action with this more electron deficient hydroperoxide involves O–O bond heterolysis. It is possible, in the aprotic solvent CH_2Cl_2, that all hydroperoxides react via a heterolytic mechanism. Also, possible in aprotic solvents is a homolytic rate-determining step followed by a second electron transfer to provide the epoxidizing iron-oxo porphyrin π-cation radical by what is overall a heterolytic mechanism (eq 3).

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Rate-Limiting P–O Fission in the Self-Stimulated Inactivation of Acetylcholinesterase by 4-Nitrophenyl 2-Propyl Methylphosphonate

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We wish to report that phosphonylation of the active-site serine residue of electric-eel acetylcholinesterase (AChE) by 4-nitrophenyl 2-propyl methylphosphonate (IMN), leading to irreversible inactivation of the enzyme, occurs with a bimolecular rate constant $k_i/K_i = 8130 \pm 380$ M^{-1} s^{-1}, a solvent isotope effect $k_{HOH}/k_{DOD} = 1.29 \pm 0.06$, and a leaving group ^{18}O isotope effect of $k_{16}/k_{18} = 1.06 \pm 0.03$. The value of k_i/K_i exceeds the rate constant for water-catalyzed hydrolysis[1] of IMN by a factor of $10^{10.3}$, indicating that a part of the catalytic power of the enzyme is recruited[2,3] in phosphonylation by IMN. Catalytic recruitment in the phosphonylation process, but not in the subsequent and therefore much slower dephosphonylation process,[4] is responsible for the fact that compounds like IMN are powerful, specific irreversible enzyme inhibitors, a property exploited in agriculture and warfare. The solvent isotope effect of 1.3 is similar to what has been observed for acylation[5] and phosphonylation[3] of AChEs but smaller than the value of 2.3–2.4 for deacetylation of AChEs[2a] or the values of 1.7–2.1 for phosphonylation of other serine proteases.[2b] We concur, as before,[3] with Quinn's interpretation[6] of the origin of this small effect being the recruitment of enzymic general base catalysis in a step which is not fully rate limiting. In principle, the small value of 1.3 could also come from nonspecific enzymic effects. The ^{18}O isotope effect of $6 \pm 3\%$ shows that fission of the P–O bond is very advanced[5] in the effective transition state(s) that governs the rate of inactivation. We believe that the inactivation process involves a partially rate-limiting inhibitor-induced enzyme conformational change,[3,6] probably with little or no solvent isotope effect and no ^{18}O isotope effect, and a partially rate-limiting displacement at phosphorus, with solvent isotope effect of 2–4 and ^{18}O isotope effect greater than 6%. Although the solvent isotope effect and ^{18}O isotope effect indicate bond formation and bond fission at phosphorus both to be partially rate limiting, we have no information about whether these events occur in a single transition state (concerted mechanism) or in separate transition states (stepwise mechanism).

The kinetics of irreversible inhibition of AChE by IMN was measured (25 °C, pH 7.60, pD 8.10, 0.0066 M KH_2PO_4, 0.0434 M K_2HPO_4, and 5% methanol) with the use of the substrates phenyl acetate at 3 K_m or naphthyl acetate at 0.5 K_m as monitors of enzyme activity. The first-order rate constants k_o for enzyme inactivation were obtained from the change in absorbance at 233 nm (naphthyl acetate) or 275 nm (phenyl acetate), in the presence of IMN at 10–70 μM. The rate constants were treated according to eq 1 to obtain k_i/K_i values.[7] Accurate values of k_i could not be obtained under the conditions employed.

