taminated by small amounts of the corresponding α-diketones, were isolated in the same manner as described above for the benzils.

Oxidation of 3-Hexyne with Thallium(III) Nitrate–CH_3OH–Perchloric Acid. A solution of 0.82 g (0.01 mol) of 3-hexyne in 5 ml of methanol was added to a stirred solution of 8.9 g (0.02 mol) of TTN in 25 ml of methanol and 5 ml of 70% perchloric acid. An exothermic reaction ensued, thallium(I) nitrate precipitated after a few seconds, and the reaction mixture turned pale yellow in color. Stirring was continued for 30 min at room temperature, the thallium(I) nitrate was then removed by filtration, and the filtrate was diluted with water and dichloromethane. The dichloromethane layer was separated, dried (Na_2SO_4), and carefully evaporated at 25° to leave a pale yellow oil. Distillation gave 0.90 g (70%) of slightly impure (purity *ca.* 95%) 4-methoxy-3-hexanone, bp 52–56° (11 mm) (lit.[46] bp 62–63° (40 mm)). The impurities present were shown by glpc, using genuine samples as both internal and external standards, to be 4-hydroxy-3-hexanone and hexane-3,4-dione.

The same conversion could be carried out in 85% yield by using thallium(III) acetate rather than TTN.

General Procedure for the Oxidation of Terminal Alkylacetylenes with TTN–Glyme–Perchloric Acid. The terminal acetylene (0.01 mol) was added to a stirred solution of 0.02 mol of TTN in 25 ml of glyme, 15 ml of water, and 8 ml of 70% perchloric acid and, after the initial vigorous reaction had subsided, the yellow reaction mixture was stirred at room temperature for a further hour. Complete removal of all of the thallium(I) salt was ensured by addition of 15 ml of 2 *N* potassium iodide solution, followed by stirring for a further 20 min. The inorganic salts were removed by filtration, and the filtrate was diluted with water and extracted with benzene. The extracts were dried (Na_2SO_4) and evaporated, and the residual oil was esterified by heating at 50° for 2 hr with 40 ml of methanol containing 5 ml of 70% perchloric acid. The cooled reaction mixture was diluted with water, and extracted with benzene, and the extracts were concentrated and distilled to give the ester. Methyl heptanoate was obtained in 80% overall yield in this way from 1-octyne, and methyl hexanoate in 55% overall yield from 1-heptyne.

Oxidation of 1-Hydroxyoctan-2-one with TTN–CH_3OH. 1-Hydroxyoctan-2-one was prepared by the general procedure of Pfeil and Barth[29] from glycolonitrile (10.0 g, 0.175 mol) and *n*-hexylmagnesium bromide (from 70 g, 0.425 mol, of *n*-hexyl bromide and 10.0 g, 0.42 g-atom, of magnesium turnings). Distillation of the reaction product gave a fraction of bp 57–62° (0.4 mm) weighing 13.6 g (54%), identified as 1-hydroxyoctan-2-one (lit.[47] bp 70–76° (6 mm)).

To a solution of 1.44 g (0.01 mol) of 1-hydroxyoctan-2-one in 30 mol of methanol was added 4.44 g (0.01 mol) of TTN. Thallium(I) nitrate separated rapidly after a few seconds. The reaction mixture was stirred at room temperature for 1 hr and filtered, and the filtrate was diluted with water and extracted with chloroform (2 × 20 ml). The chloroform extracts were chromatographed on a short alumina column (2 × 10 cm), using benzene as eluent, to give 1.10 g (76%) of a sweet-smelling liquid identified as methyl heptanoate by comparison of its glpc retention time with that of an authentic sample. An attempt to trap formaldehyde formed in this reaction by means of reaction with dimedone was unsuccessful, probably owing to complexation of the dimedone with Tl(I) and/or Tl(III).

General Procedure for the Oxidative Rearrangement of Alkylarylacetylenes with TTN–CH_3OH. The alkyne (0.01 mol) was added to a stirred solution of 4.88 g (0.011 mol) of TTN in 25 ml of methanol, and the mixture was heated under reflux for 2 hr. Thallium(I) nitrate was removed from the cooled reaction mixture by filtration, and the filtrate was extracted with ether or chloroform. The extracts were washed with water and 5% aqueous sodium bicarbonate solution and dried (Na_2SO_4). The solution was then filtered through a short column of Florisil (10 g) using chloroform as eluent; evaporation of the eluate gave the methyl α-alkylarylacetate which, in every case, was shown to be pure at this stage by glpc.

Acknowledgment. One of us (B. P. S.) is grateful for the receipt of an S.R.C. Scholarship.

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Use of the Vanadyl(IV) Ion as a New Spectroscopic Probe of Metal Binding to Proteins. Vanadyl Insulin[1a,b]

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Abstract: This study was undertaken to investigate the potential use of vanadyl ion, VO^{2+}, as a physicochemical marker of metal binding sites in proteins. VO^{2+} was substituted for Zn^{2+} in rhombohedral bovine insulin crystals, and the metal binding was characterized by the epr, optical, and infrared spectra of the protein bound VO^{2+}. Two types of binding sites, A and B, were found. The A sites are identical with the two sites in the zinc insulin hexamer in which the metal is bound to three imidazole groups of B10 His residues, and the B sites are the 24 carboxyl groups of the 24 Glu residues per hexamer. The coordination geometry about the VO^{2+} ion in both A and B sites was determined. In general, the binding in VO^{2+} insulin parallels that in Zn^{2+} insulin. This approach to studying metal binding should be applicable to other proteins as well.

Numerous proteins have metal ions as an integral part of their structure and in some cases these ions provide a convenient spectrochemical probe of molecular structure and enzyme kinetics. There are, however, a large number of proteins which require Zn^{2+} or Mg^{2+} ions as structurally or functionally essential constituents, but these ions provide only limited means by which to extract information about their coordination

(1) (a) Supported by the National Institutes of General Medical Sciences Grant GM 20194-01, the Petroleum Research Fund of the American Chemical Society, National Science Foundation Grants GP11387 and GP28215, and the National Science Foundation Undergraduate Research Participation and College Science Improvement Programs. (b) A preliminary account of this work was presented at the Rocky Mountain Regional Meeting of the American Chemical Society, Fort Collins, Colo., June 30–July 1, 1972. (c) Send correspondence to this author at the Department of Chemistry, University of New Hampshire, Durham, N. H. 03824. (d) Lawrence University. (e) NSF–COSIP participant, 1971. (f) NSF–URP participant, 1971. (g) University of Colorado.

environment since they are diamagnetic and lack ligand-field transitions. Often by use of a chelating agent such as EDTA, the native metal can be removed from the protein and a transition metal with more desirable spectroscopic properties can be introduced. With certain metalloenzymes catalytic activity is retained. Some well-known examples of this latter behavior are the zinc metalloenzymes carbonic anhydrase, alkaline phosphatase, and carboxypeptidase where Zn^{2+} can be replaced with Co^{2+}.[2]

This paper reports initial experiments to explore the use of vanadyl ion, VO^{2+}, as a metal substitute in proteins. This ion is a particularly attractive substitute because of its chemical and spectroscopic properties. Vanadyl forms hundreds of stable anionic, cationic, and neutral complexes with all kinds of ligands;[3,4] such a versatile metal ion should coordinate with the variety of functional groups encountered in proteins.

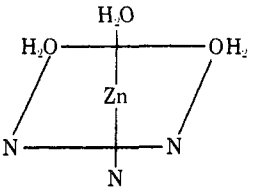
It is the spectroscopic properties of vanadyl ion that make it especially interesting as a possible protein label, however. Most ligand-field transitions of vanadyl complexes occur in the visible and near-infrared spectral regions where proteins do not absorb and can provide clues as to the nature of binding site. Since vanadyl complexes are d^1 systems, the interpretation of optical spectral data is relatively "straightforward."

