The Mechanism of Formylation of Five-Membered Cyclic Phosphorodiamidates[†]

B. A. Amato and S. J. Benkovic*

ABSTRACT: Cyclic *N,N'*-diphenylethylenephosphorodiamidates, **1**, have been found to yield up to 70% of the *N*-formyldiphenylethylenediamines when reacted in 1 *M* formate buffers (50°, 25% v/v dioxane-H_2O, μ = 1.0). The mechanism proposed for formylation on the basis of kinetic, product ratio, and ^{18}O-labeling studies apparently involves cyclization of a formyl phosphoramidate–amine adduct derived from formate attack on $1H^+$, the process probably being catalyzed by intramolecular proton transfer between the phosphoryl moiety and aniline nitrogen. An oxygen is transferred from formate to inorganic phosphate in the net reaction. The initial site of formylation with dissymmetric **1** (*p*-CH_3O, *p*-Cl) was shown by ^{18}O incorporation studies to be the *p*-anisidine nitrogen, which also corresponds to the isomer of thermodynamic control. Attack by formate on $1H^+$, therefore, results in expulsion of the more basic nitrogen. Certain mechanistic aspects of the reaction might occur in the enzyme-catalyzed ATP-dependent formylation of tetrahydrofolic acid.

Formyltetrahydrofolate synthetase (EC 6.3.4.3) catalyses the synthesis of the biologically important N-10 formyl tetrahydrofolate isomer according to the following equation:

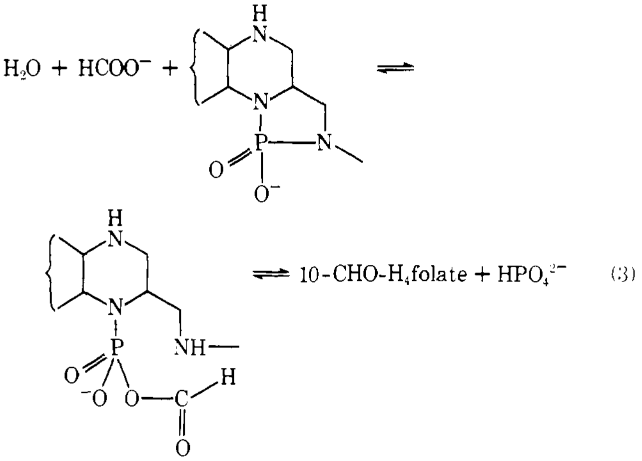
$$\mathrm{ATP^{3-} + HCOO^- + H_4folate \xrightleftharpoons{Mg^{2+}} ADP^{2-} + HPO_4{}^{2-} + 10\text{-}CHO\text{-}H_4folate} \quad (1)$$

Mechanistic investigations using the crystallized enzyme from *Clostridium cylindrosporum* have shown the reaction to proceed by a Random-Ter-Ter mechanism, probably involving a rate-determining conformational change to the active form of the enzyme in the presence of all substrates (Joyce and Himes, 1966a,b; Himes and Cohn, 1967; Buttlaire et al., 1973). The randomness of addition and the requirements for exchange are in accord with the absence of freely dissociable intermediates, but do not rule out a concerted process or pathways involving nondissociable intermediates (Himes and Harmony, 1973). The finding that ^{18}O is transferred from formate to inorganic phosphate in the course of the reaction (Himes and Rabinowitz, 1962) requires the formation of a discrete formyl phosphate or derived species participating as a transitory intermediate or as a transition state during the course of reaction.

Two basic mechanisms, without regard to the timing of bond changes, seem to satisfy these requirements. The first involves formation of formyl phosphate, with nucleophilic attack on the formyl carbon by the 10-nitrogen of H_4folate (eq 2):

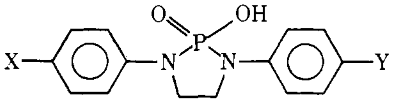
$$\begin{gathered}\mathrm{HCOO^- + ATP^{3-} \rightleftharpoons ADP^{2-} + HCOO\text{—}PO_3{}^{2-}} \\ \mathrm{HCOO\text{—}PO_3{}^{2-} + H_4folate \rightleftharpoons 10\text{-}CHO\text{-}H_4folate + HPO_4{}^{2-}}\end{gathered} \quad (2)$$

while the second involves attack of formate on an initially formed cyclic phosphorodiamidate to give a formyl phosphoramidate derivative, followed by intramolecular transfer of formate to N-10 and hydrolysis of the resulting phosphoramidate (eq 3) (Benkovic and Bullard, 1973). The



(3)

presence of the intermediate might provide a rationale for the formation of 10-CHO-H_4folate rather than the thermodynamically favored 5-CHO-H_4folate (Kallen and Jencks, 1966).



1a, X = Y = H
b, X = CH_3O; Y = Cl

Previous studies with analogs (**1a** and **b**) for the cyclic phosphorodiamidate intermediate have shown that formolysis competes with hydrolysis to yield the *N*-formyl derivatives (at the anisidine nitrogen for **1b**) under conditions where the parent diamines (**2a** and **b**) are formylated at a negligible rate (Kutzbach and Jaenicke, 1966). In this manuscript we have augmented the earlier investigations in order (1) to verify that the formylated derivatives directly result from nucleophilic attack of formate on the cyclic phosphorodiamidate, in view of the difficulty in unambiguously detecting a similar pathway in acyclic phosphoram-

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idates (Öney and Caplow, 1967), (2) to establish the presence of a formyl phosphoramidate intermediate similar to that depicted in eq 3, and (3) to determine whether the initial site of formylation in a dissymmetric system is under kinetic or thermodynamic control.

Experimental Section

All melting points are uncorrected. Microanalyses were performed by Midwest Microlab. Infrared spectra were obtained on a Perkin-Elmer 257 or 735 spectrometer; nuclear magnetic resonance (NMR) spectra on a Varian Associates A60-A spectrometer using tetramethylsilane as an internal reference. The determination of mass spectra employed an AEI MS-902 instrument equipped with a peak switching computer. Ultraviolet and visible absorbances were determined with a Cary 14 or Gilford 220 spectrophotometer. Hydrogen ion activity was measured at room temperature with a Radiometer pH meter 22 equipped with a scale expander and combination glass-calomel electrode. Thin-layer chromatograms were run using Eastman silica gel 6060 plates or preparative (1 mm) plates made from silica gel PF 254 (Brinkmann) and activated by drying at room temperature. The Chemistry Department's Modcomp II computer was used for all computations.

Materials

All chemicals and solvents were commercially available reagent grade materials used without further purification, except where noted below. Double distilled, deionized water was used throughout. *p*-Dioxane was purified by the method of Fieser (1957) and stored frozen under nitrogen prior to use. $H_2{}^{18}O$ (7.5 atom % ^{18}O) was purchased from Bio-Rad and 1.7 atom % $H_2{}^{18}O$ from Prochem. Sodium formate was powdered and dried at 100° under vacuum overnight, then stored over Drierite.

N-p-Chlorophenyl-N′-p-methoxyphenylethylenediamine (**2b**) was prepared by the method of Kutzbach and Jaenicke (1966), except that the precursor, 2-(*p*-methoxyanilino)-*p*′-chloroacetanilide (**3**), was synthesized using only a fourfold molar excess of *p*-anisidine and 250 ml of dioxane solvent: mp 108–111° (lit. mp 114–115°); NMR ($CDCl_3$) δ 3.70 (s, 3), 3.77 (s, 2), 3.87 (s, 1, broad, -NH-), 6.7 (m, 4), 7.3 (m, 4). Reduction of **3** with $LiAlH_4$ yielded 11.8 g (22% from *p*-chloroaniline) of tan powder: mp 91–93° (lit. mp 88-91°); ir (KBr) 3365, 2831, 1595 cm^{-1}; NMR ($CDCl_3$) δ 3.20 (s, 4), 3.67 (s, 3), 4.27 (s, 2, broad, disappears on shaking with D_2O), 6.8 (m, broad, 8); mass spectrum (70 eV) *m/e* (rel intensity) 276 (P, 100) and 278 (P + 2, 57).

