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ENZYMATIC OXIDATION OF DIPHENYLMETHYLPHOSPHINE AND 3-DIMETHYLAMINOPROPYLDIPHENYLPHOSPHINE BY RAT LIVER MICROSOMES

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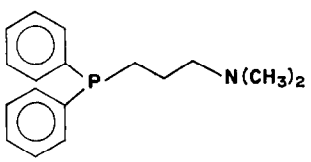
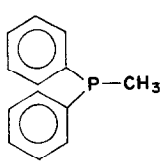
Abstract—The metabolism of two tertiary aromatic phosphines, 3-dimethylaminopropyldiphenylphosphine (a CNS depressant) and diphenylmethylphosphine, was studied. These compounds were incubated with subcellular fractions of rat liver homogenates and found to be enzymatically converted to the corresponding phosphine oxides; the aminophosphine gave rise to an *N,P*-dioxide. Enzymatic activity was localized in the microsomal fraction and shown to be associated with the cytochrome P-450 mixed-function oxidases. The substrates were also found to undergo a facile non-enzymatic reaction with thiols, a factor which portends their possible chronic toxicity.

3-DIMETHYLAMINOPROPYLDIPHENYLPHOSPHINE, compound 1, synthesized by Wiley and Godwin[1] was found to be a mammalian central nervous system depressant. It represents one of few examples of pharmacologically active trivalent phosphorous



Compound 1

compounds. Although the toxic properties of phosphine (PH_3) itself have been recorded,[2] no studies concerning the biological properties of aryl–alkyl phosphines have been reported. This dearth of information prompted the present investigation to establish whether rat liver microsomal enzymes catalyse the oxidation of phosphines. To this end, the biotransformation of 3-dimethylaminopropyldiphenylphosphine, compound 1, and diphenylmethylphosphine, compound 2, was studied by incubation of these potential substrates with subcellular fractions of rat liver homogenates.



Compound 2

The latter compound, 2, a monofunctional aromatic phosphine, was selected to

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determine if phosphines are capable of undergoing detoxication reactions, such as aromatic ring hydroxylation and dealkylation, characteristic of isoelectronic amines.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 150–200 g were used in these experiments. Animals were fasted overnight prior to use and killed by decapitation between 8 and 9 a.m.; the livers were excised immediately, washed once with ice-cold 0·02 M Tris–HCl buffer (pH 7·4), weighed and treated as described below.

Livers were homogenized with 4 vol. of cold 0·02 M Tris–HCl (pH 7·4) buffer using a glass–Teflon homogenizer driven at 2000 rpm by a portable drill. Subcellular fractions were obtained in the following way: the nuclear fraction represents washed material sedimented at 600 *g* in 20 min in a refrigerated Sorvall centrifuge; the mitochondrial fraction is the washed material obtained at 9000 *g* in a similar way; and the microsomes represent washed material sedimented at 105,000 *g* in 1 hr in a Spinco model L or IEC model B-60 ultracentrifuge. All preparations were washed by resuspending sedimented material in buffer equal in volume to the original suspension, followed by recentrifugation. All subcellular fractions were reconstituted to the original volume of the supernatant fraction from which they were obtained in order to maintain protein concentrations at the levels found in the original homogenate.

Protein content was determined by the method of Lowry *et al.*[3] Bovine serum albumin was used as the protein standard.

Typical incubation mixtures consisted of enzyme equivalent to 50 mg of liver or 5 mg of microsomal protein, NADP (1 μmole), glucose 6-phosphate (15 μmoles), glucose 6-phosphate dehydrogenase (1 i.u.), $MgCl_2$ (24 μmoles), phosphine substrate (2 μmoles), and 1·15% KCl–0·02 M Tris–HCl (pH 7·4) buffer adjusted to a final volume of 3·5 ml. Unless otherwise stated, incubations were carried out on a Dubnoff or Aminco metabolic incubator in air for 15 min at 37°, following a 5-min preincubation of the mixture less substrate.

In order to solubilize diphenylmethylphosphine, it was necessary to add 1·3 mg of Triton X-100 to each incubation mixture. Since it has been established that this concentration of Triton has no effect on the rate of aniline hydroxylation by microsomes,* it was felt that similar concentrations could be used safely in these experiments.

