Characterization of the Metal Binding Site of Phosphoglucomutase by Spectral Studies of Its Cobalt(II) and Nickel(II) Complexes†

William J. Ray, Jr.,* and Jagir S. Multani

ABSTRACT: The spectrum of the active nickel(II)–phosphoglucomutase complex contains symmetrical absorption bands at 410 mμ (ε ~23; oscillator strength about 4×10^{-4}) and 1300 mμ (ε 5; oscillator strength about 5.9×10^{-5}) and a bifurcated band centered at about 730 mμ (ε ~7; oscillator strength, 1.1×10^{-4}). The positions and intensities of these bands are consistent with an octahedral-like coordination of Ni^{2+} involving weak-field ligands such as carboxylate groups and water molecules. In fact, in the near-infrared, the visible, and the near-ultraviolet regions of the spectrum the Ni^{2+}–enzyme complex at 27° is strikingly similar to that of Ni^{2+} in saturated aqueous potassium acetate, where Ni^{2+} is coordinated to such groups. By contrast, at 0° Ni^{2+} is preferentially bound to sites on the enzyme other than the active site; under these conditions the spectrum suggests the participation of nitrogenous ligands. The spectrum of the binary Co^{2+} complex of phosphoglucomutase has unsymmetrical absorption bands centered at 564 and 1350 mμ; the visible band has maxima at 620, 595, 570, 535, and 485 (sh) mμ, while the near-infrared band has maxima at 1150, 1390, 1550, and about 1750 (sh) mμ. The main peaks in the visible band have extinction coefficients in the range of 150–210 (oscillator strength of the entire band, 3.2×10^{-3}) while those in the near infrared have extinction coefficients in the range of 15–30 (oscillator strength of the entire band, 4.6×10^{-4}). The coordination geometry of bound Co^{2+} is difficult to identify with certainty, since the spectrum is similar both to that of Co^{2+} in saturated aqueous potassium acetate, which contains the tetrahedral complex, $CoAc_4^{2-}$, and to the irregular, hexacoordinate Co(triphenylarsine oxide)$_2$(nitrate)$_2$. Changes in temperature and pH have a marked effect on both the visible and near-infrared spectra of Co^{2+}–phosphoglucomutase; decreasing the temperature from 30 to 0° decreases band intensity by about 30%. This decrease cannot be attributed to a decrease in binding of Co^{2+} at the active site of the enzyme. Although the temperature effect might be caused by a change in coordination number involving the active site Co^{2+}, *e.g.*, a tetrahedral-to-octahedral interconversion, it also might be caused by an alteration in the coordination symmetry of the bound metal without a change in coordination number.

Properties of the Mn^{2+} complex of phosphoglucomutase have been studied by nuclear magnetic resonance (nmr) techniques before and after binding of several different substrates (and inhibitors) to determine whether there is a direct interaction between the bound metal ion and the bound substrate in the ternary complex (Ray and Mildvan, 1970). The results suggest that such an interaction does exist, but that the hydroxyl groups of the sugar moiety rather than the phosphate group are involved. The present work was initiated in an attempt to confirm this suggestion by studying the Co^{2+} and Ni^{2+} complexes of the enzyme.

Although other metal ions do not elicit as high an activity in the phosphoglucomutase reaction at 30° as does Mg^{2+}, the physiologically important metal, Ni^{2+} is about 70% and Co^{2+} about 25% as efficient at their respective pH optima, and the Co^{2+}–enzyme is even more active than the Mg^{2+}–enzyme at 0° (Ray *et al.*, 1972). Moreover, the electronic spectra of Ni^{2+} and Co^{2+} complexes are sensitive to the nature of the coordinating ligands (Cotton and Wilkinson, 1966). Hence, it seemed reasonable to look for spectral changes in the Ni^{2+} and Co^{2+} complexes of the enzyme caused by substrate binding as evidence for direct replacement, by substrate, of groups in the coordination sphere of the bound metal ion. Thus, presumptive evidence for interaction between Co^{2+} and HCO_3^- in their ternary complex with carbonic anhydrase (Lindskog, 1966; Coleman, 1967), of Co^{2+} and phosphate in their ternary complex with alkaline phosphatase (Simpson and Vallee, 1969) and of Co^{2+} and peptide substrates or inhibitors in their ternary complexes with carboxypeptidase (Latt and Vallee, 1971) has been obtained in this manner. However, initial results suggested that in the phosphoglucomutase system some of the coordination properties of the bound Co^{2+} and Ni^{2+} might be illucidated by direct spectral comparison to model compounds and this is the subject of the present communication. The effect of bound substrate on these metal–enzyme complexes will be treated in a subsequent paper.

Experimental Section

Materials. Most materials, including phosphoglucomutase (phospho form), have been described previously (Ray and Mildvan, 1970; Ray *et al.*, 1972). D_2O was obtained from Columbia Organic Chemicals. Chlorides of Tris and chlorides or acetates of metals were used throughout, except for experiments in D_2O; for such experiments metal sulfates were dissolved in D_2O, evaporated to dryness, and redissolved in D_2O before use. Constants relating to enzyme activity and concentration are given in the accompanying paper (Ray *et al.*, 1972). Spectrograde dimethylformamide (Fisher) was dried with a molecular sieve before use. Tetraethylammonium acetate was prepared from the tetrahydrate by fusion under

† From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907. *Received January 24, 1972.* This work was supported by grants from the National Institutes of Health (GM-08963) and the National Science Foundation (GB-29173X).

* Public Health Service Career Development awardee.

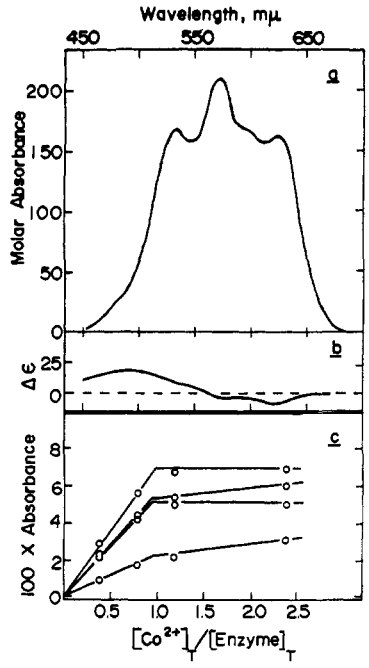

FIGURE 1: Spectra of cobalt(II)–phosphoglucomutase complexes. (a) Spectrum of the 1:1 complex. Solutions of enzyme, 42 mg/ml (0.67 mM) in 20 mM Tris-HCl (pH 8.5), were present in both sample and reference cells; 0.67 mM Co^{2+} and 0.67 mM Mg^{2+} were present in the sample and reference cells, respectively. (b) Difference spectrum of the 2:1 complex *vs.* the 1:1 complex. Conditions were the same as in part a, except that 1.34 mM Co^{2+} and 0.67 mM Co^{2+} were present in the sample and reference cells, respectively. The change in molar extinction is indicated. (c) Spectral titration of phosphoglucomutase with Co^{2+}. Solutions of phosphoglucomutase, 22 mg/ml (0.35 mM) in 50 mM Tris (pH 7.5) were present in both the sample and reference cells; in addition, Co^{2+} was present in the sample cell and Mg^{2+} in the reference at equal concentrations. At each metal:protein ratio a different pair of protein solutions was used for absorbance measurements.

vacuum. Nickel perchlorate was prepared from the hydroxide.

