The Metal Ion Catalyzed Decomposition of Nucleoside Diphosphate Sugars†

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ABSTRACT: The metal ion catalyzed decomposition of the nucleotide diphosphate sugars, uridine diphosphate glucose, uridine diphosphate galactose, uridine diphosphate *N*-acetylglucosamine, guanosine diphosphate mannose, and guanosine diphosphate fucose (UDPGlc, UDPGal, UDPGlcNAc, GDPMan, and GDPFuc, respectively), has been studied as a function of pH. UDPGlc and UDPGal decompose readily to the 1,2-cyclic phosphate derivative of the sugar and uridine 5′-phosphoric acid (UMP) in the presence of Mn^{2+}. Under all conditions tested, UDPGal decomposes two to three times more rapidly than does UDPGlc. GDPFuc is slowly degraded to free fucose under similar conditions; the other nucleotide diphosphate sugars are stable. The rate of reaction increases with increasing hydroxide ion concentration from pH 6.5 to 7.9 and with metal ion concentration from 10 to 200 mM. Several metal ions are effective catalysts; at pH 7.5 with 20 mM UDPGal and 20 mM metal ion, the following apparent first-order rate constants ($min^{-1} \times 10^4$) were obtained: Eu^{3+}, 700; Pr^{3+}, 700; Mn^{2+}, 70; Co^{2+}, 27; Zn^{2+}, 23; Ni^{2+}, 22; Ca^{2+}, 3.0; Cu^{2+}, 2.4; and Mg^{2+}, 0. It appears that Mn^{2+} concentrations that have been used in studies with nucleotide diphosphate sugars at neutral pH can catalyze significant decomposition leading to erroneous interpretation of kinetic and incorporation experiments.

The nucleoside diphosphate sugars were first isolated by Leloir and co-workers (Caputto et al., 1950). They demonstrated that uridine diphosphate glucose (UDPGlc)[1] is unstable in alkaline solution giving rise to UMP and glucose 1,2-cyclic phosphate (Paladini and Leloir, 1952). The latter was unstable in acid yielding glucose and glucose 2-phosphate. In addition, it has been shown that UDP-apiose (Kindel and Watson, 1973) and TDP-6-deoxy-L-talose (Gabriel, 1973) decompose at pH 8.0 to the corresponding sugar 1,2-cyclic phosphate and base 5′-monophosphate. To our knowledge, no further studies of the chemical stability of these or other nucleoside diphosphate sugars have been reported, although it has been demonstrated that ATP and GTP can form 3′,5′-cyclic esters on treatment with base and divalent cations (Kimura and Murad, 1974).

In this paper, we describe the metal ion catalyzed decomposition of UDPGlc and UDPGal under neutral conditions of pH utilizing concentrations of metal ions frequently employed in assays and kinetic studies of enzymes which require a metal ion for catalysis of reactions involving nucleoside diphosphate sugars as substrates. It is shown that UDPGlcNAc and GDPMan are stable under similar conditions; GDPFuc decomposed slightly to give free fucose.

Experimental Section

Materials

UDPGlc, UDPGlcNAc, GDPMan, UMP, Hepes, and Pipes were obtained from Sigma. UDPGal was prepared by the method of Moffatt (1966). GDPFuc was a gift from Mr. John O'Connor, Michigan State University, and prepared from fucose 1-phosphate (Prihar and Behrman, 1973) according to the method of Moffatt (1966). [U-^{14}C]UDPGlc was purchased from Nuclear Dynamics. [U-^{14}C]UDPGal and [U-^{14}C] GDPFuc were products of New England Nuclear. [1-^{14}C] UDPGlcNAc and [U-^{14}C]GDPMan were purchased from International Chemical and Nuclear Corp. Galactose 1,2- and glucose 1,2-cyclic phosphate were prepared according to Piras (1963).

Inorganic metal salts and organic solvents were reagent grade and were used without further purification.

Methods

Decomposition of Nucleoside Diphosphate Sugars. The decomposition was followed by high-voltage electrophoresis using a Savant horizontal paper electrophoresis apparatus. An aqueous solution of nucleoside diphosphate sugar was mixed with buffer and the metal ion stock solution was added at the desired time. The actual concentrations of these species are given in the accompanying tables and figures. In the controls, the metal ion was omitted. After a predetermined period of incubation at 37 °C, aliquots of reaction mixture were applied to electrophoretograms previously marked and dampened with triethanolamine bicarbonate buffer, 0.05 M, pH 7.5. After electrophoresis for 45 min at 100 V/cm using picrate as a visual marker, the paper was dried, cut in strips, and analyzed for phosphate (Hanes and Isherwood, 1949), uv absorbance, or radioactivity using a Packard Model 7201 strip counter.