Disappearance of phosphine substrates was followed by observing the decrease in absorbance at the characteristic u.v. maximum of 252 nm (3-dimethylaminopropyldiphenylphosphine) or 251 nm (diphenylmethylphosphine). The reactions were stopped by shaking 3·0 ml of the incubation mixture with 25 ml of spectroquality heptane for 20 min. The heptane was separated and shaken for 10 min with 3 ml of 0·05 M (pH 5·6) citrate buffer. The absorbance of the heptane solution was then read at the appropriate wavelength in order to determine the amount of substrate remaining in the incubation mixture. It was established in preliminary experiments that this extraction procedure recovered substrate nearly quantitatively from the incubation mixture (appropriate corrections for recovery were made), and that no interfering substances, including metabolites more polar than the starting substrate, were present in the final heptane solution.

* L. Sternson, unpublished observations.

Inhibition of metabolic activity by carbon monoxide was delivered as follows. Thunberg tubes containing the usual incubation mixture were alternatively gassed with either artificial air (4:1 N_2–O_2) or a 4:1 CO–O_2 mixture and evacuated. After gassing was complete, the final pressure in the tubes was adjusted to 1 atm. The reaction was initiated by adding substrate from the side arm. Incubations and assays were carried out as described above.

For metabolite identification, quantities of all ingredients in incubation mixtures were increased 10-fold over those previously stated. Incubation time was extended to 120 min. After incubation, the pH of the mixture was adjusted to 11, and the mixture was extracted twice with 250 ml of 1,2-dichloroethane (EDC). The EDC extracts were combined and dried (Na_2SO_4). The EDC was removed *in vacuo*, and aliquots of the residue were purified by thin-layer chromatography on 250 μ Silica gel plates (Analtech). The solvent system used for separation of the 3-dimethylaminopropyldiphenylphosphine reaction mixture was methanol–ammonia (99:1), and for separation of the diphenylmethylphosphine reaction mixture was chloroform–methanol (98:2) containing 1% of 28% aqueous ammonia. Bands were located under u.v. light, the Silica gel was scraped off, and the metabolite was eluted by shaking the Silica gel with anhydrous methanol for 30 min. The solvent was evaporated at 30° under a gentle stream of nitrogen and the residue introduced into a Varian-MAT CH5B or LKB model 9000 mass spectrometer operated at various temperatures for the different compounds studied, using a 70 eV electron source. For identification, mass spectra of all extracts except that of aminophosphine *p*-oxide were compared with spectra of authentic samples.

To study the reaction of phosphines with thiols, 5 μmoles of phosphine was incubated with 10 μmoles of reduced glutathione or cysteine at 37° for 15 min in 3·4 ml of 0·02 M Tris–HCl (pH 7·4) buffer. Incubations were performed in the absence of both liver protein and NADPH-generating system. Substrate disappearance was monitored as described above. Reaction products were identified using the procedure outlined under "Metabolite identification". The aqueous incubations containing cysteine were further chromatographed on cellulose acetate (paper) using a butanol–acetic acid–water (4:1:1) solvent system. Separated bands were revealed by spraying with 0·1% Ninhydrin reagent in isopropanol.

Formaldehyde was assayed by the procedure of Nash.[4] To observe the effect of sulfhydryl reagents, boiled homogenate (corresponding to 50 mg of liver) was pre-incubated with 1 mM sodium *p*-chloromercuribenzoate in water. The NADPH-generating system and phosphine were then added, and the mixture was incubated and assayed as previously described.

RESULTS

Enzymatic and non-enzymatic degradation of substrates. Phosphines are subject to spontaneous oxidation to phosphine oxides and other products.[5] Thus, in dealing with potential metabolic phenomena, it was important to determine if the results would be obscured by spontaneous chemical reactions occurring in incubation mixtures. Table 1 shows that both substrates disappear slowly when incubated in the presence of buffer alone. This loss is somewhat more rapid in the presence of the NADPH-generating system. However, the rate of disappearance in the presence of microsomal

