Catalysis of Phosphoryl Group Transfer. The Role of Divalent Metal Ions in the Hydrolysis of Lactic Acid *O*-Phenyl Phosphate and Salicylic Acid *O*-Aryl Phosphates[†]

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ABSTRACT: The spontaneous hydrolyses of lactic acid Ophenyl phosphate (I) and, to a lesser extent, 3-hydroxybutyric acid O-phenyl phosphate (II) have been investigated and compared with similar intramolecular and bimolecular reactions. Compared to bimolecular nucleophilic reactions, the reactivity of II is similar to other systems involving the formation of a six-membered ring intermediate, which suggests that the electrostatic barrier to attack of an anionic nucleophile on a phosphate diester anion is fully present in II. The reactivity of I, as compared to that of II, would suggest that at least a partial overcoming of the electrostatic barrier takes place upon closer approximation of the two reacting centers. The Mn²⁺-catalyzed hydrolysis of I exhibits saturation kinetics, consistent with the enhanced reactivity of the metal ion-substrate complex. The binding constant for this complex, determined from kinetics, is in good agreement with that obtained by electron spin resonance (ESR) titration. It is argued that the complex of Mn^{2+} with II, as observed by pulsed Fourier transform nuclear mag-

 $\mathbf{P}_{\mathrm{hosphoric}}$ acid esters in biological systems have in common their relative lack of reactivity in hydrolytic and nucleophilic processes, and their association with metal ions, particularly divalent ions, in the great majority of their biochemical transformations. The direction of a growing body of work has been toward an understanding of how phosphoryl transfer reactions may be catalyzed, in an attempt to elucidate some of the mechanistic possibilities operable in enzyme-mediated reactions. Much of this research has recently been reviewed (Benkovic, 1972; Benkovic and Schray, 1973). Some recent papers from this laboratory have considered metal ion catalyzed phosphoryl transfer (Benkovic and Miller, 1972; Steffens et al., 1973; Sampson et al., 1973). In the work described here, the hydrolytic reactions of the phosphate diesters, I and II, have been investigated and compared with previously examined diesters. IIIa and IV, in an attempt to quantify propinguity and electrostatic effects in phosphoryl transfer reactions. In addition, the examination of leaving group effects on the metal ion catalyzed reaction in the series IIIa-d, in conjunction with exploration of the influence of varying metal ions on the catalyzed hydrolysis of I, permits further detailed insight into the mechanism of the metal ion catalyzed hydrolysis.

netic resonance (NMR) techniques, is a precursor to the complex of catalytic significance. The hydrolysis of I as catalyzed by a variety of divalent metal ions suggests an optimal metal ion size. The spontaneous and metal ion catalyzed hydrolyses of salicylic acid O-aryl phosphates (IIIad) proceed through cyclic acyl phosphate intermediates after expulsion of phenol. Product studies on the parent compound have failed to detect phenyl phosphate as a product in either the spontaneous or metal ion catalyzed process. The dependence of the second-order rate constant for the metal-catalyzed hydrolysis on leaving group pK_a , β_{1g} , decreases significantly relative to β_{1g} for the spontaneous hydrolysis. From the collective data a specific interaction of the metal ion with a pentacovalent intermediate is inferred in the rate-determining step for esters I and III. The probable consequences of these mechanistic postulates for phosphoryl transfer reactions in biological systems are discussed.



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Experimental Section

Ethyl Lactate O-Diphenyl Phosphate. Diphenyl phosphorochloridate (11.8 g, 0.044 mol) was added dropwise to a stirred solution of ethyl lactate (4.7 g, 0.040 mol) in anhydrous pyridine (30 ml). After 2 hr the reaction mixture was poured into ice water (100 ml), and the resulting mixture was extracted with chloroform. The chloroform solution was dried with anhydrous MgSO4, filtered, and evaporated under vacuum. The residual oil was extracted with ether, and the ether solution was again evaporated. The crude product, obtained as a clear, viscous oil, was used for subsequent synthesis without further purification. The infrared spectrum (CHCl₃) showed absorption maxima at 1737, 1587, 1382, 1273, 1177, 1156, 1030-1000 (multiple bands), and 950 $\rm cm^{-1}$, with no hydroxyl absorption. The nuclear magnetic resonance (NMR) spectrum (neat) showed absorptions (in parts per million downfield from tetramethylsilane) at 1.17 (t, J = 6 Hz, 3 H), 1.48 (d, J = 6 Hz, 3 H), 4.10 (q, J = 6 Hz, 2 H), 5.16 (m, J = 7 Hz, 1 H), and 7.3 (s, 10 H).

Lactic Acid O-Phenyl Phosphate Dipotassium Salt (I). A suspension of ethyl lactate O-diphenyl phosphate (1.0 g, 3.5 mmol) in 0.4 N KOH (35 ml) was stirred at room temperature for 30 hr. The reaction mixture was neutralized with glacial acetic acid to pH 7, and evaporated under vacuum to a volume of 2.3 ml. Cold ethanol (10 ml) was added, followed by the dropwise addition of cold acetone (15 ml), and the mixture was stored at 0° overnight. The crystalline product was filtered, washed with acetone, and recrystallized by dissolving in a minimum volume of water and adding sequentially cold ethanol (5 ml) and cold acetone (10 ml). The product (0.40 g, 35%) was filtered and dried overnight under vacuum. The NMR spectrum (D_2O) showed absorptions at 1.97 (d, J = 7 Hz, 3 H), 5.2 (m, J = 7 Hz, 1 H), and 7.7 (m, 5 H). Complete hydrolysis (72 hr in 1 NHCl, 100°) of a sample yielded a ratio of inorganic phosphate (determined by the method of Martin and Doty (1949)) to phenol (determined as phenoxide using an extinction coefficient of 2600 at 287 mµ) of 1.02 ± 0.01 . Anal. Calcd for C₉H₉O₆PK₂·0.5H₂O: C, 32.62; H, 3.09; P, 9.34. Found: C, 32.78; H, 3.03; P, 9.74.

Ethyl 3-Hydroxybutyrate O-Diphenyl Phosphate. This ester was prepared analogously to the lactate ester. The NMR spectrum (neat) showed absorptions at 1.10 (t, J = 7 Hz, 3 H), 1.37 (d, J = 6 Hz, 3 H), 2.65 (d, J = 7 Hz, 2 H), 4.02 (q, J = 7 Hz, 2 H), 5.26 (m, J = 7 Hz, 1 H), and 7.28 (s, 10 H); infrared (ir) (chloroform) 1727, 1587, 1383, 1276, 1188, 1005, and 955 cm⁻¹.

