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Preparation and Properties of Chromium(III)–Nucleotide Complexes for Use in the Study of Enzyme Mechanisms†

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ABSTRACT: Stable complexes of Cr^{3+} with various nucleotides have been prepared by heating the nucleotide with hexaaquochromium at 80° and pH 3 for 12 min and purified by ion exchange methods. Compounds prepared were CrATP, CrCTP, CrGTP, CrUTP, CrXTP, CrITP, CrADP, CrCDP, CrGDP, and CrUDP. The resulting complexes are stable at acid pH, but are hydrolyzed above pH 7. In CrATP and the other triphosphates all three phosphates appear coordinated to chromium, and the pK of the secondary hydroxyl of the γ-phosphate appears to be 2.2. In CrADP and other diphosphates both phosphates are coordinated and the secondary phosphate pK is also low. In these and other compounds mentioned below, the remaining coordination positions are presumably taken by water. CrATP shows a magnetic moment of 3.83 BM, as expected for a mononuclear chromium complex, and an axial-looking electron paramagnetic resonance spectrum with $g_{\perp} = 1.97$ and $g_{\parallel} = 4.7$. By heating chromium complexes containing ammonia with ATP or ADP, $Cr(NH_3)_2ATP$, $Cr(NH_3)_3ATP$, $Cr(NH_3)_2ADP$, $Cr(NH_3)_3ADP$, $Cr(NH_3)_4ADP$, and $Cr(NH_3)_4(ADP)_2$ have been prepared. Other chromium complexes prepared include Cr(formate)ATP (which appears to have been the "CrADP" described by Foster, D. M., and Mildvan, A. S. (1972), *Bioinorg. Chem. 1*, 133), $Cr(oxalate)_2ADP$, Cr(PPP), Cr(PP), and $Cr(PP)_2$. Since chromium nucleotides strongly and specifically inhibit a number of enzymes which have MgATP or other nucleotides as substrates, these compounds are proving very useful in binding studies and as inhibitors for kinetic analysis of enzyme mechanisms.

It is now generally accepted that for most enzymes that have nucleotides as substrates the Mg^{2+} complex rather than the free nucleotide is the active form of the substrate. For kinetic studies it is often desirable to employ dead end inhibitors which closely resemble the substrate, but do not undergo the catalytic reaction, but for enzymes with Mg nucleotides as substrates this poses a problem. Altering the base or the sugar often produces only a poorer substrate. Changing the oxygen bridge between phosphorus atoms to $-CH_2-$ produces a compound with altered bond lengths and angles, and thus often with rather weak affinity for the enzyme, although the recently synthesized –NH– bridged compounds are much closer analogs and show good promise as inhibitors (Yount *et al.*, 1971). Changing the metal to another inactive one necessitates complex calculations of the resulting metal–nucleotide equilibria and does not allow fixing the levels of free metals or of uncomplexed nucleotides.

However, if one employs a metal that forms inert coordination complexes, these difficulties should be overcome. Cr^{3+} is a paramagnetic transition metal with octahedral coordination geometry that exchanges oxygen ligands very slowly. For example, the rate of exchange of H_2O in the inner coordination sphere is $2–5 \times 10^{-6}$ sec^{-1} at 27° depending on the ionic strength; a half-time of 39–97 hr (Hunt and Plane, 1954). This exchange rate is 10^{10}–10^{13} times slower than that seen with Co^{2+}, Ni^{2+}, Mn^{2+} (Swift and Connick, 1962), or Mg^{2+} (Eigen and Wilkens, 1965). Further, dissociation of a

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chelate can be 10^3–10^6 times slower than dissociation of its individual ligands (Basolo and Pearson, 1967). Therefore, a 1:1 complex of Cr^{3+} and a nucleotide di- or triphosphate should be a stable, essentially inert, analog of the corresponding Mg^{2+}– or Mn^{2+}–nucleotide complex. Such compounds should be useful as dead end inhibitors in kinetic studies, paramagnetic probes in nuclear magnetic resonance (nmr) and electron paramagnetic (epr) studies, and, finally, isomorphic replacements in X-ray crystallographic studies of enzymes.

By using Cr^{2+} catalysis, Hunt and Early (1960) prepared a chromium pyrophosphate complex, while Foster and Mildvan (1972) made what they thought was CrADP in yields of 1–10%. However, this "CrADP" was neither a substrate nor an inhibitor for several kinases, and it now appears from its chromatographic behavior that it also contained formate in the coordination sphere. The purpose of this paper is to describe a simple method for synthesizing and purifying complexes of Cr^{3+}–nucleotides, particularly CrATP, in yields of 50–60%, and to describe the properties of such nucleotides. Since work in this laboratory has shown that CrATP is a potent inhibitor of a number of phosphoryl transfer enzymes and shows varying affinities for the enzymes relative to their affinity for MgATP, these chromium–nucleotide complexes are useful for kinetic as well as other types of studies on a number of enzymes.

Methods

Preparation of CrATP. Crystalline Na_2H_2ATP was dissolved in deionized water to give a 20 mM solution, and an equal volume of 20 mM $Cr(H_2O)_6(ClO_4)_3$ was added with mixing to give a final solution 10 mM in each reactant, pH 3.3. The solution was immersed in boiling water until its temperature reached 80° and then placed in and out of the water as necessary to maintain 80°. After 12 min at 80° the solution was cooled in ice water. The solution rapidly changes color from blue-violet to green during heating, and the final pH is 2.7.

Chemical Assays. Total chromium was determined after conversion to chromate in 0.5 M NaOH and 1.5% H_2O_2 by heating at 100° for 4 min and then cooling in ice water (Postmus and King, 1955). Absorption at 375 nm was linear with chromium concentration (ϵ 4815). For assay of Cr in electrophoresis experiments, the pieces of paper to be assayed were shaken for 1 hr in 0.5 M NaOH and centrifuged to remove fibers, and the supernatant treated as above.

Coordinated ammonia was determined by shaking the sample in a 2-ml volume containing 2.4 M NaOH and 4.8% H_2O_2 at room temperature for 2 hr in a microdistillation apparatus (Umbreit *et al.*, 1972). The NH_3 which collected in the drop of H_2SO_4 on the center rod of the apparatus was then measured by the method of Chaykin (1969).

Hexaaquochromium was measured as the turbidity of the hydroxy species at pH 8. To a solution containing 1–10 mM $[Cr(H_2O)_6]^{3+}$ was added 0.2 ml of 2 M Tris-HCl, pH 8.4, and the sample vortexed and turbidity measured by the optical density at 800 nm. In the presence of ATP the turbidity slowly cleared, and successive readings had to be extrapolated to zero time for reproducible results. A standard curve was nonlinear. All of the stable complexes of Cr are soluble at this pH and do not interfere with the assay.

Total phosphate was determined on ashed samples (Ames and Dubin, 1960) using the ascorbic acid–ammonium molybdate method (Chen *et al.*, 1956). Determination of inorganic phosphate in the presence of acid-labile compounds such as ATP was done after extraction of the phosphomolybdate complex with benzene–isobutyl alcohol (Martin and Doty, 1949). Nucleotide concentrations were measured by their ultraviolet (uv) absorbance. Ribose was determined using an orcinal reagent (Munro and Fleck, 1966) and ribose 5-phosphate as a standard.

Paper Electrophoresis. Electrophoresis of nucleotides and Cr^{3+}–nucleotide complexes was done on Whatman No. 3MM paper for 60–75 min at 50 V/cm in 0.05 M sodium citrate, pH 3.6. The tank was cooled with running water. Compounds were located either by their uv absorption or radioactivity using a Packard 7201 radiochromatogram scanner. Counting efficiency for ^{14}C was 10%. Electrophoresis patterns were the same whether or not the sample was first dried on the paper before applying citrate buffer.

Gel Filtration Chromatography. Bio-Gel polyacrylamide beads, 100–200 mesh, were equilibrated for 24 hr at room temperature in either $HClO_4$, pH 2.5, or 0.03 M *N*-ethylmorpholine, pH 8.1, plus or minus 2 M NaCl. Columns of P2 resin (200–1800 mol wt range) and P6 (1000–6000 mol wt range) were packed and run under 20 cm of water pressure. P2 columns were 1 cm × 28 cm and had a flow rate of 0.7 ml/min, while P6 columns were 1 cm × 58 cm with a flow rate of 0.5 ml/min. Samples were applied in 0.5-ml volumes. Fraction volumes were 0.5 ml for P2 columns and 1.0 ml for P6 columns.

