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Mechanism of Non-Enzymic Transamination Reaction between Histidine and α-Oxoglutaric Acid

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Non-enzymic transamination reactions at 85° between various amino acids and α-oxoglutaric acid are catalysed by metal ions, e.g. Al^{3+}, Fe^{2+}, Cu^{2+} and Fe^{3+}. The reaction is optimum at pH 4·0. Of the 14 amino acids studied histidine is the most active. In the presence of Al^{3+} histidine transaminates with α-oxoglutaric acid, forming glutamic acid and Al^{3+}–imidazolylpyruvic acid complex as the end products. However, in the presence of Fe^{2+} or Cu^{2+} the products are glutamic acid and a 1:2 metal ion–imidazolylpyruvic acid chelate. The greater effectiveness of histidine in these reactions is attributed to the presence of the tertiary imidazole nitrogen atom, which is involved in the formation of stable sparingly soluble metal ion–imidazolylpyruvic acid complexes or chelates as end products of these reactions. Of the metal ions studied only Al^{3+}, Fe^{2+}, Fe^{3+} and Cu^{2+} are effective catalysts for the transamination reactions, and EDTA addition completely inhibits the catalytic effect of the Al^{3+}. Spectrophotometric evidence is presented to demonstrate the presence of metal ion complexes of Schiff bases of histidine as intermediates in the transamination reactions. These results may contribute to understanding the role of histidine in enzyme catalysis.

Several important enzymic reactions of amino acids, e.g. transamination, decarboxylation, dehydration of serine and threonine, cysteine desulphydrase and homocysteine desulphydrase reactions and the cleavage of β-hydroxy amino acids to glycine and carbonyl compounds, are catalysed by pyridoxal phosphate-containing enzymes, and have been reviewed by Snell (1958) and by Braunstein (1960). Most of these enzymic reactions are reported to be duplicated by non-enzymic reactions in which pyridoxal and a suitable metal salt serve as catalysts. The proposed mechanism of the reaction involves the formation of Schiff-base chelates between pyridoxal and amino acids and between pyridoxamine and keto acids (Metzler, Ikawa & Snell, 1954; Eichhorn & Dawes, 1954; Blakley, 1955). Evidence for the presence of these Schiff-base metal chelates in the transamination reaction mixtures was obtained by Fasella, Lis, Siliprandi & Baglioni (1957).

Non-enzymic transamination reactions between different α-amino acids (Herbst & Engel, 1934; Giri & Kalyankar, 1953; Giri, Kalyankar & Vaidyanathan, 1954), between α-amino acids and α-oxo acids (Mix, 1961) and between glutamic acid and 4-formyl-3-hydroxypyridine (Thanassi, Butler & Bruice, 1965) have been reported. These reports have studied transamination reactions either in the absence of metal ion or in non-aqueous solutions. Metal ion stimulation of non-enzymic transamination reactions between amino acids and glyoxylate has been reported (Metzler, Olivard & Snell, 1954). However, reactions of keto acids (rather than the more reactive glyoxylate) with amino acids were not reported by these workers. Similarly Mix (1959, 1961), Mix & Wilcke (1960), Fleming & Crosbie (1960), Dixon & Moret (1965), Dixon (1967) and Hermann & Willhardt (1968) have used metal ions to catalyse transamination reactions in aqueous solutions with or without large amounts of pyridine added to the reaction mixture. A recent report (Doctor & Oró, 1967) indicated that in the presence of metal ions histidine was more effective than other amino acids in non-enzymic transamination reactions with various keto acids. The studies reported here were carried out to define more clearly the catalytic role of metal ions and histidine in these reactions.

MATERIALS AND METHODS

Various amino acids were compared for their ability to transaminate non-enzymically with α-oxoglutaric acid in the presence of Al^{3+}. The reaction mixture included

10μmoles of α-oxoglutaric acid, 12μmoles of Al^{3+} as $Al_2(SO_4)_3$ and 25μmoles of histidine or of other amino acid in a final volume of 1ml. The solutions were made in 50mM-sodium acetate buffer, pH4·0. The reactants were heated in a screw-cap Pyrex culture tube at 85° for 5hr. and the amounts of glutamic acid were subsequently measured by paper-chromatographic separation followed by densitometric analysis (Aspen & Meister, 1958). Glutamic acid reference standard was run simultaneously with each determination.