3-Hydroxybutyric Acid O-Phenyl Phosphate Dipotassium Salt (11). The compound was prepared by the hydrolysis of the ethyl ester diphenyl phosphate (3.0 g, 7.8 mmol) in 0.8 N KOH (25 ml) for 84 hr. The product was worked up as above, and was obtained as white needles (0.60 g, 20%) after three recrystallizations from water-ethanol-acetone. The NMR spectrum (D₂O) showed absorptions at 1.33 (d, J = 6 Hz, 3 H), 2.5 (m, 2 H), 4.75 (m, J =7 Hz, 1 H), and 7.3 (m, 5 H). Anal. Calcd for C₁₀H₁₁O₆PK₂·H₂O: C, 33.89; H, 3.70; P, 8.74. Found: C, 33.62; H, 3.77; P, 8.68.

Preparation of Phosphate Esters (IIIa-d). Cyclic esters (Va, b, and d) were synthesized by standard methods (Khan et al., 1970). Vc was synthesized by the reaction of p-cyanophenol with 2-chloro-1,3,2-benzodioxaphosphorinane-2,4-dione in benzene, in the presence of a stoichiometric amount of pyridine. After filtration and removal of sol-

vent from the filtrate, the product was purified by distillation at 210° (0.05 mm): ir (CHCl₃) 2230 and 1786 cm⁻¹ with no OH absorption. Anal. Calcd for $C_{14}H_8NO_5P$: C, 55.83; H, 2.68; N, 4.65. Found: C, 54.98; H, 2.97; N, 4.29.

Redistillation was not successful in improving the purity, but the material appeared suitable for kinetics. Va, labeled in the phenoxy group with ¹⁴C and having a specific activity of 7.7×10^7 cpm/mmol, was prepared as above. The esters IIIa-d were generated from Va-d in situ (see section on Kinetics).

The dipotassium salt of IIIa was prepared in analytically pure form by dissolving Va (0.53 g, 2 mmol) in dioxane (10 ml) to which water (2 ml) was added. KOH (1 N; 4.3 ml) was added after 15 min and the solution was evaporated at room temperature under vacuum. The white solid residue was taken up in water (3 ml); ethanol was added to achieve homogeneity followed by acetone to the point of crystallization. The mixture was kept at 0° overnight; then the product was filtered, washed with acetone, and recrystallized in the same manner; yield, 0.50 g. Anal. Calcd for $C_{13}H_9K_2O_6P_{2}H_2O$: C, 39.29; H, 3.05; P, 7.80. Found: C, 38.86; H, 2.92; P, 7.84.

o-Carbohydroxamidophenyl Phosphate (VI). A solution of methyl salicylate (60 g, 0.04 mol) and pyridine (9.4 g, 0.12 mol) in ether (30 ml) was added dropwise to a stirred ice cold solution of $POCl_3$ (6.2 g, 0.04 mol) in ether (50 ml). After 3 hr water (25 ml) was added dropwise to the stirred mixture, maintained at 0°. The aqueous layer was separated and washed with ether. Barium acetate (1.0 M; 40 ml) was added, and the cold mixture was filtered through Celite. Ice cold ethanol was added in small portions to the filtrate to precipitate the product. The predominately gelatinous precipitate was centrifuged, washed with ethanol and ether, and dried under vacuum; yield, 3.12 g. The barium salt of o-carbomethoxyphenylphosphoric acid in water (20 ml) was treated with Dowex 50W-X8 (H⁺ form) in small portions until solution was complete. The mixture was filtered and hydroxylamine hydrochloride (0.7 g) and concentrated ammonia (2.0 ml) were added. The mixture was stirred overnight and filtered and the filtrate adjusted to pH 6 with glacial acetic acid. Barium acetate (1.0 M; 8.0 ml)was added, and the mixture was filtered. The product was precipitated with ethanol and worked up as above. The barium salt was mixed with water (10 ml) and treated with the Dowex 50W-X8 (H⁺ form). The mixture was filtered, and the filtrate was neutralized with 1 N KOH to pH 6, then evaporated under vacuum. The residue was twice recrystallized from water by the addition of ethanol and acetone; yield, 0.13 g. The product contained both phosphate and acyl hydroxamate groups, as evidenced by the coincidence of paper chromatographic spots active to both Haynes-Isherwood reagent and Fe³⁺ sprays. However, it appeared to be contaminated by a minor amount of salicyl phosphate (see section on Products).

Kinetics. Boiled doubly distilled deionized water was used throughout. Divalent metal perchlorates were obtained from Ventron Corporation, Alfa Products. pH-rate studies were performed at $35.0 \pm 0.1^{\circ}$, $\mu = 1.0$ (KCl). The buffers used were HCl (pH 0-2), glycine (pH 2.1-2.9), methoxy acetate (pH 2.8-3.7), acetate (4.2-5.2), phosphate (pH 6.0-7.0), Tris (pH 7.5-8.5), and carbonate (pH 9.5). In the pH region below 6 it was necessary to extrapolate rates to infinite buffer dilution, in order to eliminate small contributions from general buffer catalysis. Unless otherwise specified, the kinetic studies in the presence of divalent metal ions were performed at 35.0 \pm 0.1°, $\mu = 0.20$ (NaClO₄), pH 6.1-6.9 (Pipes buffer, obtained from Sigma)¹ or pH 5.2 (acetate), at metal ion concentrations of 0-0.20 M. Kinetics were performed by an addition of a 0.20-ml aliquot of an aqueous stock solution $(5.0 \times 10^{-3} M)$ of I, II, or IIIa or a dioxane stock solution of Vb-d to 20 ml of preequilibrated buffer solution, to give an initial substrate concentration of 5×10^{-4} M. Generation of IIIb-d from the respective cyclic anhydride is rapid; in the case of IIIa, kinetic runs commencing with the potassium salt of IIIa or the cyclic anhydride Va give identical results. Aliquots of 1 ml were removed periodically, added to 1 or 2 ml of 1 N NaOH, and centrifuged when necessary to remove metal hydroxide precipitates. Optical density was measured at a wavelength appropriate for the phenoxide released and first-order rate constants were obtained from plots of $-\log (OD_{\infty} - OD_{1})$ vs. time. Kinetics in hydroxylamine buffer (0.67 M, pH 7.2) were performed as above to determine the rate of phenol liberation, as well as monitored by the method of Stadtman (1957) to determine the rate of acyl phosphate formation

Titrations. pK_a determinations were performed on a Radiometer autotitrator, at an ionic strength of 1.0 and 35° using standard methods (Albert and Serjeant, 1971; Speakman, 1940). Manganese binding studies were done at 23° using a Varian E-4 electron spin resonance (ESR) spectrometer, by titrating a 1.0 × 10⁻⁴ M solution of Mn²⁺ with a concentrated solution of I. The substrate solution was prepared by passing it through a column of Chelex (K⁺ form).

