ml) followed by 1:1 benzene–ether (60 ml) served as the eluting solvent, with 10-ml fractions being collected. The gum (10 mg) emerging with the benzene was discarded. Thin-layer chromatography showed that all the benzene—ether fractions contained β-apopicropodophyllin ($R_f$ 0.4) and that the earlier benzene–ether fractions contained a second material as well ($R_f$ 0.51) which was different from α-apopicropodophyllin. Crystallization of the separate materials from the benzene–ether fractions although giving white fluffy needles did not improve the thin-layer chromatographic picture. The single-spot crystalline β-apopicropodophyllin (**8**) obtained from the later fractions showed mp 215–216° (lit.[1] for racemic β-apopicropodophyllin mp 214–215°), gave an infrared absorption spectrum identical with that from optically active β-apopicropodophyllin, and produced an optical rotatory dispersion curve (*c* 0.3, $CH_3OH$, or 0.12, $CHCl_3$) devoid of optical activity from 500 to 310 nm.

The decrease in observed rotation on irradiation was consistent with the presence of no optically active material in the reaction mixture other than levorotatory α-apopicropodophyllin. Thus the optical rotatory dispersion curves for α-apopicropodophyllin (*c* 0.3, 4:1 acetic acid–water plus a trace of HCl) had the same shape before and after irradiation for 0.5 hr, and gave the sample readings below.

The 30-min curve taken directly after irradiation was indistinguishable from that obtained after the mixture had been allowed to stand in the dark for several hours. Likewise, the optical rotatory dispersion curve for the α-apopicropodophyllin solution before irradiation was stable for at least this period.

| Irradn time, | [α], deg | | |
|---|---|---|---|
| min | 500 nm | 384 nm | 350 nm |
| 0 | −42 ± 7 | 0 | +306 ± 5 |
| 30 | −13 ± 6 | 0 | +90 ± 3 |

**Acknowledgment.** We are grateful for support of this work by the National Cancer Institute through Research Grant No. CA 2891 and 10529. Thanks are due to D. S. Stratouly, M. Kobayashi, and Dr. S. Hayashi of JEOLCO, Inc., Medford, Mass., for determining the 100-MHz nuclear magnetic resonance spectrum of one of our compounds, and to Professor H. H. Wotiz and Dr. R. A. Okerholm of the Medical Center, Boston University Medical School, for providing the mass spectral data. An equipment grant (GP 3618) from the National Science Foundation enabled us to acquire the 60-MHz nuclear magnetic resonance spectrometer used in this work. We are also indebted to Dr. A. von Wartburg and his colleagues at Sandoz, Ltd., Basel, for a generous gift of podophyllotoxin.

# Mechanisms of Hydrolysis of Phosphate Ester Derivatives of Phosphoenolpyruvic Acid

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**Abstract:** The hydrolytic mechanisms of dibenzylphosphoenolpyruvic acid (I), benzylphenylphosphonoenolpyruvic acid (II), and monobenzylphosphoenolpyruvic acid (III) involve cyclization by the undissociated carboxyl function to expel primarily the thermodynamically unfavorable leaving group, benzyl alcohol (80–90%). Products arising from enolic oxygen–phosphorus bond cleavage comprise the remaining pathway (10–15%). The pH–rate profiles and product studies in aqueous and hydroxylamine solutions suggest pentacovalent phosphorus intermediacy and the rapid, reversible formation of acyclic acyl phosphate or phosphonate in the reactions of I and II. III also forms acyclic acyl phosphate but apparently not reversibly under the experimental conditions. Phosphoenolpyruvic acid also cyclizes to the corresponding five-membered cyclic phosphate under these conditions. Hydrolysis in $H_2{}^{18}O$ indicates that decomposition of the cyclic acyl intermediates occurs with water attack on phosphorus rather than carbon. A rationale is offered for the product composition resulting from hydrolysis and hydroxylaminolysis for I–III and the unusual catalytic efficiency of the carboxyl function.

We have previously investigated the mechanism of hydrolysis of phosphoenolpyruvic acid.[3,4] In an effort to clarify further the effects of protonation and metal ion chelation on the transfer of the phosphoryl moiety from this substrate, we have undertaken a study of di- and monoesterified phosphate ester derivatives of phosphoenolpyruvate. Moreover, these latter systems appear to be examples of neighboring carboxyl group catalysis of phosphate ester hydrolysis. Clark and Kirby[5] originally had observed that either dimethyl- or diphenylphosphoenolpyruvic acid undergoes hydrolysis with loss of methanol or phenol at an accelerated rate relative to trimethyl or triphenyl phosphate at neutral pH. This phenomenon was attributed to intramolecular nucleophilic attack by carboxyl or carboxylate on phosphorus with displacement of alcohol or alkoxide rather than enol or enolate. Recently a similar phenomenon was noted by Blackburn and Brown[6] in the hydrolysis of diethyl-2-carboxyphenylphosphonic acid. A preliminary communication of the results reported here has appeared.[7]

## Experimental Section

Materials. Dioxane (purified by distillation over sodium), $H_2{}^{18}O$ (5% BioRad), $D_2O$ (99.8% Diaprep), and twice-distilled, deionized water were employed as solvents. Hydroxylamine hydrochloride (Fisher reagent) was recrystallized prior to use.

(1) Predoctoral Fellow of the National Institutes of Health, 1967–1969. Taken from part of the Ph.D. Thesis of K. J. S.
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**Dibenzylphosphoenolpyruvic acid** was synthesized by the method of Cramer and Voges,[8] mp 80.5–83° (lit.[8] mp 75–78°). Sodium monobenzylphosphoenolpyruvate was derived from the dibenzyl ester through reaction with sodium iodide in methyl ethyl ketone.[8]

*Anal.* Calcd for $C_{10}H_{10}PO_6Na \cdot H_2O$: C, 40.27; H, 4.03; P, 10.40. Found: C, 40.33; H, 3.82; P, 10.65.

**Ethyl dibenzylphosphoenolpyruvate** was prepared from ethyl bromopyruvate and tribenzyl phosphite and partially purified by column chromatography (1 × 10 cm) on silica gel G (acetone–methylene chloride, 1:5). Attempted purification by vacuum distillation at *ca.* 170° and 0.5 mm led to explosive decomposition. Although not analytically pure, the following spectral evidence supports our structural assignment: ir 3100–2850 (w), 1740 (s), 1640 (m, sharp), 1450 (m), 1380 (m), 1315–1365 (s), 1190–1150 (s), 1070–950 $cm^{-1}$ (s); nmr ($CCl_4$) $\tau$ 2.75 (singlet, 10 H, phenyl), 4.20 (triplet, 1 H, vinylic proton), 4.52 (triplet, 1 H, vinylic proton), 4.92 (doublet, 4 H from benzylic $CH_2$, $J$ = 15 cps), 5.88 (quartet, 2 H, $OCH_2CH_3$), 8.93 (triplet, 3 H, $-OCH_2CH_3$).

