Substrate Activity of (Adenosine triphosphato)tetraamminecobalt(III) with Yeast Hexokinase and Separation of Diastereomers Using the Enzyme†

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ABSTRACT: Yeast hexokinase has been found to catalyze the reaction: $Co(NH_3)_4ATP$ + glucose $\rightleftharpoons$ $Co(NH_3)_4$(Glc-6-P)-ADP, for which the apparent equilibrium constant at 25 °C and pH 7.0 is 0.31 mM^{-1}. Both a direct spectrophotometric assay at 585 nm and an assay using [^{14}C]glucose give a V_{max} of 0.13 s^{-1}, which is 0.06% of the V_{max} for MgATP, and a K_M for $Co(NH_3)_4ATP$ of 0.65 mM. Only one of the two diastereomers of $Co(NH_3)_4ATP$ is a substrate for hexokinase. The complex $Co(NH_3)_4$(Glc-6-P)ADP is separated from the inactive isomer by chromatography on a column of cross-linked cycloheptaamylose. The active isomer and glucose are formed by reacting $Co(NH_3)_4$(Glc-6-P)ADP with hexokinase. Solutions enriched in the two isomers have been characterized by their ^{31}P NMR and CD spectra, the active isomer having a positive CD band at 550 nm, and the inactive isomer a negative band. Both isomers exhibit inhibition vs. MgATP which is predominantly competitive, and have K_{is} values approximating the value of K_M. The K_M for $Co(NH_3)_4$(glc-6-P)ADP is 0.21 mM and that for glucose is 0.14 mM; these values are consistent with the expected Haldane relationship. The two diastereomers of $Co(NH_3)_4ATP$ have been degraded to the enantiomers of $Co(NH_3)_4H_2P_3O_{10}$ without loss of chirality. The CD of each enantiomer at 550 nm is the same in sign as that of the parent nucleotide, and X-ray studies of a crystal of $Co(NH_3)_4H_2P_3O_{10}\cdot H_2O$ from the inactive isomer of $Co(NH_3)_4ATP$ show that the active isomer has left-hand screw sense (Λ) coordination. The diastereomers of $Co(NH_3)_4ADP$ and $Cr(NH_3)_4ADP$ have been separated by chromatography on cross-linked cycloheptaamylose and their probable absolute configurations assigned, based on the similarity of their CD spectra to those of the isomers of $Co(NH_3)_4ATP$.

A great deal of effort has been directed toward understanding metal complexes of ATP. One method used in this laboratory has been to prepare complexes using substitution-inert transition-metal ions. This approach carries the advantage that species of specific configurations which can be identified and potentially separated are inert for sufficient time that their interactions with enzyme can be examined. A knowledge of which species can bind to an enzyme and which can act as substrates will contribute to understanding the most intimate details of how kinases function in solution.

The ATP complexes of chromium(III) (DePamphilis and Cleland, 1973) have proven their value as dead-end inhibitors in the study of enzyme kinetic mechanisms (Schimerlik and Cleland, 1973; Janson and Cleland, 1974; Bar-Tana and Cleland, 1974; Danenberg and Cleland, 1975; Armbruster and Rudolph, 1976). Considerable progress has been made in the separation of mono-, bi-, and tridentate isomers of CrATP (Brummond and Cleland, 1974; Cornelius and Cleland, 1977), and the complete separation of these complexes will increase their value as paramagnetic probes of enzyme active sites (Gupta et al., 1976; Mildvan et al., 1976; Gupta, 1977).

Recently, nucleotide complexes of cobalt(III)ammines have been prepared in this laboratory (Cornelius et al., 1977). Phosphorus-31 NMR aided in the characterization of these complexes and provided conclusive evidence of the coordination state of the nucleotide. The β,γ-bidentate complex $Co(NH_3)_4ATP$[1] was the most rigorously examined of the cobalt(III) complexes and exists in solution as two diastereomers which differ in arrangement around the β-phosphorus, which is an asymmetric center with a chiral AMP group attached. This paper describes the separation of these isomers and presents the results of a study of the interaction of each isomer with yeast hexokinase.

Materials and Methods

Materials. The preparation of $Co(NH_3)_4ATP$ and $Co(NH_3)_4ADP$ has been described by Cornelius et al. (1977). Glucose-6-phosphate dehydrogenase, catalase, and yeast hexokinase (type F-300) were obtained from Sigma. The concentration of active sites in hexokinase solutions was determined by measuring the amount of [^{14}C]glucose binding induced by the presence of excess CrATP (Danenberg and Cleland, 1975).

Preparation of Cycloheptaamylose Gel. Cycloheptaamylose (CHpA[2]) gel was prepared by a modification of the method of Hoffman (1970). It is important to follow the procedure exactly; attempts to increase batch size or use other apparatus have failed. To 5.0 g of CHpA is added 3.0 mL of water, and the mixture is stirred until the powder is uniformly wetted. The wet powder is dissolved in 6.0 mL of 50% (w/v) NaOH and is added to 50 mL of epichlorohydrin being rapidly stirred in the

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[1] The designation $Co(NH_3)_4ATP$ is used when specification of the state of protonation of the complex is not intended. When the actual species present in solution is to be specified, $Co(NH_3)_4HATP$ or $[Co(NH_3)_4ATP]^-$ is used. A similar convention is used for $Co(NH_3)_4$(Glc-6-P)-ADP.

[2] Abbreviations used are: CHpA, cycloheptaamylose; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); EDTA, (ethylenedinitrilo)tetraacetic acid; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP.

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uncovered metal semimicro cup of a blender. A granular mass which is thrown up on the walls of the blender cup forms after 10–30 min of blending, accompanied by a drop in pitch of the sound made by the blender. Blending is continued for 2 min, and the liquid is then decanted and the gel washed three times with acetone by decantation. The gel is scraped from the container and washed three times with 200 mL of water and once with 200 mL of 90 °C water. The washed gel from five such preparations is combined and while wet forced through a 100-mesh sieve to achieve the desired particle size. The gel is then suspended in water in a 600-mL beaker, and the fines which will not settle within 5 min are decanted. This procedure is repeated 20 times. The amount of gel remaining is sufficient to fill a column 0.7 × 235 cm. For all chromatography reported, the temperature was 4 °C, gravity-driven flow rates of 3–6 mL/h were used, and the eluting solution was 10 mM Pipes,[2] pH 7 (KOH).