^{13}C NMR. Proton decoupled spectra were obtained using a Brucker WP-60 spectrometer, equipped for Fourier transform operation, with 10-mm tubes and a capillary containing D_2O to provide lock. All chemical shifts are given in parts per million relative to Me_4Si having standardized the instrument to give the literature value for carbon 1 of β-D-glucopyranose, 97.4 ppm (Walker et al., 1976).

Calculation of Rate Constants. Values of k_{obsd} were obtained from the slope of plots similar to those shown in Figure 1 in which slope = $k_{obsd}/2.303$ (Frost and Pearson, 1961). The rate constants for the decomposition of the UDPSugar-Mn^{2+}

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[1] Abbreviations used: ^{13}C NMR, ^{13}C nuclear magnetic resonance spectroscopy; GDPFuc, guanosine diphosphate fucose; GDPMan, guanosine diphosphate mannose; Me_4Si, tetramethylsilane; UDPGlc, uridine diphosphate glucose; UDPGal, uridine diphosphate galactose; UDPGlcNAc, uridine diphosphate *N*-acetylglucosamine; UMP, uridine 5′-phosphoric acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; TDP, thymidine 5′-diphosphate.

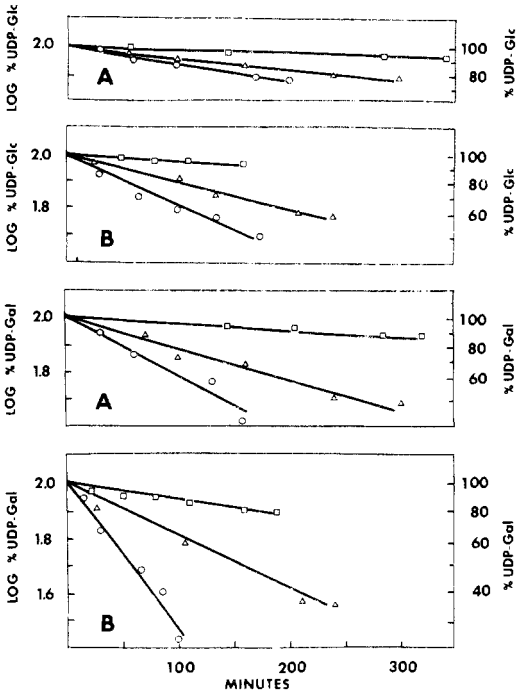


FIGURE 1: The effect of pH and Mn^{2+} concentration on the rate of decomposition of UDPGlc and UDPGal at 37 °C. The reaction mixtures contained 50 μM nucleoside diphosphate sugars, 10 mM Mn^{2+} (A) and 20 mM Mn^{2+} (B). The pH was controlled by 0.2 M Hepes at 7.9 (○) and 7.5 (△), and by 0.2 M Pipes at 7.0 (□).

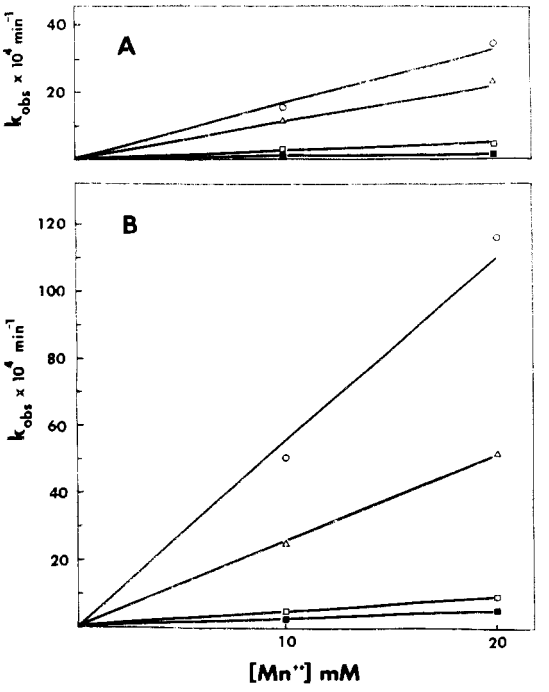


FIGURE 2: The effect of Mn^{2+} concentration on the observed rate constant for the decomposition of 50 μM UDPGlc (A) and UDPGal (B) at 37 °C. The pH was controlled by 0.2 M Hepes at 7.9 (○) and 7.5 (△), and by 0.2 M Pipes at 7.0 (□) and 6.5 (■).