*Anal.* Calcd for $C_{19}H_{21}PO_6$: C, 60.64; H, 5.59. Found: C, 59.14; H, 5.81.

**Benzyl phenylphosphonoenolpyruvic acid** was prepared from bromopyruvic acid and dibenzyl phenyl phosphite using exactly the same procedure as employed in the dibenzylphosphoenolpyruvate synthesis: mp 86–88°; ir 3330–2500 (w), 1740 (s), 1645 (m), 1580 $cm^{-1}$ (w); nmr ($CDCl_3$) $\tau$ 1.8–2.5 (multiplet, 5 H, phenyl), 2.64 (singlet, 5 H, aromatic benzyl), 4.03 (triplet, 1 H, vinylic proton), 4.52 (triplet, 1 H, vinylic proton), 4.67 (doublet, 1.6 H, benzylic $CH_2$, $J$ = 15 cps). This was not obtained in pure form even after recrystallization from ether owing to its rapid hydrolysis to phenylphosphonoenolpyruvic acid as shown by paper chromatography[9] (see section on products) and low carbon analysis.

*Anal.* Calcd for $C_{16}H_{15}O_5P$: C, 60.37; H, 4.71. Found: C, 58.05; H, 4.95.

**Phenylphosphonoenolpyruvic acid** was prepared by dissolution of impure benzylphenylphosphonoenolpyruvic acid in wet chloroform to yield crystals in several days: mp 63–65°; nmr (DMSO-$d_6$) $\tau$ 2–2.55 (5 H, multiplet, phenyl), 4.3 (1 H, triplet, vinylic proton), 4.6 (1 H, triplet, vinylic proton).

**Benzylphenylphosphonic acid** was prepared by alkaline hydrolysis of dibenzylphenylphosphonic acid, mp 56–58° (lit.[10] mp 55–57°).

**Kinetics.** The hydrolytic rates of dibenzylphosphoenolpyruvic acid, monobenzylphosphoenolpyruvic acid, ethyl dibenzylphosphoenolpyruvate, and benzyl phenylphosphonoenolpyruvic acid were obtained by following the release of benzyl alcohol by the method of Kumamoto and Westheimer.[11] Kimax tubes with Teflon-lined caps were utilized in the extraction procedure. Aliquots of the buffered reaction mixture (50:50 v/v dioxane–$H_2O$ or $H_2O$, $\mu$ = 0.2, 35°) which was 7–15 × $10^{-3}$ $M$ in substrate were withdrawn and quenched in the extraction reagents at appropriate time intervals. Buffer systems were those utilized previously.[3] Pseudo-first-order kinetics were observed to at least three half-lives for monobenzylphosphoenolpyruvic acid and benzylphenylphosphonoenolpyruvic acid. Rate constants for the dibenzylphosphoenolpyruvic acid were obtained from initial rate measurements, *i.e.*, plots of $OD_t$ *vs.* time divided by $OD_\infty/2$. $OD_\infty$ represents the loss of 2 mol of benzyl alcohol from the substrate. The difference between the rate of dibenzyl and monobenzyl ester hydrolysis is sufficiently large to permit employment of this method. Owing to partial solubility of unreacted ester in the extraction reagents, particularly at low pH, the observed error in rate constants for the dibenzyl ester ranges from ±16 to 30%. Consequently no exact kinetic analysis was attempted. Similar experimental problems were encountered with the benzyl phenylphosphonoenolpyruvic acid ester which, coupled with the impurity of the sample, forced a limited examination of its kinetics. Observed rates (error ±3–15%) for hydrolysis of monobenzylphosphoenolpyruvic acid were invariant with changing buffer concentration (formate 0.04–0.2 $M$) in the absence of air or light, or in the presence of EDTA. The large error in the determined rate constants for dibenzylphosphoenolpyruvic acid rendered similar checks unfeasible. The pH of all buffers was determined at 35° utilizing a GK 2021B Radiometer electrode. Deuterium oxide buffers were 98% $D_2O$ after correction for hydrogen acids and bases. The solvent isotope effect (pH 3.1, the monoanion being the principal species) was calculated utilizing rates measured in identical $H_2O$ and $D_2O$ buffers. Deuterium oxide effects on buffer $pK_a$'s are similar to substrate $pK_a$'s, thus allowing direct comparison of observed rates. Contribution to the observed rate by hydrolysis of the neutral species at this pH is negligible.

Acyl trapping reactions were carried out in 0.67 $M$ hydroxylamine, 0.1 $M$ buffer solutions ($\mu$ = 0.97) (Figure 2 and Table II). The hydroxylamine hydrochloride was recrystallized prior to each set of runs. Kinetic rates (error ±10%) were determined by withdrawal and development[12] of the hydroxamic acid formed at selected time intervals. Controls with the acetate and succinate buffers were run and, if necessary, any measured OD subtracted from the $OD_t$ determined for the kinetic runs. Pyruvate controls showed no significant hydroxamic acid formation at substrate concentrations. Hydroxylamine was present in large excess so that pseudo-first-order kinetics were observed.

**Dissociation Constants.** The dissociation constants ($\mu$ = 0.2) for dibenzylphosphoenolpyruvic acid and monobenzylphosphoenolpyruvic acid were determined titrimetrically in a Metrohm cell (EA 662) at 25° by the procedure outlined by Albert and Serjeant.[13] The $pK_{a_1}$ of monobenzylphosphoenolpyruvic acid is corrected for the hydrogen ion concentration.[14] All $pK_a$ values are listed in Table I.

**Apparatus.** Instrumentation used in this study has previously been described.[15] All kinetic runs were carried out in Kimax screw-cap tubes with Teflon-lined caps maintained at constant temperature (±0.1°) by a circulating water bath.

**Products.** The hydrolysis of **dibenzylphosphoenolpryuvic acid** (dioxane–$H_2O$, 50:50 v/v, $\mu$ = 0.2, 35°, pH 3.3–4.9) gave (a) benzyl alcohol, extracted[6] and identified quantitatively (85 ± 10%) by uv spectroscopy, (b) monobenzylphosphoenolpyruvic acid, as shown by the equality of its rate of hydrolysis with an authentic sample, and (c) dibenzyl phosphate, identified and determined quantitatively (13 ± 3%) by paper chromatography[16] through comparison to an authentic sample.[17]

**Monobenzylphosphoenolpyruvic acid** hydrolysis ($H_2O$ or 50:50 v/v dioxane–$H_2O$, $\mu$ = 0.2, 35°, pH 3.3–4.9) yielded benzyl alcohol (85 ± 5%), phosphoenolpyruvate, identified by qualitative paper chromatography, and monobenzyl phosphate, identified and quantitatively determined (13 ± 3%) by paper chromatography[16] through comparison to an authentic sample.[11]

