On the Mechanism of Metabolic Reactions of Amino Acids Catalyzed by Vitamin B_6 and Related Aldehydes

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Z. Naturforsch. **49c**, 571–578 (1994); received April 25, 1994

[(N-salicylidene-D,L-glutamato)(pyridine)]copper(II), Crystal Structure, INDO/2 Charges, Metabolic Reactions of Amino Acids, Vitamin B_6 Catalysis, Reaction Mechanism

The crystal structure of [(N-salicylidene-D,L-glutamato)(pyridine)]copper(II), a model for vitamin B_6-amino acid-related metal complexes, has been determined by an X-ray analysis. A close examination of the structural data on this and other related complexes combined with quantum-chemical (INDO/2) calculations enabled us to make a clear distinction between two mechanisms proposed earlier for metabolic reactions of amino acids catalyzed by the vitamin B_6 (or salicylaldehyde)-metal system. The results are consistent with a transient formation of a carbinolamine species resulting from the addition of a solvent water or alcohol molecule to the Schiff base double bond, thus supporting the mechanism of the catalysis as proposed by Gillard and Wootton.

Introduction

Metabolic reactions of amino acids are catalyzed by metaloenzymes which require pyridoxal (vitamin B_6) (formula **1,** Fig. 1) or its phosphate as a cofactor (Guirard and Snell, 1964). The common feature of these reactions is a heterolytic cleavage of one of the three bonds to the α-carbon atom of the amino acid. Of particular biochemical importance are transamination, racemization and α,β-elimination reactions which are dependent on cleavage of the $C^{\alpha}-H$ bond.

The first intermediate of the catalytic reaction is a Schiff base between pyridoxal and an amino acid which subsequently coordinates (as a tridentate ligand) to the metal ion of the enzyme to form chelate **2** (these steps may proceed in a concerted manner or even be reversed). In the next step a proton is released from the α-carbon of the amino acid moiety to form a planar, highly reactive carbanion. Then the fate of the carbanion depends on its electronic structure which can be described by canonical formulas **3a–c.** Structure **3a** predominates at pH higher than pK_a of the pyridine nitrogen when the carbanion is unprotonated so that subsequent reprotonization on the α-carbon leads to racemization of the amino acid. On the other hand, transamination, which requires protonation on the azomethine carbon, is the major reaction in the system in an acidic environment; structure **3b,** a predominant form of the protonated pyridoxal carbanion, is therefore an intermediate of the transamination reaction.

Most of these enzymatic reactions have been duplicated by non-enzymatic model reactions (Longenecker *et al.*, 1957; Holm, 1973) in which pyridoxal (or an other appropriate aldehyde) and a suitable metal ion serve as catalysts. As the phenolic and formyl groups are sufficient structural requirement for catalytic activity of the cofactor (the presence of the heterocyclic nitrogen enhances the rate of the catalytic reaction and the hydroxymethyl group functions to link the cofactor to the enzyme), pyridoxal may be replaced by salicylaldehyde so that the system salicylaldehyde–amino acid–metal (**4,** Fig. 2) represents a relatively simple model to study enzymatic reactions of amino acids. Catalytic reactions in the model system **4** also proceed through intermediate

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0939–5075/94/0900–0571 $ 06.00

