Pyridoxal Phosphate. I. Phosphonic Acid Analogs of Pyridoxal Phosphate. Synthesis *via* Wittig Reactions and Enzymic Evaluation[1]

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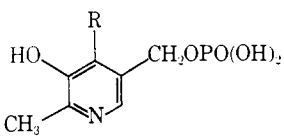
Phosphonic acid analogs (**4**–**7**) of pyridoxal (**1**) and pyridoxol (**2**) phosphates, in which the ester oxygens of **1** and **2** have been replaced by CH_2 (**4** and **5**) and CH (**6** and **7**) units, have been synthesized conveniently through the phosphonate modification of the Wittig reaction. Thus, condensation of tetraethyl methylenediphosphonate (**9**) and α^4,3-O-isopropylideneisopyridoxal (**8**) gave 2-[2,2,8-trimethyl-5-(4H-*m*-dioxino[4,5-*c*]pyridyl)]ethenylphosphonate (**10**), a reaction in which the phosphorus function and the carbon unit designed to replace the ester oxygen of **1** and **2** have been introduced *simultaneously*. The key intermediate **10** was converted through standard reactions into **4**–**7**. Enzymic evaluation of **4**–**7** showed them to possess inhibitory, but not catalytic, properties. On aspartate aminotransferase the ethenylphosphonates **6** and **7** were more inhibitory than the ethylphosphonates **4** and **5** and were approximately as inhibitory as the phosphate monoester, pyridoxol phosphate (**2**). On tyrosine decarboxylase the saturated acids **4** and **5** were the more inhibitory but were much less so than **2**. Thus, in some biological systems, phosphate monoesters may be effectively simulated by phosphonic acid analogs which contain a carbon unit replacing the ester oxygen in the parent phosphate monoester.

The design of analogs of pyridoxal phosphate (**1**, PPal[2]) required functional groups which could adequately simulate the strong enzyme-binding properties[3] of the phosphate monoester portion of PPal[4] (**1**) and of pyridoxol phosphate[5] (**2**, PPol). The phosphonic acid group (**3a**), in which a carbon unit replaces the oxygen of the parent phosphate monoester (**3b**), is an attractive potential simulator of the phosphate monoester function[6,7] and was chosen for initial evaluation.

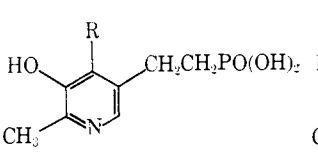


1, R = CHO
2, R = CH_2OH

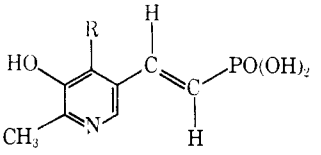
$RXPO(OH)_2$

3a, X = $-CR^1R^2-$, $=C-$, $\equiv C-$
b, X = $-O-$

This paper[8] reports the convenient synthesis and the enzymic evaluation of the phosphonic acid analogs (**4**–**7**) of PPal (**1**) and PPol (**2**), in which the methylene (**4**, **5**) and methine (**6**, **7**) units were designed to replace the ester oxygen of the parent esters **1** and **2**.



4, R = CHO
5, R = CH_2OH



6, R = CHO
7, R = CH_2OH

Chemistry.—Pyridine aldehydes and, in particular, α^4,3-O-isopropylidene isopyridoxal[9] (**8**) smoothly undergo Wittig condensations[10,11] to give α,β-unsaturated esters. In consequence, the key reaction in the synthesis of **4**–**7** was conceived to use tetraethyl methylenediphosphonate[12] (**9**) in the phosphonate modification[13] of the Wittig reaction,[14] to introduce *simultaneously* the potential methylene (methine) and phosphonate groups as a vinyl phosphonate (eq 1). In

$$RCHO + CH_2[PO(OEt)_2]_2 \longrightarrow RCH{=}CHPO(OEt)_2 \quad (1)$$

fact, **9** was known to react with benzaldehyde to give diethyl β-styrylphosphonate (eq 1, R = C_6H_5) in 67% yield.[13b] (In an independent study concurrent with ours, **9** was condensed with a variety of aldehydes and ketones to give diethyl vinylphosphonates.[15])

The synthetic sequences leading to **4**–**7** are shown in Chart I.

The sodium salt of tetraethyl methylenediphosphonate[12] (**9**) in benzene was condensed smoothly with α^4,3-O-isopropylideneisopyridoxal[9] (**8**) at room temperature to give diethyl α^4,3-O-isopropylidene-α^5-

(1) Supported in part by Grant AM-10234 from the U. S. Public Health Service.

(2) Abbreviations: PPal, pyridoxal phosphate; PPol, pyridoxol phosphate; AAT, aspartate aminotransferase; TDC, tyrosine decarboxylase.

(3) For a discussion of modes of binding of phosphate monoesters, see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," John Wiley and Sons, Inc., New York, N. Y., 1967, pp 99–100, and B. R. Baker, P. M. Tanna, and G. D. F. Jackson, *J. Pharm. Sci.*, **54**, 987 (1965).

(4) *E.g.*, H. Wada and E. E. Snell, *J. Biol. Chem.*, **237**, 127 (1962).

(5) A. Meister, H. A. Sober, and E. A. Peterson, *ibid.*, **206**, 89 (1954).

(6) The phosphonic acid function has been considered previously for the simulation of corresponding phosphate monoesters: see, for example, (a) S. Preis, T. C. Myers, and E. V. Jensen, *J. Am. Chem. Soc.*, **77**, 6225 (1955); (b) F. Kagan, R. D. Birkenmeyer, and R. E. Strube, *ibid.*, **81**, 3026 (1959); (c) B. J. Magerlein and F. Kagan, *ibid.*, **82**, 593 (1960); (d) A. F. Rosenthal, *J. Chem. Soc.*, 7345 (1965).

(7) Although the importance of inserting a carbon unit to replace the ester oxygen has been noted,[6c] most phosphonic acid analogs to date have omitted the carbon unit and have been biologically ineffective. See, for example, (a) L. Yengoyan and D. H. Rammler, *Biochemistry*, **5**, 3629 (1966); (b) A. Holy, *Tetrahedron Letters*, 881 (1967); (c) A. Holý, D. Grünberger, J. Smrt, and F. Šorm, *Biochim. Biophys. Acta*, **138**, 207 (1967); (d) D. H. Rammler, L. Yengoyan, A. V. Paul, and P. C. Bax, *Biochemistry*, **6**, 1828 (1967); (e) R. Bennett, A. Burger, and W. W. Umbreit, *J. Med. Pharm. Chem.*, **1**, 213 (1959).

