylacetate. The ability of chick microsomal enzymes to dealkylate some carbamate insecticides was found to be increased by the embryonic exposure to 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane, and 2,2-bis(*p*-chlorophenyl)ethylene.

Numerous studies have been conducted on the oxidative demethylation of *N*-methyl compounds. In the presence of NADPH and oxygen the microsomal fraction catalyzed the oxidation of some of the *N*-alkyl compounds to the aldehydes and corresponding amines (Gillette *et al.*, 1957). This enzyme system appeared to be a mixed-function oxidase or mixed-function oxygenase (Ziegler and Pettit, 1966).

A number of studies have been done on the mechanism of the oxidative N dealkylation of lipid-soluble tertiary amines (Fish *et al.*, 1955; McMahon and Sullivan, 1964; Ziegler and Pettit, 1964, 1966).

The activity of microsomal oxidases may be stimulated by exposure of the animal to certain chemicals. Current data indicate that stimulation occurs by increased synthesis of the enzyme systems normally present rather than by modification or elaboration

* From the Institute of Marine Resources, Department of Nutritional Sciences, University of California, Berkeley, California 94720. *Received May 31, 1968.*

† Present address to which reprint requests should be sent: Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843.

‡ Present address: Biology Department, Pacific Northwest Laboratories, Battelle Memorial Institute, Richland, Wash. 94352.

of a new enzymic species (Conney and Burns, 1962). Lipid-soluble compounds are particularly effective probably because of a lipid barrier between the site of enzymic action and the aqueous environment of the cell (Brodie and Maickel, 1961). Alterations in enzymic activity are further evidenced by elaboration of the smooth endoplasmic reticulum (Fouts, 1962; Remmer and Merker, 1963).

DDT[1] has been shown to stimulate microsomal activity (Hart and Fouts, 1963; Street, 1964), and the relative activity of DDT, DDD, DDE, and Kelthane on the oxidative conversion of aldrin and dieldrin by rat microsomes has also been determined (Gillette *et al.*, 1966).

In this investigation the microsomal demethylase activities for DMA and for DMAO of the liver of 1-day-old chicks have been determined. The cellular localization of the DMA demethylase activity, the requirements for its activity, and the effect of different activators and inhibitors are also reported. The activity of this enzyme system with regard to stimulation by the structurally related series, DDT, DDD, and DDE, has been examined. The ability of chick microsomal enzymes after embryonic exposure to DDT, DDD, and DDE to dealkylate some carbamate insecticides was investigated.

Experimental Procedures

Reagents. *p,p'*-DDT, *p,p'*-DDD, and DDE were obtained from the Aldrich Chemical Co., Inc., Milwaukee, Wis.

DMA from Matheson, Coleman and Bell, Berkeley, Calif., was distilled under vacuum. Aqueous solutions of DMA were prepared by dissolving the amine in 0.05 *m* HCl, then stored at −20° until needed. NADPH, NADP, NADH, NAD, glucose oxidase (type II purified), and β-D-(+)-glucose were obtained from Sigma Chemical Co., St. Louis, Mo. SKF 525-A was a generous gift from Smith, Kline and French Laboratories, Philadelphia. The following carbamate insecticides were generous gifts of the manufacturers: Carbaryl, Union Carbide 10854 or Hercules 5727, Mesurol, Pyrolan, Zectran, and Mitacil.

Preparation of Microsomal Subfraction. All manipulations were performed at 0–4°. Chicks (1-day-old) were anesthetized with diethyl ether, then exsanguinated. Livers were removed and washed free of blood and feathers in ice-cold 0.25 M sucrose. The average weight of the liver was 1 g. The tissue was minced and homogenized with four volumes of 0.25 M sucrose with an Omni-mixer (Sorvall) for 30 sec. The homogenate was filtered through cheesecloth and then subjected to differential centrifugation in a Sorvall Model RC2-B centrifuge. After centrifugation at 20,000*g* for 30 min, microsomal fractions were sedimented from the supernatant fraction by centrifugation in a Model L-2 HV Spinco centrifuge at 100,000*g* for 1 hr. The microsomal fraction was washed once with 1.25% KCl and suspended in 1.15% KCl to give approximately 0.5 g of whole liver equiv/ml.