**Hydrolysis of benzyl phenylphosphonoenolpyruvic acid** (dioxane–$H_2O$, 50:50 v/v, $\mu$ = 0.2, 35°, pH 3.5) yielded benzyl alcohol (85 ± 10%), phenylphosphonoenolpyruvic acid, which was identified by paper chromatography in two solvents (*n*-propyl alcohol–2 $N$ $NH_4OH$, 7:3, and ethanol–0.1 $M$ $K_2CO_3$, 13:7), and benzylphenylphosphonic acid. The latter was estimated (7 ± 3%) by paper chromatography through comparison of the minimum concentration visualizable as product and an authentic known amount of sample (Schleicher and Schuell orange ribbon 589c, *n*-propyl alcohol–2 $N$ $NH_4OH$, 7:3). A possible ambiguity arises in that benzylphenylphosphonoenolpyruvic acid and benzyl phenylphosphonic acid chromatograph with similar $R_f$ values in all basic solvents investigated on paper, silica gel G and aluminum oxide thin-layer chromatography, and paper electrophoresis. Thus the possibility exists that the small amount of benzylphenylphosphonic acid observed as a product may have been present initially. We do not, however, believe this to be the case. There is no reasonable synthetic pathway from starting materials or possible impurities for formation of benzylphenylphosphonic acid or any monoacidic product capable of chromatographing similarly. Also, repeated crystallizations from ether, although not yielding starting material free of phenylphosphonoenolpyruvic acid, might reasonably be expected to exclude the more ether-soluble benzylphenylphosphonic acid.

In the presence of 0.67 $M$ hydroxylamine, pH 5.1, **dibenzylphosphoenolpyruvic acid** yielded dibenzyl phosphate, isolated as dibenzyl hydrogen phosphate (67%) after acidification, extraction

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Table I. Rate Constants and Dissociation Constants of I, II, and III[a]

| Compd | $k_H^+ \times 10^2$ $M^{-1}$ min$^{-1}$ | $k_1 \times 10^3$ min$^{-1}$ | $k_2 \times 10^3$ min$^{-1}$ | p$K_{a1}$, $M^{-1}$ | p$K_{a2}$, $M^{-1}$ | Solvent |
|---|---|---|---|---|---|---|
| I | | 19 ± 3 | | | 4.35 ± 0.05 | Dioxane-$H_2O$[b] |
| II | | 230 | | | 4.35[c] | Dioxane-$H_2O$[b] |
| III | 2.36 ± 0.2 | 6.6 ± 1 | 1.8 ± 0.2[d] | 1.8 ± 0.2 | 3.73 ± 0.1 | $H_2O$, $\mu$ = 0.2 |
| | | | 0.8 | 2.50 ± 0.1 | 4.97 ± 0.06 | Dioxane-$H_2O$[b] |
| | | | 1.0 ± 0.2 | 1.9 ± 0.2[e] | 3.83 ± 0.1[e] | $D_2O$, $\mu$ = 0.2 |

[a] Rate constants were determined at 35°, dissociation constants at 25°. [b] 50:50 v/v, $\mu$ = 0.2. [c] Assumed value in analogy to p$K_{a2}$ of I. [d] Value employed in eq 2 was 1.6. [e] p$K_{a1}$ and p$K_{a2}$ = p$K_a$($H_2O$) + 0.1.

with diethyl ether, and crystallization from ether, mp 78–79° (lit.[17] mp 78°), and pyruvic acid hydroxamate. The latter was isolated as the oxime by evaporation of the above aqueous layer to dryness and dissolution of the residue in methanol, followed by precipitation of the inorganic salts by ether. Evaporation of the filtrate yielded the oxime hydroxamate, recrystallized from ether, mp 152–154° (lit. mp 143°,[18] 150–160°,[19] and 161°[20]); the ir spectrum was identical with an authentic sample. No benzyl alcohol (0 ± 5%) was detected.

In 0.67 *M* hydroxylamine solution, pH 5.1, **monobenzylphosphoenolpyruvic acid** yielded benzyl alcohol (as above) and pyruvic acid oxime hydroxamate, shown to be chromatographically identical in two solvent systems with an authentic sample (amyl alcohol–acetic acid–$H_2O$, 4:1:5, and octyl alcohol–formic acid–$H_2O$, 3:1:3). The latter presumably arises from phosphoenolpyruvate hydroxamate.

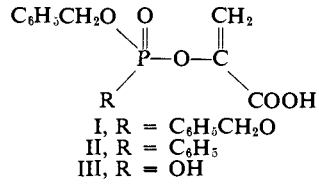
**Benzylphenylphosphonoenolpyruvic acid** in 0.67 *M* hydroxylamine solution, pH 4.9–6.0, gave benzyl phenylphosphonate isolated by acidification of the reaction solution, extraction with ether, and crystallization from benzene, mp 56–58° (lit.[10] mp 55–57°). No benzyl alcohol (0 ± 5%) was detected.

**$^{18}O$ Tracer Experiments.** Dibenzylphosphoenolpyruvic acid was hydrolyzed to completion (*i.e.*, cleavage of 2 mol of benzyl alcohol) in 0.96% $^{18}O$-enriched water (pH 2.2, $\mu$ = 0.2, 35°). The solution was evaporated to dryness and the residue (mainly phosphoenolpyruvic acid) redissolved in isotopically normal water (5 ml) and hydrolyzed with alkaline phosphatase[21] (0.6 mg). The isolated magnesium ammonium phosphate was converted to potassium dihydrogen phosphate by the method of Haake and Westheimer[22] and analyzed for $^{18}O$ content by conversion to carbon dioxide according to the procedure of Boyer, *et al.*[23] Pyruvic acid was isolated—after removal of the $MgNH_4PO_4$—by dropwise addition of $AgNO_3$ to precipitate silver pyruvate. Enzyme contamination was kept minimal by employing small enzyme concentrations and isolation of only the first formed precipitate. Pyrolytic decarboxylation yielded carbon dioxide, isolated by the method described in ref 23. Phosphoenolpyruvic acid[24] was hydrolyzed to completion in isotopically enriched buffer solution (pH 2.9, 75°) where the concentration of the monoanion species is maximal. The phosphate released was isolated and analyzed as for the dibenzyl ester.

Relative isotopic abundances occurring in the carbon dioxide were determined on an MS 902 AEI mass spectrometer by measuring peak heights directly from the instrument's collector. Controls with tank carbon dioxide were run as standard before all determinations.

## Results

**Hydrolysis.** The pH–rate profiles for the loss of 1 mol of benzyl alcohol during the hydrolysis of dibenzylphosphoenolpyruvic acid (I), benzylphenylphosphonoenolpyruvic acid (II), and monobenzylphosphoenolpyruvic acid (III) are shown in Figure 1. The solid




I, R = $C_6H_5CH_2O$
II, R = $C_6H_5$
III, R = OH

line for I and II was calculated from

$$k_{obsd} = \frac{k_1 a_H}{a_H + K_{a2}} \quad (1)$$

where $k_1$ is the first-order rate constant for hydrolysis of the neutral species, $K_{a2}$ is the dissociation constant of the carboxyl function of I or II, and $a_H$ is the hydrogen ion activity as measured by the glass electrode. The profiles for I and II suggest that $k_{obsd}$ for loss of benzyl alcohol from the anionic species is at least two orders of magnitude less than loss of benzyl alcohol from the neutral species and that the former, I, is not subject to an acid-catalyzed reaction in the pH range investigated.