(8) A brief report of part of this work has appeared [T. L. Hullar, *Tetrahedron Letters*, 4921 (1967)].

(9) (a) W. Korytnyk, E. J. Kris, and R. P. Singh, *J. Org. Chem.*, **29**, 571 (1964); (b) H. G. Brooks, Jr., J. W. Lanksa, and D. E. Metzler, *J. Heterocycl. Chem.*, **3**, 126 (1966).

(10) S. Sugasawa and H. Matsuo, *Chem. Pharm. Bull.* (Tokyo), **8**, 815 (1960); *Chem. Abstr.*, **55**, 20901a (1961).

(11) T. L. Hullar, to be published.

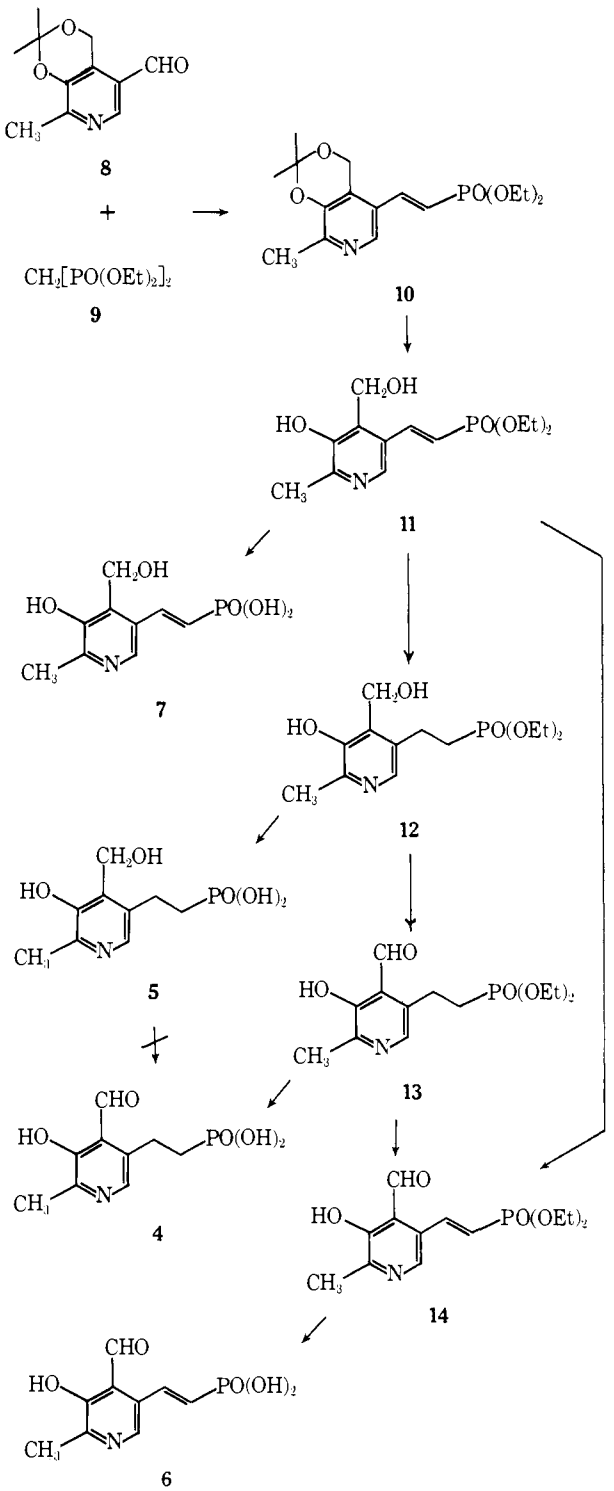
(12) G. M. Kosolapoff, *J. Am. Chem. Soc.*, **75**, 1500 (1953).

(13) (a) L. Horner, H. Hoffmann, W. Klink, H. Ertel, and V. G. Toscano, *Chem. Ber.*, **95**, 581 (1962), and preceding papers; (b) W. S. Wadsworth, Jr., and W. D. Emmons, *J. Am. Chem. Soc.*, **83**, 1733 (1961).

(14) G. Wittig and G. Geissler, *Ann.*, **580**, 44 (1953). For reviews of the Wittig reaction see (a) U. Schöllkopf in "Newer Methods of Preparative Organic Chemistry," Vol. III, W. Foerst, Ed., Academic Press, New York, N. Y., 1964, p 111; (b) H. J. Bestmann, *Angew. Chem. Intern. Ed. Engl.*, **4**, 583, 645, 830 (1965); (c) A. Maercker, *Org. Reactions*, **14**, 270 (1965); (d) S. Trippett, *Quart. Rev.* (London), **17**, 406 (1963); (e) S. Trippett, *Advan. Org. Chem.*, **1**, 83 (1960).

(15) Private communication from Professor C. E. Griffin, University of Pittsburgh; D. C. Wysocki, Ph.D. Thesis, University of Pittsburgh, 1967.

CHART I



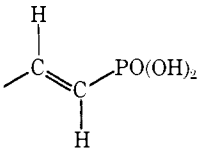
pyridoxylidenemethylphosphonate[16] (**10**) in yields up to 93%. Selective acid hydrolysis of **10**·HCl gave the key intermediate, 2-(3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridyl)ethenylphosphonate hydrochloride (**11**·HCl), in 98% yield.

The phosphonic acid analogs (**5**, **7**) of PPol (**2**) were obtained in 77–84% yield by acidic hydrolysis[17,18] of **12** and **11**, respectively.

To synthesize the saturated phosphonic acid analog (**4**) of PPal (**1**), efforts were first directed at oxidation of acid **5** in a manner analogous to the oxidation of pyridoxol phosphate (**2**) to pyridoxal phosphate (**1**).[19] Methods studied included oxidation by activated MnO_2 in water,[19,20] CrO_3–pyridine,[9a,21] and DMSO–dicyclohexylcarbodiimide.[22] Each attempt failed, presumably due to the susceptibility of the 4-formyl group to overoxidation.

Attention was then turned to a second route. Oxidation of **12** with activated MnO_2[20] in chloroform proceeded smoothly at room temperature to give **13** as yellow syrup which was hydrolyzed in acid to give **4**. The unsaturated analog (**6**) was obtained in a similar manner.