Kinetic Assays. Inhibition studies with hexokinase used the glucose-6-phosphate dehydrogenase assay in which the appearance of NADPH at 340 nm is monitored. Solutions contained 0.1 unit/mL hexokinase, 1 unit/mL glucose-6-phosphate dehydrogenase, 50 mM Pipes (pH 7.0), 100 μM NADP, 5 mM magnesium acetate, and MgATP and glucose as specified. The presence of 1 mM citrate was used to linearize the time course of the reaction (Shill and Neet, 1975). This system without the ATP was also used as an end-point assay to determine the amount of free ATP in preparations of $Co(NH_3)_4ATP$. The concentrations of MgATP and $Co(NH_3)_4ATP$ in the inhibition experiments were then corrected by this amount, which was less than 1% of the concentration of the cobalt complex.

Two methods were used to monitor the reaction when $Co(NH_3)_4ATP$ was used as a substrate for hexokinase. In the first method a 0.25-mL system containing 50 mM Pipes, up to 500 units/mL hexokinase, and the specified concentrations of [^{14}C]glucose and $Co(NH_3)_4ATP$ was held at 25 °C for a measured time interval. The reaction was quenched by the addition of 0.02 mL of 60% $HClO_4$, and the enzyme was denatured by vortexing after the addition of 1 drop of CCl_4 and 2 mL of water. The solution was filtered and charged onto a 3-mL column of AG50W-X2 cation-exchange resin, 100–200 mesh, H^+ form. Fractions of 5 mL were collected as the column was washed with 30 mL of water to elute free glucose and then with 50 mL of 1 M HCl to elute the reaction product, $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$. Aliquots from each fraction were counted in a scintillation counter, and the amount of glucose bound as glucose 6-phosphate to the cobalt was determined from the relative amounts of ^{14}C found in the eluates with water and 1 M HCl. In tests to determine the amount of substrate activity in the separated isomers, glucose levels as low as 10 mM were used to improve sensitivity. At these levels of glucose, however, the reaction does not go to completion, and a correction was made using the measured equilibrium constant of 0.31 mM^{-1} (see Results).

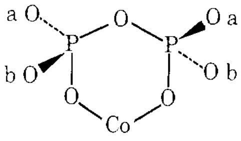
In the second method, the absorbance change at 585 nm was recorded for solutions containing hexokinase, 50 mM Pipes (pH 7.0), 10 mM glucose, and varying levels of $Co(NH_3)_4ATP$. The largest difference between the visible spectra of $Co(NH_3)_4ATP$ and $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ occurs at 585 nm, where the respective molar absorptivities are 19 and 78 M^{-1} cm^{-1}. This spectrophotometric method was also used with the sugar substrates fructose, mannose, glucosamine, and 2,5-anhydromannitol.

Degradation of $Co(NH_3)_4ATP$ to $Co(NH_3)_4H_2P_3O_{10}$. A degradation scheme similar to that applied to RNA (Steinschneider and Fraenkel-Conrat, 1966) was used to degrade $Co(NH_3)_4ATP$. A 100% excess of solid H_5IO_6 is added to 0.5 mL of 10–50 mM $Co(NH_3)_4ATP$ at isoionic pH (3.3). After 1 h of reaction in the dark, 0.01 mL of ethylene glycol is added and the solution is allowed to stand for 10 min. Next, 2.0 mL of 0.4 M aniline, adjusted to pH 5 with HCl, is added and the solution is allowed to stand for 3 h. Solid material is centrifuged off, the solution is diluted 100-fold, and cations are removed by passing the solution through a 5-mL column of AG50W-X2 resin, 100–200 mesh, H^+ form. The eluate is adjusted to pH 6 with KOH and is charged onto a 5-mL column of AG1-X2 resin, 100–200 mesh, Cl^- form. A sharp red band containing $[Co(NH_3)_4P_3O_{10}]^{2-}$ forms at the top of the column. To reduce the number of monoanions in the final product, this band is removed to a second 5-mL column of AG1-X2 and eluted with 0.05 M HCl. The yield in solution is 85%. Crystals of $Co(NH_3)_4H_2P_3O_{10}\cdot H_2O$ form upon adding 1 volume of ethanol and storing for several weeks at −20 °C (Cornelius et al., 1977).

Spectra. Proton-decoupled phosphorus-31 NMR spectra were determined using a Bruker HX90E spectrometer operating at 36.44 MHz in the pulsed mode. Transients were accumulated in and transformed by a Nicolet 1080 computer. Solutions used for the spectra contained 0.1 M EDTA to reduce the effect of paramagnetic impurities. Phosphorus chemical shifts are referenced to an external standard of 1 M H_3PO_4. CD spectra were recorded on a Jasco Model J-20 spectropolarimeter.

Nomenclature. The separation and identification of diastereomers of nucleotide complexes as described below have created a need for a nomenclature which readily identifies the screw sense of coordination without reference to the identity of the individual atoms. Only with such a nomenclature can the names of related complexes directly reflect similarities and differences in the stereochemistry of the complexes.

The nomenclature defined here adopts the symbols Δ and Λ to indicate the right- and left-hand screw sense of isomers. The helix which determines this screw sense has as its axis a line perpendicular to the chelate ring (assumed planar as shown in **1**) and passing through the cobalt atom. The sense of the helix is then determined by the bond which connects the chelate ring to the remaining part of the ligand. For ADP complexes, attachment of adenosine at either of the a positions gives the Λ isomer (that is, the P–O bond forms a skew line which defines a left-hand screw around the vertical axis through cobalt), while attachment at either b position gives the Δ isomer. Similarly, for ATP complexes attachment of AMP at a or b gives the Λ or Δ isomer, respectively. The enantiomers of the bidentate complex $Co(NH_3)_4H_2P_3O_{10}$ are similarly named.[3]



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[3] The difficulty with the *R*,*S* system for naming these complexes is illustrated by the Λ bidentate isomers of $Co(NH_3)_4ATP$, $Co(NH_3)_4ADP$, MgATP, and MgADP. The first three are *R* and the fourth is *S*, despite their similar stereochemistry. This situation results from the fact that magnesium is heavier than carbon but lighter than phosphorus, while cobalt is heavier than all of the others. Note that when the asymmetric center in the chelate ring (the β-phosphorus in the complexes discussed here) has a chiral group such as adenosine or AMP attached to it, the isomers will be diastereomers. In the tripolyphosphate complexes, where none of the groups attached to the middle phosphorus are chiral, the isomers are enantiomers.