complexes were calculated assuming that

$$\text{rate} = k_{obsd}[\text{UDPSugar}]_{total} = k_{complex}[\text{UDPSugar-Mn}^{2+}]$$

so that

$$k_{complex} = \frac{k_{obsd}[\text{UDPSugar}]_{total}}{[\text{UDPSugar-Mn}^{2+}]}$$

Using a known or assumed value for $K_{diss} = [\text{UDPSugar}][Mn^{2+}]/[\text{UDPSugar-Mn}^{2+}]$, the concentration of UDPSugar-Mn^{2+} complex at a given reaction time can be calculated and substituted in the first equation.

Results

Preliminary Observations. Incubation of [U-^{14}C]UDPGal or [U-^{14}C]UDPGlc (20 mM) with Mn^{2+} (50 mM) at pH 7.5 in 0.1 M cacodylate buffer at 37 °C results in the rapid formation of compounds having the electrophoretic mobility of UMP and of a phosphate diester. The latter contains all of the released radioactivity and has electrophoretic mobility approximately 0.6 that of the hexose 1-phosphates. Under these conditions, the reaction has a half-life of approximately 36 min for UDPGal and 70 min for UDPGlc.

The Effect of Mn^{2+} and pH. Using 50 μM UDPGal and UDPGlc, the effect of variation in the concentration of Mn^{2+} on the rate of decomposition at various pH values was evaluated. The data presented in Figure 1 demonstrate that the rate of decomposition is approximately first-order during the time of observation. The rate increases linearly as the Mn^{2+} concentration is increased (Figure 2) and as hydroxyl ion concentration increases (Figure 3). Rate constants were calculated from the data in Figure 1 assuming the reaction to be first-order and are presented in Table I.

In further experiments, it was shown that the rate increases approximately linearly as the Mn^{2+} concentration is increased to 200 mM at pH 7.5 (see Discussion).

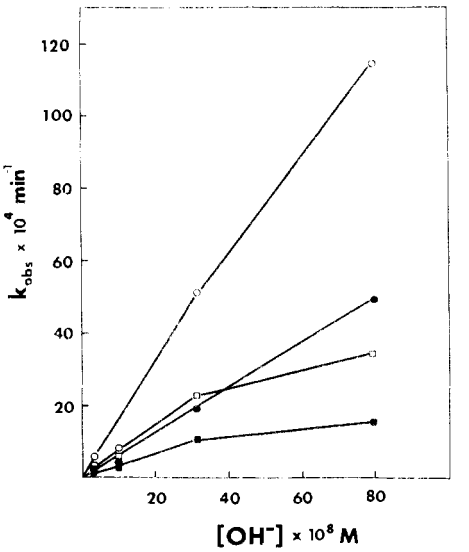


FIGURE 3: The effect of hydroxide ion concentration on the observed rate constant for decomposition at 37 °C of 50 μM UDPGlc (□) and UDPGal (○) at 10 mM Mn^{2+} (■, ●) and 20 mM Mn^{2+} (□, ○).

The Effect of Various Metal Ions. The rate of decomposition of UDPGal (20 mM) at pH 7.5 in the presence of various metal ions (20 mM) at 37 °C was determined. The values for $t_{1/2}$ and k_{obsd} are given in Table II.

Variations in Nucleoside Structure. As indicated in Figures 1, 2, and 3, the decomposition of UDPGal is more than twice as rapid as that of UDPGlc under all conditions tested. Experiments with UDPGlcNAc, GDPFuc, and GDPMan (all 20 mM) and 20 mM Mn^{2+} at pH 7.5 and 37 °C demonstrated that none of these compounds was significantly decomposed to hexose phosphate during more than 9 h. A small amount of fucose (10% of the original GDPFuc) was formed under these conditions.