Vanadyl is unlike other metal ions in that the vanadium ion has one coordination position always occupied by the vanadyl oxygen. The infrared-active stretching frequency of the vanadyl VO bond is fairly sensitive to changes in the other ligands and can be used as an additional probe of metal binding sites in crystalline proteins where the VO^{2+} concentration is sufficiently large.

Most important, vanadyl complexes exhibit eight very sharp epr lines due to the interaction of the odd electron with the $I = {}^7/_2$ spin states of the nearly 100% abundant ^{51}V nucleus. The spectrum is observable at room temperature because vanadyl has an orbitally nondegenerate ground state and no excited states nearby in energy. In fact, of all paramagnetic metal ion complexes, only those of vanadyl *consistently* give sharp epr spectra both in frozen *and* room-temperature solutions. This is an important feature since one is often interested in examining the spectrum of protein bound metal ion under conditions which mimic *in vivo* solution conditions. This cannot be achieved with many metal ions in that their epr spectra can only be observed at low temperature, conditions quite unlike those of living organisms. Furthermore, epr spectra of some transition metals naturally occurring in biological systems are complicated somewhat by overlapping spectra from different isotopes, for example, Mo(V) and Cu(II). Moreover, anisotropic g and nuclear hyperfine parameters are known for a large number of vanadyl chelates[5] and can be readily extracted from an analysis of spectra of frozen solutions and polycrystalline samples.

Since there are several cases known where vanadyl ion isomorphously replaces zinc in single crystals,[5-7] we began our studies by investigating metal replacement of zinc in bovine insulin. As an added incentive for this work, there was the possibility that these studies might open the way for investigations of vanadium in living systems. For example, hemovanadin, a metalloprotein extracted from vanadocytes in the blood of tunicates, contains V^{3+} and is believed to play a role in oxygen transport or metabolic redox reactions.[2] It is quite remarkable that the tunicates concentrate vanadium 280,000-fold over that in the surrounding sea water.[8] Vanadium has also been found in the cap of the mushroom *Amanita muscaria*.[2] Moreover, Snipes and Gordy have discovered (through epr spectroscopy) vanadyl ion associated with commercial preparations of DNA.[9]

It has long been recognized that zinc ions play an important role in the quaternary structure of insulin both in solution and crystals.[10,11] In the absence of metal ions, insulin solutions are inhomogeneous in molecular weight; however, addition of two zinc ions or other divalent first-row transition metal ions per six monomer molecular weight units results in formation of a hexamer with a molecular weight around 36,000. Metal ions in excess of two per hexamer are much less tightly bound and are easily dialyzed away. Rhombohedral insulin crystals can be grown from solutions of Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+},[11] and VO^{2+} (*vide supra*) and likewise contain two tightly bound metal ions per hexamer. Zinc and cupric insulin crystals are isomorphous, space group $R3$, with one hexamer per unit cell.[10,12] Three insulin dimers are held together by two metal ions located on the crystallographic threefold axis. The metal ions are separated by about 18 Å, each in a site of trigonal symmetry and bound to the imidazole nitrogen of three B10 histidyl residues,[10,12] *viz.*



We have prepared a vanadyl analog of bovine zinc insulin and report here the first epr, visible, and infrared spectra of protein bound vanadyl ion. The experimental techniques and methods of data analysis outlined in this model study will be useful in similar investigations of less well-understood proteins.

Experimental Section

All glassware was rendered metal free by washing in 50:50 concentrated H_2SO_4–HNO_3 followed by rinsing in doubly distilled deionized water. Reagents were rendered metal free by phase ex-

(2) A review of metalloproteins is given by B. L. Vallee and W. E. C. Wacker, "Metalloproteins" in "The Proteins," Vol. 5, H. Neurath, Ed., Academic Press, New York, N. Y., 1970.
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(11) J. Schlictkrull, *Acta Chem. Scand.*, 10, 1455, 1459 (1956); 11, 299 (1957); Thesis, Copenhagen, 1958.
(12) T. L. Blundell, G. G. Dodson, E. J. Dodson, D. C. Hodgkin, and M. Vijayan, *Recent Progr. Horm. Res.*, 20, 1 (1971), and references therein.

traction with 0.001% dithiophenol carbazone in carbon tetrachloride. All vanadyl solutions were purged with nitrogen to avoid oxidation. Aqueous $VOSO_4$ (Alfa Inorganics) and $VO(ClO_4)_2$ stock solutions were prepared and originally standardized by EDTA titration and $ZnCl_2$ back-titration with xylenol orange indicator.[13] Subsequent standardizations were done spectrophotometrically, using the decadic molar extinction coefficients of 17.6 ± 0.2 for $VOSO_4$ solutions (0.0–0.65 *M*) and 16.8 ± 0.2 for $VOClO_4$ solutions (0.0–0.75 *M*) at 750 nm.

Two methods were used to prepare vanadyl insulin. In the first (crystal soaking method), 20–40 mg of crystalline bovine zinc insulin (Sigma) was soaked in 6.0 ml of 0.1 *M* sodium EDTA, pH 7.0, for 48 hr at 4°, after which less than 0.1 g-atom of zinc per insulin hexamer remained; commercial insulin contained nominally 3.5 g-atoms of zinc per hexamer. The "metal-free" insulin crystals were collected by centrifugation and washed with two 0.5 ml portions of neutral doubly distilled deionized water and then soaked for 48 hr at 4° in 6 ml of 0.05 *M* citrate buffer, 0.01–0.02 *M* in $VOSO_4$, to yield vanadyl insulin. At times, crystals were soaked in 0.01–0.10 *M* $VOSO_4$ solution with no buffer. The pH of the citrate buffer was varied from 3.0 to 7.0 for the determination of the relative pK_a's of the metal binding sites. The product was collected and washed as above and dried under nitrogen.

In the second method (crystallization method), similar to that of Schlictkrull,[11] 20–40 mg of zinc insulin was dissolved in 3.0 ml of dilute HCl solution (pH 2) and then salted out by the addition of 3.0 ml of 15% NaCl in dilute HCl. The salted-out protein contained less than 0.02 g-atom of zinc per hexamer after washing with two 0.5-ml portions of 0.05 *M* citrate buffer, pH 6.0. In the determination of the effect of metal ion concentration on crystallization and metal binding site occupancy, the metal-free protein was partially dissolved in 0.25 ml of $VOSO_4$ of the appropriate molarity to give a VO^{2+}/hexamer molecular weight ratio of 18/1, 180/1, or 1800/1 in solution. Then 0.9 ml of reagent acetone was added to dissolve the remaining protein and followed by slow addition of 0.05 *M* citrate buffer, pH 6.0, to give a final volume of 6 ml. The final pH of the 1800/1 solution was 5.0; the pH of the other two solutions was 6.0. Vanadyl insulin can be precipitated immediately by thorough mixing, but if the insulin solution is carefully layered over the citrate buffer and allowed to diffuse slowly, better rhombohedral crystals are formed. Crystals of poor quality or amorphous precipitates are obtained from the 1800/1 solution. The cultures were soaked in the mother liquor for 48 hr at 4° and washed and dried as before. The samples from the 1800/1 solution became bluer upon soaking in the mother liquor. Both fast and slow crystallizations yield identical epr results.

Zinc[14] and vanadium contents were determined with a Perkin-Elmer Model 305 atomic absorption spectrometer and a Bausch and Lomb 1.5-m grating emission spectrograph, respectively, at the Institute of Paper Chemistry. All vanadyl insulin samples contained less than 0.03 Zn per hexamer molecular weight. Stock 1 *M* $VOSO_4$ solutions contained nominally 0.2 ppm of Zn.

