mal ligature was also increased, but no alteration was observed on either side of the distal ligature (see Fig. 2). This supports, therefore, the hypothesis of a proximal–distal transport and that retrograde transport of the enzyme does not occur. However, any surmises cannot be conclusive, for it is important to note that enzyme activity is not diminished distal to a ligature, which would be consistent with a sole proximal–distal or orthograde transport process for ChAc. It may well be that ligaturing nerves for periods of longer than 48 hr would resolve this question. Although rates of orthograde transport for ChAc in the snail were not calculated, it is clear that it is a slow process, since transport could not be detected 24 hr after ligation, and after 48 hr the enzyme activity in the first proximal segment of the analysed nerve was only 40 per cent than the control. The transport of ChAc in snail nervous tissue is thus, in principle, similar to that described for rabbit sciatic nerve [23] and mammalian cholinergic nerves [24].

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REFERENCES

1. F. Fonnum, *J. Neurochem.* **24**, 407 (1975).
2. L. T. Potter and V. A. S. Glover, *J. biol. Chem.* **243**, 3864 (1968).
3. C. Hebb, D. Morris and M. W. Smith, *Comp. Biochem. Physiol.* **28**, 29 (1969).
4. D. Morris, A. Maneckjee and C. Hebb, *Biochem. J.* **125**, 857 (1971).
5. G. Rama, C. V. Sastry and G. I. Henderson, *Biochem. Pharmac.* **21**, 787 (1972).
6. P. C. Emson, D. Malthe-Sørenssen and F. Fonnum, *J. Neurochem.* **22**, 1089 (1974).
7. S. S. Husain and H. G. Mautner, *Proc. natn. Acad. Sci. U.S.A.* **70**, 3749 (1973).
8. R. E. McCaman and M. W. McCaman, in *Biology of Cholinergic Functions* (Eds A. M. Goldberg and I. Hanin) p. 485. Raven Press, New York (1976).
9. E. Giller Jr. and J. H. Schwartz, *J. Neurophysiol.* **4**, 93 (1971).
10. R. E. McCaman and S. A. Dewhurst, *J. Neurochem.* **17**, 1421 (1970).
11. N. N. Osborne, *Nature, Lond.* **270**, 622 (1977).
12. P. C. Emson and F. Fonnum, *J. Neurochem.* **22**, 1079 (1974).
13. F. Fonnum, *Biochem. J.* **103**, 262 (1967).
14. N. N. Osborne and G. A. Cottrell, *Z. Zellforsch.* **109**, 177 (1970).
15. K. Meng, *Zool. Jb.* **68**, 539 (1960).
16. G. A. Cottrell and B. Powell, *Comp. gen. Pharmac.* **1**, 251 (1970).
17. R. R. Baker and M. T. Dowdall, *Neurochem. Res.* **1**, 153 (1976).
18. H. L. White and C. T. Cavallito, *Biochim. biophys. Acta* **206**, 343 (1970).
19. V. A. S. Glover and L. T. Potter, *J. Neurochem.* **18**, 571 (1971).
20. L. P. Chao and F. Wolfgram, *J. Neurochem.* **20**, 1075 (1973).
21. L. A. Barker and T. W. Mittag, *J. Pharmac. exp. Ther.* **192**, 86 (1975).
22. B. A. Hemsworth and T. C. Smith, *J. Neurochem.* **17**, 171 (1970).
23. S. Tučeck, *Brain Res.* **86**, 259 (1975).
24. A. Dahlström, Abstr. 5th Int. Meeting Int. Union Biochem. p. 542. Hamburg (1976).
25. L. A. Barker, M. T. Dowdall and T. W. Mittag, *Brain Res.* **86**, 343 (1975).