TABLE I: Observed Rate Constant of Decomposition and Half-Life of UDPGlc and UDPGal at 37 °C in the Presence of Mn^{2+}.

	pH[a]	$k_{obsd} \times 10^4$ (min^{-1})	$t_{1/2}$ (h)
UDPGlc (50 μM) 10 mM Mn^{2+}	6.5	1.0 ± 0.02	115.5
	7.0	3.1 ± 0.2	37.3
	7.5	10.5 ± 1.8	11.0
	7.9	15.1 ± 1.4	7.7
UDPGlc (50 μM) 20 mM Mn^{2+}	6.5	2.2 ± 0.8	52.5
	7.0	4.2 ± 2.2	27.5
	7.5	23.0 ± 2.2	5.0
	7.9	34.2 ± 3.3	3.4
UDPGal (50 μM) 10 mM Mn^{2+}	6.5	3.4 ± 0.1	34.0
	7.0	4.8 ± 0.2	24.1
	7.5	25.3 ± 1.0	4.6
	7.9	49.8 ± 0.2	2.3
UDPGal (50 μM) 20 mM Mn^{2+}	6.5	5.6 ± 0.5	20.6
	7.0	8.4 ± 0.4	13.8
	7.5	51.3 ± 3.4	2.3
	7.9	114.0 ± 9.2	1.0

[a] The pH was controlled by 0.2 M Pipes buffer except at pH 6.5 for which it was 0.2 M Hepes.

TABLE II: Observed Rate Constant of Decomposition and Half-life of UDPGal at 37 °C in the Presence of Several Metal Ions.

	Uridine Diphosphate Galactose (20 mM)[a]								
	Mn	Mg	Ca	Ni	Cu	Co	Zn	Eu	Pr
$k_{obsd} \times 10^4$ (min^{-1})[b]	70[c]	0	3.0	22.0	2.4	26.9	23.3	700	700
$t_{1/2}$ (h)	1.7		38.5	5.3	48.1	4.3	5.0	0.17	0.17

[a] The reactions were carried out at 20 mM metal ion concentration in 0.2 M, pH 7.5, Pipes buffer. [b] These values were calculated assuming that the reaction is pseudo-first-order under the conditions used. This is probably not the case. [c] This value was obtained by interpolation from k_{obsd} at 10 and 50 mM Mn^{2+}.

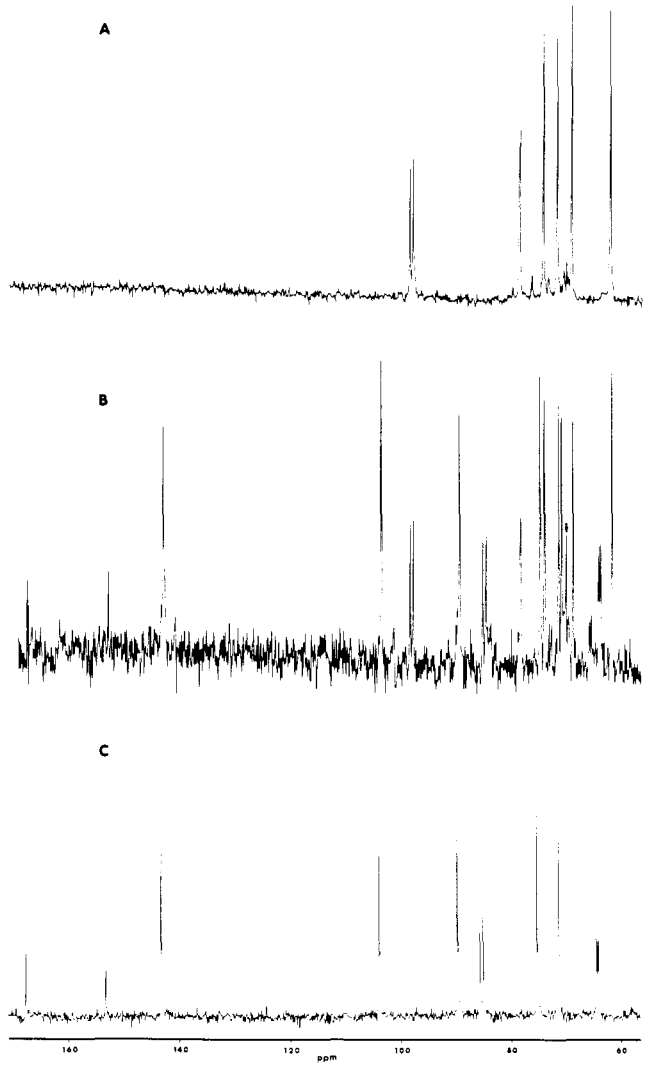


FIGURE 4: The 15.08-MHz, decoupled ^{13}C NMR spectra of (A) galactose 1,2-cyclic phosphate, (B) products of the Mn^{2+}-catalyzed decomposition of UDPGal, and (C) UMP. The arrow indicates an impurity present in the dipotassium salt of the UDPGal preparation. Chemical shifts are expressed in parts per million downfield from the resonance of tetramethylsilane.

