salts gave a dark-green Franchimont test with diethylaniline.

Anal. Calcd. for $C_2N_6O_4Pb$: N, 22.15. Found: N, 22.13, 22.34.

Lithium Nitrocyanamide.—To 0.43 g. (0.01 mole) of lithium chloride in 2 ml. of water was added 1.94 g. (0.01 mole) of silver nitrocyanamide in 10 ml. of acetonitrile. The silver chloride which formed was filtered after the reaction mixture had stood for 20 minutes. The filtrate was vacuum-concentrated to yield 0.9 g. (96.8%) of lithium nitrocyanamide containing 2 moles of water of crystallization. This salt was purified by being dissolved in absolute alcohol, from which it separated as an oily liquid. After several washings with ether, this material, when left in a vacuum desiccator overnight, gave a hard but waxy product which was quite hygroscopic. It melted at 94–95°. One part of the sample submitted for analysis was dried to constant weight at 80°, while another was dried in a vacuum desiccator overnight at room temperature. The amounts of nitrogen found in the two specimens were not significantly different.

Anal. Calcd. for $CN_3O_4Li \cdot 2H_2O$: N, 32.56. Found: N, 32.63, 32.42.

Sodium Nitrocyanamide.—The sodium salt of nitrocyanamide was prepared by a variation of the method of McKay, *et al.*[1] To a solution of 0.8 g. (0.02 mole) of sodium hydroxide, kept at 0° by external cooling, in 10 ml. of ethanol was added 2.94 g. (0.02 mole) of N-methyl-N-nitroso-N′-nitroguanidine over a 5-minute period. The reaction mixture was concentrated and the solid residue recrystallized from absolute ethanol to yield 2 g. (91.7%) of sodium nitrocyanamide. The white, crystalline salt softened with decomposition at 202°. It gave a light-green Franchimont test with diethylaniline.

Anal. Calcd. for CN_3O_2Na: N, 38.53. Found: N, 38.21, 38.27.

Strontium Nitrocyanamide.—To a solution of 1.94 g. (0.01 mole) of silver nitrocyanamide in 10 ml. of acetonitrile was added 10 ml. of 1 *N* hydrochloric acid. There was an immediate precipitate of silver chloride. After 15 minutes of shaking, the mixture was filtered and the filtrate immediately poured over 0.78 g. (0.005 mole) of strontium carbonate. Effervescence began at once and continued until no solid remained. The resulting liquid was concentrated overnight to yield 1.56 g. of a cream-colored solid. This quantity represented a 120% yield based on a non-hydrated strontium nitrocyanamide or a 99.4% yield assuming a trihydrate. When this material was dissolved in a minimum amount of methanol and petroleum ether added to the point of cloudiness, recrystallization occurred after overnight standing. The purified salt, $Sr(N(NO_2)CN)_2 \cdot 3H_2O$, melted at 77–79°, resolidified, and did not remelt below 250°. A sample submitted for analysis exploded while undergoing composition incidental to determination of carbon and hydrogen, scattering the material on the walls of the combustion tube. This occurrence may well explain the low carbon value, particularly if the solid on the walls was strontium carbonate. Strontium nitrocyanamide gave a dark-green Franchimont test with diethylaniline.

Anal. Calcd. for $C_2N_6O_4Sr \cdot 3H_2O$: C, 7.66; H, 1.91; N, 26.78. Found: C, 5.01; H, 2.01; N, 26.46.

Tin(II) Nitrocyanamide.—To 1.13 g. (0.005 mole) of stannous chloride in 10 ml. of acetonitrile was added 1.94 g. (0.01 mole) of silver nitrocyanamide in 10 ml. of acetonitrile. The reaction mixture was permitted to stand overnight then filtered. Vacuum-evaporation of the filtrate yielded 1.3 g. (89.6% of the expected yield) of a light brown salt. This material could be successively recrystallized from either water or acetonitrile without undue loss and analyzed for the pentahydrate. It gave a green Franchimont test with diethylaniline. The purified sample melted at 151° dec.

Anal. Calcd. for $C_2N_6O_4Sn \cdot 5H_2O$: N, 22.06. Found (recrystd. from water): N, 22.09, 22.23. Found (recrystd. from acetonitrile): N, 22.25, 22.31.