Mull spectra were measured on a Cary Model 14 spectrometer. Blue vanadyl insulin, precipitated from the 1800 VO^{2+}/hexamer, pH 5.0, solutions, was mulled with Kel-F grease and spread evenly on a microscope glass cover slip attached to a metal holder which mounted in the spectrometer. A similarly prepared NaCl sample served as a blank.

All epr spectra of polycrystalline samples were measured at room temperature with a Varian V-4502 spectrometer operating at X-band (9.5 GHz) with 100-kHz field modulation. The field was measured with a proton probe and the kylstron frequency with a Micro-Now Model 101 frequency multiplier and a Hewlett-Packard 5245 M frequency counter.

Infrared spectra of insulin samples in KBr pellets were measured on a Perkin-Elmer Model 337 grating spectrophotometer with an expanded scale accessory. Spectra were calibrated with a polystyrene film.

X-Ray film powder patterns of polycrystalline zinc and vanadyl insulins were measured on a General Electric XRD-5DF X-ray unit with a 71.78-mm radius A4969A camera and vanadium-filtered Cr $K\alpha$ (λ 2.2909 Å) radiation. Samples moist with mother liquor were mounted in 0.3-mm diameter capillary tubes sealed with paraffin wax.

(13) R. E. Tapscott, Ph.D. Thesis, University of Illinois, Urbana, Ill., 1967, pp 257–260.

(14) G. I. Spielholtz and G. C. Toraballa, *Analyst* (*London*), **94**, 1072 (1969).

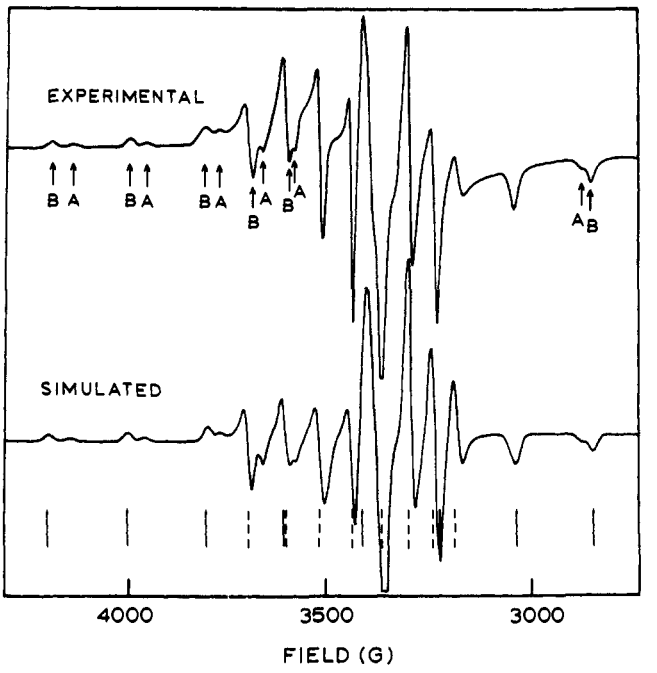


Figure 1. Experimental and simulated first-derivative X-band epr spectra of colorless polycrystalline vanadyl insulin at room temperature. The solid and dashed vertical lines denote the parallel and perpendicular resonance fields, respectively, of the B sites. $\sigma_{\parallel}$ = $\sigma_{\perp}$ = 20 G, X_A = 0.33, and X_B = 0.67. Other parameters are given in Table I. A and B designate partially resolved spectra of A and B sites. The sample was prepared by the crystal soaking method; Gaussian line-shape function.

Results and Discussion

Nature of the Binding Sites. Representative experimental and computer simulated epr spectra (see Appendix) of polycrystalline vanadyl insulin are given in Figure 1. For comparison, the spectrum of polycrystalline cupric insulin is shown in Figure 2 and is in accord with that reported earlier by Venable and Brill.[15] The sharp well-resolved features of the vanadyl insulin spectrum (Figure 1) are characteristic of randomly oriented magnetically dilute VO^{2+} groups. The most interesting feature is the partial resolution of two spectra which arise from two types of binding sites (designated A and B). Comparable evidence for more than one magnetically distinct binding site cannot be *easily* obtained from the broad cupric insulin spectrum of Figure 2. Superior resolution is, of course, one of the attractive features of vanadyl epr.

The relative occupancy of A and B sites depends markedly on sample preparation and, in conjunction with spectral data, can be used to determine site identity. The relative occupancy X_A of site A is defined as the amount of VO^{2+} occupying A sites and is expressed as a mole fraction of the total VO^{2+} bound to the protein. X_B is similarly defined. X_A and X_B are readily obtained from computer simulation of the experimental epr spectrum (see Appendix).

Since crystalline insulin is quite permeable to small ions (there are solvent channels 10 Å in diameter running between hexamers[12]), it can be titrated in the presence of VO^{2+} to determine the approximate pK_a's of site functional groups. The relative occupancy of A

(15) A. S. Brill and J. H. Venable, Jr., "The Biochemistry of Copper," P. Aisen, W. Blumberg, and J. Peisach, Ed., Academic Press, New York, N. Y., 1966, p 67.

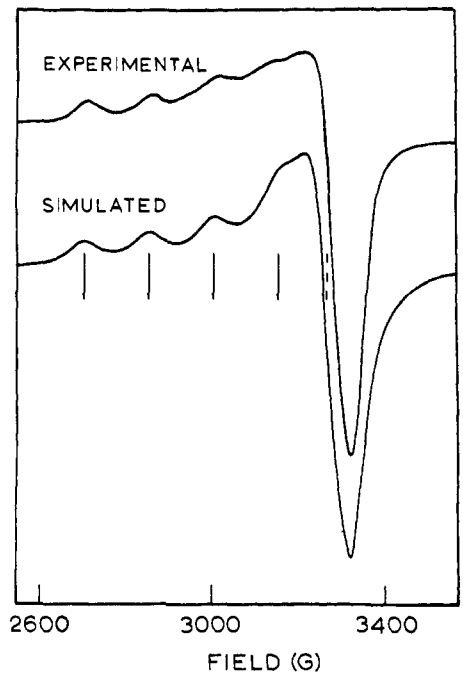


Figure 2. Experimental and simulated first-derivative X-band epr spectra of polycrystalline cupric insulin at room temperature. $\sigma_{||} = \sigma_{\perp} = 45$ G, $A_{||} = 162 \times 10^{-4}$ cm^{-1}, $A_{\perp} = 0.0$, $g_{||} = 2.038$, and $g_{\perp} = 2.07$. The solid and dashed vertical lines denote the parallel and perpendicular resonance fields, respectively. The sample was prepared by the crystallization method; Lorentzian line-shape function.

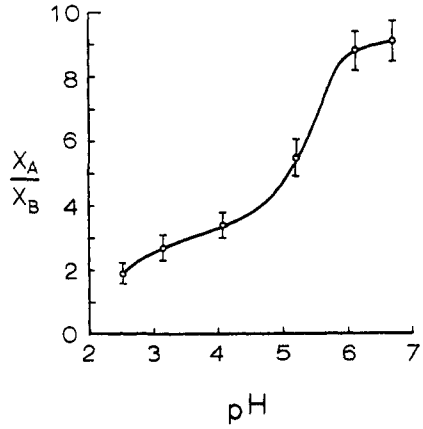


Figure 3. Relative occupancy of A and B sites, X_A/X_B, as a function of pH. See Experimental Section.

and B sites as a function of pH is monitored *directly* by epr spectroscopy. This has an advantage over conventional pH titrations in which the metal ion binding sites comprise only a small fraction of the titratable groups. The "titration" was accomplished by soaking zinc-free insulin crystals (see Experimental Section) in $VOSO_4$-citrate buffer at various pH's. The buffer was essential to avoid precipitation of vanadyl hydroxide species at pH greater than 3.7; however, this caused an overall decrease in the intensity of the spectrum with increasing pH because of the competition between citrate and insulin for available VO^{2+}. The relative occupancy of A and B sites as a function of pH is shown in Figure 3. The fact that $X_A/X_B > 1$ over the entire pH range indicates that binding is tightest at A sites. Furthermore, functional groups at A sites are seen to