Incubation Conditions. The oxidative demethylation assays were carried out in open 25-ml erlenmeyer flasks at 38° in a Dubnoff metabolic shaker. Microsomal suspension (1 ml) was added to the reaction mixture consisting of: 70 μmoles of KH_2PO_4 buffer (pH 7.4), 50 μmoles of nicotinamide, 25 μmoles of $MgCl_2$, 500 μmoles of KCl, 1 μmole of NADPH, and 15 μmoles of DMA or DMAO. After 30 min the reaction was stopped by addition of 2.5 ml of 20% trichloroacetic acid followed by centrifugation for 15 min. The deproteinized supernatant solutions were assayed for formaldehyde by the method of Nash (1953). The results are expressed as millimicromoles of formaldehyde formed per minute per milligram of microsomal protein incubated at 38° (millimicromoles of CH_2O per minute per milligram of protein).

H_2O_2-Generating System. H_2O_2 was generated by a mixture of 1 unit of glucose oxidase and 10 μmoles of glucose in phosphate buffer (pH 7.4). The oxidative demethylation assays were carried out in open 25-ml erlenmeyer flasks at 38° in a Dubnoff metabolic shaker. Microsomal suspension (1 ml) was added to the reaction mixture consisting of: 70 μmoles of KH_2PO_4 buffer (pH 7.4), 50 μmoles of nicotinamide, 25 μmoles of $MgCl_2$, 500 μmoles of KCl, and 15 μmoles of DMA. Protein was determined by the Biuret method (Gornall *et al.*, 1949).

Enzymatic Demethylation of Carbamate Insecticides. Because of the insolubility of carbamates in aqueous media, they were added to the flasks as acetone solutions and the solvent was evaporated in a stream of air. The media of incubation were the same as when DMA was used as substrate.

Injection of Eggs. Leghorn fertile eggs were injected with DDT, DDD, or DDE in peanut oil following the method of Abou-Donia and Menzel (1968).

Results

Preliminary experiments showed that nicotinamide was required for demethylation of DMA by chick liver microsomal enzymes. NADPH was proved to be required for the oxidative demethylation reaction. $MgCl_2$ as well as KCl slightly increased the enzymatic activity. Addition of the soluble fraction (Jargon) to

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: DMA, *N,N*-dimethylaniline; DMAO, *N,N*-dimethylaniline *N*-oxide; NAD, nicotinamide–adenine dinucleotide; NADH, reduced nicotinamide–adenine dinucleotide; NADP, nicotinamide–adenine dinucleotide phosphate; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; SKF 525-A, β-diethylaminoethyl diphenylpropylacetate; *p,p'*-DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; *p,p'*-DDD, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; *p,p'*-DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-*endo,exo*-dimethanonaphthalene; aldrin, 1,2,3,4,10,10-hexachloro-1,4,4a,5,-8,8a-hexahydro-1,4,5,8-*endo,exo*-dimethanonaphthalene; carbaryl, 1-naphthyl methylcarbamate; Union Carbide 10845 or Hercules 5727, 3-isopropylphenyl methylcarbamate; Mesurol, 4-(methyl sulfide)-3,5-xylenyl methylcarbamate; Pyrolan, 1-phenyl-3-methylpyrazolyl 5-dimethylcarbamate; Zectran, 4-dimethyl-amino-3,5-xylyl methylcarbamate; Mitacil, 4-dimethylamino-3-tolyl methylcarbamate.

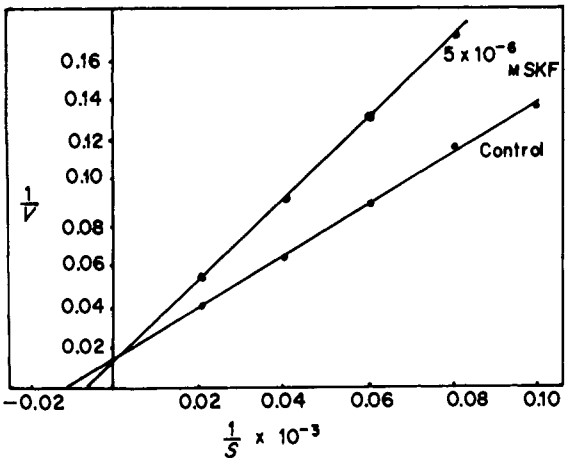

FIGURE 1: Lineweaver–Burk plots showing the competitive inhibition of SKF 525-A on the demethylation of DMA. Assays carried out as described under Methods.