TABLE 1. ENZYMATIC AND NON-ENZYMATIC DEGRADATION OF PHOSPHINES

Preparation	Substrate disappearance (nmoles/min)	
	Aminophosphine Compound 1	Phosphine Compound 2
Buffer	2·7 ± 0·6	6·3 ± 1·0
Buffer, NADPH*	22·6 ± 0·7	13·9 ± 2·5
Buffer, microsomes	15·5 ± 0·5	10·4 ± 1·2
Buffer, NADPH, microsomes†	50·5 ± 2·5	46·6 ± 0·14
Buffer, boiled whole homogenate	50·5 ± 2·0	35·0 ± 1·0
Buffer, NADPH, boiled whole homogenate†‡	50·5 ± 2·5	46·6 ± 0·14
Buffer, NADH		15·7 ± 1·0
Buffer, NADH,§ microsomes†		16·7 ± 0·8
Buffer, NADPH, boiled whole homogenate†‡ Na^+ *p*-chloromercuribenzoate (1 mM)	50·3 ± 2·5	34·9 ± 0·8

* NADPH-generating system consisting of NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and Mg^{2+}.

† Equivalent to 50 mg liver.

‡ Microsomes were boiled for 5 min and rehomogenized before use. This reaction is not time-dependent (see text).

§ NADH was generated by the enzymatic reduction of NAD by glucose 6-phosphate, glucose 6-phosphate dehydrogenase and Mg^{2+}.

protein is substantially higher and this, together with the fact that preliminary studies indicated that the rate of microsomal reaction was dependent on both time and protein concentration, indicates that enzymatically mediated reactions are involved in phosphine disappearance.

Table 1 also shows that the substrates disappear when incubated with boiled whole homogenate. This reaction occurs instantaneously (Table 2), in contrast to the far slower disappearance observed enzymatically. Furthermore, substrate disappearance in the presence of boiled liver homogenate was not affected by pretreatment of the denatured liver protein with sulfhydryl reagents, i.e. sodium *p*-chloromercuribenzoate (Table 1).

TABLE 2. REACTION OF AMINOPHOSPHINE WITH BOILED WHOLE HOMOGENATE

Preparation	Time (min)	Substrate disappearance (nmoles/min)
Fresh whole homogenate*	0	0
	20	33·8 ± 1·2
Boiled whole homogenate*	0	48·5 ± 1·8
	20	39·8 ± 1·5

* Equivalent to 100 mg liver.

The data in Table 1 also indicate that the enzymatic process is NADPH dependent and that NADH will not serve as a co-factor for the reaction.

Intracellular localization of phosphine-metabolizing enzymes. Table 3 indicates that the phosphine-metabolizing enzymatic activity is located primarily in the microsomal fraction. Substantial losses of the aminophosphine, compound 1, occur in other fractions, but reference to Table 1 reveals that these losses are comparable to those observed for incubation of this substrate in the presence of the NADPH-generating system alone. The aminophosphine, compound 1, disappears much more rapidly in the presence of microsomes than with the various supernatant fractions in which the microsomes remain in suspension (Table 3). Furthermore, the rate of this microsomal metabolism can be substantially reduced by recombination of washed microsomes with the soluble fraction (Table 4). This may indicate the presence of a compound in the soluble fraction which inhibits enzymatic alteration of the substrate. The inhibitory factor could be inactivated by boiling the soluble fraction.

TABLE 3. INTRACELLULAR DISTRIBUTION OF PHOSPHINE-METABOLIZING ENZYMES IN RAT LIVER HOMOGENATES*

Fraction	Amount of substrate metabolized (nmoles/min)	
	Aminophosphine Compound 1	Phosphine Compound 2
No enzyme protein	22·50 ± 2·50	14·00 ± 5·00
Whole homogenate	32·00 ± 2·50	45·50 ± 0·13
Nuclear fraction	20·50 ± 2·50	5·50 ± 0·28
Mitochondrial fraction	20·00 ± 0·25	5·50 ± 0·45
Microsomal fraction	50·50 ± 2·50	51·00 ± 0·75
600 *g* Supernatant	32·50 ± 3·50	50·00 ± 0·45
9000 *g* Supernatant	28·50 ± 0·15	48·50 ± 0·80
105,000 *g* Supernatant	9·50 ± 2·00	0·00 ± 0·00

* All incubations contained protein equivalent to 50 mg liver.