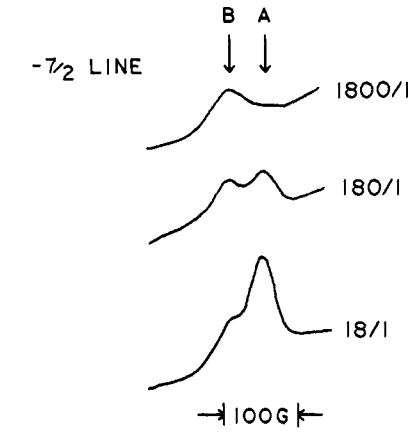


Figure 4. The dependency of the high-field parallel first-derivative resonance line of polycrystalline vanadyl insulin on the VO^{2+}:hexamer molecular weight ratio in the mother liquor from which the samples were crystallized. Only the 1800:1 sample was blue and prepared at pH 5.0. Only the *relative* intensities of the A and B components within each spectrum should be compared as different signal levels and amounts of sample were used in each spectrum.

have an apparent pK in the range of pH 6–7 which is consistent with coordination of imidazole groups of histidyl residues (pK = 6.40[16]). In contrast, B sites have an apparent pK in the range pH 3–5 which suggests carboxyl groups of glutamyl residues (pK = 4.73[16]) or C-terminal carboxyl groups (pK = 3.60[16]). The titration data in themselves do not unequivocally identify the functional groups in A and B sites; however, the results given below will confirm their identity. First we will concentrate on identification of A sites, then of B sites.

If A sites are identical with the two sites of trigonal symmetry in zinc insulin (B10 histidyl residues), then occupancy of these sites should be essential for maintaining the structural integrity of the insulin hexamer and therefore rhombohedral crystal growth. Accordingly, crystals were grown from solutions of varying VO^{2+}/insulin ratios and the relative occupancies of A and B sites in the crystalline products were determined. Figure 4 shows the high-field parallel resonance line for three samples obtained from mother liquors with various VO^{2+}/insulin hexamer molecular weight ratios. The partial resolution of spectra from A and B sites is clearly seen. Only the relative intensities of A and B components of the spectrum of each sample should be compared as different signal levels and amounts of sample were used in each case. Notice that in the sample obtained from the 18:1 mother liquor the occupancy of A sites predominates, whereas occupancy of B sites becomes appreciable only in samples prepared from solutions with large VO^{2+} to insulin ratios (*i.e.*, 180:1 and 1800:1). This result indicates that (1) occupancy of B sites is associated with binding of excess VO^{2+}, (2) binding is tightest at A sites (consistent with the previous titration data), and (3) most importantly, occupancy of A sites is essential for crystal growth, the expected result if these sites are identical with the two sites of trigonal symmetry in native Zn insulin.

Furthermore, we find that there are indeed two A sites per hexamer from a correlation of the relative occupancy

(16) pH data on insulin can be found in C. Tanford and J. Epstein, *J. Amer. Chem. Soc.*, 76, 2163, 2170 (1954); A. S. Brill and J. H. Venable, *ibid.*, **89**, 3622 (1967).

of A and B sites with the total vanadium content of the protein. Finally, the equivalence between the A sites in vanadyl insulin and Zn^{2+} sites in native zinc insulin is confirmed by the fact that *both A and B sites* become occupied when *zinc-free* insulin crystals are soaked in 0.05 *M* $VOSO_4$ solution, whereas *only B sites* become occupied when *zinc insulin* crystals are soaked in this $VOSO_4$ solution.

That VO^{2+} and Zn^{2+} have analogous structural roles in rhombohedral insulin crystals is nicely demonstrated by X-ray film powder patterns. Moist polycrystalline zinc insulin samples exhibit several well-defined low Bragg angle reflections; however, when the samples are rendered zinc-free by soaking them in 0.05 *M* sodium EDTA, pH 7.0, the reflections disappear. The same reflections reappear upon soaking the zinc-free samples in 0.01 *M* $VOSO_4$, pH 3.0, in accord with the previous data. The Bragg angles for the more prominent reflections of zinc (and vanadyl) insulin are 2.71 (2.71), 3.85 (3.83), 4.26 (4.26), 5.18 (5.15), and 6.42° (6.39). The relative intensities are also about the same for both insulins.

The epr parameters $g_{\parallel}$, $g_{\perp}$, $A_{\parallel}$, and $A_{\perp}$ for the A sites are listed in Table I and the procedure for extracting these parameters from the experimental spectrum is given in the Appendix. The magnitudes of these parameters, to be discussed later, are consistent with a moderately strong ligand field, *i.e.*, nitrogen coordination. Furthermore, since the epr parameters are identical for both A sites within resolution of the spectrum, the chemical environment of each of the two A sites must be virtually identical. A single crystal epr study of cupric insulin[10] reveals very slight differences in the coordination environment of the two sites. Likewise, the two sites in zinc insulin are related only by an *approximate* twofold crystallographic axis; however, both sites do have trigonal symmetry with the metal ion in each site bound to three imidazole nitrogens.

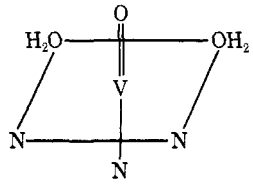
Table I. Spectral Data and Bonding Parameters[a]

	A sites	B sites	VO-$(H_2O)_5^{2+}$	VO-$(CN)_5^{3-}$
$g_{\parallel}$	1.943 (1)	1.938 (1)	1.932[b]	1.972[c]
$g_{\perp}$	1.977 (2)	1.978 (2)	1.980[b]	1.983[c]
g_0	1.965 (2)	1.965 (2)	1.964[b]	1.980[c]
$A_{\parallel}$ (10^{-4} cm^{-1})	167.1 (7)	177.0 (12)	182.8[b]	138[c]
$A_{\perp}$ (10^{-4} cm^{-1})	61.7 (10)	67.5 (10)	71.3[b]	47.2[c]
A_0 (10^{-4} cm^{-1})	96.8 (10)	104.0 (12)	108.3[b]	77.4[c]
$\Delta E_{xz,yz}$, kK		12.9	13.0[d]	15.6[e]
$\Delta E_{x^2-y^2}$, kK	17.6[f]	16.7	16.0[d]	24.8
α^2	0.93	0.95	1.00[g]	0.82[g]
β^2		0.84	0.82[g]	0.67[g]
γ^2		0.97	0.84[g]	1.06[g]
κ	0.74	0.79	0.83[g]	0.59[g]

[a] Estimated errors in the last digits given in parentheses. [b] Reference 7. [c] H. A. Kuska, Ph.D. Thesis, Michigan State University, East Lansing, Mich., 1965, as reported in ref 31. [d] C. J. Ballhausen and H. B. Gray, *Inorg. Chem.*, **1**, 111 (1962). [e] J. R. Wasson, *J. Inorg. Nucl. Chem.*, **20**, 171 (1968). [f] Estimated from κ. See text. [g] Reference 31.

Wuethrich[17] has found that for some complexes the isotropic hyperfine coupling, A_0, reflects the average environment about the VO^{2+} ion due to the four equatorial ligands. This provides a means of identifying the coordinating atoms of the equatorial ligands. If we choose as model compounds $VO(H_2O)_5^{2+}$ (A_0 = 106.4 × 10^{-4} cm^{-1})[5] with four equatorial water molecules and VO(porphyrin) (A_0 = 88.2 × 10^{-4} cm^{-1})[18] with four equatorial nitrogen atoms, then we predict for A sites with equatorial coordination of two water molecules and two imidazole nitrogen atoms: A_0 = (88.2 + 106.4)/2 × 10^{-4} cm^{-1} = 97.3 × 10^{-4} cm^{-1} in good agreement with the experimental value of (96.8 ± 1.0) × 10^{-4} cm^{-1}. Although this treatment is only approximate (since it assumes that variations in A_0 are primarily due to differences in the covalency of in-plane π bonding which is not true of all complexes[5]), it does provide reasonable evidence for coordination of two imidazole nitrogen atoms and two water molecules in the equatorial positions of A sites.

