Oxidation-Reduction Equilibrium of Cytochrome b_5[1]

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Abstract: The reduction potential of trypsin-solubilized bovine liver cytochrome b_5 has been studied as a function of pH, temperature, and ionic strength. With an optically transparent thin-layer electrode and $Ru(NH_3)_6^{2+/3+}$ as mediator, $E°_{m,7}$ was found to be 5.1 (6) mV vs. NHE [μ = 0.1 M (phosphate), 25 °C]. The dependence of $E°$ on pH indicates the presence of an ionizable functional group on the protein that undergoes a redox-linked change in pK_a from 5.7 in the oxidized protein to 5.9 in the reduced protein (25 °C, μ = 0.1 M). The dependence of $E°_{m,7}$ on ionic strength can be analyzed in terms of Debye-Hückel theory to produce a net electrostatic charge for the oxidized cytochrome of -6.2, in excellent agreement with the value of -6.5 estimated from the protein sequence. The thermodynamic parameters for cytochrome b_5 electron transfer are $\Delta S°$ = -37 (2) eu and $\Delta H°$ = -11 (1) kcal/mol [μ = 0.1 M (phosphate), pH 7.0]. All of these results may be interpreted in terms of the involvement of a heme propionate group in redox-linked binding of cations and protons as suggested by the model for cytochrome b_5 electron transfer proposed by Argos and Mathews[4e] on the basis of their X-ray crystallographic studies.

The metabolic functions[3] and structural properties[4] of cytochrome b_5 have been studied extensively, yet relatively little experimental information is available regarding the mechanism by which this cytochrome changes oxidation state. Our recent studies analyzing the kinetics of cytochrome b_5 reduction by $Fe(EDTA)^{2-}$ have provided initial insight into the nature of this process.[5] We now report a functional analysis of the cytochrome b_5 oxidation-reduction equilibrium that provides complementary information and permits evaluation of the model proposed for cytochrome b_5 electron transfer by Argos and Mathews[4e] based on their crystallographic findings.

Experimental Section

The tryptic fragment of cytochrome b_5 was prepared from fresh beef liver to an $A_{412.5}/A_{280}$ ratio of 5.7-6.0 as previously described[5] and stored in liquid nitrogen. Glass distilled water was further purified to a resistivity of 17-18 MΩ cm by passage through a Barnstead NANOpure water purification system. $Ru(NH_3)_6Cl_3$ ($E°$ = 51 mV vs. NHE[6]) was obtained from Alfa and purified by the method of Pladziewicz et al.[7] All other chemicals were of reagent grade. pH measurements were made with a Radiometer Model PHM 84 meter equipped with a Radiometer type GK2321C combination electrode.

Electrochemical measurements were made with an optically transparent thin-layer electrode (OTTLE) in a nonisothermal configuration as previously described.[8] The OTTLE cell was machined from Lucite and employed quartz windows (path length approximately 0.2 mm). The working electrode was constructed from 500-line/in. electroformed gold mesh (Buckbee-Mears Co., Minneapolis, MN). Either a Radiometer type 4112 or Bioanalytical Systems saturated calomel reference electrode was used along with a platinum wire counter electrode. Temperature was controlled to ±0.1 °C with a Lauda Model RC-3 circulating water bath in conjunction with a specially designed water-jacketed OTTLE cell holder constructed from aluminum. The temperature of the OTTLE was monitored to ±0.2 °C with a Fluke Model 2175A digital thermometer fitted with a copper-constantan subminiature thermocouple positioned as close to the working electrode as possible. The potential of the minigrid electrode was controlled with a Princeton Applied Research Model 173 potentiostat and measured to ±0.1 mV with a Keithley Model 177 digital microvoltmeter. Electronic absorption spectra were obtained with a Cary 219 spectrophotometer equipped with a thermal isolation accessory and rear beam attenuator.

