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METABOLISM OF N:N-DIALKYL CARBAMATES AND RELATED COMPOUNDS BY RAT LIVER

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Abstract—N:N-Dimethyl-*p*-nitrophenyl carbamate is metabolized by an enzyme or enzyme system of rat liver microsomes which requires TPNH and oxygen. The resulting product can be extracted into organic solvents and decomposes in strong acid to yield formaldehyde. Its stability in alkali is intermediate between that of the substrate and N-methyl-*p*-nitrophenyl carbamate. It is formed *in vivo* by rats and probably by certain insects. The rate at which this or similar systems, *in vitro*, will attack analogs of N:N-dimethyl-*p*-nitrophenyl carbamate with different alkyl groups is considerably reduced when the alkyl groups are longer than ethyl. These enzyme systems are inhibited by β-diethylaminoethyl diphenylpropyl acetate (SKF 525-A), by the methylene dioxyphenyl compounds, piperonyl butoxide and sesamex, and by N-octyl bi*cyclo*heptane dicarboximide. The metabolism of twenty-five N:N-dimethyl carbamates and twenty-three related compounds was investigated. Isolan metabolism was evident when followed both by the formation of formaldehyde-yielding material and reduction in the ability to inhibit cholinesterase *in vitro*. Metabolism of certain other carbamates gave formaldehyde-yielding materials without loss in cholinesterase-inhibiting activity.

N-ALKYL and N:N-dialkyl carbamates are widely used as insecticides[1, 2] and as medicinals for the relief of myasthenia gravis and other disorders.[3] In both cases, the ultimate mode of action appears to be the inhibition of cholinesterase. It has been shown that cholinesterase is carbamylated by carbamyl choline, N-methyl- and N:N-dimethylcarbamyl cholines[4, 5] and by N:N-dimethyl carbamoyl fluoride,[4–6] but not by all inhibitory carbamates.[2] The relationship between structure and stability, activity as inhibitors of acetyl and butyryl cholinesterases, and toxicity to houseflies and mice have recently been studied[2] in a series of thirty N-alkyl and N:N-dialkyl carbamates.

Only a few studies have been made on the metabolism of carbamate esterase inhibitors. N-Methyl-1-naphthyl carbamate (the insecticide Sevin) and N-methyl-*p*-nitrophenyl carbamate are hydrolysed, in several mammalian and avian forms, by a plasma albumin fraction which can be separated from the aliphatic, aromatic and choline esterases.[7] In a further study,[2] N-methyl, N-ethyl, N-propyl and N-*iso*-propyl-*p*-nitrophenyl carbamates were found to be hydrolysed by the same plasma albumin fraction, but not by arylesterase, cholinesterase, chymotrypsin, lipase, papain, pepsin, trypsin or egg albumin. Cholinesterase has been implicated in the hydrolysis of certain carbamates,[8] but the turnover number is so low that this cannot be considered a major degradation mechanism.

Of the N:N-dialkyl carbamates investigated, only the least stable of these, N:N-dimethyl carbamoyl fluoride, has been shown to be hydrolysed by mammalian

esterases.[9] This compound is hydrolysed in rabbit plasma by the same esterase(s) which hydrolyzes di*iso*propyl phosphoryl fluoride, as well as by other esterases. In the liver these two compounds are hydrolysed by different enzymes.[9] It is apparent that other mechanisms must be involved in the degradation of dialkyl carbamates of heterocyclic and aromatic enols, as the recovery of animals treated with sub-lethal doses is more rapid than can be accounted for on the basis of excretion of the unchanged molecule.[2]

The microsomes isolated from broken cell preparations of mammalian liver by differential centrifugation are known[10] to be the locus of activity for a large number of oxidative reactions involving drugs and other compounds foreign to the normal metabolism. These reactions include N-dealkylation and, in general, require[10] a reduced pyridine nucleotide* and oxygen. An increased synthesis of the microsomal enzymes involved in the oxidation of a particular drug can be brought about by pretreatment of the animal with the drug in question or in many cases[11–13] by other drugs, often with little apparent structural relationship. Remmer[14] has shown these enzymes to be associated with a microsomal particle of a particular density which contains, perhaps fortuitously, all the cytochrome b_5 (*m*), a substance which is known[15] to be associated with the microsome fraction. In the case of N-dealkylation the reaction yields an aldehyde and the dealkylated substrate. N:N-Dialkylcarbamates have not previously been shown to be susceptible to this type of attack and products other than the dealkylated substrate have not been posulated† except in the case of such N:N-dimethyl phosphoramidates as octamethylpyrophosphoramide.[16]

SKF 525-A and certain other phenylacetic derivatives have been shown to inhibit several microsomal reactions, including N-demethylation of mono-methyl-4-aminoantipyrine, meperidine and other alkylated amines[10, 17] and the incorporation of 1-^{14}C-DL-alanine into proteins.[18]

Carbamate-resistant and -susceptible houseflies treated with methylene dioxyphenyl compounds show an increased susceptibility to these toxicants,[19] thus raising the possibility that compounds such as sesamex and piperonyl butoxide act on the carbamate detoxification mechanism. MGK 264 is also[20] an insecticide synergist.‡ On the basis of *in vivo*-investigations with insects, Sun and Johnson[21] have suggested that the mode of action of the methylene dioxyphenyl synergists may be an inhibition of oxidative detoxification mechanisms. Piperonyl butoxide exerts a synergistic action[22] with the organophosphate, O:O-diethyl-O-(3-chloro-4-methylumbelliferone) phosphorothionate, when applied to mice, apparently correlated with an inhibition of the production of more polar metabolites.

