

Diisopropylfluorophosphate Hydrolysis by a Phosphotriesterase from *Pseudomonas diminuta*

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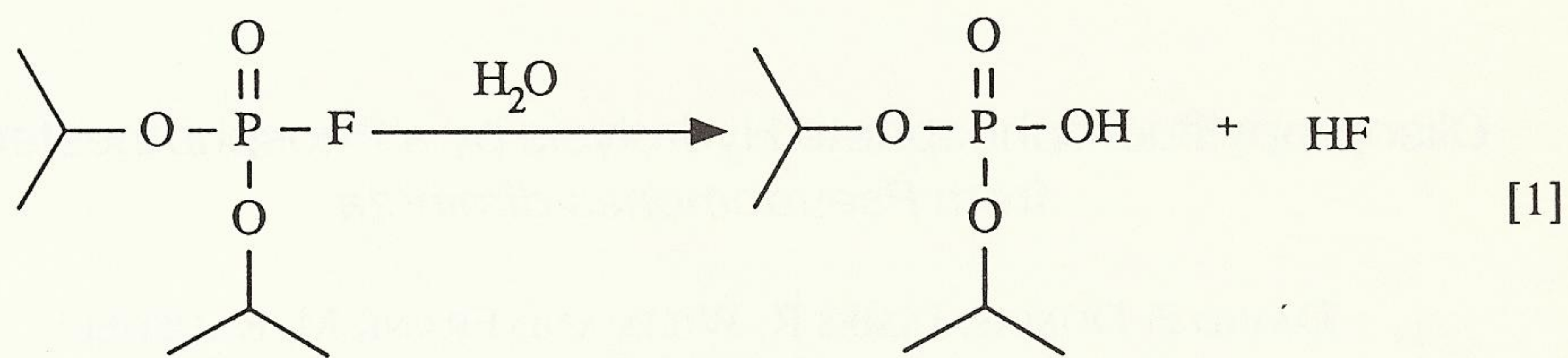
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Kinetic parameters for diisopropylfluorophosphate hydrolysis at pH 7.0 (25°C) were determined for a phosphotriesterase (parathion hydrolase) from *Pseudomonas diminuta*. The enzyme was partially purified from an *Escherichia coli* strain into which the gene (*opd*) for the phosphotriesterase had been cloned. The K_m for diisopropylfluorophosphate hydrolysis at pH 7.0 was found to be 0.12 ± 0.02 mM and the V_{max} was 3.6% of the maximal rate of hydrolysis of paraoxon. The K_m for paraoxon hydrolysis was found to be 0.012 ± 0.001 mM under the same reaction conditions. Diisopropyl-*p*-nitrophenylphosphate was shown to have a $K_m = 0.013 \pm 0.001$ mM and a relative V_{max} of 9%. Diisopropylfluorophosphate showed competitive inhibition versus paraoxon with a $K_i = 0.32$ mM at pH 9.0. Diisopropylfluorophosphate and the products of the enzymatic hydrolysis were analyzed and confirmed by ¹⁹F and ³¹P NMR spectroscopy. © 1989 Academic Press, Inc.

In the early 1930s, organophosphates were discovered by German researchers searching for a substitute for nicotine and have since had widespread use as pesticides, herbicides, and fungicides (1). In 1946, organophosphates were shown to be enzymatically hydrolyzed by an extract from mammalian blood sera by using diisopropylfluorophosphate (DFP)² as a substrate (2). DFPases have since been found in various biological sources such as squid (3) and several microorganisms (4-6). These DFPases have been categorized as Mazur-type or squid-type DFPases on the basis of activation or inhibition upon addition of Mn²⁺, respectively. An enzyme from *Pseudomonas diminuta* has been shown to hydrolyze the phosphotriesters parathion and paraoxon (7). This report describes a wider specificity range for this enzyme based on the ability of the enzyme from *Pseudomonas* to hydrolyze DFP. DFP has been shown to be a substrate (Eq. [1]) on the basis of the criteria that the rate of hydrolysis is proportional to the enzyme concentration and that it shows saturation kinetics as determined by use of a fluoride-selective electrode. The cloned phosphotriesterase of *Pseudomonas* can therefore catalyze the hydrolysis of the P-F bond in addition to the P-O bond of paraoxon and parathion.

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² Abbreviations used: DFP, diisopropylfluorophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DINP, diisopropyl-*p*-nitrophenylphosphate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.



MATERIALS AND METHODS

Enzyme assays. Throughout the purification, enzymatic activity was measured in 50 mM 2-[*N*-cyclohexylamino]ethanesulfonic acid (Ches), pH 9.0, and 1.0 mM paraoxon (Sigma) by following the change in absorbance at 400 nm over time using a Gilford 260 or 2600 spectrophotometer. The temperature was regulated at 25°C. For measurement of kinetic constants with paraoxon and diisopropyl-*p*-nitrophenylphosphate (DINP), activity was measured in 50 mM-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.0, containing 250 mM KCl at 25°C. One unit of paraoxon hydrolyzing activity is defined as the amount of enzyme needed to hydrolyze 1 $\mu\text{mol}/\text{min}$ at an initial concentration of 1.0 mM.