NMR Experiments. Measurements of ³¹P and ¹³C relaxation rates were made by Fourier transform NMR using a JEOL PS-100-FT spectrometer. Experiments were performed at 6° (³¹P) and 20° (¹³C) using samples containing 1.0 M substrate in D₂O, the solvent deuterium serving as the heteronuclear lock. T_1 measurements were carried out using a 180°- τ -90° pulse sequence. Solutions of substrate were passed through a column of Chelex (K⁺ form) immediately prior to the NMR experiments.

Product Studies. Descending paper chromatography was performed using Schleicher and Schuell 589 Orange Ribbon C paper with 2-propanol-ammonia-water (6:3:1) as the developing solvent. After drying, the chromatograms were spraved with 5% ferric chloride in 0.1 N HCl (for development of salicylate active spots) or with Haynes-Isherwood reagent followed by drying at 90-100° for 8 min and visualization with long-wavelength ultraviolet light (for development of inorganic phosphate and phosphate ester spots). Salicylic acid, which has an absolute R_f of 0.79 in this system, was consistently run as an internal standard on each chromatogram. The following are the compounds of interest and their R_f values relative to salicylic acid: inorganic phosphate, 0.11; o-carbohydroxamidophenyl phosphate, 0.26; salicyl phosphate, 0.37; phenyl phosphate, 0.53; salicylhydroxamic acid, 0.76; IIIa, 0.91.

Column chromatography of reaction mixtures was performed on DEAE-cellulose by batchwise elution with ammonium carbonate solution. Phenol was eluted with 0.01 M, salicylate and phenyl phosphate with 0.50 M, and inorganic phosphate with 1.0 M solution. Inorganic phosphate and phosphate esters were detected by the method of Bartlett (1959). Aliquots of 1.0 ml from the column eluate were combined with 3.0 ml of water and 10.0 ml of Insta-Gel Table I: Rate Constants for the Hydrolysis of the Dianions of the Phosphate Esters I-IV.



FIGURE 1: pH-rate profile for the hydrolysis of 1, 35°, $\mu = 1.0$ (KCl). The solid line is calculated from eq 3.

(Packard) and counted on a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiencies were determined by the addition of standard $[^{14}C]$ toluene. The presence of variable amounts of ammonium carbonate in the eluate caused no quenching of the samples.

Results

The hydrolysis of lactic acid O-phenyl phosphate (I) proceeds with the stoichiometric release of 1 mol of phenol over the entire pH region investigated (pH 1-10), and is pH independent in neutral and alkaline solutions. The rate of spontaneous hydrolysis in neutral solution is essentially identical with that of other phenyl phosphate diesters which are thought to proceed through the intermediacy of fivemembered and constrained six-membered ring intermediates (Table I). The hydrolysis of the similar diester, 3-hydroxybutyric acid O-phenyl phosphate (II), is considerably slower, and it was necessary to conduct kinetic runs at 75° in order to observe reasonable rates.

The intermediacy of an acyl phosphate species in the hydrolysis of I was demonstrated by carrying out the reaction in 0.67 *M* hydroxylamine buffer (pH 7.2) where the rate constant for formation of acyl hydroxamate (3.6×10^{-4} min⁻¹), as detected by the FeCl₃ assay (Stadtman, 1957), is equivalent within experimental error to the rate constant for release of phenoxide (3.2×10^{-4} min⁻¹) measured at 287 m μ . The fourfold rate enhancement in this buffer system is undoubtedly due to nucleophilic attack by hydroxylamine on the phosphate ester. Similar results were observed in trapping experiments involving the hydrolysis of IV (Sampson et al., 1973). The hydrolysis of esters (IIIa, b, and d) has been described previously and similarly proceeds through the formation of an intermediate acyl phosphate upon expulsion of phenoxide (Khan et al., 1970).

The pH-rate profile for the hydrolysis of I is shown in Figure 1. The minimal scheme necessary to describe these data is:

$$\stackrel{k_{\mathrm{H}}a_{\mathrm{H}}}{\longleftarrow} \operatorname{SH}_{2} \stackrel{Ka_{1}}{\longleftarrow} \operatorname{SH}^{-} \stackrel{Ka_{2}}{\longleftarrow} \operatorname{S}^{2-} \stackrel{k_{3}}{\longrightarrow}$$
(1)
$$\downarrow k_{1} \qquad \downarrow k_{2}$$

¹ Abbreviation used is: Pipes, piperazine-N,N-diethylsulfonate.

Table II: Rate and Dissociation Constants for the Hydrolysis of Lactic Acid *O*-Phenyl Phosphate, 35° , $\mu = 1.0$ (KCl).

Constant	Value	Method of Determination
pK _a	1.85 ± 0.07	а
pK_a	3.91 ± 0.05	а
k _H ¹	$4.9 \pm 0.1 \times 10^{-2} M^{-1} \min^{-1}$	b
k_1	$1.3 \pm 0.1 \times 10^{-3} \text{ min}^{-1}$	b
k_{2}	$1.9 \pm 0.9 \times 10^{-4} \text{ min}^{-1}$	b
k_3	$1.2 \pm 0.1 \times 10^{-4} \text{ min}^{-1}$	С

^a Potentiometric titration. ^b Multiple regression analysis. ^c From region of pH-rate profile independent of pH.

Table III: Rate Constants for the Spontaneous and Zn²⁺-Catalyzed Hydrolysis of I, II, and IIIa-d, $\mu = 0.20$ (NaClO₄).