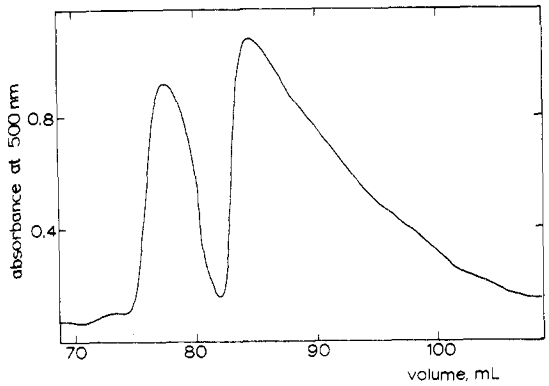


FIGURE 1: Separation of $Co(NH_3)_4ATP$ and $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ on a 0.7 × 235 cm column of CHpA gel in 10 mM Pipes, pH 7, at 4 °C. Total load, 400 μmol. The first peak is $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$.

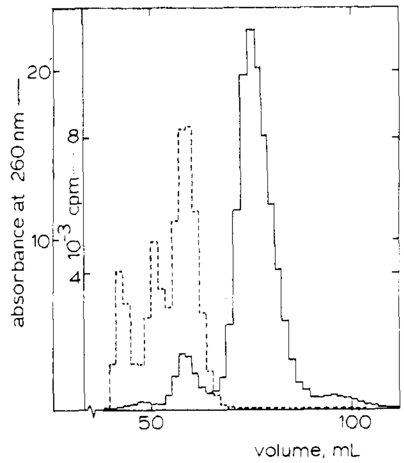


FIGURE 2: Separation of $Co(NH_3)_4ATP$ and $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ on the CHpA column under low-load conditions (20 μmol). The dashed line shows compounds containing [^{14}C]glucose. The largest absorbance peak is $Co(NH_3)_4ATP$ and the largest ^{14}C peak is $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$.

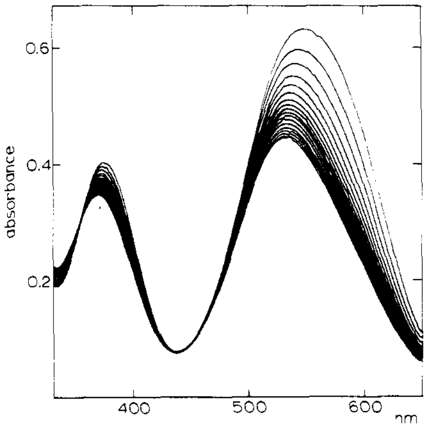
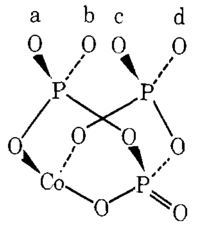


FIGURE 3: Changes in visible spectrum accompanying the hexokinase-catalyzed reaction of 6.3 mM $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ to form $Co(NH_3)_4ATP$ and glucose in 10 mM Pipes, pH 7.0, 25 °C, with glucose oxidase present. The initial solution is the top curve at 375 and 550 nm and the bottom one at 330 nm.

Tridentate–ATP complexes are considered to be generated from the corresponding bidentate complex. When the α-phosphate enters the coordination sphere, its coordination position is determined by the screw sense of the bidentate complex. Thus, the Λ-bidentate–ATP complex gives rise to two Λ-tridentate isomers, with adenosine attached at position a or b in **2**. The Δ-bidentate isomer gives rise to two Δ-tridentate isomers, with adenosine attached at position c or d. The tridentate isomers are further designated endo or exo, depending upon the configuration about the new chelate ring formed when the α-phosphate becomes coordinated. The designation is endo if the γ-phosphate and the rest of the molecule are on the same side of this ring and exo if they are on opposite sides of the ring. In **2,** attachment of adenosine at a or d puts adenosine and the phosphate on opposite sides of the ring and gives exo isomers. Attachment of adenosine at b or c puts groups on the same side of the ring and gives endo isomers. Thus, attachment at a, b, c, or d gives the Λ-exo, Λ-endo, Δ-endo, and Δ-exo isomers.



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While this nomenclature has been applied here to six-membered chelate rings formed by phosphates, it is also applicable to other five- or six-membered rings in metal chelates.

Results

Substrate Activity of $Co(NH_3)_4ATP$. The following reaction catalyzed by yeast hexokinase has been examined by several techniques.

$$[Co(NH_3)_4ATP]^- + \text{glucose} \rightleftharpoons [Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP]^{2-} + H^+ \quad (1)$$

When [^{14}C]glucose is used, a glucose-containing species binds to cation-exchange resin, showing that it contains cobalt. Under the acidic conditions of the cation-exchange resin, the product is present as $[Co(NH_3)_4(Glc\text{-}6\text{-}PO_4H)H_2ADP]^+$.

Chromatography on CHpA gel has also been used to characterize reaction 1. Figure 1 shows the resolution achieved in separating $Co(NH_3)_4ATP$ from $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ under high-load conditions. The first peak corresponds to $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$, which is lavender and is readily distinguished on the column from $Co(NH_3)_4ATP$ which is red. Figure 2 shows similar chromatography under low-load conditions monitored by the adenine absorption at 260 nm on a system containing a limiting amount of glucose labeled with ^{14}C. The constant specific activity across the first major adenine peak confirms the identity of $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$. The two peaks of ^{14}C activity which are not associated with corresponding adenine absorption could be glucose, glucose-6-phosphate, or decomposition products of $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ from which the nucleotide has dissociated.

Lastly, the changes in the visible spectrum accompanying reaction 1 have been investigated. Figure 1 shows repetitive scans as $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ isolated from chromatography on CHpA gel reacts with hexokinase to form $Co(NH_3)_4ATP$. Inspection of Figure 3 reveals the basis for selecting 585 nm as the most suitable for direct detection of substrate activity of $Co(NH_3)_4ATP$ with hexokinase.