Enzymatic degradation of diphenylmethylphosphine, compound 2, is also mediated by enzymes localized in the microsomal fraction of liver, but the oxidase activity is not affected by components in the soluble fraction.

TABLE 4. EFFECT OF SOLUBLE FRACTION ON MICROSOMAL METABOLIC RATE OF AMINOPHOSPHINE COMPOUND 1

Preparation	Substrate disappearance (nmoles/min/mg liver)
Microsomes	1·32 ± 0·04
Microsomes + 100,000 *g* supernatant	0·45 ± 0·01
Microsomes + boiled 100,000 *g* supernatant	1·20 ± 0·04

Effect of CO atmosphere on phosphine metabolism. Carbon monoxide is known to inhibit NADPH-dependent mixed-function oxidases by competitively inhibiting O_2 complexation with cytochrome P-450.[6] Since 50 per cent inhibition is observed when the substrates were incubated in a carbon monoxide atmosphere (Table 5), it is likely that this enzyme system is involved in phosphine metabolism.

TABLE 5. EFFECT OF ATMOSPHERE ON PHOSPHINE METABOLISM

Atmosphere	Substrate	Substrate disappearance (nmoles/min/ mg liver)	Inhibition (%)
O_2, N_2*	Aminophosphine, compound 1	0·74 ± 0·01	
CO, O_2†	Aminophosphine, compound 1	0·37 ± 0·01	50·0
O_2, N_2	Phosphine, compound 2	1·11 ± 0·01	
CO, O_2	Phosphine, compound 2	0·56 ± 0·02	50·4

* Mixture consisted of four parts N_2; one part O_2.
† Mixture consisted of four parts CO; one part O_2.

Reaction with thiols. Oxidative metabolic transformations may be mediated by lipid peroxidases and other hydrogen peroxide-yielding enzymes found in liver microsomes, as well as by mixed-function oxidases.[7] Such peroxide-mediated oxidations are inhibited by radical quenchers such as glutathione. When the aromatic phosphines were incubated in the presence of glutathione, immediate and substantial substrate disappearance was noted (Table 6). Incubation with cysteine also resulted in

TABLE 6. REACTION WITH THIOLS

Thiol	Substrate disappearance (nmoles/min)	
	Aminophosphine Compound 1	Phosphine Compound 2
Buffer only	2·7 ± 0·6	6·3 ± 1·0
Glutathione*	83·0 ± 3·5	103·6 ± 4·3
Cysteine	58·4 ± 1·4	72·0 ± 2·7

* Ten μmoles of thiol and 2 μmoles of substrate were used.

rapid substrate disappearance. These reactions are non-enzymatic and occur readily in the absence of microsomal protein and the NADPH-generating system. After thin-layer chromatographic separation of large-scale incubations, the reaction product obtained from the aminophosphine, compound 1, and diphenylmethylphosphine, compound 2, was identified by the mass spectral procedure outlined under "Metabolite identification" to be the corresponding phosphine sulfides, compound 3 (R_f = 0·42) and compound 4 (R_f = 0·56) (Fig. 1 and 2; equation 1). Incubation

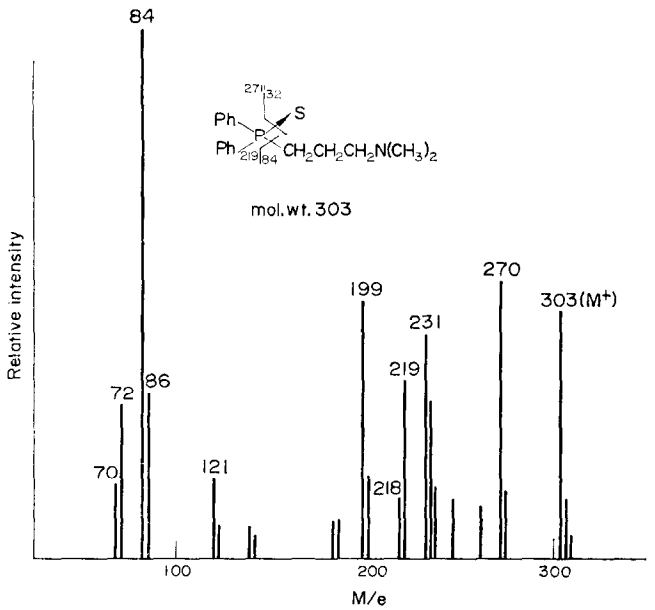


FIG. 1. Mass spectrum of extract identified as 3-dimethylaminopropyldiphenylphosphine sulfide