Metal-free enzyme at pH 5 or 8.5 was prepared by substituting 20 mM sodium acetate buffer (pH 5) or 20 mM Tris (pH 8.5) for the pH 7.5 Tris buffer in the demetallation procedure described previously (Ray and Mildvan, 1970) during the second and subsequent dialyses; the first dialysis (against pH 7.5 Tris buffer) remained the same. Unless otherwise stated, metal-free enzyme at pH 7.5 was used throughout. Metal–enzyme complexes were prepared by adding solutions of metal salts to metal-free enzyme.

Solutions of phosphoglucomutase in D_2O were prepared by lyophilizing concentrated solutions of metal-free protein (60–150 mg/ml) in 20 mM Tris (pH 7.5 or 8.5) and dissolving the residue in cold D_2O. With the best samples of enzyme, specific activity was unchanged by this treatment and essentially all of the protein dissolved within 0.5 hr.

Spectral Measurements. Visible and near-ultraviolet spectra were scanned at 24° by using a Cary 15 spectrophotometer with a 0–0.1 OD slide-wire at a rate not exceeding 1 mμ/sec. A Cary high-intensity light source was used. Final protein concentrations were between 20 and 150 mg per ml, and samples were usually centrifuged for 1 hr at 140,000*g* before addition of metal ion and for 5 min at 16,000*g* after the addition and just before use. Spectra involving Co^{2+} and Ni^{2+} forms of the enzyme were obtained by using the Zn^{2+} or Mg^{2+} form of the enzyme at the same concentration in the reference cell. Spectra were reproduced on a reduced scale by plotting readings taken from the original spectra at intervals of 10 mμ. Temperature control was provided by a Lauda K-2/R water bath-circulator in conjunction with flow-through cell holders, and the actual temperature of the cell was monitored by means of a thermister. All spectra were scanned at least twice. In temperature studies, spectra were usually scanned first at the lowest temperature and succeeding scans made at successively higher temperatures; however, the reverse process was sometimes used.

Near-infrared spectra (in D_2O solutions) and some visible spectra were scanned by using a Cary 14 spectrophotometer equipped with a 0–0.1 OD slide-wire. Because of the low optical densities measured, all spectral studies in the near-infrared region involved determining a base line with identical samples of metal-free enzyme in both sample and reference cells followed by addition of 0.03 volume of metal sulfate in D_2O to both cells (Zn^{2+} to the reference and Co^{2+} or Ni^{2+} to the sample). Both base lines and samples were scanned three times at rates not exceeding 1.5 mμ/sec, and the results averaged; difference of 0.001 OD could be reproduced. Temperature was maintained by use of jacketed cells and cell holders in conjunction with a Lauda K-2/R water bath circulator. The temperature of samples was measured as above. Spectra were reproduced by plotting readings taken from the original spectra at intervals of 25 mμ in the near-infrared region and 10 mμ in the visible region of the spectrum.

Absorbance of the Cobalt(II)–Phosphoglucomutase Complex as a Function of pH. A sample of metal-free phosphoglucomutase at pH 5 (see Materials section) was treated with 0.75 mole % Co^{2+} and pH adjustments were made by addition of very small aliquots of 1 N Tris. Samples were removed after each pH adjustment and were later centrifuged at 140,000*g* for 1 hr at 24°. Essentially all of the centrifuged samples were transferred with a pipet to a small test tube, swirled to obtain mixing, and subsequent transferred to a 1-ml cuvet with a 1.0-cm light path. The absorbance was measured *vs.* an identical sample to which Zn^{2+} had been added. The pH of samples was measured and enzymatic assays were conducted immediately after the spectra were scanned. No pH changes were observed during the experiment and all activities were the same as the original metal-free enzyme, except for the pH 5.05 sample, in which activity had decreased by 15%. (The Co^{2+} enzyme was converted to the Mg^{2+} form during the incubation prior to the actual assay.)

Results

Spectrum of the Cobalt(II)–Phosphoglucomutase Complex. Previous studies show that the binding of 1 equiv of Co^{2+} elicits essentially maximal activity and produces the maximal absorbance difference at 285 mμ obtainable with this metal (Ray *et al.*, 1972; Peck and Ray, 1969). Hence, the Co^{2+}–enzyme spectrum in Figure 1a was obtained at a Co^{2+} to enzyme ratio of 1.0. This spectrum has maxima at 620, 595, 570, 532, and 485 (sh) mμ and is somewhat similar to that of the Co^{2+} complexes of carbonic anhydrase, basic form (Lindskog and Nyman, 1964; Coleman, 1967), and alkaline phosphatase, basic form (Simpson and Vallee, 1968). The oscillator strength (Bauman, 1962) of the band is 3.2×10^{-3}.

The spectral titration shown in Figure 1c also indicates that the absorption band in Figure 1a is that of the 1:1 Co^{2+} complex of the enzyme and not to a mixture. In addition, the titration shows that spectral changes of a different type occur on addition of a second equivalent of Co^{2+}, although, as noted above, activity is not appreciably affected. The differ-

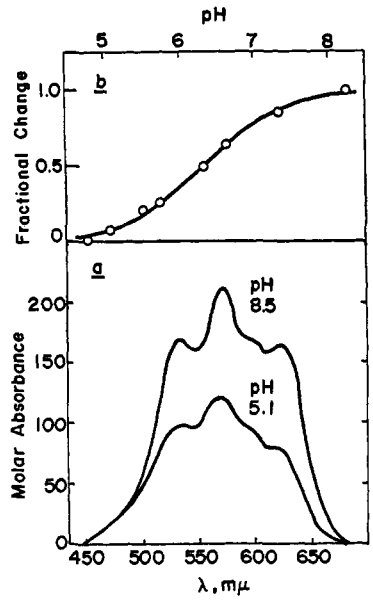

FIGURE 2: Effect of pH on the spectrum of the cobalt(II)–phosphoglucomutase complex. (a) The spectrum at pH values of 8.5 and 5.1. Solutions of enzyme, 25 mg/ml (0.4 mM), were present in both sample and reference cells; 0.32 mM Co^{2+} and 0.32 mM Zn^{2+} were present in the sample and reference cells, respectively. Buffers were 20 mM sodium acetate, pH 5.1, and the same buffer was titrated to pH 8.5 with 1 M Tris-HCl (see Results section). The molar absorbance was calculated on the basis of the total Co^{2+} present. (b) The absorbance at 575 mμ as a function of pH. For details, see Experimental Section