Identification of the Products of the Decomposition of UDPGal and UDPGlc. To an aqueous solution containing 37.5 mg of UDPGal was added 150 μl of 1.0 M $MnCl_2$ to give a final volume of 3.0 ml. Aqueous sodium hydroxide (2 M) was added at intervals to maintain pH 7.5. When consumption was complete, the reaction mixture was passed over a column containing 2 ml of Chelex 100 (Na^+ form) to remove Mn^{2+}. The solution was reduced to 1.0 ml in vacuo at 40 °C and the ^{13}C NMR spectrum obtained (Figure 4B). The spectrum of UMP was obtained and the peaks due to this compound were identified in the spectrum of the reaction mixture (Figure 4C). The spectrum of authentic galactose 1,2-cyclic phosphate was also compared with that of the reaction mixture (Figure 4A). All of the peaks in the reaction mixture are accounted for by the peaks assignable to UMP (Dorman and Roberts, 1970) and the galactose cyclic phosphate (see below). The components of the reaction mixture had electrophoretic mobilities identical with those of authentic UMP and galactose 1,2-cyclic phosphate. The same methods were used to characterize the products of the decomposition of UDPGlc in the presence of Mn^{2+}.

Treatment of the reaction mixture and authentic galactose 1,2-cyclic phosphate with acid (0.1 M HCl, 5 min, 100 °C) or with alkali (1.0 M, NH_4OH, 1 h, 25 °C) resulted in conversion of the cyclic phosphate to a compound with electrophoretic mobility identical with that of galactose 2-phosphate. ^{13}C NMR and electrophoretic analysis of the products from the acid hydrolysis of galactose 1,2-cyclic phosphate indicated that the 2-phosphate and free galactose are produced in the ratio 2 to 1.

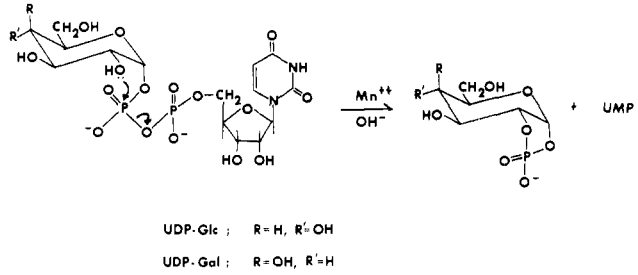
^{13}C NMR Assignments for Galactose 1,2-Cyclic Phosphate. Resonances at 98.7 ppm ($^2J_{P-C}$ = 8.8 Hz) and 62.5 ppm can be assigned to C-1 and C-6, respectively, on the basis of chemical shifts of model compounds such as methyl α-D-galactoside (Gorin, 1975) and galactose 1-phosphate (Nunez and Barker, unpublished results). A doublet at 79.0 ppm ($^2J_{P-C}$ = 2.7 Hz) is assigned to C-2; it lies 8.6 ppm downfield from that of C-2 in α-D-galactose 1-phosphate (70.4 ppm, $^3J_{P-C}$ = 7.0 Hz). The latter assignment of C-2 can be made unequivocally on the basis of the large splitting, typical of a large trans vicinal coupling in phosphate esters (Lapper et al., 1973). Resonances at 69.6, 72.1, and 74.7 ppm are attributed to C-4, C-5, and C-3, respectively. These assignments are based on

comparison with α-D-galactose (Walker et al., 1976), methyl α-D-galactopyranoside, and with galactose 1-phosphate. Relative to these standards, the C-3 resonance is significantly shifted downfield due to the proximity of the phosphate ester group in the cyclic phosphate. Vicinal coupling of P to C-3 was not observed, probably because the dihedral angle is approximately 90° leading to a small or zero value for $^3J_{P-C}$ (Lapper et al., 1973).