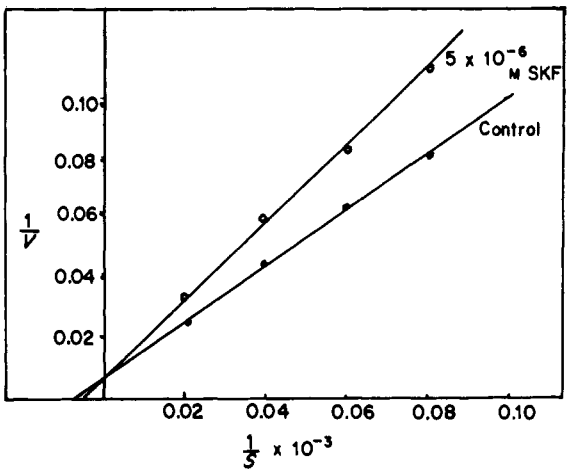

FIGURE 2: Lineweaver–Burk plots showing the competitive inhibition of SKF 525-A on the demethylation of DMAO. Assays carried out as described under Methods.

the microsomal fraction, increased the activity from 0.05 to 2.15 mμmoles of CH_2O per min per mg of protein. The same value was obtained by using NADPH and microsomal fraction. NADH participated in DMA demethylation but to a lesser extent than did NADPH. When equimolar amounts of NADP and NAD were incubated with microsomal and soluble fractions, the oxidation was less than with NADP alone due to the competition reaction with NADH.

The subcellular localization of oxidative demethylation activity in the chick liver was investigated. The specific activity was very low in the total homogenate (0.055 mμmole of CH_2/min per mg of protein). The supernatant fraction which was prepared by centrifugation of homogenate for 30 min at 20,000*g* had much higher specific activity (1.13 mμmoles of CH_2/min per mg of protein). Further fractionation of the supernatant by centrifugation at 100,000*g* for 1 hr yielded microsomal fractions and soluble fraction (Jargon) both of which alone had almost no activity for demethylation of DMA with NADP. The highest specific activity was obtained when both microsomal fractions and soluble fractions were added together (2.15 mμmoles of CH_2O/min per mg of protein) in the presence of NADP, which indicated that components from both fractions are required for the oxidative demethylation activity.

The reaction was proportional to the concentration of microsomal fraction concentration in the range of 0.5–4.0 mg of protein/ml and linear with time for at least 30 min at 38°.

The data summarized in Table I demonstrate that chick liver microsomal enzymes catalyze the demethylation of DMAO at a rate greater than the oxidative demethylation of the corresponding dimethylaniline. A graphic determination of Michaelis constant for oxidative demethylase of DMA in chick liver microsomal fraction gave a K_m of 16.0×10^{-3} M. The activity of the microsomal enzymes was inhibited by a compound frequently used to inhibit oxidative N-dealkylation reactions, SKF 525-A. The demethylation activity was almost completely inhibited with a final concentration of 10^{-4} M. The inhibitory effect appeared to be competitive with the substrate and gave a K_i value of 4×10^{-6} M. A double reciprocal plot of the enzymic demethylation of DMA alone and in the presence of SKF 525-A is shown in Figure 1.

DMAO demethylase activity was also found to be competitively inhibited with SKF 525-A. Figure 2 shows a Lineweaver–Burk plot illustrating the effect of substrate concentration and the competitive inhibition of SKF 525-A on the rate of demethylation of DMAO. The Michaelis constant, K_m, was found to be 155×10^{-3} M and the maximum velocity was 200 mμ-moles of CH_2O/min per mg of protein both in the absence and presence of the inhibitor.

The effects of some additional compounds on the demethylase activity are listed in Table II. Incubation of the microsomal fraction with NADPH gave 2.29 mμmoles of CH_2O/min per mg of protein. By omitting NADPH and incubating the microsomal enzymes with different ferric ions, *i.e.*, $FeCl_3$, $Fe_2(SO_4)_3$, and $Fe(ClO_4)_3$, DMA was oxidized, but to a lesser extent than when NADPH was present.