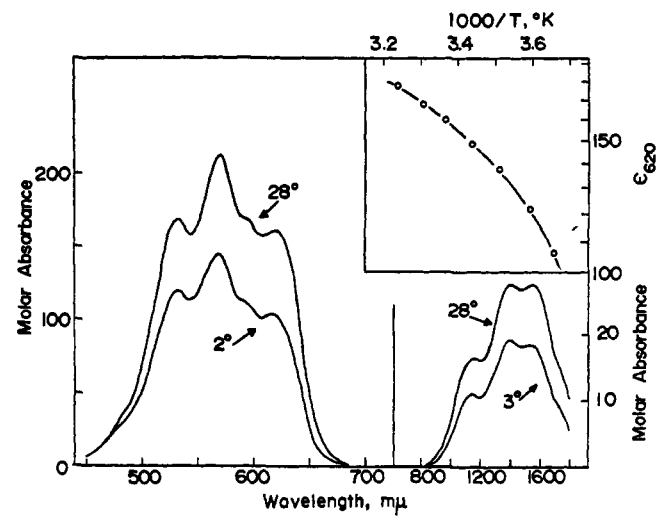

FIGURE 3: The spectrum of the cobalt(II)–phosphoglucomutase complex at 28° and near 2.5°. The visible spectrum of a 0.86 mM solution of phosphoglucomutase, 0.77 mM in Co^{2+}, was scanned against a solution with the same concentration of enzyme, 0.77 mM in Zn^{2+}, at the temperatures indicated in the figure; 20 mM Tris-HCl (pH 8.5) was present in both cells. The near-infrared spectrum was obtained under similar conditions (see Experimental Section) but with 2.1 mM enzyme and 1.75 mM metal ion in D_2O. Molar absorbance was calculated on the basis of the Co^{2+} present. Inset: the effect of temperature on the absorbance of the cobalt(II)–phosphoglucomutase complex at 620 mμ in the presence of excess metal. Solutions of enzyme, 42 mg/ml (0.67 mM) in 20 mM Tris-HCl (pH 7.5), were present in both sample and reference cells; 1.54 mM Co^{2+} and Mg^{2+} were present in these cells, respectively (enzyme:metal = 2.3:1). The molar absorbance is plotted on a logarithmic scale against the reciprocal of the temperature.

ence spectrum in Figure 1b was obtained directly as the absorption difference between solutions with a Co^{2+} to enzyme ratio of 2 and 1, and represents the spectrum for Co^{2+} at a secondary site or sites, although no evidence for a *single* species at a 2:1 ratio can be offered. The negative trough in the difference spectrum at about 620 mμ indicates that binding of the second Co^{2+} has a small effect on the spectrum of the first. However, the main change (positive) is at about 500 mμ, where octahedral complexes of Co^{2+} absorb (Cotton and Wilkinson, 1966). The difference spectrum in this region also resembles that of the tight binding but ancillary Co^{2+} sites in alkaline phosphatase (Simpson and Vallee, 1968).

Effect of pH on the Spectra of the Binary Cobalt(II)–Phosphoglucomutase Complex. Figure 2a shows the spectrum of the Co^{2+}–phosphoglucomutase complex (25% excess enzyme) at pH 5.1 in comparison to that at pH 8.5, and Figure 2b shows a plot of the fractional change in absorbance at 620 mμ *vs.* pH for the Co^{2+} complex at 25°. Within experimental error (±2%) the same fractional change was also observed at 570 and 532 mμ. At most only a small fraction of this change could have been caused by dissociation of Co^{2+} from the enzyme, as was indicated by a spectral titration at pH 5 (not shown). Although a very slight precipitation of the enzyme with concomitant light scattering was encountered at this pH, so that the end point was difficult to determine precisely, the addition of at least a twofold excess of Co^{2+} caused no significant intensity changes at 620 mμ above that produced by a slight excess of Co^{2+}, and did not produce a spectrum equivalent to that at pH 8.5.

Although the fractional change in absorbance varies with pH in a sigmoidal manner, the curve is more shallow than would be expected for a variety of simple models; hence, no attempt was made to rationalize this behavior quantitatively.

Unfortunately, the reversibility of this titration could not be demonstrated because of the tendency of phosphoglucomutase to precipitate during changes from high to low pH, although changes in the opposite direction can be made with facility. (This tendency is observed with metal-free enzyme as well as with the Zn^{2+} complex and is a function of salt concentration, identity of buffer anions, pH, and temperature.) However, since the absorbance of the Co^{2+} complex was the same whether prepared at pH 7.5 or prepared at pH 5 and subsequently titrated to pH 7.5 (see Experimental Procedure), the spectral change appears to be potentially reversible even though the problem of precipitation prevents verification of this point. Note that there is only a difference of about 10% in intensity between spectra obtained at pH 7.5 and 8.5. Because this difference is small and because most other studies of the enzyme have been conducted at the former pH, many of the spectral studies in this paper were conducted at pH 7.5 even though from a spectral standpoint, alone, pH 8.5 would have been a better pH.

Effect of Temperature on the Spectrum of the Cobalt(II)–Phosphoglucomutase Complex. The effect of temperature on the visible and near-infrared spectrum of the Co^{2+}–enzyme complex is shown in Figure 3. The pH, 8.5, was sufficiently high to be in the region where the spectrum is essentially independent of pH at room temperature. No attempt was made to control changes in pH with temperature caused by buffer effects, nor to determine the pH effect at temperature other than 25°; however, an essentially parallel temperature effect in the visible band was also observed at pH 7.5 (not shown). Note that these temperature effects and those described below are strictly reversible. The near-infrared band

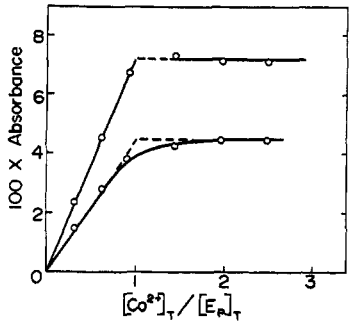

FIGURE 4: Spectral titration of phosphoglucomutase with cobalt(II) at 0 and 28°. Conditions were similar to those described in Figure 1c, except that an actual titration was performed: at 28° (○) and 0° (●). Samples were centrifuged after the third, fifth, and sixth addition of metal and returned to clean absorption cells for subsequent spectral measurements. Corrections for dilution were made before absorbances were plotted.

