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ENZYMIC HYDROLYSIS OF N:N-DIMETHYLCARBAMOYL FLUORIDE

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Abstract—The hydrolysis of N:N-dimethylcarbamoyl fluoride by rabbit tissues and by various vertebrate plasmata has been studied. The hydrolysis of this compound by rabbit liver and kidney homogenates involves an enzyme distinct from that hydrolysing di*iso*-propoxyphosphoryl fluoride. The same esterase in rabbit plasma probably hydrolyses dimethylcarbamoyl fluoride, di*iso*propoxyphosphoryl fluoride and *p*-nitrophenyl acetate. A similar result was obtained with human plasma. The major enzyme in rabbit plasma responsible for these reactions appears from electrophoretic studies to be the arylesterase (A-esterase). Aliesterase and cholinesterase of plasmata do not readily hydrolyse dimethylcarbamoyl fluoride. In addition to arylesterase, other factors of human serum may be involved in the hydrolysis reaction. The effect of certain metallic ions on the enzymic hydrolysis of dimethylcarbamoyl fluoride by rabbit plasma, liver and kidney is reported.

MANY N-alkyl and N:N-dialkyl carbamates have been extensively studied with regard to their cholinesterase-inhibiting properties,[1,2,3] clinical usefulness,[1] and applicability as insecticides.[2,3] The N:N-dialkylcarbamoyl halides provide excellent model compounds for studying the mechanism of toxic action and detoxification of the N:N-dialkyl carbamates. The halides, described by Schrader[4] and studied by Myers,[5,6] are potent *in vivo* inhibitors of cholinesterases. The inhibitory reaction between this agent and acetylcholinesterase of rat brain is similar to that observed with organophosphorus esterase inhibitors.[7] Rats treated with dimethylcarbamoyl fluoride displayed a depression of brain and serum cholinesterase and aliesterase activity which was quite marked but of short duration, indicating a biological instability for this fluorocarbamate.[7] In the present investigation, the results obtained in studies on enzymes involved in the hydrolysis of dimethylcarbamoyl fluoride are reported.

MATERIALS AND METHODS

Compounds. N:N-Dimethylcarbamoyl fluoride (DCF), a colourless fluid of moderate water solubility, was provided by Dr. G. Schrader of Farbenfabriken-Bayer, Wuppertal-Elberfeld, Germany. Di*iso*propoxy phosphoryl fluoride (DFP) was provided by Dr. L.-E. Tammelin of the Research Institute of National Defence, Sweden.

Enzyme preparations. Tissues were obtained from a 2 kg male rabbit which was first bled as completely as possible by heart puncture. The washed erythrocytes and other organs were quickly frozen and kept at −10 °C until assayed. Tissue homogenates were prepared in bicarbonate-CO_2 buffer (pH 7·6) with a glass homogenizer. Plasmata

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from rabbit and other species were prepared from heparinized blood. The various fractions of human *post-partum* serum studied were those obtained by a partly modified method no. 6 of Cohn *et al.*[15]; these fractions were prepared and provided by AB KABI (Stockholm).

Esterase activity was determined by the bicarbonate method in the Warburg apparatus.[8] The compounds studied were dissolved in distilled water to which the enzyme and bicarbonate–CO_2 buffer (pH 7·6) was added to yield a total volume of 2·0 ml in the flask. All determinations were carried out at 37 °C, except as otherwise stated. Esterase activities were expressed in b_{30} values, i.e., μl CO_2 evolved per 30 min corrected for spontaneous hydrolysis of substrates and CO_2-evolution by tissues in the absence of added substrate.

Paper chromatography was carried out according to a described procedure.[9] The solvent was a *n*-butanol–ethanol–acetic acid–water mixture (8:2:1:3), and the chromatograms were developed by spraying with ninhydrin for the detection of methylamines.

Electrophoretic separation of plasma proteins was carried out according to a recently described method[10,11] on cellulose columns (3 cm × 40 cm) in veronal buffer (pH 8·4, $I = 0{\cdot}1$) at 5–11 °C, using 5 ml buffered plasma for each run and an applied current of 260 V and 60 mA. The duration of runs was 30 hr. The protein concentration of successive 3·0 ml fractions of the eluate was estimated by a modified Folin procedure,[12] and the enzyme activity of the fractions estimated manometrically.