In conclusion, only one coordination geometry, which has "trigonal" symmetry and is also consistent with all the foregoing results, is possible for A sites, namely



With the structure of the A sites firmly established, we now turn our attention to the elucidation of the B sites. We find that the blue vanadyl insulin obtained from the 1800/1 VO^{2+}/hexamer solutions, pH 5.0, contains approximately 26 g-atoms of vanadium per hexamer. This immediately suggests that B sites are most likely the carboxyl groups of the 24 glutamyl residues per hexamer, although some coordination at other sites such as C-terminal carboxyl groups probably takes place. Glutamate coordination is further substantiated by the fact that X_B/X_A ratios of 11 ± 2 are observed for the blue samples as compared with 24/2 = 12 expected theoretically. Infrared, epr, and optical spectral results for B sites to be given later suggest that carboxyl coordination is monodentate in a position equatorial to the vanadium–vanadyl oxygen VO bond axis. In zinc insulin, zinc ions in excess of two per hexamer are also found to bind at carboxyl groups,[19] whereas in cupric insulin excess copper binds at α-amino groups.[10] Likewise, in the various heavy metal derivatives used to determine the crystal structure of insulin, metal ions were always found located in the regions of glutamyl carboxyl groups in addition to the two trigonal sites.[12]

Again, we make use of the isotropic hyperfine coupling constant A_0 to determine the identity of the equatorial ligands. With $VO(H_2O)_5^{2+}$ (A_0 = 106.4 × 10^{-4} cm^{-1}) and $VO(malonate)_2^{2-}$ (A_0 = 93.7 × 10^{-4} cm^{-1}) as model compounds, we predict for equatorial coordination of one carboxyl group and three water molecules at the B sites

$$A_0 = [3/4(106.4) + 1/4(93.7)] \times 10^4 = 103.2 \times 10^{-4}\ cm^{-1}$$

(17) K. Wuethrich, *Helv. Chim. Acta*, **48**, 1012 (1965).

(18) D. Kivelson and S.-K. Lee, *J. Chem. Phys.*, **41**, 1896 (1966).

(19) J. Eisenbrand and F. Wegel, *Hoppe-Seyler's Z. Physiol. Chem.*, **268**, 26 (1941), as reported in the thesis of ref 11.

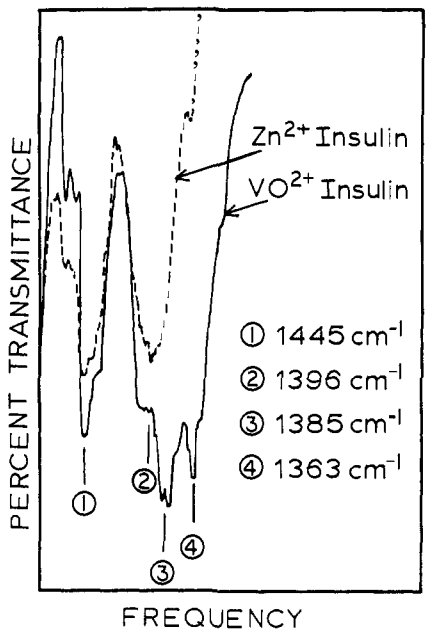


Figure 5. Infrared spectra of a KBr pellet of zinc and vanadyl insulins on an expanded scale.

which is in good agreement with the experimental value of $(104.0 \pm 1.2) \times 10^{-4}$ cm^{-1} (Table I).

The principal features of the infrared spectrum of polycrystalline zinc insulin can be readily assigned from the data on proteins given in Bellamy.[20] There are, however, two very significant differences between the spectra of zinc and vanadyl insulins. First, in blue vanadyl insulin there is a prominent new band at 993 cm^{-1} which we assign as the VO double bond stretching mode of B sites. This frequency compares with 985 ± 50 cm^{-1} for some 50 vanadyl compounds[3] and is very close to the 997 cm^{-1} observed for aqueous $VO(H_2O)_5^{2+}$.[21] Axial coordination of a variety of ligands, including acetate, to vanadyl bis(acetylacetonate) causes the VO stretching mode to shift some 48 cm^{-1} to lower energy.[3] The fact that the frequencies of $VO(H_2O)_5^{2+}$ and vanadyl insulin are nearly identical indicates that glutamate coordinates equatorially in accord with the preceding results with A_0. (Unfortunately, the VO stretching mode of A sites is too weak to be observed because of the relatively small amount of the total VO^{2+} in these sites.)

Secondly there are some rather interesting differences in the frequencies of symmetric stretching modes of carboxylate anions in vanadyl and zinc insulins (see Figure 5). Bands 2, 3, and 4 of Figure 5 disappear upon acidification of the protein, and we believe that the lower frequency bands, 3 and 4, which arise only in vanadyl insulin, are due to monodentated coordinated carboxyl groups. (Monodentate carboxyl coordination with associated frequency shifts to lower energy of 2 to 40 cm^{-1} are commonly found for transition metals.[22] In contrast, bidentate coordination exhibits frequency shifts to higher energy.[22]) The fact that we observe two prominent bands, 3 and 4, instead of only one for vanadyl insulin suggests differences in the strength of the coordinate bond at various carboxyl group binding sites. This result is corroborated by the fact that significant variations in $A_{\parallel}$ for B sites (194.0 to 197.4 G) are observed for samples with differing amounts of B site occupancy.

The epr parameters $g_{\parallel}$, $g_{\perp}$, $A_{\parallel}$, and $A_{\perp}$ for B sites (Table I) are consistent with a weak field environment about the VO^{2+} ion. Weak fields are typical of carboxyl group and water molecule coordination. Moreover, the optical spectral data, to be given in detail later, likewise indicate a weak field environment with the carboxyl group in an equatorial position.

Sufficient detail of the X-ray structure has not been reported to permit us to estimate distances between various glutamate sites. However, no two sites are likely closer than about 6 Å since we fail to observe any "forbidden" half-field $\Delta M_s = 2$ transitions around 1600 G.[23-26] These low-intensity transitions arise when two metal ions are sufficiently close, such that magnetic dipolar coupling between electron spins becomes significant and the selection rule $\Delta M_s = \pm 1$ no longer applies. In general, electron–electron dipolar interactions do not appear to significantly broaden the epr lines even in the blue samples which have up to 26 VO^{2+} per hexamer. For these samples the linewidth parameter, $\sigma = 20$ G, is comparable to that found for dilute frozen solutions of vanadyl complexes and less than the $\sigma = 30$ to 100 G found for various copper dimers.[24] If we treat the insulin hexamer as a sphere of diameter 50 Å with the 24 polar glutamate sites distributed evenly over the surface, then we estimate an "average" metal–metal distance between nearest neighbors of about 18 Å. Magnetic dipolar coupling at this distance would contribute only about 2 G to σ.

The question always arises in studies of this type as to whether the buffer also is binding at the metal site; indeed, citrate is known to form very stable complexes with a large number of metal ions.[27] Fortunately vanadyl epr hyperfine splittings are found to be sensitive to small changes in coordination environment. For example, variations in $A_{\parallel}$ and $A_{\perp}$ on the order of 16 and 4 G, respectively, are found for a series of hydroxycarboxylate chelates having apparently quite similar structures.[23] Accordingly, careful measurements of $A_{\parallel}$ were made on over ten samples prepared by the two methods with and without citrate buffer as described in the Experimental Section. Citrate does not appear to bind at either A or B sites since no correlation between $A_{\parallel}$ and the presence of buffer or between $A_{\parallel}$ and the general method of sample preparation was found.