$$k_o^{-1} = k_i^{-1} + \{(K_i/k_i)/[IMN]\}\{1 + [S]/K_m\} \quad (1)$$

The most demanding measurement was the ^{18}O isotope effect, which had to be obtained from kinetic studies with ordinary IMN and with IMN having greater than 93% ^{18}O in the phenolic oxygen site.[8] Frequently oxygen isotope effects are measured by high-precision isotope-ratio mass spectrometry with the employment of competitive techniques. This was not possible here, since the product 4-nitrophenol is formed only in stoichiometric equivalence to the quantity of AChE inactivated; direct measurements with fully labeled inhibitor were therefore essential. In one series of experiments with naphthyl acetate as substrate, identical solutions (except for the labeled-inhibitor stock solutions) were employed with both isotopic inhibitors, and the slopes of eq 1 were calculated: 10^4 slope = 2.120 ± 0.051 (^{16}O), 2.264 ± 0.082 (^{18}O). This gave the isotope effect k_{16}/k_{18} (as the inverse ratio of the slopes) as 1.068 ± 0.046. For a second series of experiments, 10^4 slope = 1.987 ± 0.044 (^{16}O), 2.108 ± 0.105 (^{18}O) for an isotope effect of 1.061 ± 0.057. Finally, with phenyl acetate as substrate, the values of k_i/K_i were independently determined for the two isotopic inhibitors. The result was k_i/K_i (M^{-1} s^{-1}) = 8260 ± 224 (^{16}O), 7790 ± 260 (^{18}O), and thus an isotope effect of 1.061 ± 0.046. Although each of these measurements has a substantial error, the mean values agree well (1.063 ± 0.026),[9] particularly since the measurements were made according to different protocols and with the use of different substrates. Furthermore, the magnitude of the effects is large enough to permit an unambiguous interpretation of at least partially rate-limiting P–O fission, even with a fairly large error.

The simplest interpretation of a solvent isotope effect of 1.3 and a large leaving group ^{18}O isotope effect is that both proton

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(1) The second-order rate constant for the water-catalyzed hydrolysis of IMN is $\sim 10^{-7}$ M^{-1} s^{-1}. Bennet, A. J.; Kovach, I. M., unpublished results.

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(5) The magnitude of k_{16}/k_{18} to be expected for completely rate-limiting P–O fission is uncertain. Gorenstein et al. (Gorenstein, D. G.; Lee, Y.-G.; Kar, D. *J. Am. Chem. Soc.* **1977**, *99*, 2264–2267) measured 1.0204 ± 0.0044 for hydrolytic decomposition of 2,4-dinitrophenyl $^{18}OPO_3^{2-}$. Knight et al. (Knight, W. B.; Weiss, P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2759–2761) and Weiss et al. (Weiss, P. M.; Knight, W. B.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2761–2762) found values of 1.01–1.02 for various secondary ^{18}O–P effects not involving P–O fission. Typical values of k_{16}/k_{18} when C–O fission is rate-limiting are around 1.06 (O'Leary, M. H. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; pp 285–316), but the fact that P has an atomic mass 2.6 times that of C means that P–O fission should produce substantially larger effects.

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(8) ^{18}O-labeled 4-nitrophenol was synthesized as described in the following: Bennet, A.; Sinnott, M.; Wijesundera, W. S. S. *J. Chem. Soc., Perkin Trans.* **1985**, *2*, 1233–1236. Mass spectral analysis indicated >93% incorporation of the ^{18}O label. Coupling of each isotopomer of 4-nitrophenol with 2-propyl methylphosphonochloridate was carried out according to ref 2. The isomeric esters were brought to >99% analytical purity by HPLC on a C-18 column (ODS Hypersil 150 × 4.7 mm, 2.5 μ particle size) with methanol–water gradient elution before each kinetic experiment and were measured spectrophotometrically at 400 nm for 4-nitrophenol equivalent after basic hydrolysis. Additional verifications of ^{18}O content in solutions prepared for kinetics were carried out by mass spectral analysis of the molecular ion ratios ($^{16}O/^{18}O$) from a 1:1 mixture of the stock solutions of the isomeric esters that had been evaporated to dryness and redissolved in dichloromethane prior to injection into the mass spectrometer.

(9) Weighted average; calculated according to $\bar{x} = \sum(w_i x_i)/\sum(w_i)$, where $w_i = 1/s_i^2$ and s_i is the propagated error in an individual isotope effect. The error in the average, $\bar{x}$, is $\bar{s} = [\sum(w_i)]^{-1/2}$.

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bridging and P–O fission are occurring in the transition state or combination of transition states that determine the rate. It is very unlikely that proton bridging to the leaving group oxygen is occurring because 4-nitrophenol is substantially dissociated at this pH.[10] Thus proton bridging is probably occurring between the enzymic histidine and the serine nucleophilic oxygen, implying that O–P bond *formation* to the nucleophile is also rate-limiting in addition to fission of the P–O bond to the leaving group. We cannot say whether these processes are concerted[11] or stepwise.