Zinc Nitrocyanamide.—To a solution of 1.94 g. (0.01 mole) of silver nitrocyanamide in 10 ml. of acetonitrile was added an excess of a saturated zinc chloride solution. The reaction mixture was filtered after aging for 20 minutes, the precipitate washed with acetone, and the filtrate evaporated to yield a viscous liquid which gave crystals upon trituration in the presence of ether. Excess zinc chloride was removed from the zinc nitrocyanamide by washing repeatedly with ether. Recrystallization from acetone–ether gave 2.34 g. (98.7%) of white hygroscopic solid which showed no apparent change nor inclination to melt up to 250°. It gave a light-green Franchimont test with diethylaniline.

Anal. Calcd. for $C_2N_6O_4Zn$: N, 35.38. Found: N, 35.37, 35.17.

Nitrocyanamide.—To a solution of 1.94 g. (0.01 mole) of silver nitrocyanamide in 10 ml. of acetonitrile was added enough anhydrous hydrogen chloride to cause complete precipitation of silver chloride. After removal of this precipitate the filtrate was evaporated *in vacuo* to give a white, crystalline material, the amount of which was about 12% more than was expected on the basis of the formula, $HN(NO_2)CN$. The 1.04-g. yield was quantitative on the basis of this formula when calculated to contain a mole of water of crystallization. It melted sharply at 137–138° and gave a light-green Franchimont test with diethylaniline. The free acid decomposed slowly but was identified by its well known potassium salt. The elemental analysis was in much better agreement with the constitution of the hydrated form than with the non-hydrated.

Anal. Calcd. for $CHN_3O_2 \cdot H_2O$: C, 11.43; H, 2.86; N, 40.00. Found: C, 12.38, 12.37; H, 2.90, 2.96; N, 40.03, 40.10.

PITTSBURGH, PA.

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

Dissociation of Copper Pyridoxylidenevaline[1]

BY HALVOR N. CHRISTENSEN

RECEIVED AUGUST 19, 1957

The chelate cupric pyridoxylidenevaline is exceedingly stable in water, more stable than copper valine$_2$. Excesses of the order of 1000 to 1 of valine can, however, displace the Schiff base from the Cu to permit evaluation of the stability constant. As the *p*H is lowered to *p*H 4.5, the reaction proceeds more slowly, showing first-order reversible kinetics. The valine anion is clearly a reactant. Part of the pyridoxylidenevaline formed then breaks down, this reaction being the slower one at higher *p*H and the more rapid one at lower *p*H. Release of pyridoxal preceding the release of copper occurs slowly if at all.

In its coenzyme function pyridoxal-5-phosphate undoubtedly forms Schiff bases with the amino acids.[2–5] Metal chelation of the Schiff base[4] may or may not be necessary for the enzymatic catalysis. We have been interested in determining

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[1] Supported in part by a Grant (C-2645) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

[2] E. Koch and W. Werle, *Biochem. Z.*, **319**, 305 (1949).

[3] J. Baddiley, *Nature*, **170**, 711 (1952).

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

[5] W. T. Jenkins and I. W. Sizer, *ibid.*, **79**, 2655 (1957).

whether tissue extracts can accelerate the exchange of the components of metal-chelated pyridoxylidene amino acids as evidence for participation by the metal.

A short but measurable interval was found to be needed for the iron of Fe(III)(PyrVal)$_2$[6] to exchange with the Fe^{59}(III) of the similar ornithine chelate in water solution.[7] In contrast the Mn(II) and Cu(II) pyridoxylidenevaline chelates exchanged their metals with pools of radioactive metal too fast for ready study. Paradoxically a slower reaction of CuPyrVal with excess free valine has been observed, which is believed to take the course

$$\text{CuPyrVal} + 2\text{Val}^- \rightleftarrows \text{CuVal}_2 + \text{PyrVal}^=$$

$$\text{PyrVal}^= \rightleftarrows \text{Pyr}^- + \text{Val}^-$$

The breakdown of CuPyrVal and of PyrVal and the formation of Pyr can be followed readily spectrophotometrically. We have explored this reaction more closely because of its possible enzymologic usefulness.

Stability of CuPyrVal in Water.—CuPyrVal proved to be remarkably stable in water solution. The molar absorbancy at various wave lengths failed to change upon dilution from 0.005 to 0.00001 *M* in a way which would indicate dissociation (Fig. 1). In agreement the molar absorbancies at 0.00005 *M* were changed only slightly and in a way not consistent with reversal of dissociation when 20 to 800 molar portions of CuVal$_2$ were added.