The effect of various metal ions in the transamination reaction between α-oxoglutaric acid and histidine was studied by including in the reaction mixture 10μmoles of Al^{3+} or other metal ion along with 10μmoles of α-oxoglutaric acid and 25μmoles of histidine. The possible role of EDTA (sodium salt) addition in reversing the catalytic effect of Al^{3+} was also investigated. The compounds were dissolved in 50mM-sodium acetate buffer, pH4·0, heated in a screw-cap Pyrex culture tube at 85° for 5hr. and tested for glutamic acid as described above. Determination of the optimum concentration of the metal ions required was evaluated by adding different amounts of metal ion (Al^{3+}, Fe^{2+} or Cu^{2+}) along with 10μmoles of α-oxoglutaric acid and 25μmoles of histidine. The amounts of glutamic acid formed were measured as described above.

The effect of pH on the transamination reaction between histidine and α-oxoglutaric acid in the presence of Al^{3+}, Cu^{2+} or Fe^{2+} was studied. Tris–HCl buffer (50mM) was used for pH6–8, sodium borate buffer (25mM) was used for pH9–11 and sodium acetate buffer (50mM) was used for pH3–5. The reaction mixture consisting of 25μmoles of histidine, 12μmoles of metal ion and 10μmoles of α-oxoglutaric acid was made up in buffers of different pH, heated for 5hr. and tested for glutamic acid as described for the above experiment. All measurements of the pH of the buffers were made at 20° after the addition of all the components.

The presence of glutamic acid in the reaction mixture was confirmed: (*a*) by t.l.c. (Peranino & Harper, 1961); (*b*) by comparing the retention time of glutamic acid standard with that of the glutamic acid present in the reaction mixture by using a Beckman–Spinco model 120 amino acid analyser. Imidazolylpyruvic acid (Calbiochem, Los Angeles, Calif., U.S.A.) alone or when formed from histidine during the transamination reaction combined with equimolar amounts of Al^{3+}, forming a complex that gave a strong maximum at 294nm. at pH4·0 ($\epsilon 12{\cdot}8\times10^3$ mole^{-1}cm.$^{-1}$) in contrast with maxima at 238 and 274nm. for imidazolylpyruvic acid. At pH1 this complex gave free imidazolylpyruvic acid and Al^{3+}. The former was converted into its 2,4-dinitrophenylhydrazone as described by Stumpf & Green (1952), and its properties, e.g. m.p. or C, H and N analysis and i.r. spectrum, were compared with those of the 2,4-dinitrophenylhydrazone prepared from imidazolylpyruvic acid. The Cu^{2+}–imidazolylpyruvic acid chelate and Fe^{2+}–imidazolylpyruvic acid chelate formed during the respective transamination reactions were sparingly soluble in water and therefore crystallized out of the aqueous solutions. The chelates were identified by comparing their i.r. spectra with those of similar chelates prepared by the interaction of imidazolylpyruvic acid with the respective metal ions. The solutions were made in 50mM-sodium acetate buffer, pH4·0. The Cu^{2+}–imidazolylpyruvic acid chelate was obtained by mixing equal volumes of two solutions in 1:2 molar ratio at 20°. Under these conditions the precipitate that formed was crystallized out of water. The Fe^{2+}–imidazolylpyruvic acid chelate was also obtained by the same procedure except that equal volumes of solutions of the metal ions and imidazolylpyruvic acid in 1:2 molar ratio were heated at 85° for 30min. to enhance the reaction.

Continuous-variation experiments (Eichhorn & Dawes, 1954) were conducted on solutions of the same total molar concentration of Cu^{2+} or Fe^{2+} with imidazolylpyruvic acid, but containing different ratios of the two. The solutions were made in 50mM-sodium acetate buffer, pH4·0. The formation of Cu^{2+}–imidazolylpyruvic acid chelate was studied with 15mM solutions of each, and that of Fe^{2+}–imidazolylpyruvic acid chelate was studied with 5mM solutions of each. The differences between the observed extinction and the extinction calculated on the assumption that no reaction occurs between the components (Vosburgh & Cooper, 1941) were plotted against the mole fraction of imidazolylpyruvic acid.