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Stereospecificity and active site requirements in a diisopropylphosphorofluoridate-hydrolyzing enzyme

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Squid nerve and hepatopancreas contain an enzyme that specifically hydrolyzes diisopropylphosphorofluoridate (DFP) [1]. This squid type DFPase is present in these tissues in other cephalopods—cuttlefish, nautilus, octopus—but is missing or present at markedly lower levels in other tissues and other classes of invertebrates [1–3]. A mammalian DFPase [4] appears to be a distinctly different enzyme, a major criterion being that the mammalian enzyme hydrolyzes another organophosphate, ethyl *N,N*-dimethylphosphoramidocyanidate (Tabun), much faster than DFP, whereas the order is reversed for squid type DFPase [1, 5]. Since DFP, Tabun, and their various analogues [6] are not naturally occurring compounds, the natural substrate for DFPase remains an important question. This is especially so for squid type DFPase because of its presence in nerve where it might have seemed, illogically, to be providing a protection against these powerfully neurotoxic agents [7].

Evidence has been presented showing Tabunase (as it was termed) in rat serum to be stereospecific, with the phosphorus atom the center of asymmetry [8]. This was confirmed [9], although the original observation and the confirmation were either indirect or at the limits of significance for the methods then available. We have now returned to this question with a different enzyme, namely squid type DFPase, a different substrate, and instrumentation for recording optical rotation in the millidegree range at wavelengths more favorable than the sodium D line of 589 nm. Some enzyme–inhibitor studies have also been made. The results suggest some structural requirements for the natural substrate, which would thus provide a physiological role for this otherwise seemingly functionless enzyme.

Squid type DFPase also hydrolyzes 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) [5], although with a K_m about ten times that for DFP [10], but does not hydrolyze

Tabun at a rate adequate for the present undertaking, especially after purification [1]. Soman, it should be noted, contains two asymmetric centers, one around phosphorus and another around C-1 of the alkoxy side-chain. Experiments in this laboratory with fluoride electrode and pH-stat show that for either DFP or Soman exactly 2 moles of acid are formed for each mole of fluoride released. Essentially the same stoichiometry had been found by other means in the original report of DFPase [11], and even more strikingly in the detoxication of isopropyl methylphosphonofluoridate (Sarin) [12], a close analogue of Soman not now available to us. Thus, enzymatic hydrolysis involves only P—F bond cleavage and possible asymmetry around phosphorus. Even in a reaction known to involve alkoxy cleavage (the "aging" of Soman-inhibited acetylcholinesterase), C-1 asymmetry seems to be unimportant [13].

In preparation for optical rotation measurements, we found that our best squid type DFPase preparation (a 2600-fold purification [1]) is of such activity that a solution capable of half-hydrolysis of 0.01 M Soman or DFP in 10–20 min has an absorbancy of 0.02 at 280 nm, and no detectable absorbancy at 300 nm. Thereafter, the procedure given in the legend to Fig. 1 was adopted for following changes in optical rotation during the enzymatic hydrolysis of racemic Soman and of DFP. Figure 1 shows that, as the enzymatic hydrolysis of Soman proceeds, thus eliminating asymmetry around phosphorus with the formation of 1,2,2-trimethylpropyl methylphosphonate, a change in optical rotation occurs, reaching a maximum of −5 or −6 millidegrees in about 10 min, and thereafter more slowly changes back toward the original reading. This does not happen with DFP, a symmetrical molecule. Thus, squid type DFPase shows some stereospecificity when the appropriate substrate is used. As far as Soman is concerned, if the fact is ignored that the hydrolysis of both isomers (either enzymatic or non-enzymatic) is going on from the instant of mixing, an approximation can be made that for the more rapidly hydrolyzed isomer, $[\alpha]^{20°}_{300\ nm} \simeq +12$ deg. The results of four other experiments, either at lower substrate concentrations or at other settings for slit width control, full chart span, and response period, while less suitable for superimposed reproduction, were all in general agreement with those shown in Fig. 1.