1,3-Diphenyl-2-hydroxy-1,3,2-diazaphospholidine 2-oxide (**1a**) was prepared in 37% yield by the method of Kutzbach and Jaenicke (1966): mp 247–248° dec (lit. mp 240–245°); ir (KBr) 3420, 2600, 2070 cm^{-1}; NMR (Me_2SO-d_6) δ 3.68 (d, 4 H, J = 8.5 Hz) and 6.8–7.5 (m, 10 H); high-resolution mass spectrum, *m/e* 274.0853 (calcd for $C_{14}H_{15}N_2O_2$, 274.0870). The sodium and potassium salts were prepared by addition of 1 equiv of sodium or potassium hydroxide to a stirred slurry of the acid followed by evaporation of the solution to dryness. The sodium salt was dried at 100° under vacuum overnight, but not otherwise purified: mp 260°; ir (KBr) 1600 and 710 cm^{-1}. The potassium salt was recrystallized by dissolving 500 mg in ca. 0.5 ml of H_2O, adding 200-300 ml of acetone, then adding ether until crystals appeared: mp 251–257° dec; ir identical with that of the sodium salt.

1-p-Chlorophenyl-3-methoxyphenyl-2-hydroxy-1,3,2-diazaphospholidine 2-oxide (**1b**) was also prepared by the method of Kutzbach and Jaenicke (1966) except that hydrolysis was allowed to proceed for 6 hr, instead of the 2 hr recommended: mp 226–230° dec (lit. mp 225–227°); ir (KBr) 2600, 2300, 735 cm^{-1}; NMR (DMSO-d_6) δ 3.65 (d, 4 H, J = 8.5 Hz), 3.74 (s, 3 H, overlays doublet peak), 6.7–7.6 (m, 8 H); high-resolution mass spectrum, *m/e* 338.0624 (calcd for $C_{15}H_{16}N_2O_3PCl$, 338.0585). The sodium and potassium salts were prepared as described above. The potassium salt was found to be extremely hydroscopic, mp (K salt) 234–260° dec.

1,3-Diphenyl-2-imidazolinium chloride or fluoroborate (**4a**) was prepared by dissolving 2.0 g (0.0094 mol) of **2a** and 2.2 g (0.14 mol) of triethyl orthoformate in a minimum amount (ca. 5 ml) of 95% ethanol, allowing it to stand for 0.5 hr, then adding concentrated HBF_4 or HCl dropwise until there was no further precipitation: yield, 1.2 g (41%); mp 209–211°; λ_{max} 313 nm (ϵ 18,100), λ_{min} 245 nm (95% ethanol) (lit. max 313, min 245 nm (Jaenicke and Brode, 1959)); ir (KBr) 3100, 1619, 1590 cm^{-1}; NMR (CF_3CO_2D) δ 4.71 (s, 4 H), 7.49 (broad singlet, 10 H), 8.90 (s, 1 H).

1-p-Chlorophenyl-3-p-methoxyphenyl-2-imidazolinium chloride or fluoroborate (**4b**) was prepared in 46% yield by the above method, except that chloroform was substituted for ethanol: mp 215–216°; λ_{max} 324 nm (1 *M* HCl) (ϵ 20,000); ir (KBr) 1620 and 1593 cm^{-1}; NMR (CF_3CO_2D) δ 4.00 (s, 3 H), 4.72 (s, 4 H), 7.05–7.7 (m, 8 H), 8.84 (s, 1, H).

N-Formyl-N,N′-diphenylethylenediamine (**5a**) was prepared by the method of Jaenicke and Brode (1959) or by stirring 1–2 g of **4a** as a slurry in 50 ml of 1.0 *M* Tris buffer at pH 9.2 overnight at room temperature. After filtration and precipitation by dropwise addition of H_2O to a warm ethanol solution of the crude material, a 41% yield of lustrous, white flakes was obtained: mp 65–66° (lit. 60–61° (Jaenicke and Brode, 1959), 65–66° (Robinson and Jencks, 1967)); λ_{max} 243 nm (ϵ 16,240) (95% ethanol); R_f 0.58 (9:1 chloroform–cyclohexane); ir (KBr) 1665 and 1600 cm^{-1}; NMR ($CDCl_3$) δ 3.34 (t, 2 H, J = 6 Hz), 4.05 (t, 2 H, J = 6 Hz), 7.0 (m, 10 H), 8.42 (s, 1 H).

N-2-(p-Methoxyphenylamino)ethyl-p-chloroformanilide (**5b**) was prepared from 0.5–1.0 g of **4b** in the same manner as **5a** with 62% yield: mp 85–85.5°; R_f 0.27 (9:1 chloroform–cyclohexane); λ_{max} 242 nm (95% ethanol) (ϵ 22,400), at pH 9.5 237 nm (ϵ 19,400); ir (KBr) 3335, 1665, 1596 cm^{-1}; NMR ($CDCl_3$) δ 3.25 (t, 2 H, J = 6 Hz), 3.70 (s, 3 H), 3.97 (t, 2 H, J = 6 Hz), 6.6 (m, 4 H), 7.2 (m, 4 H), 8.33 (s, 1 H); mass spectrum (70 eV) *m/e* (rel intensity) 304 (P, 64), 306 (P + 2, 24), 136 (100). Anal. Calcd for $C_{16}H_{17}N_2O_2Cl$: C, 63.05; H, 5.67; N, 9.19. Found: C, 63.07; H, 5.53; N, 9.08.

N-2-p-(Chlorophenylamino)ethyl-p-methoxyformanilide (**5c**) was prepared by stirring 1.0 g of **4b** in 50 ml of 1.0 *M* acetate buffer at pH 5 for 4 hr at 75°. After storage at 0° overnight, the resulting solid was filtered and recrystallized twice by dropwise addition of H_2O to an ethanol solution to give a 64% yield of white solid: mp 134.5–136.5°; R_f 0.3 (9:1 $CHCl_3$–cyclohexane); λ_{max} 253 nm (95% ethanol) (ϵ 21,400), at pH 9.5 245 nm (ϵ 17,000); ir (KBr) 3370, 1690, 1595 cm^{-1}; NMR ($CDCl_3$) δ 3.26 (t, 2 H, J = 6 Hz), 3.82 (s, 3 H), 4.00 (t, 3 H, J = 6 Hz, assumed to include -NH-), 6.4–7.2 (m, 8 H), 8.30 (s, 1 H); mass spectrum (70 eV) *m/e* (rel intensity) 304 (P, 36), 306 (P + 2, 15), 140

(100), 136 (100). Anal. Calcd for $C_{16}H_{17}N_2O_2Cl$: C, 63.05; H, 5.62; N, 9.19. Found: C, 62.86; H, 5.52; N, 9.08.

Sodium formate-^{18}O was prepared as follows. Distilled triethyl orthoformate (16.6 ml, 2.10 mol) and 4.0 ml (slight excess) of 7.5 atom % $H_2{}^{18}O$ were combined, forming two phases, in a 100-ml three-neck flask, fitted with nitrogen inlet, dropping funnel, and a reflux condenser. The reaction was initiated by addition of a drop of concentrated HCl, and upon stirring the two phases appeared to become homogeneous almost immediately. After 0.5 hr, 0.10 mol of freshly prepared sodium methoxide solution in 30 ml of methanol was added over a period of 20 min. The resulting white slurry was refluxed for 1 hr, then stirred at room temperature overnight. The white precipitate was recovered by filtration, and a second crop was obtained by addition of ether to the filtrate. The combined crop was dried at 100° under vacuum overnight, giving 5.4 g (80%) of white powder, mp 256–258° (lit. 253° (Handbook of Chemistry and Physics, 47th ed, 1966)), which was stored over Drierite. The purity was established as 95 ± 2% formate by converting a weighed sample to formic acid employing Dowex $50H^+$ ion exchange resin, followed by base titration. The ^{18}O label was determined to be 7.16 ± 0.1 atom % by the method of Rittenberg and Ponticorvo (1956), using CuCl as the oxidizing agent.

Kinetic Procedure

All kinetic experiments were performed in 25% v/v dioxane–H_2O at 50 ± 0.1°, but the reported pH measurements were made at room temperature (25°). Comparison of solutions at varying buffer ratios measured at both temperatures showed an excellent fit to the equation:

$$\mathrm{pH}_{50°} = \mathrm{pH}_{25°} - 0.12 \tag{4}$$

Buffers were either formate or HCl with $\mu = 1.0$.

Three methods were used to monitor the rate of reaction of the phosphorodiamidates: (1) release of inorganic phosphate, (2) formation of formylated product, and (3) decrease in phosphorodiamidate absorption. The three methods were generally in agreement within ca. ±5% except at pH ≤4 when the decreased solubility of **1a** prevented the accurate determination of rates by methods (1) and (2).