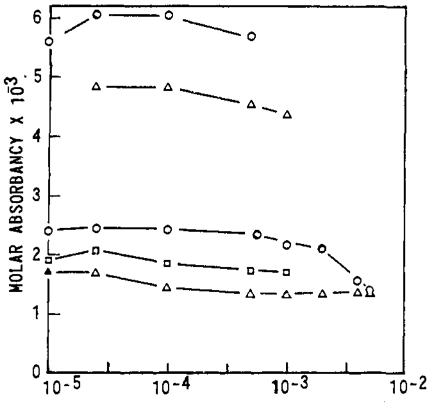

Fig. 1.—Change of molar absorbancy of an aqueous solution of CuPyrVal upon dilution. Upper curve, marked with circles, at 280 mμ; second curve marked with triangles, at 370 mμ; third curve, marked with circles, 415 mμ; fourth curve, marked with squares, 302 mμ; lowest curve, marked with triangles, 317 mμ. A release of PyrVal would increase the density at 415 mμ; a release of pyridoxal, at 317 mμ. Loss of CuPyrVal would cause loss of density at 280 and 370 mμ.

Rate of Reaction with Valine.—When a 0.0001 *M* solution of CuPyrVal was made 0.1 *M* in valine, the density at 370 mμ fell (CuPyrVal having a $\lambda_{\max}$ at about 380 mμ) whereas the densities at 415 and 317 (or 302) mμ rose according to the quantities of PyrVal and Pyr released. The reaction was complete in about 2 minutes at *p*H 8.5, the principal product being the yellow PyrVal. At *p*H 7.6 the CuPyrVal still broke down fairly rapidly but this was followed by the much slower breakdown of the formed PyrVal (Fig. 2).

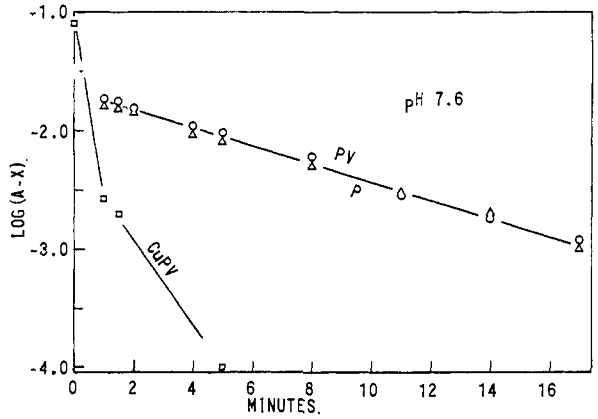

Fig. 2.—Rate of loss of CuPyrVal and PyrVal and of Pyr formation upon addition of valine to CuPyrVal at *p*H 7.6. *A* is the concentration of the substance at time *t*, *X* the concentration at equilibrium; temperature 25 ± 1°; Γ/2 = 0.050 (KHCO$_3$) plus that contributed by 0.100 *M* valine; atmosphere 5% CO$_2$, 95% N$_2$. The squares mark the loss of CuPyrVal, the triangles the loss of bound forms of Pyr and the circles the loss of PyrVal. Concentrations are in millimoles per liter.

When the *p*H was lowered to *p*H 4.5, these rate relationships were reversed: the CuPyrVal had a half-life of 16.5 minutes, whereas the intermediate PyrVal decomposed very rapidly (Fig. 3) to reach a low equilibrium level. The latter equilibrium has been described carefully by Metzler.[8] An intermediate *p*H could be selected (Fig. 4) at which CuPyrVal and the formed PyrVal broke down at very similar rates.

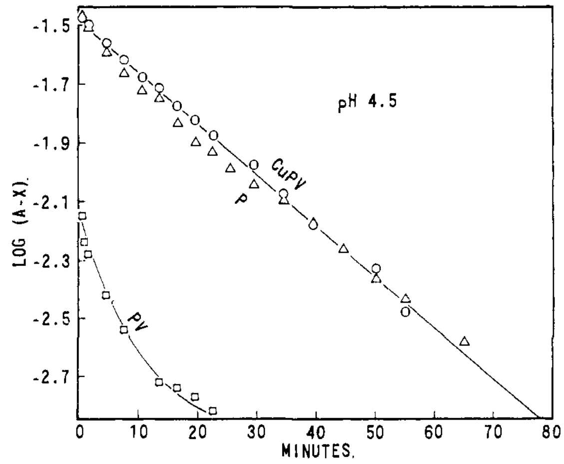

Fig. 3.—Rate of loss of CuPyrVal and PyrVal and of Pyr formation at *p*H 4.5. Conditions as in Fig. 2, except an acetate buffer, Γ/2 = 0.050, replaced the bicarbonate–carbonic acid buffer. Circles denote CuPyrVal loss; squares, PyrVal loss, and triangles the loss of bound forms of Pyr. Concentrations are in millimoles per liter.