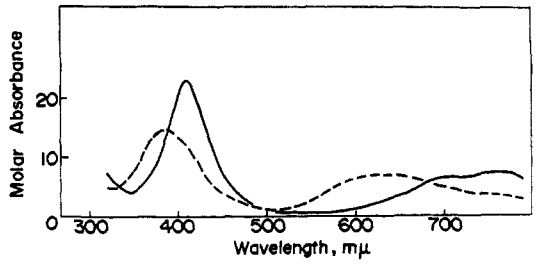

FIGURE 5: Spectra of nickel(II)–phosphoglucomutase complexes. Solutions of phosphoglucomutase at 158 mg/ml of 2.5 mM) in 50 mM Tris-HCl (pH 7.5) were present in both sample reference cells. The solid-line spectrum was obtained at 28° with 1.75 mM Ni^{2+} and Mg^{2+} present in the sample and reference cells, respectively. The dashed-line spectrum was obtained with 2 mM enzyme; 4 mM Ni^{2+} and 2 mM Ni^{2+} were present in the sample and reference cells, respectively, and the temperature was 24°.

shown in Figure 3 is centered at 1350 mμ and its oscillator strength is about 4.1×10^{-5}.

The results in the accompanying paper (Ray *et al.*, 1972) show that only a small part of the decrease in absorbance with decreasing temperature can be explained in terms of reduced active site occupancy by Co^{2+} under the conditions used, here, *viz.*, at a Co^{2+} to enzyme ratio of 1:1 (see also the following section). However, since in the 1:1 complex redistribution of Co^{2+} to ancillary sites does occur to some extent with decreasing temperature, subsequent temperature studies were conducted with a Co^{2+} to enzyme ratio of 2.3:1, where active-site occupancy is complete (Ray *et al.*, 1972). The plot in the Figure 3, inset, shows the effect of temperature on ϵ_{620} under these conditions. The ordinate is a log scale and the abscissa shows $1000/T$.

Titration of Phosphoglucomutase with Cobalt(II) at 0 and 28° as Followed by Spectral Changes. As noted above, titrations of phosphoglucomutase with Co^{2+} followed by activity regain (see Ray *et al.*, 1972) indicate that a minor redistribution from active site binding to ancillary binding occurs in the 1:1 complex as the temperature is reduced from 30 to 0° Spectral data supporting this interpretation are provided by the titrations shown in Figure 4.[1] Note again that the effect of temperature on the spectrum in the presence of excess Co^{2+} cannot be caused by either dissociation of the active-site Co^{2+} or simple partition among ancillary sites, because of the parallel disposition of the final limbs of the two titration curves.

That the active site was completely saturated at 0° with 2.3 equiv of Co^{2+} (final point on the titration curve) was shown by the results of an experiment in which enzyme equilibrated under these conditions was assayed at 28° in such a way that no increase in temperature was allowed until the enzyme was in the presence of a large excess (10 mM) of EDTA. Since no redistribution of Co^{2+} to alternative enzyme sites is expected in the presence of excess EDTA, and since the same activity was observed in an identical sample equilibrated with Co^{2+} at 28° prior to the assay, the active site must be saturated with Co^{2+} at 0° at a 2.3:1 ratio. The reverse experiment, in which enzyme samples equilibrated with excess Co^{2+} at 28 and 0°, respectively, were assayed at 0°, also produced equal activities.

Spectrum of the Nickel(II)–Phosphoglucomutase Complex. The visible spectrum Ni^{2+} in the presence of 15% excess of enzyme at 28° consists of a weak, composite peak with an ϵ_{max} of about 7 centered at about 730 mμ (oscillator strength about 1.1×10^{-4}). The two main components of the visible band have maxima in at about 700 and 760 mμ. A relatively symmetrical near-ultraviolet peak appears at 410 mμ with an ϵ_{max} of about 23 (oscillator strength $\sim 3.8 \times 10^{-4}$). The spectrum is shown in Figure 5.

Activity titrations described in the accompanying paper (Ray *et al.*, 1972) show that Ni^{2+} is bound predominately but not exclusively at the active site at a 1:1 ratio of Ni^{2+} to enzyme. The spectrum (not shown) obtained at a Ni^{2+} to enzyme ratio of 1:2 in order to minimize ancillary Ni^{2+} binding, did not appear to be significantly different from that obtained at a 1.15 ratio, although low intensity of the spectral bands prevented a thorough analysis of this point (see below). Hence, the complex predominating at a 1:1 ratio at 28° will be referred to as the Ni^{2+}–phosphoglucomutase complex.

Increasing the Ni^{2+} to enzyme ratio increases the absorbance in both the visible and near-ultraviolet (uv) regions, and the dashed line in Figure 5 approximates the absorbance of the "second" Ni^{2+} bound by the enzyme, although there is no indication that the second Ni^{2+} occupies a discrete site.

Titration of Phosphoglucomutase with Nickel(II) as Followed by Spectral Changes. A spectral titration of phosphoglucomutase with Ni^{2+} was followed at three wavelengths: 410, 610, and 780 mμ; the results are shown in Figure 6 (24°). The data are relatively imprecise because of the very small intensity changes involved; hence, the lines in the figure were drawn only to show that the spectral changes are consistent with the preferential formation of a 1:1 complex of Ni^{2+} to enzyme. In spite of the poor precision, at each wavelength the lack of a rigid stoichiometry is suggested. This was expected from results of activity titrations (Ray *et al.*, 1972).

Effect of Temperature on the Spectrum of the Nickel(II)–Phosphoglucomutase Complex. The effect of temperature on the spectrum of the mixture of complexes formed at a 1:1 ratio of Ni^{2+} and phosphoglucomutase is shown in Figure 7. Within experimental error an isosbestic point occurs in the visible spectrum at 680 mμ; however, there is no isosbestic point at other crossovers in the far-visible and near-uv re-

[1] According to this interpretation, the titration curves for absorbance in Figure 4 and activity, Figure 3 in the accompanying paper (Ray *et al.*, 1972), should be superimposable at a given temperature. That the corresponding curves have slightly different shapes probably reflects inaccuracies in the spectral data.

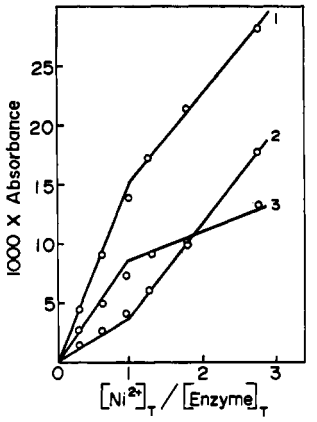

FIGURE 6: Spectral titration of phosphoglucomutase with nickel(II) at 24°. The sample and reference cells both contained phosphoglucomutase at 140 mg/ml (2.24 mM), 20 mM Tris-HCl (pH 7.5); equivalent amounts of Ni^{2+} and Mg^{2+} in 50 mM Tris-HCl (pH 7.5), were added to these cells respectively. After *each* addition the contents of the cell was briefly centrifuged at 16,000*g*. Corrections for dilution were made before plotting. Numbers 1, 2, and 3 on plots refer to absorbance changes at 410, 610, and 780 mμ, respectively.