Copper sulfate and acetate had almost the same effect as ferric ions. $HgCl_2$, $MnSO_4$, $MgCl_2$, $ZnSO_4$, $ZnCl_2$, KCN, GSH, and EDTA gave no demethylation when incubated separately with the microsomal fraction with no NADPH. Ascorbic acid and catechol when incubated separately with the microsomal fraction gave activity slightly more than when NADPH was incubated with the microsomes. In the presence of ferrous ions, *i.e.*, $FeSO_4$, $Fe(ClO_4)_2$, and $Fe(NH_4)SO_4$, the microsomal oxidative activity of DMA was higher than that in the presence of NADPH, with $FeSO_4$ being the highest.

Table III illustrates the effect of adding ascorbic acid, catechol, EDTA, KCN, and GSH to reaction mixtures containing NADPH. Ascorbic acid, catechol, and EDTA activated the oxidation reaction, while GSH inactivated it and KCN inhibited it completely. Boiled microsomes incubated with NADPH gave no reaction.

TABLE I: Demethylation of DMA and DMAO by Liver Microsomal Enzymes.

Substrate	mμmoles of CH_2O/min per mg of Protein			
	Activity[a]	V_{max}	K_m	K_i[b]
DMA	2.29 ± 0.2[c]	66.6	16.0×10^{-3}	5×10^{-6}
DMAO	3.54 ± 0.3	200	155×10^{-3}	15×10^{-6}

[a] Substrate (15 μmoles) was incubated with liver microsomes as described under Methods. [b] SKF 525-A concentration = 5×10^{-6} M. [c] Standard error.

TABLE II: Effect of Some Compounds on the Activity of Microsomal DMA Demethylase Activity in the Chick Liver.[c]

Compd[a] (2 mM)	Act. (mμmoles of CH_2O/min per mg of proteins)	Compd (2 mM)	Act. (mμmoles of CH_2O/min per mg of proteins)
NADPH	2.29 ± 0.2[b]	$HgCl_2$	0.00
$FeCl_3$	1.13 ± 0.3	$MnSO_4$	0.00
$Fe_2(SO_4)_3$	1.48 ± 0.1	$MgCl_2$	0.00
$Fe(ClO_4)_3$	1.26 ± 0.6	$ZnSO_4$	0.00
$FeCl_2$	2.72 ± 0.4	$ZnCl_2$	0.00
$FeSO_4$	3.34 ± 0.3	$Cr_2(SO_4)_3$	2.99 ± 0.1
$Fe(ClO_4)_2$	2.86 ± 0.1	EDTA	0.00
$Fe(NH_4)SO_4$	3.16 ± 0.5	Catechol	2.35 ± 0.3
$CuSO_4$	1.68 ± 0.3	Ascorbic acid	2.48 ± 0.2
$Cu(ACO_2)_2$	1.38 ± 0.2	Reduced glutathione	0.00
KCN	0.00		

[a] Controls were carried out simultaneously, utilizing heat-denatured microsomal fraction for each determination. [b] Standard error. [c] Microsomal homogenates (equivalent to 0.5 g of liver) were incubated with 15 μmoles DMA as described under Methods, except that 10 μmoles of different compounds was added instead of NADPH.

Table IV illustrates the effect of ascorbic acid, catechol, EDTA, GSH, and KCN on the oxidative demethylation of DMA in the presence of microsomal fraction and H_2O_2-generating system. Boiled microsomes incubated with the H_2O_2-generating system gave no demethylation. Ascorbic acid, catechol, and EDTA activated the demethylation reaction when each compound was incubated separately with microsomal fraction and H_2O_2-generating system. GSH greatly inactivated this reaction and KCN inhibited it completely.

The activity of the oxidative demethylase in the microsomal fraction of livers taken from 1-day-old chicks hatched from eggs injected with DDT was investigated. In general there was no change in the requirements or in the subcellular distribution of enzyme activity in chick liver. However, in all cases there was an increase of enzyme activity in the microsomal fractions taken from DDT-treated chicks.

The demethylase activity of livers taken from normal chicks and livers taken from chicks hatched from eggs injected prior to incubation of the eggs with DDT, DDD, and DDE was determined. It has been found that DDT injection caused the greatest demethylation activity (3.45 ± 0.3 mμmoles of CH_2O/min per mg of protein) and DDE the least (2.63 ± 0.2 mμmoles of CH_2O/min per mg of protein), while DDD was intermediate (3.07 ± 0.3 mμmoles of CH_2O/min per mg of protein.

