ester to the carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) and exhaustively dialyzed against 150 mM NaCl, 10 mM phosphate buffer, pH 7.5. Epitope densities ranged between 5 and 15. Balb/c mice were immunized with the KLH conjugate of **2**, and antibodies were generated by standard protocols.[6] IgG was purified from ascites fluid by affinity chromatography on protein A-coupled Sepharose 4B and judged homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis.[22]

Solutions of carboxymethylthymine dimer **1** were irradiated with 300 nm light in the presence of antibody; dimer cleavage was assayed spectrophotometrically.[23] Five out of six antibodies (IgG) were found to sensitize the photocleavage. One of these, antibody 15F1-3B1 was studied further. High pressure liquid chromatography confirmed that the reaction product was thymine monomer. The absorption and fluorescence spectra of the antibody remained unchanged upon photolysis, indicating that photodegradation of the protein was negligible. The kinetics of the antibody-catalyzed reaction are consistent with the Michaelis–Menten rate expression (Figure 1):

$$\mathrm{Ig} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{Ig{\cdot}S} \xrightarrow{k_{cat}} \mathrm{Ig} + \mathrm{P}$$

The kinetic constants k_{cat} and K_m are 1.2 min^{-1} and 6.5 μM, respectively ($k_{cat}/K_m = 1.8 \times 10^5$ M^{-1} min^{-1}). The k_{cat} value observed is comparable to the turnover number for *Escherichia coli* DNA photolyase of 3.4 min^{-1}.[24] The reaction is first order in light between incident intensities of 5×10^{-8} and 1.6×10^{-7} einsteins min^{-1}. Consequently, the k_{cat} obtained is not optimal; irradiation at higher flux should increase k_{cat} until light saturation occurs.[25] The first-order rate constant for unsensitized dimer cleavage is 5.5×10^{-3} min^{-1}. Hapten **2** is also readily cleaved by the antibody; however the K_m for this substrate is too low to be conveniently measured by direct spectrophotometric assay (<1 μM). The photocleavage of the corresponding *N,N'*-dimethyl substrate[18] is not sensitized by the antibody at substrate concentrations of up to 1.8 mM, which is consistent with the high specificity of antibody-ligand binding. In addition, the dinitrophenyl specific IgA MOPC 315, which contains a binding-site tryptophan, and the phosphorylcholine specific IgA MOPC 167 do not catalyze the photocleavage of thymine dimer under identical reaction conditions.

The wavelength dependence of the quantum yield of the antibody-sensitized photolysis of hapten **2** reveals a shoulder at 300 nm (Figure 2a). Antibody fluorescence is also quenched in the presence of thymine dimer (Figure 2b: It should be noted that the percentage of fluorescence quenched upon ligand binding does not reflect the total number of tryptophan residues in the protein due to environmental effects on Φ_F). These observations suggest that a combining site tryptophan is photosensitizing dimer cleavage. The quantum yield of the antibody-catalyzed reaction ($\Phi_{R,300}$) is 0.08. The antibody was oxidized with *N*-bromosuccinimide in 8 M urea, pH 4.5, revealing ≥10 tryptophan residues per Ig.[26] Assuming that each binding site contains only one tryptophan allows the calculation of an approximate quantum yield of ≥0.4 for photocleavage of bound dimer (based on formation of monomer). Pulse radiolysis experiments have shown that dimethyl and tetramethylthymine dimer radical anions decay to monomer with a frequency of 0.05.[27] Since the quantum yield of the antibody-sensitized reaction is significantly higher than 0.05, the antibody appears to partition the breakdown of the intermediate radical anion. Further experiments are being carried out to precisely define the mechanism of this antibody-catalyzed reaction.

This work represents the extension of antibody catalysis to a new class of reactions. Moreover, it should be possible to utilize antibody–hapten complementarity to generate antibodies with active-site amino acid residues which selectively catalyze a variety of other reactions, including hydrolyses, isomerizations, and eliminations.