Reactions were initiated by addition of 0.1–0.3 ml of a stock phosphorodiamidate solution that was prepared by dissolving 1 mmol by weight of the sodium or potassium salt of **1a** or **b** in 10 ml of H_2O. Due to the varying extent of hydration of these salts, the concentration of phosphorodiamidate in the stock was determined by the amount of inorganic phosphate released upon total hydrolysis and was generally ca. 0.09 *M*. Less than 2% of the final phosphate was apparent at initiation of the reaction. The stock was stored at 0° between experiments with no significant decomposition over a period of 2 months.

Phosphate release was monitored by assaying at intervals for inorganic phosphate by the method of Martin and Doty (1949) as modified by Jencks (Jencks and Gilchrist, 1965). Concurrently, the rate of production of formylated compound was followed in the same runs by adding 1.0-ml aliquots to 9.0 ml of 1.0 *M* HCl which was sufficient for quantitative conversion to the salt regardless of the pH, total formate, or diamine concentration. Incubation for 1 hr at 25° gave quantitative conversion of the formylated compounds to the salts, as evidenced by uv spectroscopy. Disappearance of phosphorodiamidate in formate buffers was also followed at 247 or 253 nm for **1a** and **b**, respectively, either by the above aliquot method or directly in a thermostated 1-mm cell. For runs in solutions buffered with HCl, 1-cm cells were employed to compensate for decreasing solubility of the phosphorodiamidate at lower pH, with runs being initiated with 2 µl of stock/10 ml of thermally equilibrated buffer. Addition of Na_4-EDTA at a concentration equal to that of **1b** at pH 4.10 and 1.0 *M* formate did not affect the rate, nor did addition of mercaptoethanol in a tenfold excess over **1b** at pH 3.40 at formate concentrations from 0.1 to 1.0 *M*.

For all experiments, k_{obsd} was determined from plots of $\log [(A_\infty - A_0)/(A_\infty - A_t)]$ vs. time which were linear to greater than two half-times. The error bars shown in Figures 3 and 4 are derived from the modified statistical treatment of Dean and Dixon (1951) for those experiments which were repeated three or more times with identical conditions.

Product Determination. Assay for total phosphorodiamidate was based on total phosphate measured at each time after correction for initial phosphate. Assay for the *N*-formyl product was based on quantitative conversion to the corresponding imidazolium salt by quenching in HCl as discussed above. Absolute concentrations were based on ϵ_{660} 571 for inorganic phosphate, ϵ_{313} 18,100 (1 *N* HCl) for **4a,** and ϵ_{324} 20,000 (1 *N* HCl) for **4b** at 25°; controls showed a linear dependence on concentration at these wavelengths.

The fraction of formylated product at any time was calculated from the ratio of the concentration of salt, **4**, to that of phosphate (corrected for initial phosphate). The concentration of diamine produced at t_∞ could not be determined accurately due to oxidation and overlapping absorption, therefore it was assumed to be equal to the difference between the phosphate concentration and the concentration of *N*-formyl product. The ratio of salt **4** to diamine **2** is then given by:

$$[\mathbf{4}]_t/[\mathbf{2}]_t = ([\mathbf{4}]_t/[\mathrm{P}]_t)/(1 - [\mathbf{4}]_t/[\mathrm{P}]_t) \tag{5}$$

where $[\mathrm{P}]_t$ is the total inorganic phosphate at time, *t*, minus the initial phosphate. The ratios reported in Figures 5 and 6 represent nonweighted averages and reflect the loss in precision as the value increases.

Control runs to test for production of *N*-formyl product directly from the diamine **2** were conducted at 1.0 *M* formate, which was the highest formate concentration employed, and at each of the pH's used. A typical control involved addition of 0.08 ml of 0.20 *M* KH_2PO_4 and of 0.20 *M* **2** (in ethanol) to 20 ml of thermally equilibrated buffer, and monitoring production of *N*-formyl product as above for a period based on ten half-times for the phosphorodiamidate reaction under the same conditions. The concentration of diamine included was equivalent to that released by total hydrolysis of phosphorodiamidate during the average run. The maximum concentration of the *N*-formyl derivative produced did not exceed 8% of that derived from the cyclic phosphorodiamidate at three half-times over the pH range 3.4–4.9 and at 1.0 *M* total formate.

Trapping Experiments. The effect of *N,N'*-diphenylethylenediamine (**2a**) (0.04 *M*) in kinetic runs 0.004 *M* in phosphorodiamidate (**1a**) at pH 4.10 (0.50 *M* formate) and at pH 3.40 (1.0 *M* formate) was followed by determining *N*-formyl formation as described above. The necessary correction for spontaneous formylation of the diamine was determined by the quench procedure. The observed ratio of [**4a**]/[**2a**] throughout either run remained constant and identical with that found in the absence of diamine after correction for spontaneous N-formylation.

Formate to Phosphate ^{18}O Transfer. In ca. 8 ml of H_2O was dissolved 1.36 g (0.02 mol) of sodium formate-^{18}O and the pH was adjusted to 4.47 with concentrated HCl. The volume was brought to 10.0 ml with water, and an additional 5 ml of water and dioxane were added to give a final pH of 4.90 (1 *M* formate, $\mu = 1.0$). After equilibration at 50°, reaction was initiated with 0.80 ml of phosphorodiamidate stock and allowed to proceed for ten half-times. Assay for products was as described above, using 0.25-ml aliquots.

The inorganic phosphate was isolated as follows: The organic components were removed by adjusting the pH to ca. 9 with 2 *M* NaOH and extracting with five 10-ml portions of chloroform. The aqueous portion was brought to pH ≤ 1 with concentrated HCl and lyophilized. The residue was dissolved in 10 ml of water and brought to pH ≥ 12 with 2 *M* NaOH, then transferred to a 15-ml centrifuge tube; 1 ml of saturated barium hydroxide solution was added to precipitate the inorganic phosphate. The tube was refrigerated for at least 1 hr and then centrifuged, the supernatant was decanted, and the precipitate was washed with 3 ml of absolute ethanol and 3 ml of ether. The precipitate was dissolved by addition of 0.5 ml of 0.5 *M* HBF_4 and centrifuged, and the supernatant was passed through a 1 × 8 cm column of Dowex 50W-X8-H^+ ion exchange resin (20–50 mesh). The column was eluted with ca. 12 ml of H_2O, and the eluate was then lyophilized, leaving an oily film of H_3PO_4. This residue was dissolved in approximately 1 ml of H_2O and adjusted to pH 4.5 ± 0.5, with dilute KOH. This solution was reduced in vacuo to ca. 0.5 ml and transferred to a centrifuge tube. Approximately 10 ml of absolute ethanol was added to precipitate the KH_2PO_4. After refrigeration, the precipitate was collected by centrifugation, washed with three 2-ml portions of absolute ethanol and four 2.5-ml portions of ether, and dried at 100° overnight. Recovery was 5–7 mg (60–75%) of material which assayed at $\geq 90\%$ KH_2PO_4.

The atom % ^{18}O in the recovered phosphate was determined by converting it to CO_2 and determining the ratio of *m/e* 46/*m/e* 44 peaks. The atom % ^{18}O is given by:

$$\text{atom } \%\, ^{18}\text{O} = 100R/[2(1 + R)] \tag{6}$$

where *R* is the ratio of *m/e* 46/*m/e* 44 peaks. The expected atom % ^{18}O in the phosphate is given by:

$$\text{atom } \%\, ^{18}\text{O (calculated)} = X_{\text{formyl}} \times [(\%^{18}\text{O in formate} + 0.6)/4] + 0.2X_{\text{diamine}} \tag{7}$$

where X_{formyl} is the observed mole fraction of *N*-formyl product produced and X_{diamine} is $(1 - X_{\text{formyl}})$.

Site of Initial Formylation. The interconversion of *N*-formyl compounds **5b** and **c** and salt **4b** was studied by adding 0.8 ml of 0.100 *M* **5b** or **5c** (in dioxane) to 20 ml of thermally equilibrated buffer (1.0 *M* formate, pH 4.85) and scanning aliquots at selected intervals. Identical spectra were produced starting from either compound at $t = \infty$. Calculation of the ratio of salt **4b** to formyl product **5c** at $t = \infty$ (assuming no **5b** present, see Results) showed the equilibrium to consist of 90% **5c** and 10% **4b**.