* The following abbreviations are used: DpNC for N:N-dimethyl-*p*-nitrophenyl carbamate; DEpNC for N:N-diethyl-*p*-nitrophenyl carbamate; TPN for triphosphopyridine nucleotide; TPNH for reduced triphosphopyridine nucleotide. The following common names are used as abbreviations: SKF 525-A for β-diethylaminoethyl diphenylpropyl acetate; sesamex for the 2-(2-ethylethoxyethoxy) ethyl-3:4-methylene dioxyphenyl acetal of acetaldehyde; piperonyl butoxide for α-(2-(2-butoxyethoxy)ethoxy)-4:5-methylenedioxy-2-propyltoluene; MGK-264 for N-octyl bi*cyclo*heptane dicarboximide. When other common names are used they are followed by a number referring to the chemical structure given in Table 3.

† A brief outline of some of this investigation was presented as a preliminary note[28] in which this mechanism and a new product were postulated.

‡ Use of this compound in the present investigation was suggested by Dr. Paul A. Dahm, Department of Zoology and Entomology, Iowa State College, Ames, Iowa. Dr. Dahm has shown (unpublished) that this compound is an effective inhibitor of the microsomal oxidation of methyl parathion to methyl para-oxon in mammals.

MATERIALS AND METHODS

Enzyme preparations were made as follows: 20 per cent homogenate. Male Rolfsmeyer rats (Madison, Wisconsin) were killed by a blow on the neck, the livers removed as quickly as possible, washed in distilled water and homogenized for 30 sec in ice-cold potassium phosphate buffer, pH 7·0 and 0·05 M. A Lourdes homogenizer was used with the variable resistor set at 30. All apparatus was precooled in ice prior to use. A 20 per cent homogenate was centrifuged at 15 000 **g** for 30 min at a temperature of 2 °C. The supernatant fraction was designated $S_{15\,000}$ and the residue $R_{15\,000}$. The former should contain microsomes and soluble constituents and the latter whole cells, nuclei, mitochondria and debris. $S_{15\,000}$ was centrifuged at 105 000 **g** for 1 hr at a temperature of 2 °C. The supernatant fraction was designated $S_{105\,000}$ and the residue $R_{105\,000}$.

With the exception of the experiments on oxygen requirements all incubations were carried out in 25-ml Erlenmeyer flasks on a metabolic shaker at a temperature of 37 °C. As the solubility of the substrates in water varied through extreme limits they were added to the flasks as ethanol solutions and the solvent was evaporated in a stream of air.

p-Nitrophenol was determined by adding an equal volume of acetone, removing the precipitated protein by centrifugation, making the supernatant fraction alkaline by the addition of 1 N KOH or Na_2CO_3 and determining the optical density at 400 mμ in a Bausch and Lomb Spectronic 20 colorimeter. Formaldehyde was determined by the chromotropic acid method of Frisell *et al.*,[23] following the removal of protein by trichloroacetic acid precipitation and also by the method of Tanenbaum and Bricker[24] in which the reaction is carried out at pH 7·0. Appropriate controls to correct for non-specific absorption from the enzyme preparation and non-enzymic breakdown of the substrate were carried out with each experiment.

In addition to chemical determinations of carbamate metabolic products, the ability of certain carbamates to inhibit cholinesterase was utilized as a technique for measuring their detoxification. The change in cholinesterase-inhibiting activity during their incubation with liver preparations was followed by removing aliquots at times varying from zero to 120 min, adding these to acetone, filtering, removing the acetone *in vacuo*, and assaying the samples for cholinesterase-inhibiting activity by the manometric method.[2] Appropriate controls lacking either the enzyme preparation or the carbamate indicated a high recovery of active material and no interference from liver constituents.