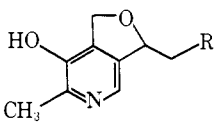
Nmr analysis of the diethyl ethenylphosphonate **10** and of the ethenylphosphonic acid **7** showed both olefinic bonds to possess the *trans* configuration. The olefinic hydrogens in both **7** and **10** had J_{HH} = 17 Hz, a value in the range expected for *trans* olefinic hydrogens.[23] For the vicinal P–H interaction, ester **10** had J_{HP} = 23 Hz and acid **7** had J_{HP} = 20 Hz. These values are in accord with the J_{HP} = ~20 Hz found previously for the vicinal *cis*-PH interaction in *trans*-ethenylphosphonates,[24] and they thus affirm the *trans* configuration of the olefinic bond in **7** and **10**. By contrast, the *trans*-PH interaction, found in *cis*-ethenylphosphonates, had J_{HP} = ~40 Hz.[24] The



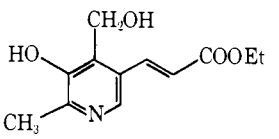
trans stereochemistry was, in fact, expected because the phosphonate modification of the Wittig reaction normally gives a high proportion of *trans* isomer[25] and was the exclusive isomer found in the independent synthesis of vinyl phosphonates.[15]

The uv spectrum of the saturated aldehyde **4** was similar to that[19] of pyridoxal phosphate. The ethenylphosphonates **6** and **7** displayed uv absorption at

product in such a hydrolysis but was not detected. Acidic hydrolysis of ii, however, furnished i (R = COOH) in excellent yield (T. L. Hullar, to be published).



i



ii

(16) The trivial names used for **8** and **10** are consistent with the current nomenclature [see W. Korytnyk, *J. Med. Chem.*, **8**, 112 (1965)] for acetonides of this type. The systematic names are 4-formyl-2,2,8-trimethyl-4H-*m*-dioxino[4,5-*c*]pyridine (**8**) and diethyl 2-[2,2,8-trimethyl-5-(4H-*m*-dioxino-[4,5-*c*]pyridyl)]ethenylphosphonate (**10**).

(17) The bicyclic phosphonic acid i (R = $PO(OH)_2$) is a potential by-

(18) Other hydrolytic methods such as the use of chlorotrimethylsilane [R. M. Rabinowitz, *J. Org. Chem.*, **28**, 2975 (1963)] should be equally efficacious.

(19) (a) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **76**, 169 (1954); (b) E. A. Peterson, H. A. Sober, and A. Meister, *Biochem. Prepn.*, **3**, 34 (1953).

(20) R. M. Evans, *Quart. Rev.* (London), **13**, 61 (1959).

(21) L. F. Fieser and M. Fieser in "Reagents for Organic Synthesis," John Wiley and Sons, Inc., New York, N. Y., 1967, p 145.

(22) (a) K. E. Pfitzner and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 5661, 5670 (1965); (b) W. W. Epstein and F. W. Sweat, *Chem. Rev.*, **67**, 247 (1967).

(23) L. M. Jackman, "Applications of Nuclear Magnetic Resonance in Organic Chemistry," Pergamon Press, London, 1959, p 85; A. A. Bothner-By, *Advan. Magn. Resonance*, **1**, 195 (1965).

(24) (a) W. M. Daniewski, M. Gordon, and C. E. Griffin, *J. Org. Chem.*, **31**, 2083 (1966); (b) G. L. Kenyon and F. Westheimer, *J. Am. Chem. Soc.*, **88**, 3557 (1966).

(25) D. H. Wadsworth, O. E. Schupp, III, E. J. Seus, and J. A. Ford, Jr., *J. Org. Chem.*, **30**, 680 (1965).

higher wavelengths in 0.1 *N* NaOH than did the ethyl phosphonates **4** and **5**. The hydroxymethyl acids **5** and **7** in buffered borate showed the hypsochromic shift characteristic[26] of the 3-hydroxy-4-hydroxymethylpyridine system.

The second ionization constant of the phosphonic acid function was somewhat less for the ethenylphosphonic acids **6** and **7** (pK = 7.1) than for the ethylphosphonic acids **4** and **5** (pK = 7.3–7.6). As expected,[27] these second ionization constants were approximately 1 pK unit greater than the second ionization constant for the phosphate monoester function of PPal (pK = 6.2[28]).

The described method for the synthesis of phosphonic acid analogs of phosphate monoesters has proved convenient and possesses several desirable features. First, the method proceeds in high yield under mild conditions on aldehydes which are readily secured by conventional means. Second, the obtained *trans* double bond can be subjected to a variety of synthetic transformations. Third, the *trans* double bond of ethenylphosphonic acids possesses potentially desirable biological characteristics. The restricted geometry of the double bond, particularly when in conjugation with an aromatic ring, may be useful in establishing enzyme selectivity. In addition, the double bond may possess electronic characteristics which could simulate the electronic nature of the oxygen in the C–O–P ester linkage and/or which could permit interaction of the charged phosphonate anion with the remainder of the molecule (such as with the aromatic ring in **6** and **7**). The difficulty in alkylating[12] **9** represents a minor synthetic limitation in the method.

Additional methods for the synthesis of phosphonic analogs of phosphate monoesters include (a) reaction of the anion of diphenyl methylphosphonate with an alkyl iodide;[29a] (b) reaction of the triphenylphosphorane, $(C_6H_5)_3PCHPO(OC_6H_5)_2$, with an aldehyde;[29b] and (c) a two-step process (eq 2) in which a derivative of the biological alcohol is converted to its next higher homolog[30] which then serves as the starting material for an Arbuzov-type synthesis.

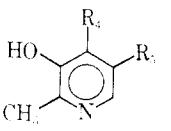
$$RCH_2OH \longrightarrow RCH_2CH_2OH \longrightarrow RCH_2CH_2PO(OR)_2 \quad (2)$$

Enzymic Evaluation.—The synthesized analogs were evaluated on two enzymes, tyrosine decarboxylase apoenzyme (TDC) and aspartate aminotransferase (AAT, EC 2.6.1.1). TDC was chosen initially because it would possibly serve as a convenient prototype for the physiologically important enzyme, aromatic acid decarboxylase,[31] and because it is a conveniently assayed.[32] PPal-dependent enzyme available commercially as the apoenzyme.[33] AAT was chosen for subsequent studies because it is a highly purified and well-characterized PPal-dependent enzyme[34] and consequently might yield detailed information about the analog–enzyme interaction.

The analog–enzyme interaction was studied as follows. *Simultaneous* incubation of PPal and analog with the apoenzyme followed by measurement of remaining enzymatic activity permitted determination of the effect of analog on (presumably) recombination of PPal and apoenzyme and, by inference, on the capacity of the inhibitors to bind to the enzymes. Using the described experimental methods the $([I]/[S])_{0.5}$ value (see Experimental Section) and K_i' value for the analogs on TDC and AAT have been obtained and are collected in Table I; each analog in Table I showed competitive inhibition (see Experimental Section).