Citrate can, however, extract VO^{2+} from insulin

(20) L. J. Bellamy, "Infra-Red Spectra of Complex Molecules," Wiley, New York, N. Y., 1958.
(21) J. C. Evans, *Inorg. Chem.*, 2, 372 (1963).
(22) K. Nakamoto, "Infrared Spectra of Inorganic and Coordination Compounds," 2nd ed, Wiley-Interscience, New York, N. Y., 1970, pp 222–224, 232–246.
(23) R. L. Belford, N. D. Chasteen, H. So, and R. E. Tapscott, *J. Amer. Chem. Soc.*, 91, 4675 (1969); N. D. Chasteen, R. L. Belford, and I. C. Paul, *Inorg. Chem.*, 8, 408 (1969).
(24) J. F. Boas, R. H. Dunhill, J. R. Pilbrow, R. C. Stivastiva, and T. D. Smith, *J. Chem. Soc. A*, 94 (1969); J. F. Boas, J. R. Pilbrow, C. R. Hartzell, and T. D. Smith, *ibid.*, 572 (1969).
(25) T. D. Smith, T. Lund, J. R. Pilbrow, and J. D. Price, *ibid.*, 2936 (1971).
(26) N. D. Chasteen and R. L. Belford, *Inorg. Chem.*, 9, 169, 2805 (1970).
(27) R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones, "Data for Biochemical Research," 2nd ed, Oxford University Press, New York, N. Y., 1969, p 431.

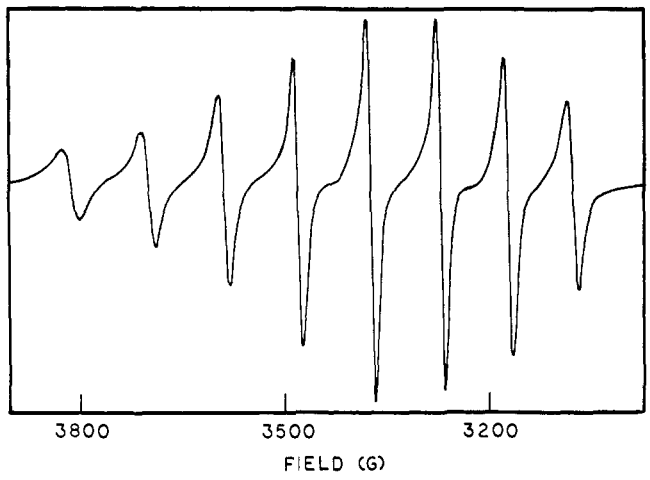


Figure 6. First-derivative X-band epr spectrum of polycrystalline vanadyl insulin stored moist with citrate buffer, pH 6.0, for several weeks. The spectrum of the moist sample arises from a vanadyl citrate complex formed in the buffer solution.

crystals. When vanadyl insulin crystals slightly damp with metal-free citrate buffer (pH 6.0) are stored for weeks, the anisotropic spectrum of protein bound VO^{2+} (Figure 1) is replaced by the isotropic spectrum of Figure 6. This spectrum, identified as that of a vanadyl citrate complex with $A_0 = (95.9 \pm 0.3) \times 10^{-4}$ cm^{-1} and $g_0 = 1.969$, exhibits no anisotropic features due to the averaging effect of molecular tumbling of the complex in solution.

Optical Spectra. Optical spectral data on vanadyl complexes can provide valuable clues as to their structure. We will summarize pertinent spectral data on a variety of complexes and finally apply these data to the spectrum of vanadyl insulin.

Vanadyl insulin samples in which the VO^{2+} ion is bound primarily at the A sites are colorless; however, when most of the B sites become occupied, the protein exhibits an intense blue color. Figure 7 shows the visible absorption spectrum of a mull sample of blue vanadyl insulin. Reasonable spectral assignments for the 775-nm (12.9 kK) and 600-nm (16.7 kK) bands are $^2B_2(xy) \rightarrow {}^2E(xz,yz)$ and $^2B_2(xy) \rightarrow {}^2B_1(x^2 - y^2)$, respectively ($C_{4v}$ notation).[28,29] (The x and y directions are taken approximately along the metal–equatorial ligand bond directions.) Presumably, the higher energy $^2B_2(xy) \rightarrow {}^2A_1(z^2)$ transition is obscured by the intense absorption tailing in from ultraviolet in Figure 7; in vanadyl complexes a third band is often observed near the ultraviolet in the 440–320-nm region. (Some low symmetry vanadyl complexes display four bands.[3,4,23]) The relative ordering of the $xy \rightarrow xz,yz$ lowest energy transition ($\Delta E_{xz,yz}$) for a variety of ligands (with the position of the band maxima in kilokaisers (kK) in parentheses) is given as follows:[30] $C_2O_4^{2-}$ (12.6) < $CH_3CO_2^-$(12.8) ~ DMSO(12.8) ~ $EDTA^{4-}$ (12.8) < H_2O(13.0) < Gly^-(13.2) < NCS^- (13.7) < His^- (14.1) < $acac^-$(15.2) < Cl^-(15.5) < CN^- (15.6) < $salen^{2-}$ (~16.0) < $acen^{2-}$ (16.5).[32]

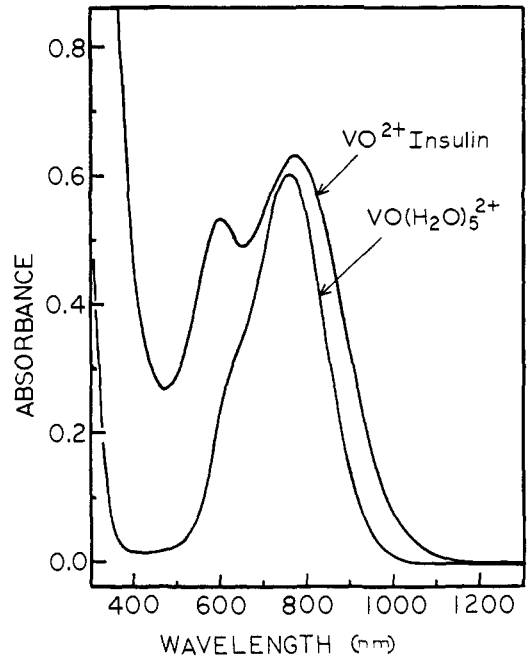


Figure 7. Optical spectra of a mull sample of blue vanadyl insulin and of an aqueous solution of $VOSO_4 \cdot 5H_2O$ (~0.35 M).

The relative positions of adjacent ligands in the series vary somewhat with different solutions because of coordination of the solvent in the axial position or hydrogen bonding of the solvent with the vanadyl oxygen. Contrary to what one would expect, the series does not follow closely the relative order of π-bonding ability of the ligands, but this can be rationalized by epr data.[31] Furthermore, we note that the lowest energy band may not correspond to $xy \rightarrow xz,yz$ for all complexes; however, our primary interest here is in the use of band positions as an empirical measure of ligand type.

Likewise, we write a ligand series for the $xy \rightarrow x^2 - y^2$ transition, ($\Delta E_{x^2-y^2}$), *viz.*, DMSO(14.8) < H_2O (16.0) < Cl^-(16.4) ~ $CH_3CO_2^-$(16.4) < $C_2O_4^{2-}$ (16.6) < $acac^-$(16.8) < $salen^{2-}$(17.0) < $EDTA^{4-}$ (17.2) < $acen^{2-}$(17.7) ~ NCS^-(17.8) < Gly^-(18.0) < His^- (19.3) < CN^-(24.8).[32] This series reflects the relative σ-bonding ability of the ligands and, except for H_2O which is abnormally displaced to lower energy, nearly parallels the spectrochemical series for transition metal ions.[33] Note that all the ligands which involve nitrogen coordination, $salen^{2-}$ through His^-, form complexes with higher energy $xy \rightarrow x^2 - y^2$ transitions; thus, we can distinguish between oxygen and nitrogen coordination in proteins.