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(18) Hapten **2** was synthesized by alkylation of thymine with chloroacetic acid in aqueous potassium hydroxide.[19] Carboxymethylthymine was treated with 1 equiv of *N,N'*-carbonyldiimidazole (CDI) in dimethylformamide (DMF) followed by addition of 0.5 equiv of ethylene glycol. Removal of solvent and trituration with water yielded the ethylene glycol diester. The diester was dissolved in minimal DMF and photolyzed in degassed 10% aqueous acetone (1 g/750 mL) through a Pyrex filter for 2 h with a 450-W Hanovia medium pressure Hg immersion lamp. Removal of solvent and trituration with water afforded the thymine dimer ethylene glycol diester as a single isomer (cis, syn). Basic hydrolysis and subsequent acidification to pH 2 gave crystalline carboxymethylthymine-*cis,syn*-cyclobutane dimer **1**. Activation with CDI in DMF followed by addition of glycine ethyl ester yielded the bis[ethyl glycinate] adduct after removal of solvent and trituration with water. Basic hydrolysis and removal of solvent gave hapten **2**. Dimethylcarboxymethylthymine-*cis,syn*-cyclobutane dimer was prepared by alkaline dimethyl sulfate treatment[20] of the carboxymethylthymine ethylene glycol diester photoproduct followed by adjustment of the pH to 11 for 1 h and subsequent acidification to pH 2 to induce crystallization.

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(23) Deoxygenated solutions of thymine dimer (5–300 μM) and antibody (3 μM) in 100 mM NaCl, 10 mM phosphate buffer, pH 7.5, were irradiated at 18–20 °C in a 1-mL quartz cuvette. All photolysis experiments were performed with a 1000-W high pressure Hg-Xe lamp equipped with a Photon Technologies International, Inc. Model Q5001 Quantacount electronic actinometer and a diffraction grating monochromator blazed at 500 nm. A bandpass of 3 nm was used for both kinetic and photon counting experiments. Kinetic experiments were performed at 300 nm with an incident flux of 1.26×10^{-7} einsteins min^{-1}. Monomer formation was assayed spectrophotometrically at fixed time intervals (λ_{max} = 272 nm, log ϵ = 3.90). Protein molarity was determined by absorbance at 280 nm by using $\epsilon^{0.1\%} = 1.37$ and a molecular weight of 150 kD for IgG.

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The Mechanism of Hydroperoxide O–O Bond Scission on Reaction of Hydroperoxides with Iron(III) Porphyrins

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An understanding of the mechanism of reaction of RCO_3H and alkyl-OOH compounds with iron(III) porphyrins is important in the understanding of the mechanisms of reactions of horseradish peroxidase, catalase, and cytochrome P-450 enzymes. Heterolytic O–O bond breaking is involved in the reactions of alkyl-OOH and RCO_3H species with (EDTA)(Fe^{III}) (in methanol) and (*meso*-tetraphenylporphinato)chromium(III) chloride ((TPP)Cr^{III}(Cl)) (in CH_2Cl_2). The log of the apparent second-order rate constants (k_{ly}) for reaction of both alkyl-OOH and RCO_3H species (YO-OH) when plotted vs the pK_a of alkyl-OH and RCO_2H (YOH) leaving groups falls on single lines with (EDTA)(Fe^{III})[1] and with ((TPP)Cr^{III}(Cl)).[2,7b] In marked contrast, a plot of log k_{ly} vs pK_a

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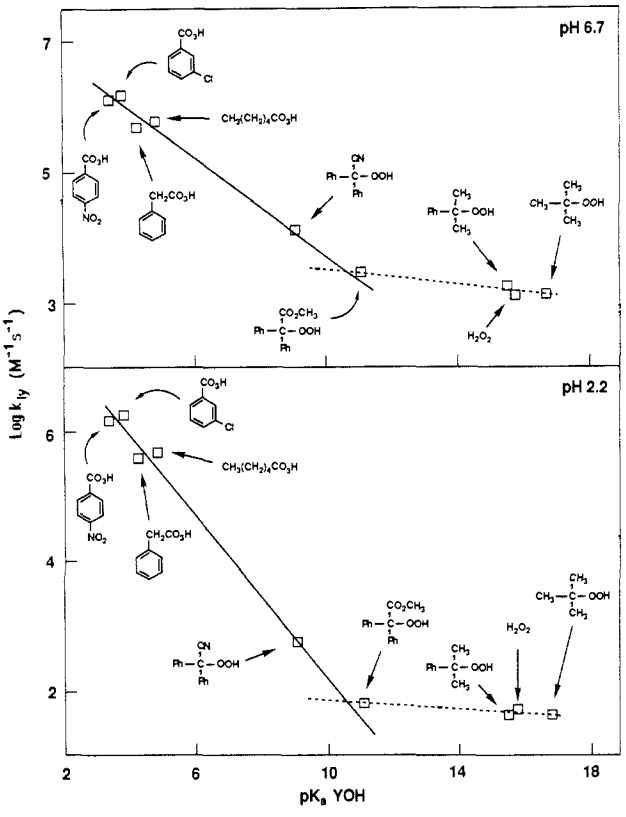