Preparation of $[^{51}Cr(H_2O)_6]^{3+}$. $Na_2{}^{51}CrO_4$ (10 μCi of 1 Ci/g) was diluted with $Na_2Cr_2O_7$ and $HClO_4$ to give 5 μCi/μmol of Cr in 1 mM $Na_2Cr_2O_7$ and 0.1 M $HClO_4$. A 100-fold excess of methanol was added and the solution heated at 100° for 1 hr to reduce the Cr and evaporate excess methanol. When experiments were done with both [^{14}C]CrATP and [^{51}Cr]CrATP, samples dried on Whatman No. 3MM paper were first counted in a γ counter where ^{14}C was not detected, and then in a scintillation counter where 8% of the recorded γ radiation was detected and subsequently subtracted from the total counts per minute to give ^{14}C counts per minute.

Hexokinase Assay. Yeast hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer. The standard assay mixture contained 5 mM glucose, 3 mM $MgCl_2$, 0.2 mM TPN, 50 mM triethanolamine-HCl, pH 8.1, 8 μg/ml of glucose-6-phosphate dehydrogenase, and 9–12 μg of hexokinase in 3.0 ml total volume. Reaction was started by adding hexokinase and followed at 340 nm.

Results

Preparation and Purification of Cr^{3+}*–Nucleotides.* Solutions of $Cr(H_2O)_6(ClO_4)_3$ and nucleotide di- or triphosphates changed color from blue-violet to green upon heating. For a solution of Cr^{3+} perchlorate and ATP, the difference spectrum before and after heating showed maxima at 445 and 630 nm. These changes are characteristic for formation of inner sphere coordination complexes of Cr^{3+}. The perchlorate salt of Cr^{3+} was usually used since perchlorate does not form stable inner sphere complexes with Cr^{3+}; however, whenever compounds were to be purified on Dowex 1-Cl, commercial $CrCl_3$ (which is $[CrCl_2(H_2O)_4]Cl$) was used because of the very tight affinity of Dowex 1 for perchlorate. The Cl leaves the coordination sphere of Cr^{3+} during the heating process, although there is little color change since the solution is green to start with, and any Cl-containing species are removed during purification. A 100 mM solution of $CrCl_3$ aged for 2 weeks becomes hexaaquochromium and its use permits the color change from blue to green upon heating to be observed.

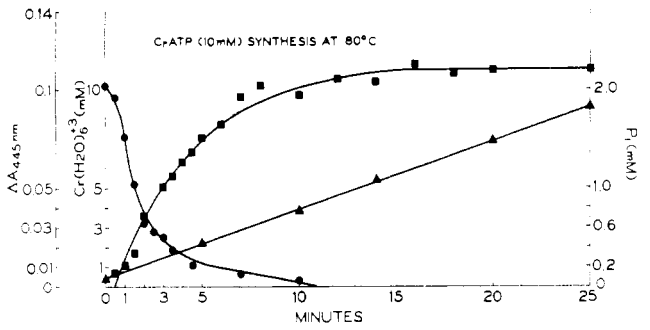


FIGURE 1: Samples of a solution containing 10 mM Cr^{3+} perchlorate and 20 mM Na_2ATP (final pH 3.2) were put in separate tubes and the reaction started by placing the tubes in an 80° bath. Tubes were removed at later times and placed in ice water to stop the reaction. Samples were then assayed for: (■) conversion to CrATP by $\Delta A_{445\ nm}$; (●) disappearance of $Cr(H_2O)_6^{3+}$; (▲) appearance of inorganic phosphate.

The synthesis of CrATP was typical of the preparation of other Cr^{3+} complexes. No reaction was detected in 1 hr at 50°. At 80°, the increase in A_{445} matched the disappearance of $Cr(H_2O)_6^{3+}$ (Figure 1). Further, paper electrophoresis at pH 3.6 of material heated for different times showed that disappearance of ATP (R_F 1.0) paralleled the appearance of a new nucleotide (R_F 0.22). This new nucleotide was 60–65% of the total [^{14}C]ATP present and was a 1:1 complex of Cr and ATP as judged by cutting out the spot and comparing the total Cr and ^{14}C. Free ATP was 10% of the total and free ADP 2%, with the rest being other presumably polymeric Cr^{3+}–nucleotide complexes (see Figures 6A and 7A).

During the first 5 min of reaction an apparent precursor (R_F 0.13) was present but rapidly disappeared. The nature of this material is unknown, but if it is a monomeric CrATP species with a normal pK for protonation of the adenine ring, the pK of the secondary phosphate is raised to 3.0 from the value of 2.2 in CrATP (see below), suggesting that the precursor may be a bidentate complex in which the β and γ, but not the α, phosphates are coordinated to Cr^{3+}. (The remainder of the six coordination positions in these and other compounds described here are presumably occupied by water.) ADP (R_F 0.78) was also present by 1 min and increased with time of heating, but AMP (R_F 0.30) was not detected until 20 min. Only about 2.5% of the acid-labile phosphate was released in 15 min at 80° (Figure 1), a maximum hydrolysis of 5% of the ATP. Variation of the ratio of ATP to Cr from 1 to 2.5 did not affect the number or position of the nucleotide spots, but did affect their relative amounts. Optimum conditions were judged by electrophoresis to be 12 min at 80° with an ATP to Cr ratio of 1.0 to 1.2.

CrATP was purified and concentrated by adsorption on a well-washed column of Dowex 50-X2 (H^-), 100–200 mesh. Unreacted nucleotides and Cr^{3+}–nucleotide complexes lacking a positive charge are not adsorbed. CrATP can be eluted from the Dowex 50 column by extensive washing with water[1] or by $HClO_4$ (0.1 M, or a gradient), but the fastest and most convenient method is elution by increased pH. When 0.1 M lithium formate, pH 3.5, is used, CrATP is focused into a concentrated band at the point in the column where the pH changes from 2 to 3.5, since the isoionic pH of CrATP is 3.0 (see below). This elution does not displace $Cr(H_2O)_6^{3+}$, and CrADP moves more slowly behind CrATP, but $Cr_3(ATP)_2$, which is formed from part of the small amount of polymeric material present in the Cr^{3+} perchlorate (Laswick and Plane, 1959), also has an isoionic point less than 3.5, and will contaminate the product unless it is removed. Fortunately, it adsorbs at the very top of the column, while CrATP, especially after some washing with water, adsorbs further down. Thus the trimer and other materials adsorbed on the top of the column can be removed by scraping off and discarding the top dark band on the column before eluting with lithium formate. Failure to take this precaution will give a product having a Cr/ATP ratio from 1.02 to 1.06. Purified CrATP contained Cr, ribose, and phosphate in a ratio of 1:1:3 and migrated during electrophoresis at pH 3.6 with an R_F of 0.22, identical with the spot seen in crude preparations.

CrATP should not be stored in formate buffer at room temperature or at 4° for more than a few days, since formate will slowly replace one or more of the remaining waters in the coordination sphere of Cr. Separation from formate and final purification are accomplished by adjusting the pH to 2, diluting at least tenfold, adsorption on Dowex 50 (H^+), washing with water to remove formate, and elution with 0.1 M aniline.[2] Again CrATP emerges as a focused band, and after immediate extraction of aniline with ether (5 × 10 vol) the product is isoionic CrATP, pH 3.0, which is stable in the icebox for weeks.[3] The solution should not be evaporated or lyophilized in its isoionic form, however, as this causes polymerization.

The same heating procedure has been used to prepare the chromium complexes of other nucleotides (initial pH of nucleotide solution must be less than 3.5). CrCTP and CrGTP are positively charged at pH 2 and can thus be adsorbed on Dowex 50-X2 (H^-) and eluted by pH focusing in the same fashion as for CrATP. Since the pK values of the base are 4.4 and 2.2 in the two cases, however, the pH of the formate used for elution should be 4.0 for CrCTP and 3.0 for CrGTP in order to be a half pH unit or so above the isoionic pH of the product. The aniline procedure also works for these compounds. CrUTP, CrITP, and CrXTP do not have a positive charge at low pH, and thus these compounds should be made by heating $CrCl_3$ and the appropriate nucleotide (avoid perchlorate when using Dowex 1), followed by dilution and adsorption on Dowex 1-X2 (Cl) or Br at pH 4 and elution with increasing levels of HCl or HBr (gradient from 0 to 0.4 M, elution at about 0.15 M). To avoid polymerization, the

[1] If washing with water is continued long enough at 4°, CrATP separates into two bands of roughly equal amounts that both have an analysis corresponding to CrATP. The visible spectra are identical. Preliminary evidence is that the two bands are slowly equilibrated even at acid pH on storage in the cold, but when freshly separated show different degrees of inhibition with hexokinase. This separation into bands in the cold was first noted by Dr. Michael Scrutton, and by Cheryl Janson in this laboratory, and has been observed only at low ionic strength. We are continuing to study this phenomenon in an attempt to discover the nature of the apparent isomers.