The time course of the substrate activity of $Co(NH_3)_4ATP$ with hexokinase has been examined using the [^{14}C]glucose assay. Such a time course appears in Figure 4 for an experiment run at 25 °C in the presence of 10 mM glucose, 0.67 mM

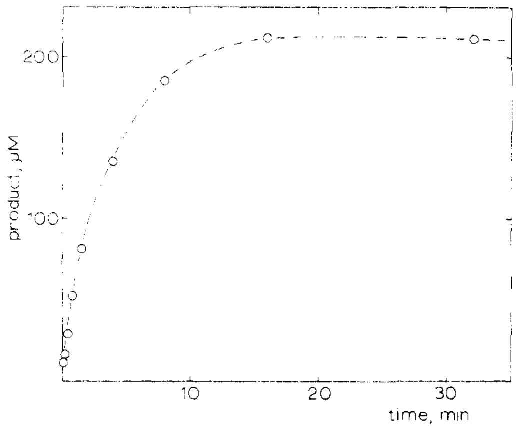


FIGURE 4: Time course for $Co(NH_3)_4ATP$ reaction using the [^{14}C]-glucose assay. Reaction mixtures contained 0.67 mM $Co(NH_3)_4ATP$, 10 mM glucose, 500 units/mL hexokinase, and 50 mM Pipes, pH 7, at 25 °C. Points at 2 min or less were run at 100 units/mL hexokinase for five times as long as indicated.

$Co(NH_3)_4ATP$, and 14 μM hexokinase. The plateau observed in the figure corresponds to an approach to equilibrium. The value of the apparent equilibrium constant has been determined by a series of 32-min experiments at glucose concentrations ranging from 2 to 100 mM. Extrapolation of these results to infinite glucose concentration reveals that only part of the $Co(NH_3)_4ATP$ present can react. The results are readily interpreted in terms of the reaction of only one of the two diastereomers previously shown by ^{31}P NMR studies to be present in this complex (Cornelius et al., 1977). Evaluation of the equilibrium constant is achieved by use of eq 2, where [P] and [G] are the equilibrium concentrations of product and glucose, respectively, and [X] is the initial concentration of active $Co(NH_3)_4ATP$.

$$K_{eq} = \frac{[Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP]}{[glucose][Co(NH_3)_4ATP]} = \frac{[P]}{[G]([X] - [P])} \quad (2)$$

This equation can be recast in the linear form of eq 3, to which data have been fitted using computer programs designed for equations of similar form (Cleland, 1967).

$$1/[P] = (1/[X]K_{eq})(1/[G]) + 1/[X] \quad (3)$$

The results are $K_{eq} = 0.31 \pm 0.05$ mM^{-1} and $[X] = 0.30 \pm 0.01$ mM at pH 7. Since the total concentration of $Co(NH_3)_4ATP$ was 0.67 mM, this value of [X] indicates that 45 ± 2% of the cobalt complex is present as the active isomer.

In an experiment designed to show the reversibility of reaction 1, a reaction mixture containing [^{14}C]glucose was allowed to come to an equilibrium calculated from the above value of K_{eq} to involve 226 μM bound glucose and was then diluted to twice the volume with a solution of the same hexokinase concentration. After the system again reached equilibrium, the concentration of bound glucose was found to be 81 μM. This value is in reasonable agreement with the value of 91 μM predicted using the above values of K_{eq} and [X].

The [^{14}C]glucose assay has also been used to determine the value of the K_M for $Co(NH_3)_4ATP$. At each concentration of $Co(NH_3)_4ATP$ used, assays were performed for four time intervals and a least-squares line was used to determine the rate at that concentration. From experiments at 10 mM glucose and 0.18 to 0.88 mM $Co(NH_3)_4ATP$, the value of K_M was 0.65 ± 0.06 mM. Using a value for the enzyme concentration determined by the CrATP-induced binding of glucose (Danenberg and Cleland, 1975), the V_{max} is 0.13 s^{-1}. This value was 0.06% of the V_{max} for MgATP under similar conditions.

TABLE I: Substrate Activity of Sugars with Yeast Hexokinase Using $Co(NH_3)_4ATP$ as the Other Substrate.

	with MgATP[b]		with $Co(NH_3)_4$-ATP
sugar[a]	K_M (mM)	V_{max} (rel)	V_{max} (rel)
mannose	0.05	0.8	0.3
glucosamine	1.5	0.7	0.5
glucose	0.1	(1.0)	(1.0)
2,5-anhydromannitol	2.4	1.1	1.9
fructose	0.7	1.8	3.4

[a] Concentration = 50 mM. [b] Values for 2,5-anhydromannitol at pH 7.0 from R. E. Viola and W. W. Cleland, unpublished results. All others at pH 7.5 from Sols et al. (1958).

The direct spectrophotometric assay at 585 nm was also used to determine K_M, and a wider range of $Co(NH_3)_4ATP$ concentrations was employed in these experiments. From experiments at 0.06 to 89 mM $Co(NH_3)_4ATP$, the value of K_M was within experimental error of the value from the [^{14}C]glucose assay, and no substrate inhibition was observed.

A variety of sugar substrates have been examined using the absorbance change at 585 nm to monitor the formation of the $Co(NH_3)_4$(sugar-6-P)ADP complexes. The relative values of V_{max} are reported in Table I. The apparent K_M for glucose in the presence of 2.5 mM $Co(NH_3)_4ATP$ was found to be 0.14 ± 0.01 mM at pH 7.

Separation of the Isomers of $Co(NH_3)_4ATP$. A 5-mL system containing 80 mM $Co(NH_3)_4ATP$, 150 mM glucose, 500 units/mL hexokinase, and 5 mM Pipes was adjusted to pH 7.0 by adding 5 M KOH to the vortexing solution. The solution was held at 25 °C, and the pH was adjusted back up to 7.0 every 30 min. After 4 h, when the absorbance at 585 nm was no longer increasing, the solution was placed on a 0.7 × 235 cm column of CHpA gel. Chromatography with 10 mM Pipes (pH 7.0) separated $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ from $Co(NH_3)_4ATP$ depleted in the active isomer as shown in Figure 1. The fractions containing $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ were combined, and 200 units/mL hexokinase, 2 units/mL glucose oxidase, and 20 units/mL catalase were added. The pH was adjusted to 6.0 and was readjusted as needed to maintain this pH as the reaction proceeded for 8 h at 25 °C. The enzymes were denatured by adjusting the pH to 2.0 with $HClO_4$ and vortexing with a few drops of CCl_4. After filtration, the solution of the active isomer of $Co(NH_3)_4ATP$ was concentrated by adsorbing the complex on a small column of AG50W-X2 at 4 °C and eluting with 0.3 M aniline. The aniline was extracted with diethyl ether, the pH adjusted to 7.0, and the solution again chromatographed on CHpA gel with 10 mM Pipes (pH 7.0). The purified active isomer can be used as it comes off the column or converted into its isoionic form by chromatography on cation-exchange resin, elution with 0.3 M aniline, and extraction of the aniline with ether.