Two sources of cholinesterase were used to obtain a suitable sensitivity to the different carbamates. Purified bovine erythrocyte cholinesterase (Winthrop Laboratories, 1450 Broadway, New York 18, N.Y.) was used at a concentration of 0·75 mg/ml and homogenates of fly heads were used at a concentration of 1·5 heads/ml. The b_{30} (μl CO_2 liberated/30 min) was 145 for the bovine erythrocyte cholinesterase and 115 for the fly head cholinesterase. Standard curves of the degree of inhibition of known amounts of the carbamate being tested were made in order to evaluate changes on incubation with the liver enzymes. The samples were diluted in order that the zero-time sample yielded a cholinesterase inhibition of 60–80 per cent. The standard carbamate solutions or experimental samples were preincubated with the cholinesterase for 30 min at 37 °C before the addition of acetylcholine chloride to a final concentration of 0·01 M. The subsequent activity of the cholinesterase was expressed as the

b_{30}. Bovine erythrocyte cholinesterase and an initial inhibitor level of 1×10^{-6} M were used for Isolan (cmpd. 17), Pyrolan (cmpd. 18), and compounds 10 and 16, and bovine erythrocyte cholinesterase and 1×10^{-7} M for physostigmine (cmpd. 31) and neostigmine (cmpd. 5). Fly head cholinesterase and an initial level of 1×10^{-6} M were used for dimetan (cmpd. 20) and compounds 15 and 19, while fly head cholinesterase and 3×10^{-7} M were used for Sevin (compd. 32).

RESULTS

Fig. 1 shows the liberation of *p*-nitrophenol from metabolites formed on incubation of DpNC with a 20 per cent liver homogenate and a number of co-factors. Heating the homogenate to 100 °C for 1 min caused a complete loss of activity.

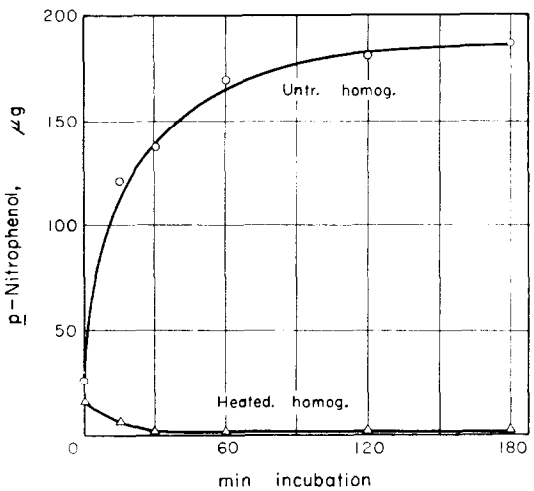


FIG. 1. Metabolism of N:N-dimethyl-*p*-nitrophenyl carbamate by rat liver homogenate: ○——○ = untreated homogenate; △——△ = heated homogenate. Flasks contained the following materials: 1 mg DpNC, 0·31 mg adenosine triphosphate, 0·41 mg diphosphopyridine nucleotide, 0·46 mg TPN, 0·075 mg nicotinamide, 4·06 mg cytochrome *c*, 0·125 mg $MgCl_2$, 0·125 mg $MnCl_2$, 0·15 mg $CoCl_2$, 2·0 ml KPO_4 buffer (0·05 M, pH 7·0) and 2·0 ml 20 per cent homogenate or homogenate which had been heated at 100°C for 1 min. Total volume = 5·0 ml. Flasks removed for *p*-nitrophenol analysis at 0, 15, 30, 60, 120, and 180 min.

By a series of similar experiments with single deletions of co-factors, it was apparent that the principal effect was due to TPN. Experiments with the addition of co-factors to limiting and optimal amounts of TPN indicated that TPNH was the effective co-factor and that other co-factors were involved in the endogenous reduction of TPN. This is illustrated in Fig. 2, which shows the effects of TPN and TPNH when a well dialysed enzyme preparation was used.

The requirement of this pathway for oxygen was evident from experiments in which the $S_{15\,000}$ fraction fortified with TPN was incubated in either air or 95% N_2: 5% CO_2. A 3·5-fold greater DpNC metabolism occurred in air than in the non-oxygen containing atmosphere, based on analysis of the *p*-nitrophenol liberated from the metabolites.

The localization of activity in subcellular fractions was reported in a preliminary communication.[28] The activity was located in the microsome fraction ($R_{105\,000}$). In

the presence of both microsomes and soluble material ($S_{15\,000}$ or $S_{105\,000}$ plus $R_{105\,000}$), either TPN or TPNH were effective, while with microsomes alone ($R_{105\,000}$) TPNH was far superior to TPN. Thus, either TPNH or the soluble enzymes necessary to effect the reduction of TPN are required.

This mechanism, located in the microsomes and requiring TPNH and oxygen, bears a striking resemblance to the N-dealkylation systems described[10] for a number of drugs, suggesting that the carbamate is attacked first at the methyl groups. If such a mechanism operates, presumably one of the products would be formaldehyde.