TABLE I

INHIBITORY CAPACITY OF PHOSPHONIC ACIDS TOWARD TYROSINE DECARBOXYLASE (TDC) AND ASPARTATE AMINOTRANSFERASE (AAT) COMPOUNDS



			TDC,[a]	AAT[a]	
No.	R_4	R_5	$([I]/[S])_{0.5}$	$([I]/[S])_{0.5}$	$K_i \times 10^6$
2	CH_2OH	$CH_2OPO(OH)_2$	87 ± 15[b]	2.0 ± 0.3[b]	0.7 ± 0.1[b]
4	CH_2OH	$CH_2CH_2PO(OH)_2$	16,700 ± 2400	4.0 ± 0.4	1.7 ± 0.2
6	CH_2OH	$CH{=}CHPO(OH)_2$	35,700 ± 6800	2.2 ± 0.5	1.0 ± 0.3
5	CHO	$CH_2CH_2PO(OH)_2$	386 ± 130	4.4 ± 0.6	2.1 ± 0.5
7	CHO	$CH{=}CHPO(OH)_2$	7900 ± 1800	1.3 ± 0.2	0.54 ± 0.08

[a] For assay details see the Experimental Section. [b] Standard deviation of the mean of three to seven determinations.

Preliminary studies showed the cofactor activity of the saturated (**4**) and unsaturated (**6**) phosphonic acid analogs of pyridoxal phosphate to be ≤7 and ≤2%, respectively, at concentrations five to ten times that required for enzyme saturation by PPal. Consequently, **4** and **6** were evaluated as inhibitors.

It was found that only analogs possessing the free phosphonic acid function were inhibitory to either TDC or AAT (Table I). Thus, the diethyl phosphonate esters **11** and **12**, pyridoxal, and 5-deoxypyridoxal[35] were without observable effect on TDC and showed no inhibition at $[I]/[S] = 100$ on AAT. This agrees with the previous work which showed that only phosphorylated analogs inhibit TDC[36] and that pyridoxal is a very inefficient coenzyme for AAT.[4]

Comparison of the inhibitory compounds (Table I) reveals differences in their inhibitory capacities. First, it is obvious that the phosphonates can effectively inhibit AAT but are largely ineffective on TDC. This lack of inhibition of TDC by the phosphonates (*e.g.*, **5**) relative to the corresponding monoester **2** (PPol) could be due to the following: weaker acidity of phosphonic

(26) J. V. Scudi, W. A. Bastedo, and T. J. Webb, *J. Biol. Chem.*, **136**, 399 (1940).

(27) L. D. Freedman and G. O. Doak, *Chem. Rev.*, **57**, 479 (1957).

(28) V. R. Williams and J. B. Nielands, *Arch. Biochem. Biophys.*, **53**, 56 (1954).

(29) (a) This was the method used in a reported synthesis of 5'-deoxyuridinemethylphosphonic acid [T. C. Myers, U. S. Patent 3,238,191 (1966); *Chem. Abstr.*, **64**, 15972 (19661)]; (b) G. H. Jones and J. G. Moffatt, Abstracts, 155th National Meeting of the American Chemical Society, San Francisco, Calif., April 1968, C40.

(30) Homoribose and 9-(homoribosyl)adenine, which could be used in such a synthesis, have been synthesized: see (a) K. J. Ryan, H. Arzoomanian, E. M. Acton, and L. Goodman, *J. Am. Chem. Soc.*, **86**, 2503 (1964); (b) J. A. Montgomery and K. Hewson, *J. Org. Chem.*, **29**, 3436 (1964); (c) *J. Med. Chem.*, **9**, 234 (1966). For a related synthesis of phosphonic acid analogs of phosphatidic acids, see A. F. Rosenthal, *J. Chem. Soc.*, 7345 (1965).

(31) W. Lovenberg, H. Weissbach, and S. Udenfriend, *J. Biol. Chem.*, **237**, 89 (1962).

(32) I. C. Gunsalus and R. A. Smith, *Methods Enzymol.*, **3**, 963 (1957).

(33) W. D. Bellamy and I. C. Gunsalus, *J. Bacteriol.*, **50**, 95 (1945).

(34) M. Martinez-Carrion, C. Turano, E. Chiancone, F. Bossa, A. Giartosio, F. Riva, and P. Fasella, *J. Biol. Chem.*, **242**, 2397 (1967).

(35) (a) D. Heyl, S. A. Harris, and K. Folkers, *J. Am. Chem. Soc.*, **75**, 653 (1953); (b) P. F. Mühlradt and E. E. Snell, *J. Med. Chem.*, **10**, 120 (1967); (c) T. L. Hullar and D. L. Failla, to be published.

(36) J. Hurwitz, *J. Biol. Chem.*, **217**, 513 (1955).

acids relative to phosphate monoesters and hence a lesser degree of the ionization which might be necessary for good enzyme–PO binding; a strong repulsion, by the enzyme, of the CH_2 unit replacing the ester oxygen; unfavorable geometry of the phosphonic acids; significant electronic differences between the phosphate monoester of **1** and **2** and the phosphonic acid function of **4–7**.

Second, on AAT the unsaturated phosphonates (**6** and **7**) show greater inhibition than do the saturated phosphonates. The reverse is true for TDC. Although reasons for the greater effectiveness of the unsaturated acids on AAT are not clear, the following should be considered in any explanation: (a) the unsaturated phosphonates possess slightly lower pK_a values ($pK_3 = 7.1$) than do the saturated acids ($pK_3 = 7.3–7.6$); (b) the higher electron density in the unsaturated side chain may approximate the unshared electrons of the ester oxygen; (c) the geometry of the *trans* double bond and its likely coplanarity with the pyridine ring may approximate more closely the biologically important conformer of PPal (or PPol) than do the saturated acids; (d) the sum of the covalent bonding radii from the pyridine ring to an acidic oxygen (estimated[37] to be 5.98 Å for the unsaturated phosphonic acids, 6.02 Å for PPal, and 6.26 Å for the saturated acids) suggests that the unsaturated acid can assume at least one conformation which will put the phosphonic acid function in almost the same location, relative to binding groups on the enzyme surface, as the phosphate function of PPal or PPol.

Third, it is clear that for AAT an unsaturated phosphonic acid function (*e.g.*, **6** and **7**) can very nearly simulate the binding capacity of the phosphate monoester side chain of PPal (**1**) of PPol (**2**).

In summary, the diverse manner in which these phosphonic acids simulate the phosphate ester function of pyridoxal phosphate suggests that both saturated and unsaturated phosphonic acids may prove of wide utility as simulators of phosphate monoesters for biochemical and chemotherapeutic purposes.