The solid line for III was calculated from

$$k_{obsd} = \frac{k_{H^+} a_H^3 + k_1 a_H^2 + k_2 K_{a1} a_H}{a_H^2 + K_{a1} a_H + K_{a1} K_{a2}} \quad (2)$$

assuming all species to be hydrolytically reactive except the dianion, where $k_{H^+}$ is the second-order rate constant associated with hydronium ion catalyzed hydrolysis of the neutral species and $k_1$ and $k_2$ are first-order rate constants for hydrolysis of the neutral and monoanion species, respectively. The dissociation constants $K_{a1}$ and $K_{a2}$ refer in order to the ionization of the phosphate and carboxyl functions of III. The values of the rate constants and dissociation constants utilized in eq 1 and 2 appear in Table I.

Inspection of Table I reveals that the neutral species of I, II, and III have relative rate constants in the ratio of 3:36:1. The neutral species of III is in turn three times more reactive than its monoanion containing the ionized phosphate moiety. For comparison the value of $k_{obsd}$ for hydrolysis of ethyl dibenzylphosphoenolpyruvate where carboxyl group participation is precluded is approximately 150-fold less than $k_{obsd}$ for I at 35° (extrapolated from data at 75°, pH 5.3, assuming $E_a$ = 20 kcal/mol). The greater rate of hydrolysis of the phosphonate II is in accord with previous results.[25] The activation parameters for hydrolysis of the monoanion III were found to be $\Delta H^{\pm}$ = 19.9 kcal/mol and $\Delta S^{\pm}$ = −16 eu in water and $\Delta H^{\pm}$ = 20.8 kcal/mol and $\Delta S^{\pm}$ = −15.4 eu in dioxane-$H_2O$, 50:50 v/v.

Investigation of the hydrolytic products reveals that although benzyl alcohol is the major product in the hydrolysis of I, II, and III (80–90% in each case), I, II, and

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III cleave at the enolic oxygen–phosphorus bond to yield 10–15% dibenzyl phosphate, 5–10% benzyl phenylphosphonate, and 10–15% benzyl phosphate (Table II). All product isolation runs were carried out at pH 3.3–4.9 in 50:50 v/v dioxane–$H_2O$.

Table II. Solvolysis Products

| Compd | Conditions[a] | Products |
|---|---|---|
| I | 50:50 v/v dioxane–$H_2O$ pH 3.3–4.9, $\mu$ = 0.2 | Benzyl alcohol (85 ± 10%)<br>Monobenzylphosphoenolpyruvic acid<br>Dibenzyl phosphate (13 ± 3%) |
| | 50:50 v/v dioxane–$H_2O$ 0.67 *M* $NH_2OH$,[b] pH 5.1 | Benzyl alcohol (0 ± 5%)<br>Dibenzyl phosphate (67%)<br>Pyruvic acid hydroxamate[c] |
| II | 50:50 v/v dioxane–$H_2O$ pH 3.5, $\mu$ = 0.2 | Benzyl alcohol (85 ± 10%)<br>Phenylphosphonoenolpyruvic acid<br>Benzyl phenylphosphonate (7 ± 3%) |
| | 50:50 v/v dioxane–$H_2O$ 0.67 *M* $NH_2OH$,[b] pH 6.0 | Benzyl alcohol (0 ± 5%)<br>Benzyl phenylphosphonate |
| III | 50:50 v/v dioxane–$H_2O$ pH 5.1, $\mu$ = 0.2 | Benzyl alcohol (85 ± 5%)<br>Phosphoenolpyruvic acid |
| | $H_2O$, pH 3.3–4.9, $\mu$ = 0.2 | Monobenzyl phosphate (13 ± 3%) |
| | 50:50 v/v dioxane–$H_2O$ 0.67 *M* $NH_2OH$,[b] pH 5.1 | Benzyl alcohol (85 ± 5%)<br>Pyruvic acid hydroxamate[c] |

[a] 35°. [b] Stoichiometric concentration. [c] As the oxime.

The results of the total hydrolysis of dibenzyl phosphoenolpyruvate to phosphoenolpyruvate in isotopically enriched water are given in Table III. The data

Table III. $^{18}O$ Tracer Studies in the Hydrolysis of Dibenzylphosphoenolpyruvic Acid[a]

| Compd | $^{18}O$ % excess in $H_2O$ | $^{18}O$ % excess in $KH_2PO_4$ | Atoms of $^{18}O$ incorporated |
|---|---|---|---|
| I | 1.60 | 1.003 | 2.51[b] |
| Pyruvate | 1.60 | 0.35 (in -COOH) | 0.44 |
| I | 1.60 | 0.185 (in -COOH) | 0.23[c] |

[a] Carried out at pH 2.2 in 50:50 v/v dioxane–$H_2O$. [b] Theoretical incorporation for two sequences of ring closure and opening at phosphorus is 1.66. [c] Theoretical incorporation for two sequences of ring closure and opening at acyl carbon is 1.5.

show that the carboxyl group of I incorporated little or no $^{18}O$ during the hydrolytic cleavage of both benzyl groups. The carboxyl moiety of the control compound, pyruvic acid, is enriched in $^{18}O$ to a greater extent than I through spontaneous exchange.[26] Although comparison to this control is not precisely valid owing to structural differences, if one allows no spontaneous carboxyl exchange in I, then incorporation amounts to only 0.23 atom in the course of the overall hydrolysis.

The results of the hydrolysis of phosphoenolpyruvic acid are shown in Table IV. In addition to the expected incorporation of one atom of $^{18}O$ due to hydrolytic phosphorus–oxygen bond scission, approximately 1.3 additional atoms are incorporated into the

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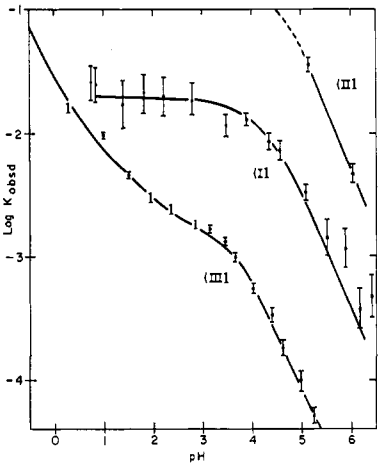



Figure 1. The pH–rate profiles for hydrolysis of dibenzylphosphoenolpyruvic acid (I), benzylphenylphosphonoenolpyruvic acid (II), and monobenzylphosphoenolpyruvic acid (III) monitoring benzyl alcohol release. Dimensions of bars indicate errors. The solid curves are theoretical (eq 1 and 2): $T$ = 35°, $\mu$ = 0.2, dioxane–$H_2O$, 50:50 v/v (I and II), and $H_2O$ (III) solvents.

phosphoryl moiety prior to hydrolysis. A control experiment revealed no $^{18}O$ incorporation into inorganic phosphate under these conditions.