RESULTS

Spontaneous hydrolysis. It was assumed that the spontaneous hydrolysis of DCF, as well as any enzymic hydrolysis yielded hydrofluoric acid, dimethylamine and carbon dioxide, by analogy with N:N-dimethylcarbamoyl chloride.[13,14] Under the conditions of the bicarbonate assay method only 1 mole of CO_2 would be expected from each mole of DCF hydrolysed. DFP yields under the same experimental conditions 2 molar equivalents of CO_2.

Neutral DCF solutions of concentrations below 0·01 M were stable for days when kept at a temperature below 20 °C. No degradation products from the solutions used could be detected by paper chromatography, and the inhibitory activity of solutions on cholinesterase was unaltered under the same conditions. Only a small amount of spontaneous hydrolysis could be detected with the manometric method at 37 °C and with high concentrations of DCF (0·1 M). When stronger alkaline solutions (0·01 M NaOH) of DCF were boiled for 4 hr, the formation of dimethylamine was ascertained by paper chromatography of the reaction mixture (Fig. 1).

Enzymic hydrolysis by various rabbit tissues. The activities of homogenates of certain rabbit tissues in the hydrolysis of DCF and DFP were compared (Fig. 2). With both substrates the plasma, liver and kidney were found to be the most active tissues. Of particular interest are the high relative activity of erythrocytes and the lowac tivity of the adrenal glands on DCF in comparison with DFP. From studies on swine and bovine tissues,[16] it was concluded that DFP and dimethylamido-ethoxy-phosphoryl cyanide (tabun) are split by the same enzyme of kidney and adrenal glands. This does not appear to be the case with the hydrolysis of DFP and DCF by rabbit kidney and adrenal glands.

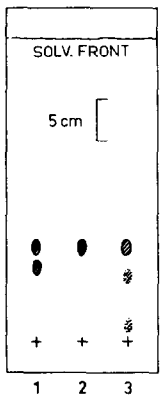

FIG. 1. Paper chromatograms of DCF and products formed on incubation with arylesterase of human serum. Solvent: *n*-butanol-ethanol-acetic acid-water (8:2:1:3). Spots developed with ninhydrin. (1) $(CH_3)_2NH$, CH_3NH_2 and DCF, fresh aqueous solns; DCF alone gave no coloured spot. (2) DCF, 1% soln. in 0·01 M NaOH, boiled for 4 hr. (3) DCF, 10 mM, incubated with arylesterase for 24 hr at 22 °C in a bicarbonate–CO_2 buffer. No $(CH_3)_2NH$ was detected after incubation of DCF with cholinesterase and albumin, under the same experimental conditions.

Enzymic hydrolysis by various vertebrate plasmata. In a survey of various plasmata for activity in DCF hydrolysis, rabbit plasma was found to be the most active, with a b_{30} of 68/100 μl plasma. Relative to rabbit plasma, the cat and sheep were 23–25 per cent as active; the dog and cow, 12 per cent; and the horse, swine, guinea pig, mouse and chicken plasmata were only from 4 to 9 per cent as active in DCF hydrlysis. This order of activity of various plasmata correlated more closely with the hydrolysis of the phosphoryl cyanide (tabun),[16] than with that of 1-naphthyl-N-methylcarbamate[17] or with the activity of the arylesterases or cholinesterases of various plasmata.[16]

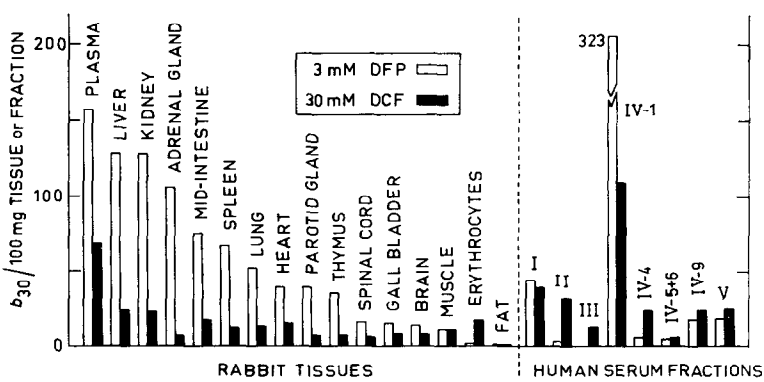

FIG. 2. Activity of rabbit tissue homogenates and human serum fractions in the hydrolysis of DCF and DFP. Determinations were made at 37 °C with 200 mg tissue or fraction for DCF, and 75 mg for DFP. Results are corrected for spontaneous hydrolysis of substrates and CO_2-evolution resulting with tissues or fractions in the absence of added substrate.