Partial purification of phosphotriesterase. The cloning of the phosphotriesterase gene from *P. diminuta* into *Escherichia coli* has been described previously (8). A culture of the phosphotriesterase-producing *E. coli* (MC4101) was grown on 22 liters of rich broth containing 10 g/liter tryptone (Difco), 5 g/liter yeast extract (Difco), and 5 g/liter NaCl to confluency at 37°C. The cells, 100 g, were harvested by centrifugation at 5000g for 10 min. The cells were suspended in 50 mM triethanolamine buffer, pH 9.0, containing 0.1 mM ZnCl₂ (buffer A) and lysed in a French pressure apparatus at 12,000 psi. The lysate was then centrifuged at 32,000g for 60 min; the supernatant fluid was decanted and diluted to give a protein concentration of approximately 100 mg/ml. Polyethyleneimine (Polysciences, Inc.) was added over 30 min with constant stirring to make a final concentration of 0.2%. The suspension was centrifuged at 13,000g for 20 min. The supernatant fluid was decanted and made to 25% saturation (134 mg/ml) with solid ammonium sulfate with constant stirring. The solution was stirred an additional 30 min before centrifugation. The supernatant fluid was then made to 45% saturation (additional 115 mg/ml) with solid ammonium sulfate and then centrifuged. The pellet was resuspended in a minimal volume of buffer A (approximately 200 ml). This sample was centrifuged at 13,000g for 20 min and then applied (in 20-ml aliquots) to a 2.5 \times 90-cm Sephadex G-150 column equilibrated with buffer A. Fractions of 15 ml were collected at a flow rate of 1 ml/min. The fractions showing enzymatic activity were pooled and applied to a 2.5 \times 30-cm Green A column (Amicon Corp.) equilibrated in buffer A at 1 ml/min. The column was washed with 500 ml of buffer A before a 1500-ml gradient of 0–500 mM KCl in buffer A was applied. The enzyme eluted at about 400 mM KCl. All purification steps were carried out at 4°C.

DFPase activity. DFP (Sigma) hydrolysis was measured with an Orion Model 96-09-00 fluoride-selective electrode and an Orion Model 601A pH/mV meter in 100 mM Hepes, pH 7.0, containing 500 mM KCl. All measurements were made in a Teflon container with constant stirring, and each assay mixture was equilibrated at 25°C before measurement. Spontaneous hydrolysis of DFP was monitored for 5 min before the addition of enzyme. The millivolt readings were recorded every 10 s for 3

TABLE I
Purification of Phosphotriesterase

Step	Volume (ml)	Activity ($\mu\text{mol}/\text{min}$)	Protein ^a (mg)	Specific activity (units/mg)	Purification fold	Yield (%)
PEI ^b	650	806	42,000	0.019	—	—
(NH ₄) ₂ SO ₄	235	1700	1,400	1.2	63	100
G-150	650	1650	330	5.0	263	97
Green-A	400	1080	32	34	1790	64

^a Protein concentrations were estimated by measuring the absorbance at 280 nm ($\epsilon_{280} = 1 \text{ ml}/\text{mg}$).

^b Polyethyleneimine.

min. The rate of DFP hydrolysis was shown to be proportional to the enzyme concentration using 1.0 mM DFP and enzyme concentrations from 0 to 0.026 unit/ml. One unit of paraoxon hydrolyzing activity is defined as the amount of enzyme required to hydrolyze 1 μmol of paraoxon per minute at pH 7.0 at 25°C.