Table IV. $^{18}O$ Exchange Studies in the Hydrolysis of Phosphoenolpyruvate

| Compd | $^{18}O$ % excess in $H_2O$ | $^{18}O$ % excess in $P_i$ | Atoms of $^{18}O$ incorporated[a] |
|---|---|---|---|
| $P_i$ | 0.96 | −0.008 to 0.002 | 0 |
| Phosphoenolpyruvate | 0.96 | 0.559 | 2.33 |
| | 1.31 | 0.758 | 2.31 |

[a] Simple phosphorus–oxygen bond cleavage hydrolysis would yield one atom $^{18}O$ incorporated.

In hydroxylamine solution the reactions of I and II yield radically different product distributions than in purely aqueous media, yielding 98 ± 2% dibenzyl phosphate and benzyl phenyl phosphonate, respectively, and the oxime of pyruvic acid hydroxamate (Table II). However, in the hydroxylamine reaction of III, benzyl alcohol remains the primary product with the apparent initial formation of phosphoenolpyruvate hydroxamate.

Investigation of the rate of hydroxamate formation in the case of I revealed that the kinetic rate law changes from a first-order dependence on hydroxylamine concentration to zero order with increasing reagent concentration. Replotting $k_{obsd}$ as a function of hydroxylamine concentration in reciprocal form (Figure 2) yields at the ordinate the maximum rate at saturating hydroxylamine concentration. At pH 7.5 this value is 6.6 $min^{-1}$, which is some 350-fold more rapid than the hydrolysis reaction. The rate constant for hydroxylaminolysis of II is equal to that of I and is thus some 30-fold more rapid than its hydrolysis. The rate of hydroxylaminolysis of III at pH 3.7 and 5.0, dioxane–$H_2O$, 50:50 v/v, is identical with that of hydrolysis under the same conditions.

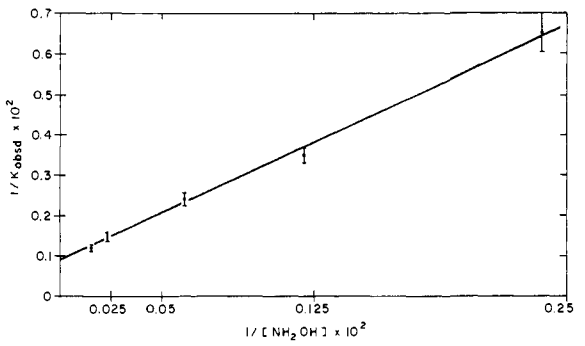



Figure 2. Double reciprocal plot of $1/k_{obsd}$ *vs.* 1/hydroxylamine concentration in the reaction of dibenzylphosphoenolpyruvic acid at pH 5.3.

## Discussion

The rate constant for loss of benzyl alcohol *via* P–O bond fission from I is approximately 150 times more rapid than generation of benzyl alcohol from IV in which the carboxyl moiety is esterified. This alone is strongly indicative of a catalytic role for the carboxyl group in the hydrolysis of I. Since trimethyl phosphate,[27] dimethyl phosphate,[28] and apparently dibenzyl phosphate[11] hydrolyze *via* C–O rather than P–O bond fission hydrolysis of the reference ester, ethyl dibenzyl phosphoenolpyruvate, most likely proceeds mainly *via* C–O bond fission in weakly acidic media with nucleophilic displacement by water on the benzylic carbon rather than attack at phosphorus. Thus, the above factor is a minimal estimate of the catalytic effectiveness of the carboxyl group. Secondly, inspection of the pH–rate profile of I reveals catalytic participation by the protonated carboxyl (or its kinetic equivalent) in the hydrolysis of the neutral species. That of III reveals that the monoanion and neutral species are hydrolytically labile also owing to catalysis by a carboxyl function. In addition, at low pH an acid-catalyzed rate typical of enol phosphates is observed.[29]

The carboxyl group can function as a catalyst by one of two general mechanisms: (1) general acid catalysis of the loss of benzyl alcohol which may or may not be concerted with the attack of water on phosphorus;[30,31] (2) nucleophilic attack by the carboxylate moiety on phosphorus, with or without concomitant proton transfer resulting in expulsion of benzyl alcohol or enol followed by hydrolysis of the cyclic or acyclic acyl phosphate intermediate.[32] The latter is clearly correct as evidenced by the formation of hydroxamate products in the reaction of I, II, and III which demands formation of activated acyl groups. The observation of minor but significant amounts of dibenzyl phosphate, benzyl phenyl phosphonate, and monobenzyl phosphate as well as the predominant product, benzyl alcohol, in the hydrolysis of I, II, and III, respectively, suggests the possibility of at least *two* different acyl phosphate species.

(27) P. W. C. Barnard, C. A. Bunton, D. R. Llewellyn, C. A. Vernon, and V. A. Welch, *J. Chem. Soc.*, 2670 (1961).

(28) C. A. Bunton, M. M. Mhala, K. G. Oldham, and C. A. Vernon, *ibid.*, 3293 (1960).

(29) Presumably at lower pH's than investigated, I and II also would reveal an acid-catalyzed rate as is observed with other enol phosphates.

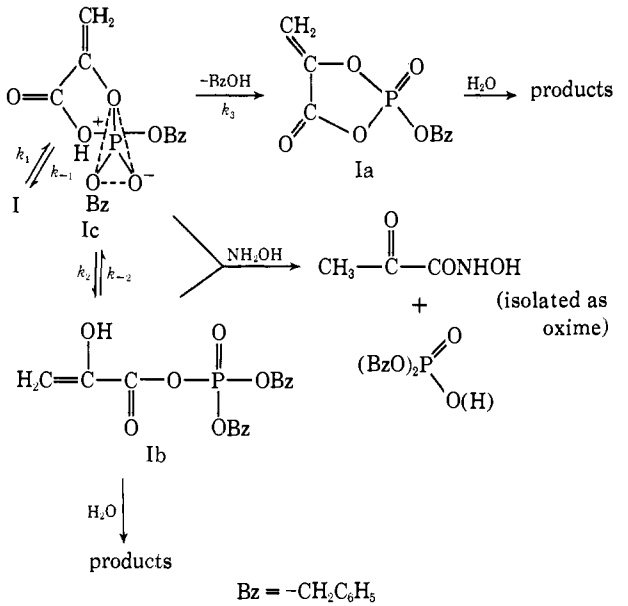
(30) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. II, W. A. Benjamin, New York, N. Y., 1966.

(31) A. J. Kirby and S. G. Warren, "The Organic Chemistry of Phosphorus," Elsevier, Amsterdam, 1967, p 324.

(32) V. M. Clark and A. J. Kirby, *J. Amer. Chem. Soc.*, 85, 3705 (1963).