While enzyme–substrate studies would be the most direct means of exploring the active site requirements of squid type DFPase, compounds analogous to DFP are not readily available. Instead, substituted phosphates have been tested as possible inhibitors. Two compounds, monoisopropylphosphoric and diisopropylphosphoric acids, were separated from the commercially obtained materials (Pfaltz& Bauer), which is normally a mixture of about equal parts of the two [14–16]. Monophenylphosphate (sodium salt, Eastman) and diphenylphosphate (Aldrich) were represented as pure and were found essentially so by us. Methyl-, ethyl- and butylphosphoric acids (Pfaltz & Bauer, Eastman) are also mixtures of about equal parts of the respective mono- and dialkyl compounds. The virtual lack of inhibition by the first two, and the results with the separated isopropyl and phenyl members suggested that the separations of these methyl, ethyl and butyl pairs were not essential to the subsequent conclusions. The enzymatic hydrolysis of DFP in the presence and absence of these compounds was measured over a suitable range of concentrations using both pH-stat and fluoride electrode as reported previously [7, 10]. K_i values were calculated from slopes and intercepts taken from Lineweaver–Burk and Eadie–Hofstee plots. Where significant inhibition was found, direct linear plots were also made [17]. The differences between the various methods of determination and plotting were insignificant, and average results are given in Table 1. It can be seen that monosubstituted phosphates in which the substituent is branched or bulky fit the DFPase active site about as well as DFP. The second substituent seems less important. Thus, there appears to be an attachment or hydrophobic interaction involving the enzyme and the alkoxy group, and orienting the P—F bond or its natural equivalent for subsequent cleavage.

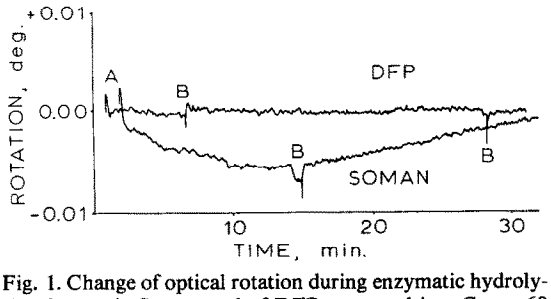

Fig. 1. Change of optical rotation during enzymatic hydrolysis of racemic Soman and of DFP measured in a Carey 60 spectropolarimeter set at 300 nm, slit width 0.5 mm, full chart span 20 millidegrees, response period 30 sec, light path 5 cm. Substrate concn, 0.01 M; squid-type DFPase concn, sufficient to cause half-hydrolysis in 10–20 min; medium 0.01 M PIPES (1,4-piperazinediethanesulfonate), pH 7.1 at start of reaction, 6.3 or slightly higher at end of recording; room temperature. Enzyme and substrate were mixed at $t = 0$; the record was started 1–2 min later. Key: A, artifact at start of recording; B, artifact caused by bubble forming on face of cuvette; recording was interrupted, cuvette was tapped lightly, and recording was recommenced.

Table 1. Michaelis constant and competitive inhibitor constants for squid type DFPase

Substrate	K_m (m-mole l^{-1}) ± S.D.
DFP	5.5 ± 1.3
Inhibitor	**K_i (m-mole l^{-1}) ± S.D.**
Monoisopropylphosphate	2.8 ± 0.8
Diisopropylphosphate	4.3 ± 0.7
Monophenylphosphate	4.6 ± 2.0
Diphenylphosphate	1.9 ± 0.3
Butylphosphate *	1.5 ± 0.2
Ethylphosphate *	30 to > 100
Methylphosphate *	> 100
Trimethylphosphate	Little
Triethylphosphate	or no
Phosphites in general	inhibition

* Mixtures of about equal parts of the mono- and dialkyl compounds; concentrations are based on phosphorus.