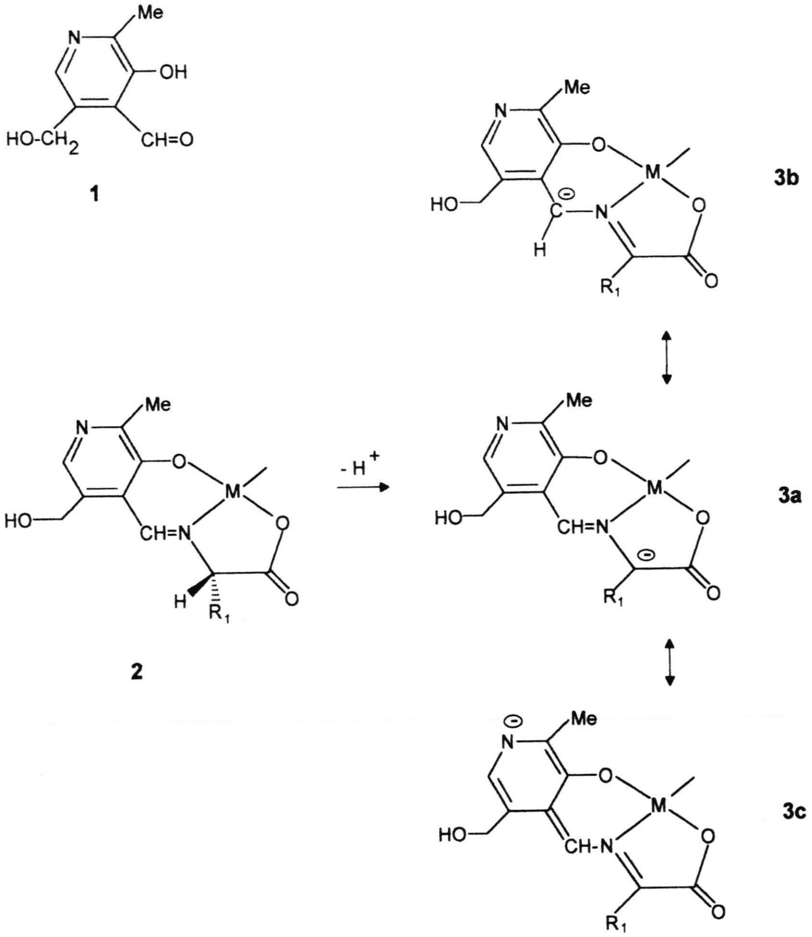
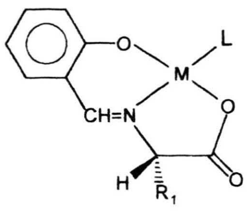


Fig. 1. General structures of the compounds derived from pyridoxal.

formation of the carbanion **5** with the only exception that the transamination reactions are not observed. This is most likely caused by the fact that the carbanion **5** cannot exist, due to the lack of the ring nitrogen, in the form analogous to **3b** (or **3c**).

The key question is: What is the mechanism of the rate determining step of the catalytic reaction, *i.e.* formation of the carbanion from chelate **2** or **4.** There have been a number of reports dealing with this question. The first attempt to rationalize the literature data on these systems was made by Metzler *et al.* (1954), who proposed a mechanism (hereafter mechanism A) according to which the labilization of the C^{α}–H bond was attributed to the direct displacement of an electron pair from the C^{α}–H bond to the α-C atom, a displacement which is facilitated by the presence of the planar conjugated system and further intensified by the electron-accepting capability of the metal ion. This idea was then modified by Perault *et al.* (1961) in the sense that the driving force for the release of the α-proton was an increase in the delocalization energy of the system, since after formation of the carbanion the α-C atom became a part of the conjugated system.

Several years later, Gillard and Wootton (1970) have found that: a) in nucleophilic solvents the rate of the racemization reaction of amino acid esters **6** is by several orders of magnitude higher relative to the corresponding free acids, b) by using deuterioethanol exchange of hydrogen for deuterium takes place at position C^{α}, and c) the rate of the reaction can be enhanced by addition of a base. Based on these facts the authors concluded that in nucleophilic solvents (water, alcohols) the nucleophile can add accross the azo-



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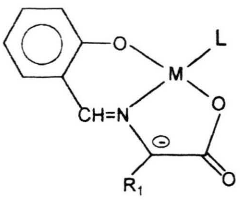