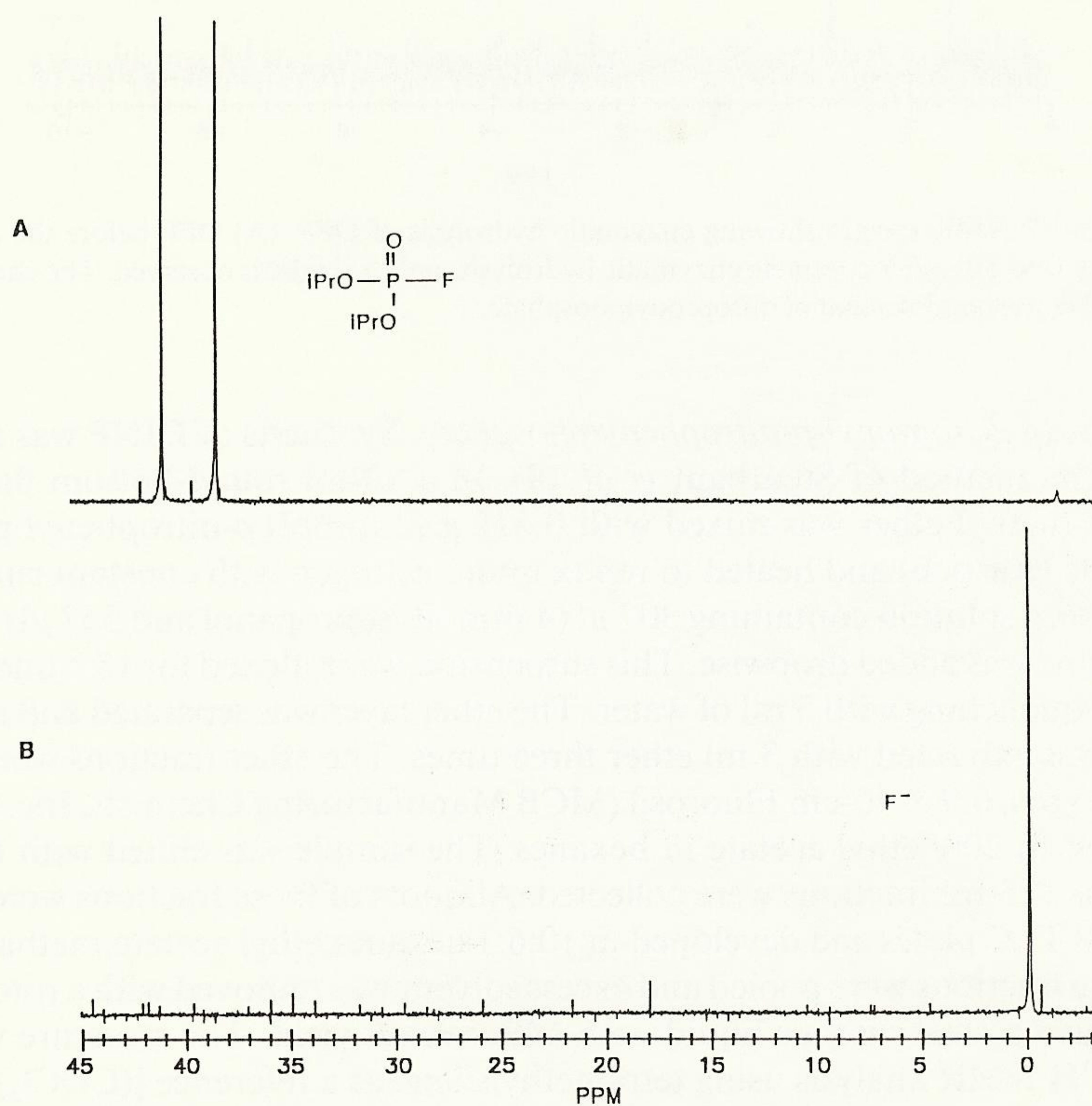


FIG. 1. The ¹⁹F NMR spectra showing enzymatic hydrolysis of DFP. (A) DFP before the addition of phosphotriesterase. (B) Two hours after the addition of phosphotriesterase, complete hydrolysis of DFP is observed as a single signal corresponding to the chemical shift for F⁻. Only a small F⁻ signal representing 0.5% of the reaction is observed over 2 h when no phosphotriesterase is added.

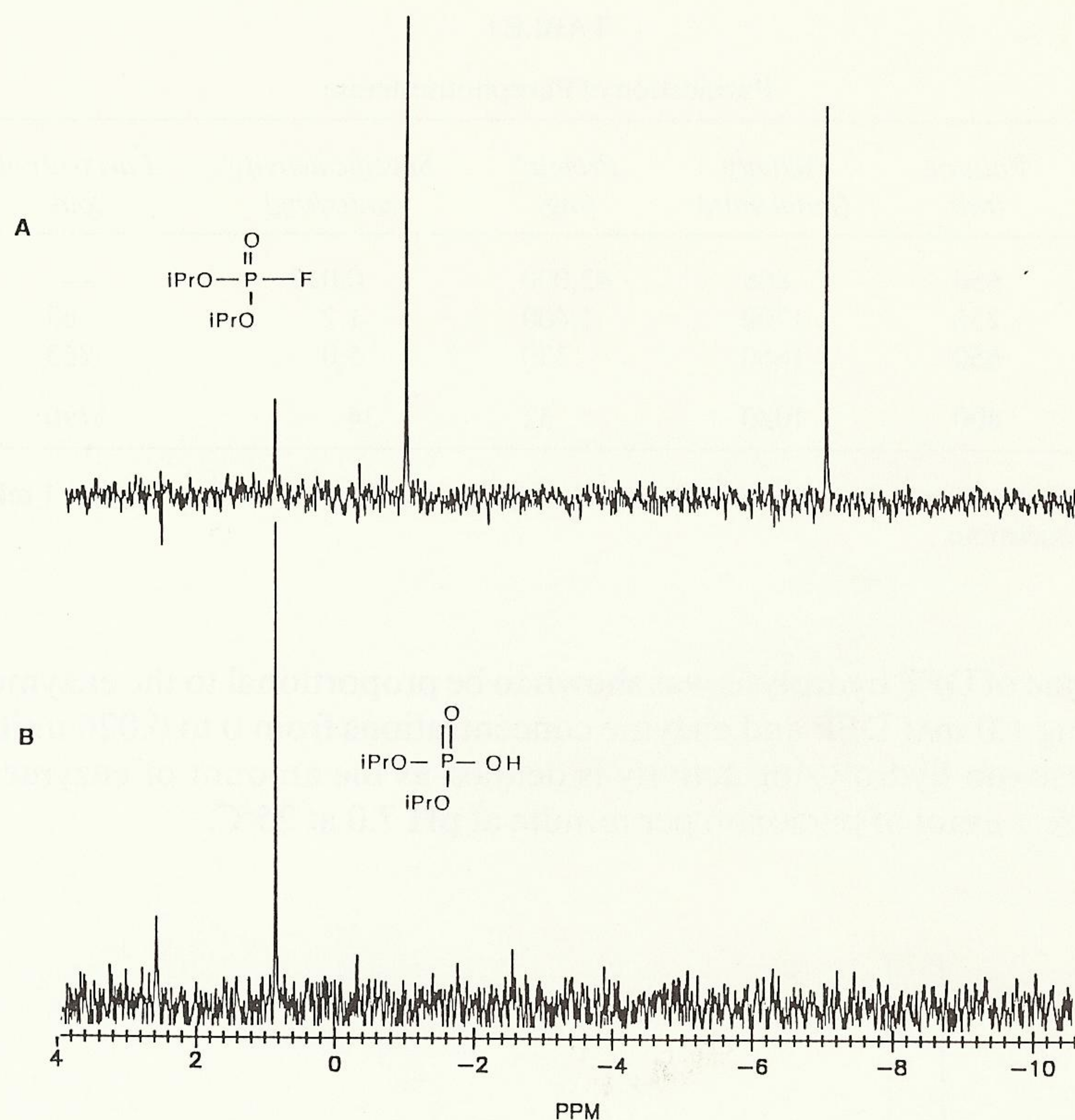


FIG. 2. The ^{31}P NMR spectra showing enzymatic hydrolysis of DFP. (A) DFP before the addition of phosphotriesterase. (B) After complete enzymatic hydrolysis, only a singlet is observed. The chemical shift for this signal corresponds to that of diisopropylphosphate.