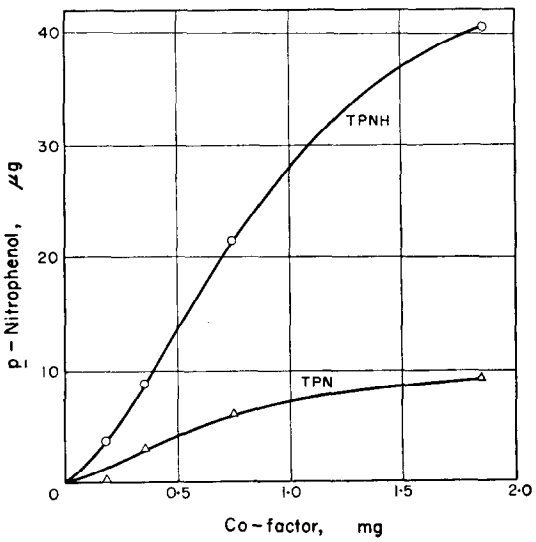


FIG. 2. Effect of TPN and TPNH on the production of *p*-nitrophenol from N:N-dimethyl-*p*-nitrophenyl carbamate. △——△ = +TPN, ○——○ = +TPNH. Each flask contained 1 mg DpNC, 0·0 to 1·85 mg TPN or TPNH, 0·5 ml dialysed $S_{15\,000}$, and 1·0 ml KPO_4 buffer (0·05 M, pH 7·0). Total volume = 3·0 ml. The $S_{15\,000}$ was dialysed against four changes KPO_4 buffer (0·02 M, pH 7·0) over a period of 24 hr, at a temperature of 4 °C. Flasks were incubated at 37 °C for 1 hr before *p*-nitrophenol analysis.

DpNC, 1 mg, was incubated for 1 hr with 1·0 ml $S_{15\,000}$, 1·0 ml KPO_4 buffer and 3·7 mg TPN. Formaldehyde analysis[23] showed the formation of 15 μg of formaldehyde in the presence of TPN and only 2 μg when TPN was omitted.

The molar ratio of total formaldehyde[23] to *p*-nitrophenol production was determined with the metabolites from both DpNC and its N-methyl, N-ethyl analog. The results indicated that only one methyl group of DpNC was attacked (ratios of 0·93:1 and 0·97:1 in separate studies) and that only the methyl group of N-methyl,N-ethyl-*p*-nitrophenyl carbamate was attacked (ratio of 0·98:1).

The observation that on the addition of alkali, during the determination of *p*-nitrophenol, the color developed slowly indicated that formaldehyde and N-methyl-*p*-nitrophenyl carbamate may not be the immediate products, as the latter compound is unstable and would be broken down to free *p*-nitrophenol by the end of incubation. The product of this metabolic reaction was recovered by ether extraction, the ether evaporated and an equal volume of acetone and 0·5 M KPO_4 buffer of pH 8·0 or 9·0 was added. By following the optical density at 400 mμ it was found that the product

had a half life of 37 min at pH 8·0 and 7·0 min at pH 9·0. Under these same conditions the half life of N-methyl-*p*-nitrophenyl carbamate is less than 5 sec and that of N:N-dimethyl-*p*-nitrophenyl carbamate is more than one year.[2]

Further proof of the existence of this product of intermediate stability between the mono and dimethyl compounds was obtained by extracting incubation mixtures with chloroform and determination of *p*-nitrophenol and formaldehyde in the chloroform layer as well as in the water layer. The results indicated 90 per cent extraction into the organic phase from water of both *p*-nitrophenol-yielding material and formaldehyde-yielding material. Molar ratios of formaldehyde:*p*-nitrophenol were 0·93:1 in the water layer before extraction and in the chloroform layer after extraction. Since formaldehyde cannot be extracted into chloroform from water, the product is a compound stable under the conditions of incubation, which liberates formaldehyde under the acid conditions of formaldehyde determination,[23] yields *p*-nitrophenol under alkaline conditions and can be extracted into chloroform from water. In several experiments using DpNC as the substrate, formaldehyde analysis by the method of Tanenbaum and Bricker,[24] which is carried out at pH 7·0, showed less than one-tenth the amount shown by the chromotropic acid method.[23] Further experiments indicated that the metabolite could be extracted into ether and benzene.

Table 1 shows the breakdown of other N:N-dialkyl-*p*-nitrophenyl carbamates by the $S_{15\,000}$ fraction of rat liver and the effects of TPN on this breakdown.

TABLE 1. METABOLISM OF N:N-DIALKYL-*p*-NITROPHENYL CARBAMATES BY RAT LIVER MICROSOMES

Each flask contained 5·0 μmoles substrate, 1·5 ml $S_{15\,000}$, 0·5 ml KPO_4 buffer (0·05 M, pH 7·0), with or without 3·7 mg TPN. Total volume = 3·0 ml. Flasks were incubated at 37 °C for 1·5 hr prior to *p*-nitrophenol determination. The results were compared to those for DpNC, which was arbitrarily given a value of 100 in the presence of TPN. The amount of *p*-nitrophenol released by alkaline degradation of the metabolite from DpNC was 113·0 μg.

N-Alkyl groups	*p*-Nitrophenol production	
	No TPN added	TPN added
Dimethyl	4	100
Methyl, ethyl	8	82
Diethyl	8	93
Di-*n*-propyl	8	23
Di*iso*propyl	3	5
Di-*n*-butyl	1	6

The inhibition due to SKF 525-A, piperonyl butoxide, sesamex or MGK 264 was investigated and the results, shown in Table 2, indicate that all were effective inhibitors of the metabolism of DpNC and its diethyl analog (DEpNC).