Spectrophotometric studies were conducted on standard solutions of Cu^{2+} (7·5mM), Fe^{2+} (7·5mM) and histidine (25mM), singly or in combination, in 50mM-sodium acetate buffer, pH4·0. The absorption spectrum was measured for each solution with a Bausch and Lomb Spectronic 505 recording spectrophotometer. All solutions in this experiment were freshly prepared and heated at 85° for various times. The spectra of the final reaction mixtures (after heating for 5hr. at 85°) were compared with freshly made mixtures of solutions of imidazolylpyruvic acid, Cu^{2+} or glutamic acid in 50mM-sodium acetate buffer, pH4. All spectra were recorded against 50mM-sodium acetate buffer as the reference.

RESULTS AND DISCUSSION

The results presented in Table 1 show that, in the presence at Al^{3+}, eight out of 14 amino acids transaminate non-enzymically with α-oxoglutaric acid and of these histidine was the most effective amino donor. Further studies carried out in the presence of Cu^{2+} or Fe^{2+} showed that here also the same eight amino acids transaminate with α-oxoglutaric acid, histidine again being the most effective amino donor.

The results of the transamination reactions at pH4·0 between α-oxoglutaric acid and histidine in the presence of various metal ions are presented in Table 2. Of the metal ions tested Al^{3+}, Cu^{2+}, Fe^{2+} and Fe^{3+} had stimulatory effects, with Al^{3+} showing the highest stimulation. The possibility that Fe^{2+} is converted into Fe^{3+} during the reaction cannot be excluded and therefore the results with Fe^{2+} and Fe^{3+} could be considered equivalent. EDTA addition in amounts equimolar to the Al^{3+} completely reversed the stimulatory effect of this metal ion.

The results of the addition of different concentrations of Al^{3+}, Cu^{2+} or Fe^{2+} on the transamination of histidine with α-oxoglutaric acid are presented in Fig. 1. Al^{3+} were more effective than Fe^{2+} or

Table 1. *Non-enzymic transamination of α-oxoglutaric acid by amino acids in the presence of* Al^{3+}

The reaction mixture contained 25 μmoles of amino acid, 10 μmoles of α-oxoglutaric acid and 12 μmoles of Al^{3+} in a total volume of 1 ml. The compounds were dissolved in 50 mM-sodium acetate buffer, pH 4·0, and the mixture was heated in a screw-cap Pyrex culture tube at 85° for 5 hr. Glutamic acid concentrations were measured on a portion of the reaction mixture by paper chromatography followed by densitometric analysis.

Amino acid	Glutamic acid formed (μmoles)
None	0·0
Histidine	6·9
Aspartic acid	3·0
Arginine	1·8
Proline	0·0
Alanine	1·6
Serine	0·0
Cysteine	0·0
Leucine	2·3
Phenylalanine	3·8
Lysine	2·2
Threonine	0·0
Tyrosine	0·0
Valine	0·0
Tryptophan	2·7

Table 2. *Effect of metal ions on the transamination of α-oxoglutaric acid with histidine*

The reaction mixture contained 25 μmoles of histidine, 10 μmoles of metal ion and 10 μmoles of α-oxoglutaric acid in a total volume of 1 ml. The compounds were dissolved in 50 mM-sodium acetate buffer, pH 4·0, heated at 85° for 5 hr. and tested for glutamic acid as described in Table 1.

Metal salt	Glutamic acid formed (μmoles)
None	0·8
$Al_2(SO_4)_3$	9·2
$CoCl_2$	0·6
$CuSO_4$	3·0
$Fe(NH_4)_2(SO_4)_2,6H_2O$	8·7
$FeNH_4(SO_4)_2,12H_2O$	7·6
$MgSO_4$	0·7
$MnSO_4$	0·6
$NiSO_4,6H_2O$	0·6
$ZnSO_4,7H_2O$	0·8
$Al_2(SO_4)_3$ + EDTA (10 μmoles)	0·0

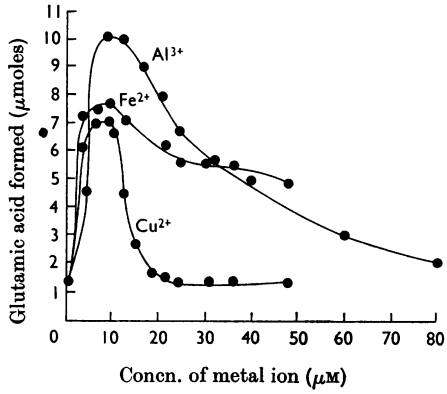


Fig. 1. Effect of different concentrations of Cu^{2+}, of Fe^{2+} or of Al^{3+} on the formation of glutamic acid. The reaction mixture contained different concentrations of metal ion, 10 μmoles of α-oxoglutaric acid and 24 μmoles of histidine in a total volume of 1 ml. The compounds were dissolved in 50 mM-sodium acetate buffer, pH 4·0, heated at 85° for 5 hr. and tested for glutamic acid as described in Table 1.