Solutions of cytochrome b_5 were prepared in sodium phosphate buffer to a concentration of 120 μM assuming the published extinction coefficients.[9] $Ru(NH_3)_6Cl_3$ concentration was 12 μM in all measurements except for those evaluating the effect of mediator concentration. This mediator was selected because it has a negligible absorbance over the wavelength range of interest and has a reduction potential close to that anticipated for cytochrome b_5. Solutions containing protein and mediator (approximately 0.5 mL) were electrochemically deoxygenated in situ by reduction for 15 min at -360 mV (vs. NHE) and subjected to one oxidation/reduction cycle prior to data collection. The absorbance/potential data were analyzed as previously described[8b] with a weighted linear least-squares fit to the Nernst equation. The resulting midpoint potentials were referenced to the normal hydrogen electrode as described by Dutton.[10] Thermodynamic parameters were calculated as described by Taniguchi et al.[8c]

Results

The spectra generated in a typical measurement are illustrated in Figure 1. Isosbestic points were observed at 353.0, 414.8, 437.7, 513.0, 534.2, 544.0, and 565.8 nm. Equilibration was determined at each potential by monitoring the change in absorbance at 556 nm and was usually achieved within 15 min. The Nernst plot ($E_{applied}$ vs. log ([O]/[R]) obtained from these spectra is shown in Figure 2. The protein was fully reversible and was cycled between reduced and oxidized states up to 8 times with no detectable change in behavior. As cytochrome b_5 has a net negative electrostatic charge and the mediator is positively charged, the effect of mediator concentration on $E°_{m,7}$ was evaluated to determine whether the use of $Ru(NH_3)_6^{2+/3+}$ perturbs the behavior of the protein. Variation of the [cytochrome b_5]/[$Ru(NH_3)_6^{2+/3+}$] ratio over several values in the range from 0.1 to 15 had no effect on the observed reduction potential [25 °C, pH 7.0 (phosphate) μ = 0.1 M]. This result also indicates that aquation of $Ru(NH_3)_6^{2+/3+}$ which is known to have no effect on the reduction potential of the complex also has no adverse effect on our measurements.

(1) A preliminary report of this work has been presented: Reid, L. S.; Mauk, A. G. *Fed. Proc.* **1982**, *41*, 1206. Contribution No. 6603 from the Division of Chemistry and Chemical Engineering, California Institute of Technology.

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Table I. Parameters Calculated from Analysis of the Ionic Strength Dependence of the Cytochrome b_5 Reduction Potential[a]

	f(μ)				
	$\mu^{1/2}$ [b]	$\mu^{1/2}/(1+\mu^{1/2})$ [b]	$\mu^{1/2}/(1+2\mu^{1/2})$ [b]	$\mu^{1/2}/(1+5.2\mu^{1/2})$ [c]	$\mu^{1/2}/(1+5.6\mu^{1/2})$ [d]
slope, V	0.039	0.080	0.132	0.377	0.411
$E°$, V	−0.009	−0.016	−0.021	−0.040	−0.042
q_{ox}	−0.1	−0.8	−1.7	−5.7	−6.2

[a] pH 7.0, phosphate buffer, 25 °C. [b] Reference 15b. [c] The constant 5.2 is the product[16] of the Debye-Hückel B term (0.329) and the radius (15.2 Å) of cytochrome b_5 estimated[5] from an assumed M_r of 11 000. [d] The constant 5.6 is the product[16] of the Debye-Hückel B term and the average radius (17 Å) of cytochrome b_5 estimated from crystallographic measurements.[4]

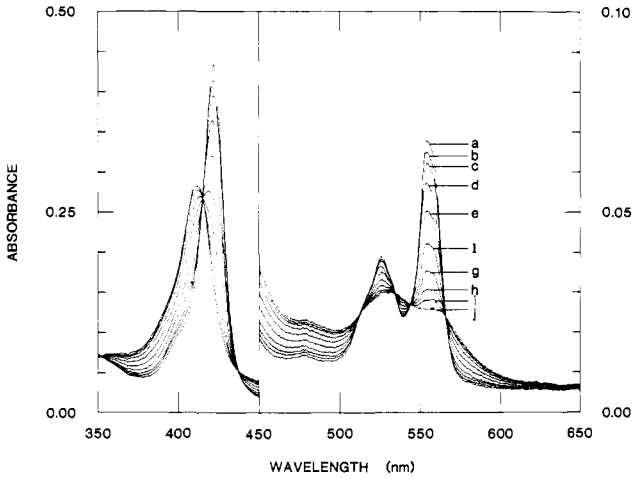

Figure 1. Representative family of thin-layer spectra of cytochrome b_5 at different values of applied potential, E_{app} (mV vs. NHE). Cytochrome b_5 (120 μM), $Ru(NH_3)_6^{2+/3+}$ (12 μM), 25 °C, pH 7 (phosphate), μ = 0.1 M: (a) −255.6, (b) −55.6, (c) −35.6, (d) −15.6, (e) 4.4, (f) 24.4, (g) 44.4, (h) 64.4, (i) 84.4, (j) 244.4.