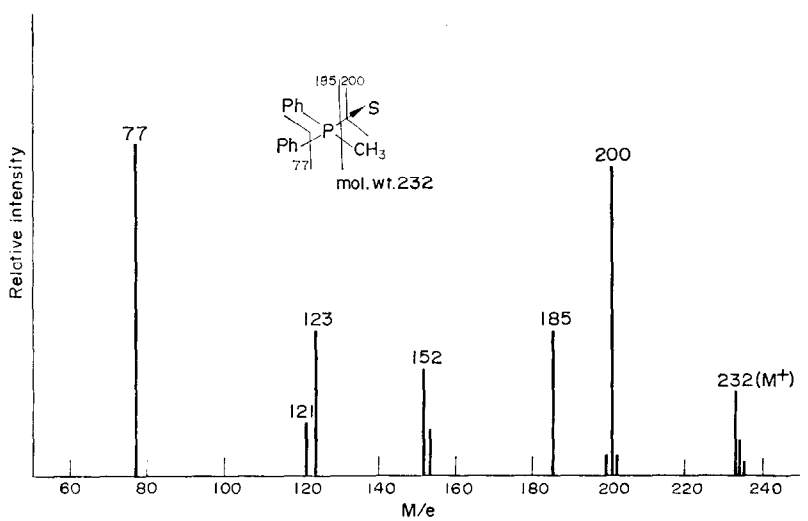
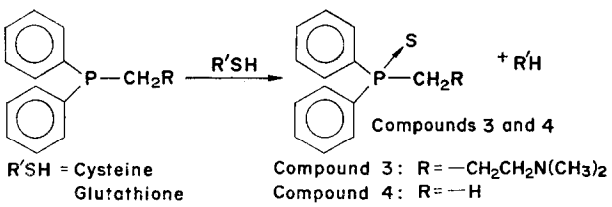


FIG. 2. Mass spectrum of extract identified as methyldiphenylphosphine sulfide.



(1)

mixtures containing the phosphine and cysteine were also subjected to paper chromatography. Two spots were manifested (R_f 0·05; R_f 0·42) after spraying with Ninhydrin reagent. These were identified as cysteine and alanine, respectively, by chromatographic comparison with known compounds.

Formaldehyde production. Tertiary methylamines are commonly demethylated by microsomal oxidases (equation 2), as the methyl group is

$$R_2N\text{–}CH_3 \xrightarrow[\text{enzyme}]{\text{liver}} R_2NH + CH_2O \qquad (2)$$

converted to formaldehyde. In order to ascertain whether similar *p*-methyl cleavage occurs with diphenylmethylphosphine, assays for formaldehyde were performed. No formaldehyde was detected, indicating that neither oxidative-*p*-methyl cleavage nor *N*-demethylation is a likely detoxication pathway for the compounds studied.

Identification of metabolites from 3-dimethylaminopropyl-diphenylphosphine. Aminophosphine, compound 1, was incubated in the presence of buffer alone, and with the NADPH-generating system, alone and with microsomal protein. Thin-layer chromatography of the residues obtained from the protein-free incubations indicated one spot (R_f 0·30) in addition to the one attributable to unreacted substrate (R_f 0·46). This substance was identified by mass spectrometry to be the phosphine oxide corresponding to the substrate, compound 5 (Fig. 3). It was clear that less of the oxide