[2] If the top of the original Dowex 50 column is removed as mentioned above, the formate elution can be eliminated and CrATP eluted directly from the first column with aniline. This should only be done, however, if the column has been washed with sufficient water to move CrATP far enough down the column so that the top bands can be completely removed.

[3] Upon longer standing, the CrATP solution slowly deposits a small amount of a gray-green insoluble powder, which is only slightly soluble in acid, but has the expected analysis of CrATP when dissolved in base. The nature of this insoluble material is not known, but it is presumably a polymer of CrATP. The CrATP remaining in solution retains its ability to inhibit enzymes, so in practice one simply removes the insoluble material and continues to use the supernatant; this can be done for over 6 months. This process apparently is related to the low ionic strength in the isoionic solution, and it has never been seen in samples of CrATP or other Cr nucleotides which contained appreciable ionic strength.

TABLE I: Visible Spectra of Chromium Nucleotides at pH 3.[a]

Compound	λ_{max} (nm)	ϵ	λ_{max} (nm)	ϵ	Color
CrATP	430	20	610	20	Green
$Cr(NH_3)_2ATP$	403	26	565	24	Violet
$Cr(NH_3)_3ATP$	397	29	543	27	Red-violet
CrADP	428	19	598	18	Green
$Cr(NH_3)_2ADP$	404	25	555	25	Violet
$Cr(NH_3)_3ADP$	400	28	545	28	Red violet
$Cr(NH_3)_4ADP$	382	35	516	42	Red
$Cr(NH_3)_4(ADP)_2$	390	35	530	42	Red
$Cr(PP)_2$[b]	441	21	626	20	Apple green

[a] The spectra of other di- and triphosphates including CrPP and CrPPP are essentially the same as those of CrADP and CrATP. See Figures 2 and 4. [b] In 1 M HCl. There is also a peak at 283 nm (ϵ 8.6).

TABLE II: Rates of Chromium Nucleotide Breakdown at 25°.[a]

Nucleotide	k (min^{-1}) pH 7.0	pH 8.0
CrATP	0.0004	0.028
$Cr(NH_3)_2ATP$		0.013
$Cr(NH_3)_3ATP$		0.004
CrADP	0.0005	0.039
$Cr(NH_3)_2ADP$	0.0003	0.023
$Cr(NH_3)_3ADP$	0.0002	0.008
$Cr(NH_3)_4ADP$	0.0001	0.0008

[a] Breakdown of ATPs (50 μM) measured with hexokinase assay (see Methods). Breakdown of ADPs (50, 500, or 625 μM) measured with pyruvic kinase (0.003 mg/ml)–lactic dehydrogenase (0.02 mg/ml) assay containing 67 μM phosphoenolpyruvate, 100 μM DPNH, and 3 mM Mg^{2+}. Buffers used were 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.0, or Tris, pH 8.0.

resulting eluates should not be taken to dryness, but rather the mineral acid should be removed by extraction with methyldioctylamine in $CHCl_3$ or by distillation into 1 M NaOH in a desiccator *in vacuo*, and the resulting solution concentrated to a convenient volume (but not above 100 mM to avoid polymerization; the pH should also be adjusted to between 2.5 and 3) and stored in the icebox. Compounds prepared in this way do not contain Cl in the coordination sphere.

CrADP is prepared by heating Cr^{3+} perchlorate and NaADP in the same fashion as for CrATP and then adsorbing the product on Dowex 50-X2 (H^+). Having two positive charges at pH 2, it sticks tightly and, after washing with water, is eluted by a linear gradient from 0 to 1 M $HClO_4$ (coming off about 0.4 M). Gradient chromatography is necessary to avoid a small amount of a $Cr(ADP)_2$ complex which resembles CrATP in charge and is eluted at about 0.1 M H^+ and $Cr(H_2O)_6^{3+}$, which is eluted shortly after CrADP. The CrADP fractions are combined, diluted 1:10, and readsorbed on a small column of Dowex 50 (H^+) just large enough to hold the sample. After washing well with water, the CrADP is eluted with 1 M $HClO_4$, and the eluate is neutralized carefully to pH 3 with $KHCO_3$ and placed in the icebox to complete precipitation of $KClO_4$. The filtered solution is then stable if stored in the icebox.

CrADP can also be purified by pH focusing by eluting $Cr(ADP)_2$ first with 0.1 M lithium formate, pH 3.5, and then switching the pH of the buffer to 4.5 to elute CrADP. Because of the single positive charge remaining on chromium, however, formate replaces water and enters the coordination sphere quite rapidly. Incubation of CrADP in 0.1 M formate, pH 4.5, at room temperature overnight converts most of it into Cr(formate)ADP, which resembles CrATP in chromatographic behavior and was probably the "CrADP" isolated by Foster and Mildvan (1972).[4] If this method of purification is used, the eluted CrADP must be immediately adjusted to pH 2, diluted, readsorbed on Dowex 50 (H^+), and washed free of formate.

CrCDP and CrGDP have been prepared by procedures similar to that for CrADP. CrUDP resembles CrATP in charge and can be purified by pH focusing with formate, pH 3.5, but in view of the ease of substitution of the chromium diphosphates with formate, gradient elution with $HClO_4$ is preferable. The aniline procedure is not safe for diphosphates (a precipitate forms during ether extraction), and the final step in all cases should be adsorption on a small column of Dowex 50 (H^+), elution with $HClO_4$, neutralization to pH 2.5–3 with $KHCO_3$, and filtration after storage overnight in the icebox.

Preparation of Ammonia-Substituted Cr^{3+}–Nucleotides. By replacing Cr^{3+} perchlorate with a Cr^{3+} salt containing ammonia in the coordination sphere one obtains ammine derivatives of Cr nucleotides when the above procedures are used. The compounds used were: $[Cr(NH_3)_2(H_2O)_2Br_2]Br$ (Werner and Klein, 1902), $[Cr(NH_3)_3(H_2O)Cl_2]Cl$ (Werner, 1906), and $[Cr(NH_3)_4(H_2O)Cl]Cl_2$ (Pfeiffer, 1905). During the heating process the Br or Cl leaves the coordination sphere, but NH_3 remains as indicated by analysis for coordinated NH_3 in the final products. Coordination positions not occupied by NH_3 or nucleotide will contain water. $Cr(NH_3)_2ATP$ and $Cr(NH_3)_3ATP$ were purified by the normal procedure used for CrATP, including the aniline elution, to give isoionic compounds. $Cr(NH_3)_2ADP$, $Cr(NH_3)_3ADP$, and $Cr(NH_3)_4ADP$ were purified by the normal procedure for CrADP, and in the latter case, a small amount (~5% of the main peak) of $Cr(NH_3)_4(ADP)_2$ was also isolated. The spectral properties of these compounds are listed in Table I and their stabilities to hydrolysis in Table II.

Preparation of Related Compounds. $Cr(oxalate)_2ADP$ was formed by heating *cis*-$K[Cr(oxalate)_2]$ (Werner *et al.*, 1914) with NaADP in the usual fashion and chromatographing the product on Dowex 1-X2 (Cl), 100–200 mesh. Elution with 0.1 N HCl removed a small amount of green $Cr(oxalate)(H_2O)_2ADP$, followed by violet $[Cr(oxalate)_2]^-$. Green $Cr(oxalate)_2ADP$ was eluted with 0.1 M HCl–1 M LiCl, neutralized to pH 7 with LiOH, evaporated to a small volume, and crystallized by addition of ethanol. Freshly prepared material is soluble, but after some months it appears to polymerize and become insoluble. Blue $Cr(oxalate)_3^{3-}$ remains on the column and can be eluted by raising the LiCl concentration.

CrPPP can be prepared by heating PPP_i (pH adjusted to 3.5 with HCl) with $CrCl_3$. The product was adsorbed on

[4] The "CrADP" of Foster and Mildvan (1972) was eluted from Dowex 50 by 1 M formic acid ($[H^+] < 0.02$ M), while authentic CrADP requires about 0.4 M H^+ for elution. Since these authors used Dowex 50 columns preequilibrated with 1 M formic acid, it is not surprising that insertion of formate into the CrADP occurred.

