Novel Mechanisms in the Activation of L-Alanine by Copper(II)[1]

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In the presence of dioxygen, alkaline solutions containing copper(II) ions and alanine yield pyruvate, which catalyses the loss of optical activity from the alanine.

CERTAIN transition-metal ions, notably Co^{3+}, Cu^{2+}, and Pt^{2+}, bring about a substantial increase in [2] the rate of racemisation of co-ordinated amino-acids, particularly in alkaline solution. Such reactions are perhaps most conveniently followed using the kinetically inert (with regard to ligand displacement) and diamagnetic cobalt(III) complexes $[Co(en)_2L]^{2+}$ (L = amino-acid anion, en = ethylenediamine) where, in mildly alkaline deuterium oxide solution, ligand racemisation occurs at a rate very similar to that of the incorporation of deuterium at the methine position. These results have been interpreted as favouring racemisation *via* the formation of an intermediate carbanion (step 1) and a similar mechanism has been widely accepted for exchange in a variety of other complexes of Co^{III} with amino-carboxylate ligands.

However, when we examined [3a] the effect of Cu^{II} on the reactivity of amino-acids it was unfortunately not possible to follow the incorporation of deuterium directly, since the paramagnetism of the Cu^{2+} ion renders simple ^1H n.m.r. spectroscopy unavailable, at least on the intact complex. Nevertheless, it could be shown that, as for Co^{III}, racemisation induced by Cu^{2+} ion is accompanied by incorporation of deuterium, although the two processes were necessarily measured separately. The degree of racemisation was estimated by measuring the optical rotation of the copper complex in solution † (a standard method for determining the optical purity of amino-acids [4]), whilst the extent of deuteriation was subsequently estimated from the ^1H n.m.r. spectrum of the free alanine (Ala), after removing the copper. From those measurements it was found that the rate of exchange exceeded the rate of racemisation, although this was difficult to reconcile with the simple carbanion mechanism.

Recent attempts [5] ' to repeat ' these observations were unsuccessful. The difficulties, chiefly the precipitation of the copper, seem to result partly from the limited experimental detail available in our earlier report [3a] (the only condition specified was pH *ca.* 12), but chiefly from a failure [5] to appreciate that at high pH the copper may readily be kept in solution if the L-Ala : Cu^{2+} ratio is raised to $\geqslant 4:1$. Indeed,[3b] at such high pH it is only by this means that the simple copper–amino-acid complex may be formed at all. Therefore, in view of this confusion,[5] coupled with the obvious and growing importance of the metal-ion induced activation, not only in organic synthesis [2,6,7] but also in geochemistry,[8] we have extended our earlier work to specify in more detail the conditions to be used and the mechanism of the reaction.

RESULTS

Our initial observations quickly confirmed the earlier findings. Namely, if a solution of Cu^{2+} : L-Ala (1 : 4 or greater) is made alkaline, pH >10, then the optical activity of the solution rapidly diminishes and when deuterium oxide is used as solvent the DL-alanine isolated from the reaction is extensively deuteriated in the methine position. The extent of deuterium incorporation was shown to increase with time (Figure 1), but as found previously [3] the

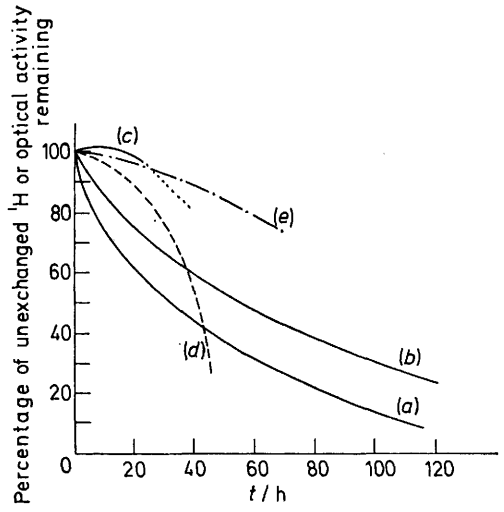

FIGURE 1 Racemisation and deuteriation of L-alanine in the presence of Cu^{II}: (*a*) uncorrected rate of deuterium incorporation; (*b*) racemisation, measured for the free amino-acid remaining after removal of the copper (curve is identical to the corrected rate of deuterium incorporation); (*c*)—(*e*) the change in overall rotation of the reaction mixture. Conditions: (*a*)—(*c*) pH 11.1, 100 °C, Cu^{II} : L-Ala = 1 : 8; (*d*) pH 11.8, 56 °C, Cu^{II} : L-Ala = 1 : 4; (*e*) pH 11.5, 56 °C, Cu^{II} : L-Ala 1 : 4