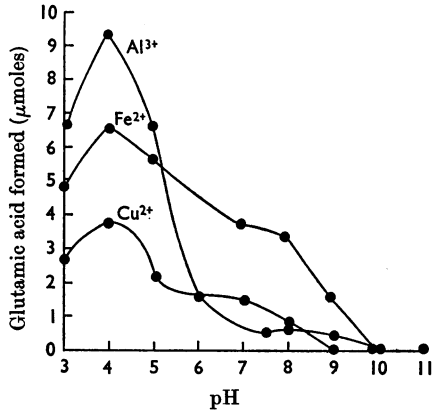


Fig. 2. Effect of pH on the formation of glutamic acid. The reaction mixture contained 10 μmoles of α-oxoglutaric acid, 12 μmoles of metal ion and 25 μmoles of histidine in a total volume of 1 ml. The compounds were dissolved in buffers of different pH: 50 mM-sodium acetate buffer for pH 3–5, 50 mM-tris–HCl buffer for pH 6–8 and 25 mM-sodium borate buffer for pH 9–11. The mixture was heated at 85° for 5 hr. and tested for glutamic acid as described in Table 1.

Cu^{2+}. All three cations showed a sharp increase in the stimulatory effect as the concentration was increased to an optimum value. As the concentration was increased further there was a gradual decrease in the stimulatory effect with Fe^{2+} and Al^{3+} and a sharp decrease in the stimulatory effect with Cu^{2+}. Doctor & Oró (1967) reported that Cu^{2+} and Fe^{3+} were not effective in the transamination reaction because the concentrations of the cations used were higher than the optimum and under these conditions the stimulatory effect was not significant (Fig. 1). The inhibitory effect of metal ions at higher concentrations was investigated further. Determinations of unchanged histidine were made by the chromatographic method described above

for glutamic acid. The results of this study showed that at higher concentrations of metal ions there was less free histidine available to form the complex with keto acid and to form the ternary complex. An alternative explanation for the inhibitory effect of metal ions at higher concentrations is that the metal ions may remove the keto acid and therefore less of it is made available to form the ternary complex.

The influence of pH on the transamination of α-oxoglutaric acid with histidine in the presence of Al^{3+}, Cu^{2+} or Fe^{2+} is shown in Fig. 2. All three metal ions showed an optimum stimulatory effect at pH 4·0; however, the decrease in the stimulatory effect at higher pH is gradual with Fe^{2+} and sharp with Al^{3+} and Cu^{2+}.

The presence of free glutamic acid formed during the reaction between histidine and α-oxoglutaric acid in the presence of Al^{3+}, Cu^{2+} or Fe^{2+} was confirmed by t.l.c. and ion-exchange chromatography on a portion of the final reaction mixture. Imidazolylpyruvic acid formed during the transamination reaction presumably undergoes complex-formation with Al^{3+} and formation of stable chelates with Cu^{2+} or Fe^{2+}. At pH 1·0 imidazolylpyruvic acid was liberated from the Al^{3+} complex, but not from the chelates of Cu^{2+} or Fe^{2+}. The free imidazolylpyruvic acid was then converted into the 2,4-dinitrophenylhydrazone (Stumpf & Green, 1952) and its i.r. spectrum, m.p. and elemental analysis were compared with those given by an authentic sample (Calbiochem). Both the compounds had m.p. 239° (decomp.) and gave essentially identical i.r. spectra. The C, H and N analyses of the two compounds reported were also closely similar (Found for synthetic compound: C, 42·5; H, 3·4; N, 24·0. Found for isolated compound: C, 43·1; H, 3·5; N, 23·0. Calc. for $C_{12}H_{11}N_6O_6$: C, 43·0; H, 3·3; N, 25·0%).