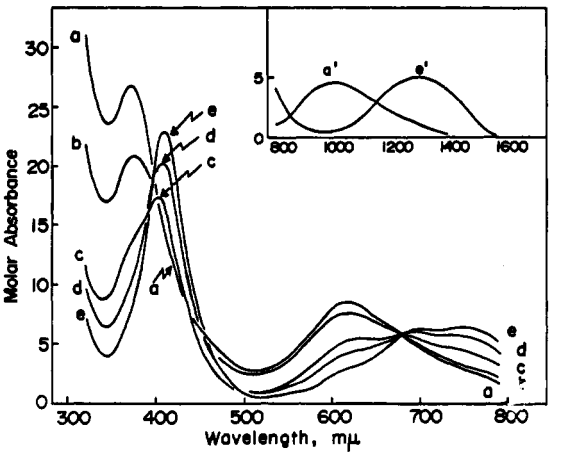

FIGURE 7: The effect of temperature on the visible and near-ultraviolet spectrum on the nickel(II)–phosphoglucomutase complex. Conditions were similar to those described for the solid-line spectrum in Figure 5, except that the enzyme concentration was 140 mg/ml (2.24 mM); 1 equiv of Ni^{2+} and Mg^{2+} was used. Temperature was controlled and measurements were made as described in the Experimental Section: a, 1°; b, 6.4°; c, 11.8°; d, 18.3°; e, 24.4°. Inset: the effect of temperature on the near-infrared spectrum of the nickel(II)–phosphoglucomutase complex. The near-infrared spectrum was obtained under similar conditions, but in D_2O (see Experimental Section); also the enzyme concentration was 148 mg/ml (2.37 mM) and 1.0 equiv of Ni^{2+} was used; temperatures were: a', 2°; e', 27°.

gions. The increased absorbance at less than 350 mμ at the lower temperatures was not caused by precipitation of the Ni^{2+}–enzyme since (a) extensive centrifugation at 0° did not alter the spectrum, (b) the same spectral changes were observed whether the study began at 0° or at room temperature and hence are strictly reversible, and (c) the absorbance in this region was not noticeably time dependent. However, light scattering was apparent in solutions of both the Mg^{2+}– and Ni^{2+}–enzyme and a slight reversible aggregation that was more extensive for the Ni^{2+}– than the Mg^{2+}–enzyme could have caused such an effect. Also shown in this figure (inset) are the near-infrared bands of the 1:1 complex at 0 and 27°; the oscillator strength of the high-temperature, near-infrared band is about 5.9×10^{-5}.

The band positions in the low-temperature spectrum, which involves Ni^{2+} bound predominately at ancillary sites (see Ray *et al.*, 1972), are rather typical of Ni^{2+} in an octahedral environment in which there are strong-field ligands (nitrogenous ligands in the present context). The absorbance from the residual high-temperature form probably causes the long-wavelength tail in both the visible and near-infrared bands of the low-temperature spectrum. The absorbance at less than 330 mμ, is not typical of simple Ni^{2+} complexes of the above type; however, this absorbance could be produced either by light scattering (see above) or by a differential perturbation of aromatic residues of the protein by Ni^{2+} and Mg^{2+} (the Mg^{2+} complex was present in the reference cell). In this respect, the fact that the optical density at 290 mμ from the protein alone is greater than 100 while the absorbance in question at only a slightly longer wavelength, 320 mμ, is less than 0.05 is worth pointing out.

The spectrum of the Ni^{2+} complex predominating at high temperature (the active-site complex) may be affected in the region below 380 mμ by the presence of a minor amount of the low-temperature species or by light scattering, or both, and we have discounted absorbance in this region in comparing the high-temperature spectrum to spectra of models. Like the low-temperature spectrum the high-temperature spectrum also resembles Ni^{2+} in an octahedral environment; however, in contrast to the low-temperature spectrum, the high-temperature spectrum indicates an environment composed of weak-field ligands (see following section).

Model Systems for Co^{2+} and Ni^{2+} Binding. Spectra were examined for a large variety of Co^{2+} and Ni^{2+} complexes in which oxygen and nitrogen ligands of the type present in proteins were involved. (Sulfur ligands at the metal binding site in the phosphoglucomutase system are ruled out by Milstein's data on the effect of mercuribenzoate on Mg^{2+} binding (1961).) The spectral correspondence between the enzymatic system and the best overall model system which was found, saturated aqueous potassium acetate (33.3 mole % KAc), was remarkably close for both Co^{2+} and Ni^{2+}. Figure 8 shows this comparison: solid lines, spectra of the Ni^{2+} and Co^{2+} complexes of phosphoglucomutase; dashed and dotted lines, those of Ni^{2+} and Co^{2+}, respectively, in the model system. Not only are band positions nearly identical in the enzyme and model systems, but intensities are also similar.

The strongly absorbing species in the Co^{2+}-saturated KAc solution probably in $Co(Ac)_4^{2-}$ (see Discussion), which probably is partially dissociated in this medium. Hence, Figure 8b also shows the spectrum of a mixture of anhydrous $CoAc_2$ and 2 equiv of KAc in anhydrous dimethylformamide. A cobalt concentration of 10 mM was used in order to minimize dissociation of the $CoAc_4^{2-}$. The molar extinction under these conditions was about 15% less than in a dilute solution of $CoAc_2$ in dimethylformamide, 0.2 M in tetraethylammonium acetate. In both the aqueous solution and in dimethylformamide the visible band is centered at about 565 mμ while the near-infrared band is centered at about 1300 mμ. The analogous bands for the Co^{2+}–enzyme are centered at about 565 and 1350 mμ. Band splitting, at least in the visible band, is more severe in the enzyme system.

Table I shows the λ_{max} and ϵ_{max} values of the near-ultra-

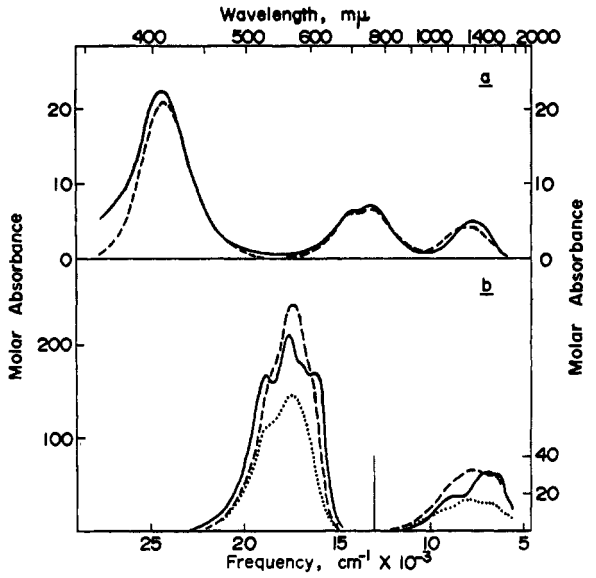

FIGURE 8: Comparison of the spectra obtained with the nickel(II) and cobalt(II) complexes of phosphoglucomutase with the same metals in a model system. (a) The nickel(II) complexes. The spectrum of the Ni^{2+}–phosphoglucomutase complex, solid line, was taken from Figure 5 (solid line spectrum) and Figure 7 (spectrum e′) and the spectrum indicated by the dashed line is that of Ni^{2+} in saturated aqueous potassium acetate at room temperature (near-infrared spectrum obtained in D_2O at the same mole fraction KAc). (b) The cobalt(II) complexes. The spectrum of the Co^{2+}–phosphoglucomutase complex, solid line, was taken from Figure 3 (28° spectrum). The dotted line spectrum is that of Co^{2+} in saturated aqueous potassium acetate (near-infrared spectrum obtained in D_2O); the dashed line spectrum was obtained by dissolving a dried mixture of Co^{2+}–acetate plus 2 equiv of potassium acetate in anhydrous dimethylformamide.