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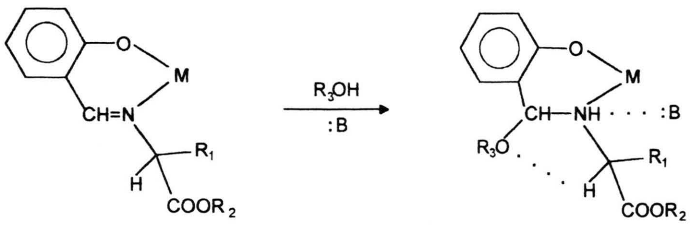

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Fig. 2. General structures of the compounds derived from salicylaldehyde. :B represents any base present in solution.

methine double bond to form a reactive carbinolamine **7** and subsequent base-catalyzed elimination led to formation of the carbanion. Gillard and Wootton advanced a hypothesis that in system **4** a similar addition-elimination mechanism (mechanism B) may also be involved in formation of the carbanions, the higher rate of racemization in chelate **6** (compared to that in **4**) being explainable in the following way: in the carbinolamine **7** free rotation around the $N-C^{\alpha}$ bond can bring the α-proton into a position which favours its removal by the leaving alkoxy group during the elimination step; on the other hand, in the carbinolamine which would result from **4** coordination of the carboxy group fixes the conformation of the groups on the chelate ring so that the α-proton is in a less favourable position for removal by the leaving OR_3 group.

Although the experimental data available in the literature seem to be consistent with mechanism B, there are still two groups of workers which incline to either of the above mechanisms (see, *e.g.*, Bkouche-Waksman *et al.*, 1988). Consequently, the aim of this work was to test which of the two mechanisms fits reality. To achieve this, we have adopted a simple strategy based on the fact that upon chelation of the Schiff base the rate of racemization is enhanced by several orders of magnitude; for divalent cations the reaction rate in system **4** (or **6**) increases in the following order: $Zn^{2+} < Co^{2+} < Pd^{2+} < Ni^{2+} < Cu^{2+}$ (O'Connor *et al.*, 1968; Nunez and Eichhorn, 1962). It is also well documented that the catalytic effect of the metal ion is observed just after (and not before) the formation of the Schiff base (Nunez and Eichhorn, 1962; Martell, 1982).

It is obvious that in the case of mechanism A the system (Schiff base) shifts, due to metal activation, along the reaction coordinate towards the final state **5**, *i.e.* the complex should be closer to the species **5** than the free, uncoordinated Schiff base. This should be reflected in redistribution of the charge density (enhanced electron density on the α-carbon accompanied by an adequate lowering of this density on the α-hydrogen) and in the geometry around the α-C atom (change of the bond angles due to rehybridization from sp^3 to sp^2, a tendency of the $C^{\alpha}-H$ bond to become perpendicular to the salicylaldimine plane coupled with a planarization of the $C^{\alpha}-C^{\beta}$ bond).

On the other hand, according to mechanism B the Schiff base is in the first (rate determining) step activated by the metal ion towards nucleophilic attack by R_3OH so that, in agreement with the theory of charge controlled Ad_N2 reactions (Fleming, 1976), one would expect an enhancement of the positive charge on the azomethine carbon upon introduction of the metal ion.

From the above it is clear that in order to study the mechanism of the catalytic (*e.g.* racemization) reaction it is necessary to monitor changes in geometry and distribution of the electron density associated with the transfer of the Schiff base into the chelate. In this work, single-crystal X-ray diffraction and quantum-chemical calculations were chosen for this purpose. The crystal structure of one derivative belonging to class **4** was investigated in this paper and the remaining were retrieved from the Cambridge Structural Database (Allen *et al.*, 1979). The salicylaldehyde chelates were chosen over pyridoxal chelates since the former more readily provide good crystals, and Cu^{II} was chosen over other metals because of the high reaction rate for copper-catalyzed reactions (Nunez and Eichhorn, 1962; Martell, 1982).

