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THE METABOLISM OF HEXAMETHYLPHOSPHORAMIDE AND RELATED COMPOUNDS

A. R. JONES and H. JACKSON

Unit of Reproductive Pharmacology, University of Manchester, Manchester 13

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Abstract—The metabolism of hexamethylphosphoramide (HMPA) has been studied in the rat and mouse. Three urinary metabolites were isolated from both species and identified as pentamethylphosphoramide (PMPA), *N',N',N'',N'''*-tetramethylphosphoramide and *N',N'',N'''*-trimethylphosphoramide. Hexamethylthiophosphoramide (thio-HMPA), besides producing HMPA, is degraded by an alternative pathway to PMPA *via* pentamethylthiophosphoramide (thio-PMPA). Distribution studies with ^{32}P-HMPA indicated no specific localisation of labelled material in the tissues of either species.

Metabolism of the homologues hexa-ethylphosphoramide (HEPA) and hexa-*n*-propylphosphoramide (HPPA) to *N',N'',N'''*-tri-ethylphosphoramide and *N',N'',N'''*-tri-*n*-propylphosphoramide respectively suggests general detoxification of such compounds by a stepwise series of dealkylations.

Degradation of the phosphoramides by rat liver slices *in vitro* indicates that their metabolism occurs by a process of oxidative dealkylation.

THE DEMONSTRATION that hexamethylphosphoramide (HMPA, I) has a marked antispermatogenic effect on rats and mice[1] necessitated an investigation into its metabolism in the event that the biological activity was due to more active metabolites. Apart from a recent paper[2] on the metabolism of HMPA in the housefly, little is known about the detoxification of hexa-alkylphosphoramides in animals. Compared with the rapid and complete metabolism of tri-ethylene-phosphoramide (XI) to inorganic phosphate in the mouse[3], a preliminary investigation of the fate of ^{32}P-HMPA reported no degradation to phosphate in either rat or mouse, the majority of administered compound being rapidly excreted in both species "apparently unchanged"[4].

This matter has now been re-investigated together with a study of the metabolism of hexa-ethylphosphoramide (HEPA, VII), hexa-*n*-propylphosphoramide (HPPA, VIII) and hexamethylthiophosphoramide (thio-HMPA, V). Some preliminary results have been published elsewhere[5].

MATERIALS AND METHODS

^{32}P-*labelled hexamethylphosphoramide*

Phosphorus oxychloride (274 g) labelled with ^{32}P (23·3 mc) was diluted with unlabelled material (1·9 g) in *n*-hexane (20 ml). This was added dropwise over 1 hr to a well stirred solution of anhydrous dimethylamine (2·8 g) and triethylamine (5 g) in *n*-hexane (30 ml) at 0°. After refluxing for 15 hr, the mixture was filtered, the filtrate sealed under nitrogen with dimethylamine (2 g) and triethylamine (2 g) and kept at 90° for 18 hr. Filtration, followed by removal of the solvent *in vacuo* and addition of unlabelled HMPA (B.D.H., 2 g), gave the product as a colourless liquid, b.pt 68°

(0·6 mm). It was homogeneous by TLC and GLC. The sp. act. was 1·4 mc/g representing an isotope recovery of 63 per cent.

Pentamethylphosphoramide (II)

A solution of *N*-methylaminodichlorophosphine oxide (15 g)[6] in dry ether (50 ml) was added dropwise with stirring over 1 hr to anhydrous dimethylamine (22 g) in dry ether (100 ml) at − 70°. After keeping the mixture overnight at room temperature, filtration and removal of the solvent gave a colourless oil, purified by distillation. B.p. 119–121° (2·5 mm) (55%); reported[7] b.p. 137–143° (3 mm).

Hexamethylthiophosphoramide (V)

This was prepared from phosphorus sulphochloride and dimethylamine in 75 per cent yield as for hexamethylphosphoramide. It was recrystallized from chloroform as white prisms m.p. 28–29°, reported[8] m.p. 29°.

