Influence of Alkali- and Alkaline-earth-metal Cations on the 'Salt-induced Peptide Formation' Reaction *

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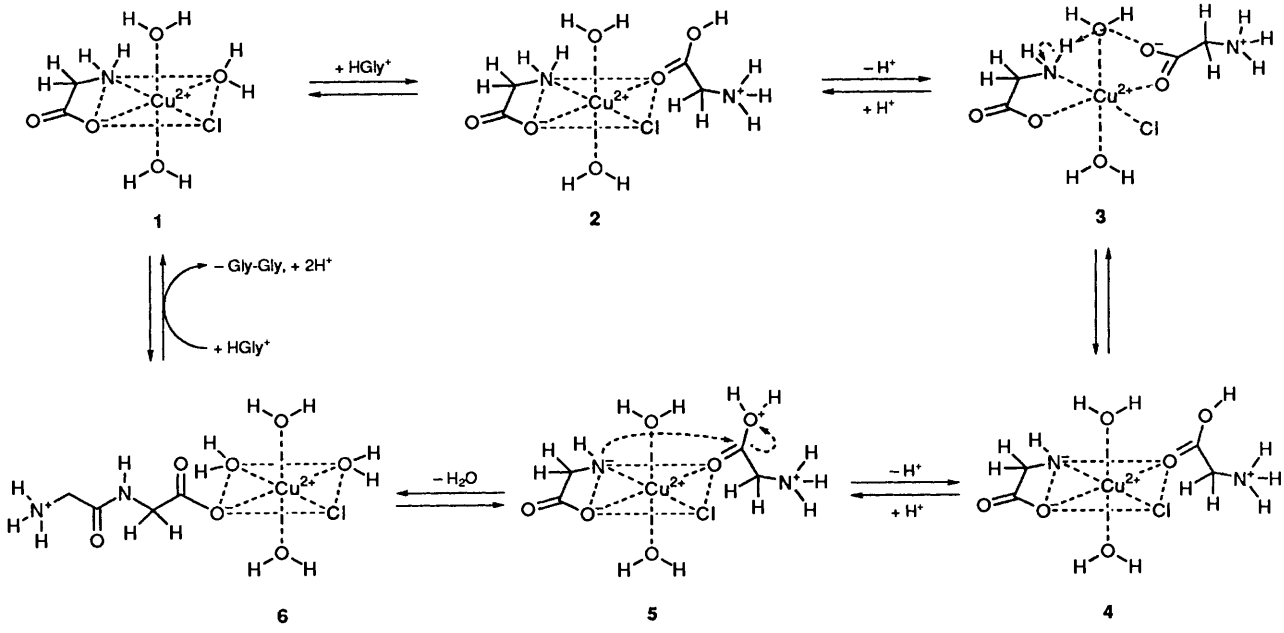
The reaction mechanism of the salt-induced peptide formation from amino acids has been investigated by variation of the inorganic salt delivering Cl^- ions and providing the dehydrating effect. Chloride anions proved to be essential to prevent chelate complexation of the second amino acid. Upon exchange of sodium by other alkali- or alkaline-earth elements, peptide formation is still observed. The dipeptide yields are mainly determined by two factors: on the one hand the pH of the solution should be below 3 to prevent Cu^{II}-catalysed peptide hydrolysis and give an optimum species distribution for peptide formation, and above 2 to keep proton-catalysed peptide hydrolysis as low as possible; on the other hand by the concentration of the inorganic salt for removing water from the reaction and thus shifting the equilibrium towards the peptide side. The hydration enthalpies of the cations are the determining factor for the initial rate of peptide formation and lead to the series $Mg^{2+} > Ca^{2+} > Ba^{2+} > Na^+ > NH_4^+ > K^+ > Cs^+$. In the long run the initial advantage of divalent cations is overruled by stronger hydrolysis due to the lower pH of their solutions. The ion NH_4^+ is atypical, apparently due to its buffering ability.