The formation of products yielding formaldehyde,[23] in the presence of $S_{15\,000}$, from a large number of N:N-dimethyl carbamates, N-methyl carbamates and related compounds, was investigated and the effect of TPN on this production determined (Table 3). In certain cases, the enol moiety of the molecule contained methyl groups which could have given rise to formaldehyde. Where possible, the enol product of hydrolysis was used alone; the results obtained appear within parentheses after those of the parent compound (Table 3). In all cases in which formaldehyde was produced, TPN was stimulatory. Some of the products of hydrolysis yielded formaldehyde, although

dimethylamine and monomethylamine, formed spontaneously from the carbamic acids resulting from a splitting of the ester bond of N:N-dimethyl and N-methyl carbamates, gave negative results. Free formaldehyde and products which are degraded in acid to yield formaldehyde cannot be distinguished by the chromotropic acid procedure.[23] The enzyme preparations used were effective in converting free formaldehyde to a form which cannot be detected either in strong acid[23] or at neutral pH.[24] The rate of free formaldehyde loss was such that the formaldehyde detected under acid conditions must have resulted from acid degradation of metabolites.

TABLE 2. PERCENTAGE INHIBITION OF METABOLISM OF N:N-DIALKYL-*p*-NITROPHENYL CARBAMATES BY SKF 525-A, MGK 264, PIPERONYL BUTOXIDE AND SESAMEX

Each flask contained 5 μmoles substrate, 1·5 ml $S_{15\,000}$, 3·7 mg TPN, 0·5 ml KPO_4 buffer (0·05 M, pH 7·0) and a series of concentrations of the inhibitors. Total volume = 4·0 ml. MGK 264, piperonyl butoxide and sesamex were added in ether solution and the solvent evaporated in a stream of air. Flasks were incubated at 37 °C for 90 min prior to *p*-nitrophenol determination. Results shown are those of a single set of determinations which are, however, in agreement with several other determinations which have been made. Accurate determination of inhibition levels were not made as three of the inhibitors and both substrates are relatively insoluble in water. The amount of *p*-nitrophenol released in the absence of inhibitors was 98 μg in the case of DpNC and 89 μg in the case of DEpNC.

Inhibitor (μmoles)	SKF 525-A		MGK 264		Piperonyl butoxide		Sesamex	
	DpNC	DEpNC	DpNC	DEpNC	DpNC	DEpNC	DpNC	DEpNC
0·00	0	0	0	0	0	0	0	0
0·03	17	13	0	2	29	5	22	0
0·30	35	36	46	31	38	31	58	47
3·00	65	69	66	76	48	56	67	63
30·00	80	90	68	87	60	69	90	77

TABLE 3. METABOLISM OF N:N-DIMETHYL CARBAMATES AND RELATED COMPOUNDS BY RAT LIVER MICROSOMES

Each flask contained 1 mg substrate, 1·5 ml $S_{15\,000}$, 0·5 ml KPO_4 buffer (0·05 M, pH 7·0), with or without 3·7 mg TPN. Total volume = 3·0 ml. Substrates were added as ethanol solutions and the solvent evaporated in a stream of air. Flasks were incubated for 1·5 hr at 37 °C prior to formaldehyde estimation. Determinations were made at least twice with each substrate and the results compared to that from DpNC, which was determined with each enzyme preparation, the value for which was arbitrarily set at 100. When hydrolysis products were studied, the relative value for formaldehyde production from these is placed in parenthesis after that for the parent compound. The amount of formaldehyde release from the DpNC metabolite in different experiments varied from 17 μg to 40 μg.

Compound* N:N-Dimethyl carbamates (R = $Me_2NC(O)$—)	Source†	Formaldehyde production	
		No TPN added	TPN added
1. R—O—Ph	G	0	65
2. R—O—Ph—NO_2-3	R	9	50
3. R—O—Ph—NO_2-4 (DpNC, model compound)	G	12	100
4. R—O—Ph—NH_2-3	R	15	39
5. R—O—Ph—NMe_3 . Br-3 (neostigmine, parasympathomimetic agent)	R	9 (8)	61 (25)
6. R—O—Ph—CH_2NMe_2-2	R	6	39
7. R—O—Ph—Ph-4	G	14	23
8. R—O— 	G	14	33

