ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, THE UNIVERSITY OF MICHIGAN]

Metal Chelates of Pyridoxylidene Amino Acids

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RECEIVED MARCH 15, 1957

The writer has prepared numerous metal chelates of the pyridoxylidene amino acids in crystalline form, usually with the composition M (pyridoxal·amino acid)$_2$, although in the case of copper the composition was M·pyridoxal·amino acid. For the more strongly catalytic Fe(III) and Cu chelates the titration attributed to the pyridine N was found to be shifted downward, relative to pyridoxal, by 3 *p*H units or more, presumably because of linkage of the metal to the phenolic oxygen. In the Mn(II) and Ni(II) chelates this titration was near its usual location, arguing against extensive linkage in this position. These conclusions are supported by infrared spectra showing a neutral structure for the Cu chelate and a zwitterionic structure for the Mn(II) and Ni(II) chelates. Chelation to the phenolic oxygen is probably not necessary for catalytic activity.

Amino acids undergo several metabolically important structural changes in the presence of pyridoxal and transition-group metals, under conditions permitting formation of chelated pyridoxylidene derivatives of the amino acid,[2,3] many of which now have been isolated.[4-6] Although the destabilizing action of carbonyl compounds on amino acids is well known, the role of the metal here is not well defined. Four possible actions have been suggested by Longenecker and Snell[7]: (a) preliminary proton-displacement to facilitate Schiff-base formation, (b) stabilization or destabilization of the Schiff base, (c) maintenance of planarity in the conjugated system of double bonds, and (d) reinforcement of electron-displacement from the α-carbon by the metal ion.

Whether each of these effects actually contributes to a given degradation will depend upon the structure of the catalytic intermediate and the extent of its formation. In particular, atoms of the amino acid will not be held in the plane of the aromatic ring unless the chelation joins them to this ring in a planar structure. Snell and his associates have generally written a 1:1:1 chelate (*e.g.*, formula II), which for copper probably yields 3 coplanar rings. On the other hand several metals with strong catalytic activity may or may not tend to maintain coplanarity; these may not even be chelated to the aromatic ring.

Eichhorn and Dawes[3] postulated the 3-point attachment to the Schiff-base for the Ni(II) as well as the Cu chelate on the basis of spectral changes upon adding pyridoxal to nickel plus alanine solutions. Evidence will be presented here that Mn(II), Zn(II), Fe(II) and Ni(II), in chelates crystallizing from ternary mixtures, instead are largely unbonded to the phenolic oxygen, in contrast to Fe(III) and Cu(II).

(1) Supported in part by a Grant (C-2645) from National Cancer Institute, U. S. Public Health Service. The scintillation-counting equipment was obtained through the Faculty Research-Research Equipment Fund of the Horace H. Rackham School of Graduate Studies, University of Michigan.

(2) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

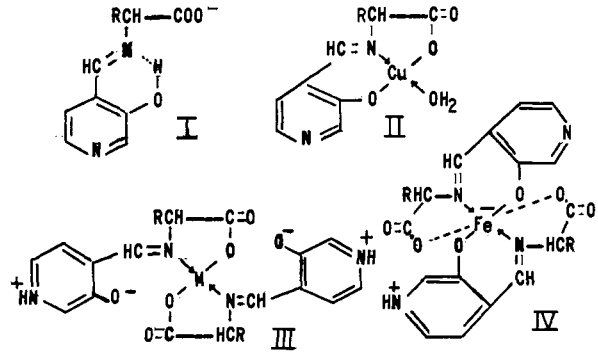
(3) G. L. Eichhorn and J. W. Dawes, *ibid.*, **76**, 5663 (1954).

(4) J. Baddiley, *Nature*, **170**, 711 (1952).

(5) H. N. Christensen and S. Collins, *J. Biol. Chem.*, **220**, 279 (1956).

(6) H. N. Christensen, *Biochem. Preparations*, **6**, in press (1957).

(7) J. B. Longenecker and E. E. Snell, THIS JOURNAL, **79**, 142 (1957).



Chelate structures: I, Schiff base anion, proposed by Metzler; II to IV, proposed here for metal chelates. The 6-methyl and 5-hydroxy methyl groups have been omitted.

The procedures used have permitted the crystallization of chelates of a large variety of amino acids; of these the chelates of L-valine were selected for this comparison; these were uniformly crystalline. The investigation actually began as an attempt to explain the paradox that an electrically neutral chelate crystallized out when either Mn(II) or Fe(II) combined with two pyridoxylidenevaline anions (formula I) or when Cu(II) combined with only one pyridoxylidenevaline. If one Mn(II) suffices to neutralize the charge of two pyridoxylidenevaline anions, then one Fe(III) should yield a positively charged product, and one Cu(II) combining with a single Schiff-base anion should also form a cation.