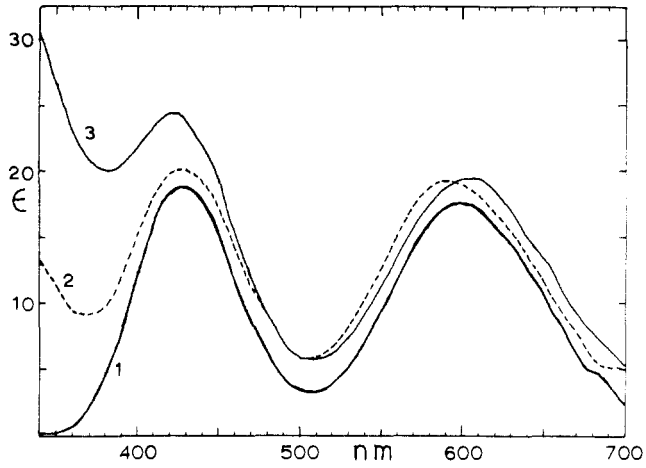


FIGURE 2: Visible spectra at pH 3 of: 1, CrADP; 2, $Cr_2(ADP)_2$; 3, $Cr_3(ADP)_2$.

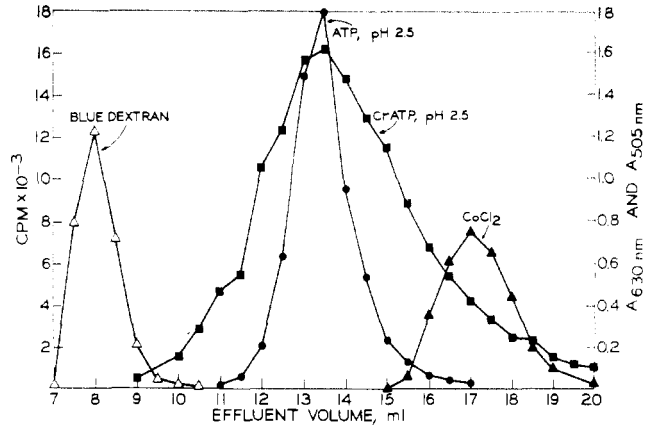
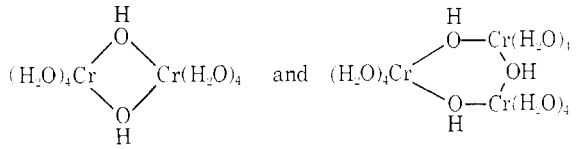


FIGURE 3: Gel filtration chromatography on Bio-Gel P2 (200–1800 mol wt range) equilibrated in $HClO_4$, pH 2.5. (△) Blue Dextran 2000 (mol wt 2×10^6) assayed by absorbance at 630 nm; (▲) $CoCl_2 \cdot 6H_2O$ (mol wt 238) assayed by absorbance at 505 nm; (●) [^{14}C]-Na_2ATP (mol wt 551); (■) [^{14}C]CrATP (mol wt 610). CrATP (1 mM) and ATP (4 mM) titrated to pH 2.5 were separately applied to the column in 0.5-ml samples. The eluent was $HClO_4$, pH 2.5.

Dowex 1-X2 (Cl) and eluted with 0.2 M HCl. The eluate was neutralized to pH 6.5 with LiOH, filtered to remove a small amount of insoluble material, and diluted with ethanol to turbidity. On standing in the icebox, a crystalline precipitate formed. A small amount of $Cr(PPP)_2$ also forms and requires 5 M HCl for elution; its spectrum resembles that of $Cr(PP)_2$ (peaks at 285, 442, 628, 646, shoulder at 680 nm), so all six coordination positions are probably not filled with phosphate.

CrPP can be prepared similarly. If an excess of PP_i is used (33 mM PP_i, 10 mM Cr^{3+}), the major product is $Cr(PP)_2$ (no $Cr(PP)_3$ was obtained) which is eluted from Dowex 1 (Cl) by 1 M HCl and gives a crystalline salt after neutralization with LiOH to pH 6.5 and addition of ethanol. Its spectrum shows the red shift expected from the presence of four phosphate ligands (Table I), as well as a shoulder on the long wavelength peak at 650 nm that is more prominent than in the di- or triphosphate spectra. The shoulder seen at 680 nm in the other spectra (see Figure 5) is also present.

Preparation of Polymeric Species. Aqueous solutions of $Cr(H_2O)_6^{3+}$ contain some dimer and trimer of presumed formulas



with charges of 4+ and 6+. A solution containing 10% of each of these can be obtained by heating 0.1 M Cr^{3+} perchlorate at 100° for 1 hr (Laswick and Plane, 1959). After cooling, the solution was passed through a small column of Dowex 50 (H^+) (the column should be small enough to become overloaded, so that most of the $Cr(H_2O)_6^{3+}$ passes through), residual hexaaquochromium was eluted with 1 M $HClO_4$ and the polymers with 4 M $HClO_4$. The latter solution was neutralized to pH 3 with K_2CO_3, placed in the icebox overnight, and filtered. The resulting solution (5.3 mM in Cr) was heated with NaH_2ADP (7.3 mM) at pH 3 in the usual fashion and adsorbed on Dowex 50-X2 (H^+). Elution with 0.4 M $HClO_4$ gave a band of $Cr_2(ADP)_2$ (7% yield), while gradient elution from 0.4 to 2 M $HClO_4$ gave a band of $Cr_3(ADP)_2$ (8% yield). The spectra of these compounds are shown in Figure 2. It should be noted that the absorbance at 340 nm appears to be a good measure of the presence of polymers of this type; the near absence of absorbance at 340 by Cr^{3+}–nucleotides prepared by methods outlined in this paper is evidence that they do not contain polymeric species.

$Cr_3(ATP)_2$ has been isolated from the material removed from the top of the first column during the purification of CrATP by elution with 0.1 M formate, pH 3.5.

Evidence on the Structure of CrATP. At low pH CrATP behaves as expected for a stable monomeric 1:1 complex of Cr^{3+} and ATP. Paper electrophoresis at pH 3.6 of freshly prepared but unpurified CrATP (Figure 6A) showed only one major peak which contained a 1:1 complex of CrATP that accounted for 60–65% of the original nucleotide. A smear of more anionic material was also present. Electrophoresis of an unheated solution of Cr^{3+} perchlorate and ATP gave a single peak of ATP with 100% recovery.

Descending chromatography of freshly prepared but unpurified CrATP on Whatman No. 1 paper in isobutyric acid, 1 M NH_3, and 0.1 M EDTA (100:60:1.6) also gave a single major peak containing CrATP (R_F 0.54) that migrated between ADP (R_F 0.48) and AMP (R_F 0.65). In addition, a trailing smear of more anionic material similar to that seen in electrophoresis was present.

Gel filtration chromatography in $HClO_4$, pH 2.5, of freshly prepared CrATP on Bio-Gel P2 showed that the bulk of the nucleotide had a molecular volume similar to ATP (Figure 3). The trailing of CrATP seen in Figure 3 indicates some nonspecific adsorption between Cr and the polyacrylamide matrix of the gel. This is even more prominent with $Cr_3(ATP)_2$, which despite its larger size was eluted after CrATP from a P-2 column.

Further evidence for the nature of the coordination in CrATP comes from the pK values of the molecule. The pK values of the base in CrATP, CrCTP, and CrGTP were obtained by measuring the change in optical density with pH at 284, 295, and 295 nm, respectively. The values obtained were 3.7, 4.4, and 2.2, which are closer to the pK values for AMP (3.8), CMP (4.5), and GMP (2.4), than to those of ATP (4.1), CTP (4.8), or GTP (3.3), or the diphosphates which have intermediate values near those of the triphosphates. These values, and the measured isoionic pH of 3.0 for CrATP prepared by the aniline procedure, suggest that CrATP (and CrCTP and CrGTP) has a single negative charge on one

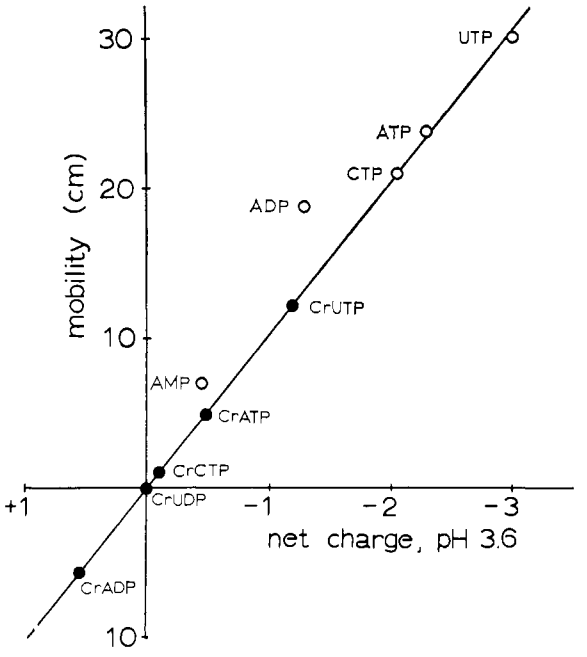


FIGURE 4: Mobility of several Na^+ and Cr^{3+} nucleotides on paper electrophoresis plotted as a function of their calculated net charge at pH 3.6.

of the phosphates at the pH where the adenine loses its proton, as is the case with AMP. This negative charge presumably comes from ionization of the secondary hydroxyl on the terminal phosphate, and the pK of this group must be about 2.2 in CrATP. This very low value points out the largely covalent character of the coordinate bonds between Cr^{3+} and phosphate, since the pK values of this group in MnATP and MnADP are 4.5 and 4.6 and those in MgATP and MgADP are 5.0 and 5.1 (Smith and Alberty, 1956). The d orbitals available in Mn^{2+} for bonding apparently permit a form of binding in which less negative charge resides on the coordinated oxygens than is the case with Mg^{2+}, but Cr^{3+} nearly eliminates this negative charge, since the pK in CrATP is almost that expected of a primary phosphate hydroxyl. Further evidence that at least the β- and γ-phosphate groups are strongly coordinated in CrATP comes from the fact that hydrolysis (1 M $HClO_4$, 80°) of the acid-labile phosphates from CrATP was three times slower than from free ATP.