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(6) Abbreviations: Pyr = pyridoxal; Val = L-valine; PyrVal = pyridoxylidenevaline; CuPyrVal = its copper chelate.

(7) H. N. Christensen, This Journal, **79**, 4073 (1957).

(8) D. E. Metzler, *ibid.*, **79**, 485 (1957).

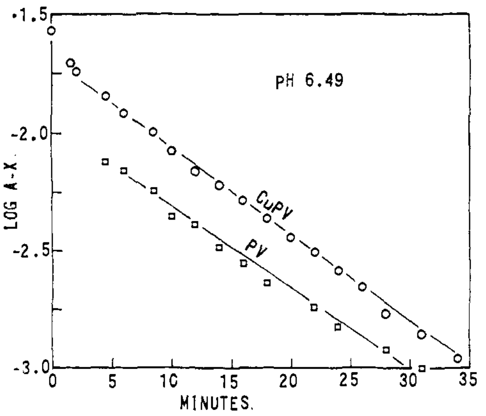

Fig. 4.—Rate of loss of CuPyrVal and PyrVal at *p*H 6.49. Conditions as in Fig. 2, except that the atmosphere was 100% CO_2, saturated with water vapor. Circles designate CuPyrVal loss; squares PyrVal loss. The Pyr gain steadily equalled the sum of the losses of CuPyrVal and PyrVal. Concentrations are in millimoles per liter.

Equilibrium State.—CuPyrVal solutions, 0.0005, 0.0002 and 0.0001 *M* in 0.05 *M* $KHCO_3$-H_2CO_3 buffer, *p*H 7.70 containing 0.12 *M* $KClO_4$, were made 0.05 and 0.02 *M* in L-valine. After 1.5 hr. the quantity $[CuVal_2][Pyr]/[CuPyrVal][Val]$ was 0.0032 (S.D. = 0.0003), the quantity $[CuVal_2]\cdot[PyrVal]/[CuPyrVal][Val]^2$ was 0.055 (S.D. = 0.005). These quantities were also fairly constant (although different) at *p*H 5.00. Each bracketed concentration includes all ionic species of the compound.

At *p*H 7.70 a valine to pyridoxylidenevaline ratio of about 1000 to 1 caused the Cu to be equally divided between CuPyrVal and $CuVal_2$, showing the far greater stability of CuPyrVal. A stability constant ($K_1 \times K_2$) for $CuVal_2$ of $10^{14.45}$ has been reported.[9] Using this value a constant $(CuPyrVal)/(Cu^{++})(PyrVal^{=})$ of $10^{14.4}$ can be calculated for $PyrVal^{=} + Cu^{++} \rightleftarrows CuPyrVal$ from the present results.

The extent of reaction of CuPyrVal and Val was obviously a complex function of the *p*H. In spite of the instability of PyrVal at lower *p*H values, the extent of CuPyrVal breakdown decreased with the *p*H throughout the range 8.5 to 4.5 showing the dominant influence of the valine anion concentration. Solutions of 0.0002 *M* CuPyrVal in 0.1 *M* valine were made up to $\Gamma/2 = 0.10$ with $KClO_4$ and small amounts of KOH or $HClO_4$ to produce various *p*H values. After 3 hr. CuPyrVal, PyrVal and Pyr were determined spectrophotometrically and $CuVal_2$ calculated from the loss of CuPyrVal.