† The assumption that only $[Cu(L\text{-}AlaO)_2]$ (AlaO = alaninate) contributed to the optical activity of such solutions is now shown to be unjustified.

[1] This paper is considered as Part 3 of the series ' The Isomers of α-Amino-acids with Copper(II);' Part 2, R. D. Gillard, R. Mason, N. C. Payne, and G. B. Robertson, *J. Chem. Soc.* (*A*), 1969, 1864.

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[5] L. G. Stadtherr and R. J. Angelici, *Inorg. Chem.*, 1975, **14**, 925.

[6] J. R. Brush, R. J. Magee, M. J. O'Connor, S. B. Teo, R. J. Geue, and M. R. Snow, *J. Amer. Chem. Soc.*, 1973, **95**, 2034.

[7] J. Dabrowiak and D. W. Cooke, *Inorg. Chem.*, 1975, **14**, 1305.

[8] J. L. Bada and R. A. Schroeder, *Naturwiss.*, 1975, **62**, 71.

rate of deuteriation appeared to be faster than the loss of optical activity. The rate of this loss diminishes with pH and temperature, so that at pH *ca.* 10 the rate of racemisation (as judged by the change in optical rotation of the solution) was very slow: 10% in 10 d at room temperature; this is still considerably faster than for free alanine.[8] However, on increasing the pH the racemisation occurred much more readily, and some typical times for 50% racemisation are given in Table 1.

TABLE 1

Half-times of racemisation at 50 °C, $[Cu^{II}] = 8 \times 10^{-3}$ mol dm^{-3}, and [L-Ala] = 3.2×10^{-2} mol dm^{-3}

pH	$t_{\frac{1}{2}}$/h
11.3	135
11.5	90
11.7	40
11.8	35

The course of the reaction was followed in some detail, particularly during the early stages. The faster reactions did not exhibit the simple pseudo-first-order rate profile which was expected for racemisation at constant pH. Instead, there was an anomalous, and initially puzzling, period in which the optical rotation, α (546.1 nm), of the solution remained constant or in some cases even increased. This was only later followed by the expected decrease. Moreover the pattern of this later stage of the reaction still did not follow the typical first-order profile exactly, but exhibited the characteristics of an autocatalytic path, particularly at lower temperatures (Figure 1). The duration of the initial phase (which under some conditions showed the characteristics of an induction period) depended on the reaction conditions (pH, $[Cu^{2+}]$: [L-Ala], and temperature) but was greatest at pH 10.5—11 in systems with a large excess of alanine ($[Cu^{2+}]$: [L-Ala] 1 : 8). At higher pH the induction period diminished, and it was also shorter at higher temperature. Further, as racemisation proceeded, the pH of the solution decreased and red copper(I) oxide formed. This precipitation was also temperature and pH dependent, being most rapid at high pH and high temperature.

The presence of reduced copper suggested that the amino-acid had been oxidised. For all the solutions at pH >10 it was indeed possible to isolate pyruvate within a few minutes of starting the reaction, although no significant amount of it was found in the absence of Cu^{II}. For very alkaline solutions, *i.e.* at pH >12, which were heated for extended periods, acetaldehyde could also be detected, although blanks showed that under these conditions pyruvate itself was decarboxylated, even in the absence of copper. In each case ammonia could also be detected (in the copper-containing solutions). However, in the absence of copper, ammonia was not formed. The production of pyruvate during the course of this aerobic reaction was assayed,[7,9,10] although because of self-condensation and further degradation of the keto-acid the results are not quantitative (Figure 2). Nevertheless, although the reduction in optical activity of the solution paralleled the formation of pyruvate, at least at low temperature, the yield of pyruvate (and hence the loss of L-alanine) was not sufficient in itself to account for the measured reduction in optical rotation. Therefore, the role of pyruvate in the reduction of the optical activity of the system was examined in more detail.