These difficulties appear to be avoided if we may freely place protons on the pyridine nitrogens or not as is required for electroneutrality. The isoelectric form of the chelate may be much less soluble than the anionic or cationic forms, so that it crystallizes out, enriching the solution with or depriving it of a hydrogen ion as the case may be. If so the Fe(III) chelate should require for optimal precipitation a more alkaline solution than the Mn(II), Fe(II) or Ni(II) chelate. This is by no means the case. Indeed one can separate the Fe(II) from the Fe(III) chelate by raising the *p*H to 6.0, whereby the Fe(III) chelate, crystallizing at *p*H 4.5 to 5, is redissolved. In order to explain this paradox the *pK* of at least one dissociating group must have very different values in some of these chelates than in others.

Evidence for the Structure of the Chelates from Titrations.—The titration of each of the chelates showed a sigmoid area with a slope approximately appropriate to the dissociation of a free group, taken to be the pyridine N (Figs. 1, 2). In the

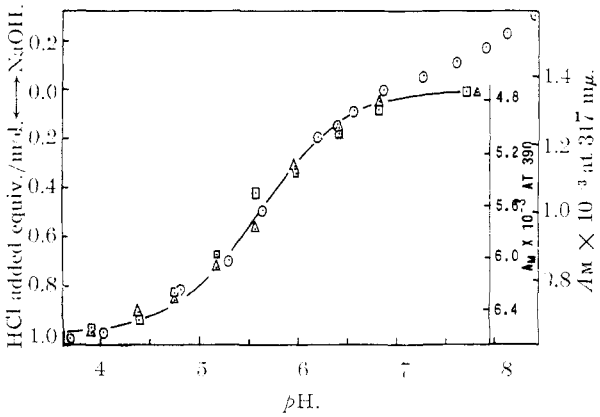


Fig. 1.—Titration of CuPyrVal: ○, titration with 0.1 *N* HCl (and NaOH) of a 0.023 *M* solution, no salt added; △, change with *p*H of the molar absorbancy at 390 mμ of a 0.0005 *M* solution of CuPyrVal containing 0.007 *M* $CuVal_2$; ionic strength = 0.016; □, molar absorbancy at 317 mμ for the same solution. The line is drawn according to the mass action law for $pK = 5.6$.

copper chelate HCl was needed to show this group, titrating with a mid-point at *p*H 5.6 (Fig. 1). One equivalent of OH^- per mole of the Fe(III) chelate showed for it a similarly situated dissociation, centered at *p*H 5.4 and having the theoretical slope. The other pyridine N known to be present

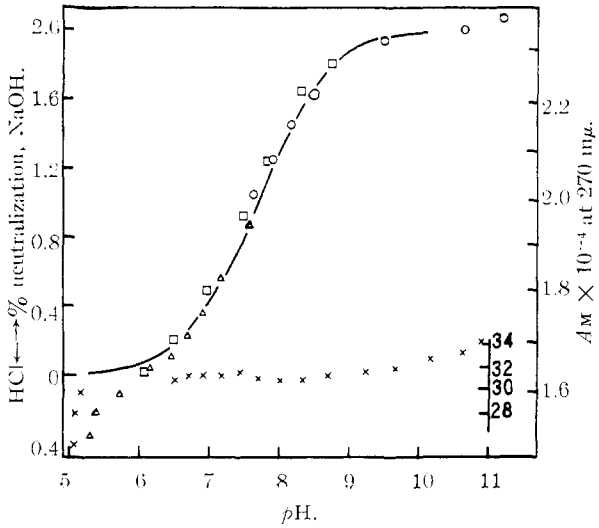


Fig. 2.—Titration of $Ni(PyrVal)_2$: circles, % neutralization *versus* *p*H for 0.05 *M* $Ni(PyrVal)_2$ prepared by dissolving the chelate in 0.45 equivalent NaOH; scale at left. Triangles, titration curve continued into region of lower solubility using 0.005 *M* $Ni(PyrVal)_2$. Squares, the molar absorbancy index for $Ni(PyrVal)_2$ 0.0001 *M* in 0.01 *M* $Ni(Val)_2$, scale at right. Crosses, the molar absorbancy index at 940 mμ *versus* *p*H, 0.005 *M* $Ni(PyrVal)_2$, scale at lower right. Ionic strengths 0.05. The solid line is drawn according to the mass action law for $pK_1 = 7.3$, $pK_2 = 8.1$.

in this chelate presumably associated a proton below *p*H 4.5, but the titration could not readily be followed because of the intense destabilization of the chelate in this region.