TABLE 3.—*continued.*

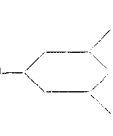
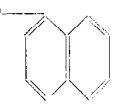
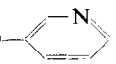
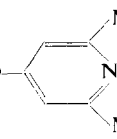
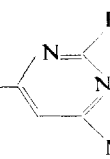
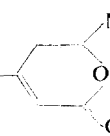
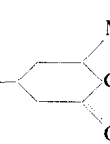
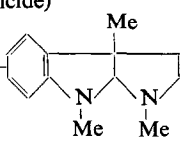
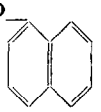
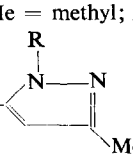
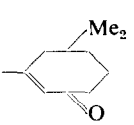
Compound* N:N-Dimethyl carbamate (R = $Me_2NC(O)$—)	Source†	Formaldehyde production	
		No TPN added	TPN added
9. R—O— 	G	9	32
10. R—O— 	G	0	25
11. R—O— 	G	0	12
12. R—O— 	R	12	41
13. R—O— 	G	27	74
14. R—O—  (Pyramat, insecticide)	G	18	32
15. R—O—Pyr—H-1	G	6 (0)	33 (0)
16. R—O—Pyr—Et-1	G	11 (0)	98 (0)
17. R—O—Pyr—*i*—Pr-1 (Isolan, insecticide)	G	13 (0)	98 (3)
18. R—O—Pyr—Ph-1 (Pyrolan, insecticide)	G	9 (0)	59 (6)
19. R—O—Pyr—$C(O)NMe_2$-1	G	11	60
20. R—O—Res (dimetan, insecticide)	G	20 (9)	55 (20)
21. R—O— 	G	7	40
22. R—O— 	G	21	37
23. R—O—$C_2H_4NMe_3$. Br	G	15 (11)	34 (7)
24. R—Cl	G	0‡	0‡
25. R—F	B	0	0

TABLE 3.—*continued.*

Compound* N:N-Dimethyl carbamates (R = $Me_2NC(O)$—)	Source†	Formaldehyde production	
		No TPN added	TPN added
Other carbamates			
26. (Me)(Et)NC(O)OPh—NO_2-4	G	15	64
27. (Me)(H)NC(O)OPh—NO_2-4	G	0‡	0‡
28. (Me)(H)NC(O)OPh—*i*—Pr-3 (compound 5727, insecticide)	H	0	0
29. (Me)(H)NC(O)OPh—NMe_2 . HCl-3	R	15	42
30. (Me)(H)NC(O)OPh—Me_2-3:5-NMe_2-4 (Zectram, insecticide)	Dow	0 (0)	18 (18)
31. (Me)(H)NC(O)O—  (physostigmine, sympathomimetic agent)	—	15	45
32. (Me)(H)NC(O)O—  (Sevin, insecticide)	C	2	11
33. (Me)(Et)NC(O)ORes	G	9 (9)	34 (20)
34. (Me)(*n*-Bu)NC(O)ORes	G	13 (9)	35 (20)
35. $Me_2NC(S)ORes$	G	9 (9)	26 (20)
36. $Me_2NC(S)SRes$	G	0 (9)	9 (20)
37. $H_2NC(O)OC_2H_4NMe_3$. Cl (carbachol, parasympathomimetic agent)	—	4 (11)	21 (7)
38. (Me)(H)NC(O)$OC_2H_4NMe_3$.Br	G	6 (11)	0 (7)
Non carbamates			
39. Me_2NSO_2Ph—Cl-4	G	3	0
40. $Me_2NC(O)NHPh$ (fenuron, herbicide)	D	0	8
41. $Me_2NC(O)NHPh$—Cl-3 (monuron, herbicide)	D	2	10
42. $Me_2NC(O)NHPh$—Cl_2-3:4 (diuron, herbicide)	D	2	10
43. $MeNH_2$.HCl	—	0	0
44. Me_2NH.HCl	—	0	0
45. $(MeO)_2P(O)OCMe{=}CHC(O)NMe_2$ (compound 3562, α-isomer, insecticide)	S	0	14
46. $(Me_2N)_2P(O)F$ (dimefox, insecticide)	F	0	0
47. $(Me_2N)_3P(O)$	M	0	0
48. $(Me_2N)(MeO)P(S)$—OPh—Cl_3-2:4:5	Dow	0	0

* Abbreviations: Me = methyl; Et = ethyl; Pr = propyl; Bu = butyl; Ph = phenyl; Pyr = 3-methyl-5-pyrazolyl  ; Res = 5:5-dimethyl dihydrorescrorcinyl = 

† The authors gratefully acknowledge the following sources for the compounds studied: B = Dr. G. Schrader, Farbenfabriken Bayer, Wuppertal-Elberfeld, Germany; C = Dr. R. Back, Union Carbide Chemicals Co., New York, New York; Dow = Dr. J. Johnson, Dow Chemical Co., Midland, Michigan; D = Dr. J. Carnahan, E.I., Du Pont de Nemours and Co., Wilmington, Delaware; F = Dr. G. Hartley, Fisons Pest Company Ltd., Saffron Walden, Essex, England; G = Dr. H. Gysin, J. R. Geigy, Basle, Switzerland; H = Dr. A. Lohr, Hercules Powder Co., Wilmington, Delaware; M = Monsanto Chemical Co., St. Louis, Missouri; R = Dr. J. Aeschlimann, Hoffman-La Roche Inc., Nutley, New Jersey and Dr. E. Silberschmidt, Hoffman-La Roche Inc., Basle, Switzerland; S = Dr. R. Whetstone, Shell Development Co., Modesto, California.