The concentration relationships among these substances are shown in Fig. 5 in the form of logarithms of $K_{1pH} = [CuVal_2][PyrVal]/[CuPyrVal]\cdot[Val]^2$ and $K_{2pH} = [CuVal_2][Pyr]/[CuPyrVal]\cdot[Val]$, all ionic species of each substance being included. Assuming that the reaction constants for the following reactions are stable

$$CuPyrVal + 2Val^- \rightleftarrows CuVal_2 + PyrVal^= \quad (1)$$

$$CuPyrVal + Val^- \rightleftarrows CuVal_2 + Pyr^- \quad (2)$$

the solid lines in Fig. 5 show the way in which the log K_{pH} values should change with *p*H, taking into account the anticipated depletion of reactants by the following reactions: Association by CuPyrVal of a proton, $pK' = 5.6$[7]; association by Val^- of a proton $pK' = 9.72$; association by $PyrVal^=$ of one or two protons, $pK' = 10.49$ and 5.88[8]; and association by Pyr^- of one or two protons, pK''s = 8.66 and 4.20.[10] $CuVal^+$ was considered a minor component. Therefore, following the lead of Metzler[8]

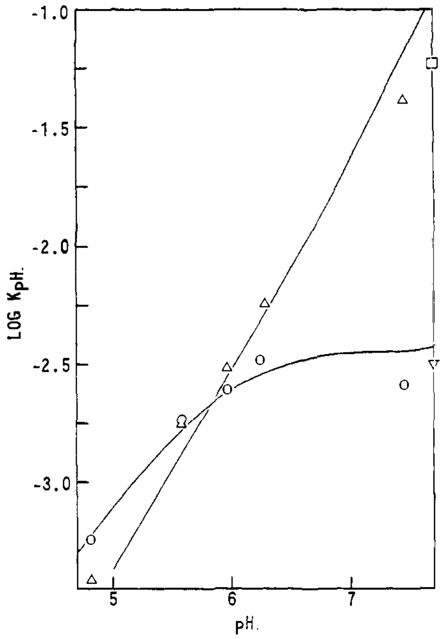

Fig. 5.—Theoretical changes of the values of log K_{pH} with *p*H for two equilibria. The straighter line shows how log K_{1pH} theoretically should change with *p*H where K_{1pH} is $[CuVal_2][PyrVal]/[CuPyrVal][Val]$.[3] The triangles Δ show how log K_{1pH} actually changed with *p*H at $\Gamma/2 = 0.10$. The square represents the average of 6 other determinations at $\Gamma/2 = 0.17$. The more curved line shows how log K_{2pH} theoretically should change with *p*H, where $K_{2pH} = [CuVal_2][Pyr]/(CuPyrVal][Val]$. The circles indicate the actual changes of log K_{2pH} with *p*H at $\Gamma/2 = 0.10$. ∇, the average of 6 determinations of log K_{2pH} at $\Gamma/2 = 0.17$.

$$\log K_{1\,pH} = \log K_1 - 2\log[1 + \text{antilog}(9.72 - pH)] - \log[1 + \text{antilog}(5.6 - pH)] + \log[1 + \text{antilog}(10.49 - pH) + \text{antilog}(16.37 - 2\,pH)]$$

and

$$\log K_{2\,pH} = \log K_2 - \log[1 + \text{antilog}(9.72 - pH)] - \log[1 + \text{antilog}(5.6 - pH)] + \log[1 + \text{antilog}(8.66 - pH) + \text{antilog}(12.86 - 2\,pH)]$$

The values for log K_{pH} are seen to change between *p*H 4.8 and 7.5 approximately as predicted. The results do not prove, however, that both degradative reactions occur, because the equilibrium relationships in the reaction $Pyr^- + Val^- \rightleftarrows PyrVal^=$ are also fixed; these we found to correspond to Metzler's plot of the corresponding log K_{pH} *versus* *p*H.[8] Addition of this reaction to reaction 2 yields reaction 1. Hence either of the two degradations could take place and its products

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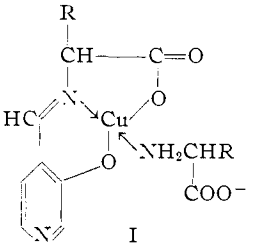
[9] L. E. Maley and D. P. Mellor, *Nature*, **165**, 453 (1950).

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

could then react to produce the remaining substances.

The kinetic studies show that the reaction CuPyrVal + $Val^- \rightarrow CuVal_2 + Pyr^-$ if it occurs at all is comparatively slow. That is, over the *p*H range studied the quantity [PyrVal]/[Pyr][Val] always ran far ahead of the equilibrium value for that *p*H, until the late stages of the reaction. Therefore the principal source of Pyr must have been PyrVal rather than the reverse being true. This does not mean that the reverse reaction, $Pyr^- + CuVal_2 \rightarrow Val^- +$ CuPyrVal, did not participate. In experiments at about *p*H 7.5 CuPyrVal was observed to break down only very slightly after the first few minutes despite the continuing loss of PyrVal; this suggests that the products of PyrVal cleavage were nearly as effective as PyrVal in stabilizing CuPyrVal.