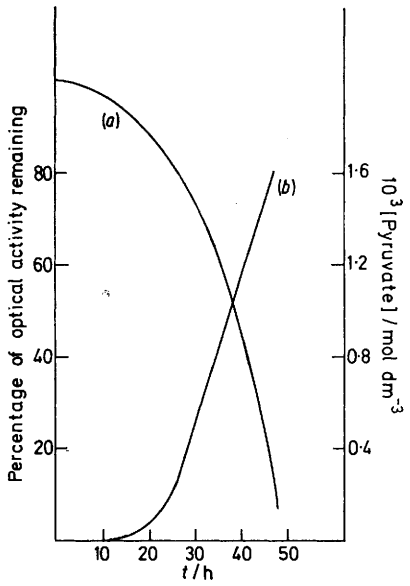


FIGURE 2 Loss of optical activity (*a*) and pyruvate production (*b*) at pH 11.7, 51 °C, $[Cu^{II}] = 1 \times 10^{-2}$ mol dm^{-3}, and [L-Ala] $= 4 \times 10^{-2}$ mol dm^{-3}

The addition of pyruvate to the alkaline copper(II)–L-alanine solution resulted in an increase in the observed optical rotation, which reached a maximum at a pyruvate : L-alanine ratio of slightly more than 1 : 1 (Figure 3). However, the wavelength of maximum rotation was almost unaffected, although the visible-absorption spectrum changed and finally came to resemble that of authentic aqua[*N*-(1-carboxylatoethylidene)-L-alaninato]copper(II).[11]

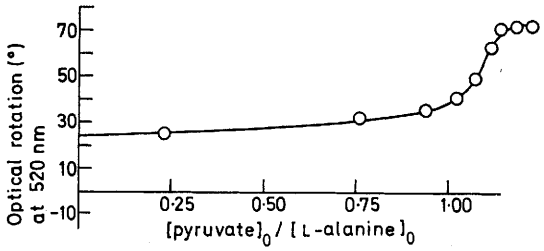


FIGURE 3 Effect of added pyruvate on the optical rotation of the copper(II)–L-alanine system at $[Cu^{II}] = 1.25 \times 10^{-2}$ mol dm^{-3}, [L-Ala] $= 5.0 \times 10^{-2}$ mol dm^{-3}, pH 10.6, and cell length = 1.0 cm

While the visible spectrum of these solutions then remained essentially constant, although it did alter finally due to the precipitation of copper(I) oxide, its optical rotatory dispersion (o.r.d.) spectrum, measured at time intervals, showed a rapid decrease in the optical rotatory power of the solution, which for various values of pH in the range 9.0—10.5 exhibited reasonable first-order kinetics; at higher pH the precipitation of copper(I) oxide was sufficiently rapid to interfere (Table 2).

[9] E. M. Case, *Biochem. J.*, 1932, **26**, 753.
[10] T. E. Friedman and G. E. Haugen, *J. Biol. Chem.*, 1943, **147**, 15.
[11] Y. Nakao, K. Sakuri, and A. Nakahara, *Bull. Chem. Soc. Japan*, 1966, **39**, 1471.

Schiff bases may undergo transamination [12-16] and by a further series of deuterium-labelling experiments it was shown to occur in this system. After racemisation at room temperature in the presence of pyruvate, the product DL-alanine contained deuterium only in the methine position in accordance with our earlier report,[3a,b] and as noted by other workers.[11] However, in a similar experiment at 100 °C it was possible to isolate DL-alanine deuteriated in both methine and methyl positions, as shown by changes in the characteristic C–H bands of the i.r. spectrum.[17]

TABLE 2

Effect of 0.01 mol dm^{-3} pyruvate on the racemisation of copper(II)–L-alanine systems at 40 °C