Since Miller's [1] famous experiments attempting to discover and explain how precursors of amino acid-based life formed and evolved on Earth, various models, depending on the state of knowledge about the conditions on the primitive Earth (4.5–3.6 billion years ago), have been proposed. It was found that the initially assumed reducing methane–ammonia atmosphere would have been very short-lived because of the intense UV irradiation (NH_3 would have been destroyed within *ca.* 3×10^4 years and even more rapidly washed out by rain; CH_4 would be converted into higher hydrocarbons, which have not been found in geological rock samples).[2] Therefore, a neutral to mildly oxidizing primitive secondary atmosphere, consisting of the same compounds as present day volcanic gases (H_2O, CO_2 and in lower concentrations N_2, SO_2, HCl, Ar, H_2S, NH_3, CH_4 and H_2),[3,4] is believed to have prevailed during the initial evolution of life. The formation of amino acids in this atmosphere can still be explained by Miller-type or Fischer–Tropsch-type experiments.[5–10]

Former proposals for peptide formation in aqueous solution under primitive Earth conditions are not really satisfactory because of the need for larger amounts of condensation agents,[11–16] the formation and stability of which on the primitive Earth are questionable. The condensation of amino acids in the molten state[17] within a well defined (for amino acids) non-harmful temperature range and the absence of other compounds such as inorganic salts requires unrealistic conditions, too. Condensation of amino acids on the surface of clays[18–27] leads to strongly varying and often ambiguous results, depending on the method of peptide identification. Some attempts were also made to form peptides in hypothetical 'sea media'[28–31] at elevated temperatures over longer times. The resulting products were polymers, named 'marisomes', which however have barely any similarity with biologically relevant compounds.

The initial idea for 'salt-induced peptide formation' came from theoretical considerations. Monte Carlo simulations of concentrated sodium chloride solutions[32–35] had shown that, at salt concentrations above 3 mol dm^{-3}, sodium is partially unsaturated in water in its first hydration shell, forming thus a potential dehydrating force able to induce the condensation of amino acids by binding the water formed in its hydration shell. Furthermore, *ab initio* calculations on model complexes of metal cations binding suitable ligands had shown that such a complexation could considerably lower the activation barrier for the ligand–ligand interaction.[36–38] As sodium chloride ought to have been the most abundant salt in the primitive ocean, experiments at high concentration of this salt containing other metal ions possibly providing a catalytic effect were carried out. Amongst a large number of cations, including alkaline-earth- and transition-metal ions, Cu^{II} was found to be the most active,[39,40] leading to a series of further investigations on this new peptide formation reaction.[41–45] The optimum conditions for the condensation of glycine were found at high concentrations of glycine (1.0 mol dm^{-3}), and 5 mol dm^{-3} NaCl with a ratio of glycine:Cu^{II} of 2:1. Variation of temperature showed that 80–90 °C is optimal both under air and in an inert (argon) atmosphere. Under such conditions, also condensation of alanine, aspartic acid and glutamic acid takes place. In mixed systems of Gly and another amino acid, formation of all mixed dipeptides of Gly with Ala, Val, Asp, Glu and Pro with 0.1–10% of the initial amino acid incorporated into the peptide could be observed. The relative reactivity and thus the peptide yield is influenced by several factors. Glycine, the simplest amino acid, reacts most readily, apparently because of its mobility and ease of complex formation with copper(II), whereas valine does not form divaline, probably because of its immobility in the very polar solution matrix caused by its apolar side-chain and for steric reasons in the copper complex formation. Another factor determining peptide yield is the relative electro- and nucleo-philicity of the two amino acids in the system. Glycine is the electrophilic reaction partner in systems with Ala and Val, but the nucleophilic one in systems with Asp and Glu, thus Gly-Ala, Gly-Val, Asp-Gly and Glu-Gly are formed primarily by salt-induced peptide formation. Subsequent sequence inversion *via* substituted diketopiperazines forms Ala-Gly, Val-Gly, Gly-Asp and Glu-Asp to a certain extent, depending on the kinetic and

* *Non-SI units employed:* atm = 101 325 Pa, cal = 4.184 J.