The preparation of the inactive isomer made use of the $Co(NH_3)_4ATP$ depleted in the active isomer which was a by-product of the preparation of the active isomer. This $Co(NH_3)_4ATP$ solution was diluted to 5 mM with 50 mM Pipes (pH 7.0) and made 0.5 mM in glucose. For each micromole of $Co(NH_3)_4ATP$ present, 5 units of hexokinase was added, and the reaction was allowed to proceed for 24 h at 25 °C. The enzyme was denatured with $HClO_4$ and CCl_4 and filtered off. The solution was concentrated by the usual procedure on cation-exchange resin, the pH adjusted to 7.0, and the complex chromatographed on the CHpA column. The inactive isomer may be converted into its isoionic form or used at pH 7.0.

Characterization of the Isomers of $Co(NH_3)_4ATP$. The extent of isomeric enrichment of solutions obtained in the manner just described has been determined by measuring the fraction of the $Co(NH_3)_4ATP$ present which could act as a substrate for hexokinase in the [^{14}C]glucose assay. Solutions enriched in the active isomer were found to contain 71–89% active isomer, while solutions enriched in the inactive isomer contained only 7% active isomer. In an attempt to reduce the amount of substrate activity in the inactive isomer, the extraction of active isomer by reaction with hexokinase and glucose and chromatography on CHpA gel was repeated twice more on some samples. The level of substrate activity remained at 5–7% in spite of these further treatments. This level of substrate activity also remains constant when the length of incubation time is increased. Thus, if the inactive isomer is a substrate for hexokinase at all, its rate under the conditions of the assay is less than 1% of the rate of the active isomer.

The CD spectra of solutions enriched in the active and inactive isomers of $Co(NH_3)_4ATP$ are shown in Figure 5. The concentration (based on A_{260}) of inactive isomer used for the CD spectrum was 84% that of the active isomer. These solutions gave spectra of equal magnitude and correspond very closely to equal concentrations of the isomers as described later under Discussion. The spectra are nearly equal and opposite but are shifted from each other slightly. The λ_{max} for the active isomer is 550 nm, while that for the inactive one is 545 nm. This small difference is shown in the spectrum of the mixed isomers, which is also shown in Figure 5.

The ^{31}P NMR spectrum of $Co(NH_3)_4ATP$ previously reported (Cornelius et al., 1977) shows small chemical-shift differences between the two isomers for the tightly coupled α- and β-phosphorus atoms, and simulated spectra of the individual isomers were generated to correspond to the observed spectrum. Figure 6 shows experimental spectra of the α,β region for solutions enriched in the individual isomers. For each isomer, the γ-phosphorus appears at −4.0 ppm as a doublet with small virtual coupling to the α-phosphorus. The experimental spectra are in excellent agreement with the simulated spectra previously reported.

When $Co(NH_3)_4ATP$ is present, the time course of hexokinase-catalyzed reaction between MgATP and glucose changes shape to become more linear. This is much the same effect as induced by citrate (Shill and Neet, 1975). The inhibition by $Co(NH_3)_4ATP$ was therefore measured in the presence of citrate. Under this condition, solutions enriched in either the inactive or active isomers give noncompetitive inhibition vs. MgATP in which the slope effect is much larger than the intercept one. For the inactive isomer, $K_{is} = 0.65 \pm 0.08$ mM and $K_{ii} = 4.5 \pm 0.7$ mM, while for the active isomer $K_{is} = 0.41 \pm 0.02$ and $K_{ii} = 2.4 \pm 0.1$ mM. The K_M of $Co(NH_3)_4ATP$ enriched in the active isomer was determined with the spectrophotometric assay at 585 nm to be 0.27 ± 0.01 mM. Although K_M values were determined in the absence of citrate and K_i values in its presence, citrate at the level used (1 mM) was found to have no effect on K_M.

Characterization of $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$. The preparation of $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ in solution was described above. The complex exhibits limited stability, decomposing at the rate of 10%/h at 25 °C, as measured by the change in absorbance at 585 nm of solutions at pH 7. The rate of decomposition is markedly slower at 4 °C, and solutions were always kept cold until use and were used within 8 h of the time they came off the CHpA column. Enzymatic assays for free glucose, glucose 6-phosphate, and ADP in aged solutions of the complex revealed that ADP was lost from the coordination sphere of the cobalt somewhat faster than was glucose-6-phosphate. At a detection level equal to 1% of the complex, no free glucose was found. Thus, no nonenzymatic reversal of reaction 1 occurs.