Experimental Section

Melting points were taken with a Fisher-Johns melting block and those below 230° are corrected. Ir spectra were determined in KBr disks, unless otherwise indicated, with a Perkin-Elmer 237 spectrophotometer; only some of the more characteristic strong bands are noted. Uv spectra were determined with a Perkin-Elmer Model 202 or a Beckman DB-G spectrophotometer. Nmr spectra were determined on a Varian A-60 spectrometer; chemical shifts are reported in hertz downfield from the internal standard; the J values are accurate to ±1.0 Hz; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Elemental analyses were performed by A. Bernhardt, Mülheim, West Germany. Petroleum ether used throughout was a fraction of bp 30–60°. Tlc was done on silica gel G with $CHCl_3$–Me_2CO (4:1, v/v); the compounds were detected by exposing the plates to I_2 vapor, and the relative proportions of the components were estimated visually. Paper chromatography was done in descending form using Whatman no. 1 paper and *t*-BuOH–H_2O–90% HCO_2H (70:15:15, v/v)[19] as eluent. $CHCl_3$ extracts were dried over anhydrous $MgSO_4$. All solutions were concentrated by spin evaporation at 60–70° under reduced pressure (aspirator) unless otherwise indicated. pK_a values were determined in aqueous solution ($\mu \sim 0.05$) at *ca.* 25° with a Radiometer TTT-1 titrator pH-Stat equipped with a SBR-2, SBU-1 recorder; the given values were read directly from the titration curves except where otherwise noted.[38]

Diethyl α^4,3-O-Isopropylidene-α^5-pyridoxylidenemethylphosphonate[16] (**10**). **A.**—To a stirred mixture of NaH (0.265 g of 50% hydride in oil, 5.5 mmoles) in C_6H_6[39a] (10 ml) at room temperature was added tetraethyl methylenediphosphonate[12] (1.58 g, 5.5 mmoles) in C_6H_6 (10 ml). When a clear solution had resulted, α^4,3-O-isopropylideneisopyridoxal[9] (1.045 g, 5 mmoles) in C_6H_6 (10 ml) was added dropwise with stirring at room temperature. A heavy, gummy precipitate formed when about one-half the aldehyde had been added. The gummy mixture was stirred 1 hr at room temperature,[39b] diluted with $CHCl_3$–H_2O (30:15 ml), and the aqueous layer was extracted with $CHCl_3$ (three 20-ml portions). The combined $CHCl_3$ solutions were dried and concentrated to a very light yellow syrup (1.835 g) which crystallized spontaneously on standing overnight at room temperature. Trituration with petroleum ether (three 2-ml portions) gave crude **10** (1.385 g, 81%). Recrystallization of this solid from Et_2O[40]–petroleum ether (2:20 ml) at room temperature, then at −5°, gave very pale yellow plates of **10** (1.100 g, 65%), mp 66–67°, suitable for further transformation. Decolorization of the plates in EtOH followed by crystallization from Et_2O–petroleum ether gave pure **10**: mp 67–68°; ν_{max} 1620, 1590, 1550, 1280, 1250, 1200, 965, and 930 cm^{-1}. *Anal.* ($C_{16}H_{22}NO_5P$) C, H; P: calcd, 9.07; found, 9.54. The nmr spectrum ($CDCl_3$, TMS internal standard) showed 85 (t, OCH_2CH_3, J = 7 Hz), 97 (s, CH_3 of acetonide), 148 (s, 2-pyridyl-CH_3), 256 (apparent quintet, OCH_2CH_3, J = 7 Hz), 313 (s, 4-pyridyl-CH_2), 383 (t (possibly two doublets), -CH=C*H*PO, $J_{HH} = J_{HP} = 17.5$ Hz), 413 (q (two doublets), C*H*=CHPO, $J_{HH} = 17$ Hz, $J_{HP} = 23$ Hz), and 513 Hz (s, 6-pyridyl *H*). The assignments of the ethenyl hydrogens were based on the known, relative chemical shifts of vinylic hydrogens in β-substituted styryl systems[41] (wherein **H** α to the aromatic ring is at lower field than H β to the ring) and by analogy with vinyl phosphonates[24] (wherein H α to P is at higher field relative to H β to phosphonate).

To recover the remainder of **10**, an ethereal solution (20 ml) of the combined, concentrated mother liquors was treated with Et_2O–HCl until no more white precipitate formed. The ice-cooled mixture was filtered and the white solid was air-dried to give **10**·**HCl** as needles (0.500 g, 26%), mp 138–139°, suitable for further transformations. Recrystallization of 0.200 g of **10**·**HCl** twice from EtOH (0.2 ml)-EtOAc (1.0 ml)-Et_2O (6–10 ml) gave pure **10**·**HCl** (75–80% recovery): mp 147–149°, softening at 141°; ν_{max} 2500 (broad), 2275 (broad), 2025 (broad), 1630, 1540, 1380 (d), 1270, 1250, 1220, 1135, 1060–1000, 970, and 950 cm^{-1}. *Anal.* ($C_{16}H_{25}ClNO_5P$) C, H, Cl, N.

B.—To isolate **10**·**HCl** rather than the free base, an ethereal solution (100 ml) of the pale yellow syrup from the above reaction was treated with Et_2O–HCl as above to give white needles of **10**·**HCl** (3.507 g, 93%), mp 138–140°.

Diethyl 2-(3-Hydroxy-4-hydroxymethyl-2-methyl-5-pyridyl)-ethenylphosphonate (**11**).—A solution of **10**·**HCl** (0.500 g) in 10% HCO_2H (2.5 ml) was refluxed 1 hr and then concentrated to a very pale pink syrup. The syrup was decolorized in EtOH to give a colorless syrup which crystallized very readily from EtO**H** to give a colorless syrup which crystallized very readily from EtOH–EtOAc (0.7:8 ml) to give **11**·**HCl** (0.446 g, 98%), mp 124–126°. Recrystallization from the same proportions of solvents gave the analytical sample of **11**·**HCl**: mp 128–130°; ν_{max} 3250, 2900, 2550 (broad), 1625, 1525, 1215, 1065–1015 (broad), and 980–950 cm^{-1} (broad). *Anal.* ($C_{13}H_{21}ClNO_5P$) C, H, Cl, N, P.

To obtain **11**, a solution of **11**·**HCl** in aqueous $NaHCO_3$ was extracted with $CHCl_3$ and the dried extracts were concentrated

(37) Based on bond distances reported in (a) J. D. Dunitz and J. S. Rollett, *Acta Cryst.*, **9**, 327 (1956); (b) J. Kraut and L. H. Jensen, *ibid.*, **16**, 79 (1963); (c) W. L. Kolton, *Biopolymers*, **3**, 665 (1965).