Pentamethylthiophosphoramide (VI)

Phosphorus sulphochloride (34 g) in dry ether (100 ml) was added with stirring over 0·5 hr to anhydrous dimethylamine (36 g) in dry ether (200 ml) at − 70°. The mixture was stirred a further hour, filtered and the filtrate reduced *in vacuo*. Distillation gave *N',N',N'',N''*-tetramethyldiaminochlorophosphine sulphide as a colourless oil, b.p. 102–104° (4 mm) (70%).

Found, C, 25·96, H, 6·40, N, 14·98%.

$C_4H_{12}N_2PSCl$ requires C, 25·73, H, 6·48, N, 15·01%. A solution of the sulphide (15 g) in dry ether (50 ml) was added dropwise with stirring over 0·5 hr to anhydrous dimethylamine (12 g) in dry ether (100 ml) at − 70°. The mixture was stirred 3 hr at room temperature, the hydrochloride filtered off and the ether removed *in vacuo*. From the residue, pentamethylthiophosphoramide (VI) distilled as a colourless oil, b.p. 95–97° (0·5 mm) (50%).

Found, C, 33·20, H, 8·75, N, 22·90%.

$C_5H_{16}N_3PS$ requires C, 33·14, H, 8·90, N, 23·18%.

Other Phosphoramides

N',N',N'',N'''-tetramethylphosphoramide (III) was synthesized in 75 per cent yield from methylamine and *N',N'*-dimethylaminodichlorophosphine oxide[9], while the tri-alkylphosphoramides and hexa-alkylphosphoramides were prepared by the methods of Arceneaux *et al.*[7], Stuebe and Lankelma[10] and Mark[11] respectively.

Chromatography

The procedure for TLC is given in Table 1. Radioactive chromatograms were scanned either on a B.T.L. Chromatogram Counter or a Packard model 7201 Radiochromatogram Scanner and developed by contact autoradiography on Ilford X-ray film. Liquid scintillation counting was carried out in an I.D.L. Tritomat 6020.

BIOLOGICAL STUDIES

In drinking water experiments, the phosphoramides were given to rats at the following concentrations; 0·1 per cent using HMPA, PMPA and HEPA; 0·025 per cent for thio-HMPA and thio-PMPA. HPPA was given by gavage at 50 mg/kg/day in 50% aqueous dimethyl sulphoxide. Animals were housed in metabolic cages, the collected urine being filtered and stored at − 10°.

Extraction of urine. Samples (100–200 ml) were diluted to about 500 ml with Analar methanol, kept at − 10° overnight and the precipitated salts removed by filtration. The liquid was reduced *in vacuo* to about 20 ml, diluted with methanol (100 ml), filtered and again reduced to low volume. This concentrate was continuously extracted with chloroform for 24 hr, the chloroform phase dried (Na_2SO_4) and reduced to a brown oil (1–2 ml).

TABLE 1. R_f VALUES FOR SYNTHETIC AND 'METABOLIC' PHOSPHORAMIDES ON SILICA GEL *G* THIN-LAYER CHROMATOGRAMS

Phosphoramide	Structure	R_f synthetic	R_f metabolite
Hexamethylthio-	V	1·00	—
Pentamethylthio-	VI	0·95	0·94
Hexamethyl-	I	0·85	0·84
Pentamethyl-	II	0·73	0·73
Tetramethyl-	III	0·62	0·62
Trimethyl-	IV	0·53	0·52
Hexa-ethyl-	VII	0·81	—
Penta-ethyl-		0·78	—
Tri-ethyl-	IX	0·57	0·58
Hexa-*n*-propyl-	VIII	0·82	—
Penta-*n*-propyl-		0·78	—
Tri-*n*-propyl-	X	0·52	0·52

Immediately before use thin-layer plates were activated at 110° for 3hr. The developing solvent system was chloroform:ethanol 3:1, phosphoramides being detected by the molybdate reagent of Hanes and Isherwood[15].