Experimental

Selection of the compounds

All compounds selected for this study are listed in Table II. They differ in the nature of the amino acid and of the additional ligand L. The glycinato chelates were not included in the analysis because of uncertainty which of the two C^{α}–H bonds is actually cleaved in the catalytic reaction. Since, to our knowledge, no Schiff base of salicylaldehyde and an amino acid has been studied crystallographically, we have chosen for this purpose the known structure of chloro-triphenyl-(O-ethyl-N-salicylideneglycinato) Sn^{IV} (Lee *et al.*, 1990) in which the O-ethyl-N-salicylideneglycinato ligand is monodentately coordinated *via* the phenolate oxygen to the tin atom, *i.e.* it contains an uncoordinated azomethine nitrogen and hence may be regarded, to a good approximation, as the metal-free Schiff base.

X-ray structure of **4e**

The complex [Cu(sal–glu)(pyridine)] (**4e**) (sal–glu = N-salicylideneglutamate) was prepared by allowing of [Cu(sal–L-glu)$(H_2O)_2$]. H_2O to react with an equimolar amount of pyridine in a stirring ethanolic solution at 60–65 °C for 0.5 h. After cooling to room temperature the precipitate was collected by filtration and recrystallized from ethanol to give well-developed dark-green crystals.

Analysis for $C_{17}H_{16}N_2O_5Cu$ (394.89)

Calcd	C 51.71	H 4.08	N 7.09,
Found	C 51.52	H 4.12	N 6.99.

A crystal of size 0.3×0.2×0.2 mm was selected for the structure analysis. The unit cell parameters were refined by a least-squares fit of positional angles of 15 reflections with $5 < 2\theta < 26°$. The intensities were measured on a Syntex $P2_1$ diffractometer using graphite-monochromatized CuKα radiation and the θ–2θ scanning technique in the range $0 < 2\theta < 100°$. The scan speed in the interval 4.88–29.3° min^{-1} was controlled automatically, with each reflection scanned 1° (in 2θ) above and below the Kα doublet. The background was measured at each end of the scan for one half of the scan time. Two standard reflections monitored after every 98 scans showed that no correction for instrument instability or crystal decay was required. Of the 1696 unique reflections recorded, 1410 with $I > 1.96\sigma(I)$ were classified as observed and used for the structure analysis.

Crystal data: Formula weight M_r = 394.9, monoclinic, space group $P2_1/n$, a = 8.128(2), b = 10.649(3), c = 19.113(5) Å, β = 95.47(2)°, V = 1646.7(7) Å^3, Z = 4, μ = 2.19 mm^{-1}, ϱ_x = 1.59 g·cm^{-3}, F(000) = 804, MoKα, λ = 0.71069 Å, room temperature.

The structure was solved by Patterson and Fourier methods and refined on F by block-diagonal least-squares with anisotropic thermal parameters assigned to all non-hydrogen atoms. Positions of the H atoms H1–H9, which were found on a difference Fourier map, were refined while the remaining (H10–H16) were fixed at calculated positions; thermal parameters of all the H atoms were set to 0.5 Å^2 higher than B_{eq} of the associated C or O atoms. The function $\Sigma w(|F_o| - |F_c|)^2$ was minimized by using unit weights (w = 1) for all the observed reflections. Final residuals were R = 0.062, wR = 0.059, S = 1.22. In the final cycle $(\Delta/\sigma)_{max}$ = 0.043, $(\Delta\varrho)_{max}$ = 0.30, $(\Delta\varrho)_{min}$ = −0.45 e·Å^{-3}. The scattering factors for the neutral atoms were taken from International Tables, 1974. All crystallographic calculations were carried out with the XRC83 program package (Pavelčík *et al.*, 1985). Preliminary results of the crystal structure of **4e** have been reported previously (Krätsmár-Šmogrovič *et al.*, 1985).

MO calculations

Electronic structures in terms of total atomic charges, Wiberg indices and orbital energies were obtained from a local version of the INDO/2 program (Boča, 1989) by using a fixed geometry – constructed on the basis of known X-ray structures – for all six chelates studied. Initial geometries of the metal-free Schiff bases were derived from the X-ray structure of the corresponding chelate parents by removal of the copper atom and completing the freed valencies on the phenolate and carboxylate oxygens by H atoms. Subsequent optimizations using AM1 (Dewar *et al.*, 1985) have revealed only slight torsional rearrangement indicating that the planar conformation of the Schiff base ligand observed in the chelate is also a low-energy conformation for the ligand itself.