The bands at 12.9 and 16.7 kK observed for vanadyl insulin correspond nicely with carboxyl coordination in the above two ligand series. Furthermore, in Figure 7 the relatively large difference between the position of the $xy \rightarrow x^2 - y^2$ transition in $VO(H_2O)_5^{2+}$ and vanadyl insulin, 625 nm (16.0 kK) *vs.* 600 nm (16.7

(28) M. H. Valek, W. A. Yeronas, G. Basu, P. K. Hon, and R. L. Belford, *J. Mol. Spectrosc.*, 37, 228 (1971).
(29) C. J. Ballhausen, B. F. Djursinskij, and K. J. Watson, *J. Amer. Chem. Soc.*, 90, 958 (1968).
(30) Band maxima taken from ref 3 and 31. Band maxima for His^-, Gly^-, and $CH_3CO_2^-$ were determined in the authors' laboratory.
(31) L. J. Boucher, E. C. Tynan, and T. F. Yen in "Electron Spin Resonance of Metal Complexes," T. F. Yen, Ed., Plenum Press, New York, N. Y., 1969, pp 111–130.
(32) Abbreviations used: DMSO, dimethyl sulfoxide; $EDTA^{4-}$, ethylenediaminetetraacetate; Gly^-, glycine; His^-, histidine; $acac^-$, acetylacetonate; $salen^{2-}$, bis(salicylaldehyde)ethylenediimine; $acen^{2-}$, bis(acetylacetone)ethylenediimine.
(33) B. N. Figgis, "Introduction to Ligand Fields," Interscience, New York, N. Y., 1966, p 243.

kK), certainly suggests a significant change in in-plane σ bonding and therefore equatorial coordination of the carboxyl group. The increase in the *relative* intensity of the $xy \rightarrow x^2 - y^2$ transition on going from $VO(H_2O)_5^{2+}$ to vanadyl insulin (Figure 7) may indicate a lowering of the local symmetry from C_{4v} upon displacement of one equatorial H_2O molecule with the carboxyl group. Axial coordination of the carboxyl group opposite the vanadyl oxygen would preserve the local symmetry.[34]

Epr Parameters and Bonding. The epr parameters of copper and iron enzymes are quite unusual when compared with those of simple model compounds and indicate unusual coordination geometries of low symmetry. Vallee and Williams have suggested metal sites in enzymes are in an "entatic state" poised for catalytic action.[35] In contrast, the epr parameters for the binding sites in vanadyl insulin (Table I) are not unusual when compared with those of model compounds. Most vanadyl complexes have $g_\parallel$, g_0, $A_\parallel$, $A_\perp$, and A_0 values intermediate between those of strong-field $VO(CN)_5^{3-}$ and weak-field $VO(H_2O)_5^{2+}$ (Table I). The epr parameters generally fall in the range: $g_\parallel = 1.952 \pm 0.020$, $g_\perp = 1.981 \pm 0.006$, $g_0 = 1.972 \pm 0.008$, $A_\parallel = (160 \pm 23) \times 10^{-4}$ cm^{-1}, $A_\perp = (60 \pm 13) \times 10^{-4}$ cm^{-1}, and $A_0 = (93 \pm 16) \times 10^{-4}$ cm^{-1}. ($g_\perp$ is the least sensitive parameter to changes in the environment of the VO^{2+} ion.)

Boucher, *et al.*, have correlated optical spectral transitions and epr data with ligand type for a variety of vanadyl complexes.[31] Among other things, they find that $\Delta g_\parallel (= 2.002 - g_\parallel)$, $\Delta g_0 (= 2.002 - g_0)$, $g_\perp - g_\parallel$, $A_\parallel$, $A_\perp$, and A_0 all decrease with increasing $\Delta E_{x^2-y^2}$, *i.e.*, increasing ligand-field strength. The relative magnitudes of the epr parameters for A and B sites of vanadyl insulin (Table I) are consistent with this correlation; *i.e.*, a greater field strength is anticipated for coordinating imidazole groups than for coordinating carboxyl groups.

To characterize the metal–ligand bonds in vanadyl insulin, we have calculated from the epr parameters and the ligand-field transitions the coefficients (α, β, and γ) of the xy, $x^2 - y^2$, and xz,yz metal orbitals, respectively, in the antibonding molecular orbitals, and κ, the Fermi contact term. The details of these calculations will not be given here as they have been amply discussed elsewhere.[6,31,36] We have assumed values for the spin–orbit coupling constant of 170 cm^{-1} and the electron–nuclear dipolar interaction of 0.0125 cm^{-1} to make our results comparable with those for other vanadyl compounds.[6,31]

The bonding parameters for A and B sites as well as for $VO(H_2O)_5^{2+}$ are summarized in Table I. Only α^2 and κ are given for A sites since the optical transitions could not be observed. One should only attach significance to the relative magnitudes of these parameters. (A value for α^2, β^2, or γ^2 of 0.5 corresponds to a completely covalent bond in this treatment.) The high values of $\alpha^2 (= 0.93)$ and $\gamma^2 (= 0.97)$ compared with $\beta^2 (= 0.84)$ for B sites (Table I) indicate that π bonding involving the xy and xz,yz orbitals is considerably more ionic than σ bonding involving the $x^2 - y^2$ orbital. This is typical of many vanadyl complexes.[6,31]

The squared coefficient α^2 for the xy orbital in which the unpaired electron resides is rather large for both A and B sites, 0.93 and 0.93, respectively. This is consistent with the general belief that this orbital is largely nonbonding and accounts for the fact that the observation of ligand superhyperfine splittings in vanadyl complexes is relatively rare. It is not surprising that we fail to observe any nitrogen splittings from the coordinated imidazole groups of A sites. With $VO(CN)_5^{3-}$ in which there is considerable in-plane π bonding ($\alpha^2 = 0.82$[31]) isotropic ^{13}C superhyperfine splittings of 11.3 G have been observed.[37]

The Fermi contact term, κ, can be used to estimate the energy of the $xy \rightarrow x^2 - y^2$ transition in a vanadyl labeled protein when optical spectral data are not available as is the case with the A sites. Boucher, *et al.*,[31] obtain a nearly linear plot of $1/\Delta E_{x^2-y^2}$ *vs.* κ for different ligands, *i.e.*, $\Delta E_{x^2-y^2}$ (kK) $\cong 13.0/\kappa$. For A sites, substitution of $\kappa = 0.74$ from Table I yields $\Delta E_{x^2-y^2} = 17.6$ kK which falls in the range of larger transition energies, 17.0–19.3 kK, expected for nitrogen coordination as given in the previously discussed ligand series. Similarly, substitution of $\kappa = 0.79$ for B sites yields $\Delta E_{x^2-y^2} = 16.5$ kK as compared with the experimental value of 16.7 kK from the optical spectrum in Figure 7. The uncertainty in transition energies derived from this procedure is probably not larger than ± 0.5 kK.

Conclusion

The application of VO^{2+} spectroscopy to insulin has clearly demonstrated the utility of this technique for determining (1) the total number of metal binding sites per protein molecule, (2) which of these sites are essential to the quaternary structure of the protein, (3) the chemical equivalence or difference between various sites, and (4) more specifically, the functional groups and coordination geometry of these sites. From this approach we have found that metal binding in vanadyl insulin parallels that in native zinc insulin. This is most reassuring in that the greatest value of the VO^{2+} labeling technique will be derived if the VO^{2+} ion mimics the native metal itself. To further establish the value of this technique, additional studies on other proteins are needed. Indeed, there are numerous proteins available in crystalline form which should be amenable to this type of study.[38] Furthermore, while there is a great deal of structural and spectroscopic data in the literature on vanadyl complexes, practically no work on complexes of amino acids or polypeptides has been reported. More data on model complexes are definitely needed to aid in the interpretation of results with proteins.