[Cu^{II}]	[L-Ala]		Percentage
mol dm^{-3}		pH (25 °C)	racemisation [a]
	0.10	9.38	0
	0.10	11.80	5 [b]
0.01	0.04	9.38	0
0.01	0.04	10.80	80
0.01	0.04	11.35	100
0.01	0.04	11.73	*c*
0.01	0.04	11.98	*c*

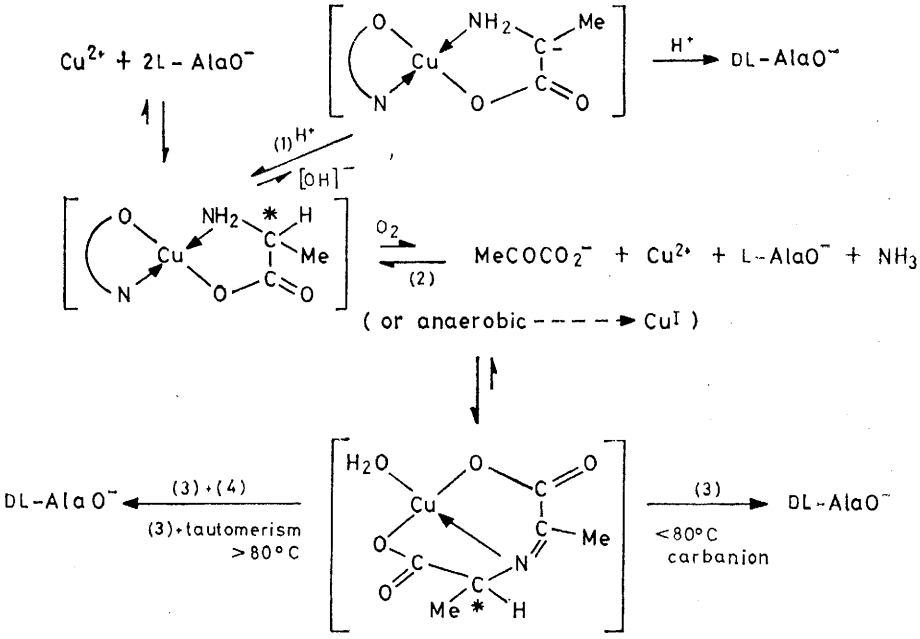
[a] After 24 h. [b] This small amount of racemisation could possibly be attributed to contamination by trace elements. [c] Copper(I) oxide precipitated after 1 h.

The rate of 'racemisation' was re-examined, not by measuring the optical rotation of the copper-containing solution (which is clearly inappropriate) but by isolating the alanine and measuring its optical activity directly (Figure 1).

Role of Atmospheric Oxygen.—The role of atmospheric oxygen in this ligand oxidation was examined. A series of copper(II)–L-alanine solutions was deoxygenated, and made alkaline using oxygen-free sodium hydroxide solution. Under these anaerobic conditions the behaviour of the system differed from that observed previously. Here, at pH *ca.* 11.5, racemisation was minimal, even after 3 d at 100 °C; neither pyruvate nor copper(I) oxide were found and no ammonia could be detected. Finally, the pH remained unchanged. However, when O_2 was admitted the character of the reaction changed completely, reverting to that described previously; ammonia and pyruvate were both detected within 30 min, the precipitation of copper(I) oxide soon became apparent, the optical activity of the solution began to diminish, and the pH gradually decreased.

Interestingly though, at 'higher' pH (>11.5), despite the most stringent precautions to remove oxygen, the anaerobic system reacted like the aerobic system, with the production of pyruvate, copper(I) oxide, and ammonia and a gradual decrease in pH (Table 3). However, by admitting oxygen the rate of formation of these products was much accelerated.

These results show that the loss of optical activity in the alkaline copper(II)–L-alanine system cannot properly be described as a 'racemisation' and instead of the single simple carbanion-type mechanism, which was previously



SCHEME Racemisation paths

TABLE 3

Anaerobic deamination of alanine *

pH		Presence in product of	
initial	final	pyruvate	Cu_2O
12.18	10.70	Positive	Positive
12.18	10.60	Positive	Positive
11.40	11.40	None	Negative

* Solutions (Cu^{II} : L-Ala = 1 : 4, [Cu^{II}] = 0.1 mol dm^{-3}) in sealed ampoules were set aside overnight at 40°C.