violet and near-infrared bands for some of the Ni^{2+} complexes examined here or reported elsewhere. The table is arranged in order of increasing value of the λ_{max} value for the near-infrared band; this order is similar to that which would be obtained if the λ_{max} values for the near-ultraviolet band had been used. For complexes involving simple ligands, those with a predominance of strong-field ligands occur toward the top of the table; those with a predominance of weak-field ligands are toward the bottom.

TABLE 1: Position and Intensity of the Near-Ultraviolet and Near-Infrared Bands of Nickel(II) Complexes.

Ligands	λ_{max}, mμ (ϵ_{max})
$(H_2NC_2H_4NH_2)_3$[a]	343 (8.6), 890 (7.2)
$(H_2NC_2H_4NH_2)(H_2O)_4$[a]	370 (6.4), 970 (5.0)
$EDTA^{4-}$[a]	382 (16), 990 (30)
Phosphoglucomutase (0°)	~370 (--), ~1000 (4.5)
$(NH_2CH_2CO_2^-)(H_2O)_4$[a]	383 (6.5), 1040 (5.1)
$NTA^{3-}(H_2O)_2$[a]	390 (13), 1050 (14.7)
$(O_2CCO_2^{2-})(H_2O)_4$[a]	393 (10), 1120 (4.4)
$(O_2CC_2H_2CO_2^{2-})(H_2O)_4$[a]	394 (6.3), 1160 (2.9)
$(Ac^-)_2(H_2O)_4$[b]	394 (6.3), 1165 (2.9)
$(H_2O)_6$[c]	392 (5.3), 1180 (2.4)
$(CH_3CO_2H)_6$[d]	405 (12), 1230 (4.7)
$(DMF)_6$[c]	413 (16), 1240 (6.3)
$(Ac^-)_2(DMF)_4$[e]	402 (16), 1250 (5.2)
$(NO_3^-)_6$[f]	408 (19), 1250 (4.0)
$(Ac^-)_x(H_2O)_{6-x}$[g]	410 (21), 1250 (4.0)
$(Ac^-)_6$[h]	414 (19), 1280 (2.4)
Phosphoglucomutase (28°)	410 (23), 1300 (5)
$(Ac^-)_x(DMF)_{6-x}$[i]	415 (25), 1325 (4.9)
$(SO_4^{-2})_6$[j]	433 (21)

[a] Complex identified from the ratio of Ni^{2+} to ligand used, together with published stability constants both for the complex in question and other possible complexes of Ni^{2+} and that ligand (Sillen and Martel, 1964). The number of water ligands is taken as six minus the maximum number of ligands that could be contributed by the chelator. [b] Dilute solutions of Ni^{2+} in 1 M KAc were used. According to published constants (Sillen and Martel, 1964), Ni^{2+} should be associated with a minimum of two Ac^- groups under these conditions, although more Ac^- groups might be involved. [c] Sufficiently dilute solutions of $Ni(ClO_4)_2$ were used (less than 5 mM) so that the same spectra were obtained, on a molar basis, after a further 10-fold dilution. DMF = dimethylformamide. [d] Duffy and Ingram (1969). [e] Solution in dimethylformamide was prepared from anhydrous $NiAc_2$. Spectra increased in intensity by about 5% and λ_{max} shifted by about 2 mμ on diluting from 25 to 2.5 mM; hence the identity of this complex is questionable. [f] Nitrate glass; Duffy and Ingram (1969). [g] Ni^{2+} in saturated aqueous KAc. [h] Acetate glass; Duffy and Ingram (1969). [i] Anhydrous $NiAc_2$ plus 0.1 M tetraethylammonium acetate in anhydrous dimethylformamide. [j] Duffy *et al.* (1968).

Discussion

The spectral titrations of phosphoglucomutase with Co^{2+} and Ni^{2+} (Figures 1c and 6) that are reported here confirm the activity titrations described in the accompanying paper (Ray *et al.*, 1972): at room temperature and pH 7.5 the titration curves have break points at a 1:1 stoichiometry, although in the case of Ni^{2+} the break is not sharp because of binding at ancillary sites. However, with both metals a substantial preference is shown for active site binding. Hence, in the following discussion we will take the properties of the 1:1 complex to be those of the active-site complex.

Both the intensity and relative positions of the spectral bands of the 1:1 Ni^{2+}–phosphoglucomutase complex are those expected for an octahedral or octahedral-like Ni^{2+} complex. These spectral properties are in contrast with those of Ni^{2+} complexes involving coordination geometries such as tetrahedral or square planar (Cotton and Wilkinson, 1966). Moreover, the Ni^{2+}–enzyme is a high-spin paramagnetic complex,[2] as would be expected for an octahedral (or tetrahedral) Ni^{2+} complex. Since Mg^{2+}, like Ni^{2+}, prefers octahedral coordination (Matwigoff and Tanbe, 1968; Nakamura and Meiboom, 1967), it would not be surprising if the Mg^{2+}–

[2] The bulk magnetic susceptibility of Ni^{2+}–phosphoglucomutase is essentially the same as that of aquo Ni^{2+}, since at 17° and a concentration of 1.5 mM both produce essentially the same frequency shift, 6 cps, for methyl protons of dioxane, tetramethylammonium chloride, and *tert*-butyl alcohol. Measurements were made with a 220-mHz nmr spectrometer operated with a Fourier transform program and a direct frequency readout: private communication from Drs. George H. Reed and Jack S. Leigh, Jr., of the Johnson Foundation.

enzyme also had an octahedral-like coordination—especially in view of the similarity in the catalytic properties of these two forms of the enzyme (Ray *et al.*, 1972). On the other hand, the coordination symmetry of the Co^{2+}–phosphoglucomutase complex (Figure 8b) cannot be easily identified from its spectrum (see below), although in intensity it more nearly resembles that of a tetrahedral than an octahedral complex (see Carlin, 1965; Cotton and Wilkinson, 1966).