These two titrations were bounded above and in the case of Cu also below by regions of much weaker buffering so that their positions were not equivocal. The Fe(III) chelate consumed little OH^- from *p*H 7 to 9, and the spectral transmittance between 270 and 600 mμ was scarcely altered. Above *p*H 10 it became highly unstable to OH^-. CuPyrVal[8] consumed OH^- all the way from *p*H 7 to 11. This behavior could be differentiated from the group titration under study not only by its low slope, but also by two other observations: (a) changes in the absorbancies at 317 and 390 mμ which occurred symmetrically about the pK' of about 5.6 were terminated at about *p*H 7; (b) the absorbancy at the peak at 670 mμ was nearly constant from *p*H 4 to 7, but was decreased steadily by the further addition of alkali. This titration was attributed to displacement of other ligands from the Cu by OH^-.

The writer has not determined why the absorbancy changes at 317 and 390 mμ occurred when a proton was associated at *p*H 5.6. Possibly a part of the uncharged chelate takes the form of a chelated aminoacetal[9] absorbing at 317 mμ and is converted to the aldimine chelate absorbing at 390 mμ upon proton-addition. Titration of the 0.5 m*M* CuPyrVal in the presence of 20 m*M* $Cu(Val)_2$ (Fig. 1) led to essentially the same results, except that the very rapid destabilization which otherwise occurred just below *p*H 4.0 was prevented. Accordingly the titration behavior was attributed to CuPyrVal and not to its dissociation products, pyridoxal or PyrVal. Clearly CuPyrVal hydrolyzes only slightly even in dilute solution.

The pK's of the dissociating group of the Ni(II) and Mn(II) chelates were not as readily fixed. The Ni chelate gave a dissociation curve centered at about *p*H 7.5; the upper limits of the titrations were quite distinct (Fig. 2), especially when shown in fairly concentrated solutions. The lower limit was also distinguishable, although rapid breakdown took place below *p*H 5.3. H^+ consumption by the group-titration and by the cleavage of the chelate were differentiated as follows. The absorption at 940 mμ, characteristic of the Ni-chelated aldimine structure,[8] remained constant from *p*H 6 to 10; it increased moderately above *p*H 10 (exaggerated in Fig. 3 by the use of a dilute solution) and decreased rapidly below *p*H 6.3, in association with the extra OH^- and H^+ requirements. The titration of the free chemical group in the Ni(II) chelate also was described by an increase in absorbancy at 270 mμ (Fig. 3). The shape of the dissociation curve for $Ni(PyrVal)_2$ suggested that the two dissociating groups had pK's of approximately 7.3 and 8.1 (Fig. 3).

The much less stable Mn(II) and Zn(II) chelates split enough in saturated watery solution to raise the *p*H to 7.8 and 7.4, respectively, and after adjustment to a *p*H below 7.2 and 7.0, respectively,

(8) Abbreviations: Pyr = pyridoxylidene; Val = valine; Orn = ornithine; Pro = proline; Aib = α-aminoisobutyrate.

(9) D. E. Metzler, This Journal, **79**, 485 (1957).

H^+ consumption continued for many minutes. Accordingly the limit and position of the lower part of the pyridine N dissociation was not obtained accurately; $MnVal_2$ and $ZnVal_2$ were themselves not sufficiently stable to serve as stabilizers above *p*H 8. By dissolving in slightly more than an equivalent of NaOH per mole, the concentration of the chelate could be brought to 0.05 *M*, thereby improving the stability; the part of the titration obtained by adding more alkali to this solution supported a mid-point of about 8.2 for the entire titration attributed to the two pyridine N's in each chelate. In addition the titration above *p*H 7.3 was marked by much less change in stability (as indicated by change in absorbancy at 390–412 mμ) than below *p*H 7.3. These results, together with the infrared spectral demonstration that these two chelates are zwitterionic, indicate that they resemble the Ni(II) chelate.

Interpretation.—The foregoing titration behaviors are the ones required by the isoelectric formulations shown above for the crystalline chelates: the Cu chelate without a proton on the pyridine N; the Fe(III) chelate with a proton on only one pyridine N, and the Ni(II), Mn(II) and Zn(II) chelates with protons on both pyridine N's. Accordingly, the Ni(II), Mn(II) and Zn(II) chelates lose H^+ from their pyridine N's at much higher *p*H values than the Cu and Fe(III) chelates. What is the significance of this difference?