Enzymic hydrolysis by various fractions of human serum. Fractions from human *post-partum* serum were assayed for activity in the hydrolysis of DCF and DFP (Fig. 2). Although the highest activity against both substrates appeared in the fraction containing arylesterase (fraction IV-1), the activity in DCF hydrolysis was more generally distributed among the fractions than was the activity against DFP, tabun, tetraethyl pyrophosphate or diethyl *p*-nitrophenyl phosphate.[18]

Formation of dimethylamine during the enzymic hydrolysis. When DCF (10 mM) was incubated with a purified arylesterase preparation of human serum for 24 hr at 20 °C in a bicarbonate–CO_2 buffer, dimethylamine was detected after chromatography on paper of the reaction mixture (Fig. 1). No other nitrogen compounds were observed. Dimethylamine could not be detected when DCF was incubated under the same experimental conditions with purified preparations of cholinesterase or albumin of human serum.

Enzymic hydrolysis of mixtures of DCF and DFP. When mixed DCF and DFP of varying concentrations was incubated with rabbit plasma, the hydrolysis rates of the mixtures were found to be approximately equal to the theoretical values calculated on the assumption that the same enzyme acted on the two substrates (Table 1).

TABLE 1. HYDROLYSIS OF MIXTURES OF DCF AND DFP BY RABBIT PLASMA

(Determinations were made at 37 °C at 67 μl plasma per 2·00 ml reaction mixture).

mM DCF (*a*)	Rate of enzymic hydrolysis (b_{30})		
	DCF alone	3 mM DFP (*b*) + varying concs. of DCF	
		determined	calculated*
0	—	115	115
0·3	4	106	115
1	8	104	113
3	16	97	109
6	20	104	104
10	26	98	99
12	29	96	96·5
24	38	88	87·5
48	49	88	78·5

*On the assumption that DCF and DFP were hydrolysed by the same enzyme, applying the equation (for its derivation, see ref. 27), we get

$$\frac{V_a \frac{a}{K_a} + V_b \frac{b}{K_b}}{\frac{a}{K_a} + \frac{b}{K_b} + 1}$$

where V_a, V_b are the maximum velocities; a, b the concentrations; and K_a, K_b the Michaelis constants for DCF and DFP, respectively. V_a and K_a, deduced from cols. (1) and (2), are 60 and 12×10^{-3}. K_b for DFP and plasma *A*-esterase is 3×10^{-3}, which gives $V_b = 230$ when $a = 0$. For $b = 3 \times 10^{-3}$, the hydrolysis rate of the mixture is

$$\frac{a \times 60 + 230 \times 12 \times 10^{-3}}{a + 27 \times 10^{-3}}$$

However, at constant DFP concentration (3 mM) the values determined deviated from those calculated when the DCF concentration was higher than equimolar to

DFP. A possible explanation for this discrepancy would be that at higher DCF concentration a second enzyme (cf., Fig. 3) was coming in, and this hydrolysed DCF faster than DFP. In similar experiments with rabbit plasma pre-incubated with 0·1 mM DFP to inhibit all but the arylesterase activity, it was also found that the subsequent hydrolysis rates for DCF and *p*-nitrophenyl acetate in mixtures were approximately equal to those calculated, assuming that the two reactions were catalysed simultaneously by the same enzyme and provided the DCF-concentration was not higher than equimolar to the aromatic ester.

Electrophoretic separation of esterase active components in rabbit plasma. On electrophoretic separation of rabbit plasma on cellulose columns, it was found that probably two (A_1 and A_2) or more enzymes are involved in DCF hydrolysis and that these migrate coincidentally with a portion of the activity in the hydrolysis of DFP and *p*-nitrophenyl acetate (Fig. 3). The major enzyme involved seems to be identical with