In attempting to learn something more about the metal activating site in phosphoglucomutase, systems were sought which would produce spectra with *both* Ni^{2+} and Co^{2+} that resemble the analogous metal–enzyme spectra. Only systems involving oxygen and nitrogen ligands of the type likely to be present as coordinating groups in proteins were examined. The spectra in Figure 8 indicate a striking success in mimicing the spectra of both the Co^{2+} and Ni^{2+} complexes of the enzyme with a *single* system—saturated aqueous potassium acetate. Unfortunately, because of the paucity of spectral comparisons between metalloenzymes and simple model complexes it is not possible to state that a correspondence such as that in Figure 8 allows firm conclusions to be drawn about the metal binding site of the enzyme. If the latter involved an irregular coordination structure, such as is the case for carboxypeptidase (Hartsuck and Lipscomb, 1971) spectral comparisons to simple complexes could be misleading. On the other hand, in such cases it is not certain that simple complexes could be found whose spectra would sufficiently resemble that of the metalloenzyme to suggest that such comparisons be made in the first place. If, however, the metal coordination site of the enzyme were arranged so that complexes with a reasonably symmetrical ligand field were formed, such comparisons might well suggest some of its properties. Although we do not know in advance which of the above situations exists in the case of phosphoglucomutase, an estimate of zero-field splitting obtained from the *solution* electron paramagnetic resonance (epr) spectrum of the Mn^{2+}–phosphoglucomutase complex indicates that at least this complex of phosphoglucomutase is not highly distorted (Reed and Ray, 1971). Moreover, we are sufficiently impressed by the Figure 8 comparisons—especially in the case of the Ni^{2+} complexes—to consider what might be postulated about the metal binding site of the enzyme on the assumption that extrapolation from simple metal complexes to a metalloenzyme is valid, at least in the present case. In order to do this, questions regarding both the identity of the complexes of Co^{2+} and Ni^{2+} present in the model system and the extent to which the model system is exclusive in its ability to mimic the enzyme system must be considered, although both are difficult to answer.

Saturated aqueous KAc is not unique in its ability to produce Ni^{2+} and Co^{2+} spectra similar to those of the analogous enzyme complexes. Thus, a saturated solution of KAc in methanol produces a relatively good but slightly poorer match for both metals (not shown). Acetate glass (rapidly quenched melts of LiAc and KAc) produces a somewhat poorer match (Duffy *et al.*, 1968; Duffy and Ingram, 1969) while excess acetate in dimethylformamide produces a relatively good match for Co^{2+} (Figure 8b) but a rather poor match for Ni^{2+} (not shown). A number of other Co^{2+} complexes exhibit visible and near-infrared spectra with intensities and band positions similar to that of Co^{2+} phosphoglucomutase but have not been examined in the case of Ni^{2+}, $Co(CF_3CO_2)_4^{2-}$ (Bergman and Cotton, 1966), $(Phe_3AsO)_2Co(NO_3)_2$ (Cotton *et al.*, 1963), and $Co(NO_3)_4^{2-}$ (Cotton and Dunne, 1962). In all of these cases the metal ligands are oxygens and in all cases two or more weak-field, CO_2^- ligands (or the spectrochemically similar ONO_2^- ligands) are present.

Although several simple Co^{2+} complexes containing nitrogenous ligands exhibit visible spectra similar to that of Co^{2+}–phosphoglucomutase, *e.g.*, Co(histidine)$_2$ (Morris and Martin, 1970), there is essentially no spectral correspondence between these complexes and the enzyme in the near infrared. In addition, the presence of nitrogen in the coordination sphere of octahedral Ni^{2+} usually produces spectral bands at shorter wavelengths than those shown in Figure 8. This tendency can be seen in terms of the position of the absorption bands of a number of such complexes (Table I), and is expected from the increased field strength of nitrogen as opposed to hydroxyl or carboxylate ligands (Cotton and Wilkinson, 1966). In view of these observations we suggest that to the extent that spectral comparisons based on simple models are valid, the metal activating site in phosphoglucomutase contains a predominance of weak-field hydroxyl and carboxylate ligands—as opposed to nitrogen ligands. (The seryl phosphate group of the enzyme also would be classed as a weak-field ligand and thus could be involved in metal binding.)

By comparison to the high-temperature (28°) spectrum of the Ni^{2+} enzyme, the low-temperature (0°) spectrum is substantially blue shifted (Figure 7) as is the spectrum of the second Ni^{2+} in the 2:1 complex at room temperature (Figure 5). In both cases ancillary binding predominates—as opposed to active-site binding. Thus the change from active site to ancillary binding is consistent with an increased importance of strong field (nitrogenous) ligands.

The question of the identity of the absorbing species for Ni^{2+} and Co^{2+} in saturated KAc poses additional problems. In the case of Ni^{2+} the spectrum indicates the presence of octahedral-like complexes of the type $Ni(Ac)_x(H_2O)_{6-x}$, where x is at least equal to 2 and probably is 3 or more (see Table I). In the case of Co^{2+} the complex is probably $Co(Ac)_4^{2-}$ since its spectrum is similar to that of $Co(CF_3CO_2)_4^{2-}$ in acetonitrile (Bergman and Cotton, 1966);[3] however in the aqueous solvent the tetraacetate undoubtedly dissociates to some extent, as is indicated by the increased intensity of $Co(Ac)_4^{2-}$ in dimethylformamide (Figure 8). Hence, the correspondence between the spectra of Co^{2+} and Ni^{2+} in the model and enzyme systems involves a comparison of the Ni^{2+}–enzyme to an octahedral-like complex and of the Co^{2+}–enzyme to a tetrahedral complex. Since Co^{2+} has a greater preference than Ni^{2+} for tetrahedral coordination (Cotton and Wilkinson, 1966), especially in anionic environments, the difference in coordination in the model system is not surprising, and it is conceivable that the metal activating site of phosphoglucomutase actually is sufficiently flexible to allow it to assume a different geometry depending on whether Co^{2+} or Ni^{2+} is bound. Thus, the effect of temperature on the Co^{2+} phosphoglucomutase complex (Figure 3), which does not involve migration of the metal to an ancillary site, might be rationalized in terms of a tetrahedral–octahedral equilibrium such as the one studied by Shaife and Wood (1967): $CoCl_4^{2-} + 3H_2O \rightleftharpoons CoCl_3(H_2O)_3^- + Cl^-$. In such a case a more highly absorbing tetrahedral-like species would be expected to predominate at the higher temperatures and absorbancy should

[3] Except for intensity, the spectrum of Co^{2+} in saturated KAc is also quite similar to that of Co^{2+} in acetate glass (Duffy and Ingram, 1969), and of $CoAc_2$ plus excess tetramethylammonium acetate in anhydrous chloroform, acetone, dimethylacetamide, and dimethyl sulfoxide (W. J. Ray, Jr., unpublished results).

decrease with decreasing temperature, as was found (Figure 3).[4]