In CuPyrVal and Fe(III)$(PyrVal)_2$ the shift in the position of the pK_a' of the pyridine N from its normal position at about *p*H 8.6 in pyridoxal to about *p*H 5 is a predictable consequence of linkage to the phenolic oxygen. For comparison, O-methylation of pyridoxal lowers this *pK* to 4.75.[10] Similarly, Metzler[9] has concluded recently that this *pK* is lowered to 5.9 in pyridoxylidenevaline by bonding of a proton to the phenolic O, the proton being stabilized in this position by H-bonding to the imine N.[9] Displacement of this proton by a metal ion should reproduce this effect, unless the metal remains unbonded to the phenolic O.

Three explanations may be considered for the absence of the expected downward shift in the *pK* of the pyridine N in the Ni(II), Mn(II) and Zn(II) chelates:

1. Bonding between the metal and the phenolic oxygen may be totally absent.
2. The bonding may be so unstable that the *pK* shift is small.
3. The bonding may have less influence on the acidity of the pyridinium ion whenever it makes the metal electronegative.

According to the last alternative the Fe(III) chelate also ought to have a high *pK* for at least one pyridinium group. The second explanation, which is considered the most likely, means that the linkage of metal to the phenolic oxygen is broken most of the time in solution.[11]

According to this interpretation the most abundant form of the chelates of Mn(II), Ni(II) and zinc at isoelectric *p*H values is zwitterionic, and simply a double Schiff-base of the amino acid–metal chelate. The presence of the proton on the pyridine N is attested by the infrared spectra (see below).

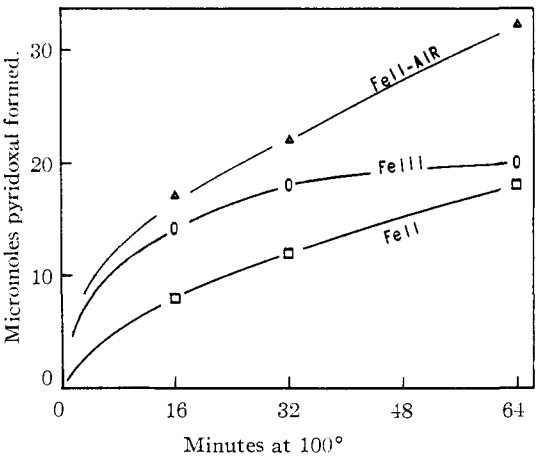


Fig. 3.—Comparative catalysis of transamination by ferrous and ferric ions. Ten millimoles each of pyridoxamine and α-ketoglutarate in 10 ml. of solution buffered at *p*H 4.8, heated in N_2-filled Thunberg tubes under boiling water. Fe, 0.000125 *M* added as ferrous ammonium sulfate or ferric chloride. The triangles show values we obtained with Fe(II) in open tubes.

The Fe(II) chelate was not readily prepared free of Fe(III); its titration was difficult because of intense instability to O_2 and OH^-; presumably it resembled the Mn(II), and Ni(II) chelates in structure. The much greater stability of the Ni than the Mn and Zn chelates discourages one from attributing the structural difference merely to low chelate stability.

The somewhat lower catalytic activity in transaminations and similar reactions of the Mn(II), Zn(II) and Ni(II) chelates suggests a rather limited role for linkage to the phenolic O in this function. These results do not permit us to conclude whether bonding to the phenolic O facilitates catalysis by producing planarity or by stabilizing the aldimine link or not at all. Incidentally, a low pK_a for the pyridine N, other factors being equal, might appear to handicap the catalytic activity of the chelate at higher *p*H values, if the proton carried on this group must receive an electron in the electromeric shift believed to underly the catalysis.[2]

The illustrated bonding of both carboxyl groups to Fe(III) has not been proved, but is proposed for the following reason: If an amino acid–metal chelate reacts with pyridoxal, bonding of the metal to the phenolate ion requires at above *p*H 6, as shown here, the displacement of a proton from the pyridinium group. Accordingly, the link to the carboxylate group probably is more stable than that to the phenolic O. The chelate does not contain a pyridine N titrating in the *p*H range 7–9; accordingly, Fe(III) is taken to be bonded to both phenolic oxygens; hence both carboxyl groups also may well be bonded. Incidentally, the bonding of a second Schiff-base to the metal shown here has been proposed to explain the excess of L-glutamic

(10) D. E. Metzler and E. E. Snell, THIS JOURNAL, **77**, 2431 (1955).

(11) The finding that the zinc chelate has the composition $Zn(PyrVal)_2$ provides additional support for assigning structure III to this group of chelates. With a coördination number of 4 it is unlikely that the zinc ion is joined to each of the two Schiff base residues at three points.

acid obtained catalytically from α-ketoglutarate and L-alanine.[12]

The activity of Fe(II) in non-enzymic catalysis, recently reported[13] to be just slightly less than that of Fe(III), seems unaccountably high by analogy with Mn(II), Zn and Ni(II). Therefore, the catalytic activity for transamination between pyridoxamine and α-ketoglutarate was estimated with careful exclusion of O_2 in a Thunberg tube, the conditions otherwise being as described by Longenecker. Under these conditions Fe(II) showed less than half as much activity as in air (Fig. 3), it now falling between Ni(II) and Co(II) in catalytic activity. (This experiment was kindly made by Mr. Walter B. Dempsey of this Laboratory.)