Compd	$k_0 (\min^{-1})$	$k_{Zn} (M^{-1} \min^{-1})$	pН	<i>T</i> (°C)
I	8.90 × 10 ⁻⁵	0.55 ± 0.02	6.5 <i>a</i>	35
I	4.7×10^{-4}	0.047 ± 0.002	2.5^{b}	35
II	8.60×10^{-6}	0.021 ± 0.002	6.2 <i>a</i>	75
IIIa	$1.38 \pm 0.60 \times 10^{-4}$	0.159 ± 0.008	6.1^{a}	35
IIIb	$1.62 \pm 0.29 \times 10^{-2}$	5.46 ± 0.44	6.1 <i>a</i>	35
IIIc	$4.23 \pm 0.25 \times 10^{-2}$	4.46 ± 0.29	6.1 <i>a</i>	35
IIId	$2.55 \pm 0.08 \times 10^{-1}$	11.7 ± 1.2	6.1 <i>a</i>	35

from which may be calculated the observed rate constant, k_{obsd} , from the equation:

$$k_{\text{obsd}} = \frac{k_{\text{H}}a_{\text{H}}^3 + k_1a_{\text{H}}^2 + k_2Ka_1a_{\text{H}} + k_3Ka_1Ka_2}{a_{\text{H}}^2 + Ka_1a_{\text{H}} + Ka_1Ka_2}$$
(2)

The dissociation constants, Ka_1 and Ka_2 , were evaluated at 35° from pH titration data by the method of Speakman (1940), and the rate constant, k_3 , could be obtained from the pH-independent region above pH 6. From these data the rate constants, $k_{\rm H}$, k_1 , and k_2 , could be evaluated from the kinetic data by multiple regression analysis (Bevington, 1969) using a rearranged form of eq 2:

$$\frac{k_{obsd}a_{H}^{2} + Ka_{1}k_{obsd}a_{H} + Ka_{1}Ka_{2}k_{obsd} - k_{3}Ka_{1}Ka_{2}}{a_{H}} = \frac{a_{H}}{k_{H}a_{H}^{2} + k_{1}a_{H} + k_{2}Ka_{1}}$$
(3)

The dissociation constants and rate constants obtained in this way are given in Table II. No differences in rate were observed when lithium perchlorate or guanidinium chloride was used as the inert salt in place of sodium perchlorate.

The rates of hydrolysis of I and II are dramatically increased by the presence of Zn^{2+} (Table III), as well as by a variety of other divalent metal ions (Table IV). In all cases the reactions proceed with the liberation of 1 mol of phenol. The rate constant for release of phenol from I (5.3×10^{-3}) min^{-1} at 0.020 M Zn²⁺) is in fair agreement with the rate constant for formation of acyl phosphate (4.5 \times 10⁻³ min^{-1}) under the same conditions with the exception of a fourfold higher initial substrate concentration (2.0×10^{-3}) M). Maximal metal ion catalysis is observed in the pH region associated with the dianionic form of I (Table III). Although the pH-rate profile for the metal ion catalyzed reaction was not defined in detail, at least half of the effects observed at pH 2.5 can be explained in terms of the metal ion catalyzed hydrolysis of the dianion, present to the extent of 4% of the total substrate at this pH. With the exception of

Table IV: Apparent Second-Order Rate Constants for the Metal Ion Catalyzed Hydrolysis of Lactic Acid *O*-Phenyl Phosphate, 35° , $\mu = 0.20$ (NaClO₄).

Metal Ion	Ionic Radius (A)	$k_2 (M^{-1} \min^{-1})$	pН
Ba	1.35	0.00157 ± 0.00028	6.9
Са	0.99	0.0140 ± 0.0012	6.9
Mg	0.65	0.0221 ± 0.0009	6.9
Ni	0.69	0.103 ± 0.003	6.9
Со	0.72	0.206 ± 0.004	6.2
Zn	0.74	0.547 ± 0.023	6.8
Mn	0.80	0.204 ± 0.006	6.8
Cu	0.92	9.01 ± 0.18	5.2



FIGURE 2: Mn^{2+} -catalyzed hydrolysis of l, 35°, pH 6.2 (Pipes), $\mu = 1.0$ (NaClO₄). The solid line is calculated from eq 5.

the studies in hydroxylamine buffer, most metal ion studies were performed at low ionic strength ($\mu = 0.20$) and low metal ion concentration (0-0.02 *M*), and the rate constants are reported as second-order coefficients. However, data obtained for the Mn²⁺-catalyzed hydrolysis of I at 1.0 ionic strength clearly demonstrate that a saturation phenomenon is involved, as illustrated in Figure 2. These data may be interpreted in terms of the typical saturation scheme:

$$\stackrel{k_0}{\longleftarrow} S + M \stackrel{K}{\Longrightarrow} C \stackrel{k_M}{\longrightarrow}$$
(4)

from which the observed rate constant may be calculated according to the equation:

$$k_{\text{obsd}} = \frac{k_0 + k_{\text{M}} K[\mathbf{M}]_{\text{T}}}{1 + K[\mathbf{M}]_{\text{T}}}$$
(5)

where $[M]_T$ represents the total metal ion concentration. Analysis of the data in Figure 2 by multiple regression techniques yielded values of k_M and K equal to $1.2 \pm 0.4 \times 10^{-2}$ min⁻¹ and $11 \pm 3 M^{-1}$, respectively. This binding constant compares favorably with the value of $20 \pm 6 M^{-1}$, determined at 23° under conditions of varying ionic strength, by ESR titration of a $1.0 \times 10^{-4} M$ solution of Mn^{2+} with the dipotassium salt of I.

The hydrolysis of IIIa and IIIb in the presence of 0.02 MZn²⁺ likewise proceeds via the cyclic acyl phosphate intermediate. In the case of IIIa, salicyl hydroxamate is obtained as the product arising from trapping of the intermediate (VII) by hydroxylamine at 10-20% reaction and at completion for the Zn²⁺-catalyzed hydrolysis (eq 6; Table V); in the case of IIIb, the appearance and disappearance of the acyl phosphate species in the metal ion catalyzed reaction may be directly monitored at 246 m μ . The identity of the hydroxamate was established by chromatographic compari-

Table V: Reaction	Products f	from the l	Metal I	lon Catalyzed
Hydrolysis of IIIa,	35°, pH 6	.1, 0.02 M	/ Zn ²⁺	-

Conditions	Products
Pipes buffer, 1 hr	IIIa, salicyl phosphate
Pipes buffer, 24 hr	Salicyl phosphate, salicylic acid, inorganic phosphate
Hydroxylamine buffer, 1 hr	IIIa, salicyl hydroxamate
Hydroxylamine buffer, 24 hr	Salicyl hydroxamate
$\begin{array}{c} C_{e}H_{5}OH \\ + \\ IIIa \longrightarrow \bigcup_{\substack{O \\ O \\ O \\ VII}} O \\ VII \end{array}$	(6)

son to the hydroxamate (VI) synthesized via hydroxylaminolysis of o-carbomethoxyphenyl phosphate. Possible formation of phenyl phosphate in either the spontaneous or metal ion catalyzed reaction of IIIa was investigated by employing the labeled starting material, O-phenyl salicyl phosphate-U-phenyl-1⁴C, under conditions of analysis where phenyl phosphate would have been separated and observed at the level of 1%. In either case the only radioactive material in the product mixture is phenol; thus, one concludes the products of the metal ion catalyzed and spontaneous reaction are identical within limits of detection.