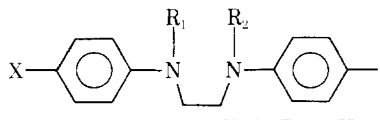
Buffers containing $H_2{}^{18}O$ (1.0 *M* formate, $\mu = 1.0$) were prepared by dissolving 0.68 g (0.01 mol) of sodium formate in ca. 3 ml of $H_2{}^{18}O$ (1.7 atom %) and adjusting the pH to 4.45 with concentrated HCl. The volume was brought to 5 ml with $H_2{}^{18}O$, and an additional 2.5 ml each of $H_2{}^{18}O$ and dioxane were added to give a final pH of 4.83 ± 0.02. Reaction was initiated by addition of 16 mg (0.047 mmol) of **1b** to the thermally equilibrated buffer followed by vigorous shaking. After 90 min (one half-time) the reaction was cooled in ice-water and extracted with three 10-ml portions of chloroform. The chloroform solution was reduced in vacuo to ca. 1 ml and chromatographed on a 20 × 20 cm preparative thin-layer chromatography plate (4:1 ethyl acetate–cyclohexane). The *N*-formyl product band was excised, extracted by stirring with 20 ml of methanol for 10 min, filtered, and washed with an additional 20 ml of warm methanol. The filtrate was reduced in vacuo to ca. 1 ml, and the product was precipitated by dropwise addition of ice-cold H_2O. The precipitate was collected by centrifugation and dried at room temperature under vacuum for 2 hr. A melting point determination confirmed the product as the expected **5c.** A sample was saved for mass spectroscopy, and the remainder was dissolved in ca. 1 ml of warm methanol, rechromatographed, and reprecipitated as above and dried to provide a second sample. Control runs with **5b, 5c,** and **4b** were carried out in the same manner, except that an amount of material was introduced equal to approximately one-half the amount of **1b** used.

The ^{18}O label was determined from the ratio of the *m/e* 306 (P + 2) to *m/e* 304 (P) peaks as described above. The calculated natural abundance ratio of 0.345 was determined by adding the natural abundance ratio of ^{37}Cl, ^{35}Cl (Silverstein and Bassler (1967), p 29) to the expected (P + 2)/(P) ratio for a compound of the formula of **5c** minus chlorine (Willard et al., 1965). Excellent agreement (0.346) was found upon analysis of a known, unlabeled sample of **5c.** The expected ratio on inclusion of one atom of oxygen from solvent was then calculated for **4b** and **5b** by addition of 0.017 (the fraction of ^{18}O in the labeled water) to the natural abundance ratio. These results are in excellent agreement, within experimental error, of the calculated value.

Results

Structure Assignments. The phosphorodiamidates (**1a** and **b**) have been previously prepared by Kutzbach and Jaenicke (1966). Their cyclic structure is confirmed by the NMR chemical shift equivalence of the four methylene protons in addition to the superimposed symmetrical P-H coupling of 8.5 Hz observed in each case (Jackman and Sternhell, 1969). Moreover, the agreement of the parent *m/e* peak observed with high-resolution mass spectrometry with that calculated for the molecular weight of **1a** and **b** is consistent with the assignment.

The formamidinium structures, **4a** and **b**, are supported by the observation of a strong band at 1620 cm^{-1} attributable to a conjugated formamidinium moiety and of single proton NMR resonance at δ 8.90 and 8.84, in accord with a strongly deshielded formamidinium proton. The appearance of uv absorbance at longer wavelengths relative to the unformylated diamines is expected for an increased π system (Silverstein and Bassler, 1967); moreover the observed spectrum of **4a** agrees with that reported previously (Jaenicke and Brode, 1959).



5a, X = Y = H; R_1 = CHO; R_2 = H
b, X = CH_3O; Y = Cl; R_1 = H; R_2 = CHO
c, X = CH_3O; Y = Cl; R_1 = CHO; R_2 = H

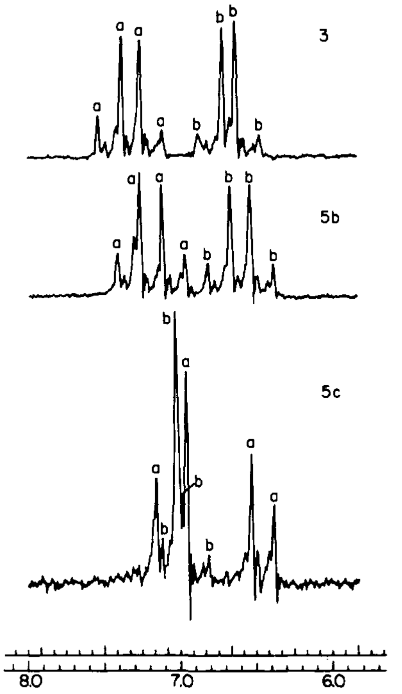
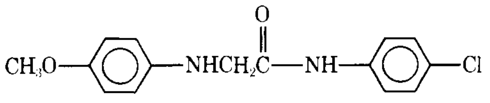


FIGURE 1: Aryl proton region NMR spectrum (60 MHz) of **3**, **5b**, and **5c** in $CDCl_3$ relative to tetramethylsilane. (a) Peaks assigned to the *p*-chloroaniline ring protons; (b) peaks assigned to the *p*-anisidine ring protons.

Hydrolysis of the unsymmetrical formamidinium salt, **4b**, can lead to either of two *N*-formyl derivatives. The product isolated depends markedly on pH, i.e., **5b**, containing the *p*-chloroformanilide moiety, is the product of kinetic control at pH 9, whereas **5c**, containing the *p*-methoxyformanilide species, is the product of thermodynamic control at pH 5 (Benkovic et al., 1972). Structure proof for **5b** and **c** is derived from examination of the aryl proton NMR resonances (Figure 1). In the spectrum of 2-(*p*-methoxyanilino)-*p*′-chloroacetanilide (**3**) two aromatic pseudo-AB quartets, each integrating to four protons (J = 9 Hz), appear centered at δ 7.3 and 6.7. Upon reduction with $LiAlH_4$, the quartet at δ 6.7 remains essentially unchanged and thus is assigned to the *p*-anisidine system, whereas the center of the lower field resonances (δ 7.3) is shifted upfield to δ 6.8 accompanied by an increased chemical shift difference (0.4-5) between the pairs of ring protons in the AA′BB′ pattern. This is consistent with the greater upfield shift found for ortho (ca. 0.8 ppm) vs. meta (ca. 0.3 ppm) protons upon reduction of acylated aniline derivatives (Jackman and Sternhell, 1969). Formylation of **2b** at the *p*-chloroaniline nitrogen thus would give a spectrum similar to **3**. Formylation at *p*-anisidine nitrogen, on the other hand, would have little effect on the *p*-chloroaniline resonances centered at δ 6.8 but correspondingly would cause a downfield shift as well as a collapse of the AA′BB′ pattern for the *p*-anisidine protons centered at ca. δ 6.7. This effect is noted in the spectrum assigned to **5c** relative to **5b**. Comparison of the aryl proton spectra for *p*-anisidine (Sadtler Index no. 94) and 4′-hydroxy-*N*-methylacetanilide (Sadtler Index no. 14485M) likewise shows the anticipated changes.



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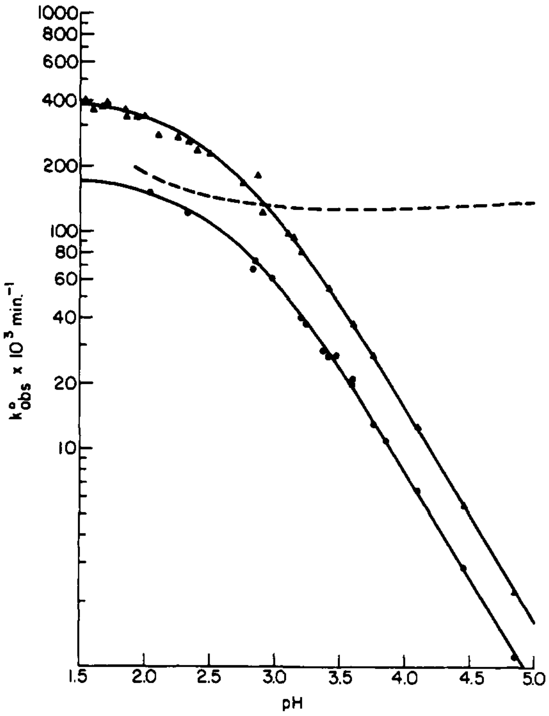


FIGURE 2: Plots of k_{obsd}^0 vs. pH for the hydrolysis of **1a** (●) and **1b** (▲) (25% v/v dioxane-H_2O, 50°, μ = 1.0). The solid lines are calculated from eq 8, using the coefficients given in Table I. The dashed line is calculated for the hydrolysis of *N*-*p*-chlorophenylphosphoramidate (Chanley and Feageson, 1958) under these conditions.