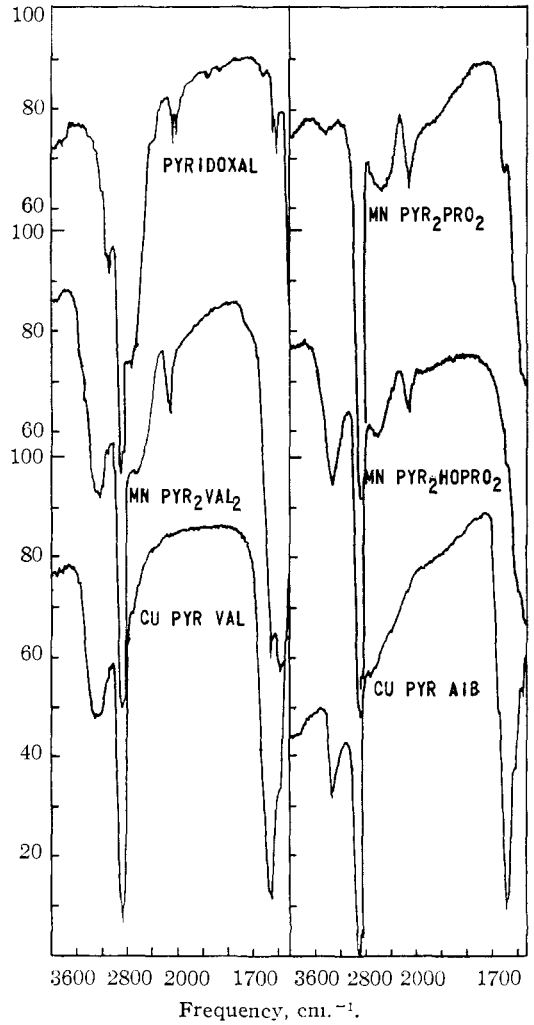


Fig. 4.—Portions of infrared spectra of chelates compared with that of pyridoxal. One frequency scale interval is 400 cm.$^{-1}$ from 4000 to 2000 cm.$^{-1}$ and 100 cm.$^{-1}$ below 2000 cm.$^{-1}$. Attention is directed to: (a) increased aliphatic hydroxyl absorption (*ca.* 3200 cm.$^{-1}$) and appearance of the aldimine absorption (*ca.* 1600 cm.$^{-1}$) upon formation of the amino acid but not the imino acid derivatives; and (b) the ionic properties (1900 to 2600 cm.$^{-1}$) of Mn but not of Cu chelates. Ni(II) and Zn(II) chelates gave curves resembling those of Mn(II) chelates.

(12) J. B. Longenecker and E. E. Snell, *Proc. Nat. Acad. Sci.*, **42**, 221 (1956).

(13) J. B. Longenecker and E. E. Snell, THIS JOURNAL, **79**, 142 (1957).

Infrared Absorption.—The infrared spectrum of CuPyrVal on one hand is very different from the spectra of the corresponding Mn(II), Zn(II), Ni(II) chelates, on the other. The latter two resemble pyridoxal in having prominent peaks at about 2100 and 2700 cm.$^{-1}$ (Fig. 4), thus supporting their zwitterionic structure. The isoelectric copper chelate in contrast, shows essentially no absorption in this region, revealing it to be uncharged. This result confirms the presence of protons on the pyridine N atoms in crystals of the Mn and Ni chelates precipitating at neutral *p*H, and the absence of such a proton on CuPyrVal precipitated at similar *p*H values. The spectrum of the Fe(III) chelate in the indicated region has an appearance intermediate between the non-ionic Cu and the strongly ionic Mn chelate. Figure 4 also shows portions of the infrared spectra of chelated pyroxylidene derivatives of proline and α-aminoisobutyric acid, showing also the non-ionic nature of the copper chelates and the decreased absorption attributable to the hydroxymethyl group and to an aldimine group, undoubtedly arising from their being largely in aminoacetal forms.