Scheme 1 Postulated reaction mechanism for the salt-induced peptide formation

thermodynamic stability of the peptides. In addition to this sequence-inversion reaction, the relative concentrations of the amino acids have a considerable influence on the product distribution.[42]

In order to be ascribed prebiotic relevance, a chemical reaction has to fulfil some conditions, such as availability of educts and reaction conditions on the primitive Earth (especially temperature, solvent and atmosphere), in agreement with current knowledge of the environment present during molecular evolution. The salt-induced peptide formation reaction seems to fulfil these conditions: the availability of Cu^{II} is indicated by the presence of the so-called 'green zones' consisting of azurite and malachite in precambrian rock formations. Sodium chloride and water were ubiquitously present; temperatures around 80 °C are most reasonable at times after water had condensed on Earth, and the presence of 10^{-35} atm oxygen needed to guarantee a considerable amount of Cu^{II} (ref. 46) is also in agreement with estimations of the oxygen content of the primitive atmosphere, ranging from 10^{-1} to 10^{-15} pal (present atmospheric level).[47–50] Results of experiments with α- and β-alanine and α-, β- and γ-aminobutyric acid[45] supported the prebiotic relevance, since α-amino acids, dominant in all natural peptides, are preferentially incorporated into peptides due to the higher stability of their complexes with Cu^{II}.

Theoretical and experimental investigations were performed to obtain more knowledge about the underlying reaction mechanism. Determination of complex-formation constants for Cu^{II} with amino acids in 5 mol dm^{-3} NaCl solution[51] demonstrated the presence of chlorocuprate species in agreement with the observation that the 'active' solutions are green and not blue, as they should be if they contained only copper(II)–amino acid chelate complexes. Furthermore, a Monte Carlo simulation of a solution containing 0.5 mol dm^{-3} $CuCl_2$ and 5 mol dm^{-3} NaCl supplied a detailed species distribution, from which it could be seen that the majority of Cu^{II} is present in the form of a hydrated $CuCl^+$ species.[52] These findings lead to the postulation of the reaction mechanism in Scheme 1. The hydrated chlorocuprate species binds one amino acid in chelate form (**1**), and a second one in its protonated form only through the carbonyl oxygen (**2**), as further chelate bonding is hindered by the chloride ligand. *Ab initio* calculations of this reactive species showed in addition that it is very stable compared to other species.[53] In such a complex the amino acids are already polarized in a suitable way to enable nucleophilic attack of the chelate-bonded amino nitrogen at the carboxyl carbon (**5**). Since the complexation of the peptide is weaker than that of the amino acid, the peptide formed in that way is released to the solution, and the reaction can start with two new amino acids or an amino acid and a peptide. As sodium does not play a distinct role in the postulated reaction mechanism it should be exchangeable by other cations. In this work the salt influence was investigated in order to get more data about the variability of the reaction. A possible perturbation of the reaction by cations other than sodium, decreasing the reaction's prebiotic probability, was examined too. Furthermore, the water-removing effect, not definitely proven in former experiments, could be investigated easily considering the different hydration energies of the cations.

Experimental

The compounds NH_4Cl, KCl, CsCl, $MgCl_2·6H_2O$ and $CaCl_2·2H_2O$ were obtained from Fluka, NaCl, $BaCl_2·2H_2O$, $CuCl_2·2H_2O$ and KH_2PO_4 from Merck, glycine, di- and triglycine in analytical grade quality from Senn Chemical, and sodium hexanesulfonate from Sigma. All chemicals were used without further purification.

Solutions were prepared in distilled water and experiments carried out in two different ways. In long-time experiments the solutions were heated in a glass flask without stirring on a thermostatically controlled sand-bath with reflux cooler under air, the cooler ends being closed with aluminum foil to prevent contamination. For short-time experiments ten vials (2 cm^3) containing reaction solution (1 cm^3) were sealed gas-tight and heated in an oven at 80 °C. At regular intervals 20 μl of the solution were taken out, diluted with distilled water (1 cm^3) and analysed using a Hewlett-Packard 1090M liquid chromatography system with diode-array detection. Amino acids and peptides were monitored after separation on a Shannon Hypersil column (ODS 200 × 2.1 mm, 5 μm) at 195 nm using a mobile phase consisting of 50 mmol dm^{-3} KH_2PO_4 + 7.2 mmol dm^{-3} sodium hexanesulfonate, pH 2.5 adjusted by H_3PO_4, with a flow rate of 0.35 cm^3 min^{-1} and at a

Table 1 Peptide formation in the system 0.5 mol dm^{-3} Cu^{II}, 1.0 mol dm^{-3} Gly at varying concentrations of different monovalent cations at temperatures between 80 and 90 °C after selected reaction times