Results and Discussion

Final atomic coordinates for **4e** are given in Table I. Relevant structural parameters and the total atomic charges in the catalytically interesting part of the chelates studied are compared in Tables II and III, respectively. As the atomic charges calculated for the six metal-free Schiff bases were almost identical, only the mean values are given in Table III.

Table I. Final atomic coordinates ($\times 10^4$) and equivalent isotropic thermal parameters B_{eq} (Å^2) with E.S.D.'s in parentheses*. $B_{eq} = 4/3 \sum_i \sum_j B_{ij} \vec{a}_i, \vec{a}_j$.

Atom	*x*	*y*	*z*	B_{eq} [Å^2]
CO(1)	1450(2)	1301(1)	4547(1)	6.52(4)
O(1)	2117(7)	61(5)	5238(3)	5.57(18)
O(2)	986(7)	2585(5)	3813(3)	5.85(17)
O(3)	1122(7)	2983(6)	2683(3)	6.51(19)
O(4)	–3793(7)	–412(6)	2671(3)	7.18(21)
O(5)	–1977(8)	–1393(8)	2100(4)	9.82(28)
N(1)	512(8)	2430(6)	5251(3)	5.07(20)
N(2)	2223(8)	358(6)	3781(3)	5.41(21)
C(1)	–617(11)	3315(8)	5036(5)	5.92(31)
C(2)	–1231(11)	4117(8)	5523(6)	6.77(35)
C(3)	–745(13)	4017(9)	6219(5)	7.38(33)
C(4)	399(13)	3106(9)	6435(5)	7.13(35)
C(5)	1001(11)	2333(8)	5941(5)	5.97(29)
C(6)	1299(10)	2303(8)	3201(4)	5.63(27)
C(7)	1930(11)	949(8)	3082(4)	5.82(28)
C(8)	668(11)	233(8)	2591(4)	5.66(27)
C(9)	–1079(11)	346(9)	2843(5)	6.31(30)
C(10)	–2300(12)	–581(9)	2477(5)	6.50(30)
C(11)	3102(10)	–876(8)	5146(4)	5.23(25)
C(12)	3604(10)	–1263(8)	4496(4)	5.15(25)
C(13)	4672(11)	–2305(8)	4456(5)	6.06(26)
C(14)	5227(11)	–2968(9)	5049(6)	6.70(32)
C(15)	4732(11)	–2611(9)	5692(5)	6.55(31)
C(16)	3694(11)	–1600(8)	5756(4)	5.81(28)
C(17)	3108(11)	–630(8)	3847(4)	5.58(26)

* Anisotropic thermal parameters, H-atom coordinates, F_o/F_c tables, and complete lists of geometrical parameters are available at the authors.

The overall geometry of **4e** (Fig. 3) shows the usual features of this type of complex, perhaps with the exception that the coordination around the Cu atom is exactly square-planar. This is sub-

Table II. List of compounds[a] studied here and their relevant geometric parameters[b].

Compound	$N-C^{\alpha}-C$	$N-C^{\alpha}-C^{\beta}$	$C-C^{\alpha}-C^{\beta}$	C=N	$C{=}N-C^{\alpha}-C^{\beta}$	$C{=}N-C^{\alpha}-H$	Ref.
4a	107.4	109.5	109.1	1.270	83.3	–39.8[c]	18
4b	108.5	112.3	110.2	1.275	70.6	–46.6[c]	19
4c	107.3	112.7	108.3	1.286	73.7	–48.8	20
4d	107.5	111.7	109.1	1.285	72.8	–47.7[c]	21
4e	109.6	112.6	110.0	1.267	75.9	–50.3	this work
4f	105.3	108.3	114.8	1.288	74.0	–41.8	22
usb[d]	111.8			1.303			13

[a] The compounds are designated as follows (see also Fig. 2): **4a:** $R_1 = CH_3$, L = pyrazole; **4b:** $R_1 = CH_3$, L = NCO; **4c:** $R_1 = CH_2Ph$, L = NCO; **4d:** $R_1 = (CH_2)_2COOH$, L = H_2O; **4e:** $R_1 = (CH_2)_2COOH$, L = pyridine; **4f:** $R_1 = CH(CH_3)_2$, L = H_2O.