(38) Where analyses are indicated by only symbols of the elements and functions, analytical results obtained for those elements and functions were within ±0.4% of the theoretical values.

(39) (a) Reagent grade benzene was used throughout. C_6H_6 distilled from NaN gave equivalent results. (b) Ir analysis of the mixture showed the aldehyde C=O to be almost absent immediately after the addition of aldehyde and to be absent after 1 hr of stirring. When refluxed for 1 hr, the mixture turned yellow or brown and the product was less readily isolated in pure form. Because of the ready reaction at room temperature, heating the mixture was inadvisable.

(40) The product is very soluble in Et_2O and exceptionally soluble in more polar organic solvents. Et_2O is the solvent of choice, but only a minimum amount must be used to obtain even modest recoveries by recrystallization.

(41) "Varian Spectra Catalog," Vol. 1, Varian Associates, Inc., Palo Alto, Calif., 1962, Spectra 230 and 257.

to a crystalline solid. Recrystallization of the solid from EtOAc–petroleum ether gave pure **11**: mp 97–98°; ν_{max} 3300–2400, 1630, 1600, 1550, and 1230 cm^{-1}. *Anal.* ($C_{13}H_{20}NO_5P$) C, H, N, P.

Diethyl 2-(3-Hydroxy-4-hydroxymethyl-2-methyl-5-pyridyl)-ethylphosphonate (12). A.—A solution of **11**·**HCl** (1.00 g) in EtOH (25 ml) with 5% Pd–C (0.200 g) was shaken with 2.1 kg/cm^2 of H_2 for 1 hr (uptake constant) and then concentrated to a yellow syrup. Saturated aqueous $NaHCO_3$ (10 ml) was added, and the mixture was extracted with $CHCl_3$ (three 10-ml portions). Concentration of the combined extracts gave a pale yellow syrup (0.787 g, 89%) which crystallized spontaneously. The crude product was decolorized in EtOH and then very readily recrystallized from hot EtOAc–Et_2O to give **12** (0.718 g, 81%), mp 106–108°. Further recrystallization of **12** from EtOAc–Et_2O gave 90% recovery of pure **12**: mp 108–109°; ν_{max} 3000 (broad), 2600 (broad), 1250, 1230, 1210, 1050, 1025, 955, 835, and 785 cm^{-1}. *Anal.* ($C_{13}H_{22}NO_5P$) C, H, N, P.

Reduction of the free base **11** gave **12**, mp 105–107°, in 70% yield.

B.—A solution of **10** (0.171 g) in 10% HCO_2H (2 ml) was refluxed 1 hr and then concentrated to a syrup. Hydrogenation of the syrup in EtOH (5 ml) with 5% Pd–C (0.035 g) for 3 hr at 2.1 kg/cm^2 gave, after filtration and concentration, a syrup which crystallized spontaneously. Decolorization in EtOH followed by crystallization from EtOAc–Et_2O gave white needles (0.110 g, 72%) of **12**, mp 105–107°.

C.—A solution of **10** (0.200 g) in EtOH (5 ml) was shaken with H_2 (2.1 kg/cm^2) for 2 hr over 5% Pd–C (0.040 g). A solution of the obtained syrup in 10% HCO_2H (2 ml) was refluxed 0.5 hr and then concentrated to dryness. Crystallization was very slow. Drying *in vacuo* followed by decolorization and crystallization as above gave a slow deposition of **12** (0.109 g 72%), mp 104–106°. The isolation of **12** from this route was more difficult and the product was obviously less pure than in A or B above; consequently this route is not recommended.

2-(3-Hydroxy-4-hydroxymethyl-2-methyl-5-pyridyl)ethylphosphonic Acid (5).—A solution of **12** (5.26 g) in concentrated HCl (55 ml) was refluxed for 12 hr and then concentrated to a yellow syrup. The syrup was dissolved in H_2O (20 ml) and the solution was concentrated to a syrup; this was repeated three times. All attempts to crystallize **5**·**HCl** before or after the reconcentrations were unsuccessful. A solution of the syrup in H_2O (200 ml) was then stirred with Ag_2CO_3 (9.5 g) for 8 hr at room temperature protected from light. The mixture was filtered through Celite, and the Celite was washed with H_2O (three 20-ml portions). The combined clear solutions were percolated through a column (1.1 × 52 cm) of carboxylic acid ion-exchange resin (Rexyn 102, H form, 16–50 mesh) to give a colorless, clear effluent (270 ml, fraction 1). The column was eluted with H_2O to give fractions 2 (100 ml) and 3 (250 ml). Fraction 1 was concentrated (45°) to *ca.* 10 ml at which point crystallization occurred. The mixture was cooled in ice for 2 hr, diluted with EtOH (30 ml), cooled in ice 1 hr, filtered, and washed with EtOH to give short, dense needles of **5** (1.78 g, 42%). Fraction 2 was concentrated to 8 ml and handled as above to give very pale gray needles (0.96 g, 22%) of **5**. Fraction 3 was concentrated to a solid residue (0.30 g) which was suspended in H_2O (*ca.* 1 ml) and handled as above to give more **5** (*ca.* 0.12 g). The mother liquors of fractions 1 and 2 upon concentration and handling as above gave 0.44 g of **5** (total yield of 3.30 g, 77%). Recrystallization was achieved by dissolving **5** (0.105 g) in hot H_2O (3 ml), concentrating the solution to about 0.5 ml to permit crystallization, then handling as above to give 85% recovery of pure **5**: mp >270°; ν_{max} 3200–2000 (little resolution), 1635, 1545, 1345, 1300, 1225, and 1100–900 cm^{-1} (broad); λ_{max} 0.1 *N* HCl, 292 mμ (ε 9300); 0.067 *M* phosphate (pH 7.5), 254 (3300), 324 (8500); 0.06 *M* phosphate–0.01 *M* borate (pH 7.5), 297 (7500); 0.1 *N* NaOH, 242 (7000) and 305 (8400); pK_2 = 5.42, pK_3 = 7.57. *Anal.* ($C_8H_{12}NO_5P$) C, H, N, P.