Conditions		t/h	% $(Gly)_2$	% $(Gly)_3$	Conditions		t/h	% $(Gly)_2$	% $(Gly)_3$
Na^+	3.33 mol dm^{-3}	97.3	2.77	0.064	NH_4^+	3.00 mol dm^{-3}	91.4	2.55	0.101
	81 °C	245.8	5.60	0.170		89 °C	264.7	4.09	0.138
		509.2	6.73	0.298			503.8	4.58	0.165
	5.00 mol dm^{-3}	97.6	4.16	0.178			744.9	3.31	0.131
	82 °C	246.2	6.89	0.323		5.00 mol dm^{-3}	91.4	3.94	0.173
		509.6	7.56	0.425		87 °C	264.7	5.92	0.250
K^+	3.33 mol dm^{-3}	101.4	2.78	0.077			504.8	6.29	0.297
	85 °C	195.3	4.59	0.155			745.0	4.90	0.250
		533.4	7.66	0.323	Cs^+	3.00 mol dm^{-3}	91.4	0.383	0.023
	5.00 mol dm^{-3}	91.4	3.94	0.206		80 °C	264.6	1.41	0.106
	90 °C	264.8	6.46	0.257			503.7	2.32	0.199
		503.8	7.88	0.406			744.9	2.05	0.181

column temperature of 40 °C. Peptides were identified and quantified by comparison with the retention times and peak areas of analytical grade reference substances.

Results and Discussion

The System Cu^{II}–Gly–MCl (M = Na, K, NH_4 *or* Cs).—Experiments with different monovalent cations were carried out at concentrations of 3.0, 3.33 and 5.0 mol dm^{-3} inorganic salt. The peptide yield at selected reaction times is shown in Table 1. In all experiments formation of di- and tri-glycine could be observed. Caesium, which has the lowest hydration enthalpy,[54] gives the lowest peptide yield over several weeks, whereas the yield obtained with Na, K and NH_4 are similar. The higher the concentration of inorganic salt the higher is the amount of peptide formed, in accordance with the results found for sodium chloride. The maximum peptide concentration at 80–90 °C was found after approximately 500 h of reaction, depending on the pH of the reaction solution and thus on the kind of cation used. This influence is small in the case of monovalent cations, except for ammonium, which is a special case due to its buffering ability. In order to separate the contributions of pH, acidic hydrolysis and the dehydrating effect, short-time experiments were carried out. Under the assumption that at the very beginning of the reaction the diglycine concentration is zero, the second term of the rate law, which describes the acidic hydrolysis of the peptide, can be neglected, and all complexes can be described in terms of concentration of Cu^{II}, Gly, Cl^- and H^+. Hence the rate law simplifies to equation (1). As the experiments were carried

$$d[Gly–Gly]/dt = k'[Cu^{2+}]^a[GlyO^-]^b[Cl^-]^c[H^+]^d \quad (1)$$

out at constant concentrations of Cu^{II}, Cl^- and Gly and no additional species are formed in the pH region of interest (1.0–3.0), the slope of [Gly–Gly] *versus* time just depends on the rate constant, which is also a function of the dehydrating ability of the cation and the pH. The exponent *d* of $[H^+]$ in the rate law was determined from a series of experiments with sodium chloride at different pH. Calibration of the measured pH to real pH was achieved by titration of a 3 mol dm^{-3} sodium chloride solution with 0.1 mol dm^{-3} HCl in 3 mol dm^{-3} NaCl to correct for the high ionic strength of the system. The results of these experiments are shown in Fig. 1. The initial slopes were plotted *versus* the pH of the system, and the dashed line in Fig. 2 representing the dependence of the diglycine formation per hour on pH was obtained. The plot of [Gly–Gly]/time unit *versus* $[H^+]^2$ is as shown by the solid line in Fig. 2. After pH calibration of KCl and CsCl solutions, a linear correlation between hydration enthalpy and peptide formation per hour (which is directly proportional to the rate constant *k'*) could be obtained as shown by the solid line in Fig. 3. This finding