[12] A. Nakahara, H. Yamamoto, and H. Matsumoto, *Bull. Chem. Soc. Japan*, 1964, **37**, 1137.
[13] O. A. Gansow and R. H. Holm, *J. Amer. Chem. Soc.*, 1969, **91**, 573.
[14] E. H. Abbot and A. E. Martell, *J. Amer. Chem. Soc.*, 1970, **92**, 5845.
[15] G. A. Auld and A. Davison, *Inorg. Chem.*, 1968, **7**, 306.
[16] R. B. Johns and D. J. Whelan, *Austral. J. Chem.*, 1966, **19**, 2143.
[17] T. Oshima and N. Tamiya, *Spectrochim. Acta*, 1961, **17**, 384.

assumed [3a] to account entirely for the loss in optical activity, the formation of DL-alanine, and deuterium incorporation, at least five different routes must now be considered for a complete description of these reactions, since they are now shown not necessarily to be simultaneous (see Scheme).

DISCUSSION

The rate of racemisation of L-alanine in the presence of Cu^{II} is clearly the result of competition between the various paths outlined (Scheme), the reactive species leading to racemisation being either the Schiff base derived from pyruvate, L-alanine, and Cu^{II} or the bis(L-alaninato)copper(II) complex. The relative importance of each path in the overall mechanism is then a function of such conditions as pH and temperature, and by careful control it is possible to alter the relative contribution of each and hence the predominant path.

Simple Carbanion Mechanism.—The reaction is simplest in the absence of oxygen and at 'low' pH [path (1)]. Under these anaerobic conditions and at pH <11.5, the formation of DL-alanine and deuterium incorporation (using ^2H_2O as solvent) occur concurrently, albeit extremely slowly. Since ligand oxidation appears to be negligible, Schiff-base formation can be discounted. Therefore, it seems that the carbanion mechanism cannot be excluded, although its importance, except under these rather mild conditions, is minimal. This view is supported by the observation that the methylene groups of $[Cu^{II}(edta)]^{2-}$ (edta = ethylenediaminetetra-acetate) also exhibit activation,[18] although in this case ligand oxidation or Schiff-base formation is precluded by the absence of primary amino-groups.

Interestingly though, deuterium incorporation into edta appears faster than for alanine, although strictly quantitative studies have not yet been carried out. This is contrary to expectation since the edta complex nominally has a more negative overall charge which should reduce the activation.[3,19,20] However, two factors may be of importance here. First the overall negative charge of the alanine complex may possibly be increased, either by axial addition of hydroxide or in some other way; secondly, the overall rates of ring opening and ligand exchange are likely to be greater in the bidentate alanine complex.

The rate of reaction in $[Cu(L\text{-}AlaO)_2]$ * at moderate pH is at least three orders of magnitude [2] [path (1)] lower than for the cobalt(III) complex $[Co(en)_2(L\text{-}AlaO)]^{2+}$. However, in addition to having a substantially higher positive charge, the cobalt(III) complex is also kinetically inert and will neither add $[OH]^-$ nor readily undergo ring opening, all of which should favour reaction *via* the carbanion intermediate.

Schiff-base Racemisation.—The increased rate of disappearance of optical activity which becomes particularly apparent at pH >12 (anaerobic/aerobic) cannot be accounted for simply by the increase in concentration of hydroxide ion, although of course this must certainly be considered. Instead, the oxidation of ligand, clearly indicated by the formation of pyruvate and ammonia, must necessarily be important (Table 1) since the products are themselves optically inactive.