2-(3-Hydroxy-4-hydroxymethyl-2-methyl-5-pyridyl)ethenylphosphonic Acid (7).—A solution of **11** (1.01 g) in concentrated HCl (12.5 ml) was refluxed 12 hr to give a gummy solid. Trituration with H_2O (*ca.* 20 ml) gave white crystals. The mixture was concentrated to 8 ml, EtOH (20 ml) was added, the mixture was filtered, and the crystals were washed with EtOH–Et_2O to give white needles (0.623 g, 84%). Recrystallization of the needles from hot H_2O or from hot 0.1 *N* HCl followed by dioxane (1 vol) gave, after washing with EtOH then petroleum ether, pure **7**: mp >270°; ν_{max} 3270, 3000, 1635, 1600, 1530, 1250, 1220, 1135, 1110, 1050, and 1015 cm^{-1}; λ_{max} 0.1 *N* HCl, 226 mμ (ε 18,700), 303 (8000); 0.067 *M* phosphate (pH 7.5), 238 (17,300), 334 (6300); 0.07 *M* phosphate–0.01 *M* borate (pH 7.5), 238 (21,200), 310 (7200), 340 (1500); 0.1 *N* NaOH, 233 (20,800) and 325 (6500); nmr absorption (1 *N* NaOD, *t*-BuOH as internal standard), 70 (s, 2-pyridyl-CH_3), 212 (s, 4-pyridyl-CH_2OH), 311 (q, CH=C*H*PO$_3$, J_{HH} = 17 Hz, J_{HP} = 16 Hz), 360 (q, C*H*=CHPO$_3$, J_{HH} = 17 Hz, J_{HP} = 20 Hz), and 390 Hz (s, 6-pyridyl *H*); pK_2 = 5.01, pK_3 = 7.14, and pK_4 = 9.54. *Anal.* ($C_9H_{12}NO_5P$) C, H, N, P.

2-(4-Formyl-3-hydroxy-2-methyl-5-pyridyl)ethylphosphonic Acid (4).—A solution of pure **12** (5.00 g) in $CHCl_3$ (400 ml) was stirred with activated MnO_2[42] (20.0 g) at room temperature for 1.5 hr at which time tlc showed no starting material remaining. The mixture was filtered, the Mn solids were washed with $CHCl_3$ (400 ml), and the bright yellow filtrate was concentrated to a yellow syrup (4.70 g). Attempts to crystallize this syrup or convert it to a crystalline hydrochloride were unsuccessful. If syrupy **13** was kept at room temperature for several days, it gradually discolored to give a dark amber syrup. Consequently a mixture of the syrup in 5.7 *N* HCl (100 ml) was refluxed 12 hr, and the resulting amber solution was concentrated (*ca.* 50°) to a syrup. On standing overnight, yellow crystals formed. The highly soluble crystals and the syrup were applied to a column (3.5 × 105 cm, 1000 ml) of carboxylic acid ion-exchange resin (Amberlite CG-50, H form, 100–200 mesh).

The column was eluted (H_2O) at about 1 ml/min and 20-min fractions were collected. The fractions were analyzed by obtaining their uv spectra in 0.1 *N* HCl and 0.1 *M* K_2CO_3 and plotting the absorptions at 297 and 340 mμ (0.1 *N* HCl) and at 270 and 390 mμ (0.1 *M* K_2CO_3). Fractions 67–104 comprised the major peak and were concentrated (40–45°) to 10 ml. The bright yellow solution was filtered, diluted to 20 ml, and lyophilized to give **4** as a bright yellow solid (1.87 g, 46% from **12**).

To obtain additional pure **4**, fractions 56–65 and 105–114, which contained brown impurities, were concentrated and lyophilized to a dark yellow powder (1.21 g). Column chromatography as above of the powder permitted isolation of fractions 91–103 which after lyophilization furnished additional **4** (0.73 g, 18%; total yield of 64%). The analytical sample, prepared as above, was homogenous by paper chromatography and gave λ_{max} 0.1 *N* HCl, 258 mμ (ε 1750), 295 (6050), 340 (2000); 0.067 *M* phosphate (pH 7.5), 220 (10,000), 327 (2430), 380 (3440); 0.1 *N* NaOH, 232 (11,750), 268 (2750), 301 (1650), and 391 (5260); pK_1 = 2.87, pK_2 = 4.35, pK_3 = 7.35,[43] and pK_4 = 8.75.[43] *Anal.* ($C_9H_{12}NO_5P$) C, H, N, P, C–CH_3.

2-(4-Formyl-3-hydroxy-2-methyl-5-pyridyl)ethenylphosphonic Acid (6).—The free base of **11** (0.780 g) was oxidized, the resulting yellow syrup hydrolyzed, and the hydrolysis solution was processed exactly as described above for **4** to give, after rechromatography, a bright yellow solid (0.062 g, 10% from **11**): λ_{max} 0.1 *N* HCl, 227 mμ (ε 15,200), 258 (8400), 313 (7500); 0.067 *M* phosphate (pH 7.5), 216 (13,700), 244 (13,200), 307 (6300), 380 (900); 0.1 *N* NaOH, 225 (20,200), 296 (5000), and 402 (3550); pK_2 = 4.55, pK_3 = 7.12, and pK_4 = 8.57. *Anal.* ($C_9H_{10}NO_5P$) C, H, N, P.

General Procedures for Enzymatic Evaluation. A. General.—The inhibition data were plotted as v_0/v *vs.* [I], where v_0 = uninhibited velocity, v = inhibited velocity, and [I] = concentration of inhibitor.[44] If several substrate concentrations are employed, competitive inhibitors give a family of straight lines all interesting at v_0/v = 1.[44] From a resulting straight line for a given concentration of substrate, the value of [I] corresponding to 50% inhibition (v_0/v = 2) can be obtained. The value of $([I]/[S])_{0.5}$, the ratio of the concentration of inhibitor necessary for 50% inhibition to concentration of substrate employed,[45,46] can then be calculated.[47]

(42) Beacon Chemical Co. The commercial product uniformly gave satisfactory results.

(43) Calculated from the titration curves by the method of A. A. Noyes [*Z. Physik. Chem.*, **11**, 495 (1893)] as described by A. Albert and E. P. Sergeant, "Ionization Constants of Acids and Bases," John Wiley and Sons, Inc., New York, N. Y., 1962, pp 51–56.

(44) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford University Press, London, 1958, pp 80–87.

(45) It is important to stress the substrate dependency of $([I]/[S])_{0.5}$ values. See, for example, ref 46.

(46) J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. 1, Academic Press, New York, N. Y., 1963, pp 106–108.

(47) B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez, and E. Tong, *J. Med. Chem.*, **2**, 633 (1960).