Synthesis of diisopropyl-p-nitrophenylphosphate. Synthesis of DINP was a modification of the method of Steurbaut *et al.* (9). In a 10-ml round-bottom flask, 3 ml anhydrous diethyl ether was mixed with 0.512 g (2 mmol) *p*-nitrophenyl phosphodichloridate (Aldrich) and heated to reflux under nitrogen with constant stirring. To this mixture, a solution containing 307 μl (4 mmol) isopropanol and 557 μl (4 mmol) triethylamine was added dropwise. This suspension was refluxed for 18 h under nitrogen before quenching with 5 ml of water. The ether layer was separated and the aqueous layer was extracted with 3 ml ether three times. The ether fractions were pooled and applied to a 0.9×40 -cm Fluorosil (MCB Manufacturing Chemists, Inc.) column equilibrated in 20% ethyl acetate in hexanes. The sample was eluted with the same solvent and 125-ml fractions were collected. Aliquots of these fractions were spotted on silica gel TLC plates and developed in 10:6:1 hexanes:ethyl acetate:methanol. The appropriate fractions were pooled and excess solvent was removed with a rotary evaporator to give a clear viscous liquid with 64% overall yield. The structure was confirmed by ^1H NMR analysis using tetramethylsilane as a reference [(CDCl_3) 1.34 (d, 6H, $J = 6.3$ Hz), 1.39 (d, 6H, $J = 6.3$ Hz), 4.77 (septet, 2H, $J = 6.3$ Hz), 7.39 (d, 2H, $J = 10.8$ Hz), 8.29 (d, 2H, $J = 10.8$ Hz)].

Inhibition of paraoxon hydrolysis by DFP. The inhibitory effect of DFP at varying concentrations of paraoxon at pH 9.0 in 50 mM Ches buffer was analyzed using the