The spontaneous and Zn^{2+} -catalyzed rate constants for hydrolysis of IIIa-d at pH 6.1 are given in Table III and plotted as a function of leaving group pK_a in Figure 3. Owing to the high metal ion concentrations required to achieve saturation, the rate constants are reported as second-order coefficients. Given the proviso that the substituents do not perturb the binding constants of Zn^{2+} with the substrates to any appreciable extent, the second-order rate constants may then be internally compared. At pH 6.1, $k_{obsd} \simeq k_3$ so that hydrolysis of the dianionic species effectively accounts for the hydrolytic reaction.

Calculations of internuclear distances in the complex of I with Mn^{2+} were carried out as described by Mildvan and Cohn (1970) and Mildvan and Engle (1972). The identity of $1/pT_{1p}$ (the paramagnetic contribution to the longitudinal relaxation rate) and $1/T_1M$ (the longitudinal relaxation rate in the metal ion coordination sphere) was assumed. The correlation time of the metal ion-substrate complex was taken as the tumbling time of the complex, derived from Stokes' law:

$$\tau_{\rm r} = 4\pi \eta a^3 / 3kT \tag{7}$$

where η is the viscosity of the medium and *a* is the radius of a rigid sphere, taken as 6.8 ± 0.8 Å as estimated from molecular models. The viscosities of the NMR samples were compared with those of pure water at the two temperatures, and were found to be identical.

The distance, r, in angstroms from the nucleus in question to the paramagnetic ion was calculated from the equation:

$$r = C[T_{1M}f(\tau_{c})]^{1/6}$$
(8)



FIGURE 3: Second-order rate constants for the spontaneous (\bullet) and Zn²⁺-catalyzed hydrolysis of IIIa-d as a function of leaving group pK_{a} .

Table VI: Internuclear Distances in Mn^{2+} -Lactic Acid *O*-Phenyl Phosphate Complex.

$1/pT_{1p}$ (sec ⁻¹)	$\tau_{\rm c}$ (sec)	r (Å)
$2.39 \pm 0.15 \times 10^{-4} \\ 1.67 \pm 0.37 \times 10^{-3}$	$\begin{array}{c} 3.2 \pm 1.1 \times 10^{-10} \\ 5.1 \pm 1.7 \times 10^{-10} \end{array}$	4.0 ± 0.2 6.9 ± 0.4
	$\frac{1/pT_{1p} (\sec^{-1})}{2.39 \pm 0.15 \times 10^{-4}}$ 1.67 ± 0.37 × 10^{-3}	$\frac{1/pT_{1p} (\text{sec}^{-1})}{2.39 \pm 0.15 \times 10^{-4}} \qquad \frac{\tau_c (\text{sec})}{3.2 \pm 1.1 \times 10^{-10}} \\ 1.67 \pm 0.37 \times 10^{-3} \qquad 5.1 \pm 1.7 \times 10^{-10}$

where $C = 601 ({}^{31}\text{P})$ or 512 (${}^{13}\text{C}$) and $f(\tau_c)$ is given by the equation:

$$f(\tau_{o}) = \frac{3\tau_{o}}{1 + (3.94 \times 10^{13})v_{I}^{2}\tau_{o}^{2}} + \frac{7\tau_{o}}{1 + (1.71 \times 10^{19})v_{I}^{2}\tau_{o}^{2}}$$
(9)

where v_I is the NMR frequency in megahertz. Deviations in the calculated values for r arise from the uncertainty in the a value. Coordination (1:1) between metal ion and ligand was assumed. The data from the calculations are summarized in Table VI.

Discussion

The hydrolytic reactions of phosphate diesters are extremely slow, the half-life for hydrolysis of diphenyl phosphate being greater than 1000 years under neutral conditions (Kirby and Younas, 1970a,b). Although a similar lack of reactivity is exhibited in bimolecular nucleophilic reactions, a number of examples have recently appeared which demonstrate remarkable intramolecular nucleophilic catalysis in suitably substituted phosphate esters (Khan et al., 1970; Bromilow et al., 1971, 1972; Sampson et al., 1973). Analogously, the spontaneous hydrolysis of I under neutral or alkaline conditions is at least 106-fold more rapid than that of diphenyl phosphate. The hydrolysis of the lactate model proceeds with the stoichiometric release of phenol under all conditions (pH 1-10), with the concomitant formation of an intermediate cyclic acyl phosphate, as demonstrated by hydroxylamine trapping experiments. Thus, the mechanism of the reaction may be postulated as shown in eq 10, wherein is included the formation of a pentacovalent



intermediate. Such pentacovalent species are generally thought to occur in intramolecular reactions of phosphate diesters (vide infra), whereas, for bimolecular reactions of these compounds, the evidence is suggestive, but by no means conclusive, that a single step in-line displacement mechanism is involved (Khan et al., 1970; Benkovic and Schray, 1973). In the latter case the pentacovalent phosphorus would have only a transition state lifetime. Presumably the trapping experiments do not involve an acyclic acyl phosphate. The poorer leaving group tendencies of the alkoxide vs. phenoxide ion make this an unlikely intermediate.

The hydrolysis of II is much slower than that of I, and it was necessary to run kinetic experiments at 75° in order to observe appreciable rates. This model is greater than 1000 times less reactive than I, although it still hydrolyzes at least 1000 times more rapidly than diphenyl phosphate. The bimolecular rate constant for nucleophilic attack of acetate on diphenyl phosphate or methyl phenyl phosphate has not been determined, but an estimate from published linear free-energy relationships (Kirby and Younas, 1970a,b) would be $10^{-10} M^{-1} \min^{-1}$ at 35°. The ratio of the rate constant for II to that for the bimolecular reaction is $10^3 M$, comparable to intramolecular efficiencies in acyl transfer reactions involving six-membered ring transition states (Gaetjens and Morawetz, 1960; Bruice and Benkovic, 1963). Thus, it would appear that the electrostatic barrier to attack by an anionic nucleophile is as large in the intramolecular reaction of II as it is in the bimolecular reaction. In other words, the electrostatic work involved in bringing the nucleophile to the vicinity of the phosphoryl group is insignificant, compared to the work involved for the ensuing nucleophilic attack.