Lineweaver–Burk plots for microsomal demethylase activity of DMA isolated from livers taken from chicks hatched from eggs injected with DDT, DDD, and DDE prior to incubation were plotted. Table V lists the Michaelis constants, maximum velocities, and K_i for the competitive inhibitor SKF 525-A calculated from the plots.

Table VI shows the demethylation of six carbamate insecticides by microsomal fractions isolated from liver of normal chicks and from liver of chicks hatched from eggs injected with DDT prior to incubation. It shows that all carbamate compounds were greatly demethylated with microsomal fractions isolated from chick livers hatched from DDT-treated eggs.

Discussion

Data obtained in this work indicate that there is an enzyme system in 1-day-old chick liver microsomal

TABLE III: Effect of Some Compounds on the Microsomal DMA Demethylase Activity when Incubated with NADPH.

Compounds[b]	Act.[a] (mμmoles of CH_2O/min per mg protein)
	2.30 ± 0.2[c]
Ascorbic acid	3.45 ± 0.4
Reduced glutathione	1.46 ± 0.3
Catechol	2.98 ± 0.1
EDTA	3.90 ± 0.1
KCN	0.00
Boiled microsomes	0.00

[a] Microsomal homogenates (equivalent to 0.5 g of liver) were incubated with 15 μmoles of DMA as described under Methods, except that 10 μmoles of different compounds was added to the reaction mixture. [b] Controls were carried out simultaneously, utilizing heat-denaturated microsomal fraction for each determination. [c] Standard error.

TABLE IV: Effect of Some Compounds on the DMA Demethylation with Microsomal Fraction and H_2O_2-Generating System.

Compounds[b]	Act.[a] (mμmoles of CH_2O/min per mg of protein)
	2.10 ± 0.2[c]
Ascorbic acid	3.96 ± 0.3
Reduced glutathione	0.71 ± 0.1
Catechol	5.67 ± 0.4
EDTA	3.43 ± 0.2
KCN	0.00
Boiled microsomes	0.00

[a] DMA (15 μmoles) was incubated with 10 μmoles of glucose, 1 unit of glucose oxidase, and 1 ml of microsomal homogenate as described under Methods. [b] Controls were carried out simultaneously, utilizing heat-denatured microsomal fraction for each determination. [c] Standard error.

fraction which catalyzes the oxidative demethylation of DMA and DMAO to produce formaldehyde. The enzyme system requires for its activity NADPH, nicotinamide, and to less extent $MgCl_2$ and KCl and is competitively inhibited by SKF 525-A. The observed Michaelis constant for DMA demethylase was 16.0×10^{-3} M, compared with 1.42×10^{-3} M for rat liver microsomes (Pettit and Ziegler, 1963). The high K_m value of DMAO demethylase (155×10^{-3} M) was comparable to that reported in both rat and pork liver microsomal fractions, 139×10^{-3} M (Pettit and Ziegler, 1963), and may be attributed to the very limited lipid solubility of the polar *N*-oxide as was suggested by Brodie *et al.* (1958).

Our data demonstrate that chick liver microsomal enzymes catalyze the demethylation of DMAO at a rate greater than the oxidative demethylation of the corresponding dimethylaniline, as is indicated by the maximum velocity of 200 μmoles of CH_2O/min per mg of protein and 66.6 mμmoles of CH_2O/min per mg of protein for DMAO and DMA, respectively. They demonstrate that the rate of DMAO demethylation by chick liver microsomal fraction is sufficiently high for the *N*-oxide to be considered as an intermediate in the oxidative demethylation of DMA. This conclusion is in contrast with that of McMahon and Sullivan (1964), who concluded that the microsomal demethylation of 1-propoxyphene does not proceed through an *N*-oxide intermediate. However, it is in agreement with other work (Fish *et al.*, 1955; Pettit and Ziegler, 1963; Ziegler and Pettit, 1964, 1966; Machinist *et al.*, 1966), which suggests that rat and pork liver microsomal enzymes dealkylate DMA by an initial oxidative attack on the nitrogen atom of the tertiary amine to yield an intermediate *N*-oxide, which is consequently dealkylated to give formaldehyde and secondary amine. The DMA demethylase activity is concentrated in the chick liver microsomal fraction and is activated when ferrous ions are incubated with the microsomal fraction and NADPH. EDTA catalyzed the microsomal demethylation of DMA in the presence of NADPH but it could not substitute for NADPH. GSH inactivated the oxidative demethylation reaction and KCN inhibited it. Ferric and cuprous ions could substitute for NADPH but with less activity than ferrous ions. Thus it was indicated that iron ions are probably effective only in the reduced state.