Table I: Coefficients for Reaction of Phosphorodiamidates, 1a and b (from Scheme I).

	1a	1b
k_w	0.183 min^{-1}	0.415 min^{-1}
k_b	4.84 × 10^{-2} min^{-1}	6.27 × 10^{-2} min^{-1}
K_{a_1}	2.21 × 10^{-3} M	2.59 × 10^{-3} M
K_{a_2}	1.0 × 10^{-2} M^a	1.0 × 10^{-2} M
K_F	11.13 M^{-1}	21.00 M^{-1}
K_{a_f}	1.34 × 10^{-4} M^b	1.03 × 10^{-4} M^b

[a] This represents a lower limit (see Results). [b] As determined from product distribution results.

Kinetics. Plots of k_{obsd}^0, the pseudo-first-order rate constants at zero buffer concentration for hydrolysis of phosphorodiamidates **1a** and **b**, as a function of pH, are shown in Figure 2. Values for k_{obsd}^0 at pH >4 were obtained by extrapolation to zero buffer concentration. The pH-rate profile is described by the rate equation:

$$k_{obsd}^0 = k_w a_H/(Ka_1 + a_H) \qquad (8)$$

where k_w represents the rate constant associated with spontaneous hydrolysis for the netural phosphorodiamidate and K_{a_1} represents the dissociation constant for the neutral species. Values for k_w and K_{a_1} are listed in Table I.

Plots of k_{obsd}, the pseudo-first-order rate constants for the reaction of **1a** and **b**, as a function of formate concentration are shown in Figures 3 and 4. With decreasing pH the degree of catalysis, which is proportional to $(k_{obsd} - k_{obsd}^0)/[F]_T$, increases to approximately pH 4.0 and then abruptly decreases. Inhibition by formate is actually observed in the

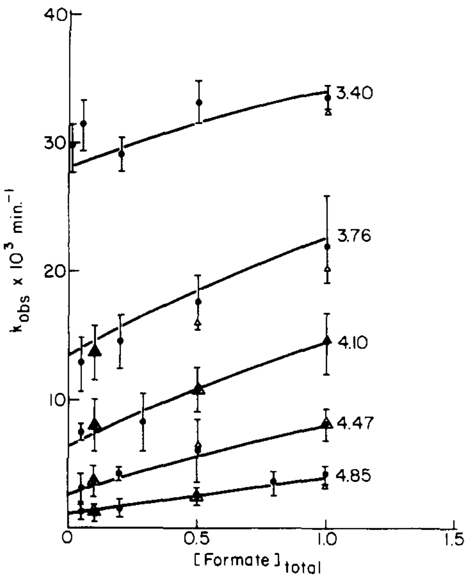


FIGURE 3: The observed rate constant (k_{obsd}) for the reaction of **1a** vs. total formate concentration at the pH values shown. Conditions as in Figure 2. The solid lines are calculated according to eq 9, using the coefficients shown in Table I. Rates determined by assay for inorganic phosphate (△) or spectrophotometrically (●).

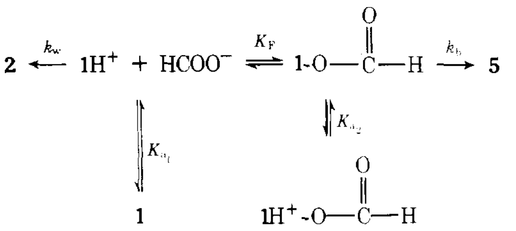
unsymmetrical model, **1b,** at pH 2.82. The data are described by the following equation:

$$k_{\text{obsd}} = \frac{k_w + k_b K_F[F^-]}{(K_{a_1} + a_H)/a_H + (K_F(K_{a_2} + a_H)/K_{a_2})[F^-]} \quad (9)$$

where $[F^-]$ is the calculated concentration of formate anion based on the dissociation constant for formate, K_{a_f}, determined from the observed product distribution (see below), and the remaining constants are defined in terms of Scheme I for both **1a** and **b**.

Although Scheme I represents one of several kinetically indistinguishable formulations, the stipulation of $[1H^+][HCOO^-]$ rather than $[1][HCOOH]$ seems reasonable in view of the required protonation of acyclic phosphorodiamidates for nucleophilic reactivity (Chanley and Feageson, 1958, 1963; Jencks and Gilchrist, 1965; Öney and Caplow, 1967; Jameson and Lawler, 1970; Benkovic and Sampson, 1971) and the greater nucleophilicity of formate vs. formic acid.

Scheme I



The behavior of eq 9 can be analyzed in terms of the relative magnitudes of the two denominator terms keeping in mind that $k_b < k_w$. Beginning at high pH (5.0), $K_{a_1}/a_H > (K_F(K_{a_2} + a_H)/K_{a_2})[F^-]$, so that catalysis by formate is small owing to the decreased concentration of **1**H$^+$. Since the observed catalysis initially increases with decreasing pH, K_{a_2} is greater than 10^{-5}, otherwise inhibition would be observed throughout the pH range investigated. As the pH is decreased, the increase then decrease in the slopes in Figures 3 and 4 arises from a noncompensating increase in $[1H^+]$ and decrease in $[HCOO^-]$, respectively, owing to their differing pK_a values. At low pH (<3.0) the decrease in k_{obsd} as a function of total formate may be attributed to inhibition of hydrolysis by accumulation of the formylated species of **1** since $K_{a_1}a_H \simeq (K_F(K_{a_2} + a_H)/K_{a_2})[F^-]$ but $k_b < k_w$. The determination of a precise value for K_{a_2} for **1a** is prevented by experimental limitations so that the reported number is merely a lower limit. A satisfactory check on the reported value for **1b,** however, was achieved by measuring k_{obsd} for **1b** at pH 2.60 at 1 *M* total formate. The coefficients shown in Table I were determined by use of an iterative, nonlinear least-squares program (Bevington, 1969). A check on the evaluation of $k_b K_F$ is obtained from the observed product distribution, as noted below.

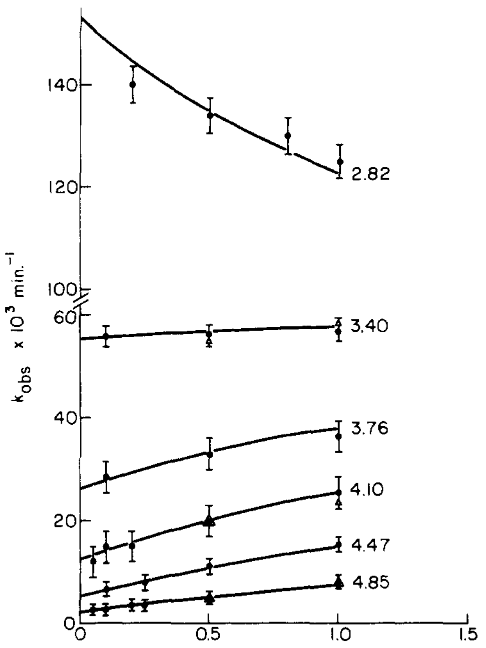


FIGURE 4: The observed rate constant (k_{obsd}) for the reaction of **1b** vs. total formate concentration at the pH values shown. Conditions as in Figure 2. The solid lines are calculated according to eq 9, using the coefficients shown in Table I. Rates determined by assay for inorganic phosphate (△) or spectrophotometrically (●).

Product Distribution. Measurement of the ratio of formolysis to hydrolysis showed this quantity to remain constant during the time course of a given experiment. Plots of $[5]/[2]$ at varying total formate and pH are shown in Figures 5 and 6, for both **1a** and **1b.** It is apparent that $[5]/[2]$ is linear with increasing total formate and that the slope approaches a limiting value at high pH. The results are consistent with Scheme I, for which the ratio $[5]/[2]$ is given by:

$$[5]/[2] = \frac{k_b K_F K_{a_f}[F]_{\text{total}}}{k_w(K_{a_f} + a_H)} \quad (10)$$

Expression 10 exhibits the necessary linearity in total formate as well as the sigmoidal dependence on pH observed in Figures 5 and 6. Graphical evaluation of a rearranged form of eq 10, i.e.

$$Ra_H = K_{a_f}(k_b K_F/k_w) - RK_{a_f} \quad (11)$$

where R is defined as $([5]/[2])/[F]_T$ at a given pH gives values for $(k_b K_F/k_w)$ and K_{a_f} as intercept and slope, respectively.