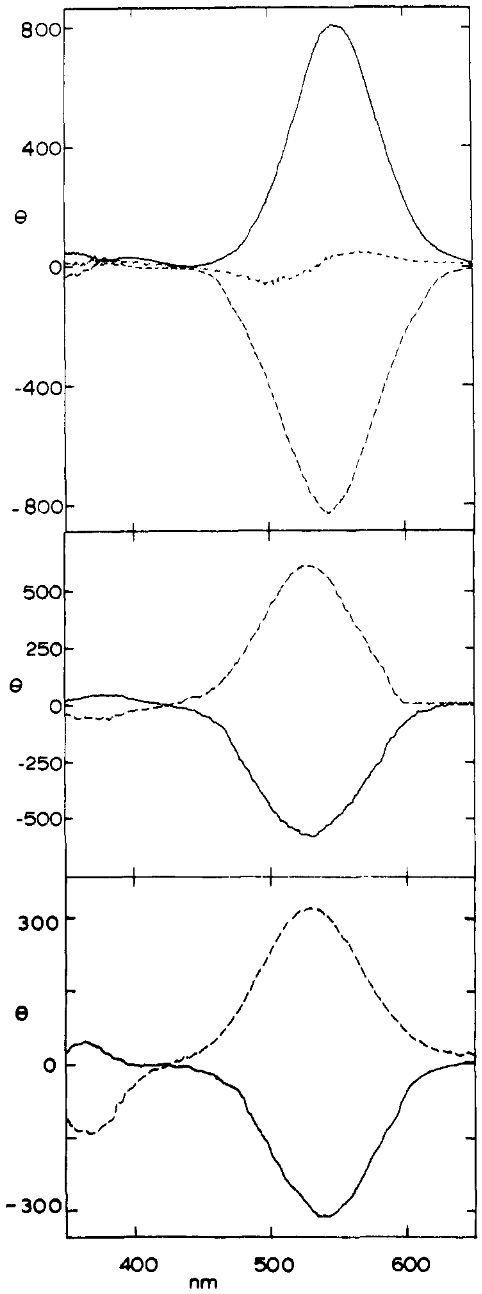


FIGURE 5: CD spectra of tetraamminecobalt(III) complexes. Top: $Co(NH_3)_4ATP$ in 10 mM Pipes, pH 7. The curve with positive ellipticity is the active isomer, the one with negative ellipticity is the inactive one, and the middle trace is the unresolved mixture of isomers. The spectra have been scaled to correspond to ellipticities (in deg cm^2/dmol) calculated for pure solutions. Middle: $Co(NH_3)_4H_2P_3O_{10}$ isomers in 50 mM HCl. The curve with positive ellipticity was prepared from active $Co(NH_3)_4ATP$, and the one with negative ellipticity from the inactive isomer. Spectra have been scaled to correspond to pure solutions. Bottom: $Co(NH_3)_4ADP$ isomers in 10 mM Pipes, pH 7. The curve with negative ellipticity is the first isomer eluted from the CHpA column, and the curve with positive ellipticity is the second isomer eluted.

The visible spectrum of $Co(NH_3)_4(Glc\text{-}6\text{-}P)ADP$ at pH 7 is shown as the initial curve in Figure 3. As the pH is lowered to 3, the maxima in the spectrum change reversibly from 550

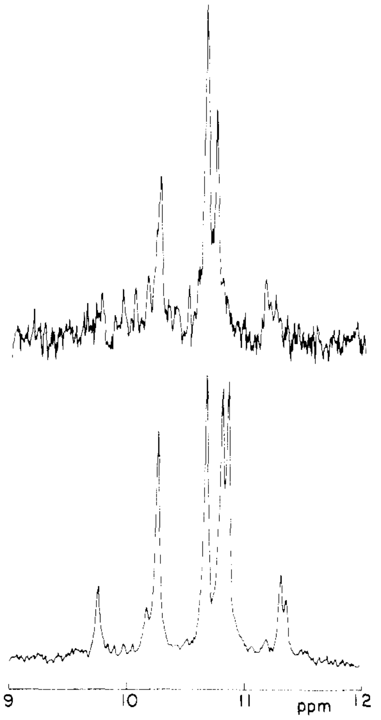


FIGURE 6: Proton-decoupled 36.4-MHz phosphorus-31 NMR spectra of the α,β region of $Co(NH_3)_4ATP$ isomers at pH 8 in the presence of 0.1 M EDTA. Upper curve is 5 mM active isomer, 4000 scans. Lower curve is 75 mM inactive isomer, 250 scans. Chemical shift scale relative to 1 M H_3PO_4.

(ϵ 102 M^{-1} cm^{-1}) and 373 (ϵ 59 M^{-1} cm^{-1}) to 523 (ϵ 77 M^{-1} cm^{-1}) and 364 nm (ϵ 52 M^{-1} cm^{-1}). The spectrum at pH 3 is very similar to that of $Co(NH_3)_4ATP$, which at pH 3.3 has maxima at 518 and 366 nm (Cornelius et al., 1977) and at pH 7 is negligibly different. The pK associated with the spectral changes of $Co(NH_3)_4$(Glc-6-P)ADP was calculated to be 5.03 ± 0.03.

For fructose, 2,5-anhydromannitol, glucosamine, and mannose, the spectral changes accompanying the reaction of $Co(NH_3)_4ATP$ to $Co(NH_3)_4$(sugar-6-P)ADP were also examined. Within experimental error, the spectral changes in the region 350 to 650 nm were identical with those which occur with glucose as the substrate.

Substrate Activity of $Co(NH_3)_4$(Glc-6-P)ADP. The spectrophotometric assay at 585 nm was used to determine the K_M for $Co(NH_3)_4$(Glc-6-P)ADP at pH 7.4. From experiments in the concentration range 0.04 to 0.3 mM, K_M was calculated to be 0.21 ± 0.09 mM, $V_{max} = 0.096 \pm 0.023$ mM/min, and $V/K = 0.45 \pm 0.09$ min^{-1}. For the forward reaction using the mixed $Co(NH_3)_4ATP$ isomers under the same conditions and at 33 mM glucose, $K_M = 1.33 \pm 0.19$ mM, $V_{max} = 0.056 \pm 0.004$ mM/min, and $V/K = 0.042 \pm 0.004$ min^{-1}. The ratio of the two V/K values in forward and reverse directions is 0.19 ± 0.05, assuming that half of $Co(NH_3)_4ATP$ is the active isomer, and thus the true V/K value in the forward direction is twice that observed.

The appropriate Haldane relationship in eq 4,

$$K_{eq} = \frac{(V/K)_1}{(V/K)_2 K_{i,Glc}} \qquad (4)$$

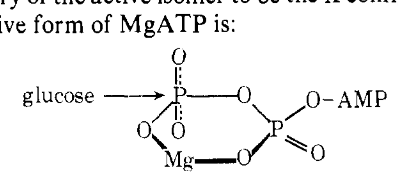
where the subscripts 1 and 2 refer to values for $Co(NH_3)_4ATP$ and $Co(NH_3)_4$(Glc-6-P)ADP, together with a value for K_{eq} of 0.75 ± 0.12 mM (corrected to pH 7.4 from the value determined at pH 7), allows calculation of $K_{i,Glc}$ as 0.25 ± 0.08 mM at pH 7.4, in reasonable agreement with the measured K_M for glucose of 0.14 mM at pH 7.

Degradation of $Co(NH_3)_4ATP$ to $Co(NH_3)_4H_2P_3O_{10}$. To determine the absolute stereochemistry of the active and inactive isomers of $Co(NH_3)_4ATP$, a method using periodate and aniline has been used to remove the adenosine from the complex and thus prepare the bidentate tripolyphosphato complex. The asymmetric center at the β-phosphorus remains intact and the crystal structure of either enantiomer of $Co(NH_3)_4H_2P_3O_{10}$ will establish the absolute configuration of the parent ATP complex. The crystal structure (Merritt, et al., 1978) of $Co(NH_3)_4H_2P_3O_{10}\cdot H_2O$ derived from the inactive isomer of $Co(NH_3)_4ATP$ shows the Δ configuration, and establishes that the isomer of $Co(NH_3)_4ATP$ which is a substrate for hexokinase has the Λ configuration. The CD spectra of solutions of $Co(NH_3)_4H_2P_3O_{10}$ prepared from solutions enriched in the active and inactive isomers of $Co(NH_3)_4ATP$ are shown in Figure 5. For a 3 mM solution of $Co(NH_3)_4H_2P_3O_{10}$ prepared from $Co(NH_3)_4ATP$ which had not been treated to enrich the isomer ratio, no CD spectrum could be detected. The limit of detection for this experiment corresponds to a limit of 3% on the extent to which one diastereomer may predominate in $Co(NH_3)_4ATP$.