The mobility of Cr^{3+} complexes at pH 3.6 on paper electrophoresis also agrees well with the expected monomeric structures and the net charges calculated from them and from the measured pK values of the molecules (Figure 4). The anomalously high mobilities of free AMP and ADP in this system are not explained, but all of the other compounds show a linear relationship between calculated charge at pH 3.6 and measured mobilities.

The uv spectra of Cr^{3+} nucleotides are essentially unchanged from those of the uncomplexed nucleotides, but the visible spectra consist of two major bands with maxima around 425 and 600 nm and molar absorbances for both peaks around 20 (Figures 2 and 5; Table I). The maxima for triphosphates are slightly higher (430, 610 nm) than those for diphosphates (425, 600 nm), in agreement with the expected coordination of three oxygens in the triphosphates and only two in the diphosphates. There is no sign of any effect of the adenine ring on the visible spectrum, and the visible spectra of all of the nucleotides are essentially the same, regardless of the nature of the base. Substitution of NH_3 for coordinated water does

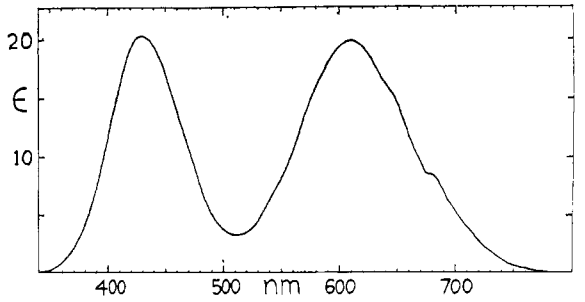


FIGURE 5: Visible spectrum of CrATP at pH 2.6.

move both visible peaks toward the uv and intensify them as expected (Table I).

In Table III are shown the effects of various Cr complexes on the relaxation rates of water protons as measured by pulsed nuclear magnetic resonance (nmr) techniques. The molar relaxation rates do seem to increase with the number of coordinated waters, but it seems that the net charge of the complex and the resulting acidity of the protons of coordinated water are also important factors. In addition, coordinated nucleotides may promote relaxation by themselves (thus addition of ADP to the dioxalate complex actually increases relaxation slightly, even though there is no longer any coordinated water and the complex has one more negative charge). However, the equivalent effect of CrATP, CrCTP, and Cr(formate)ADP, all of which should have at pH 1 three coordinated waters, one positive charge on the base, and no net charge in the vicinity of chromium, shows that for complexes with similar ligands and charge the effects are reproducible and predictable. Note that CrADP has a greater effect than CrATP, as one expects from having four rather than three waters coordinated and a net positive charge on Cr as well as the one on the base.

TABLE III: Proton Relaxation Rates of Water Protons in the Presence of Chromium Complexes.[a]

Compound	$1/T_{1p}$[Cr] (M^{-1} sec^{-1})	$1/T_{2p}$[Cr] (M^{-1} sec^{-1})	T_{1p}/T_{2p}
$[Cr(en)_3]Cl_3$	390	460	1.2
$NH_4[Cr(NH_3)_2(SCN)_4]$	410	540	1.3
$K_3[Cr(oxalate)_3]$	480	610	1.3
trans-$NH_4[Cr(oxalate)_2(H_2O)_2]$	720	870	1.2
trans-$[Cr(H_2O)_4Cl_2]Cl$	1600	5300	3.3
$[Cr(H_2O)_5Cl]Cl_2$	4100	12000	2.9
$[Cr(H_2O)_6](ClO_4)_3$	6200	15400	2.5
$Cr(oxalate)_2ADP$	800	1100	1.4
Cr(formate)ADP	2800	3700	1.3
CrADP	3400	4400	1.3
$Cr_2(ADP)_2$	590	780	1.3
$Cr_3(ADP)_2$	2500	3900	1.6
CrATP	2800	3500	1.3
CrCTP	2900	3900	1.3

[a] The longitudinal ($1/T_{1p}$[Cr]) and transverse ($1/T_{2p}$[Cr]) molar relaxation rates were determined at 24.3 MHz and 25° in the presence of 0.1 M $HClO_4$ by the methods of Mildvan and Cohn (1963). These experiments were carried out in the laboratory of Dr. A. S. Mildvan by M. Schimerlik and K. Danenberg.

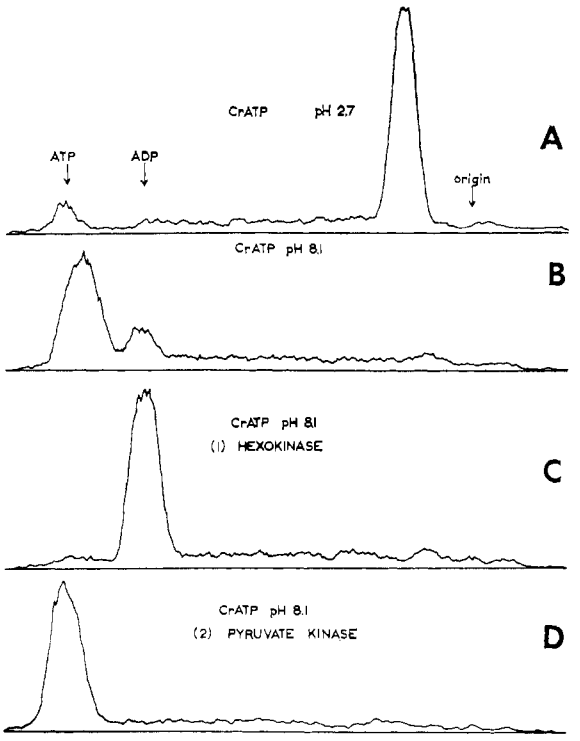


FIGURE 6: (A) Paper electrophoresis at pH 3.6 of 0.2 mM [^{14}C]-CrATP (pH 2.7 as prepared). (B) Same as A except [^{14}C]CrATP was first incubated 1 hr at 25° in a hexokinase reaction mix, pH 8.1, minus TPN, glucose-6-phosphate dehydrogenase, and glucose. (C) Sample used in B was treated for 30 min at 25° with hexokinase (5 μg/ml) and glucose. (D) Sample used in C was vortexed with 0.25 vol of CCl_4 to precipitate hexokinase. The sample was centrifuged to separate phases and bubbled with N_2 to remove any residual CCl_4. The solution was then adjusted to 0.1 M KCl, 0.35 mM phosphoenolpyruvate, and 5 μg/ml of pyruvate kinase, and incubated for 30 min at 25°. All electrophoresis was done at pH 3.6. Markers show where pure samples of ATP and ADP migrated. The peak of CrATP in A contained 2900 cpm.

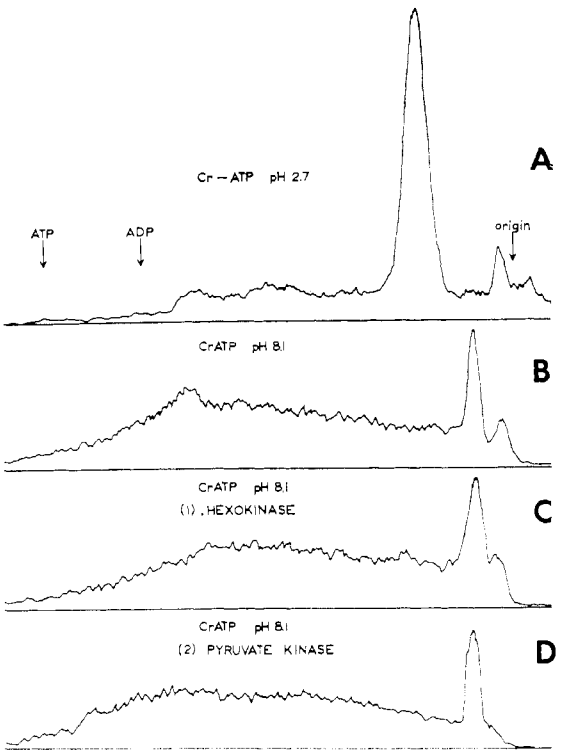


FIGURE 7: The same experiment as in Figure 6 except that 0.2 mM [^{51}Cr]CrATP was used. The peak of CrATP in A contained 3900 cpm.