Isotopic Study of Metal Exchange.—The usual procedure is to mix the chelate under investigation with a labeled complex of the same metal (the latter complex being known to exchange rapidly) and then to reseparate the two complexes. Instead, in the cases of Fe(III) and Mn(II) use was made of the soluble monopyridoxylideneornithine chelates (which appear to have below *p*H 7.5 the usual structure involving only one amino group). A large portion of the less-soluble pyridoxylidenevaline chelate readily was reisolated for radioactive counting. (More could have been recovered but with an undesirable delay.) The method yields information on the exchange rate between the two chelates without telling which exchange is rate-limiting. For the copper chelate, exchange between $Cu^{64}Val_2$ and CuPyrVal was studied, the latter being reisolated upon concentration of the solution.

The Fe of Fe(III)(PyrVal)$_2$ recrystallized from a solution also containing half the molar quantity of Fe^{59}(PyrOrn)$_2$ by lowering the *p*H from 7.3 to 4.7, and collected in a minimal operating time of 5 min., had only 11% of the specific activity shown by the 46% of the Fe remaining in solution. When the solution was first incubated 30 min. at 0°, the exchange was 43% complete; in 15 min. at 25°, 58% complete.

Mn(PyrVal)$_2$ in contrast exchanged completely with the corresponding labeled ornithine chelate in the minimal 20 min. (mostly at 0°) required for mixing, concentrating to reprecipitate it, and collecting the precipitate. The proportions taken and reisolated were similar to those used with Fe. The copper of CuPyrVal likewise exchanged very rapidly with Cu^{64} of $CuVal_2$ (representing $^1/_5$ of the total Cu), being completely exchanged in the 15 minutes at 0–15° required to reisolate the CuPyrVal.

These results appeared to limit to the Fe(III)

the possibility of testing for biological acceleration of exchange. Another method, however, gave a lower rate of exchange for CuPyrVal. When a 0.0005 *M* solution was brought to 0.1 *M* in valine at *p*H 7, the decrease in absorbancy at 380 mμ due to the reaction CuPyrVal + Val → Cu^{+}Val + Pyr^{-}Val, continued for more than an hour at 25°, with a half-time of over 5 min.

Acknowledgments.—The writer is grateful to Miss Jane LeFever for assistance with some of the experiments, to Miss Lois Walker for copper and amino acid analyses, and to Mr. Walter B. Dempsey for performing the experiments of Fig. 3. He has benefited from discussing the infrared spectra with Dr. J. L. Johnson of the research laboratories of the Upjohn Company.

Experimental

Apparatus and Materials.—A Beckman model G *p*H meter and model DU spectrophotometer were used. To permit reading solutions as concentrated as possible (to diminish dissociation) 0.5 cm. photometer cells usually were used. Highly active Fe^{59} and Cu^{64} were obtained from Carbon and Carbide Co., Oak Ridge, and Mn^{54} from Nuclear Science and Engineering Corporation, Pittsburgh; all were used as the chlorides. Disintegrations were counted in a Nuclear-Chicago well-type scintillation counter, at 20 to 200 times background rates. Infrared spectra were obtained in a Nujol mull using a Perkin-Elmer instrument.

Pyridoxal hydrochloride (Merck and Co.) was converted to the free base by adjusting a strong aqueous solution to *p*H 6.5 with potassium hydroxide; the crystals were washed, and dried over P_2O_5 at room temperature. The inorganic salts were of analytical reagent grade. The amino acids, when optically active, were the L-forms, and were obtained from Nutritional Biochemicals, Inc., or in a few cases from the H. M. Chemical Company. Reagent grade methanol and ethanol were used without purification.

General Procedure for Preparation of Chelates.—The amino acid was dissolved in an equivalent quantity of methanolic or aqueous sodium hydroxide, and a mole of pyridoxal dissolved in this solution to form the sodium salt of the Schiff base, which remained in solution. The metal salt was then added in aqueous or methanol solution, using the metal acetate in the latter case. If crystallization did not follow from methanolic solutions, ethanol (for small-molecular amino acids) or water (for large-molecular amino acids) was added, often by way of the vapor phase. Acetate added either as the metal salt, or separately as an acetate buffer, in several cases served to accept hydrogen ion from the chelate to facilitate crystallization in the isoelectric form.