The ratio of reactivities of I and II, however, is markedly different from the factor of 2.5-150 observed for five-membered vs. six-membered ring transition states in acyl transfer reactions (Gaetjens and Morawetz, 1960; Bruice and Benkovic, 1963). It is possible that the additional rate factor may be accounted for by at least a partial overcoming of the electrostatic barrier. It may be calculated that the rate of a chemical reaction in water will change by about 100fold for a unit change in charge of the reacting species, depending on the reaction volume one assumes, given no change in the enthalpy of activation (Laidler, 1965). This simple argument considers only entropy effects: changes in charge will produce differences in solvation of the reactants, and presumably of the transition state as well. It is possible that an unpredictable contribution to the activation enthalpy and entropy results from, for example, dipolar effects in the transition state.

Electrostatic effects which have been reported in the literature vary from very small to large. If one of the charges is some distance from the reaction site, the attractive or re-

pulsive effects on the rate are usually in the range of 1.3- to 30-fold (Kirby and Jencks, 1965; Holmquist and Bruice, 1969; Loudon et al., 1974). However, the rate constants for nucleophilic attack on the carbonyl group of acetyl phenyl phosphate by formate, and the phosphate and carbonate dianions are depressed by factors of 16, 2500, and 30,000, respectively, relative to rate constants for primary and secondary amines (Di Sabato and Jencks, 1961), in part indicating the effect of multiple charge approximation. In cases where the charges are in close proximity in the transition state, the effects are appreciable. For example, the rate of reaction of acetate ion with methyl-2,4-dinitrophenyl phosphate is 830 times slower than that with pyridine (Kirby and Younas, 1970b), although it is not clear how much of the effect may be ascribed to electrostatic interactions. One is attempting to compare here the rates of formation of different phosphorus-heteroatom bonds, and this bond energy may be reflected, to an unknown and perhaps variable extent, in the kinetic processes. Perhaps the example most relevant to the present model systems is the comparison of rates of reaction of a phosphate triester with that of a phosphate diester anion, for a given nucleophile, and in particular the intramolecular reactions of VIII and IIIa (Khan et



al., 1970; Bromilow et al., 1972). For these two compounds the rate constants for expulsion of phenol differ by a factor of 120, all of which, however, cannot be attributed to electrostatics. Phosphate tri- and diesters differ in their inherent susceptibility to nucleophilic attack (Khan and Kirby, 1970; Kirby and Younas, 1970b), and the literature data are not sufficient to evaluate this effect for a phenoxide leaving group.

It would appear, therefore, that the unusually rapid rate of hydrolysis of I, relative to II, is due to a combination of normal propinquity effects, and the effect of overcoming the barrier to association of like charges, to an extent which is in accord with theoretical predictions as well as previous model studies. The application of electrostatic considerations to biological reactions must be cautionary, since the effect of a highly structured enzymic active site of lower dielectric constant, but one in which very specific charge neutralizations and hydrogen bond formations occur, remains to be appraised.

The pH-rate profile for the hydrolysis of I is identical in form with that observed for the hydrolysis of IV (Sampson et al., 1973), although in the latter case a mechanism involving P-N bond cleavage is operable under acidic conditions. It differs from the profile observed for the hydrolysis of IIIa, for which the term associated with the monoanionic form of the substrate is less by at least an order of magnitude (Khan et al., 1970). The interpretation of this term is kinetically ambiguous, although k_2 probably represents carboxylate attack on the neutral phosphate ester. The reason for the difference in relative rates of k_2 and k_3 for I and IIIa is unclear, particularly in view of the similarities of the two pH-rate profiles in all other respects.

Recent examples of metal ion catalyzed phosphoryl transfer reactions in model systems have led to a number of questions concerning the mechanisms of these reactions (Lloyd and Cooperman, 1971; Sampson et al., 1973). The present model reactions allow detailed comment on some of these. Kinetic studies with the Zn²⁺-catalyzed hydrolysis of lactic acid and 3-hydroxybutyric acid O-phenyl phosphates demonstrated that, once again, a stoichiometric release of phenol is observed, and that a similar proportional amount of catalysis is observed with both model compounds. Experiments performed in hydroxylamine buffers reveal that the mechanism involving intramolecular nucleophilic attack by the carboxylate group is the same in both the spontaneous and metal ion catalyzed reactions. The observation of saturation kinetics indicates the presence of a highly reactive metal ion-substrate complex. The substantial agreement of the formation constant for this complex, determined by ESR-monitored titration of Mn²⁺, with the constant evaluated from the kinetic data confirms the saturation model. However, the apparent association constant, determined by any method, will be the sum of all association constants for the reactive and unreactive complexes in solution. To consider the example of one reactive and one abortive complex:

$$\begin{array}{c}
\overset{k_{n}}{\leftarrow} & \mathbf{S} + \mathbf{M} \\
\overset{K'}{\leftarrow} & \mathbf{C'}
\end{array}$$
(11)

$$k_{\text{obsd}} = \frac{k_0 + k_M K[M]_T}{1 + (K + K')[M]_T}$$
(12)

Thus, from either thermodynamic or kinetic measurements it may not be possible to evaluate the binding constant associated with the reactive complex, and as a consequence to determine the true value of the rate constant associated with reaction of that complex. This is entirely analogous to the problem of abortive substrate complexation in enzymecatalyzed reactions.

The results of the pulsed Fourier transform NMR experiments suggest that the primary locus for Mn²⁺ binding to the lactic acid O-phenyl phosphate molecule is at the carboxylate, rather than the phosphate position. A distance of 4.0 Å from the carboxylate carbon is somewhat ambiguous, however, since this is greater by as much as 0.5 Å from what would be anticipated for inner sphere coordination. The observed distances may reflect an equilibration among several forms of the complex. The results are to be contrasted to the Mn-P distance of 2.8-2.9 Å in the Mn²⁺-phosphoglycolate complex, where complexation to the dianionic phosphate group is clearly demonstrated (Nowak and Mildvan, 1972). This is in accord with an association constant of 480 M^{-1} (Miller et al., 1968) for Mn²⁺ with lactic acid Ophosphate, reflecting the stronger association of the metal ion with the more tightly bound phosphate monoester ligand. With the lactic acid O-phenyl phosphate, on the other hand, the available data suggest that the Mn²⁺-phosphate and Mn²⁺-carboxylate forms of the complex would be of comparable stability (Sillen and Martell, 1964).