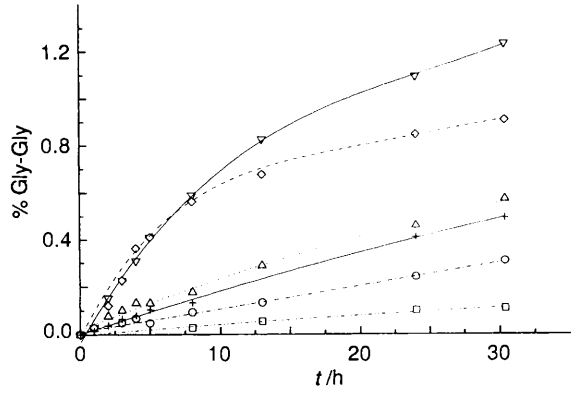


Fig. 1 Diglycine formation in the system 1.0 mol dm^{-3} glycine, 0.5 mol dm^{-3} $CuCl_2$ and 3 mol dm^{-3} NaCl at different pH and 80 °C. pH 1.93 (◇), 2.18 (▽), 2.52 (△), 2.60 (+), 2.74 (○) and 3.04 (□)

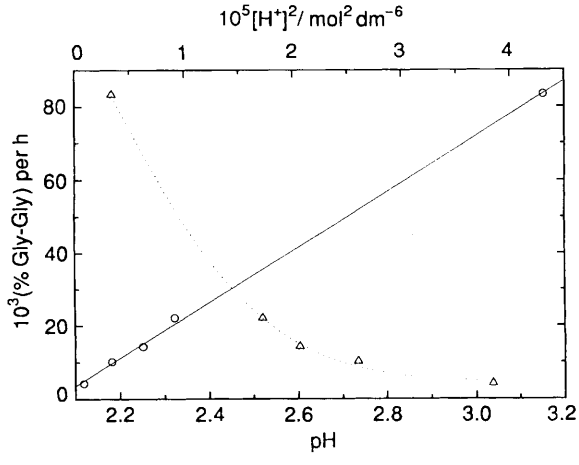


Fig. 2 The pH dependence of diglycine formation (– – –) and a plot of diglycine formation per hour against $[H^+]^2$ (——)

demonstrates the water-removing effect of the cations, the initial idea for the salt-induced peptide formation reaction postulated from theoretical work.

The System Cu^{II}–Gly–MCl_2 (M = Mg^{2+}, Ca^{2+} *or* Ba^{2+}).—As the chloride concentration had to be kept constant, the experiments with divalent cations were carried out at concentrations of 1.5 and 1.67 mol dm^{-3}, in the case of $CaCl_2$ additional experiments were done at a concentration of 2.5 mol dm^{-3}. In Table 2 the results of long-time experiments at temperatures from 80 to 90 °C are shown. No Gly-Gly-Gly

Table 2 Peptide formation in the system 0.5 mol dm^{-3} Cu^{II}, 1.0 mol dm^{-3} Gly at varying concentrations of different cations at temperatures between 80 and 90 °C after selected reaction times

Conditions		t/h	% $(Gly)_2$	Conditions		t/h	% $(Gly)_2$
Mg^{2+}	1.67 mol dm^{-3}	97.52	0.849	Ca^{2+}	1.50 mol dm^{-3}	91.6	1.99
	83 °C	246.1	1.16		89 °C	264.9	3.01
		509.5	1.32			504.0	3.22
Ca^{2+}	1.67 mol dm^{-3}	101.3	2.57		2.50 mol dm^{-3}	101.6	1.47
	85 °C	195.2	3.51		80 °C	195.5	1.81
		533.3	4.79			533.6	2.47
Ba^{2+}	1.67 mol dm^{-3}	97.3	1.82		2.50 mol dm^{-3}	91.6	1.54
	80 °C	245.9	4.33		87 °C	265.0	1.84
		509.3	5.75			504.0	2.07

Table 3 Stability constants used to calculate the species distribution of Fig. 4

Species [51]	log K	Species [51]	log K	Species	K
Gly	10.60	$CuCl^+$	-0.28	$[CaCl]^+$ [57]	0.60
$HGly^+$	13.58	$CuCl_2$	-1.34	$CaCl_2$*	0.90
$[Cu(GlyO)]^+$	8.72	$[Cu(GlyO)Cl(H)]^+$	11.48	$[Ca(GlyO)]^+$ [55]	$10^{0.55}$
$[Cu(Gly)]^{2+}$	11.40	[Cu(GlyO)Cl]	8.68		
$[Cu(GlyO)_2]$	15.75	$[Cu(GlyO)_2Cl]^-$	15.66		

* This value, not available in the literature, was estimated at 0.3 units higher than the $[CaCl]^+$ stability constant, because this is the usual difference for the complexation of the second ligand in other MX_2 systems.