In I and II the change of products from predominantly benzyl alcohol to entirely dibenzyl phosphate and benzyl phenyl phosphonate, respectively, in 0.67 *M* hydroxylamine solution infers, however, that hydroxamate formation cannot simply be trapping of cyclic tetravalent phosphorus as this requires prior release of benzyl alcohol which is not observed. The required prior species can reasonably be either the cyclic pentacovalent intermediate Ic[33] or the acyl phosphate Ib, provided in the latter case that the enol formation step in the absence of hydroxylamine is reversible. These and the above facts are incorporated into the mechanism shown only for I in Scheme I.

Scheme I




In terms of Scheme I the observed products in the presence of hydroxylamine may arise in the following manner. Hydroxylamine attack on the acyl carbon of Ic would generate an acyclic pentacovalent phosphorus which is anticipated to rapidly decompose through loss of the thermodynamically favored enol[34] rather than benzyl alcohol yielding the observed products. On the other hand, hydroxylaminolysis of Ib would expel dibenzyl phosphate directly.[35] The fact that in aqueous solution only a small fraction of the total product is dibenzyl phosphate would be a consequence of the ratio of hydroxylaminolysis to hydrolysis for acyl esters, *i.e.*, Ib, with excellent leaving groups.[36,37] Efficient trapping by hydroxylamine of Ib in effect causes the step characterized by $k_2$ or an earlier one to become rate determining. In either case interception of Ic or Ib by hydroxylamine alters the product composition only if the rate of hydroxylaminolysis is at least *ca.* 20-fold greater than the loss of benzyl alcohol. Both explana-

(33) We have envisioned initial formation of the zwitterionic species in line with recent calculations indicating that this is a more stable form than the neutral species. Pseudorotation probably cannot occur through such a zwitterionic form, however. We at present have no evidence to clarify which of the several possible pathways is actually utilized in forming this species.

(34) P. Ballinger and F. A. Long, *J. Amer. Chem. Soc.*, 82, 795 (1960).

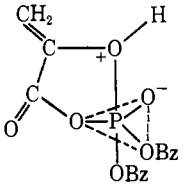
(35) G. DiSabato and W. P. Jencks, *ibid.*, 83, 4393 (1961).

(36) Calculation of the ratio of rate constants for hydroxylaminolysis to hydrolysis of acyl phosphate based on product composition is $10^8$–$10^9$ and is similar to the ratio of rate constants for the identical nucleophilic reactions of 2,4-dinitrophenyl acetate ($10^8$).[37]

(37) W. P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, 90, 2622 (1968).

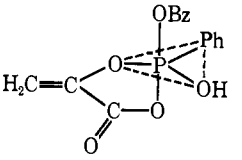
tions would satisfy the observed saturation kinetics with increasing hydroxylamine solution since in either case rate steps are available prior to trapping to become rate determining. Two questions thus remain: (1) is the hydroxylamine reaction the result of interception of acyl phosphate Ib or the pentacovalent intermediate Ic, and (2) if the acyl phosphate is being trapped, is a pentacovalent intermediate required mechanistically?

Let us consider the latter question first. A considerable accumulation of data has developed concerning the formation and chemistry of pentacovalent phosphorus.[38] In particular, five-membered cyclic phosphates are thought to form pentacovalent species as metastable intermediates similar to Ic in their hydrolyses.[39] An alternative formulation of Ic is its existence merely as a transition state structure. The latter postulate, however, would require an apical–equatorial displacement (accepting the preference rules) of enolic oxygen to account for acyclic phosphate formation. This process requires an activation energy of at least 16 kcal/mol above that of apical–apical displacement.[40-43] Moreover, if one argues that the time required for pseudorotation is greater than the lifetime of the transition state between I and Ib, then Ic is necessary as an intermediate which pseudorotates to form Id (below).[43-45] The latter can then expel enolic oxygen from an apical position as suggested by the preference rules.[39] Note that in order to expel the enol, it is necessary to postulate a proton transfer at some stage in the process from the attacking carboxyl to the leaving enolic oxygen.



Id

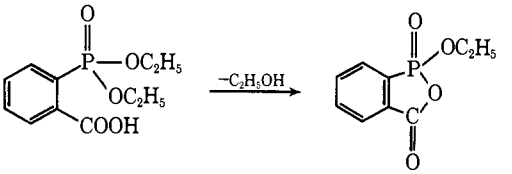
The observation that II follows an identical reaction course to I, both in the presence and absence of hydroxylamine, suggests that any generated pentacovalent intermediates must freely pseudorotate. Although the presence of the phosphorus–carbon bond lowers the number of attainable pentacovalent species because of the increased free energy required to locate an aryl group apical (Muetterties rule) intermediates such as IIe must be readily accessible. There apparently is no large difference in free energy between IIe and a zwitterion pentacovalent intermediate which features a protonated apical position with the phosphoryl oxygen ionized. The latter would not be expected to pseudorotate to species resulting in acyclic acyl phosphate formation. The data thus imply that the energy difference between the zwitterion and neutral form is not as large as that calculated.[46] In short, the inclusion of a



IIe

pentacovalent species in Scheme I appears justified.

A possible means of resolving question 1 is to study the rate of hydroxylaminolysis of a similar system which is incapable of forming acyclic acyl phosphate. The hydrolysis of diethyl 2-carboxyphenyl phosphonate proceeds through carboxyl group cyclization with expulsion of ethanol. The cyclic phosphonate may be intercepted with hydroxylamine to yield the corresponding hydroxamate. The rate of hydroxylaminolysis is approximately the same as ethanol loss; thus, it appears that the cyclic phosphonate and not a precursor pentacovalent species is being trapped.[47] Insofar as the analogy is appropriate, one would thus argue that in the present case (I and II) hydroxylamine is trapping an acyclic acyl phosphate like Ib albeit at a rate faster than benzyl alcohol loss.



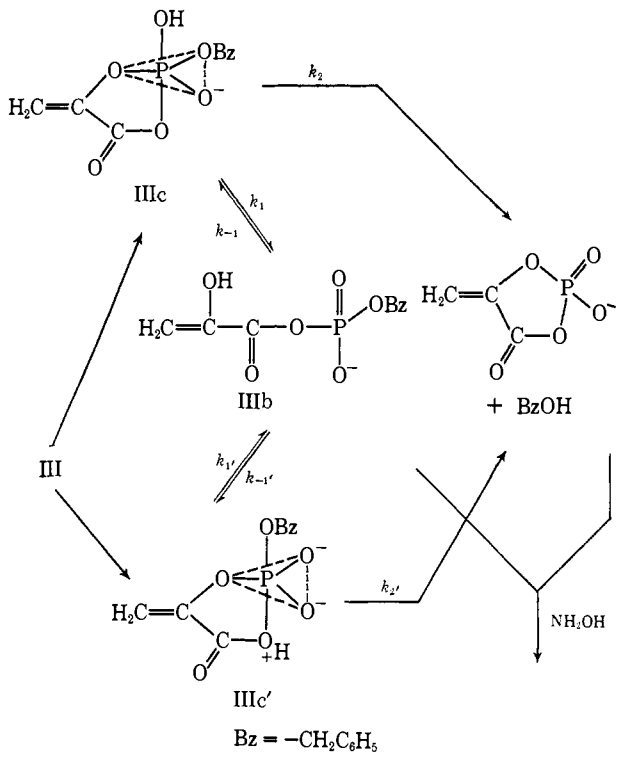
From the saturation kinetics observed with I and II the observed rate constants for formation of Ib are 350- and 30-fold more rapid than the hydrolysis to benzyl alcohol. This difference is in accord with expectations based on comparison of leaving group $pK_a$'s (enol *vs.* alcohol).[48] The 12-fold inequality in $k_{obsd}$ for both hydrolysis and hydroxylaminolysis of I and II arises from previously observed differences in the magnitude of nucleophilic attack, $k_1$, on phosphonate and phosphate esters. In conclusion, the experimental data are readily interpretable in terms of trapping an acyclic acyl phosphate by hydroxylamine.