[b] E.S.D.'s for the bond angles range from 0.2 to 0.6°, for the C=N bond length from 0.003 to 0.011 Å, and for the torsion angles from 0.3 to 0.7° ($C{=}N-C^{\alpha}-C^{\beta}$) and from 0.6 to 1.0° ($C{=}N-C^{\alpha}-H$).

[c] Position of the hydrogen calculated, *i.e.* not actually found in the difference Fourier synthesis.

[d] Uncoordinated Schiff base (see text).

Table III. INDO/2 net atomic charges.

Compound[a]	q_C	q_N	$q_{\alpha-C}$	$q_{\alpha-H}$
4a	0.26	−0.02	0.05	−0.01
4b	0.25	−0.01	0.06	−0.02
4c	0.25	−0.03	0.07	−0.02
4d	0.26	−0.05	0.05	−0.01
4e	0.26	−0.05	0.03	−0.02
4f	0.26	−0.02	0.06	−0.01
usb[b]	0.20	−0.22	0.11	−0.02

[a] For designation of the compounds see the footnote of Table II.
[b] Atomic charges for the uncoordinated Schiff base (usb) are means of six values (see text).

stantiated by the fact that no atom occurs at a distance shorter than 3.3 Å in the axial direction and by the coplanarity of Cu with the four donor atoms. The protonated side chain carboxyl group is not involved in the coordination to the copper atom, but it forms a strong hydrogen bond with the carbonyl oxygen O3 [O···O = 2.593(5) Å] of another molecule at (−0.5 − *x*, −0.5 + *y*, 0.5 − *z*), thus forming chains going along the diagonal of the unit cell.

As the space group ($P2_1/n$) is centrosymmetrical, the crystal structure of **4e** is a racemic mixture of [Cu(sal−D,L-glu)(pyridine)] even though an optically active parent complex [Cu(sal−L-glu)$(H_2O)_2$] was used in the reaction with pyridine (see above). This again demonstrates that the racemization of the Schiff base ligand readily occurs under mild conditions even in neutral or weak acidic aqueous or alcoholic solutions (Krätsmár-Šmogrovič *et al.*, 1991).

As can be seen in Table II, bond angles at the α-C atom for the six chelates studied range from 105.3 to 114.8° with the mean value of 109.7° corresponding exactly to the tetrahedral (sp^3) value. Moreover, the N−C^α−C angle in all six chelates is even smaller than that found in the uncoordinated Schiff base (111.8°). Similarly, as shown by the torsion angles presented in Table II, it is the C^α−C^β bond rather than the C^α−H bond which tends to be oriented perpendicularly to the π system of the molecule. Inspection of Table III further shows that although chelation of the Schiff base causes a lowering of the positive charge on the α-carbon, the lowering is not at the expense of reduced elec-