Separation of Isomers of $Co(NH_3)_4ADP$ and $Cr(NH_3)_4ADP$. While the CHpA column does not appreciably separate $Co(NH_3)_4ATP$ isomers, the diastereomers of $Co(NH_3)_4ADP$ are well separated by chromatography of the freshly prepared complex on a 0.7 × 235 cm column of CHpA gel in 10 mM Pipes (pH 7.0). Following elution of several minor bands of undetermined composition, two major peaks were eluted from the column at volumes of 160 and 235 mL. The CD spectra of these solutions are shown in Figure 5. The spectra are slightly displaced, with the isomer which is eluted first having a peak at 540 nm, while the second one eluted has a maximum at 530 nm. The ^{31}P NMR spectra of the isomers (a pair of doublets in each case) were also recorded for comparison with the spectrum of the mixture of isomers reported by Cornelius et al. (1977), which clearly showed the presence of both isomers. For the isomer in the band eluted second, the α-phosphorus peak is upfield 0.2 ppm from that for the other isomer. The CHpA column also separates the diastereomers of $Cr(NH_3)_4ADP$, again with the first isomer eluted showing a negative CD band at 500 nm, and the second isomer a positive band (θ about 200 deg cm^2/dmol).

Discussion

This study of the interaction of the substitution-inert complex $Co(NH_3)_4ATP$ with hexokinase has yielded information not presently obtainable by examining the natural substrate MgATP. The fact that this cobalt complex is a substrate is an unambiguous determination that hexokinase can use a β,γ-bidentate metal complex of ATP. Furthermore, the enzyme has been shown to be stereospecific for one of the two possible diastereomers. The crystal structure (Merritt, et al., 1978) of the tripolyphosphate complex resulting from degradation of the inactive isomer of $Co(NH_3)_4ATP$ has revealed the absolute stereochemistry of the active isomer to be the Λ configuration. Thus, the active form of MgATP is:



This information will be an invaluable aid for the understanding of the microscopic mechanism based on the crystal structure of hexokinase. In addition, the stereochemical information is being extended to other enzymes which use ATP by simple experiments which determine the sign of the CD spectrum of unreacted Co(NH$_3$)$_4$ATP. For phosphoribosyl pyrophosphate synthetase, the Δ form of Co(NH$_3$)$_4$ATP is the active isomer (Li et al., 1978).

Isomer Purity. The Λ and Δ isomers of Co(NH$_3$)$_4$ATP are present initially in nearly equal abundance. Equilibrium measurements reveal 45 ± 2% of the Λ (active) isomer, but this value may be subject to systematic errors. In particular, dissociation of glucose-6-phosphate from Co(NH$_3$)$_4$(Glc-6-P)-ADP during the experiments could result in a low experimental value. Measurements from the CD spectrum of Co(NH$_3$)$_4$-H$_2$P$_3$O$_{10}$ prepared from the mixed isomers indicate that neither isomer predominates by more than 3%, corresponding to a mixture of 48.5% of one isomer and 51.5% of the other. Both results are consistent with nearly equal concentrations for the two isomers.

Neither isomer could be prepared in 100% purity. At least two problems appear to be involved. For the inactive isomer, slow racemization about the β-phosphorus may be the limiting factor. For the active isomer, an impurity other than the inactive isomer also appears to be present. The equilibrium constant for conversion of the Λ isomer to Co(NH$_3$)$_4$(Glc-6-P)ADP is sufficiently large that at 0.5 M glucose less than 1% of the active isomer initially present should be present at equilibrium, but in spite of successive treatment of Co(NH$_3$)$_4$ ATP to deplete it in the active isomer, the amount could not be reduced below 5%. Inhibition by the inactive isomer does decrease the rate as the isomer ratio increases, but this inhibition was not large enough to prevent the observation of residual activity in the [^{14}C]glucose assay. The logical conclusion is that racemization occurs, and the conditions under which this occurs are a subject of continuing investigation.

The purity of samples of the active isomer ranged from 71 to 89% as measured by the [^{14}C]glucose assay. The remaining species in the solutions appear to include unidentified complexes which are not the inactive isomer. The CD spectra in Figure 5 were obtained from solutions diluted to have equal and opposite spectra but which had different concentrations based on A_{260}. Since the inactive isomer used contained 7% active and 93% inactive material, the net inactive isomer contributing to the CD spectrum was 86%. Assuming the CD spectra of the two isomers to be of equal magnitude and opposite in sign at 545–550 nm, the active isomer solution contained 72% active isomer. This result is consistent with the 71% found by direct assay only if the remaining 29% does not contribute to the CD spectrum. Attempts to remove the impurity by chromatography on cation-exchange resin under conditions which would have removed Co(NH$_3$)$_4$ADP were unsuccessful. The identity of the contaminant remains unknown.

Kinetic Parameters. The values of $V_{\max}$, K_{M}, and K_{is} presented under Results are all apparent values based on the total concentration of Co(NH$_3$)$_4$ATP determined from A_{260}. If the true K_{M} for the active isomer and K_{is} for the inactive one are equal, the observed K_{is} and K_{M} values will be the true ones. These conditions are sufficiently close to being true in the present case that more detailed calculations are not warranted, and the observed values of K_{M} and K_{is} may for practical purposes be taken as equal to the intrinsic values for the individual isomers.

For $V_{\max}$, the observed value is less than the true one by the factor $(1 + K_{\mathrm{M}}/K_{\mathrm{is}})$ for a mixture containing equal levels of active and inactive isomers. Although this correction when applied to the observed value gives 0.26 s^{-1} as the true $V_{\max}$, the relative maximum velocity, 0.06% that for MgATP, is probably still valid because a similar correction would certainly have to be applied to obtain the true $V_{\max}$ for MgATP. The worth of such a comparison is however clouded by the differences in the products of the reaction, since when Co(NH$_3$)$_4$ATP is a substrate both products remain together as a single complex. A comparison of the rates in the reverse direction of reaction 1 is more meaningful because the nucleotide and sugar products are independent entities for the reaction using either magnesium or cobalt.[4] The effects of nonproductive binding of inactive isomers are also eliminated. Using the ratios of V_1/V_2 for Co(NH$_3$)$_4$ATP and MgATP of 1.17 and 5.3, respectively, the $V_{\max}$ for Co(NH$_3$)$_4$(Glc-6-P)ADP is 0.26% that with MgADP and glucose-6-phosphate. This number shows the rate using cobalt(III) still to be very slow, although Co(NH$_3$)$_4$ATP reacts 60 times faster than Cr(NH$_3$)$_4$ATP and 200 times faster than CrATP (Danenberg and Cleland, 1975).