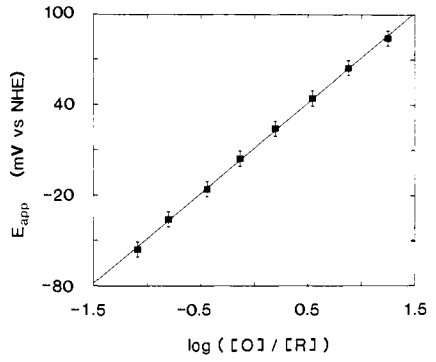

Figure 2. Nernst plot calculated from the spectra shown in Figure 1 by using ΔA_{556}. The midpoint potential determined from this analysis is 5.1 (6) mV and the slope is 59.7 (4) mV. The error bars represent an uncertainty of 0.5 mV in the observed potential.

The effect of pH on the reduction potential of cytochrome b_5 is shown in Figure 3. These data have been analyzed by the methods of Clark[11] and Ricard et al.[12] as described by Moore et al.[13] for cytochrome c_{551} by a weighted nonlinear least-squares fit of the reduction potentials to the relationship:

$$E_m = E + \frac{RT}{2.303nF} \ln \frac{K_{red} + [H^+]}{K_{ox} + [H^+]} \quad (1)$$

This approach assumes the existence of an ionizable functional group that undergoes a change in pK_a when the protein changes

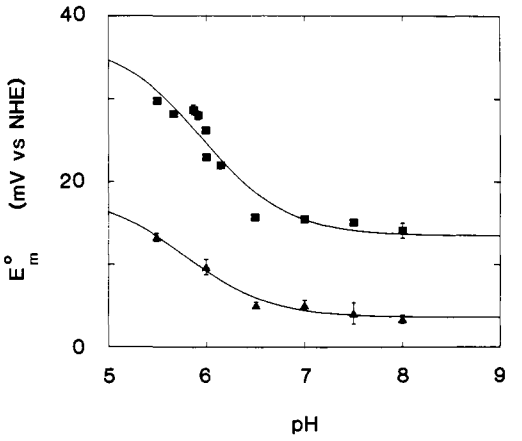

Figure 3. pH dependence of the cytochrome b_5 reduction potential (μ = 0.5 M (phosphate), 25 °C). The solid line is the theoretical fit of the data to eq 1 described in the text. (■) μ = 0.5 M; (▲) μ = 0.1 M.

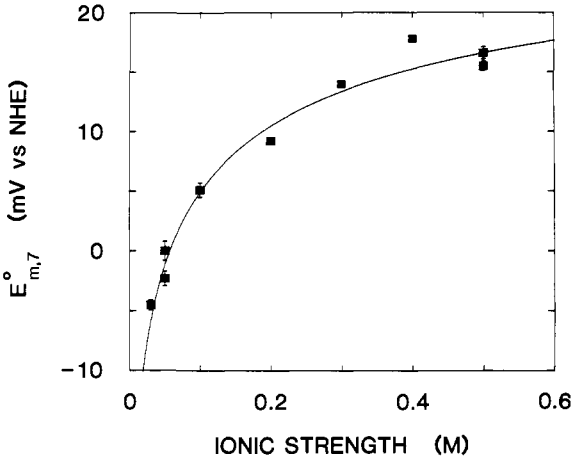

Figure 4. Variation in the reduction potential of cytochrome b_5 with ionic strength in sodium phosphate buffer at pH 7.0 and 25 °C. The solid line is the theoretical fit of the data to eq 2 where $f(\mu) = \mu^{1/2}/(1 + 5.2\mu^{1/2})$ (see Table I).

oxidation state. The data for cytochrome b_5 produce values of $K_{ox} = 2.1 \times 10^{-6}$ M (pK_{ox} = 5.7) and $K_{red} = 1.2 \times 10^{-6}$ M (pK_{red} = 5.9) with an E value of 11 mV at an ionic strength of 0.1 M. At μ = 0.5 M, we found $K_{ox} = 1.7 \times 10^{-6}$ M (pK_{ox} = 5.8), $K_{red} = 6.9 \times 10^{-7}$ M (pK_{red} = 6.2) and E = 25 mV. The spectra of cytochrome b_5 were invariant with changes in pH.