In the v_0/v *vs.* [I] plot, the slope of a line for a competitive inhibitor is given by eq 3[44] where K_i' = apparent inhibition constant, K_m' = apparent Michaelis constant, and [S] = substrate

$$m = \frac{1/K_i'}{1 + 1/K_m'[S]} \quad (3)$$

concentration used. The K_m' was determined from substrate (PPal) saturation curves (see below in B and C) and corresponds to concentration of PPal necessary to secure $^1/_2V_{max}$. From eq 3 the K_i' was then calculated. The method of Dixon[48] was used sometimes, to obtain independent evaluation of K_i'.

B. Tyrosine Decarboxylase (TDC).—Tyrosine decarboxylase apoenzyme from *Streptococcus faecalis*[32,33] was obtained from Sigma Chemical Co. and from Worthington Biochemical Corp. Attempts to prepare cell-free preparations[49] of TDC apoenzyme from these commercial materials were unsuccessful in that no activity was recovered in the supernatant after the autolysis step.[49b] The enzyme preparation was obtained as follows. A suspension of commercial enzyme (1.6 mg/ml) at room temperature was briefly homogenized manually in a Thomas tissue grinder to give a suspension which could be pipetted conveniently. To the ice-cooled, stirred suspension of enzyme was then added $NaBH_4$ (0.5 mg/ml of final solution) in H_2O (2 ml). Stirring was continued for 1 hr in an ice bath before the enzyme was used for assay.

Decarboxylate activity was determined by the standard manometric assay[32] conducted as follows. All solutions and suspensions, except those of inhibitors, were made with 0.2 *M* acetate buffer (pH 5.5). The side arm of a Warburg flask was charged with 0.03 *M* L-tyrosine (1.0 ml) and the main chamber was charged sequentially with 10^{-6} *M* pyridoxal phosphate (0.1 ml), inhibitor (concentration and amount variable), 0.2 *M* (pH 5.5) acetate buffer to give a final volume (including enzyme) in the main chamber of 2.0 ml, and enzyme suspension (sufficient to give 4–7 μl of CO_2/min). The flask was equilibrated for 30 min at 37°, the tyrosine then was tipped in, and the evolved CO_2 was measured directly in a Gilson differential respirometer at 5-min intervals for 20 min. Preliminary runs showed (a) that the concentration of PPal (5×10^{-8} *M*) employed during incubation in the above assay was below saturation and (b) that 30 min of incubation was sufficient to achieve maximum activity and inhibition. Each concentration of a compound was run in triplicate. The $([I]/[S])_{0.5}$ values were the average of at least five separation determinations. Using the v_0/v *vs.* [I] plot or Lineweaver–Burk plot, PPal and selected inhibitors showed competitive kinetics.

C. Aspartate Aminotransferase (AAT).—Commercial aspartate aminotransferase was obtained from Sigma Chemical Co. and Boehringer Mannheim Corp. The apoenzyme was prepared as follows using a resolution step[50] followed by $NaBH_4$[51] to reduce remaining enzyme–PPal Schiff bases to enzymically inactive secondary amines. To a suspension of AAT (0.2 ml containing enzyme to convert about 500 μmoles of α-ketoglutarate to L-glutamate/min at pH 7.4 at 37°) was added 0.6 *M* (pH 7.4) L-aspartate (0.2 ml) and H_2O (0.4 ml). The resulting pale yellow solution was kept at room temperature for 15 min, treated with 1.0 *M* (pH 4.75) phosphate buffer (0.8 ml), and then kept at 37° for 30 min. The colorless solution was dialyzed successively at 5° against 0.05 *M* (pH 5.4) acetate buffer for 4–6 hr, 0.005 *M* $NaBH_4$[51b] for *ca.* 8 hr, and against 0.50 *M* (pH 5.4) acetate buffer for 8–12 hr. Immediately before use the enzyme solution was diluted with 0.05 *M* (pH 7.4) Tris–HCl buffer to give an activity of *ca.* 10^{-4} moles of oxalacetate formed/l. per min.

The following assay procedure was used routinely and is based on the procedure of Martinez-Carrion, *et al.*[34] Ice-cooled, diluted enzyme (1.00 ml) was added to an ice-cold solution (0.40 ml) of 0.5×10^{-5} *M* PPal and an appropriate concentration of inhibitor; the solution was kept at 0° for 30 min. To aliquots (0.50 ml) in 1-cm cuvettes was then added a 37° solution (2.20 ml) of 0.009 *M* α-ketoglutarate–0.135 *M* phosphate (pH 7.40), and each cuvette was warmed ~15 sec in a 37° water bath. The assay was started by addition of 0.2 *M* (pH 7.40) aspartate (0.10 ml); H_2O (0.10 ml) was added to the second cuvette serving as the blank. The reaction was followed at 257 mμ[52] with a Beckman DB-G spectrophotometer, equipped with temperature control maintained at 37° and a Beckman Model 100502 10-in. recorder using a chart speed of 2 in./min. The initial velocity was measured from the slope of the trace obtained during the first 1–2 min.

Each run used for determining $([I]/[S])_{0.5}$ and K_i included a PPal control to give V_{max}, a PPal control for v_0, and at least four concentrations of inhibitor; duplicate assays of each concentration were run. From five PPal saturation curves, the concentration of PPal necessary to give $^1/_2V_{max}$ was found to be 0.60 ± 0.10[53] $\times 10^{-6}$ *M* and this value is taken as the K_m'. Preliminary runs showed maximal activity was obtained after 15–30 min of PPal–enzyme incubation. Thus in all assays a 30-min incubation time was maintained. Control experiments showed, as found previously,[34] that all recombination of enzyme with PPal ceased when the phosphate was added to the incubation mixture. The inhibitors and PPal showed competitive kinetics using the v_0/v *vs.* [I] plot, the Dixon plot, and the Lineweaver–Burk plot.

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(48) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

(49) (a) J. Hurwitz, *J. Biol. Chem.*, **205**, 935 (1953); (b) H. M. R. Epps, *Biochem. J.*, **38**, 242 (1944).

(50) V. Scardi, P. Scotto, M. Iaccarino, and E. Scarano, *ibid.*, **88**, 172 (1963).

(51) (a) Y. Matsuo and D. M. Greenberg, *J. Biol. Chem.*, **234**, 507 (1958); (b) M. Martinez-Carrion and D. Tiemeier, *Biochemistry*, **6**, 1715 (1967).

(52) This represents the maximum of the difference spectrum of oxaloacetate and α-ketoglutarate [C. P. Henson and W. W. Cleland, *Biochemistry*, **3**, 338 (1964)]. For calculation purposes the respective extinction coefficients were taken as 1090 and 57 [B. E. C. Banks, A. J. Lawrence, E. M. Thain, and C. A. Vernon, *J. Chem. Soc.*, 5799 (1963)].

(53) Standard deviation.