Isomers of Co(NH$_3$)$_4$ADP and Cr(NH$_3$)$_4$ADP. The absolute configuration of the separated isomers of Co(NH$_3$)$_4$-ADP and Cr(NH$_3$)$_4$ADP cannot be assigned by means of any direct evidence. A comparison of the CD spectra in Figure 5, however, reveals a striking resemblance among the spectra of the isomers of Co(NH$_3$)$_4$ATP, Co(NH$_3$)$_4$H$_2$P$_3$O$_{10}$, and Co(NH$_3$)$_4$ADP. The resemblance among the first two sets of spectra is known to correspond with the structures in that in each set the upper curve corresponds to the Λ isomer. In the absence of evidence to the contrary, the isomer of Co(NH$_3$)$_4$-ADP which has a positive CD spectrum at 530 nm and is the second one eluted from the CHpA column is assumed to be the Λ configuration. For Cr(NH$_3$)$_4$ADP, the band eluted second again has positive CD at 500 nm and is probably the Λ isomer as well.

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[4] We are comparing $V_{\max}$ values, and the binding steps (and thus the number of reactants) are unimportant. The only steps which affect $V_{\max}$ are phosphate transfer and product release, and any accompanying conformation changes in the enzyme reactant complexes.

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In Vitro and in Vivo Age-Related Modification of Human Erythrocyte Phosphoribosyl Pyrophosphate Synthetase[†]

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ABSTRACT: Upon storage, human erythrocyte phosphoribosyl pyrophosphate synthetase (PRibPP synthetase, EC 2.7.6.1) from normal individuals was found to undergo a spontaneous dissociation into active enzyme components of much smaller molecular mass (60 000–90 000). These modified forms of enzyme exhibit kinetic properties different from the original large molecular weight enzyme (over 200 000). The small active components can be reversibly associated to form larger molecules in the presence of purine ribonucleotides as well as phosphoribosyl pyrophosphate (PRibPP). ATP was found to be most effective in associating PRibPP synthetase, while guanylate nucleotides seem to have no effect. The large molecular weight components, once separated from the milieu, were not able to undergo further dissociation. Fresh or stored human white cell or tissue homogenates were found to lack the low-molecular-weight enzyme under all our experimental conditions. A characteristic enzyme modification similar to that observed in stored erythrocyte was also noted in erythrocytes of increasing ages. The physiological significance of these findings to the regulatory function of PRibPP synthetase in purine metabolism in vivo is discussed.

Phosphoribosyl pyrophosphate synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase) catalyzes the synthesis of PRibPP from ATP and ribose 5-phosphate. This enzyme has been shown to be regulated by a complex mechanism in bacteria (Switzer, 1969, 1970, 1971), in rat liver (Roth et al., 1974a,b), and in man (Fox and Kelley, 1972). The enzyme is able to exist in many active forms of various molecular weights (Schubert and Switzer, 1975; Roth et al., 1974a,b; Fox and Kelley, 1971). A detailed study on the relationship of the PRibPP synthetase activity to its subunit association has been reported recently (Becker et al., 1977; Meyer and Becker, 1977). Reassociation of the enzyme molecules in vitro can be achieved by the addition of ATP. The current study is designed to clarify whether aggregation or dissociation of the native enzyme occurs in normal erythrocytes during the in vitro aging process and to explore the physiological significance of these changes as well as the regulatory nature of purine nucleotides and other metabolites on PRibPP synthetase.

Materials and Methods

All the nonradioactive chemicals were obtained from Sigma Chemical Co., St. Louis, Mo. Radioactive substrates were purchased from New England Nuclear, Boston, Mass.

PRibPP Synthetase Enzyme Assay. Heparinized blood obtained from normal subjects was centrifuged at 1400*g* in an International refrigerated centrifuge for 10 min at 4 °C. The cells were washed several times with isotonic saline. After each washing, the buffy coat and the top layer of erythrocytes were carefully removed. The erythrocytes were hemolyzed by mixing with 2 volumes of distilled water, freezing in a dry ice–ethanol bath, and thawing three times. The mixture was then centrifuged at 25 000*g* at 4 °C in a Sorvall refrigerated centrifuge for 10 min, and the supernatant solution was used for the enzyme assay. The enzyme activities were measured by: (1) the amount of PRibPP produced (Yip and Balis, 1975a), (2) the conversion of [^{14}C]ATP to [^{14}C]AMP (Yip and Balis, 1975a) in the presence of ribose 5-phosphate, and (3) the reverse reaction of the conversion of [^{14}C]AMP to [^{14}C]ATP in the presence of phosphoribosyl pyrophosphate (Johnson et al., 1974). The assay mixture of (1) contained 70 mM Tris buffer (pH 7.4), 0.7 mM ethylenediaminetetraacetate, 1.7 mM mercaptoethanol, 7 mM magnesium ion, 33 mM potassium phosphate (pH 7.4), 0.17 mM ribose 5-phosphate, 0.17 mM ATP, 0.125 mM [^{14}C]adenine (sp act. 3.3 Ci/mol), and 5 μL of partially purified APRibTase (sp act. of 0.2 IU/mg, ca. 850-fold purified from human erythrocytes and completely void of PRibPP synthetase activity) in a total volume of 125 μL. Each sample was incubated in a 37 °C shaker bath for 10 to 60 min, depending on the amount of enzyme used. The reaction was terminated by immersion of the test tubes in an ethanol solid CO$_2$ bath. The procedure for the recovery of the radioactive nucleotide thus formed has been described (Yip and Balis, 1976). The assay mixture in 2 is essentially the same as that in 1, except [^{14}C]ATP (sp act. 0.8 Ci/mol) was used in place of [^{14}C]adenine and no purified APRibTase was added. Anion-exchange paper chromatography was used to separate the radioactive product from the reactants. After the

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