Composition of the chelates could be determined by analyzing colorimetrically a solution in 0.1 *N* sulfuric acid for pyridoxal,[14] for amino acids[15] and for the metal, Cu being determined as the diphenyldithiocarbamate derivative,[16] manganese after oxidation to permanganate,[17] iron as the complex ferric thiocyanate ion,[18] and nickel as the dimethylglyoxime chelate.[19]

Procedure for Cu Chelates, Illustrated by CuPyrVal.—One hundred and fifty micromoles of the Schiff-base in 0.5 ml. of methanol was treated with 0.36 *M* (approximately saturated) aqueous cupric acetate, followed by 2 ml. of ethanol. In a few minutes the solution filled with long green needles. After cooling at 5° overnight, the crystals were removed and washed with ethanol, and dried at 56° over P_2O_5. The product, weighing 35 to 40 mg., contained one valine in 343 g., one pyridoxal in 338 g. and one copper in 329 g.; the theoretical 1:1:1 composition $C_{13}H_{16}O_4N_2Cu$ corresponds to a molecular weight of 328. The chelate dissolved in water to about 0.025 *M* at 25°. Similarly, 1:1:1 compositions were shown by the isoleucine, methionine, β-alanine, phenylalanine, α-aminoisobutyric and sarcosine chelates. The latter two probably were largely aminoacetals rather than aldimines[9] (see infrared spectra, Fig. 5); the α-aminoisobutyric derivative was dark brown rather than green. Crystalline chelates obtained with several other amino acids showed moderate excesses of copper over the theoretical composition.

Chelates could also be obtained from aqueous solution, often using an additional equivalent of NaOH to render them less soluble; the chelates of alanine and proline were obtained in this way although in amorphous form.

Procedure for Manganous, Nickelous, Zinc and Ferrous Chelates, Illustrated for Mn(PyrVal)$_2$.—One hundred and fifty micromoles of the Schiff base in 0.5 ml. of methanol was treated with 0.15 ml. of a fresh 0.5 *M* manganous acetate solution in methanol. The brown solution crystallized out square brown plates after a few minutes. After chilling 2 or 3 hours the crystals were removed, washed with methanol, and dried at 56° over P_2O_5. The product, weighing about 25 mg., contained one valine in 310 g., one pyridoxal in 305, and one manganese in 603. The theoretical values for Mn(PyrVal)$_2$ are 292, 292 and 583. Similar compositions were obtained for chelates from alanine, serine, threonine, isoleucine, proline and hydroxyproline. The latter two probably were aminoacetals, the Schiff bases and the chelates being light yellow in color. For Mn, glycine is the only amino acid so far obtained in a 1:1:1 chelate, and that from a non-aqueous solution.[5]

Manganous pyridoxylidenevaline and zinc pyridoxylidenevaline also were obtained readily by dissolving 200 micromoles each of L-valine and pyridoxal in 0.2 ml. of *N* NaOH, and then adding 0.1 ml. of *M* aqueous manganous acetate or zinc chloride. The square plates were washed with water, in which the solubility was, for Mn(II), about 5.6 m*M* at 0°, 8.5 m*M* at 25°, and 12 m*M* at 37°; for Zn, about 4 m*M* at 25°.

Ni(PyrVal)$_2$ was obtained readily by the latter procedure (clear yellow rectangular blocks), as was Fe(II)(PyrVal)$_2$ (clear blue square and octagonal plates) at 0° although oxygen had to be excluded strictly in the latter case to prevent autoöxidation to the red Fe(III) chelate.

Fe(III)(PyrVal)$_2$.—0.2 ml. of a molar aqueous solution of the sodium salt of the Schiff base, formed as usual, was treated with 0.1 ml. of a molar aqueous solution of ferric chloride. A deep red color resulted, and crystallization began at once. 0.1 ml. of an acetate buffer (1.5 molar each in acetic acid and sodium acetate) was then added. After standing overnight in the refrigerator the crystals were separated and washed with cold water to yield 45 mg. of chelate. This was recrystallized by dissolving carefully in 0.3 *N* NaOH, keeping the *p*H below 8 and centrifuging to clarify the deep red solution. Upon adding 0.1 ml. of the acetate buffer the chelate recrystallized in beautiful red complexly truncated right-triangular prisms (orthorhombic hemimorphic). The crystals upon redissolving in water produced a *p*H below 4.5 which rose rapidly because of the low stability at this *p*H, until the dissociation products reached levels which stabilized the chelate.

The crystalline product contained one pyridoxal in 290, one valine in 287, one Fe in 579 mg. (theoretical m.w. = 583).

Titrations were made at 25–26° at the ionic strengths indicated in the figures, disregarding the ionic strength arising from any dipolar ion.

Isotopic Exchange.—0.5 ml. of an aqueous solution, *p*H 7.3, containing 80 micromoles of previously crystallized Fe(III)(PyrVal)$_2$ chilled to 0° was mixed with 0.25 ml. of a similarly cold solution of 40 micromoles of Fe^{59}(III)(PyrOrn)$_2$ (prepared in solution by mixing the components) also at *p*H 7.3. Instantly 0.15 ml. of 3 *M* acetate buffer *p*H 4.7 was mixed with the solution. Within 40 sec. of the original mixing centrifuging was begun and continued 4 min. at high speed. The crystalline precipitate was washed three times with water at 0°. In other experiments an interval at 0 or 25° was allowed before addition of the acetate buffer.