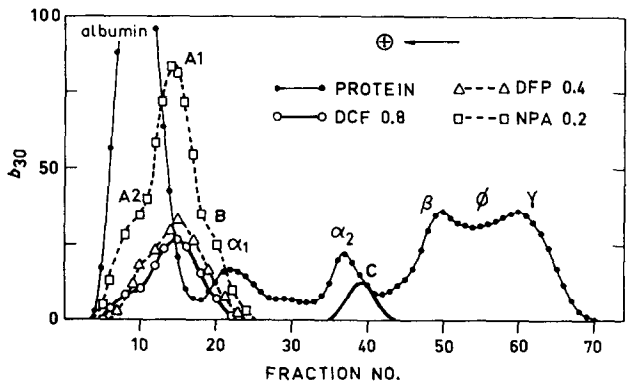

FIG. 3. Electrophoretic pattern of rabbit plasma showing elution position of fractions active in the hydrolysis of DCF, DFP and *p*-nitrophenyl acetate (NPA). b_{30}, esterase activity expressed in μl CO_2 evolved in 30 min with ml aliquot indicated within the graph. A_1, A_2, B and C represent elution positions for two arylesterases, aliesterase and cholinesterase, respectively. Protein curve is based on relative concentration values, and peaks are indicated with conventional symbols.

the arylesterase (A-esterase), which has been shown to hydrolyse dimethylamidoethoxyphosphoryl cyanide as well (AUGUSTINSSON, unpublished). The results indicate also that the DFP/DCF activity ratio differs for the two enzymes being higher for A_1 than A_2. Neither aliesterase nor cholinesterase hydrolysed DCF or DFP.

Effect of Mn^{2+} *and* Co^{2+}. The effect of Mn^{2+} and CO^{2+} on the hydrolysis of DFP and DCF by rabbit plasma, kidney and liver was investigated (Table 2). Mn^{2+} and Co^{2+} activated the DFP-hydrolysing enzymes of liver and kidney as was previously noted with many species,[19] but resulted in inhibition of the DFP-hydrolysing esterases of rabbit plasma as has also been previously noted by Mounter.[20] The tabun-hydrolysis was similarly influenced by those ions.[21] With DCF as substrate, Mn^{2+} failed to activate, and Co^{2+} was inhibitory to, DCF hydrolysis by liver and kidney. However, with plasma the degree of inhibition by Mn^{2+} and Co^{2+} was similar for both substrates. These results indicate that different enzymes in liver and kidney are primarily responsible for DCF and DFP hydrolysis, whereas the same plasma enzyme(s) may contribute to the hydrolysis of both substrates.

TABLE 2. EFFECT OF Mn^{2+} AND Co^{2+} ON THE HYDROLYSIS OF DCF AND DFP BY RABBIT PLASMA, LIVER AND KIDNEY

Whole plasma or 20 per cent tissue homogenates were dialysed against distilled water before assay. Buffer for assay was 0·04 M $NaHCO_3$–CO_2 at pH 7·6. $MnSO_4$ or $CoCl_2$ was incubated with the enzyme for 60 min at 37 °C before adding the substrate. Results are corrected for spontaneous hydrolysis of substrates in the presence and absence of added cations and for CO_2 evolution from tissues in the absence of added substrate. Enzyme activity (b_{30}) refers to 100 μl plasma or 100 mg tissue. Ratios refer to activity with 1·0 mM metal ion divided by activity with no added metal ion.

Tissue	DCF (30 mM)			DFP (3 mM)		
	b_{30}	Ratios		b_{30}	Ratios	
		Mn^{2+}	Co^{2+}		Mn^{2+}	Co^{2+}
Plasma	75	0·79	0·02	120	0·79	0·02
Liver	15	0·92	0·59	67	1·64	1·68
Kidney	15	0·99	0·64	79	2·00	1·75

Effect of various metallic ions on the enzymic hydrolysis by rabbit plasma. A more complete study was made of the rabbit plasma systems hydrolysing DCF and DFP, since rabbit plasma was most active in DCF hydrolysis of those examined, and Mounter[19] has concluded the A-esterase (arylesterase) of rabbit plasma is responsible for DFP hydrolysis. The relative effects were determined of several possible