Figure 1. Plots of the log of the second-order rate constants (k_{ly}) for the reaction of RCO_3H and ROOH species (YOOH species) with (**1**)-$Fe^{III}(X)(H_2O)$ vs the pK_a of the leaving groups (YOH species) at pH 2.2 and 6.7. For alkyl hydroperoxides, the value of $k_{ly} = k_{obsd}/[(\mathbf{1})Fe^{III}(X)(H_2O)]$ at the given pH, where k_{obsd} is the pseudo-first-order rate constant when [YOOH] ≫ [iron(III) porphyrin]. Reactions were followed by trapping the hypervalent iron-oxo porphyrin species with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate). The values of k_{ly} for the acyl hydroperoxide ([YOOH] ≫ $[(\mathbf{1})Fe^{III}(X)(H_2O)]$) reactions were calculated from the linear plots of k_{obsd} vs [YOOH], where k_{obsd} is the pseudo-first-order rate constant for the disappearance of $(\mathbf{1})Fe^{III}(X)(H_2O$ in the absence of a trapping agent.

of YOH (in methanol) using $(TPP)Fe^{III}(Cl)$ provides two distinct linear plots. One of greater slope for RCO_3H and another for alkyl-OOH.[7]

To this date such studies have been carried out in organic solvents so that pH is unknown as are the acid dissociation constants of the reactants and intermediate complexes. We have now[3,4] determined the pH (2–12) dependence of the second-order rate constant for the reaction of YOOH compounds with the water soluble and non-μ-oxo dimer forming (*meso*-tetrakis(2,6-dimethyl-3-sulfonatophenyl)porphinato)iron(III) hydrate [(**1**)-$Fe^{III}(X)(H_2O)$; X = H_2O or HO^-]. There are three complexes which break down in committing steps. These are the following:[3] (A) $(\mathbf{1})Fe^{III}(OH_2)(HOOY)$; (B) $(\mathbf{1})Fe^{III}(OH_2)(^-OOY)$; and (C) $(\mathbf{1})Fe^{III}(^-OH)(^-OOY)$. At pH's 2.2 and 6.7 complexes A and B are the reactive species. The plots of log k_{ly} vs pK_a of YOH (Figure 1) show a change in slope which may be accounted for by a change from heterolytic to homolytic O–O bond breaking.

That the reactions of alkyl-OOH are radical in nature are supported by the observation that reaction of $Ph(Me)_2C$-OOH with $(\mathbf{1})Fe^{III}(X)(H_2O)$ provides, in 90% yield, the fragmentation products of $Ph(Me)_2C$-O• (i.e., Ph-CO-Me plus MeOH). No PhOH nor $(Me)_2CO$ could be detected.[3b,4] Also in support of a rate-limiting homolysis with alkyl-OOH is the sensitivity to O_2 of the formation of an observable $(\mathbf{1})Fe^{IV}(O)$ intermediate.[4,5] Formation of $(\mathbf{1})Fe^{IV}(O)$ with RCO_3H does not require the presence of O_2. However, $(\mathbf{1})Fe^{IV}(O)$ is seen with alkyl-OOH only in the presence of O_2. Formation of iron(IV) porphyrin with RCO_3H involves a comproportionation reaction (eq 1), while the

$$(\mathbf{1})Fe^{III}(X)(H_2O) + YOOH \rightarrow (^{+\bullet}\mathbf{1})Fe^{IV}(O) + X + YOH + H_2O \quad (1a)$$

$$(\mathbf{1})Fe^{III}(X)(H_2O) + (^{+\bullet}\mathbf{1})Fe^{IV}(O) \rightarrow 2(\mathbf{1})Fe^{IV}(O) + H_2O + H^+ \quad (1b)$$

dependence upon O_2 with alkyl-OOH requires the formation of an iron(II) species through a homolytic reaction (eq 2). In the absence of O_2 the $(\mathbf{1})Fe^{IV}(O)$ (eq 2a) would be expected to react with $(\mathbf{1})Fe^{II}(H_2O)$ (eq 2c) to provide 2 × $(\mathbf{1})Fe^{III}(X)(H_2O)$. The $(\mathbf{1})Fe^{II}$ intermediate has been spectrally characterized after trapping with CO.