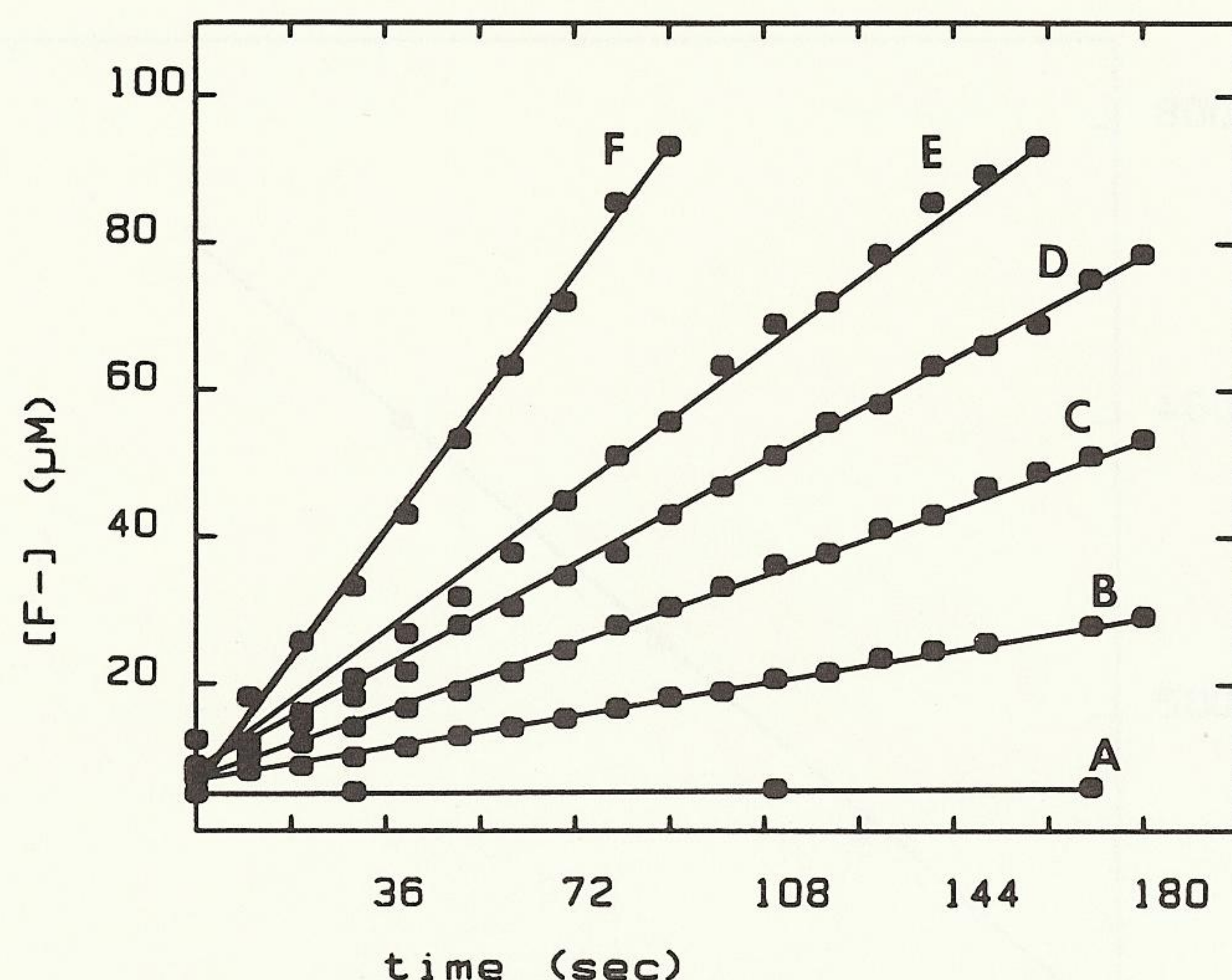


FIG. 3. The time-course plot of the enzymatic hydrolysis of 1.0 mM DFP at pH 7.0. (A) No enzyme added. (B) 0.064 paraoxon unit added. (C) 0.128 unit added. (D) 0.192 unit added. (E) 0.256 unit added. (F) 0.32 unit added.

competitive inhibition computer program of Cleland (10). In steady-state kinetics, competitive inhibition can be described by

$$v = \frac{V_m A}{K_m(1 + I/K_i) + A}, \quad [2]$$

where v is the velocity, V_{max} is the maximum velocity, A is the substrate concentration, I is the inhibitor concentration, K_m is Michaelis constant, and K_i is the inhibition constant.

Nuclear magnetic resonance. A concentration of 1.0 mM DFP in 50 mM HEPES, pH 7.0, and 20% D₂O was used for the NMR analysis. The ¹⁹F and ³¹P NMR spectra of DFP were obtained shortly after the addition of enzyme using Varian XL-400 and Varian XL-200 spectrometers, respectively. The reference standard for ¹⁹F NMR spectroscopy was NaF, while H₃PO₄ was used as a standard for the ³¹P NMR spectra. The NMR spectra after complete enzymatic hydrolysis were obtained with both probes after 2 h of incubation with the phosphotriesterase.

RESULTS AND DISCUSSION

Purification of phosphotriesterase. The phosphotriesterase from *P. diminuta* was partially purified from an *E. coli* host containing the cloned gene for the enzyme (8). The purification scheme included ammonium sulfate fractionation, gel filtration through Sephadex G-150, and dye-matrix chromatography. The results are summarized in Table I. The enzyme has been purified over 900-fold with an overall yield of 64%. However, at least three bands of M_r 31,000, 39,000, and 52,000 were observed after SDS-polyacrylamide gel electrophoresis and Coomassie staining, indicating that only a partial purification was achieved. No significant paraoxonase or DFPase (<1%) could be detected in the *E. coli* host that did not contain the plasmid with the