An advantage of the present model system is the fact that hydrolysis is catalyzed by a wide variety of divalent metal ions, which allows one to consider properties of ions with respect to their catalytic efficiency. Significantly, no correlation is observed between the second-order rate constants for the metal ion catalyzed processes and the binding constants of the respective ions with oxygen monoanion ligands such as acetate (Sillen and Martell, 1964). There is, however, an interesting correlation with the Pauling ionic radius of the metal ions, which shows a maximum effect in the neighbor-



FIGURE 4: The dependence of the second-order rate constants for the hydrolysis of I on the ionic radius of the metal ion. The data are taken from Table IV.

hood of 0.75 Å (Figure 4). The only appreciable deviation is the second-order rate constant for the Cu^{2+} -catalyzed reaction. This is not unusual, in the sense that Cu^{2+} is often the only effective metal ion, or vastly superior, in reactions of phosphate esters where metal ion effects are observed (Hofstetter et al., 1962; Murakami and Takagi, 1969; Benkovic and Dunikoski, 1971; Benkovic and Miller, 1972). The basis for the unusual efficiency of Cu^{2+} is not understood.

A correlation with metal ion radius has been observed in other systems, for example ion exchange experiments involving the elution of tervalent metal ions from ion exchange resins with phosphoric acid (Genge and Salmon, 1957), or the metal ion catalyzed bromination of β -dicarbonyl systems (Pedersen, 1948), The correlation with ionic radius immediately suggests the importance of bidentate coordination with strict geometrical requirements. For example, the rates of metal ion catalyzed bromination of 2carboethoxycyclopentanone (Pedersen, 1948), which probably proceeds through the intermediate IX, show good corre-



lation with metal ion radius, whereas decarboxylation of the sterically flexible complex X (Prue, 1952; Leong and Lister, 1972) exhibits a very poor correlation. The experimental evidence does not support a mechanism utilizing a hydroxo-

metal complex such as XI that acts through the hydroxo function either as a nucleophile or as a general base. The involvement of XI is incompatible with the observation of intermediate cyclic acyl phosphate formation in the metal catalyzed reaction. Furthermore, the hydrolysis of the *m*nitrophenyl derivative IIIb is not catalyzed by hydroxopentaamminecobalt(III) ion, at concentrations of the complex far greater than the base forms of the simple aquo metal ions which would exist in the neutral pH region investigated here (J. J. Steffens, I. J. Siewers, and S. J. Benkovic, unpublished results).

Experimental evidence derived from the spontaneous and Zn²⁺-catalyzed hydrolysis of the series IIIa-d also bears on the locus of the metal ion in the reactive complex. A Brönsted coefficient of -1.2 for the spontaneous process has been reported previously (Khan et al., 1970), and the value of -1.17 ± 0.07 (r = 0.996) reported here is in excellent agreement. In view of the fact that the β_{eq} for the equilibrium transfer of the phosphoryl moiety $[PO_3^{2-}]$ to oxygen bases is estimated at ca. -1.2 (Benkovic and Sampson, 1971) then this observation suggests that the critical transition state features nearly complete bond cleavage of the phosphorus-phenoxide bond. In addition, expulsion of the exocyclic phenoxide group always is observed, even in those cases where loss of the endocyclic phenoxide is favored on the basis of pK_a (Khan et al., 1970). A general explanation for the latter observation is the intermediacy of a pentacovalent species (XII) which undergoes phenoxide loss only from an apical position.



Equilibration of XII with XIII through pseudorotation is obviated by the substantial energy barrier for placing an oxyanion apical (Westheimer, 1968). Significantly the Brönsted coefficient is 0.7 ± 0.1 (r = 0.96) for the Zn²⁺catalyzed reaction whose products are identical with those observed for the spontaneous process as described in eq 6. Subject to the assumption of identical binding within the series it appears that considerably less charge, ca. 0.7-1.2units, is on the phenolic oxygen suggesting a transition-state structure with the metal ion proximal to leaving group, lowering the latter's charge density. This reasoning, in conjunction with the metal ion size requirements, leads to structures such as XVa and XVb, illustrated for the lactate ester, as being involved in the metal ion catalyzed reactions.

The catalytic effect of divalent metal ions presumably arises from a stabilization of the pentacovalent phosphorus intermediate (Farrell et al., 1969) and the concomitant fa-



cilitation of the breakdown of this intermediate, at least, although not necessarily exclusively, in the direction of products. The observed rate enhancement at saturating Mn²⁺ concentrations is 120-fold, and even greater for Zn²⁺ and Cu²⁺ catalysis for I, although as noted above, calculation of the actual magnitude of the catalytic effect is uncertain. Complexation of the metal ion with the phenoxide oxygen could make this a better leaving group by about 2 pK_a units (Sillen and Martell, 1964), in agreement with the observed rate acceleration. It is difficult to assess accurately the function of the metal ion to act as an electrophilic catalyst, i.e., to facilitate nucleophilic attack on the phosphoryl group simply by neutralizing its negative charge, but as argued above, the electrostatic barrier to nucleophilic attack may in large part already be overcome for I and IIIa-d. Nevertheless, one cannot unequivocally comment on the nature of the initial cyclization step, i.e. whether it involves nucleophilic attack on a phosphoryl-metal ion complex by carboxylate, analogous to the metal ion facilitated hydrolysis of carboxylate esters and amides (Kroll, 1952; Meriwether and Westheimer, 1956; Buckingham et al., 1968, 1967) or conversely to attack by the metal-complexed carboxylate group on the phosphoryl center, since the cyclization step is a preequilibrium step. The latter process likewise would lead to the postulated intermediate structures XVa and XVb and would assign a role to the thermodynamically more stable metal ion-substrate complex as determined by the NMR experiments. A mechanism involving nucleophilic attack by a carboxylate-metal ion complex has been proposed recently to rationalize the results from a study of the nickel acetate catalyzed oxidation of benzoin (Hammond and Wu, 1973).