A modified Varian X-band electron paramagnetic resonance (epr) spectrometer (Palmer, 1967) was used to obtain epr spectra of solutions (1–10 mM, pH ~2) of the Cr^{3+} complexes at room temperature and at 80 and 11°K. In the liquid state $Cr(H_2O)_6^{3+}$ gave a single line of *ca.* 170 G peak-to-peak width, centered at $g = 1.97$, in agreement with earlier reports (Levanon *et al.*, 1970, and references therein), while the nucleotide complexes gave greatly reduced (4–8%) signal amplitudes. At the lower temperatures the following details emerged: the spectra had an axial appearance, with $g_\perp = 1.97$ and $g_\parallel = 4.7$. The ratio of the amplitude of the $g = 1.97$ resonance to that of the $g = 4.7$ feature decreased in the order $Cr(H_2O)_6^{3+} > CrPP \simeq CrADP > CrPPP \simeq CrATP$ from a value of 19 to a value of 1.6, and the overall amplitude of the $g = 1.97$ feature also decreased by a factor of about 10 in the above order. Part of the latter effect may be due to dipolar broadening accompanying salting out of solute during freezing, as suggested by Ross (1964), since the inclusion of 25% methanol in CrATP samples increased the signal amplitudes by a factor of 2. However, at the lower temperatures the relative tetragonal distortion of the ligand field in the phosphate complexes was apparent, and this along with enhanced electron relaxation in these complexes (Levanon *et al.*, 1970) appears to account for the diminished size of the signals at all temperatures.

This reduction in signal amplitude could also be accounted for by antiferromagnetic interaction of Cr^{3+} ions (Selwood, 1956) *via* oxygen bridges in polynuclear complexes with the nucleotides, but the following magnetic susceptibility experiments seem to rule this possibility out. Liquid samples (15 ml) were suspended from a single pan Mettler balance in a thermostat at 22.5° and were weighed to 0.01 mg in zero field and at 6420 G (Gouy method). Diamagnetic corrections were obtained with solvent minus the Cr complex. CrATP (41 mM in 0.5 M lithium formate, pH 3.5) had a magnetic moment of 3.83 BM, whereas the value found for $Cr(H_2O)_6 \cdot K(SO_4)_2$ was 3.70 BM. Both measurements are in the expected range for $S = {}^3/_2$ ions (Selwood, 1956).

On this basis then, no magnetic coupling between ions at room temperature was detected, ruling out this mechanism for the observed epr phenomena. The magnetic susceptibility measurements are thus consistent with the formulation of these species as mononuclear, and the epr properties evidently reflect the expected lowered symmetry of the ligand field in comparison to $Cr(H_2O)_6^{3+}$ as additional phosphoryl oxygens are coordinated to Cr^{3+}. Further studies of the magnetic properties of these compounds are in progress.

Breakdown of CrATP to ATP. When 50–200 μM CrATP was added to a reaction mix at pH 8.1, 25°, containing yeast hexokinase but no free ATP, the resulting time course was a curve concave upward, suggesting that CrATP slowly changed into a form that acted as a substrate for the enzyme. The rate of this conversion was measured in two ways. First, 65 μM CrATP was added to a reaction mix minus hexokinase, incubated for varying times up to 11 min, and then hexokinase was added and the reaction followed spectrophotometrically as usual. The result was an initial burst equivalent to the amount of substrate that had accumulated, followed by a much slower, nearly linear rate. Assuming first-order kinetics, CrATP was converted to a substrate with a rate constant of 0.046 min^{-1}. In the second method, CrATP (0–140 μM) was added to a reaction mixture containing 15–60 μg/ml of hexokinase. Since

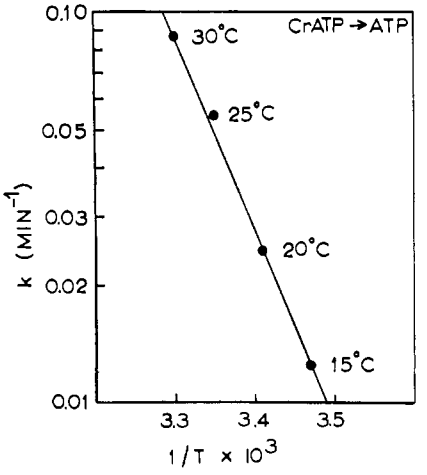


FIGURE 8: The rate of conversion of 65 μM CrATP to ATP as a function of temperature was plotted according to the Arrhenius equation. ATP was measured as a function of time using the hexokinase assay at the indicated temperature.

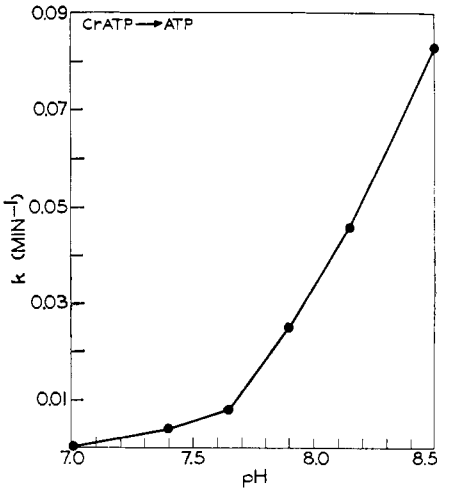


FIGURE 9: The rate of conversion of 65 μM CrATP to ATP plotted as a function of pH. The amount of ATP as a function of time was measured with the hexokinase reaction run at the same pH as the sample being assayed.

CrATP strongly inhibits hexokinase, several concentrations of hexokinase were used for each CrATP concentration, and the resulting initial rates were extrapolated to infinite hexokinase concentration. The extrapolated initial rates were linear with CrATP concentration and gave a rate constant of 0.052 min^{-1} ($t_{1/2}$ = 13.4 min) at pH 8.1, 25°, for the conversion of CrATP into a substrate.

The ability of CrATP to act as a substrate for hexokinase could be entirely accounted for as a base-catalyzed hydrolysis releasing ATP which then combined with the Mg^{2+} present to give the normal substrate, MgATP. Electrophoresis at pH 3.6 of 200 μM freshly prepared and unpurified [^{14}C]CrATP (pH 2.7 after the heating process) gave a single major peak containing CrATP and 60% of the ^{14}C label (Figure 6A). When this same concentration of CrATP was present in a hexokinase assay mix (pH 8.1) minus enzyme, CrATP was quantitatively converted to a nucleotide that migrated like ATP (Figure 6B). This assay mix was then treated with hexokinase and again subjected to electrophoresis, with the result that the ATP was quantitatively converted to ADP (Figure 6C). Hexokinase was then destroyed by lowering the pH to 2.5 with $HClO_4$ and extracting the solution with CCl_4. After residual CCl_4 was removed by bubbling N_2 through the solution and pyruvic kinase plus the necessary materials for its activity were added, electrophoresis showed that all of the ADP was quantitatively converted back to ATP (Figure 6D). The same experiment was repeated using [^{51}Cr]CrATP (Figure 7) with the result that after exposure to pH 8.1 all of the chromium appeared on electrophoresis as an anionic smear. Successive treatment with hexokinase and pyruvic kinase showed no effect on the electrophoresis pattern; therefore, the observed substrate activity was not the result of hexokinase acting on some form of CrATP, but rather on its normal substrate, MgATP.

Conversion of CrATP to ATP occurred upon titration to pH 8 and did not require any cofactors. No differences in the resulting electrophoresis patterns were found whether the pH was raised with NaOH, KOH, Tris, triethanolamine, or *N*-ethylmorpholine. The appearance of ATP was faster in the presence of Mg^{2+}, but this is expected, since another multivalent cation should chelate phosphoryl ligands as they are released from Cr^{3+} and thus inhibit re-formation of phosphoryl–Cr bonds.