However, distinctions among Co^{2+} complexes with symmetries substantially lower than those of more or less regular octahedral complexes can be difficult to make on the basis of spectra, alone (Carlin, 1965). Thus while the high-temperature spectrum of the Co^{2+}–phosphoglucomutase complex does indeed resemble that of $Co(CH_3CO_2)_4^{2-}$, it is also quite similar to that of $(Phe_3AsO)_2Co(ONO_2)_3$ in the visible range (except for more severe band splitting in the case of the enzyme) and passably resembles the spectrum of the latter complex in the near-infrared region. The $(Phe_3AsO)_2Co(ONO_2)_3$ complex is not tetrahedral but has a distorted hexadentate coordination involving six oxygens (by analogy with $((CH_3)_3PO)_2Co(ONO_2)$, Cotton and Soderberg, 1963). Moreover, small temperature-dependent changes in the symmetry of a hexacoordinate complex such as this also might give rise to temperature effects of the type observed.[5] Thus, there seems to be no firm basis for a decision as to the coordination of the Co^{2+}–enzyme. The fact that the Mn^{2+}–enzyme appears to be octahedral like (Reed and Ray, 1971) and that the Mn^{2+}– and Co^{2+}–enzymes have rather similar catalytic properties (see Ray *et al.*, 1972) makes us rather reluctant to suggest a tetrahedral-like coordination for the latter complex at this time, although this possibly certainly cannot be ruled out.

It might be noted again that the visible spectrum of the Co^{2+}–enzyme is rather similar to that of the Co^{2+} complex of carbonic anhydrase (Lindskog and Nyman, 1964; Coleman, 1967) as well as that of alkaline phosphatase (Simpson and Vallee, 1968). In the case of carbonic anhydrase, nitrogen ligands predominate at the metal binding site, and by analogy with the Zn^{2+} complex, these are arranged in a somewhat irregular array (Kannen *et al.*, 1972). However, the near-infrared band for the carbonic anhydrase complex is sufficiently different from that of the corresponding phosphoglucomutase complex that further comparison does not appear to be warranted at this time.

Conclusions

While few firm conclusions can be drawn from this study, we would like to suggest as a working hypothesis that the coordination geometry of the reactive Ni^{2+} complex of phosphoglucomutase is octahedral like and that the metal ligands are predominately weak-field hydroxyl and carboxylate groups. In view of its preference for octahedral coordination the physiologically active metal ion, Mg^{2+}, may be bound similarly. However, we are unable to make suggestions about the active-site binding of Co^{2+} other than to point out that its complex with phosphoglucomutase does not involve regular octahedral coordination.

Acknowledgment

The authors acknowledge the excellent technical assistance of Mrs. Lily Ng. The unusually helpful suggestions and criticisms of Dr. R. J. P. Williams, who originally suggested an examination of the effect of temperature on the Co^{2+} phosphoglucomutase complex, are gratefully acknowledged. The authors are also indebted to Drs. R. T. Simpson and B. L. Vallee for making available a copy of their manuscript prior to publication, to Dr. R. B. Martin for helpful discussions, and Drs. S. E. Ostroy and L. E. Mortenson for the use of their Cary 14 spectrophotometers.

References

Bauman, R. P. (1962), Absorption Spectroscopy, New York, N. Y., Wiley, pp 238–243.
Bergman, J. G., Jr., and Cotton, F. A. (1966), *Inorg. Chem. 5*, 1420.
Carlin, R. L. (1965), *Transition Metal Chem. 1*, 1.
Coleman, J. E. (1967), *J. Biol. Chem. 242*, 5212.
Cotton, F. A., and Dunne, T. G. (1962), *J. Amer. Chem. Soc. 84*, 2013.
Cotton, F. A., Goodgame, D. M. L., and Soderberg, R. H. (1963), *Inorg. Chem. 2*, 1162.
Cotton, F. A., and Soderberg, R. H. (1963), *J. Amer. Chem. Soc. 85*, 2402.
Cotton, F. A., and Wilkinson, G. (1966), Advanced Inorganic Chemistry, 2nd ed, New York, N. Y., Wiley, Chapters 25, 26, and 29.
Duffy, J. A., Glasser, F. P., and Ingram, M. D. (1968), *J. Chem. Soc. A*, 551.
Duffy, J. A., and Ingram, M. D. (1969), *J. Chem. Soc. A*, 2398.
Hartsuck, J. A., and Lipscomb, W. (1971), *Enzymes 3*, 1.
Kannen, K. K., Liljas, A., Waara, I., Bergstén, P.-C., Lövgren, S., Strandberg, B., Bengtsson, U., Carlbom, U., Fridborg, K., Jarup, L., and Petef, M. (1972), *Cold Springs Harbor Symp. Quant. Biol. 36*, 221.
Latt, S. A., and Vallee, B. L. (1971), *Biochemistry 10*, 4263.
Lindskog, S. (1966), *Biochemistry 5*, 2641.
Lindskog, S., and Nyman, P. P. (1964), *Biochim. Biophys. Acta 85*, 462.
Matwiyoff, N. A., and Tanbe, H. (1968), *J. Amer. Chem. Soc. 90*, 2796.
Milstein, C. (1961), *Biochem. J. 79*, 591.
Morris, P. J., and Martin, R. B. (1970), *J. Amer. Chem. Soc. 92*, 1543.
Nakamura, S., and Meiboom, S. (1967), *J. Amer. Chem. Soc. 89*, 1765.
Peck, E. J., Jr., and Ray, W. J., Jr. (1969), *J. Biol. Chem. 244*, 3748.
Ray, W. J., Jr., Goodin, D. S., and Ng, L. (1972), *Biochemistry 11*, 2800.
Ray, W. J., Jr., and Mildvan, A. S. (1970), *Biochemistry 9*, 3886.
Reed, G. H., and Ray, W. J., Jr. (1970), *Biochemistry 10*, 3190.
Schaife, L. G., and Wood, K. P. (1967), *Inorg. Chem. 6*, 358.
Sillen, L. G., and Martel, A. E. (1964), Stability Constants of Metal-Ion Complexes, 2nd ed, London, Burlington House.
Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7*, 4343.

[4] Since the visible spectrum of the Co^{2+}–phosphoglucomutase complex resembles that of the Co^{2+}–carbonic anhydrase complex, basic form (Lindskog and Nyman, 1964) the possibility of a temperature-dependent spectral change was also examined in this system by comparing the spectra of the binary Co^{2+}–carbonic anhydrase complex and the ternary complex of Co^{2+}, carbonic anhydrase, and cyanide at 0 and 30°; however, in neither case was a significant change observed (J. S. Multani and W. J. Ray, Jr., unpublished results).

[5] Duffy and coworkers (Duffy *et al.*, 1968; Duffy and Ingram, 1969) suggest that temperature-induced spectral intensity changes for Co^{2+} complexes involving ligands such as acetate or sulfate may be produced by a temperature-dependent change between monodentate and bidentate coordination of these ligands.