In summary, these results suggest that the natural substrate for squid type DFPase is a phosphate triester with the phosphorus atom a center of asymmetry. One substituent should probably be branched; while the second substituent seems less important, it should probably not be —OH since this would be the steric equivalent of P=O; the third should have an electron withdrawing tendency, perhaps comparable to the electronegativity of fluorine. It may be speculated that nucleotide phosphate triesters or phospholipid triesters could fulfill these requirements. The former, with nearby hydroxyl groups, are likely to be unstable [18], and the latter have had a doubtful history [19–22]. However, just such instability factors may suggest a regulatory role for such compounds and a physiological role for a hydrolyzing enzyme, in this instance squid type DFPase.

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REFERENCES

1. J. M. Garden, S. K. Hause, F. C. G. Hoskin and A. H. Roush, *Comp. Biochem. Physiol.* **52C**, 95 (1975).
2. F. C. G. Hoskin and M. Brande, *J. Neurochem.* **20**, 1317 (1973).
3. F. C. G. Hoskin, in *Jerusalem Symposium on Molecular and Quantum Pharmacology* (Eds. E. D. Bergman and B. Pullman), p. 205. Reidel, Dordrecht, Holland (1974).
4. L. A. Mounter, in *Handbuch der experimentallen Pharmakologie, Cholinesterases and Anticholinesterase Agents* (Ed. G. B. Koelle), p. 486. Springer, Berlin (1963).
5. F. C. G. Hoskin, *Science* **172**, 1243 (1971).
6. B. Holmstedt, in *Handbuch der experimentallen Pharmakologie, Cholinesterases and Anticholinesterase Agents* (Ed. G. B. Koelle), p. 428. Springer, Berlin (1963).
7. F. C. G. Hoskin, *J. Neurochem.* **26**, 1043 (1976).
8. F. C. G. Hoskin and G. S. Trick, *Can. J. Biochem. Physiol.* **33**, 963 (1955).
9. K-B. Augustinsson, *Acta chem. scand.* **11**, 1371 (1957).
10. F. C. G. Hoskin and R. J. Long, *Archs Biochem. Biophys.* **150**, 548 (1972).
11. A. Mazur, *J. biol. Chem.* **164**, 271 (1946).
12. F. C. G. Hoskin, *Can. J. Biochem. Physiol.* **34**, 75 (1956).
13. J. H. Keijer and G. Z. Wolring, *Biochim. biophys. Acta* **185**, 465 (1969).
14. F. W. Plapp and J. E. Casida, *Analyt. Chem.* **30**, 1622 (1958).
15. B. V. Ramachandran, *Acta chem. scand.* **21**, 609 (1967).
16. C. W. Stanley, *J. Chromat.* **16**, 467 (1964).
17. R. Eisenthal and A. Cornish-Bowden, *Biochem. J.* **139**, 715 (1974).
18. D. M. Brown, D. I. Magrath and A. R. Todd, *J. chem. Soc.* 4396 (1955).
19. F. D. Collins, *Nature, Lond.* **188**, 297 (1960).
20. A. J. DeKoning, *Nature, Lond.* **200**, 1211 (1963).
21. D. S. Galanos and V. M. Kapoulas, *Biochim. biophys. Acta* **93**, 313 (1965).
22. J. B. Davenport, *Nature, Lond.* **210**, 198 (1966).

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Properties of a carboxypeptidase from aloe

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Aloe (*Aloe arborescens* Mill var. *natalensis* Berger) has been valued in Japan as family medicine for skin injury and burns. We have been studying this plant from pharmacological [1] and immunological [2] aspects to evaluate its therapeutic use as an anti-inflammatory agent. In a previous report we reported that aloe contains enzyme(s) hydrolyzing bradykinin, which might explain its anti-inflammatory effect and also that the enzyme seems to split bradykinin between Gly^4 and Phe^5 [1]. However, while attempting to purify the enzyme, we found that one of the hydrolysis products from bradykinin was arginine, the amino acid located at both terminals. We, therefore, reinvestigated the action of the enzyme on bradykinin and found that it may be regarded as a "serine carboxypeptidase" [3].