The Cu^{2+}–imidazolylpyruvic acid chelate and the Fe^{2+}–imidazolylpyruvic acid chelate formed during the transamination reactions with the respective ions were sparingly soluble and were precipitated out of solution at room temperature (20°).

The i.r. spectra of Fe^{2+}–imidazolylpyruvic acid chelate isolated from the final reaction mixture and of the synthetically prepared compound were essentially identical. The i.r. spectrum of the Cu^{2+}–imidazolylpyruvic acid chelate precipitated out of the final reaction mixture was also essentially identical with that of the synthetic compound. Comparison of the i.r. spectra of the Fe^{2+}–imidazolylpyruvic acid chelate and of the Cu^{2+}–imidazolylpyruvic acid chelate with that of imidazolylpyruvic acid showed that the last-named compound exhibits two strong bands at 6·1 and 6·3 μm. corresponding to the carboxyl ion and carbonyl of the α-oxo group respectively, whereas the two chelates show one band at 6·1 μm. indicating co-ordination of the keto group with the metal ion. Determination of keto function on the chelates by the dinitrophenylhydrazine reaction (Metzler & Snell, 1952) showed negative results, confirming that the chelates do not liberate free keto groups in acid solution. The involvement of the tertiary nitrogen atom of the imidazole ring in the chelate formation was suggested by the absence of diazo reaction by the chelates, in contrast with the distinct positive reaction with imidazolylpyruvic acid (Pauly, 1904; Ames & Mitchell, 1952).

The results of continuous-variation experiments with different solutions of Cu^{2+} or of Fe^{2+} with imidazolylpyruvic acid are presented in Fig. 3. At two different wavelengths the extinction values pass through a maximum at a point corresponding to a solution containing 2 moles of imidazolylpyruvic acid to 1 mole of Cu^{2+} or Fe^{2+}. The effect of the addition of different concentrations of imidazolylpyruvic acid on the complex-formation with 5 mM each of Cu^{2+} or Fe^{2+} was also studied. The unchanged imidazolylpyruvic acid was assayed by the dinitrophenylhydrazine method of Metzler & Snell (1952). The results showed that imidazolylpyruvic acid at concentrations lower than twice that of the metal ions was completely used up in complex-formation, whereas concentrations above this value remained unchanged. These results are interpreted to suggest that 1:2 metal ion–imidazolylpyruvic acid chelates with the structural formula shown in Fig. 4 may be formed under these conditions. In these molecules the histidine anions

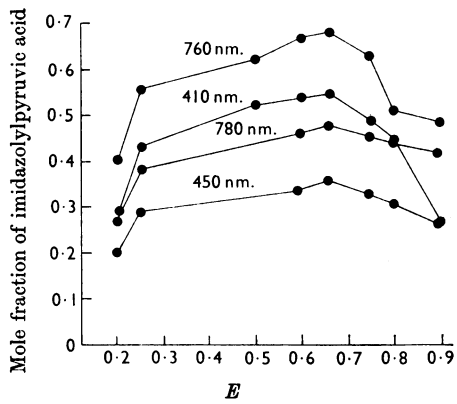


Fig. 3. Continuous-variation experiments with Cu^{2+} or Fe^{2+} with imidazolylpyruvic acid. The concentration of the two solutions was 15 mM for the Cu^{2+} experiment and 5 mM for the Fe^{2+} experiment. Extinctions were measured at 410 and 760 nm. for the Cu^{2+}–imidazolylpyruvic acid mixture and at 450 and 780 nm. for the Fe^{2+}–imidazolylpyruvic acid mixture.

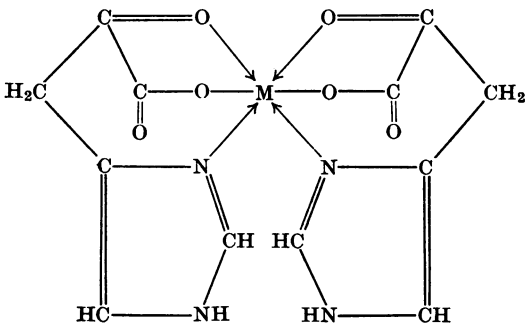


Fig. 4. Postulated structure of metal ion–imidazolylpyruvic acid (1:2) chelate. M represents the metal ion.