Discussion

All the nucleoside diphosphate sugars tested are stable in aqueous solution buffered at neutral pH values. In the presence of metal ions, only UDPGlc and UDPGal undergo decomposition by a mechanism apparently involving the attack of the 2-OH of the pyranose sugar on the β-phosphate group of the nucleotide to form a 1,2-cyclic phosphate and UMP (Scheme I). The presence and position of the hydroxyl group determine

Scheme I



whether or not the reaction can proceed. In UDPGal and UDPGlc, the pyranose ring has the 2-OH group equatorial, and the pyranosyl ring can be distorted to facilitate the formation of the five-membered ring of 1,2-cyclic phosphate. The more rapid decomposition of the galactose derivative is probably due to the presence of the axial 4-OH group which acquires a less hindered orientation in the product cyclic phosphate which must approximate a half-chair conformation. Similar effects are observed in the hydrolysis of glycosides (BeMiller, 1967). In GDPMan, the 2-OH group is axial and consequently the formation of the mannose 1,2-cyclic phosphate is not expected (Khorana et al., 1957). In the case of GDPFuc, decomposition with the formation of cyclic phosphate was not observed. The 2-OH group of α-L-fucose 1-phosphate has a similar configuration with resepct to the phosphate group of that of β-D-glucose 1-phosphate. Both sugar phosphates have been reported to give the 1,2-cyclic phosphate in the presence of dicyclohexylcarbodiimide (Prihar and Behrman, 1973; Khorana et al., 1957). It appears either that GDPFuc does not form a Mn^{2+} complex which facilitates the loss of GMP, or that the steric constraints required for formation of the cyclic phosphate are too severe. A small amount of free fucose is formed from GDPFuc in the presence of Mn^{2+}, however, which may indicate that hydrolytic cleavage of the glycosyl phosphate bond is possible in this case. It is unlikely that a 1,2-cyclic phosphate is involved as an intermediate since such compounds are hydrolyzed to give the 2-phosphate as the principal product. The latter was not observed in the reaction mixture. In UDPGlcNAc, the hydroxyl is replaced by *N*-acetyl which does not participate in the displacement.

The reaction depends on hydroxide ion concentration, the species of metal ion, and its concentration. Paladini and Leloir (1952) demonstrated that at sufficiently high pH values in the absence of metal ions, UDPGlc and UDPGal decompose according to Scheme I. We find that, at pH values as low as 6.5, decomposition can occur if an appropriate metal ion is present at a sufficiently high concentration. Under the conditions used, i.e., 50 μM nucleotide diphosphate and $Mn^{2+} > 10$ mM, the rate of decomposition appears to be first-order in nucleotide diphosphate, as shown in Figure 1. It is probable, however, that the reaction involves a complex formed in a rapid, reversible equilibrium such as:

$$\text{UDPGal} + Mn^{2+} + OH^- \rightleftarrows \text{UDPGal} \cdot Mn^{2+} \cdot OH^- \rightarrow \text{UMP} + Mn^{2+} + \text{Gal 1,2-cyclic-P} + OH^-$$

in which the stoichiometry in the complex is not specified.

Complexes of Mn^{2+} and UDPGal (Berliner and Wong, 1976; Khatra et al., 1974) and UDPGlc (Dwek, 1973) have been described previously. These were not proposed to involve hydroxide ions. If, however, it is assumed that a complex is involved in the UDPGal decomposition which has $K_{diss} = 7.5$ mM at pH 7.4, as reported by Khatra et al. (1974), rate constants for the decomposition via that complex can be computed from the data at pH 7.5 of 44 min^{-1} at 10 mM Mn^{2+} and 73 min^{-1} at 20 mM Mn^{2+} (see Methods for calculations). These values do not agree well and indicate that a complex other than that determined by Khatra et al. (1974) or Berliner and Wong (1976) may be involved in the intermediate.