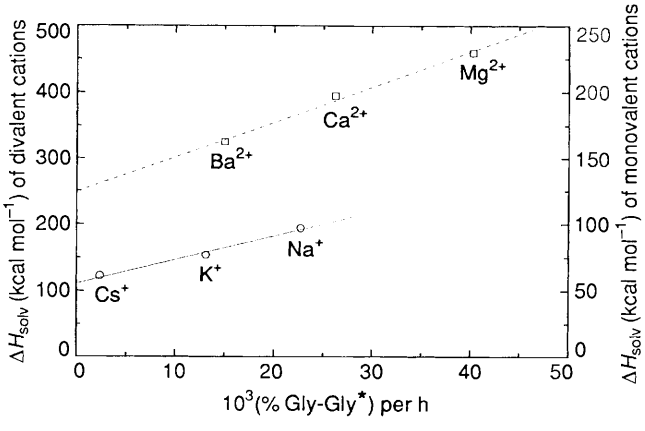


Fig. 3 Correlation between the rate of diglycine formation and hydration enthalpy of the system 1.0 mol dm^{-3} glycine, 0.5 mol dm^{-3} $CuCl_2$ and 3.0 mol dm^{-3} of NaCl, KCl or CsCl (——) and 1.5 mol dm^{-3} $MgCl_2$, $CaCl_2$ or $BaCl_2$ respectively (– – –)

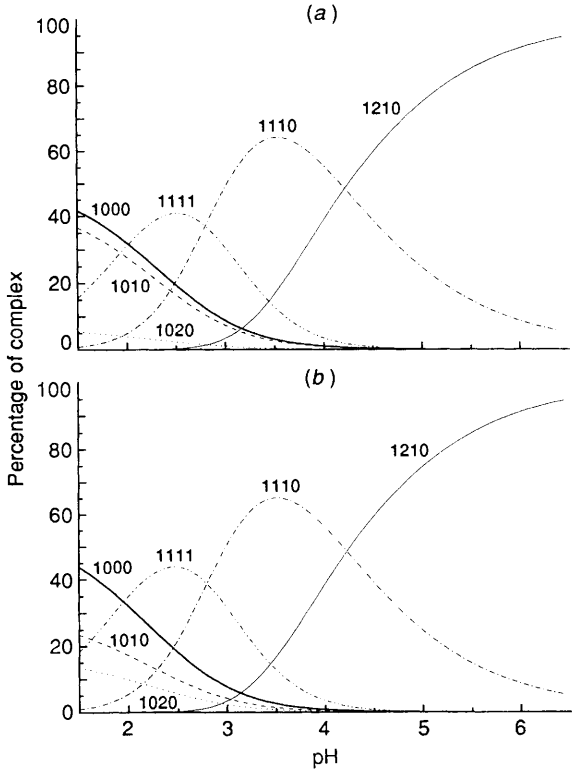


Fig. 4 Species distribution in the systems 1.0 mol dm^{-3} glycine, 0.5 mol dm^{-3} $CuCl_2$ and (*a*) 3 mol dm^{-3} NaCl or (*b*) 1.5 mol dm^{-3} $CaCl_2$; the numbers indicate *pqrs* as in $Cu_p(GlyO)_qCl_rH_s$

yields are listed because they were below 0.15% for all samples. The lowest and highest yields were obtained in the cases of Mg^{2+} (< 0.06%) and Ba^{2+} (0.152%, Gly–Gly–Gly after 509 h). The lower yields of diglycine in long-time experiments obtained with divalent cations can definitely not be ascribed to the lack of dehydrating power, they are clearly a consequence of the lower pH of the solutions compared to those of the monovalent cations. The lower yield obtained with 2.5 compared to 1.5 mol dm^{-3} $CaCl_2$ is in contrast to the results found for all monovalent cations, as a higher cation concentration should induce the binding of more water and in this way shift the amino acid peptide equilibrium more towards the peptide side. However, an increase in the concentration of the divalent cations decreases the pH, which is not the case for monovalent cations, resulting in a higher amount of acidic hydrolysis. In addition, the lower pH produces a different species distribution, which is influenced also by the existence of M^{2+}–Gly [55] complexes, negligible in the case of monovalent cations. Fig. 4(*a*) and 4(*b*) show the difference in the species distributions of sodium and calcium chloride systems using the stability constants given in Table 3 and calculated with the program SPE.[56] A larger difference can be seen in the concentration of chlorocuprate species without glycine. The concentration maximum of the 1111 [CuCl(GlyO)H] complex, which is assumed to be the reactive species, is 7% higher in the sodium system. In addition to this lower maximum (located at pH 2.5), the equilibrium pH of the calcium system is much lower, thus shifting the actual species distribution from the almost optimum value realized in the sodium chloride system (pH 2.6) to pH 2.0, where only 31% of