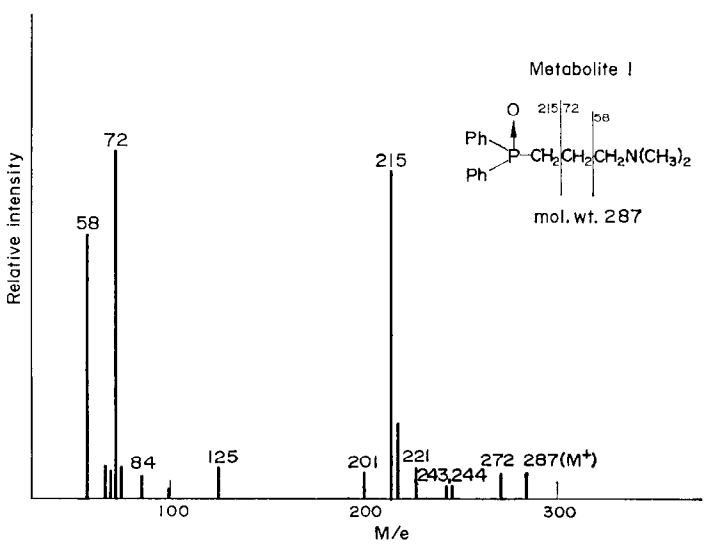
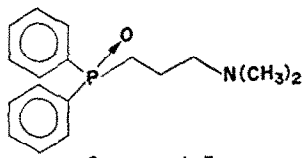


FIG. 3. Mass spectrum of extract tentatively identified as 3-dimethylaminopropyldiphenylphosphine oxide.



Compound 5

was produced in the buffer incubation, but decisive quantitative distinction between relative amounts of substrate and oxide present in the other two systems was not possible. In addition to the phosphine and its *p*-oxide, the microsomal residue contained a third substance (R_f 0·40), whose mass spectrum is shown in Fig. 4. Although

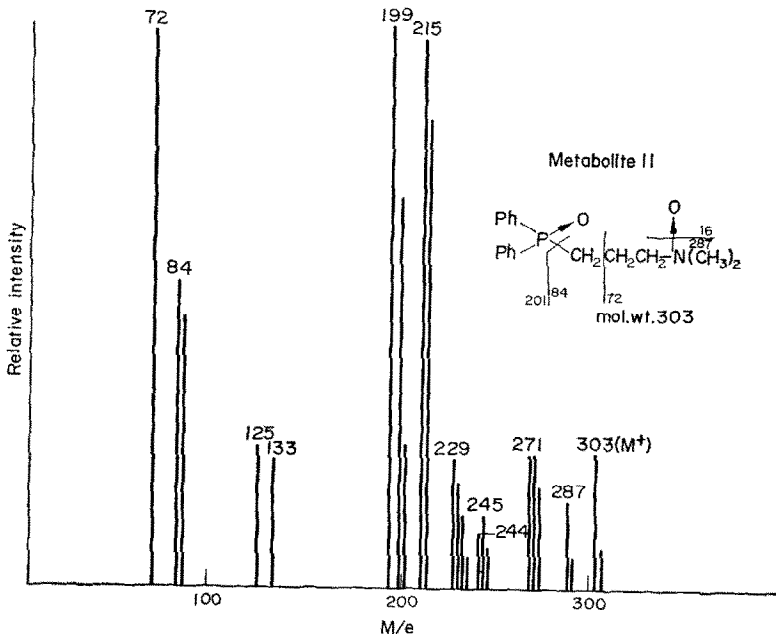
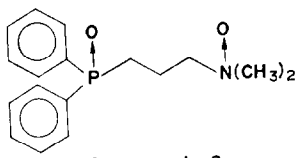


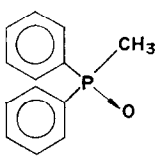
FIG. 4. Mass spectrum of extract identified as 3-dimethylaminopropyldiphenylphosphine *N,P*-dioxide.

this material has the same molecular ion (weight) as the aminophosphine sulfide, compound 3, the mass spectrum is easily differentiable from the sulfide. The presence of very prominent M-16 and M-32 peaks indicates that two oxygen atoms rather than one sulfur atom have been introduced. The presence of characteristic phosphine oxide peaks at m/e 199 and 215 established that no aromatic hydroxylation has occurred. Similarly, characteristic alkylamino peaks at m/e 72 and 84 indicate that the alkyl chain has not suffered hydroxylation. It was thus presumed that oxidation of the tertiary aliphatic amine had occurred to form an amine oxide. This assumption was supported by the fact that mass spectral fragmentations of *N*-oxides are known to involve extensive *N—O* cleavage,[8] thus explaining the essentially unchanged character of the peaks at m/e 84 and 72 from those observed in the mass spectrum of the *p*-oxide. The second metabolite was therefore identified as 3-dimethylaminopropyl diphenylphosphine-*N,P*-dioxide, compound 6.