Throughout this discussion we have assumed that the hydroxylamine studies on I and II have probed the hydrolysis reaction and tacitly have argued that the same mechanism is operative in pure aqueous media. Furthermore, a pentacovalent intermediate is implied as the species which rapidly loses benzyl alcohol, and/or pseudorotates to form acyclic acyl phosphate. It is necessary to point out that loss of benzyl alcohol may occur directly in a displacement process which is not sensitive to our present mechanistic probes, although acyclic acyl phosphate formation may not.

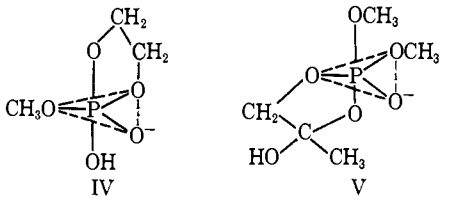
A mechanism similar to Scheme I appears to apply to the monoanion of III (Scheme II). A direct displacement mechanism must again invoke apical–equatorial displacement to explain the monobenzyl phosphate (10–15%) product and is disfavored for the above reasons. The finding of monobenzyl phosphate thus requires the presence of IIIb and IIIc or IIIc′. The hydroxylamine reaction of III, however, differs from that

(38) F. Ramirez, *Accounts Chem. Res.*, **1**, 168 (1968).
(39) F. H. Westheimer, *ibid.*, 1, 70 (1968).
(40) D. W. Allen and I. T. Millar, *J. Chem. Soc. B*, 263 (1969).
(41) G. Aksnes and K. Bergesen, *Acta Chem. Scand.*, **19**, 931 (1965).
(42) K. L. Marsi, *Chem. Commun.*, 846 (1968).
(43) D. A. Usher, *Proc. Nat. Acad. Sci. U. S.*, **62**, 661 (1969).
(44) Pseudorotation[45] can be envisioned by holding one of the equatorial positions fixed while the two apical bonds are pushed back and shortened and the two equatorial bonds are pulled forward and lengthened.
(45) R. S. Berry, *J. Chem. Phys.*, **32**, 933 (1960).
(46) D. B. Boyd, *J. Amer. Chem. Soc.*, **91**, 1200 (1969).
(47) We wish to thank Dr. G. M. Blackburn for disclosing his results to us prior to publication.
(48) The $pK_a$'s of the enol of ethyl pyruvate and benzyl alcohol are 12 and 16, respectively. See ref 34.

**Scheme II**




of I in that the products remain the same as the hydrolysis reaction. This demands that breakdown of IIIc or IIIc′ to expel benzyl alcohol and form the cyclic phosphate is at least tenfold faster than benzyl phosphoryl migration to yield the acyclic acyl phosphate, since there is no reason to believe that hydroxylaminolysis of the acyl phosphate IIIb would be markedly different from Ib. The pH–rate profile (not shown) for hydroxylaminolysis of monobenzylphosphoenolpyruvic acid (III) is identical with that of hydrolysis. Thus there is no experimental requirement that hydroxylamine is trapping a prior pentacovalent intermediate (IIIc or IIIc′). The lack of product change may be simply ascribed to $k_2$ or $k_2' > k_1$ or $k_1'$. The greater rate of benzyl alcohol expulsion for III relative to I is in accord with the expected decrease in stability of a monoanionic relative to a neutral pentacovalent intermediate, in which case the zwitterion possesses a greater potential driving force for alcohol expulsion and collapse to cyclic phosphate. Indeed, Kluger, *et al.*,[49] have argued that species IV, similar to IIIc, which is formed in the alkaline hydrolysis (pH 7–11) of methyl ethylene phosphate decomposes so rapidly that pseudorotation cannot occur since the products arise entirely by ring opening. However, in the hydroxide ion catalyzed hydrolysis of dimethylphosphoacetoin such an intermediate V does apparently pseudorotate because the loss of methoxide ion comprises only 3% of the reaction.[50,51] Since the unifying feature for the above cases appears to be ring opening rather than methoxide expulsion, a possible explanation for the behavior of the present system is a strong preference for the electronegative carboxyl moiety to remain apical. Thus the favoring of benzyl alcohol expulsion ($k_2 > k_1$ or $k_2' > k_1'$) may result from the rate of pseudorotation being competitive




with the rate of decomposition of IIIc or IIIc′ leading fortuitously to a hydrolytic product distribution similar to I and II. The composite activation entropy (−15 to −16 eu) observed in the reaction of III is in accord with a transition state featuring a constrained ring structure.[52] Although we have offered one rationale for our product distribution, it is necessary to note that at present *a priori* predictions of the favored decomposition pathway of a pentacovalent intermediate are tenuous. Much of the available data is complicated by the unknown extent of internal return, *i.e.* $k_{-2}$ or $k_{-1}$ in Schemes I and II, and the degree of sensitivity of particular pentacovalent species decomposition rates to the $pK_a$ of the leaving group.

It is not clear which form of IIIc shown in Scheme II would initially be formed. Although the proton transfers and pseudorotations involved in the pathways of these intermediates are different, neither appears distinctly preferable. In this case, however, a proton transfer must accompany either pentacovalent phosphorus formation, IIIc, or the pseudorotation process leading to acyl phosphate from IIIc′. The solvent isotope effect, $k_{H_2O}/k_{D_2O} = 1.8$, may be a reflection of the above or the rapid rate of breakdown of the ionized pentacovalent species, such that proton transfer is partially rate determining in the expulsion of benzyl alcohol ($k_2$ or $k_2'$). Note that the available proton is an important feature since its presence allows the monobenzyl ester to simulate a diester. This apparently is the first case in which a diester monoanion has been observed to intramolecularly transfer the phosphoryl moiety.[53] The failure to observe significant intramolecular general base catalyzed hydrolysis by carboxylate or intermolecular nucleophilic attack by water with the dibenzyl ester monoanion indicates that the monobenzyl phosphate product does not arise from these processes. It is interesting to realize that benzyl alcohol loss from the monoanion is only *ca.* threefold slower than the neutral species, indicating that the decreased stability of the former pentacovalent species and thus probable lower concentration is compensated for by an increased rate of benzyl alcohol expulsion.