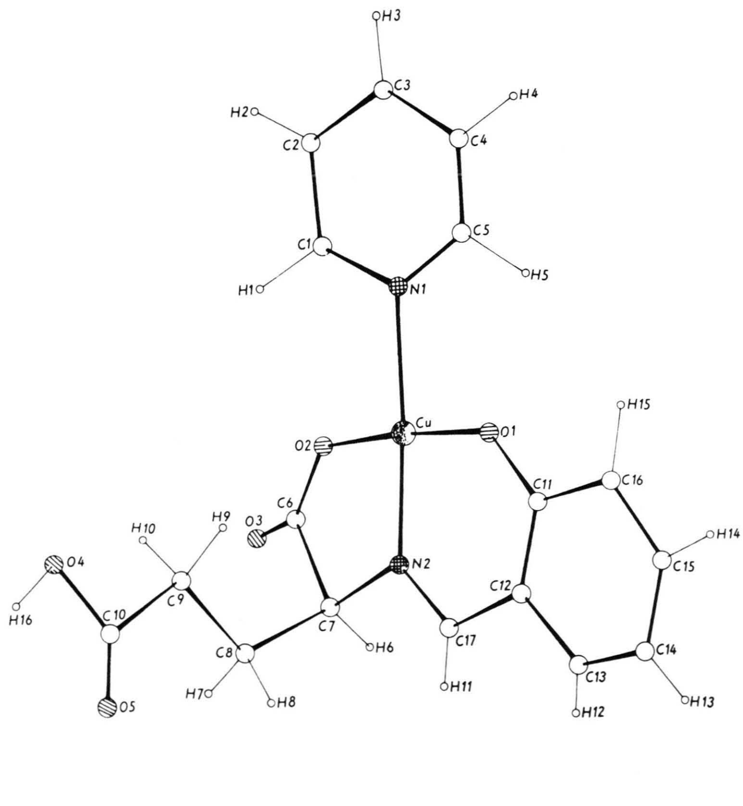

Fig. 3. A perspective drawing of the *S*-enantiomer of **4e** derived from the X-ray coordinates.

tron density on the α-hydrogen. However, as the semiempirical quantum-chemical methods of INDO type are generally known to underestimate the charge polarization, the results of the charge distribution should be taken with caution. Nevertheless, this drawback of the INDO methods is supposed to affect the C–H polarization in both the metal-free Schiff bases and the corresponding chelates in a similar manner.

From the above it is clear that our results are not consistent with reaction mechanism A, which predict a direct activation of the amino acid by the π system of the Schiff base and the metal ion. In contrast, according to mechanism B the catalytic reaction is initiated by nucleophilic attack of R_3OH on the azomethine carbon of the Schiff base. As shown in Table III, the characteristic feature of all the complexes studied is an 0.05 to 0.06 increase in the positive charge on this atom relative to the metal-free Schiff bases. Comparison of the charges on the azomethine nitrogen (Table III) shows that the enhanced positive charge on the azomethine carbon results from electron withdrawal by the copper atom. It is notable that the azomethine bond shortens from 1.303 to 1.267–1.288 Å upon copper introduction (Table II). This interval corresponds to the range 1.27–1.29 Å generally accepted for a pure C=N double bond (Burke-Laing and Laing, 1976). It should be emphasized in this context that INDO/2 was unable to reproduce the observed increase in the C=N bond order so that the metal-induced enhancement of the positive charge on the azomethine carbon is probably even higher than that indicated by INDO/2.

The above electron distribution coupled with the high double-bond character in the azomethine group is in line with enhanced susceptibility of this carbon toward nucleophilic attack by a solvent water or alcohol molecule (Fleming, 1976). That the addition of the nucleophile to the Schiff base double bond is indeed charge controlled was verified by comparison of the frontier orbital energies: the LUMO energy for the six chelates studied here was calculated by INDO/2 to range from 1.7 to 2.0 eV while the HOMO energy of water or alcohols is known to be < -10 eV (Fleming, 1976); this indicates that the HOMO/LUMO energy separation is large enough for the frontier orbital interactions to be neglected.

In conclusion, the results of this study strongly indicate that the catalytic effect of the copper ion resides in electron withdrawal from the azomethine linkage and in increase in the C=N bond polarization. Both these electronic effects enhance the electrophilicity of the imine carbon. This in turn implies that activation of the amino acid by the π system of the Schiff base, if any at all, is insufficient and instead it requires an active influence of the nucleophilic solvent as originally suggested by Gillard and Wootton (1970).

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