Compound 6

Identification of metabolites from diphenylmethylphosphine. The substrate was incubated in the presence of buffer alone and with the NADPH-generating system both in the presence and absence of microsomal protein. TLC analysis of the residue of all three incubation mixtures revealed only one spot (R_f 0·17) in addition to unreacted diphenylmethylphosphine, compound 2 (R_f 0·63). This material was identified by mass spectrometry to be diphenylmethylphosphine oxide, compound 7 (Fig. 5). It



Compound 7

showed a molecular ion at 216 and a prominent M-16 peak arising from loss of an oxygen atom. Identification of the phosphine oxide was substantiated by comparison with an authentic sample synthesized by the *m*-chloroperbenzoic acid oxidation of diphenylmethylphosphine.[1]

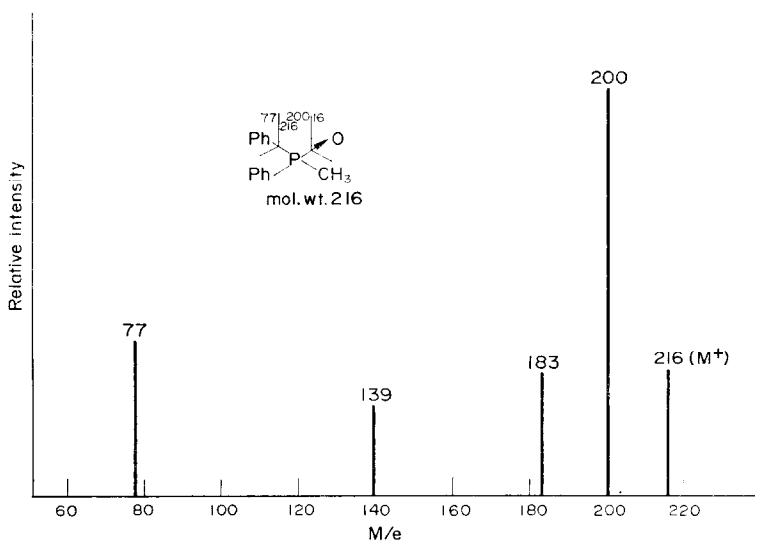
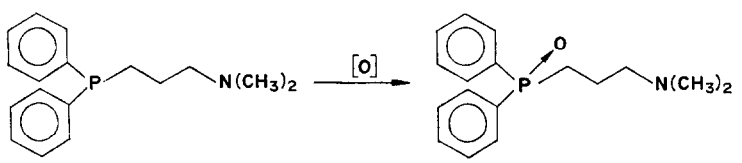


FIG. 5. Mass spectrum of extract identified as methyldiphenylphosphine oxide.

DISCUSSION

Experiments have shown that 3-dimethylaminopropyldiphenylphosphine, compound 1, and diphenylmethylphosphine, compound 2, are metabolized by rat liver. The biotransformations are NADPH-dependent, inhibited by carbon monoxide, and mediated by enzymes localized in the microsomal fraction. It is therefore presumed that their metabolism is associated with the cytochrome P-450 mixed-function oxidases. This stands in contrast to results obtained in certain other cases, where enzymatic *N*-oxygenation is not dependent on the cytochrome P-450 system.[9] In fact, according to the Arrhenius concept,[10] *N*-oxygenation is an indication of a disturbed flavoprotein–cytochrome P-450 interaction.

Tissue distribution profiles revealed that maximal oxidase activity for the aminophosphine, compound 1, resided in the microsomal fraction of liver. This activity was significantly greater than that observed with whole liver or supernatant fractions in which microsomes remained in suspension. Such results may indicate the presence of a substance in the soluble fraction which inhibits detoxication by either interfering with enzyme function or with substrate binding. This postulation was substantiated, since microsomal activity could be significantly reduced by recombination of the microsomes with the soluble fraction (Table 4). The inhibitor appears to be heat labile since no reduction in oxidase activity is observed when the microsomes are combined with boiled soluble fraction. Since diphenylmethylphosphine metabolism was not affected by components in the soluble fraction, it was concluded that this was probably not a general inhibitor of phosphine oxidation. Since preliminary studies have shown compound 6 to be stable toward incubation and extraction procedures used in these studies, it is unlikely that compound 5 arises from spontaneous reduction of 6.