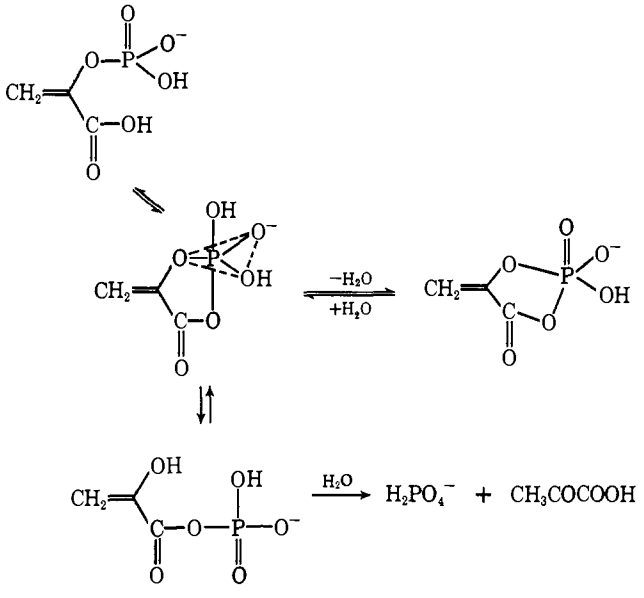
**$^{18}O$ Studies.** Clark and Kirby[5] originally postulated that attack by water on the cyclic acyl phosphate occurs at phosphorus and not carbon. Similarly, in the hydrolysis of diethyl 2-carboxyphenyl phosphonate Blackburn and Brown[6] observed water attack only at phosphorus in their postulated cyclic phosphonate. Our data in Table III are in agreement with both of the above observations. Two ring closures and hydrolysis at phosphorus would yield a theoretical incorporation of 1.66 atoms in the phosphate moiety. Incorporation of 2.5 atoms is observed, indicating some exchange without ring opening at the cyclic phosphate level as expected[22] in addition to the hydrolytic incorporation. Little or no cleavage occurs at carbon. Consequently there is

(49) R. Kluger, F. Covitz, E. Dennis, L. D. Williams, and F. H. Westheimer, *J. Amer. Chem. Soc.*, 91, 6066 (1969).
(50) F. Ramirez, B. Hansen, and N. B. Desai, *ibid.*, 84, 4588 (1962).
(51) D. S. Frank and D. A. Usher, *ibid.*, 89, 6360 (1967).
(52) T. C. Bruice and S. J. Benkovic, *ibid.*, 85, 1 (1963).
(53) D. M. Brown and D. A. Usher, *J. Chem. Soc.*, 6547 (1965).

no net transfer of oxygen from the carboxyl to phosphorus during the entire course of hydrolysis from dibenzylphosphoenolpyruvic acid to phosphoenolpyruvic acid. This also may be viewed as evidence against attack by water on the double bond of Ib leading to 1,4 expulsion of dibenzyl phosphate or displacement by a hydrated carboxyl function. These arguments may be extended by analogy to the hydroxylaminolysis.

The finding of exchange in phosphoenolpyruvate hydrolysis indicates that the carboxyl group of the phosphoenolpyruvate monoanion (predominant species at this pH) attacks phosphorus in a mechanism presumably analogous to that for the monobenzyl phosphoenolpyruvate monoanion (Scheme III). Nonreversible hydrolysis of the acyclic product to pyruvate and inorganic phosphate can be estimated at 10–15% based on data from the above monobenzyl phosphoenolpyruvate. Consequently this pathway contributes *ca.* 25% to the overall rate of hydrolysis for the phosphoenolpyruvate monoanion (estimated contribution $2\text{–}3 \times 10^{-3}$ min$^{-1}$; observed rate $8.8 \times 10^{-3}$ min$^{-1}$). The existence of this cyclic phosphoenolpyruvate may be involved in several enzyme-catalyzed reactions of phosphoenolpyruvate. The main advantages occurring with this intermediate would be: (1) rapid group interchange through the level of pentacovalent phosphorus which has a large kinetic advantage over acyclic systems and (2) ring opening to an acyl phosphate which as the enol (enolate) may readily undergo condensation with an electrophile, like carbon dioxide or a proton.

**Scheme III**




## Conclusion

The hydrolysis of dibenzyl- and monobenzylphosphoenolpyruvic acid and benzylphenylphosphonoenolpyruvic acid occurs through participation by the carboxyl function leading to formation of pentacovalent intermediate (s). These break down with loss of benzyl alcohol or pseudorotate and expel enol to give the corresponding acyclic acyl phosphates and phosphonates in all cases, resulting in intramolecular phosphoryl group migration. Acyclic acyl derivative formation for dibenzylphosphoenolpyruvic acid and benzylphenylphosphonoenolpyruvic acid is more rapid (350- and 30-fold, respectively) than benzyl alcohol loss and is reversible. This is revealed by the dramatic alteration in product composition and rate of formation in the presence of hydroxylamine which intercepts acyclic acyl phosphate. Thus, net hydrolysis is primarily benzyl alcohol loss. In contrast, monobenzylphosphoenolpyruvic acid loses benzyl alcohol more rapidly than the rate of rearrangement to the acyclic acyl phosphate since hydrolysis in the presence of hydroxylamine does not change the product composition. Intramolecular migration of the monobenzylphosphoryl moiety does occur, however, owing to the presence of a carboxyl-derived proton allowing the required pseudorotations. The overall hydrolysis from the dibenzyl substrate to phosphoenolpyruvic acid proceeds *via* net retention of oxygen in the carboxyl function through two sequences of cyclization and ring opening by nucleophilic attack of water on phosphorus.

The question may be raised as to why the carboxyl group participates nucleophilicly to form a relatively strained five-membered cyclic phosphate. Clearly the high strain energy[54] of the product might offset the propinquity effect favoring the reaction in the initial ground state.[30] However, formation of the pentacovalent intermediates involves little or no ring strain[55] and apparently the transition state for collapse to cyclic phosphate does not reflect product strain. Indeed it is probable that these transition states are actually stabilized by the presence of the sterically less crowded five-membered ring[56] in relation to the transition state of a bimolecular reaction. Thus both $\Delta S^{\pm}$ and $\Delta H^{\pm}$ may be favorably altered in these systems.

**Acknowledgment.** We wish to acknowledge the generous support of the National Institutes of Health (GM 13306) and the assistance of Miss Carol Martin in the preparation of some of the above compounds.

(54) J. R. Cox, Jr., R. E. Wall, and F. H. Westheimer, *Chem. Ind. (London)*, 929 (1959).
(55) D. A. Usher, E. A. Dennis, and F. H. Westheimer, *J. Amer. Chem. Soc.*, 87, 2320 (1965).
(56) D. Swank, C. N. Caughlan, F. Ramirez, O. P. Madan, and C. P. Smith, *ibid.*, 89, 6503 (1967).