Four ml. of a saturated Mn(PyrVal)$_2$ solution (50 micromoles) was concentrated to 1.5 ml., and chilled in ice; then 25 micromoles of Mn^{54}(PyrOrn)$_2$ was added in 0.3 ml. of water (both solutions at *p*H 7.0). Concentration *in vacuo* 4 min. (10–15°) started crystallization; this was continued 10 min. at 0°. After 5 min. of centrifuging the liquor was removed and the precipitate washed 3 times. Forty per

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cent. each of the Mn and of the radioactivity was precipitated; the precipitate contained the appropriate amounts of pyridoxal and amino acid.

To 4 ml. of ice-cold 0.020 *M* CuPyrVal, previously crystallized, was added 0.33 ml. of 0.060 *M* $Cu^{64}Val_2$, both solutions at *p*H 7.2 and the solution concentrated quickly in the cold to about 0.5 ml. After holding 5 min. at 0°, the suspension was centrifuged 4 min. at high speed, and the crystals washed twice with water. In a representative test, 32.2% of the copper and 33.1% of the radioactivity had been precipitated.

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The Hydrogen Ion Equilibria of a Single Group Attached to Serum Albumin: Some Implications as to the Surface Characteristics of Protein Molecules

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RECEIVED MARCH 18, 1957

The ionization equilibria of the dimethylamino group of $(CH_3)_2N$—⟨⟩—N=N—⟨⟩—Hg— attached to bovine serum albumin have been studied in various aqueous solvents. In water the pK_a of this dye when attached to cysteine is 3.3; when linked to protein, 1.8. In 8 *M* urea, the pK_a is approximately 3.3 for the dye on the protein or amino acid, near 5 in 0.03 *M* dodecyl sulfate in both cases. These results and the more detailed behavior of the ionization equilibria are not in concordance with electrostatic expectations. It is suggested that the pK_a's reflect the structure of the water envelope of the protein. A variety of observations can be described in terms of changes in the order in this aqueous framework.

Introduction

Proton equilibria of acid–base groups on proteins have been the subject of numerous investigations for over 50 years. The early literature has been summarized by Cohn and Edsall[1] and more recent work has been reviewed by Steinhardt.[2] In general, the maximum uptake or release of protons corresponds well with the number of acidic and basic groups presumed to be on the protein from analyses of its content of specific amino acids. The course of the *p*H-titration curve, however, usually differs markedly from what one might calculate for the corresponding number of comparable proton-dissociating groups in an ordinary aqueous environment.

The modified acidity of groups on a protein molecule has been ascribed in part to electrostatic interactions between successively dissociating sites.[3–6] These interactions account reasonably well for the titration curves of some proteins[4,7] but more often there are large deviations, in both directions, from electrostatic predictions. Such discrepancies have been attributed to changes in molecular weight or shape,[8–10] to binding of small ions[6] and to the influence of hydrogen bonding between polar groups on the pK's of proton-dissociating groups.[7,11]

The interpretation of acid–base ionizations of protein molecules is complicated in part by the fact that the measurements reflect the composite behavior of a large number of groups. Even in limited regions of *p*H, for example, 3–5, where only one type of equilibrium is involved, —COOH ⇌ —COO^-, the number of participating carboxyl groups is very large. There are usually fewer imidazole or tyrosine groups in a protein, but even these are commonly present in appreciable number and their proton equilibria overlap those of amino groups, so that a sharp separation cannot be made by electrometric methods.

The proton-donor group usually present in smallest number in proteins is the sulfhydryl. A direct study of its dissociation by electrometric methods is not feasible, however, since the equilibria of amino and tyrosine groups would obscure the contribution of –SH. In principle, it might be possible to adapt the optical method used by Benesch and Benesch[12] for the determination of pK_{SH} of cysteine, a method which takes advantage of the appearance of an absorption peak at 240 mμ when –SH is converted to $-S^-$. In most proteins, however, absorption of light at this wave length is very strong even in dilute solution and even when the mercaptan group is not ionized. It would be difficult, therefore, to measure the small differences in absorption accompanying ionization of the –SH, against the strong background absorption of the relatively concentrated protein solution which would be needed to supply an appreciable concentration of mercaptan groups.

An alternative approach which could still take advantage of the small number of –SH groups on a protein would be to introduce a molecule which reacts specifically with the mercaptan side-chain, and which contains a substituent whose proton uptake, or release, is accompanied by a marked change in visible absorption spectrum. A class of such molecules are the azomercurials, (I), in which

X—⟨⟩—N=N—⟨⟩—Hg—Ac

I

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