The dependence of the cytochrome b_5 reduction potential on ionic strength is illustrated in Figure 4. These data have been fitted to the equation (pH 7, 25 °C):

$$E°_{obsd} = E° - \frac{RT}{F} A(q_{ox}^2 - q_{red}^2) f(\mu) \quad (2)$$

where $E°_{obsd}$ is the reduction potential at a given ionic strength, $E°$ is the standard reduction potential extrapolated to an ionic strength of zero, A is the Debye-Hückel constant, and q_{ox} and q_{red} are the net electrostatic charges of the cytochrome in the oxidized and reduced states. Various forms of the function $f(\mu)$

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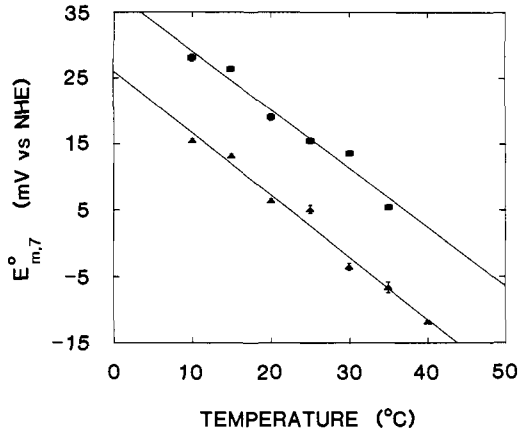

Figure 5. Temperature dependence of the cytochrome b_5 reduction potential at pH 7.0 (phosphate): (▲) μ = 0.1 M; (■) μ = 0.5 M.

were evaluated as previously suggested.[14] In each case, linear plots were obtained with approximately equivalent fits to the data. The resulting output parameters from these analyses are set out in Table I.

The temperature dependences of the cytochrome b_5 reduction potential at two ionic strengths (pH 7) are shown in Figure 5. The thermodynamic parameters obtained from these data are ΔS° = −37 (2) eu and ΔH° = −11 (1) kcal/mol (μ = 0.1 M), and ΔS° = −36 (4) eu and ΔH° = −10 (1) kcal/mol (μ = 0.5 M).

Discussion

Although several measurements of the cytochrome b_5 reduction potential have been reported,[16] none has evaluated the effects of pH, ionic strength, and temperature on this equilibrium. The measurements available (0–30 mV) are in reasonable agreement with the values determined here. Some efforts[16c,d] have been directed toward comparing the properties of the membrane-binding form of the cytochrome[17] with those of the solubilized form used here. As the result of this and other[18] work, it is currently thought that the heme-binding and membrane-binding domains of this protein function independently of each other, so the present results are likely to be relevant to the behavior of the insoluble as well as the soluble form of the cytochrome.

On the basis of their X-ray crystallographic studies, Argos and Mathews have proposed[4e] a mechanism for the change in oxidation state of cytochrome b_5. Specifically, they suggest that in the oxidized cytochrome, the carboxyl group from one of the heme propionate groups[19] is oriented toward the heme iron to help offset its net positive charge. This stabilization of the oxidized form of the protein presumably accounts for the relatively low reduction potential of the cytochrome. On reduction, the net formal charge on the heme iron changes to zero, and the negative charge on the propionate carboxyl group is neutralized by cation binding to the protein at this site. The conformations of reduced and oxidized cytochrome b_5 are essentially identical.[4e] The present study constitutes the first experimental evaluation of this model.