Our data should be considered in the light of the finding of Ottolenghi (1959) that the addition of ferrous salts to a microsomal suspension caused lipid peroxidation (confirmed by Beloff-Chain *et al.*, 1965). Hence it may be suggested that the oxidative demethylation of DMA occurred in connection with lipid peroxidation. Also the dissimilar activating effects of Fe^{3+} and Fe^{2+} are generally consistent with Beloff-Chain's suggestion (Beloff-Chain *et al.*, 1965) that the role of the enzyme system involved in peroxidation of microsomal lipids is to transport electrons from NADPH to Fe^{3+}, giving Fe^{2+} which initiates the peroxidation reaction. Microsomal enzymes (Mason *et al.*, 1965) catalyze a variety of reactions which require oxygen and $NADPH_2$. It is assumed that the enzyme functions to form an active oxygen moiety which in turn serves to introduce the hydroxyl group by an unknown mechanism. The nature of the active oxygen is not fully understood. Our data support the postulations that the active oxygen is hydroxyl radical (·OH) as suggested by Buhler and Mason (1961) rather than hydroxonium ion (OH^+) as suggested by Brodie *et al.* (1954). It has been reported that hydrogen peroxide is formed during microsomal oxidation of $NADPH_2$ in the absence of an added substrate (Gillette *et al.*, 1957). Thus it may be assumed that ferrous ion and hydrogen peroxide

TABLE V: The Effect of DDT, DDD, and DDE on the Michaelis Constant and Maximum Velocity of the Chick Microsomal DMA Demethylase.

Treatment[a]	K_m (M)	K_i (M)	V_{max} (mμmoles of CH_2O/min per mg of protein)
Normal	16×10^{-3}	5×10^{-6}	66.6
DDT	16.2×10^{-3}	5.1×10^{-6}	156
DDD	16.4×10^{-3}	5.2×10^{-6}	90
DDE	16.1×10^{-3}	4.9×10^{-6}	75

[a] Incubation conditions are described under Methods.

TABLE VI: Effect of DDT, DDD, and DDE on the Microsomal Demethylation Activity of Some Carbamate Insecticides in Chick Liver.

Carbamates[c]	Act.[a] (mμmoles of CH_2O/min per mg of protein)	
	Normal	DDT[b]
Carbaryl	0.80 ± 0.1[d]	0.98 ± 0.1
Zectran	2.40 ± 0.3	3.00 ± 0.3
Metacil	2.90 ± 0.3	3.27 ± 0.3
UC 10854	0.91 ± 0.1	0.98 ± 0.2
Mesurol	0.95 ± 0.3	1.13 ± 0.2
Pyrolan	0.98 ± 0.4	1.23 ± 0.4

[a] Carbamates (10 μmoles) were incubated for 30 min with microsomal fraction as described under Methods. [b] Livers were taken from chicks hatched from eggs injected with DDT as described under Methods. [c] Controls were carried out simultaneously, utilizing heat-denatured microsomal fraction for each determination. [d] Standard error.

generate ferric hydroxide ion and hydroxyl radical, which attacks the substrate in the usual fashion. The function of ascorbic acid when present is to reduce ferric ion back to ferrous ion being converted into the ascorbate anion radical, thus accelerating the demethylation reaction. The same function can be speculated about catechol which also catalyzes this reaction.

In order to examine the hypothesis of the hydrogen peroxide oxidation mechanism, the microsomal fraction was incubated with H_2O_2-generating system. The formaldehyde formation was found to be linearly proportional to the microsomal concentration. Also there was no formaldehyde formed when the microsomal fraction was boiled. These data show the definite requirement for some components in the active microsomal enzymes and that the H_2O_2-generating system alone cannot oxidize DMA.