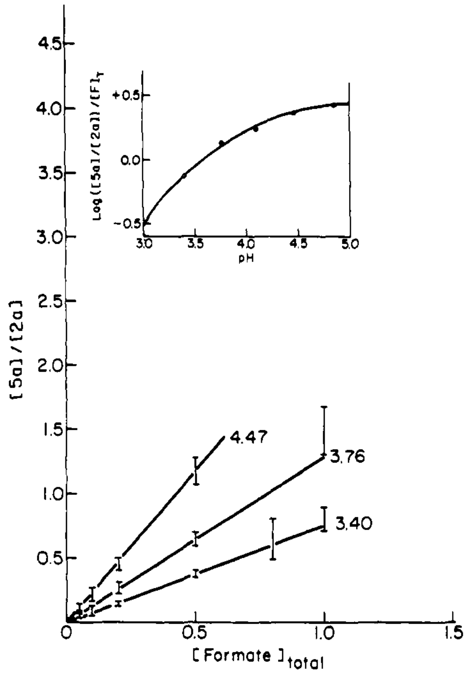


FIGURE 5: Ratio of formylated product (**5a**) to diamine (**2a**) vs. total formate concentration at the indicated pH values for **1a**. The solid lines are calculated from eq 10, using the coefficients given in Table I. Inset: ([**5a**]/[**2a**])/[F]$_T$ vs. pH. The line is calculated from eq 10 using the coefficients given in Table I.

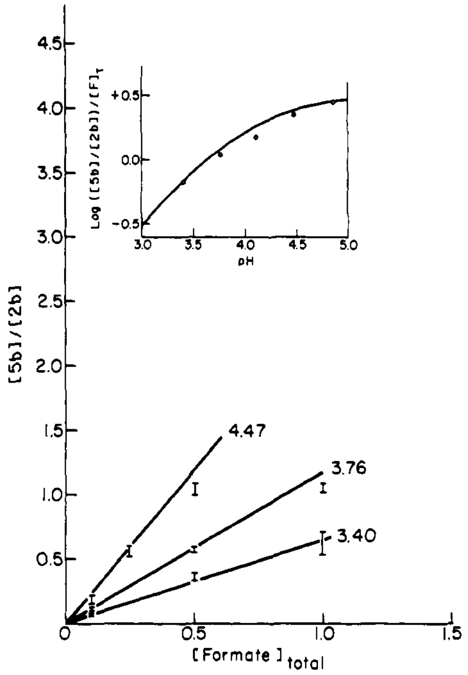
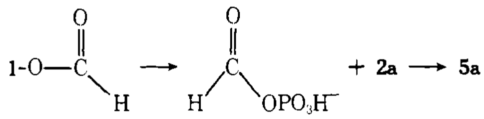


FIGURE 6: Ratio of formylated product (**5c**) to diamine (**2b**) vs. total formate concentration for **1b** at the pH values shown. The solid lines are calculated from eq 10, using the coefficients given in Table I. Inset: ([**5c**]/[**2b**])/[F]$_T$ vs. pH. The line is calculated from eq 10 using the coefficients given in Table I.

In order to exclude the possibility that formyl phosphate is the actual formylating agent, two runs with **1a** were conducted in the presence of a tenfold excess of diamine, **2a**,



$$\text{1-O—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—H} \longrightarrow \text{H—}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{—OPO}_3\text{H}^- + \mathbf{2a} \longrightarrow \mathbf{5a} \qquad (12)$$

relative to **1a**. It was anticipated that additional diamine might lead to a significant increase in the apparent yield of formylated product over the ca. 40% expected under the conditions chosen. Although formylation was increased by ca. 4%, this gain could be attributed entirely to reaction between formate and diamine as shown by a control experiment.

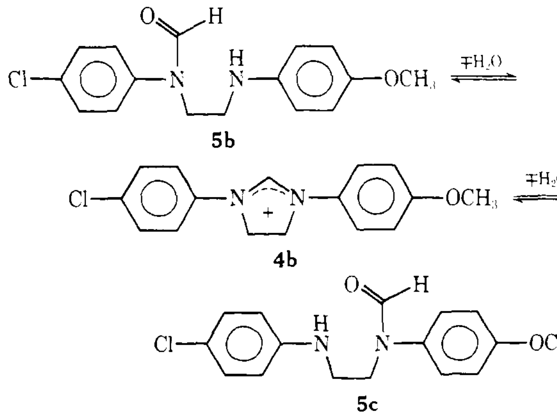
Formate to Phosphate ^{18}O Transfer. To determine whether formylation of phosphorodiamidates **1a** and **b** involves transfer of oxygen from formate to phosphate, experiments were conducted with both **1a** and **1b** using formate-^{18}O. Conditions were chosen to maximize both the yield of formylated product and the solubility of the phosphorodiamidate at the high initial concentrations required for these experiments. The results are shown in Table II. The calculated atom % ^{18}O is obtained from eq 7, which assumes that ^{18}O is transferred to phosphate quantitatively in the formylation reaction but not in the hydrolysis. The close agreement between the calculated and experimentally determined values of the atom % ^{18}O in the recovered phosphate indicates that formylation involves quantitative transfer of one atom of oxygen from formate to inorganic phosphate.

Table II: Formate to Phosphate ^{18}O Transfer.

Material	Atom % ^{18}O Found	Atom % ^{18}O Calculated
Sodium formate-^{18}O	7.16	
Phosphate derived from **1a**	1.34[a]	1.31
Phosphate derived from **1b**	1.39[a]	1.40

[a] Average of two samples.

Site of Initial Formylation. Spectroscopic determination of the site of initial formylation of the unsymmetrical model, **1b**, was precluded by the rapid conversion of **5b** to salt, **4b**, under the conditions employed for kinetic and product ratio experiments, the latter partitioning to an equilibrium mixture consisting of ca. 90–94% **5c** and 6–10% **5b** at pH 4.85 (eq 13). Similar results have been observed by Benkovic and coworkers (1972) with the tetrahydroquinoxoline models for folate cofactors.



5b

4b

5c

(13)

The isomerization of **5b** to **5c** has been used to determine the site of formylation by an isotopic labeling method involving $H_2{}^{18}O$. Isomerization of **5b** to **5c** via **4b** must involve exchange of oxygen with solvent water, and should re-

Table III: Incorporation of ^{18}O into Recovered 5c.

Starting Material	Ratio of m/e 306 to m/e 304^a
5c	0.342 ± 0.002
5b	0.361 ± 0.002^b
4b	0.361 ± 0.001^b
1b	0.348 ± 0.002

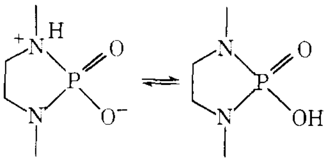
^{a}Calculated for natural abundance (see Experimental Section): 0.346. ^{b}Calculated (see Experimental Section): 0.362.

sult in quantitative labeling of **5c** produced via this pathway. On the other hand, the exchange of the formyl oxygen of **5c** with water might be slow at pH 4.85, so that little or no equilibration with solvent would occur over a limited interval. Control runs starting with **4b, 5b,** and **5c** that were allowed to react for the ca. $t_{1/2}$ for the reaction of **1b** with formate at pH 4.85 show the expected quantitative incorporation of ^{18}O into the formyl moiety of **5c** commencing with **5b** or the salt **4b,** but not with **5c** (Table III). With the phosphorodiamidate, **1b,** no significant level of ^{18}O is found in the **5c** produced; thus one concludes that the immediate product of formylation of **1b** is **5c,** the thermodynamically more stable product.

Discussion

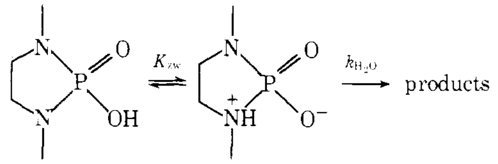
The total hydrolysis of **1a** and **b** proceeds via a stepwise pathway with the acyclic phosphoramidate presumed as the intermediate species. Thus there are two possibilities in regard to the rate-determining step, i.e., either P-N bond fission to open the ring or loss of inorganic phosphate from the acyclic phosphoramidate after rapid initial ring cleavage. Interpolation of the data of Chanley and Feageson (1958) for the hydrolysis of *p*-chlorophenylphosphoramidate in dioxane-water mixtures permits construction of its pH-rate profile under our conditions. Inspection of Figure 2 reveals that hydrolysis of the acyclic species is more rapid than that of **1a** or **b** at pH >2-3, so that in this region attack by water and/or ring opening of the cyclic phosphorodiamidate probably constitute the rate-determining step. At lower pH both steps are competitive, although the possibility of general acid catalysis or electrostatic effects stemming from the β-amino group in the hydrolysis of the acyclic neutral species cannot be discounted (Lloyd and Cooperman, 1971; Kirby and Jencks, 1965).