The potentiometric data reported here are largely consistent with this model. For example, the pH dependence of the reduction potential is readily accounted for by the presence of a single ionizable functional group on the protein that undergoes a slight change in pK_a on electron transfer. The functional group most likely to respond to the change in oxidation state in this manner is the heme propionate that stabilizes the heme iron charge in the oxidized protein. According to the model, protonation of this group should stabilize the reduced form of the protein and produce an increase in potential with a decrease in pH as seen in Figure 3. A similar effect of pH on the rate of cytochrome b_5 reduction by $Fe(EDTA)^{2-}$ also lends itself to this interpretation.[5] NMR experiments of Keller et al. provide further support for the protonation of this heme propionate group between pH 5 and 6.[20b]

The increase in reduction potential with ionic strength can be explained at least partially in terms of increased cation binding to the reduced form of the protein at high ionic strength. Moreover, the generalized increase in reduction potentials seen for all values of pH at high ionic strength (Figure 3) may be explained on the basis of a similar binding of protons and other cations (e.g., Na^+) to the same site on the protein. Our failure to demonstrate a dependence of the reduction potential on $Ru(NH_3)_6^{2+/3+}$ concentration presumably results from the inability of this redox-linked site to accomodate cations of this size. From the analysis of the ionic strength dependence data shown in Table I, it is apparent that the modified Debye–Hückel expression proposed by Beetlestone and Irvine[15] produces a net charge for cytochrome b_5 that is very similar to the charge estimated from sequence data.[5] In this regard, our results are in line with previous findings for horse heart cytochrome *c* and at variance with those reported for cytochrome *c* from *Euglena*. No explanation for these different types of behavior is available at present. Although the experiments reported here were performed by necessity at ionic strengths outside the range over which Debye–Hückel theory is normally regarded to be valid, some recent work suggests[21] that this type of analysis may not be as inappropriate at higher ionic strengths as previously believed. The ability of eq 2 to fit our data support this notion.

The thermodynamic parameters for cytochrome b_5 electron transfer permit a more detailed analysis of the mechanism by which this protein changes oxidation state. If we restrict the comparison of our data to those measurements obtained for six-coordinate, low-spin hemeproteins using a similar experimental method, then the discussion can be limited to our previous measurements on *c*-type cytochromes.[8c] The most striking feature of these data is that ΔH° for cytochrome b_5 is 4–5 kcal/mol more positive than for these other proteins. This finding is consistent with a Coulombic stabilization of the oxidized state of cytochrome b_5 that is significantly greater than previously appreciated. The fact that ΔS° for cytochrome b_5 is approximately 6 eu more negative than for the other cytochromes accords with the Argos–Mathews model in which a cation binds to a heme propionate group in the reduced protein.

In summary, a relatively complete functional characterization of the cytochrome b_5 oxidation–reduction equilibrium has been undertaken in which the pH, ionic strength, and temperature dependences of the cytochrome reduction potential have been determined with an optically transparent thin-layer electrode. The results are accounted for by the model of cytochrome b_5 electron transfer proposed on the basis of X-ray crystallographic studies.

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The implications of these data in the interpretation of cytochrome b_5 electron-transfer kinetics are currently being evaluated.

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Supplementary Material Available: Listing of reduction potentials and Nernst slopes (2 pages). Ordering information is given on any current masthead page.

Electrochemical and Spectral Investigations of Ring-Substituted Bipyridine Complexes of Ruthenium

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Abstract: A spectroelectrochemical study of a series of Ru complexes has been carried out by using an optically transparent thin-layer electrode (OTTLE). The visible spectra of the reduced complexes $Ru(Bp5COOEt)_3^n$ (Bp5COOEt = 5,5′-bis(ethoxycarbonyl)-2,2′-bipyridine) and $Ru(bpy)_3^n$ (bpy = bipyridine) appear to resemble the spectra of the corresponding ligand radical anion whereas the spectrum of $Ru(Bp4COOEt)_3^n$ (Bp4COOEt = 4,4′-bis(ethoxycarbonyl)-2,2′-bipyridine) does not. In the near-IR two types of spectral behavior are observed once the complexes are reduced beyond the 2+ oxidation state: Type A complexes (e.g., $Ru(bpy)_3$, $Ru(Bp4Me)_3$ (Bp4Me = 4,4′-dimethyl-2,2′-bipyridine)) exhibit low-intensity ($\epsilon < 2500$) bands which are similar to the spectra of the reduced free ligand. Type B complexes (e.g., $Ru(Bp4COOEt)_3^n$, $Ru(Bp4CONEt_2)_3^n$ ($Bp4CONEt_2$ = 4,4′-bis(diethylcarbamyl)-2,2′-bipyridine)) exhibit broad bands of greater intensity ($1000 < \epsilon < 15000$). Possible origins for type B behavior are discussed. Examination of electrochemical results reveals an almost perfect linear correlation when ligand reduction potentials are plotted against the 2+/1+ couple of the corresponding ruthenium complex (correlation coefficient = 0.9993). The thermodynamic implications of this observation are considered. Both the spectral and electrochemical data support a model of the reduced metal complex having electrons localized in ligand orbitals.