These observations suggest that oxidation of L-alanine to pyruvate is followed by condensation of pyruvate and alanine to give a Schiff base. This then co-ordinates to the copper ion, yielding the [*N*-(1-carboxylatoethylidene)-L-alaninato]copper(II) fragment, with the fourth co-ordination position occupied by a water or, more likely, a deprotonated water molecule, $[OH]^-$ (see Scheme). Such complexes are well known, as is the additional activating effect of Schiff-base formation. Indeed, [*N*-(1-carboxylatoethylidene)glycinato]copper(II) is [21] considerably more effective than is simple $[Cu(GlyO)_2]$ in the synthesis of new amino-acids, by condensation of aldehydes with the activated methylene group of co-ordinated glycine (Gly). A similar activation in the *N*-(1-carboxylatoethylidene)-L-alaninato-complex would result in an increased rate of racemisation of the alanine which will offset the greater optical rotatory power of this complex.[11]

Oxidative deamination of co-ordinated amino-acids is not unknown, but is more normally associated with neutral or acid pH.[22-24] Oxidation of the amino-acid ligand under the conditions described here is unusual and appears not to have been reported previously, other than when induced photochemically.[25] However, such redox behaviour is quite common in copper complexes of α-hydroxyacids, although again generally at significantly lower pH. Nevertheless the hydroxy- and amino-acids are isoelectronic and the different conditions necessary to bring about the reaction may simply reflect the greater difficulty of deprotonating the nitrogen, which is likely to be the key step in the reaction (see Scheme). The possible existence of such an intermediate is supported † by the observation [26] that the co-ordinated amino-groups in $[Ni(GlyO)_2]$ may both be deprotonated (although under rather more drastic conditions, using potassium–liquid ammonia).

The Schiff-base intermediate may of course be derived in two distinct ways: aerobically by a homogeneous redox reaction [path (2)] involving Cu^{II} and O_2, or anaerobically by an intramolecular redox reaction

* This apparently first-order rate, of great importance in oceanography and geochemistry,[8] is at present under study.

† Although a novel equilibrium (pK_a *ca.* 11.8) has been found for $[Cu(L\text{-}AlaO)_2]$, it is not certain that this arises from a conjugate base rather than hydroxide complexation.

[18] P. R. Norman and D. A. Phipps, *Inorg. Chim. Acta*, 1976, **17**, L19.

[19] D. A. Buckingham, L. G. Marzilli, and A. M. Sargeson, *J. Amer. Chem. Soc.*, 1967, **89**, 5133.

[20] P. R. Norman and D. A. Phipps, unpublished work.

[21] T. Ichikawa, T. Okamoto, S. Maeda, S. Ohdan, Y. Araki, and Y. Ishido, *Tetrahedron Letters*, 1971, **1**, 79.

[22] D. E. Metzler and E. E. Snell, *J. Amer. Chem. Soc.*, 1952, **74**, 769.

[23] H. Mix, *Z. physiol. Chem.*, 1961, **323**, 171.

[24] A. Marx, M. Sendrea, and M. Petcovice, *Experientia*, 1970, **26**, 35.

[25] G. A. Shagisultanova, L. A. Ilyukevich, and L. I. Burdyko, *Zhur. fiz. Khim.*, 1965, **19**, 2730.

[26] G. W. Watt and J. F. Knighton, *Inorg. Chem.*, 1967, **7**, 1010.

involving a Cu^{I}–Cu^{II} L-alanine redox couple, the rate of the aerobic reaction being much greater. It is probably worth noting that this mechanism has been the significant one in a large number of copper(II) blanks used in studies of vitamin B_6 or model studies of this vitamin.

Effect of Schiff Base on Racemisation.—The effect of pyruvate on the observed optical activity must be treated in two parts: first the increase in rotation caused by Schiff-base formation (corrected, of course, for loss of alanine) and secondly the increased *rate* of racemisation, which can be demonstrated simply by adding extraneous pyruvate to any alkaline copper(II)–L-alanine system. Thus the kinetic behaviour is dominated by the balance between these two factors and when the rate of formation of pyruvate matches, or even exceeds, the enhanced rate of racemisation the optical rotation of the solution will remain constant or increase, thus accounting for the induction period (Figure 2). [At most wavelengths, the molecular rotation of the *N*-(1-carboxylatoethylidene)-L-alaninato-complex is considerably larger than that of the simple bis(L-alaninato)-copper(II) species; thus the loss of L-alanine is outweighed by the formation of the Schiff base.]