Conversion of CrATP to ATP at pH 8.1 was found to be sharply temperature dependent with an E_a of 22,600 cal/mol, a 4.4-fold decrease in the rate of hydrolysis in going from 25 to 15° (Figure 8). The appearance of ATP in dilute solutions of CrATP was also markedly dependent on pH (Figure 9). The rate of conversion was barely detectable at pH 7.0, 25°, giving $k = 3.8 \times 10^{-4}$ min^{-1}, a value 210 times lower than the rate observed at pH 8.5. Therefore, CrATP can be effectively used to study enzymes by keeping the pH at 7.0 or less and the temperature and free concentration of Mg^{2+} as low as is practical.

CrADP is somewhat more readily hydrolyzed than CrATP, as one might expect from having only two rather than three phosphates coordinated (Table II), but is is quite practical to use it at pH 7.0. Both CrATP and CrADP become more resistant to hydrolysis as ammonia replaces water in the coordination sphere (Table II), although the pH variation of the hydrolysis rate is similar. Unfortunately the molecules become less potent inhibitors at the same time in the few systems in which they have so far been tried, so it is questionable whether their increased stability conveys sufficient advantage in most cases to offset their higher K_i values.

The amount of free ATP released after 1 hr of incubation of CrATP in the absence of Mg^{2+} at pH 8.1, 25°, depended on the initial concentration of CrATP. This was demonstrated in two ways. First, the amount of ATP was assayed using hexokinase and measuring the initial burst size as described above (Figure 10). Second, electrophoresis of CrATP which has been incubated at low concentrations for 1 hr at pH 8.1, 25°, showed conversion of CrATP to ATP (Figure 6), while electrophoresis of CrATP incubated at high concentrations under the same conditions showed conversion of CrATP to an anionic form with only a 1–2% increase in the amount of free ATP already present (Figure 11).

The nature of the Cr^{3+}–nucleotide species formed under basic conditions at high CrATP concentrations is not clear. Both Cr and nucleotide were present in varying amounts. Magnetic susceptibility measurements on 40 mM CrATP, pH 8.1, gave a reduced magnetic moment of 3.30, which could be interpreted as due to the presence of 25% of the diamagnetic dimer, Cr–O–Cr (Garver and House, 1970).

We attempted to define the size of the Cr^{3+}–nucleotide species present at pH 8.1 using gel filtration chromatography. Both CrATP (20 mM, 1 hr at pH 8.1, 25°) and 20 mM ATP chromatographed one fraction behind Blue Dextran on both

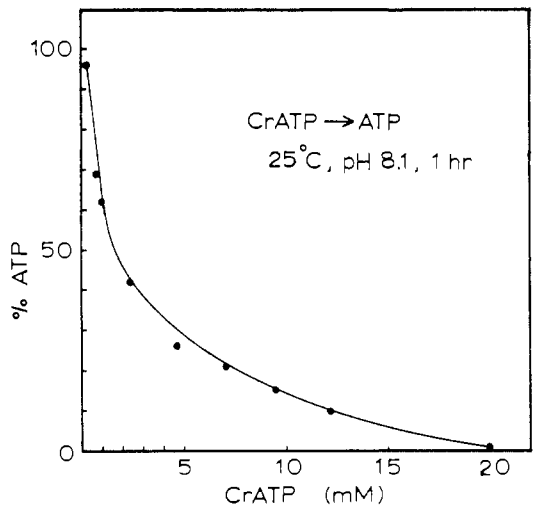


FIGURE 10: The amount of free ATP present in a given concentration of purified CrATP after a 1-hr incubation in 30 mM triethanolamine-HCl, pH 8.1, at 25° was assayed by adding an aliquot to a hexokinase assay mix contaning enzyme and calculating the initial burst of activity as ATP.

Bio-Gel P2 and P6 equilibrated with 30 mM *N*-ethylmorpholine, pH 8.1. Repeating the chromatography in the presence of 2 M NaCl caused both nucleotides to cochromatograph (Figure 12). Therefore, either high salt causes dissociation of polynuclear Cr species or the hydrated volume of such species was not significantly different from that of ATP at pH 8.1.

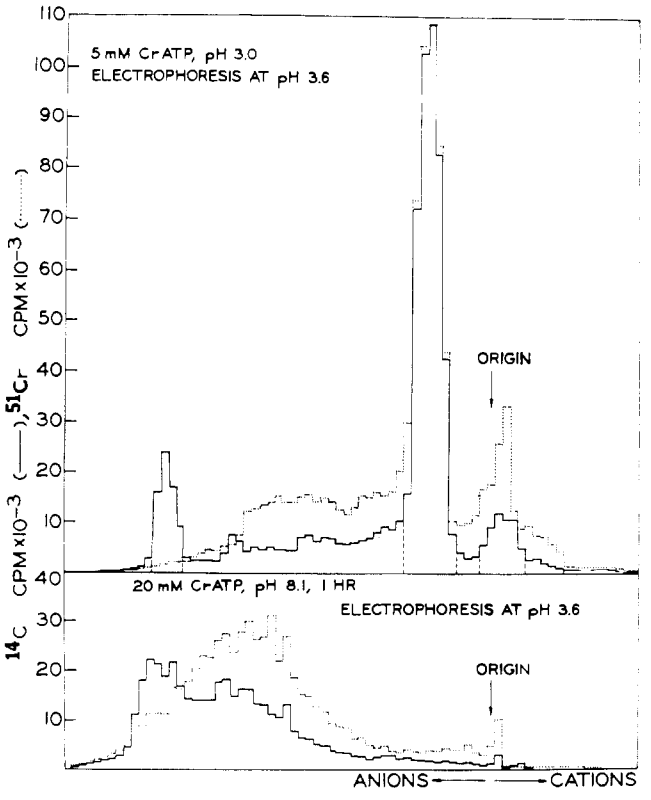


FIGURE 11: (top) Paper electrophoresis at pH 3.6 of freshly prepared and unpurified CrATP. The large peak is CrATP; the small one to the left containing ^{14}C, but no ^{51}Cr, is free ATP. (bottom) Similar material (20 mM) incubated at pH 8.1 for 1 hr before electrophoresis. The propertion of free ATP has not appreciably increased.

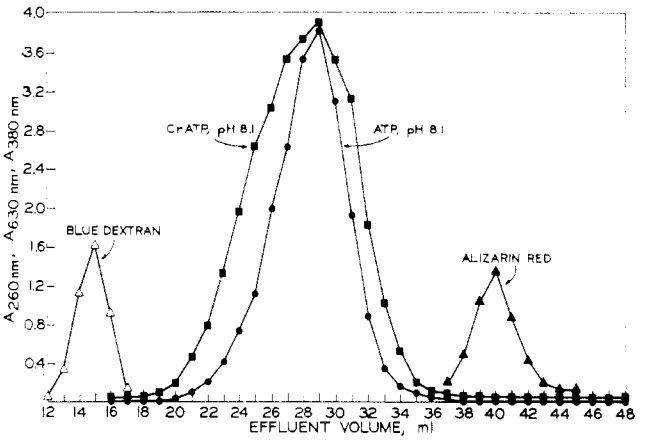
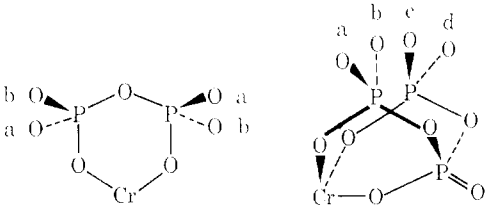


FIGURE 12: Gel filtration chromatography on Bio-Gel P6 (mol wt 1000–6000 range) equilibrated in 30 mM *N*-ethylmorpholine, pH 8.1, and 2 M NaCl: (△) Blue Dextran 2000 (mol wt 2×10^6), assayed by absorbance at 630 nm; (▲) Alizarin Red S (mol wt 280) assayed by absorbance at 380 nm; (●) Na_3ATP (mol wt) 595) assayed by adsorption at 260 nm; (■) CrATP assayed by absorbance at 260 nm. Purified CrATP (20 mM) and ATP (4 mM) were titrated to pH 8.1 in 30 mM *N*-ethylmorpholine and incubated for 1 hr at 25° before putting separate 1.0-ml fractions on the column. Eluent was 30 mM *N*-ethylmorpholine–2 M NaCl, pH 8.1.