A crude enzyme preparation was obtained as follows. The exudate from fresh leaves of aloe was treated with 3 vol. of acetone and the precipitate was dissolved in 0.05 M NaOAc buffer, pH 6.0. The solution was brought to 70 per cent saturation of $(NH_4)_2SO_4$ and the resulting precipitate was dissolved in 0.05 M NaOAc buffer, pH 6.0. Precipitates were successively obtained by the addition of $(NH_4)_2SO_4$ to make 35, 55 and 75 per cent saturations and were assayed for bradykininase activity. The highest activity was found in the precipitate at 35–55 per cent saturation of $(NH_4)_2SO_4$ which was dissolved in 0.05 M NaOAc buffer, pH 6.0 and dialyzed against the same buffer.

Bradykininase activity was estimated by a semi-quantitative method. The enzyme was incubated with 1 mM bradykinin in 0.05 M NaOAc buffer, pH 6.0 at 37° for 1 hr and the amount of amino acid released (arginine) was determined by the ninhydrin method [4]. Effects of various reagents and metals on the bradykininase activity of the enzyme were examined as follows: the enzyme was preincubated with a reagent or a metal ion at a final concentration shown in Table 1 for 20 min at 37° in 0.05 M NaOAc buffer, pH 6.0. The substrate bradykinin was then added to make a final concentration of 1 mM and the mixture was incubated at 37° for 30 min. The reaction was terminated by heating the mixture in a boiling water bath. The amount of arginine liberated was determined with a Shimadzu Dual-Wavelength TLC Scanner CS-900 after high-voltage paper electrophoresis at 3,600 V in a pyridine–acetic acid buffer, pH 3.5 and ninhydrin staining. Optimum pH of the enzyme for bradykinin and Z-Gly-Pro-Leu-Gly were estimated by using 0.05 M acetate buffers, pH 4.0–6.5 and 0.05 M phosphate buffers, pH 6.0–7.5 with pH intervals of 0.5. Synthetic bradykinin, angiotensin I and Z-Gly-Pro-Leu-Gly were purchased from Protein Research Foundation, Osaka, Japan.

The hydrolysis of bradykinin by the crude enzyme preparation was followed by analyzing the amounts of free amino acids released after various incubation times. As shown in Fig. 1, the peptide was split sequentially from the COOH-terminal. The release of Arg, Phe, Pro and Ser was further confirmed by high-voltage paper electrophoresis and by paper chromatography. The enzyme also hydrolyzed a decapeptide angiotensin I, thus releasing the C-terminal amino acids Leu, His, Phe and Pro. Action of the enzyme on a smaller synthetic peptide Z-Gly-Pro-Leu-Gly was then studied and the products were found to be Gly and Leu. These results indicate that the enzyme from aloe is a carboxypeptidase rather than an endopeptidase.

Properties of the enzyme were then investigated in order to compare with those of other carboxypeptidases. The optimum pH of the enzyme for bradykinin and Z-Gly-Pro-Leu-Gly was approx. 6 and 5, respectively. The effects of various reagents and metal ions on the enzyme activity are summarized in Table 1. Enzyme activity was little affected by L-cysteine or 2-mercaptoethanol which can activate plant thiol proteases such as papain [5] and ficin [6]. Although inhibited to some extent by *p*-chloromercuriphenylsulfonic acid, a potent SH reagent, the enzyme was not inhibited by iodoacetamide, an inhibitor of thiol proteases [5, 6]. Most of the metal ions examined exhibited more or less inhibitory effects on the enzyme, but chelating agents such as EDTA and 1,10-phenanthroline did not affect much the enzyme activity. Diisopropyl phosphofluoridate which is known to inhibit "serine carboxypeptidase" [3] completely inactivated the enzyme. From these data the enzyme from aloe should be classified as a serine protease, but neither a metal nor an acid protease according to the classification of proteases by Hart-