The pH-rate profile for hydrolysis of **1a** and **b** is apparently generated by protonation of **1** followed by water attack. The reactive species of **1** presumably involves protonation at nitrogen in order to avoid expulsion of the thermodynamically unfavorable aniline anion. The former is in equilibrium with the O-protonated form, which may constitute the major fraction of the neutral species on the basis of the low kinetic pK_a observed (2.66 for **1a,** 2.59 for **1b**). Similar low pK_a values have been found for several related compounds, for example 2.81 for monomethyl phosphoramidate (36.8°, μ = 0.2, interpolated to 25% dioxane) (Öney and Caplow, 1967) and 3.1 in water for methyl *N*-cyclohexylphosphoramidate (Hamer, 1965). Garrison and Boozer (1968) have suggested that the favorability of O-protonation is due to the tendency for p_π-d_π bonding between phosphorus and nitrogen. The small difference in apparent pK_a between **1a** and **1b** may also be a reflection of the predominance of O-protonation.



(14)

It is probable that the *p*-anisidine moiety is being expelled in the initial step of hydrolysis of **1b** (see below), so that $\beta \simeq 0.5$ employing a $\Delta pK_a = 0.71$ for the difference between the proton dissociation constants of the *p*-anisidine and aniline nitrogens (Albert and Serjeant, 1971). This may be accounted for by defining k_w in terms of k_{H_2O} and K_{zw} as given in eq 15 (Benkovic and Sampson, 1971). Presuming the predominance of O-protonation, i.e., $K_{zw} < 1$, then $k_w = k_{H_2O}K_{zw}$. The structure-reactivity correlation, thus, is the sum of the Brönsted slope for k_w and the dependency of K_{zw} on the pK_a of the aniline ($\beta_{K_{zw}} = +1$). The β_{lg} for water attack consequently is ca. −0.5, indicative of a transition state with roughly 50% diminution of the positive charge on the leaving nitrogen. This result contrasts with the lack of rate sensitivity toward the nature of the leaving group for the hydrolysis of acyclic phenylphosphoramidate monoanions where the mechanism involves metaphosphate expulsion and little participation by solvent. Moreover, the greater reactivity of the acyclic phosphoramidate vs. the cyclic phosphorodiamidate toward hydrolysis (Figure 2) is not expected, in view of the higher hydrolytic reactivity for endocyclic cleavage of cyclic five-membered phosphate diesters relative to acyclic monoesters of comparable pK_a (Kluger et al., 1969; Khan and Kirby, 1970).



(15)

The mechanism of formylation of **1a** and **1b** must be in accord with the following salient observations: (1) the absence of appreciable formylation in controls involving formate plus **2** (<8% of the amount of formylation observed with **1** at t_∞), (2) the reactive species are $[HCOO^-][1H^+]$ or their kinetic equivalent, (3) inhibition of the rate of reaction of **1** by formate at low pH, (4) the linear dependence of the product ratio, i.e., *N*-formyl to diamine, on formate concentration, (5) the lack of an increase in formylated product or in rate upon addition of diamine **2** to the reaction, and (6) the transfer of ^{18}O from formate-^{18}O to inorganic phosphate as well as the lack of ^{18}O incorporation from solvent into the formylated product formed in the reaction of **1b.** Several potential pathways are assessed below on the basis of these findings.

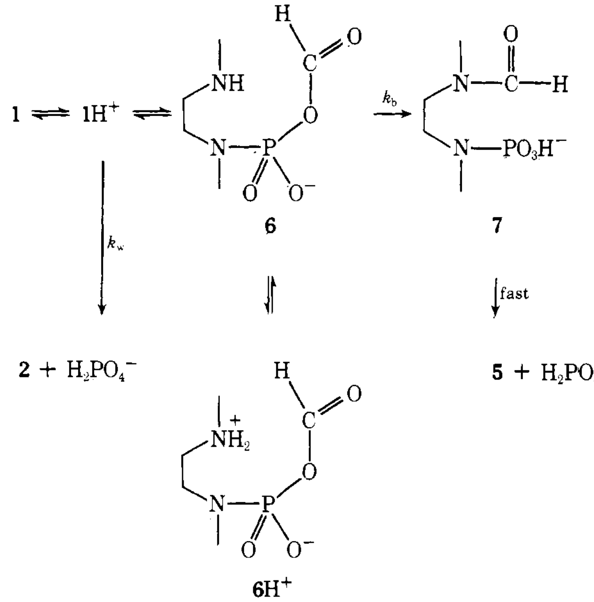
Direct formylation of either diamine, **2a** or **b,** rather than the respective phosphorodiamidate has been eliminated by the control runs noted above as well as the observation that transfer of oxygen from formate to inorganic phosphate accompanies formylation. Moreover, the rate of production of **5** does not exhibit a lag as would be required in order to accumulate diamine (Kutzbach and Jaenicke, 1966).

A second alternative involves attack of formate on either cyclic phosphorodiamidate followed by hydrolysis of both P-N bonds to release formyl phosphate, which serves as the actual formylating agent. One can calculate that the rate of hydrolysis of formyl phosphate at pH 3-5 under our conditions is approximately 0.1 min^{-1} (Jaenicke and Koch, 1963) whereas the rate of aminolysis is estimated at 0.3-0.4

min^{-1} at 10^{-3} M diamine.[1] Consequently formyl phosphate would be a viable intermediate but only near t_∞ would the amine concentration approach 10^{-3} M. Therefore, the failure to observe an increase in formylated product (**5a**) in the trapping experiments which were done under conditions where the yield of **5a** should be responsive to added diamine suggests that formyl phosphate is not involved. Even if amine attack is not rate determining, the product ratio should reflect the added amine concentration. Moreover, the constancy of the product ratio ([**5**]/[**2**]) throughout each experimental run rather than its parabolic increase as the concentration of **2** available for formylation increases is not in accord with a free formyl phosphate pathway.

A scheme consistent with the results stated above is given in Scheme II. The inferred structures for **1**-CO_2H and **1**H^+-CO_2H are denoted as **6** and **6**H^+. Several features of this scheme should be noted. First, the rate-determining step is assigned to the cyclization of **6** to product, and an equilibrium is established among species **1**, **1**H^+, **6**, and **6**H^+. As demonstrated in Results the kinetic expression (eq 9) derived on this basis adequately describes both the behavior of k_{obsd} and the product distribution as a function of formate concentration and pH. Inhibition at low pH results from accumulation of **6** and **6**H^+ (at pH 3.4, ca. 31% for **1a** and 38% for **1b**) as well as the fact that $k_b < k_w$. If the initial formylation step were rate limiting, no rate inhibition by formate would be expected. Likewise dephosphorylation of **7** should be faster than **6** → **7** and therefore not rate determining for reasons cited earlier. Formation of **6** is described as stemming from nucleophilic attack by formate on N-protonated **1**, an interpretation that avoids expulsion of an unfavorable aniline anion.

Scheme II



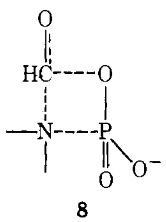
Second it is necessary for ([**6**] + [**6**H^+]) to be at a non-steady-state concentration in order to rationalize the inhibition. However, attempts to detect **6** spectrophotometrically were frustrated by the apparent similarities in the uv absorption spectra of **1** (**a**, λ 247 nm, ε 30,000; **b**, λ 253 nm, ε 30,000) and **5** (**a**, λ 240 nm, ε 16,300; **c**, λ 253 nm, ε 21,400). The concurrence of rates monitored by uv or measurement of inorganic phosphate release suggests that the former procedure follows the cleavage of both P–N bonds. From the kinetic data for **1b** a value for pK_{a_2} may be assigned to the dissociation of **6**H^+ that is similar to those reported for **1a** and **b**. This macroscopic constant should be comprised of protonation at all three basic sites; it is evident that protonation is not principally at the p-anisidine nitrogen. The requirement for the intermediate, **6**, eliminates a four-center mechanism whose transition state for N-formylation is composed of the cyclic phosphorodiamidate and formate (Kutzbach and Jaenicke, 1966).