Discussion

The preparation and purification of Cr^{3+}-nucleotide complexes as described in this paper is relatively easy and the resulting compounds are reasonably stable in the cold at low pH. Their structures appear to involve the coordination of either two (for diphosphates) or three (for triphosphates) phosphoryl oxygens in the primary coordination sphere of chromium, and there is no evidence to support the coordination of any other groups from either the sugar or base. What is as yet uncertain is how many coordination isomers exist in the compounds as prepared, and whether they can be interconverted. Thus, for CrADP there should exist two isomers and for CrATP four isomers,[5] corresponding to attachment of adenosine to oxygens a, b, c, or d in the following structures (for CrADP, the two oxygens marked a are equivalent, as are the two marked b)



Since adenosine is asymmetric itself, none of these isomers are enantiomers, and all are diastereoisomers and theoretically separable. No evidence for isomers has been seen for CrADP, but as indicated above CrATP does separate into two similar bands at low ionic strength on Dowex 50 (H^+). It is possible that these bands represent isomers (or pairs of isomers) that differ in their ability at zero ionic strength for the protonated base to approach one of the oxygens of the terminal phosphate. Such a difference would alter the p*K* values enough to permit separation, but further speculation will have to await

[5] We are ignoring the possibility that the three coordinated oxygens of the tripolyphosphate are arranged equatorially around chromium, since a model of this structure suggests that considerable strain would be present.

more experimentation. Since the compounds prepared as described here give reproducible results in inhibiting various enzymes, the presence of more than one isomer is no disadvantage for most studies.

There are three major uses that can be made of chromium nucleotides. First, they are good dead end inhibitors of a number of enzymes for which MgATP or other nucleotides are substrates. These include enzymes that release pyrophosphate and transfer the AMP (gentamycin and streptomycin adenylating enzymes and methionyl-tRNA synthetase) as well as several kinases. Thus, Cr^{3+}–nucleotides can be generally used to deduce or confirm kinetic mechanisms. For example, the inhibition patterns at pH 7 with yeast hexokinase confirm that the mechanism is random (Cleland *et al.*, 1972), while with glycerokinase the mechanism appears ordered with glycerol adding first (Janson and Cleland, 1973). CrATP binds very tightly to both of these enzymes ($K_i < 1$ μM), thus making possible binding studies to determine the number of active subunits. With creatine kinase at pH 7 the addition of MgATP followed by creatine is equilibrium ordered, while CrATP, and to a lesser extent CrADP and MgADP, also permit creatine binding (Cleland *et al.*, 1972). In the reverse direction the mechanism appears random as it is at pH 8.

Furthermore, for some enzymes Cr^{3+}–nucleotides are very tight inhibitors, while for others they bind less tightly than MgATP. The lack of strong inhibition *vs.* pyruvic kinase, for example, makes the pyruvic kinase–lactic dehydrogenase assay for ADP practical for determining the inhibition of chromium–nucleotide complexes *vs.* other kinases with MgATP as substrate. The very strong inhibition by CrATP *vs.* hexokinase rules out its use in following the reverse reactions of most kinases but the 3-phosphoglycerate kinase–glyceraldehyde-3-phosphate dehydrogenase assay can be used, or an assay specific for the other product released.

Inhibition by Cr^{3+}–nucleotide complexes is specific since inhibition constants for CrATP and CrADP can differ 100-fold for some enzymes (hexokinase), while no inhibition was detected using Cr(citrate) or CrPP. The observation of competitive inhibition patterns *vs.* Mg^{2+}–nucleotide complexes in the above enzyme studies also argues for specificity in the effects of CrATP and CrADP. The results of the above and other studies which have been carried out in this and other laboratories and will be published elsewhere clearly indicate the usefulness of these molecules for kinetic analysis. The only precautions that need to be observed are: (1) that the compounds be used at pH 7 or below (for kinetic studies one can use somewhat higher pH values if the products of the slow breakdown do not interfere, initial rates are observed, and temperatures above 25° are avoided; thus phosphofructokinase has been studied at pH 7.4 in this laboratory without difficulty (if higher pH values or temperatures must be used, the ammonia-substituted compounds could be tried)); (2) that they not be stored in the presence of any ligands such as formate that could enter the coordination sphere. Perchlorate is safe, as are levels of chloride or bromide below 50 mM.

As a second major use, the paramagnetic properties of these complexes should permit investigation of the distances between the chromium and various other substances adsorbed on the enzyme, where the chromium nucleotide is very tightly bound. Thus, the distance between CrATP bound at one site and the Mn^{2+} bound at the other site of pyruvic carboxylase has been shown to be greater than 15 Å.[6] Third, if any of these complexes can be crystallized, we will be able to deduce the shape of the active sites in which it is adsorbed. So far crystals suitable for X-ray analysis have not been obtained of any of these materials, but, particularly if any success is obtained in isolating isomers of CrATP, this situation may change. In any case the availability of rigid, paramagnetic, tightly bound analogs of MgATP and other nucleotides offers a wealth of new possibilities for the study of enzymatic reactions.

[6] Scrutton, M. C., Reed, G. H., and Mildvan, A. S., manuscript in preparation.

Acknowledgments

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Arrangement of the Phosphate- and Metal-Binding Subsites of Phosphoglucomutase. Intersubsite Relationships by Means of Inhibition Patterns†

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ABSTRACT: Dissociation constants were determined for the complexes of the competitive inhibitors, inorganic phosphate and methylphosphonate, with both the phospho and dephospho forms of phosphoglucomutase. Kinetic procedures were used for measurements involving the Mg^{2+} complexes of the enzyme while water proton relaxation rates were used with the Mn^{2+} complexes. The results show that methylphosphonate can be used as a phosphate analog in studies of the phosphate-binding subsites of phosphoglucomutase. The dissociation constants for complexes of inorganic phosphate and of a series of α,ω-alkanediol bisphosphates with the Mg^{2+} forms of both the phospho- and dephosphoenzymes were determined kinetically. These and other results show that the dephosphoenzyme has a weak phosphate-binding subsite as well as a strong one; the strong subsite is also present in the phosphoenzyme and appears to be involved in the binding of the phosphate group of the normal substrates, glucose 1- and 6-phosphates. By contrast, the weak phosphate-binding subsite of the dephosphoenzyme is apparently absent in the phosphoenzyme, probably because it is occupied by the phosphate group esterified with the active-site serine residue. Thus, the weak phosphate-binding subsite appears to be the subsite at which the catalytic ($-PO_3$) transfer occurs. In such a case glucose 1,6-bisphosphate probably binds to the dephosphoenzyme in two different ways—one with the 6-phosphate at the weak subsite and the other with the 1-phosphate group at this subsite—and the interconversion of the two different complexes thus formed must be part of the catalytic cycle. However, this interconversion must occur by means of a process that does not require the complete dissociation of either complex.

If a paramagnetic metal ion, usually Mn^{2+}, is bound at the metal-activating site of an enzyme, an estimate of the distance between this site and various functional groups of a bound substrate or inhibitor sometimes can be deduced by measuring the effect of the bound metal ion on the nuclear magnetic resonance (nmr) signal of these groups (Mildvan and Cohn, 1970). Such a study involving phosphoglucomutase, Mn^{2+}, and the phosphate analog, methylphosphonate, is described in the accompanying paper (Ray and Mildvan, 1973). However, phosphoglucomutase has two phosphate-binding subsites, and the thermodynamic properties and kinetic roles of these sites must be understood in order to interpret the results of such a study. In the present paper, inhibition by inorganic phosphate and methylphosphonate is evaluated in order to show that the latter acts as a phosphate analog in the phosphoglucomutase system. Inhibition by inorganic phosphate also is contrasted with that produced by a series of α,ω-alkanediol bisphosphates, as an indication of how the binding strength varies between the phosphate-binding subsites. In addition, the observed binding patterns for inorganic phosphate are used to place restrictions on the manner in which ($-PO_3$) transfer can occur within the central complexes.

Experimental Section

Materials. The phospho form of phosphoglucomutase was isolated by means of a scaled-up and modified version of the previously described procedure (Ray and Koshland, 1962). The dephosphoenzyme was prepared from the phosphoenzyme by inducing ($-PO_3$) transfer to water by means of Xyl-1-P followed by extensive dialysis. These procedures will be described elsewhere (E. J. Peck, Jr., J. W. Long, L. Ng, J. D. Owens, and W. J. Ray, Jr., manuscripts in preparation); in the meantime, details are available on request (from W. J. R.). Enzyme activity was at least 900 units/mg for the phosphoenzyme and 850 for the dephosphoenzyme, as measured in the standard assay (National Academy of Sciences, 1972). The phosphoenzyme contained less than 7% dephosphoenzyme; the dephosphoenzyme contained less than 5% and was probably free of phosphoenzyme. The purification of the sugar phosphate substrates of phosphoglucomutase has been described (Ray and Roscelli, 1964). α-D-Xylose-1-P and α-D-2-deoxyglucose-1-P were obtained from Sigma and used without further purification. Methylphosphonate was generously supplied by Dr. Alexander Hampton, Institute for Cancer Research. ^{32}P-Labeled glucose-1-P was prepared in the manner described previously (Ray and Koshland, 1962). The prep-

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