Clearly then, this formation of the Schiff base must mean that the measurements of optical purity and hence the rate of racemisation estimated previously cannot be correlated directly with the rate of deuterium incorporation, since the enhancement of the optical rotation must lead to a lower apparent rate of racemisation, thus accounting for the 'stereospecificity' reported previously.[3] However, if the true rate of racemisation at the L-alanine chiral centre were measured independently (say by isolating the amino-acid) then it should correlate well with deuterium exchange. Initially, therefore, it was surprising when this was found not to be the case, but instead as shown previously (Figure 1) racemisation appeared to be faster than the exchange.

The appearance of the Schiff base does however allow an explanation both of this and also of the β-deuteriation [17] noted earlier. Although a considerable body of evidence confirms the additional activating effect of Schiff-base formation, the origin of this effect remains ambiguous. Two mechanisms may be postulated: one merely invoking the electron-withdrawing effect of the >C=N linkage, which will assist carbanion formation particularly if reinforced by further conjugation; the other calling for tautomerism of the C=N double bond. The former has been demonstrated in salicylidene Schiff bases,[15,16] the latter with pyridoxylenes.[13,14] The two mechanisms may be partly distinguished by deuterium labelling and tautomerism may be demonstrated *via* β-deuteriation of the amino-acid. In this case the isolation of β-deuteriated alanine implies that part at least of the effectiveness of the pyruvate arises from a transamination, but this does not rule out any other activating effect. Presumably, since β-deuteriated alanine was found only at the higher temperatures, the two routes operate simultaneously but the tautomerism has a higher activation energy and is therefore significant only when the reaction mixture is heated.

This tautomerism can now be invoked to account for the apparently inequivalent processes leading to racemisation and deuteriation. The β-deuteriation will reduce the effective methyl-group signal and thus inflate the $C-H : CH_3$ ratio. When these effects are allowed for it can be seen that the formation of DL-alanine and ^{1}H–^{2}H exchange at the *methine* position are apparently synchronous.

One further mechanism must be considered if the reaction is carried out aerobically and at pH >12. Under these conditions, and particularly at high temperatures, the pyruvate is decarboxylated to form acetaldehyde. Other workers [27] have shown that under such conditions, when copper(I) oxide is also present, L-alanine is rapidly racemised and that the aldehyde is a necessary adjunct. The detailed mechanism of this heterogeneous route is not known.

Conclusion.—The naturally occurring L-(+)-amino-acids are normally considered to be optically stable, except when subjected to such drastic treatment as heating in concentrated acid or alkali. Nevertheless, even at room temperature, amino-acids do racemise, albeit slowly, and recently this process has been quantified (typically $t_{\frac{1}{2}}$ >2 000 years). On the basis of such results, the estimation of the optical purity of amino-acids in biogenic deposits has been used for both geochronological and palaeothermometric analysis.[2] Clearly, therefore, if samples have been subjected to conditions of high pH during their history some enhancement of the rate of racemisation is likely to have occurred which may lead to an overestimate of their age. Whilst present results do not seem to have suffered in this fashion, since they have been validated by comparison with other methods, *e.g.* ^{14}C and ^{40}K dating, the risk of enhanced racemisation should nevertheless be borne in mind in this approach to dating.

The importance of Schiff-base formation demonstrated here also provides reinforcement for accounts of amino-acid synthesis based on *N*-(1-carboxylatoethylidene)-glycinato-complexes rather than on the previously used simple amino-acid complexes, since from our qualitative studies it appears that the formation of the Schiff base is a substantial additional favourable factor in the activation of the 2-carbon of amino-acids.

EXPERIMENTAL

Materials.—All the reagents used were AnalaR grade with the exception of the amino-acids which were chromatographically homogeneous (B.D.H.), and were used without further purification.

Reaction Mixtures.—Racemisation studies were carried out under a variety of conditions; typically, volumetric solutions of copper(II) sulphate and L-alanine were prepared. Appropriate amounts of these solutions were mixed and the pH was adjusted with 1 mol dm^{-3} sodium hydroxide. Concentrations of copper in such mixtures were between 8×10^{-3} and 0.24 mol dm^{-3}.