Our data are in contrast to the finding of Ziegler and Pettit (1964) that a small amount of H_2O_2 or H_2O_2-generating systems such as the D-amino acid oxidase could not replace NADPH in oxidizing DMA to the *N*-oxide by rat and pork liver microsomal fraction. This disagreement may be attributed to species differences between mammals and birds.

In this investigation on the effect of DDT and some of its metabolites on chicks, we have followed the capacity of liver microsomal fraction of chicks hatched from eggs which had been injected with DDT, DDD, and DDE to undertake oxidative N demethylation of DMA. We have determined the capacity of the same fractions to demethylate some carbamate insecticides. Preliminary experiments, done by injecting DDT only, have indicated that there was no change in the requirements of the chick liver microsomal demethylase activity to demethylate DMA.

However, in all cases the demethylase activity of cellular fractions isolated from chick livers hatched from eggs injected with DDT was always greater than those isolated from chick livers hatched from untreated eggs.

Our results show that DDT, DDD, and DDE are all capable of markedly stimulating the hepatic microsomal N dealkylation of DMA. Injected DDT produced the greatest increase in enzyme action (56%), DDE the least (19%), while DDD was intermediate in activity (34%). This result contrasts with the stimulatory effect of these compounds observed by feeding them to rats (Gillette *et al.*, 1966) in that while DDT had the greatest effect, DDD had the least effect, with DDE intermediate in activity. However, this observation is probably a reflection of a species difference similar to the finding (Hart and Fouts, 1963) that the mouse appears to be more resistant to DDT stimulatory effects than the rat. It is interesting to note that there were no changes in the enzymic properties of the system as measured by the effect of substrate concentration and the competitive inhibition of SKF 525-A, the calculated Michaelis constants and K_i values being the same for the activity isolated from normal or treated chicks.

Although the mechanism by which these compounds stimulate DMA demethylase in chick liver is not known, the present work suggests that there was no change in the qualitative nature of the microsomal enzymes. However, it has been suggested that DDT stimulation of drug-metabolizing enzymes is in some way related to the proliferation of the hepatic cell (Hart *et al.*, 1963). Since then much evidence has been gathered to support this hypothesis (Ortega, 1962; Hart *et al.*, 1963; Hart and Fouts, 1965; Morello, 1964). Agosin *et al.* (1965) have found that protein biosynthesis is stimulated by DDT, but not by DDE in nymphal *Triatoma infestans*, which appeared to be consistent with the observations of Ilevicky *et al.* (1964) that DDT stimulated NAD-kinase activity in *T. infestans*. Our results indicated an increased synthesis of the demethylase system in the chick as well.

Carbaryl and *p,p'*-DDT have different types of toxic

action. While the actual target of DDT and carbamates in insects and mammals is considered to be the nervous system, DDT acts by blocking the transport of cations with an unknown mechanism (O'Brien and Matsumura, 1964); however, carbamates inhibit acetylcholinesterase activity (Kolbezen *et al.*, 1954). In addition, SKF 525-A inhibited the metabolism of dimethylcarbamates by rat liver microsomes (Hodgson and Casida, 1961). In this work we have found that carbamates similarly were demethylated by chick liver microsomal fractions. The reaction is also inhibited by SKF 525-A. Table VI summarizes the results of the demethylation of six carbamate insecticides by microsomal fractions from normal chicks hatched from DDT-injected eggs prior to incubation. Zectran and Metacil yielded the greatest amount of formaldehyde because these compounds possess a *N,N*-dimethylamine group which is liable to demethylation in addition to the carbamoyl group of the carbamate compounds. Although Pyrolan has an *N,N*-dimethylcarbamoyl group, it yielded much less formaldehyde than both Zectran and Metacil, indicating that probably only one methyl group in the *N,N*-dimethylcarbamoyl moiety was demethylated. UC 10854, Mesurol, and Carbaryl were more stable to chick liver microsomal demethylation.

Although DDT is metabolized primarily to DDD and DDE in the chick (Abou-Donia, 1967; Abou-Donia and Menzel, 1968), it is possible that a metabolite other than DDD or DDE might be the actual inducer of the increased demethylase activity. Induction by another metabolite would, however, seem unlikely since DDT induced more activity than either DDE or DDD. Injected DDE is poorly metabolized by the chick embryo, while DDD is metabolized in the same manner as DDT.

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