Metabolism of HMPA. The concentrate from administration of HMPA was chromatographed on a column (40 × 2 cm) of Whatman SG-31 Chromedia. Successive elution with chloroform containing 5, 10 and 15% methanol gave HMPA, PMPA and *N',N',N'',N'''*-tetramethylphosphoramide respectively. Purification of the two metabolites was carried out by preparative TLC on silica gel G (Table 1) and elution with methanol. Their identities were confirmed by comparison of the mass spectral splitting patterns and gas chromatographic behaviour[12] with authentic samples. Chromatography of the urine extract from PMPA administration and elution with chloroform containing 15% methanol gave, besides *N',N',N'',N'''*-tetramethylphosphoramide, crystals of *N',N'',N'''*-trimethylphosphoramide (IV), crystallizing from chloroform as white prisms m.p. 103°, mixed m.p. 102–103° with an authentic sample[7].

Metabolism of other phosphoramides. The concentrate from administration of each phosphoramide was similarly purified by chromatography on SG-31 Chromedia. Metabolites of thio-PMPA, HEPA and HPPA were isolated by elution with chloroform containing 10, 12 and 15% methanol respectively. Preparative TLC further purified the metabolites which were identified by mass spectroscopy.

In control experiments with the compounds added to normal rat urine, there was no chromatographic evidence of any induced change following the same extraction process.

Isolation of ^{14}C-formaldehyde-dimedone. Uniformly labelled ^{14}C-HMPA (0·55 mM) in water (1 ml) was added to oxygenated rat liver slices (20 g) in 0·075 M phosphate buffer (50 ml) at 37°. After 1 hr inactive aqueous formaldehyde (2%, 1·5 ml) was

added and the mixture filtered into aqueous dimedone (0·4%, 100 ml). The precipitate of formaldehyde-dimedone[13] was collected and recrystallized from aqueous methanol to constant specific activity.

Liver oxidation of HEPA. Unlabelled HEPA (300 mg) was incubated with rat liver slices (60 g) as described for active HMPA. Acetaldehyde was isolated as its dimedone derivative (m.p. 141°) by the method of Mackenzie *et al.*[14] The filtrate was continuously extracted with chloroform to give *N',N'',N'''*-triethylphosphoramide (IX), purified by preparative TLC.

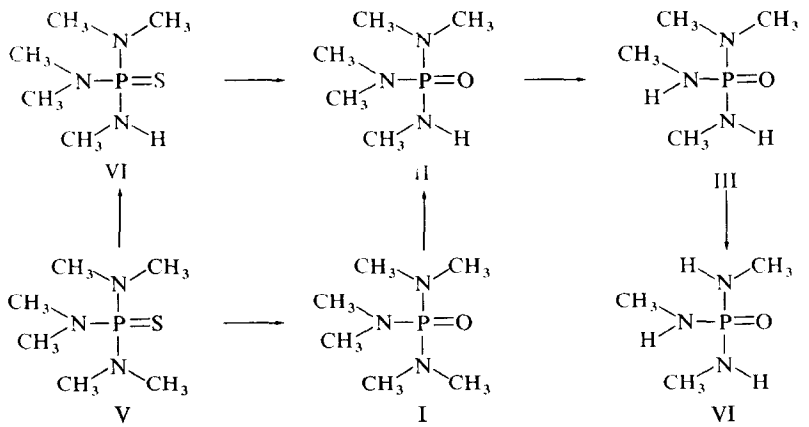

FIG. A. Metabolic Pathways of HMPA and thio-HMPA in the rat and the mouse.

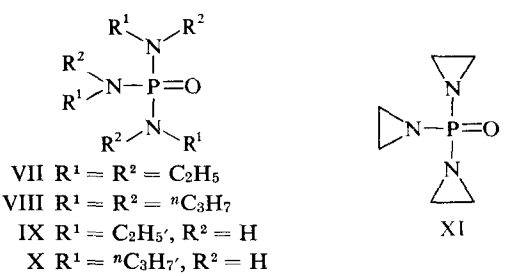

FIG. B.