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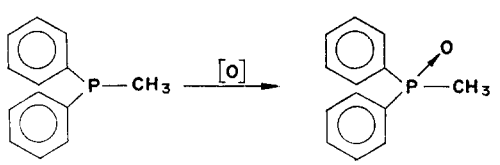


(4)

Some non-enzymatic oxidation of the organo-phosphines occurs, especially in the presence of the NADPH-generating system, but the extent of oxidation was significantly less than is observed with the mixed-function oxidases.

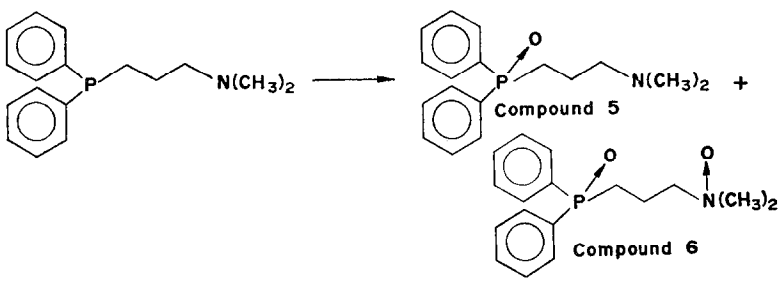
An immediate, non-enzymatic disappearance of substrate occurred in the presence of boiled whole liver homogenate. It was felt that perhaps sulfhydryl groups are liberated during the denaturation process and subsequently react with the phosphine. Both substrates were shown to react rapidly with thiols in aqueous media at room temperature, to yield the phosphine sulfide (equations 3 and 4), apparently by sulfur abstraction.

Although triphenylphosphine is known to react with episulfides[11] and octyl mercaptan with triethyl phosphite,[12] this is the first indication that tertuary aralkyl

phosphines react with biologically significant thiols. Such reactivity indicates a possible chronic toxicity hazard.

Subsequent experiments failed to support the hypothesis that protein-bound sulfhydryl groups are involved in the non-enzymatic degradation of phosphines. Examination of the reaction products involving boiled homogenate revealed only one product, the corresponding phosphine oxide. In addition, phosphine oxide formation in the presence of boiled homogenate was not inhibited by sulfhydryl reagents (*p*-chloromercuribenzoate). If oxidation involved intermediate reaction with thiol groups, the rate of phosphine oxide formation should be reduced in the presence of compounds that covalently combine with thiols. Since the rate of *p*-oxide formation in the presence of boiled homogenate was significantly greater than oxidation in the presence of the NADPH-generating system alone, the denatured protein must play some yet unknown role in facilitation of oxidation.

3-Dimethylaminopropyldiphenylphosphine, compound 1, was metabolized to yield a mixture of two products: the corresponding phosphine oxide, compound 5;



(5)

and the *N,P*-dioxide, compound 6 (equation 5). No evidence could be found to indicate that this *N*-oxide undergoes a Polonovskii-type demethylation, unlike chlorpromazine where both *N*-oxide and *N*-demethylated product have been identified.[13]

The oxygen atom of the mono-oxide is bonded to phosphorous (not nitrogen) indicating that trivalent phosphorus is more susceptible to microsomal oxidation than nitrogen. This may be due to the fact that aromatic rings do not reduce the basicity of phosphines as they do with amines, since the unshared electrons on phosphorous cannot be as efficiently delocalized by the aromatic system.[14] The highly localized electron density on trivalent phosphorus, therefore, may make phosphines very susceptible to attack by an electrophilic oxygenating species.

Diphenylmethylphosphine, compound 2, was metabolized by microsomal enzymes to yield only one product, diphenylmethylphosphine oxide, compound 7.

Careful analysis of incubation mixtures revealed no evidence for oxidative *p*-methyl cleavage analogous to *N*-demethylation, or for other possible metabolic products, such as phenols, which would have resulted from aromatic hydroxylation.

Perhaps this is a consequence of the remarkably high water solubility of phosphine oxides, which are rapidly formed by microsomal enzymes. Oxidation to this state may produce a metabolite sufficiently polar as to be rapidly removed from further interaction with microsomal enzymes.

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