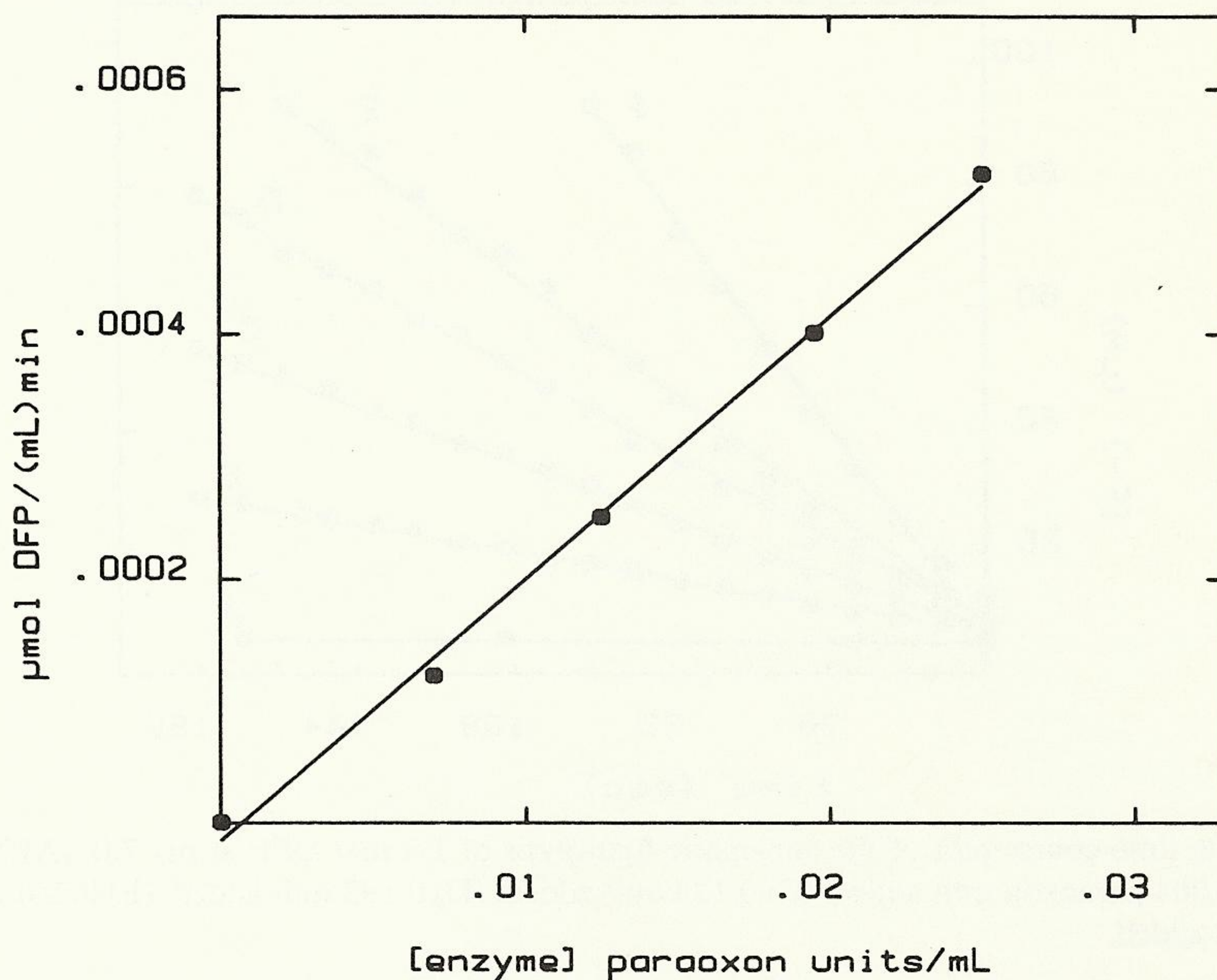
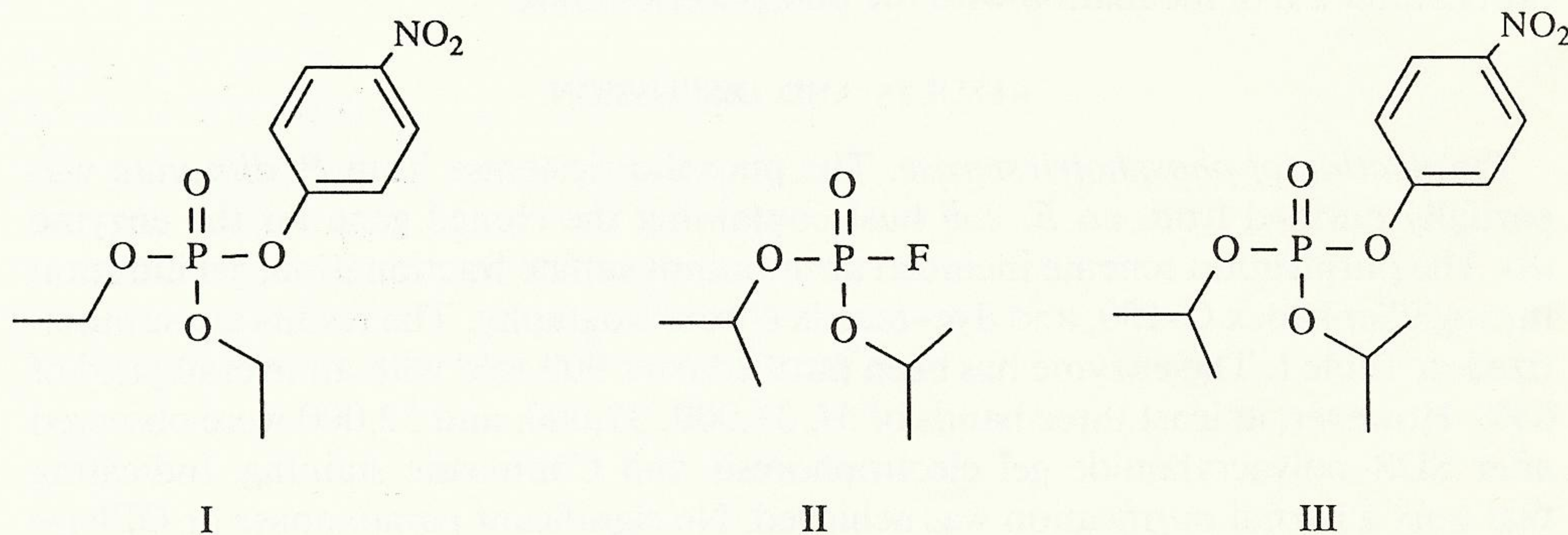


FIG. 4. The rate of DFP hydrolysis by phosphotriesterase is proportional to the rate at which paraoxon hydrolyzed under the same buffer conditions described under Materials and Methods.

phosphotriesterase gene. Therefore, the DFPase activity must be due to the same protein that catalyzes the hydrolysis of paraoxon in the original strain of *P. diminuta*.

Hydrolysis of diisopropylfluorophosphate. The hydrolysis of DFP, as catalyzed by the phosphotriesterase enzyme, was initially detected with ^{19}F and ^{31}P NMR spectroscopy. Shown in Fig. 1A is the ^{19}F NMR spectrum for DFP. The spectrum consists of a simple doublet with a fluorine-phosphorus coupling constant of 970 Hz. After enzymatic hydrolysis is complete, the doublet disappears and is replaced by an upfield singlet that corresponds to the signal for F^- (Fig. 1B). The corresponding ^{31}P NMR spectra are shown in Fig. 2. The ^{31}P NMR spectrum for DFP (Fig. 2A) consists of a doublet due to the phosphorus-fluorine coupling. After the phosphorus-fluorine