The principal reaction is therefore considered to be a displacement of the Schiff base from its combination with the cupric ion by valine. The valine may well first displace water from the fourth coordinate position on the copper atom, to yield I. A mixed Schiff base II in which one valine of $CuVal_2$ still carries a pyridoxylidene group is a plausible second intermediate; probably neither of these hypothetical substances would be distinguished from CuPyrVal by the method used. The Pyr added as CuPyrVal was recovered within 4% as CuPyrVal plus PyrVal plus Pyr. Presumably the loss of pyridoxal from the mixed chelate is then so slow that the entire Schiff base is more rapidly displaced from the chelate by a second valine.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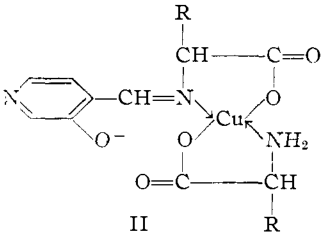
II

Addition of CuPyrVal to tissue extracts, and to serum albumin, showed the presence of components more active than valine in destabilizing the chelate. Another communication deals with reactions of peptides and proteins with pyridoxal and pyridoxal phosphate.[11]

Acknowledgment.—The valuable assistance of Miss Jane D. Le Fever is gratefully acknowledged.

Experimental

CuPyrVal was prepared as described previously[7] and recrystallized from alcohol. The crystals, dried over P_2O_5 at 56°, were weighed freshly, and the buffered solution let stand for an hour before use. The L-valine was from a familiar lot from the Nutritional Biochemicals Company; it was dissolved freshly to 0.5 *M* concentration and $^1/_4$ volume of this added to the CuPyrVal solution. A Beckman model DU spectrophotometer and a Beckman Laboratory Model *p*H meter were used, the temperature being maintained at 25 ± 1°. During the early rapid stages of reaction each of 3 wave lengths was followed in separate runs, but after a few minutes readings could be made on all 3 wave lengths in repeating sequence. "Infinite time" readings were taken when no further change could be observed in 10 minutes, but within 2 hr. of the beginning, further slow changes being attributed to other reactions.

For the kinetic studies acetate buffer was used to secure *p*H values from 4.5 to 5.5 and bicarbonate–carbonic acid buffers from *p*H 6.5 to 8.0. Appropriate CO_2 pressures were obtained with 100% CO_2, 5% CO_2–95% N_2, or air from out-of-doors, in each case first passed through a column of water. The ionic strength was 0.050 plus that contributed by 0.100 *M* valine.

To estimate the equilibrium concentrations at various *p*H values under conditions as constant as possible buffers were omitted and the desired *p*H values approximated by adding small amounts of KOH or $HClO_4$, then using $KClO_4$ to bring the ionic strength to 0.100, plus the ionic strength contributed by 0.1 *M* zwitterionic valine. The temperature was maintained at 25.0 to 25.5°. Values read about 3 hr. later were used for the calculations. Parallel lots of CuPyrVal with a similar *p*H spread were incubated in the absence of valine to provide values at each *p*H for the relative densities at 415, 370 and 317 (or 302) mμ. The densities at 317 and 370 change with *p*H around a pK' of about 5.6, as recorded previously.[7] For the molar absorbancies of Pyr and PyrVal the figures of Metzler[8,10] were used.

First simultaneous equations were used to estimate the CuPyrVal and PyrVal from the optical densities at 370 and 415 mμ. The densities which the resulting amounts of these 2 substances contribute at 317 (or 302) mμ were then deducted from the found densities and the Pyr concentration calculated from the residual density[10] in each case. The densities at 370 and 415 mμ were then corrected for the presence of this amount of pyridoxal. New values of CuPyrVal and PyrVal were then calculated from the corrected densities at 370 and 415 mμ. The $CuVal_2$ formed was taken to equal the loss of CuPyrVal.

In calculating the constant for $Cu^{++} + PyrVal^{=} \rightleftarrows$ CuPyrVal, $[Cu^{++}]$ was calculated from the equation[9] $[CuVal_2]/[Cu^{++}][Val^-]^2 = 10^{14.45}$. $[Val^-]$ and $[PyrVal^=]$ were evaluated using the pK' values cited earlier.

ANN ARBOR, MICHIGAN

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[11] H. N. Christensen, THIS JOURNAL, **80**, 99 (1958).