An alternate formulation of the observed kinetic behavior ascribes the depression in k_{obsd} at low pH (<3.0) to medium effects introduced by the change in buffer composition. If this be the case, then it is no longer necessary for ([**6**] + [**6**H^+]) to be at a non-steady-state concentration nor is the presence of [**6**H^+] required in order to rationalize the inhibition. Scheme I may be solved with a steady-state approximation for [**6**] yielding the following expression for k_{obsd}:

$$k_{obsd} = \frac{k_w(k_b + k_{-F}) + k_b k_F[F^-]}{(k_b + k_{-F})(K_{a_1} + a_H)/a_H} \tag{16}$$

where $K_F = k_F/k_{-F}$. At low pH, $k_b k_F[F^-] < k_w(k_b + k_{-F})$ and $K_{a_1} < a_H$ so that $k_{obsd} = k_w$. Salt effects that depress k_w approximately 20% would rationalize the kinetics as well as the inability to detect **6**. Salt effects encompassing that order of magnitude in mixed solvents have been described by Salomaa et al. (1971) and Grunwald and Butler (1960). In addition, with this formulation it is not possible at present to assign unequivocally the step denoted by k_b as rate limiting, since the ratio k_b/k_{-F} is unknown. Thus the value of k_w remains that reported in Table I whereas that assigned $k_b K_F$ would be equivalent to $k_b k_F/(k_b + k_{-F})$.

Third, the observation of ^{18}O transfer from formate to phosphate follows from the proposed cyclization of **6** → **7**. Since there is excellent agreement between the calculated and observed amounts of ^{18}O transfer between formate and phosphate, no competing hydrolysis of either **6** or **6**H^+ has been included in Scheme II. Hydrolysis of **6** or **6**H^+ via C–O bond cleavage would result in enrichment of the ^{18}O content of the inorganic phosphate relative to the amount of formyl product (Di Sabato and Jencks, 1961b). Moreover, the calculated value for the rate constant for hydrolysis of acetyl phenylphosphate under our conditions is less than 10% of k_b (Di Sabato and Jencks, 1961b). The 70% yield of formylated product at 1 M formate (pH 4.85) also attests to the efficiency of the reaction. In the case of the dissymmetric phosphorodiamidate, **1b**, the absence of incorporation of ^{18}O from solvent water into isolated **5c** requires that the initial ring opening involves expulsion of the more basic p-anisidine nitrogen which is then formylated in the cyclization step. The alternative, that the p-chloroaniline nitrogen leaves followed by a four-centered reaction employing the anisidine nitrogen as shown in **8**, is less likely on steric



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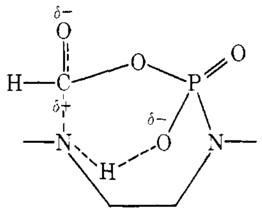
[1] The rate of aminolysis is based on that observed for the attack by aniline on acetyl phosphate dianion (Di Sabato and Jencks, 1961a) increased by factors of 300 and 2–3 for the substitution of acetyl by formyl (Taft, 1959) and the higher temperature.

grounds since the formyl phosphate is required to adopt transiently a highly strained cis conformation (Marsden and Sutton, 1936; Huisgen and Ott, 1959). The preferred description is in accord with the generality that the proton acidity is the dominant influence on leaving group order in amines (Bullard et al., 1974; Jencks, 1969). Although the attack by formate on **1** may proceed via pentacovalent species the available evidence does not warrant their inclusion.

It is possible that the transition state for the cyclization of **6** → **7** might involve an intramolecular proton transfer as illustrated in **9**. This process would serve to favor C–O bond cleavage by lowering the pK_a of the phosphoramidate leaving group as well as disfavoring reversion to **6**. The relative ΔpK_a between the amine and phosphate moieties should decrease as the reaction proceeds, thereby favoring proton transfer in the desired direction. Moreover, the required six-membered ring conformation can be achieved readily within the larger seven-membered ring framework when viewed with CPK models.



9

The results of this study lend support to earlier suggestions that a similar mechanism might be operating in certain enzyme-catalyzed ATP-dependent formylations of tetrahydrofolate, utilizing the enzyme from *Micrococcus aerogenes* (Whiteley and Huennekens, 1962) and from pigeon liver (Jaenicke and Brode, 1961a,b; Jaenicke, 1961) although the purity of the preparation employed has been criticized (Himes and Harmony, 1973). Indeed the pattern of ^{18}O exchange between formate and the inorganic phosphate produced in the *Clostridium cylindrosporum* enzyme is identical (Himes and Rabinowitz, 1962). The observation that no ^{18}O is incorporated from solvent water into inorganic phosphate in the reaction catalyzed by this enzyme (Benkovic and Bullard, 1973), however, is evidence against such a mechanism unless water produced upon formation of the cyclic phosphorodiamidate from tetrahydrofolate and ATP does not exchange with water in the medium. The failure of the model reaction to yield **5b** in analogy to the enzymic production of 10-CHO-H$_4$folate is not entirely unexpected since other reactions designed to mimic processes involving H$_4$folate generally lead to products initially derived from expulsion of the more basic nitrogen (Benkovic et al., 1972, 1973). Thus, it appears that a major role of the folate requiring enzyme is to create an environment for switching this preference. A related reaction is the ATP-requiring carboxylation of biotin (Bruice and Benkovic, 1966) which involves a similar transfer of an oxygen atom from carbonate to the inorganic phosphate derived from ATP (Ochoa and Kaziro, 1961; Kaziro et al., 1962). Although it is doubtful that a highly strained four-membered cyclic phosphate is formed from the nitrogen and oxygen atoms of the ureido group in this reaction, the phosphoryl moiety may act in a manner similar to that illustrated in **9**, with an enzyme bound phosphorylated carbonate functioning as the carboxylating agent (Polakis et al., 1972).

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Terbium Binding to Ribosomes and Ribosomal RNA[†]

Thomas D. Barela,[‡] Stuart Burchett,[§] and Donald E. Kizer*

ABSTRACT: Terbium binding to rat liver ribosomes and ribosomal RNA (rRNA) was examined by equilibrium dialysis and fluorescence spectroscopy. Upon binding to ribosomes and rRNA, the enhancement of terbium fluorescence emission at both 488 and 541 nm was dependent only upon the amount of bound terbium and independent of ionic strength. Binding profiles for ribosomes and rRNA suggested that terbium was bound to ribosomes primarily through rRNA interactions. Data suggested that terbium mimicked characteristics previously described for interactions between ribosomes and magnesium. It is proposed, therefore, that fluorescence of terbium bound to ribosomes may prove useful in studies on the nature and extent of interactions between ribosomes and magnesium.

Eucaryotic ribosomes require magnesium to maintain both structure and function (Petermann, 1964; Spirin, 1969; Maden, 1971; Haselkorn and Rothman-Denes, 1973). Ultracentrifugation studies, coupled with bound magnesium quantitation, showed sedimentation properties and protein content of ribosomes to be markedly altered by changes in bound magnesium (Petermann, 1960, 1964). Most magnesium binding involved rRNA (Edelman et al., 1960; Petermann, 1960) probably through interaction with phosphate diester linkages between adjacent ribose moieties (Wiberg and Neuman, 1957; Felsenfeld and Huang, 1959; Edelman et al., 1960). Despite the numerous functions of magnesium in protein synthesis catalyzed by ribosomes, direct studies concerning the role of magnesium have been hampered by a lack of easily examined spectral properties for magnesium.

Lanthanide metal ions recently were used to probe alkaline earth metal binding sites in proteins and nucleic acids (Darnall and Birnbaum, 1970; Luk, 1971; Smolka et al., 1971; Sherry and Cottam, 1973; Starcher and Urry, 1974; Secemski and Lienhard, 1974). The rationale for this approach was based on the abundance of easily examined spectral properties for lanthanides and a paucity of similar properties for alkaline earth metals. Lanthanide–macromolecule complexes were examined by fluorescence (Luk, 1971; Sherry and Cottam, 1973; Formoso, 1973; Kayne and Cohn, 1974), magnetic resonance (Reuben, 1971a,b; Sherry and Cottam, 1973; Valentine and Cottam, 1973; Jones and Kearns, 1974), difference spectroscopy (Birnbaum et al., 1970; Secemski and Lienhard, 1974), and circular dichroism (Smolka et al., 1971; Starcher and Urry, 1974). In several instances, lanthanide ions replaced normal alkaline earth metal ions in macromolecules and the biological function of the macromolecules was preserved. For instance,

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[‡] Present address: Medical School, University of New Mexico, Albuquerque, New Mexico 87131.

[§] Permanent address: Department of Chemistry, Southwestern Oklahoma State University, Weatherford, Oklahoma 73096.