SCHEME 1

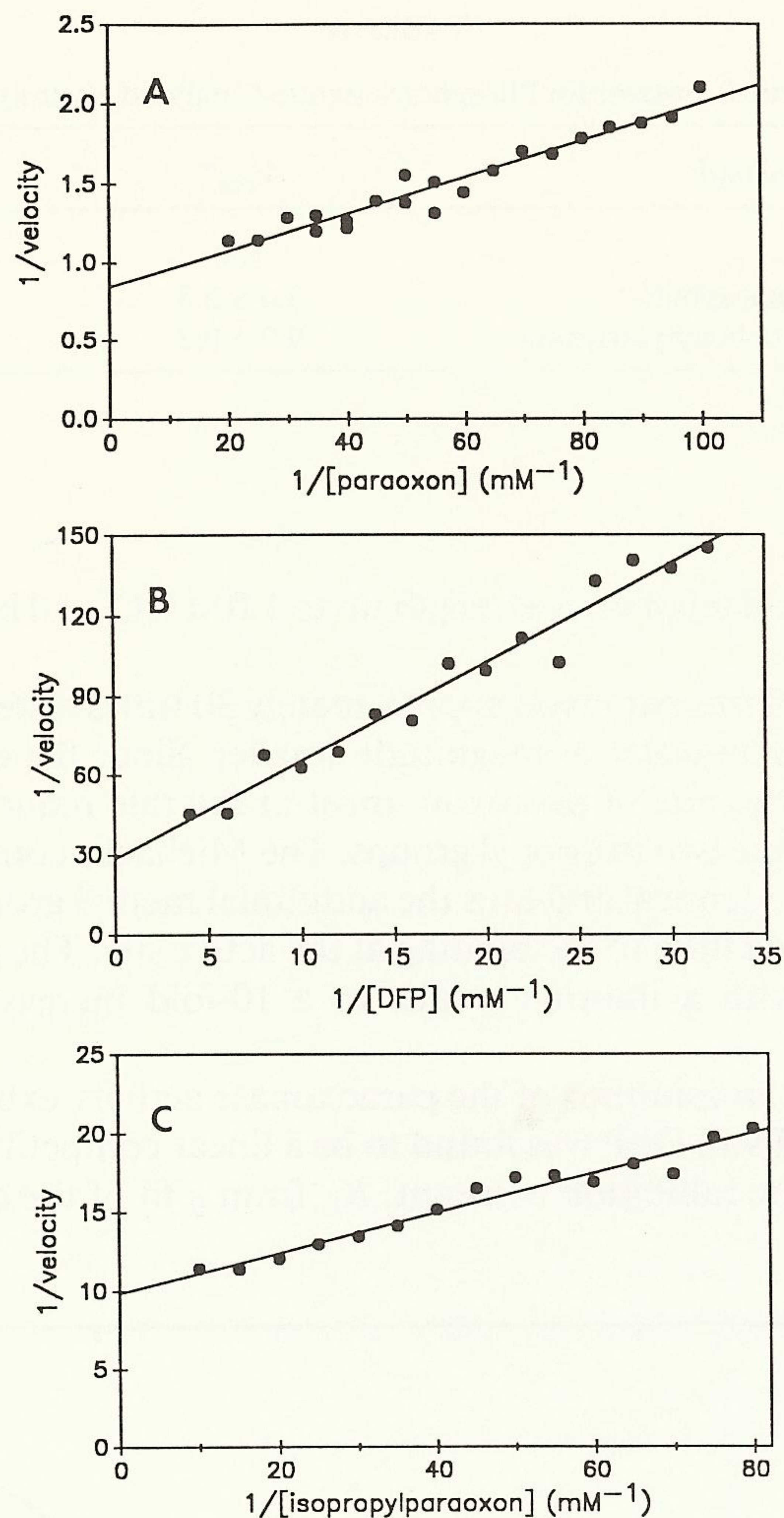


FIG. 5. Double-reciprocal plots for the three substrates: (A) paraoxon, (B) DFP, and (C) DINP. The ordinate is in units of $\text{min} \cdot \text{ml enzyme} / \mu\text{mol substrate}$.

bond is hydrolyzed by the phosphotriesterase, the spectrum consists entirely of a downfield singlet from the diisopropylphosphate. Therefore, the phosphotriesterase from *Pseudomonas* effectively hydrolyzes DFP to F^- and diisopropylphosphate.

Kinetic constants for DFP hydrolysis. A quantitative analysis of DFP hydrolysis, as catalyzed by the phosphotriesterase, was made using an ion-selective electrode. Shown in Fig. 3 are the time courses for the appearance of F^- after the addition of phosphotriesterase to a 1.0 mM solution of DFP at pH 7.0. No significant uncatalyzed hydrolysis occurred within the indicated time period and the rate of F^- production was linear with time for at least 10% of the complete reaction. The rate of hydrolysis was also proportional to the amount of added enzyme (Fig. 4).

The kinetic constants for the phosphotriesterase hydrolysis of paraoxon (I), DFP (II), and DINP (III) were determined at pH 7.0 (see Scheme 1). The double-reciprocal plots are shown in Fig. 5, and the results are summarized in Table II. Phosphotriester-

TABLE II
Kinetic Constants for Phosphotriesterase-Catalyzed Hydrolysis^a

Compound	V_{\max}^b	K_m (mM)
Paraoxon	100	0.012 ± 0.001
Diisopropylfluorophosphate	3.6 ± 0.3	0.12 ± 0.02
Diisopropyl- <i>p</i> -nitrophenylphosphate	9.0 ± 0.2	0.013 ± 0.001

^a Determined at pH 7.0.

^b Relative velocities.

ase activity is independent of ionic strength up to 1.0 M KCl and buffer concentration up to 500 mM.