This conclusion is supported by the observation that the rate of reaction increases approximately 12-fold when Mn^{2+} concentration is increased from 10 to 50 mM using 20 mM UDPGal at pH 7.5. Values of k_{obsd} are 16×10^{-4} and 190×10^{-4} min^{-1}, respectively. If a complex with $K_{diss} = 7.5$ mM was involved, the rate constant at 50 mM Mn^{2+} should be 40.5 $\times 10^{-4}$ min^{-1} relative to $k_{obsd} = 16 \times 10^{-4}$ min^{-1} at 10 mM Mn^{2+}. The $k_{obsd} = 190 \times 10^{-4}$ min^{-1} implies that the complex has much larger dissociation constants. This is borne out by the demonstration that Mn^{2+} concentrations up to 200 mM do not saturate the system. At Mn^{2+} concentrations of 50, 100, 150, and 200 mM using 50 μM UDPGal (pH 7.5), values of k_{obsd} are 220, 530, 720, and 1400×10^{-4} min^{-1}, respectively, leading to the conclusion that the association between Mn^{2+} and UDPGal responsible for the decomposition is a weak one. Plots of $1/k_{obsd}$ vs. $1/[Mn^{2+}]$ or of $1/k_{obsd}$ vs. $1/[OH^-]$ are not linear indicating that the assumption that a simple UDPGal-Mn^{2+} complex is an intermediate in the decomposition is not valid. The possibility must be considered that more than one metal ion is involved as has been shown for the ATP-Mg^{2+} complex by Fogt and Rechnitz (1974).

The results shown in Table II demonstrate that several cations effectively catalyze the decomposition of UDPGal. Similar effects are expected with UDPGlc. It should be noted that Mg^{2+}, even at high concentrations, does not catalyze the decomposition of UDPGal.

The most effective metal ions studied are Eu^{3+} and Pr^{3+} which catalyze the decomposition of UDPGal approximately ten times better than Mn^{2+} at pH 7.5. It would be of interest to know if Eu^{3+} can replace Mn^{2+} as a cofactor for the galactosyl transferase of whey. The finding that these lanthanides are effective catalysts indicates that they must be employed with caution as shift reagents in studies of sugar nucleotides and other potentially labile compounds.

Many kinetic studies of glycosyl transferases and studies involving estimates of enzyme activity have utilized concentrations of Mn^{2+}, pH values, and times of incubation similar to those used in this study (Brew et al., 1968; Morrison and Ebner, 1971; Khatra et al., 1974). It will be necessary to reassess the conclusions based on data obtained assuming that UDPGlc and UDPGal were stable. For example, the use of 40 mM Mn^{2+} at 37 °C and pH 7.5 with an incubation time of 30

min will lead to decomposition of approximately 50% of the UDPGal initially added. If the enzymatic conversion being studied results in utilization of a small proportion of the UDPGal during the incubation, the substrate concentration assumed will be seriously in error.

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Spectroelectrochemical Investigations of Stoichiometry and Oxidation–Reduction Potentials of Cytochrome *c* Oxidase Components in the Presence of Carbon Monoxide: The "Invisible" Copper†

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ABSTRACT: Spectroelectrochemical studies are presented for the carbon monoxide complex of isolated, purified cytochrome *c* oxidase (EC 1.9.3.1) in solutions saturated with carbon monoxide. The results indicate a stoichiometry of three equivalents per oxidase-carbon monoxide complex molecule. Formal reduction potentials ($E°'$) of the two copper and one heme component at pH 7.0 were obtained by means of quantitative absorbance-charge titrations in the absence and presence of cytochrome *c*, and by means of a Nernstian "Minnaert" plot in the presence of cytochrome *c*. Analysis of the absorbance-charge curves from these titrations gave an indirect determination of the high potential, "invisible" copper component. The copper potentials in the carbon monoxide complex were found to be relatively unchanged with respect to those of the native enzyme. The $E°'$ values obtained were: high potential ("invisible") copper (340 ± 20 mV (NHE)), low potential copper (190 ± 20 mV), and low potential heme (250 ± 10 mV).

Numerous papers have appeared concerning the carbon monoxide complex of cytochrome *c* oxidase (EC 1.9.3.1) since the early work of Keilin and Hartree (1939). Both equilibrium properties, such as redox potentials (Tzagoloff and Wharton, 1965; Tsudzuki and Wilson, 1971; Wilson et al., 1972; Wilson and Leigh, 1972; Lindsay and Wilson, 1972, 1974; Cusanovich and Wharton, private communication, 1973; Lindsay, 1974; Lindsay et al., 1975), and kinetic properties (Gibson and Greenwood, 1963, 1964; Greenwood et al., 1974) have been investigated.

This work was initiated in order to consider the effects of CO on the formal reduction potentials at pH 7 of the two copper and two heme prosthetic groups in cytochrome *c* oxidase. Special consideration was given to the potentials of the copper centers, and in particular, the "invisible" copper, to ascertain how they were affected by CO addition. The absorbance-charge approach (Heineman et al., 1972, 1973; Hawkridge and

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