[27] A. Tai, K. Okada, T. Masuda, and Y. Izumi, *Bull. Chem. Soc. Japan*, 1976, **49**, 310.

pH *Measurements*.—pH was determined throughout at room temperature (20 °C) using glass electrodes and is hence uncorrected to the reaction temperatures which varied between 50 and 100 °C.

Determination of Optical Rotation.—The rotation of the complex was measured using either a spectropolarimeter or a polarimeter. Polarimetric measurements were of α (546.1 nm), each the mean of at least three measurements. Alternatively, the decay of rotation was followed at the maximum of the dispersion curve (526 nm); here the ratio of observed rotation to optical density was utilised. Substantially the same results were obtained by both methods.

Analysis for Pyruvate.—Pyruvate was detected as its 2,4-dinitrophenylhydrazone (dnph) by the method of Case.[9] To acidified reaction mixture (10 cm^3) was added an equal volume of dnph reagent (*ca.* 5%). The solution was then shaken for several minutes and extracted three times with ethyl acetate (3 × 15 cm^3). The combined extracts were then treated with excess of anhydrous calcium carbonate. The ethyl acetate extract was then decanted and reduced in volume to 2—3 cm^3 *in vacuo*. This solution was then taken up in toluene (20 cm^3) and extracted with a 25% solution of $Na_2[CO_3]$ (5 cm^3). The pyruvate 2,4-dinitrophenylhydrazone is extracted into the aqueous layer, making it brown, and it was precipitated by dropwise addition of hydrochloric acid. A reagent blank was carried out and no pyruvate could be detected in the alanine as supplied. Pyruvate 2,4-dinitrophenylhydrazone, m.p. 184 °C (lit.,[9,27] 186—187.4 °C), was further characterised by its i.r. spectrum and by paper and thin-layer chromatography.

The determination of pyruvate in the reaction mixture was carried out by a modification of the method of Friedman and Haugen.[10] Standard aliquot portions (2—5 cm^3) were allowed to react with 0.1% dnph solution ([HCl] = 1 mol dm^{-3}) followed by successive extraction into toluene and a 15% solution of $Na_2[CO_3]$, and colorimetric analysis in 1.5 mol dm^{-3} Na[OH]. A linear calibration curve was obtained for 0.2×10^{-3}—2.5×10^{-3} mol dm^{-3} pyruvate.

Degassed Experiments.—Two techniques were used. Water was degassed by heating under reflux under dried argon and degassed solutions were then prepared in a dry-box (under dry argon): kinetic runs were refluxed under dry argon. Alternatively, solutions were made up in Pyrex ampoules on a conventional vacuum line, the water used being outgassed to better than 10^{-3} Torr.*

N.M.R. Experiments.—Solutions in ^2H_2O having Cu^{II} : L-Ala = 1 : 4 ([Cu^{II}] = 2.5×10^{-2} mol dm^{-3}) were used. These were heated under reflux at pD 11.5, samples being periodically taken for analysis. The samples were acidified (to pH 4.5) and the copper precipitated with hydrogen sulphide; the n.m.r. spectrum and optical rotation (in 5 mol dm^{-3} HCl at 365 nm) of the alanine thus isolated were determined quantitatively.

Instrumentation.—Infrared spectra were recorded in KBr discs on a Perkin-Elmer 457 spectrophotometer, u.v.–visible spectra on a Pye-Unicam SP 1800 spectrophotometer, o.r.d. spectra on a Bellingham and Stanley Polarmatic 62 spectropolarimeter, with single-wavelength rotation measurements made on the related N.P.L. type 243 polarimeter at 546 nm (mercury green filter), and n.m.r. spectra on a Perkin-Elmer R.32 spectrometer (90 MHz) using 3-(trimethylsilyl)propane-1-sulphonic acid as internal standard. Measurements of pH were made with a Jenway 300 meter.

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* Throughout this paper: 1 Torr = (101 325/760) Pa.