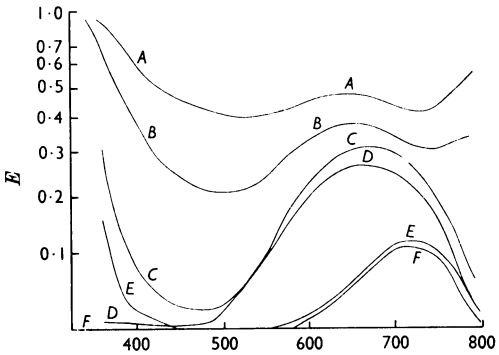


Fig. 5. Absorption spectra of solutions containing Cu^{2+} (7·5mM), histidine (25mM) and α-oxoglutaric acid (10mM), singly or in combination. The compounds were dissolved in 50mM-sodium acetate buffer, pH4·0, and heated at 85° for various times. *A*, Cu^{2+}–α-oxoglutaric acid–histidine mixture after 30min.; *B*, Cu^{2+}–α-oxoglutaric acid–histidine mixture after 15min.; *C*, Cu^{2+}–α-oxoglutaric acid–histidine mixture after 0min.; *D*, Cu^{2+}–histidine mixture after 0min. or 15min.; *E*, Cu^{2+}–α-oxoglutaric acid after 0min. or 15min.; *F*, Cu^{2+} alone.

are co-ordinated primarily through the tertiary imidazole nitrogen atom and α-oxo group, forming stable six-membered rings. The two carboxyl oxygen atoms approach the Fe^{2+} ion closely enough to be considered as loosely co-ordinated. The structural formula suggested in Fig. 4 is based on conclusions by Kretsinger, Cotton & Bryan (1953) and by Chakravorty & Cotton (1963) on the structural formula of metal ion complexes with imidazole derivatives based on stability constants. The proposed structural formula of the complex would be more reasonable with Fe^{3+} or Al^{3+} because these are hexa-co-ordinate. Also the *trans* form of the isomer would be more stable than the *cis* isomer shown in Fig. 4.

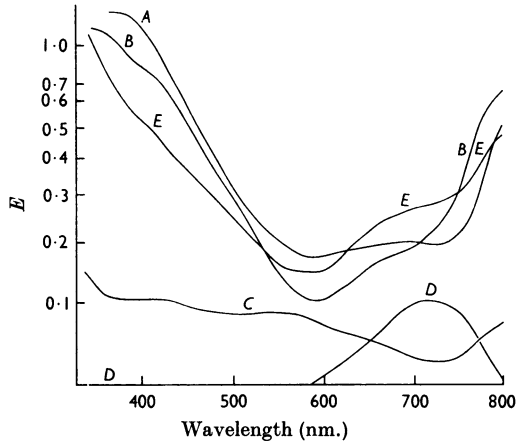
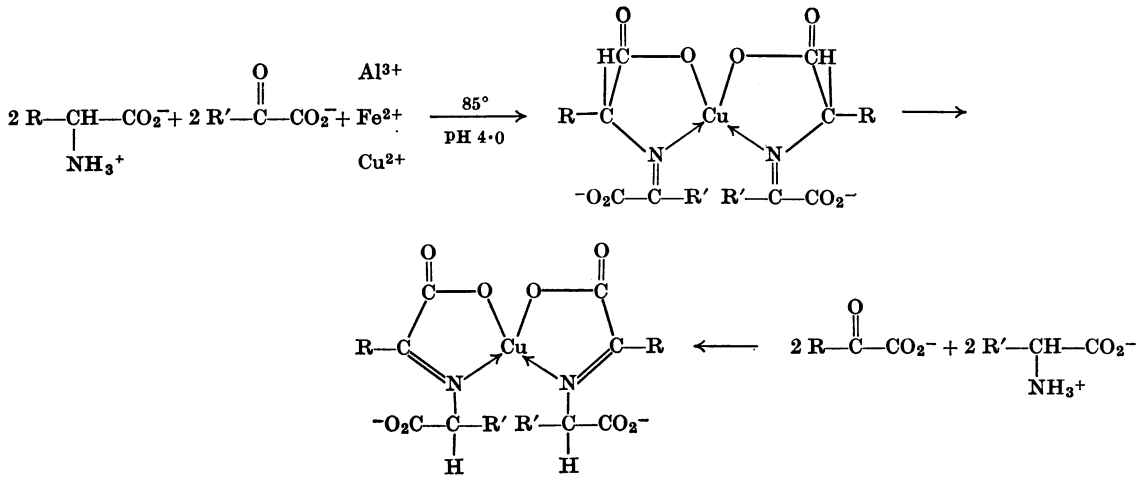