Bipyridine complexes of ruthenium possess several especially interesting chemical and spectral properties. For example, their strong visible absorptions, arising from a metal-to-ligand charge-transfer transition, produce a long-lived excited triplet capable of affecting important redox chemistry.[1a–d] Also, the inertness of ruthenium to ligand substitution allows the redox chemistry of both the ground and excited state to be studied without complications from ligand exchange reactions.[1a–d] Developing an understanding of the relationship between the detailed ligand structure and various kinetic and thermodynamic properties of the respective metal complexes is important if desirable properties of these metal complexes are to be optimized. The excited-state electron-transfer rates, the wavelength maximum of the charge-transfer absorption, and the redox potential are all examples of properties which are dependent on the exact nature of the ligand.

Herein we report the systematic spectral and electrochemical study of a variety of bipyridine ligands and their complexes, primarily with ruthenium. A series of substituted bipyridines were employed which differ in the position of substitution, the type of substitution, and reduction potential. The bipyridines most important to this study are shown in Figure 1. The results of our investigations reveal that modest changes in ligand substitution sometimes have dramatic effects on the spectral properties of the metal complexes.

Experimental Section

Equipment. The electrochemical and spectroelectrochemical equipment and cells have been described elsewhere.[2] Unless otherwise stated all potentials are reported with respect to SCE.

Reagents. Acetonitrile and dimethylformamide (Burdick and Jackson Distilled in Glass) were used without further purification. Tetra-*n*-butylammonium hexafluorophosphate was used as supporting electrolyte.

Coulometry. Coulometric data used for calculating n values were obtained by one or both of the following methods: (1) bulk reduction in a three-compartment H-cell inside a nitrogen atmosphere box or (2) thin-layer coulometry on the reverse potential step (reoxidation) obtained in the optically transparent thin-layer electrode (OTTLE) cell. (Forward potential step data could not generally be used since the generation of highly charged species and the accompanying solution density change causes appreciable stirring at the edges of the OTTLE cell.)

Spectroelectrochemistry. Before each potential step a new solution was drawn into the OTTLE cell and a spectrum of the solution was taken at 0.0 V. The solution was then electrolyzed at the appropriate potential and a spectrum of the reduced species was recorded. After the reverse potential step a spectrum was taken of the reoxidized solution. All reduced species reported exhibit superimposable initial and final spectra, substantiating the chemical reversibility of the electron-transfer processes observed.

Preparation of Free Ligands and N-Alkylated Ligands. Structures and abbreviations of the ligands are shown in Figure 1.

(i) **4,4′-Dimethyl-2,2′-bipyridine (2).** This compound was prepared by the method described by Sasse and Whittle.[3] The only modification was the distillation of the 4-picoline from KOH prior to use in the synthesis. Distillation of 100 mL of picoline in the presence of 3 g of KOH yields 80–90 mL of pyrrole-free picoline satisfactory for the bipyridine coupling reaction, mp 170–171 °C.

(ii) **4,4′-Dicarboxy-2,2′-bipyridine (3).** The diacid was obtained following the procedure of Sprintschnik et al.[4] During the oxidation with potassium permanganate, the starting material steam-distilled into the reflux column. Yields were increased by washing the starting material back into the reaction mixture with permanganate solution. The potassium permanganate initially added to the reaction mixture plus the additional aliquots total approximately a 6-fold molar excess of permanganate.

(iii) **4,4′-Bis(ethoxycarbonyl)-2,2′-bipyridine (4).** Esterification of **3** was done in the presence of sulfuric acid in absolute ethanol. The solution

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