‡ These compounds are unstable and readily hydrolyse in aqueous solution.

When assayed for loss of cholinesterase-inhibiting activity by the method described, the greatest loss occurred with Isolan (cmpd. 17) (Fig. 3). In the presence of TPN over 95 per cent of the inhibitory activity was destroyed in 1 hr; in its absence little loss occurred. None of the other carbamate-inhibitors of cholinesterase approached this rapid loss when treated in the same way. Some loss of inhibiting ability was noted in the case of dimetan (cmpd. 20), Pyrolan (cmpd. 18) and compound 16, none in the case of physostigmine (cmpd. 31), Sevin (compd. 32), and compounds 10, 15 and 19.

DpNC was administered to rats at an oral dosage of 200 mg/kg. After 4 hr the liver was removed and homogenized in acetone. The acetone was removed *in vacuo* and the residue extracted with ether. The ether was removed in a stream of air and the residue extracted with acetone. The acetone solution was filtered and examined for the presence of metabolites of the same type as those produced by isolated microsomes. A compound of intermediate stability in alkali could be demonstrated with a half time for degradation of 40 min at pH 8·0 and 8 min at pH 9·0.

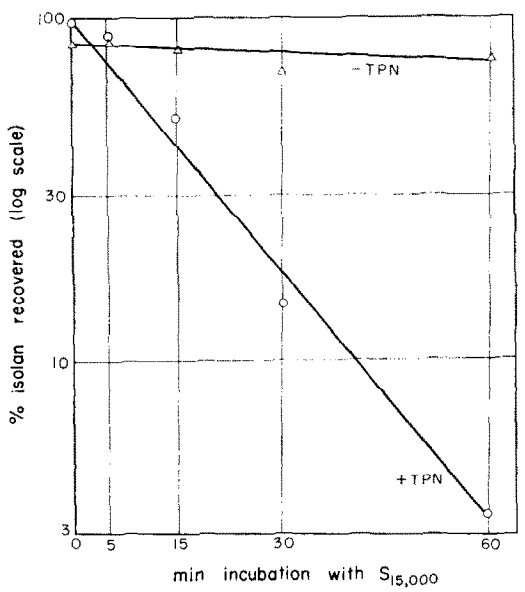


FIG. 3. Loss of cholinesterase-inhibiting activity on incubation of Isolan with $S_{15\,000}$ from rat liver. Each flask contained 1×10^{-5} M Isolan, 2·5 ml $S_{15\,000}$, 0·5 ml KPO_4 buffer (0·05 M, pH 7·0), with or without 3·7 mg TPN. Total volume, 5·0 ml. Flasks were incubated at 37 °C, samples taken at 0,5,10,15,30 and 60 min and prepared for cholinesterase inhibition determination at 1×10^{-6} M eqivalent of Isolan by the method previously described.

Houseflies (*Musca domestica* L.) were treated topically and American roaches (*Periplaneta americana* L.) by injection with DpNC at a dosage of 1 mg/g. After 4 hr the whole insects were extracted in the same manner as was the rat liver. A metabolite of the same type as that produced by rat liver microsomes or intact rats appeared to be present, but interference from an opalescent material in the extracts prevented accurate determinations of the half time for degradation.

DISCUSSION

The N:N-dialkyl-*p*-nitrophenyl carbamates are not effective cholinesterase inhibitors, but their chemical stability and the relative ease with which their breakdown products can be analysed make them valuable model compounds for metabolic studies.

The data support the hypothesis that a microsomal enzyme system, requiring TPNH and oxygen, acts on many N:N-dialkyl carbamates. In the case of DpNC only one alkyl group is attacked and the reaction cannot properly be referred to as a dealkylation, since the methyl group is not released as free formaldehyde. The product of the enzyme reaction is relatively stable at pH 7·0–8·0, but breaks down to yield *p*-nitrophenol at higher hydroxyl ion concentrations. The product will yield formaldehyde under strong acid conditions[23] but not in neutral aqueous solution.[24]

N-Dealkylation systems localized in the microsomes and requiring TPNH and oxygen have been described for a number of drugs.[10] Although only one previous report[16] indicates such an intermediate product in the dealkylation, the fact that formaldehyde determinations are normally done under strong acid conditions could mean that such intermediates are degraded during analysis.

By analogy with the mechanism proposed for the metabolism of N:N-dimethyl tryptophane[25] and the oxidation of octamethylpyrophosphoramide,[16] one possible structure would be N-methyl-N-methylol-*p*-nitrophenyl carbamate. The possibility that one methyl group is oxidized to the level of CO_2 and the other to the methylol level is extremely unlikely as the metabolite yields 1 mole of formaldehyde per mole of *p*-nitrophenol and N-methyl carbamates are not subject to microsomal N-methyl oxidation. DpNC appears to be stable to many chemical oxidations, but will react with chlorine in carbon tetrachloride solution to form a small amount of a product which yields formaldehyde in acid and can be extracted into organic solvents from water.