DISCUSSION

Metabolism. Hexamethylphosphoramide (I) has been shown to undergo a sequence of demethylations *in vivo* to *N',N'',N'''*-trimethylphosphoramide (IV). On the basis of the anti-spermatogenic effect of the parent compound, these dealkylations constitute an inactivation since the metabolites show negligible activity in the rat and mouse[16]. Borkovec's recent finding that HMPA, an effective insect chemosterilant, is metabolized only to inactive PMPA (II) in the housefly[2] and the fact that a similar pathway is known to occur in the turnip[17] suggests that this is a general metabolic process.

A related derivative of phosphoric acid tri-amide, tri-ethylenephosphoramide (XI), is rapidly and completely degraded to inorganic phosphate by the mouse but even at a low dose level shows high pharmacological activity[18]. This suggests primary chemical reaction with cellular components either by a direct alkylation mechanism

or possibly through a protonated ring-opened species[19]. That a similar alkylating mechanism might occur with the hexa-alkylphosphoramides is indicated by the nature of the metabolites produced by HMPA and the isolation of *N',N'',N'''*-tri-ethylphosphoramide (IX) and *N',N'',N'''*-tri-*n*-propylphosphoramide (X) as the only metabolites of the hexa-ethyl and hexa-*n*-propyl analogues. Whereas HMPA can be found in the urine of rats 2 days after administration, the homologues are rapidly metabolized; e.g. no unchanged HEPA or HPPA could be detected 24 hr after dosing. That their detoxification proceeds in a stepwise manner (analogous to that of HMPA) is indicated by the metabolism of penta-ethylphosphoramide and penta-*n*-propylphosphoramide, which were also rapidly converted to the corresponding *N',N'',N'''*-tri-alkylphosphoramides.

If the biological activity of HMPA is the result of a direct alkylation mechanism, the higher alkyl homologues might be even more effective since they are dealkylated more rapidly. This, however, is not the case for fertility studies with HEPA, at levels comparable to sterilizing doses of HMPA, show only minimal antispermatogenic activity.[16]

An initial step in the metabolism of the sulphur analogues (thio-phosphoramides) is replacement of sulphur by oxygen. Thus triethylenethiophosphoramide is rapidly converted to triethylenephosphoramide (XI) in the rat, dog and rabbit[20] and morpholinodiethylenethiophosphoramide yields morpholinodiethylenephosphoramide in both rat and man.[21] Thio-HMPA (V) is also converted to HMPA in the rat and mouse but the competitive process of dealkylation produces a more toxic intermediate, thio-PMPA (VI) which, in turn, is converted to biologically inactive PMPA.

Mechanism of Dealkylation. The metabolites produced from HMPA and its homologues are indicative of a sequence of oxidative dealkylations which are known to occur in the metabolism of tertiary amines.[22] As the enzyme systems responsible for tertiary amine dealkylations can be isolated from liver preparations, ^{32}P-HMPA was incubated with rat liver slices and the filtrate chromatographed. Autoradiography demonstrated the presence of all three urinary metabolites. The *in vitro* isolation of formaldehyde (as its dimedone derivative) and the detection of the metabolites in this system suggest that the hexa-alkylphosphoramides are degraded *in vivo* in the liver. This metabolic pathway appears to parallel that of octamethylpyrophosphoramide ("Schradan") which is mainly transformed (by loss of one methyl group) to its heptamethyl derivative.[17] Although the mechanism for the conversion of Schradan to its major metabolite is not clear, it is known that an initial unstable but active intermediate is produced, which is responsible for the induced anticholinesterase activity. Since this conversion can be carried out with oxygenated liver slices, by oxidation with permanganate or hydrogen peroxide, the following pathway (A → B → C → D) has been suggested[17] with the methylol (C) as the active intermediate.

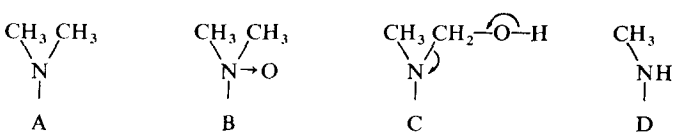

FIG. C.