The efficiency of metal ion catalysis, at least in the intramolecular nucleophilic reactions of phosphate diesters, thus may be attributed to stabilization by the metal ion of the pentacovalent intermediate and the respective transition states for its partitioning. A similar mechanism recently has been proposed for the Zn²⁺-catalyzed hydrolysis of oligonucleotides (Ikenaga and Inoue, 1974), where a neighboring hydroxyl group is the nucleophile in the formation of nucleotide cyclic phosphate. On the other hand the absence generally of efficient metal ion catalysis in phosphate monoester reactions is a consequence of the dissociative or metaphosphate type mechanism that is operative in the transfer of $[PO_3^{2-}]$ to a nucleophilic species in these systems rather than the associative mechanism described above (Benkovic and Schray, 1973). Since much of the driving force for the dissociative process is derived from the high electron density on the phosphoryl oxygens, coordination of these ligands to a metal ion effectively inhibits this pathway. Only in cases where specific coordination of the metal ion to the leaving group to increase its stability or to a negatively charged nucleophile to decrease unfavorable electrostatic interactions is catalysis effective. In the latter case the metal ion also may act as a template orienting the nucleophile and ester species (Lloyd and Cooperman, 1971). It is noteworthy that the metal ion interaction with the phosphoryl moiety is insufficient in the case of IIIa to alter the direction of breakdown of the intermediate so as to favor acyclic acyl phosphate (XIV) formation by catalyzing the pseudorotation of XVI to XVII where M represents the



metal ion. Despite a careful search for phenyl phosphate, which should be formed from XIV in the presence of hydroxylamine, none was found under conditions where as little as 1% would have been detected. Evidently the required pseudorotation apparently is still slow relative to direct breakdown of XVI to the normal exocylic products.

Extrapolation of the above results to phosphodiesterase enzymes suggests that a primary function of the metal ion required in these systems is catalytic and, more precisely, may involve interaction with a metastable pentacovalent intermediate particularly when two of the phosphorus ligands are constrained as in the model systems. It is possible, however, that the role of the metal ion may depend on its identity-in terms of ionic radius considerations, Mg²⁺ and Mn²⁺ but not Ca²⁺ are capable of interacting in this fashion with such intermediates. The inability to detect evidence for metal ion catalyzed pseudorotation, of course, does not eliminate its possible importance in other cases. However, viewed in terms of minimizing structural reorganization within the enzyme-substrate complex, metal ion facilitation of an apical-apical displacement process may be the more favorable pathway for enzyme-catalyzed reactions involving phosphate diesters even in those situations where a pentacovalent intermediate may be reasonably involved, i.e. ribonuclease action (Usher, 1969; Usher et al., 1970).

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Messenger RNA Complexity in Drosophila melanogaster[†]

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ABSTRACT: Complementary DNA was synthesized as a copy of polyadenylated RNA from the cytoplasm of *Drosophila* cultured cells. The kinetics of hybridization of cDNA with the RNA used as template revealed a complex distribution of frequencies in the population of polyadenylated RNA. Computer simulation suggested three frequency classes containing about 4, 190, and 6700 different RNA molecules of mean molecular weight 4×10^5 . About 15% of

During the recent debate concerning the number of genes constituting the eucaryotic genome (Ohno, 1972), particular attention has been directed toward Drosophila melanogaster. For this organism, in which the DNA content is only 5% of that of mammals, independent estimates of gene number are possible from cytological, genetic, and biochemical data (Beerman, 1972; Bishop, 1974; Lewin, 1974). The number of cytologically observable bands is between 5000 and 6000 (Bridges, 1938). Since, in limited regions of the chromosome at least, the number of chromomeres correlates well with the number of complementation groups (Judd and Young, 1973; Hochman, 1973), it is tentatively concluded that 5000-6000 is also a reasonable estimate for the total genetic potential of the organism. Other estimates of the number of genes by counting recessive lethals are in reasonable agreement (Shearn et al., 1971; Shearn and Garen, 1974). However, this line of reasoning may be criticized since mutations may not always lead to recessive lethals or to observable morphological effects.

For these reasons, it is valuable to approach the question through biochemical methodology. Molecular hybridization experiments have already established the fact that a major fraction of the *Drosophila melanogaster* genome is transcribed in larvae, pupae, and adults as well as tissue culture cells (Turner and Laird, 1973; McCarthy et al., 1973). However, these data do not bear directly on the number of genes since most of the RNA which hybridized was Hn-RNA,¹ an unknown fraction of which appears in the cytoplasm as messenger RNA. The question of the complexity of the messenger population in *Drosophila* can now be approached directly using the method of Bishop et al. (1974) this complementary DNA reacted with repetitive sequences of *Drosophila* DNA. The most frequent polyadenylated RNA is preferentially enriched in its content of repetitive sequences. Comparative experiments using cDNA synthesized as a complement of larval polyadenylated RNA demonstrated some stage specific changes in the populations of polyadenylated RNA.

in which polyadenylated messenger RNA is hybridized with complementary cDNA¹ synthesized in vitro.

Using this approach we present data concerning the complexity of mRNA in larvae, pupae, adults, and Schneider's cells, the repetitive content of this RNA, and the differences which exist among the populations of the RNA of various cells.

Experimental Section

Materials. Drosophila tissue culture cells (Schneider's cells, line 2) were grown as described elsewhere (McCarthy et al., 1973). Third instar larvae, pupae, and adult Drosophila melanogaster were kindly provided by Galvin Swift. The AMV DNA polymerase was kindly supplied by Dr. W. J. Rutter.

The radioactive materials were from Schwarz/Mann. Hydroxylapatite (DNA grade) was from Bio-Rad Laboratories.

Methods. PREPARATION OF SCHNEIDER CELLS CYTO-PLASMIC RNA. The RNA was prepared as described by Penman (1969). For a typical preparation, 1000 ml of a suspension culture of *Drosophila* cells was collected at 2000 rpm for 20 min at 4°. The cells were washed with an isotonic saline solution and resuspended in 40 ml of 10 mM NaCl-10 mM Tris-HCl (pH 8.5)-3 mM MgCl₂. One percent diethyl pyrocarbonate was added to inhibit nucleases. NP40 (Shell Oil Co.) was added to the suspension to a concentration of 0.5% and cells were lysed in a Dounce homogenizer. Nuclei were removed by centrifugation at 3500 rpm for 5 min at 0°.

The cytoplasmic supernatant was made 0.1 M in NaCl, 0.01 M in EDTA, and 0.5% in sodium dodecyl sulfate

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¹ Abbreviations used are: Hn-RNA, heterogeneous nuclear RNA; cDNA, cytoplasmic DNA; SDS, sodium dodecyl sulfate; PEB, phosphate equimolar buffer containing 1 mM EDTA; SSC, standard sa-line-citrate.