Fig. 6. Absorption spectra of solutions containing Cu^{2+} (7·5mM), glutamic acid (10mM) and imidazolylpyruvic acid (5mM), singly or in combination, heated for 5hr. at 85°. *A*, Cu^{2+}–α-oxoglutaric acid–histidine mixture after 5hr.; *B*, Cu^{2+}–imidazolylpyruvic acid mixture after 0min.; *C*, imidazolylpyruvic acid alone; *D*, Cu^{2+} alone; *E*, Cu^{2+}–imidazolylpyruvic acid–glutamic acid mixture after 0min.

The absorption spectra of the solutions of Cu^{2+}, histidine and α-oxoglutaric acid, singly and in combination, are presented in Fig. 5. The Cu^{2+}–α-oxoglutaric acid solution exhibits the same absorption spectrum as the non-co-ordinated Cu^{2+}, indicating a very low degree of complex-formation or little change in the environment of Cu^{2+} when in the complex. On the basis of spectral changes the measurement of affinity constants of various keto acids for the formation of complexes with Cu^{2+} or Fe^{2+} showed that α-oxoglutaric acid had the lowest affinity of all the keto acids tested and that imidazolylpyruvic acid had the highest value. The Cu^{2+}–histidine solutions on the other hand clearly demonstrate complex-formation (Eichhorn & Dawes, 1954). These curves may now be compared with the spectra of solutions of Cu^{2+}, α-oxoglutaric acid and histidine together. The characteristics of simple complexes were clearly significantly altered by a shift in the maximum. The extinction of the three-component solution increased rapidly on heating at 85°, indicating an increase in the amounts of the ternary complex formed. After the solution had been heated for 5hr. (Fig. 6), the spectrum changed again and resembled that of solutions of Cu^{2+} with imidazolylpyruvic acid or a three-component system consisting of Cu^{2+}, imidazolylpyruvic acid and glutamic acid. The results shown in Fig. 6 indicate a change from the ternary complex to the products, namely Cu^{2+}–imidazolylpyruvic acid chelate and glutamic acid. Results similar to



Scheme 1. Postulated mechanism for the non-enzymic transamination of amino acids with keto acids in the presence of metal ions.

those presented in Figs. 5 and 6 were also obtained for the reactions between Fe^{2+}, histidine and α-oxoglutaric acid.

The mechanism shown in Scheme 1 is postulated to explain the role of metal ions in catalysing the transamination reaction between amino acid and α-oxoglutaric acid. The greater effectiveness of histidine in these reactions may be attributed to the presence of the tertiary imidazole nitrogen atom, which is involved in the formation of stable, sparingly soluble, metal ion–imidazolylpyruvic acid complexes or chelates. On a molar basis imidazolylpyruvic acid was 15–20 times as effective in forming metal ion co-ordination complexes as any of the other α-oxo acids tested (V. M. Doctor & J. Oró, unpublished work). These results may account for the shift in the reaction towards glutamic acid formation when imidazolylpyruvic acid was the end product instead of one of the other α-oxo acids. The role of imidazole as a base in removing the α-proton during the rearrangement of double bonds (Scheme 1) was investigated by adding different concentrations of imidazole to the medium when histidine or other amino acid was being tested. The results showed no stimulatory effect by imidazole. However, this would not rule out an intramolecular base catalysis when histidine was used as an amino donor. Mix (1961) reported that Cu^{2+}-catalysed transamination reactions between α-amino acids and α-oxo acids were greatly accelerated by pyridine. Piperazine and acetate were reported by Dixon (1967) to accelerate Cu^{2+}-catalysed transamination of *Pseudomonas* cytochrome *c*-551 at pH 7·0. Whether the transition complex has a 1:1 ratio between Schiff base and metal ion or a 2:1 ratio as shown in Scheme 1 cannot be decided on the available information. Studies of the ratio of concentrations of amino acids and metal ions required for optimum transamination indicate that a 2:1 Schiff-base transition complex may be formed before being transformed into a 2:1 product complex.

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