N:N-Dialkyl-*p*-nitrophenyl carbamates with alkyl groups larger than methyl are also metabolized by the same or similar enzyme systems. The data do not allow any conclusions as to whether one or more than one enzyme system is involved. The data presented indicate that only the methyl group of N-methyl,N-ethyl-*p*-nitrophenyl carbamate is attacked. It is apparent that activity toward compounds with alkyl groups larger than ethyl is markedly reduced. The metabolites formed were less stable than that from DpNC, although the original substrates are more stable.[2] SKF 525-A will inhibit demethylation but not de-ethylation of derivatives of 4-amino antipyrine,[17] whereas in the present case the metabolism of both the N:N-dimethyl and N:N-diethyl compounds was inhibited.

Methylene dioxyphenyl compounds have been implicated as affecting the metabolism of carbamates in insects in which they are known to have a synergistic action *in vivo*.[19] In the present study, piperonyl butoxide and sesamex are seen to be inhibitors of the metabolism of some N:N-dialkyl carbamates by rat liver microsomes. The oxidative attack on the N-methyl group of the α-isomer of SD 3562 (compd. 45) by rat liver microsomes is interesting, since this compound acts synergistically with sesamex in the insect;[21] this is one of the compounds shown to be an inhibitor of oxidative reactions in rat liver microsomes. MGK 264 is an insecticide synergist[20] and has been shown to inhibit the oxidation of methyl parathion to methyl paraoxon in mammalian

microsomes. In the present study, MGK 264 is shown to be an effective inhibitor of N:N-dialkyl carbamate oxidation by rat liver microsomes.

The survey of carbamates for formaldehyde production with the $S_{15\,000}$ fraction was designed to show the possible range of chemical structures, within the carbamates and related compounds, which might be susceptible to this type of metabolism. Careful comparison of the rates of enzymic reactions was not possible as the solubility of the compounds in water varied through extreme limits. One source of error, the variability of the enzyme preparations, was reduced by comparing the formaldehyde production with that from DpNC, which was determined with every preparation.

Certain general conclusions can be made. In all cases in which formaldehyde was produced, TPN was stimulatory. This finding supports the view that these compounds are metabolized by the same or a similar mechanism to that shown for DpNC. All the compounds yielding appreciable amounts of formaldehyde were N:N-dimethyl carbamates; the N-methyl carbamates are apparently not readily metabolized by this system. N:N-Dimethyl carbamoyl fluoride and chloride (compds. 24 and 25) do not yield formaldehyde. It has been demonstrated that N:N-dimethyl carbamoyl fluoride is readily hydrolysed by liver esterases.[9] This finding and the fact that dimethylamine and monomethylamine, the products of enzymic[9] and non-enzymic[26,27] hydrolysis of N-methyl and N:N-dimethyl carbamates, do not yield formaldehyde, rules out the possibility that the reaction mechanism is an initial hydrolysis followed by the production of formaldehyde from the products of hydrolysis.

Considerable variation in the enol portion of the molecule can be made without destroying activity, since the two types of carbamate which yield formaldehyde most readily are the *p*-nitrophenyl carbamates (cmpds. 3 and 26) and the 1-R-3-methyl-5-pyrazolyl carbamates (cmpds. 15–19). In some cases, methyl groups on the enol moiety of the molecule yielded formaldehyde when tested alone, such as 5:5-dimethyldihydroresorcinol from dimetan (cmpd. 20) and compounds 33–36, and the enol moieties of neostigmine (cmpd. 5) and Zectran (cmpd. 30). In these cases, it is likely that methylol groups were formed in the oxidation.

If the oxygen of the ester linkage is sustituted by a nitrogen atom, as in the change from a carbamate to a substituted urea, or if either of the oxygen atoms around the carbamate carbon is substituted by a sulphur atom, the compound does not yield formaldehyde to an appreciable extent. Since these compounds are particularly insoluble in water, the explanation could lie in their not being available to the enzymes.

Degradation of Isolan was evident based on loss of cholinesterase-inhibiting-potential and on formaldehyde released from the metabolite at acid, but not at neutral, pH. Certain other carbamate cholinesterase inhibitors which yielded formaldehyde at acid pH did not lose the ability to inhibit cholinesterase. This apparent discrepancy is susceptible to two explanations; first, that sites on the molecule other than the carbamoyl group yielded the formaldehyde; the second, that the metabolites which yield formaldehyde under strong acid conditions may, themselves, be effective cholinesterase inhibitors. The first explanation seems to be the less likely since the enol products of hydrolysis when tested alone, never yielded as much formaldehyde as the parent compound. However, the hypothesis cannot be completely ruled out, since the presence of the carbamoyl group may make other sites more susceptible to methyl oxidation. If the second hypothesis is correct the fact that formaldehyde is produced under strong acid conditions would not necessarily correlate with loss of cholinesterase-inhibiting

capacity, as the relatively mild conditions used in preparing samples for cholinesterase determination would presumably not degrade the metabolites.

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