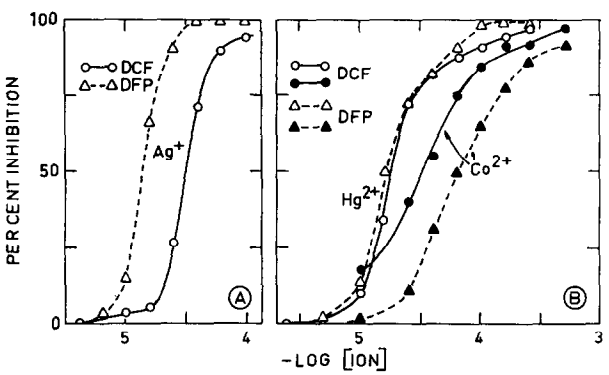

FIG. 4. Inhibitory activity of certain metallic ions on the enzymic hydrolysis of DCF and DFP by rabbit plasma. The plasma was dialysed against distilled water and used at 100 μl per 2·00 ml reaction mixture for assay against 30 mM DCF and at 50 or 100 μl against 3 mM DFP. The buffer solution used was 0·04 M $NaHCO_3$–CO_2 at pH 7·6. The metallic salts ($AgNO_3$, $HgCl_2$ and $CoCl_2$) were incubated with the enzyme for 60 min at 37 °C before adding the substrate. Results are corrected for spontaneous substrate hydrolysis. A: enzyme concentration for DCF twice that for DFP. B: the same enzyme concentration for DCF and DFP. Values for pI_{50} are as follows:

Substrate	Ag^+	Hg^{2+}	Co^{2+}
DCF	4·5	4·75	4·2
DFP	4·85	4·8	4·5

cationic inhibitors of the enzymes hydrolysing DCF and DFP. For DCF determinations the enzyme concentration was usually twice and the substrate concentration ten times that for DFP. At 1·0 mM, Ca^{2+} and Mg^{2+}, gave slight inhibitory effects, Mn^{2+}, Ba^{2+} and Sr^{2+} were more active inhibitors, and Co^{2+}, Cu^{2+}, Ag^{+} and Hg^{2+} gave almost complete inhibition. Similar results were obtained with both substrates. On further dilution of the more active inhibitors, it was found that the effect on the enzymes hydrolysing both substrates increased in the order: $Cu^{2+} < Co^{2+} < Hg^{2+}, Ag^{+}$. Inhibition curves for the three most active cations are shown in Fig. 4. Where the same enzyme concentration was used for assay against DCF and DFP, the inhibition curves with Hg^{2+} were almost coincidental, but when twice the enzyme concentration was used for DCF as for DFP it required twice as much Ag^{+} to effect a similar degree of inhibition of DCF hydrolysis. However, where the same enzyme concentration was used to assay against the two substrates, different inhibition curves resulted with Co^{2+}. This effect was not further investigated nor was the high inhibitory effect of Co^{2+} found in these experiments compared with a much lower Co^{2+} inhibition of DFP hydrolysis by rabbit serum reported previously.[20] The differences in enzyme or substrate levels, or the nature of the substrate, may be contributing factors. It has been shown recently[21] that Sr^{2+} will activate the human serum enzyme hydrolysing tabun while this cation inhibits the reaction on DFP by what appears to be the same enzyme. However, as the experiments in Fig. 4 were carried out with a mixture of the two enzymes A_1 and A_2 (Fig. 3), the discrepancies noted would be explained if A_2 were less sensitive to Co^{2+}, but more sensitive to Ag^{+}, than A_1, and if A_1 and A_2 were equally sensitive to Mg^{2+}.

CONCLUSIONS

Several different enzyme systems appear to be involved in the detoxification of N-alkyl and N:N-dialkylcarbamates. Although cholinesterases will apparently hydrolyse certain carbamates,[7,22–27] the turn overnumber is so low that these enzymes might not be considered of importance as a major detoxification mechanism. Plasma albumin has been implicated as a factor of possible importance in the detoxification of 1-naphthyl-N-methylcarbamate[17] and *p*-nitrophenyl-N-alkylcarbamates.[24] However, neither plasma albumin nor plasma arylesterase readily hydrolyse the more stable N:N-dialkylcarbamates.

As shown in the present investigation, one of the least stable of these N:N-dialkyl carbamates, dimethylcarbamoyl fluoride, is hydrolysed by enzyme(s) in human and rabbit plasmata which may also be involved in the hydrolysis of di*iso*propoxy-phosphoryl fluoride and *p*-nitrophenyl acetate. One of these enzymes is probably identical with arylesterase (A-esterase). Aldridge[25] and Mounter[20] have previously concluded that this esterase of rabbit plasma also hydrolyses tetraethylpyrophosphate and diethyl-*p*-nitrophenyl phosphate. The hydrolysis of DCF by human plasma is probably determined by more factors than the hydrolysis of organophosphorus compounds.[26] In rabbit liver and kidney, different enzymes are primarily involved in the hydrolysis of dimethylcarbamoyl fluoride and di*iso*propoxy-phosphoryl fluoride.

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