$$(\mathbf{1})Fe^{III}(X)(H_2O) + (Me)_3COOH \rightarrow (\mathbf{1})Fe^{IV}(O) + (Me)_3CO^\bullet + 2H_2O \quad (2a)$$

$$(Me)_3CO^\bullet \rightarrow Me^\bullet + (Me)_2CO \quad (2b)$$

$$(\mathbf{1})Fe^{III}(OH)(H_2O) + Me^\bullet \rightarrow (\mathbf{1})Fe^{II}(H_2O) + MeOH \quad (2c)[10]$$

$$(\mathbf{1})Fe^{II}(H_2O) + \tfrac{1}{2}O_2 \rightarrow (\mathbf{1})Fe^{IV}(O) + H_2O \quad (2d)$$

We find the second-order rate constants for reactions of *t*-BuOOH and $Ph(Me)_2C$-OOH with $(\mathbf{1})Fe^{III}(X)(H_2O)$ are insensitive to the concentrations of oxygen or nitrogen acid and base species. In contrast, the reaction of $(TPP)Fe^{III}(X)$ with YOOH species in methanol are markedly catalyzed by (1:1) collidine/collidine·H^+.[6,7] Traylor has proposed that general base catalysis of oxygen transfer from YOOH compounds to $(TPP)Fe^{III}(Cl)$ is diagnostic of a heterolytic reaction.[6a,8] In methanol the pK_a associated with the formation of the reactive species [$(TPP)Fe^{III}(X)(HOOY) \rightarrow (TPP)Fe^{III}(X)(^-OOY) + H^+$] should be markedly increased when compared to H_2O as solvent. Rate limiting homolysis of the hydroperoxide O–O bond could, therefore, be general base catalyzed (eq 3a). The 2e$^-$ oxidation

$$(Porph)Fe^{III}(X)(H_2O_2) + B: \xrightarrow{slow} (Porph)Fe^{IV}(X)(O) + {}^\bullet OH + BH \quad (3a)$$

$$(Porph)Fe^{IV}(X)(O) + {}^\bullet OH + BH \rightarrow (^{+\bullet}Porph)Fe^{IV}(X)(O) + H_2O + B: \quad (3b)$$

of HR peroxidase and catalases by hydroperoxides may be attributed to rate-determining homolysis followed by a rapid 1e$^-$ transfer reaction (eq 3b). It has been reported that (*meso*-tetrakis(2,6-dichlorophenyl)porphinato)iron(III) chloride ($(Cl_8TPP)Fe^{III}(Cl)$) in CH_2Cl_2 with *t*-BuOOH is capable of epoxidation of norbornene.[11] The log of the second-order rate constant for reaction of $Ph_2(CN)COOH$ with $(\mathbf{1})Fe^{III}(X)(H_2O)$ in the plot of Figure 1 falls with the acyl hydroperoxides. Perhaps the re-

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(5) For $(\mathbf{1})Fe^{IV}(O)$ at pH 6.7, λ_{max} = 421 nm and ϵ is about 1.3 × 10^5 M^{-1} cm^{-1}. The identity of $(\mathbf{1})Fe^{IV}(O)$ was established by (i) titration with 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) which showed an oxidation level 1e$^-$ above $(\mathbf{1})Fe^{III}(X)$ and (ii) its spectroelectrochemical generation under controlled-potential conditions.
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(8) There is no precedence for such an expectation. Heterolytic O–O bond scission with transfer of oxygen from YOOH species to I$^-$, dimethyl sulfide, and tertiary amines (to provide IO$^-$, sulfoxide, and amine *N*-oxide, respectively) is not subject to general catalysis (see ref 9).
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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 $k_i/K_i\ (M^{-1}\ s^{-1}) = 8260 \pm 224$ (^{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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(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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