Our results with HMPA tend to support methylol formation as the conversion of

HMPA to PMPA with permanganate involves the liberation of formaldehyde (Table 2). However, hydrogen peroxide did not accomplish this conversion although trace

TABLE 2. PERCENTAGE OF RADIOACTIVITY IN FORMALDEHYDE-DIMEDONE ISOLATED FROM LIVER* AND PERMANGANATE† OXIDATION OF UNIFORMLY LABELLED ^{14}C-HMPA

pH	liver oxidation	permanganate oxidation
7	5	36
8	22	61
9	0	117

* Oxygenated rat liver slices (20 g) in 0·075M phosphate buffer (50 ml).

† 0·55mM $KMnO_4$ in 0·075M phosphate buffer (50 ml). Incubated for 1 hr at 37° with 0·55mM ^{14}C-HMPA of s.a. 0·36 μc/mM. 100 per cent corresponds to the loss of one sixth of the total methyl groups.

amounts of a crystalline product, thought to be HMPA *N*-oxide (B), were found which could not be converted to PMPA. Therefore it appears that the detoxification of HMPA proceeds A → C → D. Should there by an analogy with Schradan, perhaps the unstable methylol (C) is responsible for the antispermatogenic activity.

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REFERENCES

1. H. JACKSON and A. W. CRAIG in *Proc. 5th I.P.P.F. Conf.*, p. 49 (1967).
2. S. C. CHANG, P. H. TERRY, C. W. WOODS and A. B. BORKOVEC, *J. Econ. Entomol.* **60**, 1623 (1967).
3. M. V. NADKARNI, E. I. GOLDENTHAL and P. K. SMITH, *Cancer Res.* **17**, 97 (1957).
4. H. JACKSON and A. W. CRAIG, *Nature, Lond.* **212**, 86 (1966).
5. A. R. JONES and J. S. BERTRAM, *Experientia* **24**, 326 (1968).
6. E. Y. SPENCER, R. D. O'BRIEN and R. W. WHITE, *J. agric. Fd. Chem.* **5**, 123 (1957).
7. R. L. ARCENEAUX, J. G. FRICK, E. K. LEONARD and J. D. REID, *J. org. Chem.* **24**, 1419 (1959).
8. H. J. VETTER and H. NOETH, *Chem. Ber.* **96**, 1308 (1963).
9. J. B. DICKEY, T. E. STANIN and H. W. COOVER, U.S. Patent 2,487,859 (*Chem. Abs.* **44**, P2546i (1950)).
10. C. STUEBE and H. P. LANKELMA, *J. Am. Chem. Soc.* **78**, 976 (1956).
11. V. MARK, *Org. Synth.* **46**, 42 (1966).
12. J. S. BERTRAM, MSc. Thesis, Manchester (1968).
13. J. M. JOHNSTON and C. G. MACKENZIE, *J. biol. Chem.* **221**, 301 (1956).
14. C. G. MACKENZIE, J. M. JOHNSTON and W. R. FRISELL, *J. biol. Chem.* **203**, 743 (1953).
15. C. S. HANES and F. A. ISHERWOOD, *Nature, Lond.* **164**, 1107 (1949).
16. H. JACKSON, E. R. A. COOPER and A. R. JONES, unpublished.
17. D. F. HEATH, D. W. J. LANE and P. O. PARK, *Trans. R. Soc. Lond.* **239B**, 191 (1955).
18. A. W. CRAIG and H. JACKSON, *Br. J. Pharmac.* **10**, 321 (1955).
19. C. BENCKHUIJSEN, *Biochem. Pharmac.* **17**, 55 (1968).
20. A. W. CRAIG, B. W. FOX and H. JACKSON, *Biochem. Pharmac.* **3**, 42 (1959).
21. R. K. MALLER and C. HEIDELBERGER, *Cancer Res.* **17**, 296 (1957).
22. R. T. WILLIAMS, *Detoxication Mechanisms*, p. 148. Chapman & Hall, London (1959).