Acknowledgments. We wish to thank Dr. D. B. Easty, Dr. L. L. Winton, and Dr. D. G. Williams of the Institute of Paper Chemistry for their valuable assistance and use of facilities. Acknowledgment is made to the donors of the Petroleum Research Fund, admin-

(34) In C_{4v} and C_{2v} symmetries, the $xy \rightarrow x^2 - y^2$ transition is electric dipole forbidden in all polarizations by static selection rules but is allowed in lower symmetries; however, careful single crystal studies have shown that static selection rules do not completely dominate spectral intensities of vanadyl complexes. See ref 28 and 29.

(35) R. J. P. Williams and B. L. Vallee, *Proc. Nat. Acad. Sci. U. S.*, **59**, 498 (1968).

(36) B. R. McGarvey, *Transition Metal Chem.*, 3, 89 (1966).

(37) H. A. Kuska and M. T. Rogers, *Inorg. Chem.*, 5, 313 (1966).

(38) We have prepared vanadyl analogs of the zinc metalloenzymes bovine carboxypeptidase A and carbonic anhydrase: N. D. Chasteen, R. J. DeKoch, and C. E. Baldwin, to be submitted for publication.

istered by the American Chemical Society, the National Science Foundation, and the National Institute of General Medical Sciences for support of this research.

Appendix

To a good approximation, the anisotropic magnetic properties in VO^{2+} insulin have axial symmetry in which the VO bond direction presumably corresponds to the symmetry z axis. The usual axially symmetric $S = {}^1/_2$ spin-Hamiltonian is given by

$$\mathcal{H} = \beta[g_\parallel H_z \hat{S}_z + g_\perp(H_x\hat{S}_x + H_y\hat{S}_y)] + A_\parallel \hat{S}_z\hat{I}_z + A_\perp(\hat{S}_x\hat{I}_x + \hat{S}_y\hat{I}_y) \quad (1)$$

where x, y, and z refer to the molecular fixed axis system with the principal axes of the electron **g** and the vanadium nuclear hyperfine **A** tensors assumed to be coincident. H_i, $\hat{S}_i$, and $\hat{I}_i$ ($i = x, y, z$) are the components of the applied magnetic field and of the electron and nuclear spin operators, respectively. β is the Bohr magneton. The eight resonance fields H_M ($M = \pm{}^7/_2, \pm{}^5/_2, \pm{}^3/_2, \pm{}^1/_2$) to second-order perturbation theory are given by[39] eq 2, where the klystron frequency ω_0,

$$H_M(\theta) = \left\{\omega_0 + KM - \frac{A_\perp^2(A_\parallel^2 + K^2)}{4\omega_0 K^2} \times [I(I+1) - M^2] - \frac{1}{2\omega_0}\left(\frac{A_\parallel^2 - A_\perp^2}{K}\right)^2 \times \sin^2\theta\cos^2\theta\, M^2\right\} \Big/ g\beta \quad (2)$$

$A_\parallel$, $A_\perp$, and K are expressed in cm^{-1}, $I = {}^7/_2$, $\beta = 4.6686 \times 10^{-3}$ cm^{-1}/G, and

$$g = (g_\parallel^2\cos^2\theta + g_\perp^2\sin^2\theta)^{1/2} \quad (3)$$

and

$$K = (A_\parallel^2 g_\parallel^2\cos^2\theta + A_\perp^2 g_\perp^2\sin^2\theta)^{1/2}/g \quad (4)$$

θ is the angle between the symmetry axis (parallel direction) and the applied magnetic field. The isotropic g_0 and A_0 values are calculated from

$$g_0 = (g_\parallel + 2g_\perp)/3 \quad (5)$$

and

$$A_0 = (A_\parallel + 2A_\perp)/3 \quad (6)$$

Equation 2 gives the angular dependence of the resonance field $H_M(\theta)$ for a given hyperfine line; however, in frozen solutions or polycrystalline samples the epr spectrum is a superposition of spectra arising from molecules randomly oriented with respect to the applied magnetic field direction. In order to obtain the parameters $A_\parallel$, $A_\perp$, $g_\parallel$, and $g_\perp$ from polycrystalline samples, one needs to know which features of these rather complicated spectra correspond to the principal resonance fields, *i.e.*, the $H_M(\theta)$ for $\theta = 0$ and 90°.

(39) B. Bleany, *Phil. Mag.*, 42, 441 (1951).

We have written a Fortran IV computer program[40] which simulates the spectrum by summing spectra, each composed of $2I + 1$ first-derivative Lorentzian or Gaussian hyperfine lines, over θ values between 0 and 90°. The first-derivative Lorentzian and Gaussian functions are given by

$$F_M(H,\theta) = \frac{-\sigma(H - H_M(\theta))}{[\sigma^2 + (H - H_M(\theta))^2]^2} \quad (7)$$

and

$$F_M(H,\theta) = -\frac{(H - H_M(\theta))}{\sigma^3}\exp\left(-\frac{(H - H_M(\theta))^2}{2\sigma^2}\right) \quad (8)$$

respectively, where σ is an anisotropic line-width parameter adjusted to give the best fit to the spectrum, *viz.*

$$\sigma = (\sigma_\parallel^2 g_\parallel^2\cos^2\theta + \sigma_\perp^2 g_\perp^2\sin^2\theta)^{1/2}/g \quad (9)$$

The program works for any system with a single nuclear spin and axially symmetric or isotropic magnetic properties and takes into account contributions to the total spectrum from more than one magnetically distinct binding site, each having different values of $A_\parallel$, $A_\perp$, $g_\parallel$, and $g_\perp$. The total spectral function $G(H)$ is of the form

$$G(H) = \sum_i\sum_\theta\sum_M X_i P(\theta) F_{iM}(H,\theta) \quad (10)$$

where

$$P(\theta) = g_\perp^2(g_\parallel^2/g^2 + 1)\sin\theta\Delta\theta \quad (11)$$

M sums over the $2I + 1$ hyperfine lines. θ sums over all molecular orientations, 0 to 90°, in increments $\Delta\theta$ (usually 1°), and $\sin\theta\Delta\theta$ is proportional to the number of molecules with orientations between θ and $\theta + \Delta\theta$. The expression $g_\perp^2(g_\parallel^2/g^2 + 1)$ is proportional to the transition probability for molecules with orientation θ. X_i is the fractional population of the ith magnetically distinct binding site. The resultant spectrum is drawn by a Calcomp plotter. Simulation of a typical vanadyl spectrum with two magnetic sites requires about 3.5 min (not including plotting time) on an IBM 360/44 computer.

Figures 1 and 2 show experimental and computed curves from vanadyl and cupric insulins. The large line-width parameter, $\sigma = 45$ G, required to fit the experimental cupric insulin spectrum in part reflects unresolved nitrogen superhyperfine splittings[10] not taken into account in the simulation program. While simulated epr spectra of polycrystalline samples rarely give a perfect fit to the experimental curves (the curves differ primarily in amplitude), they do enable us to locate the principal resonance fields quite accurately and thus obtain reliable epr parameters (Table I).[41]

(40) Copies and instructions are available on request from N. D. C.

(41) Approaches to simulation of epr spectra can be found in L. D. Rollman and S. I. Chan, *J. Chem. Phys.*, 50, 3416 (1969); R. Neiman and D. Kivelson, *ibid.*, 35, 156 (1960); J. H. Mackey, M. Kopp, E. C. Tynan, and T. F. Yen, ref 31, pp 33–57.