These and other data are beginning to delineate the structural features that the enzyme–phosphonylation transition state must possess. In the near future, these structural features will be introduced by molecular-modeling techniques[12] into the known active-site structures of serine hydrolases, to deduce the molecular origins of the phenomenon of catalytic recruitment.

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Biosynthesis of 3,6-Dideoxy Hexoses: C-3 Deoxygenation May Proceed via a Radical Mechanism

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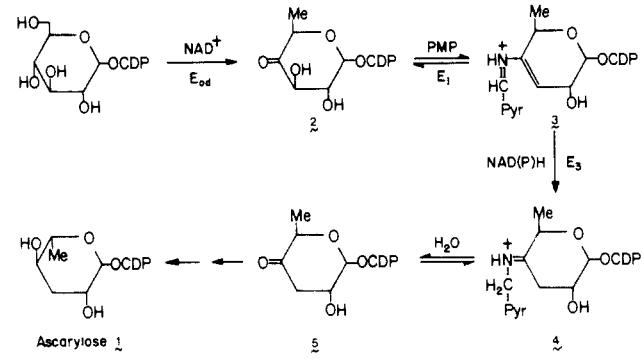
The 3,6-dideoxyhexoses are found in the lipopolysaccharide of gram-negative bacteria where they have been shown to be the dominant antigenic determinants.[1] On the basis of the pioneering efforts of Strominger and his co-workers,[2] the pathway for the biosynthesis of ascarylose, a 3,6-dideoxy-L-*arabino*-hexose **1**, had been put forward as shown in Scheme I. The key reaction of this proposed sequence is the C-3 deoxygenation step catalyzed by enzymes E_1, a pyridoxamine-5′-phosphate linked enzyme, and E_3, a NADPH dependent catalyst, both of which had been purified from *Pasturella pseudotuberculosis*.[3] Although the catalytical roles of these enzymes have been well defined,[2] the intimate mechanism of these steps is still disputable. For instance: (1) the hypothesized 3,4-glucoseen product **3** has never been identified; therefore, the actual mechanism of E_1 remains unresolved;[2c,4] (2) the E_3-catalyzed reduction of **3** which is still E_1 bound is believed to be a hydride-transfer process; however, such a reduction demands that their active sites be brought into closer proximity than is possible;[5] (3) incubation of E_3 with [4-$^3H_2$]NADPH resulted

Scheme I



in no tritium incorporation at either the product **5** or the regenerated PMP coenzyme; moreover, both the 4R and 4S hydrogens of NADPH were found to be labile in this reduction step;[2d] (4) E_3 can also catalyze the direct electron transfer from NADH to O_2;[2d,3b] albeit it contains no chromophoric groups. In an effort to clarify these mechanistic ambiguities, we have isolated an "E_3 equivalent" from *Yersinia pseudotuberculosis*[7] which is known to have ascarylose as the nonreducing terminal sugar in its lipopolysaccharide structure.[8] Reported herein is the preliminary characterization of the catalytic properties of this enzyme as a NADH oxidase and the consequent implication on its mechanism as a 3,4-glucoseen reductase.[9]

The purified enzyme[10] consists of a single polypeptide chain with a molecular weight of 41 000 and contains no metals.[11] Its UV–vis spectrum is that of a simple polypeptide with an absorption maximum around 280 nm. This result unequivocally demonstrates that this enzyme is not a flavoprotein and possesses none of the common electron carriers to mediate the electron transfer from NADH to O_2. The nature of the oxygen metabolite was determined to be H_2O_2 based on the ferrous-ferric procedure of Thurman et al.[12] and the oxidation of *leuco*-2′,7′-dichlorofluorescein (*leuco*-DCF) to DCF of Kochi and Wartburg.[13] Since the ratio of NADH oxidized to H_2O_2 produced is approximately one, this enzyme-catalyzed NADH oxidation is clearly a two-electron redox process overall. A variety of alternate electron

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(10) A highly selective sequence incorporating DEAE cellulose, phenyl-Sepharose, DEAE Sephadex, and Sephadex G-100 chromatography was developed in this study which led to a 2000-fold purification of this protein. Overall yield was ca. 4 mg of enzyme per 480 g of wet cells.
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