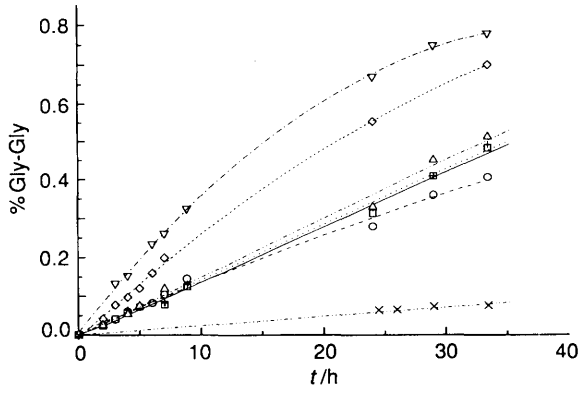


Fig. 5 Rate of peptide formation at 80 °C in the system 1.0 mol dm^{-3} glycine, 0.5 mol dm^{-3} $CuCl_2$ and 1.5 mol dm^{-3} MCl_2 or 3.0 mol dm^{-3} MCl for all cations: NH_4^+ (□), Na^+ (+), K^+ (○), Cs^+ (×), Mg^{2+} (▽), Ca^{2+} (◇) and Ba^{2+} (△)

Cu^{II} is present as the 1111 complex. As the square of the proton concentration is proportional to the reaction rate, this unfavourable species distribution is initially compensated by the lower pH, increasing the rate of formation by a factor of 1.8. This refers only to the first hours of reaction, afterwards peptide decomposition by acidic hydrolysis dominantly influences the yield.

Short-time experiments with 1.5 mol dm^{-3} solutions of the divalent cations were performed in order to compare the reaction rate with that in the case of monovalent ions. Fig. 5 shows that peptide formation occurs faster for all M^{2+}, Ba^{2+} having a value similar to that of sodium. In the case of Mg^{2+} the pH is already sufficiently low that a linear slope can only be observed for the first 5 h of reaction; after that hydrolysis of diglycine becomes more and more important so that the equilibrium value of 1% diglycine is reached after 50 h of reaction. The dependence of the peptide production per hour (not corrected for pH) on the hydration enthalpy can be recognized from the dashed line in Fig. 3.

First experiments with Cu^{II} and L- or D-Ala, but under different conditions, had indicated a slight preference of L-Ala in reaction rate and yield; this result did not prove statistically significant in a limited series of parallel experiments,[44] but seems worthwhile for further studies under the conditions used in the investigations presented here.

Conclusion

Peptide formation is observed in all systems containing Cu^{II}, glycine and higher concentrations of chloride salts of alkali- and alkaline-earth metals, showing the generality of this reaction. The diglycine yields at 80 °C and 1.0 mol dm^{-3} Gly, 0.5 mol dm^{-3} $CuCl_2$ and 1.5–3 mol dm^{-3} MCl vary from 1 to 9%, mainly depending on the pH of the solution and thus on the cation. Sodium, which should definitely have been the most abundant element in solution on the primitive Earth, provides the optimum pH favouring the formation of the reactive $[Cu(Gly)Cl]^+$ complex and preserving the highest yields over a long time.

The rate of the reaction is proportional to the square of the proton concentration. This explains the observation that the initial rate of peptide formation with divalent cations is faster than in the case of monovalent cations owing to their lower pH. Subsequent acidic hydrolysis annihilates this initial advantage.

The dehydrating effect of the cation, as postulated from theoretical investigations, could be demonstrated to be directly related to hydration enthalpies within the series Na^+, K^+, Cs^+ and Mg^{2+}, Ca^{2+}, Ba^{2+}.

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