The enzyme hydrolyzes paraoxon approximately 30 times faster than DFP and the Michaelis constant is an order of magnitude smaller. Since the enzyme also hydrolyzes DINP at 1/10 the rate of paraoxon, most of the rate reduction observed with DFP must be due to the two isopropyl groups. The Michaelis constants for paraoxon and DINP are nearly identical and thus the additional methyl groups in the isopropyl substituents contribute little to the binding at the active site. The replacement of a *p*-nitrophenyl group with a fluorine results in a 10-fold increase in the Michaelis constant.

DFP was tested as an inhibitor of the paraoxonase activity exhibited by the phosphotriesterase. At pH 9.0, DFP was found to be a linear competitive inhibitor versus paraoxon (Fig. 6). The inhibition constant, K_i , from a fit of the data to Eq. [2], was

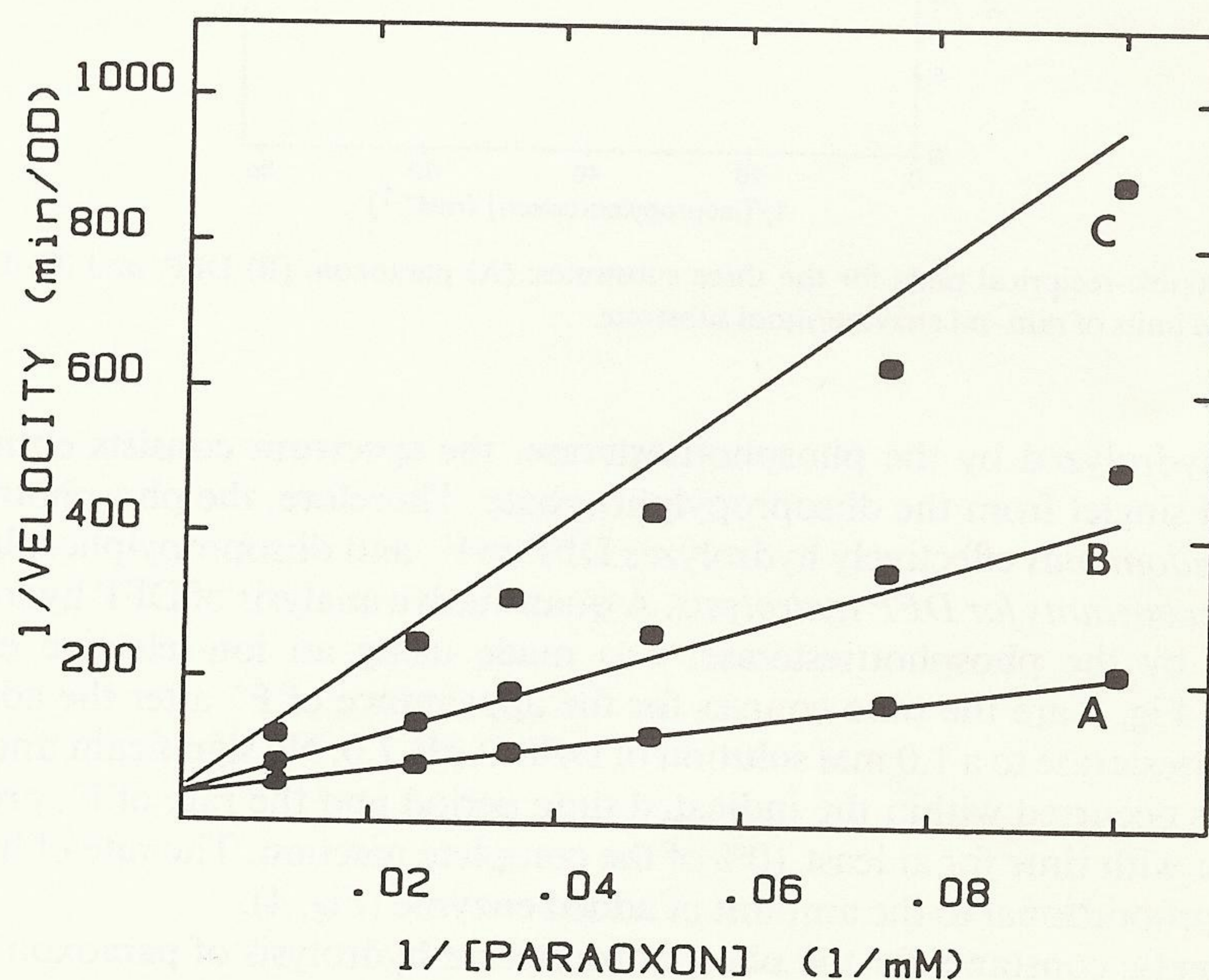


FIG. 6. Competitive inhibition of DFP versus paraoxon at pH 9.0. The DFP concentrations (A) 0 mM, (B) 0.4 mM, and (C) 1.4 mM were used, and the data were fit to Eq. [2].

0.32 ± 0.01 mM. The K_m for paraoxon was determined to be equal to 0.05 mM at pH 9.0. The competitive inhibition pattern strongly suggests that the hydrolyses of paraoxon and DFP are occurring at the same site of a single enzyme.

The phosphotriesterase from *Pseudomonas* hydrolyzes DFP at 3.6% of the maximal paraoxon hydrolysis rate. This report demonstrates the DFP hydrolyzing activity and the paraoxon hydrolyzing activity are due to the same enzyme and not representative of some contaminating DFPase. The hydrolytic cleavage of the P-F bond by this enzyme suggests that this enzyme may be useful in the hydrolysis of a wide variety of highly toxic phosphotriesters.

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