EVAPORATION CYCLE EXPERIMENTS – A SIMULATION OF SALT-INDUCED PEPTIDE SYNTHESIS UNDER POSSIBLE PREBIOTIC CONDITIONS

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Abstract. Evaporation cycles applied to dilute solutions of amino acids, Cu(II) and NaCl lead to peptides within 1–3 days. This simulation of possible coastal or laguna processes in a primitive earth environment gives further indications towards the relevance of the salt-induced peptide formation reaction in chemical evolution. The experiments were successfully applied to glycine, alanine, aspartic and glutamic acid. Besides isolated amino acids, also their mixtures with glycine as reaction partner were studied, leading to peptides for all of the aforementioned substances, as well as for valine and proline, which do not dimerize alone. Sequence preferences and some conservation of optical purity were observed.

1. Introduction

The recently discovered peptide formation in aqueous solution, induced by copper(II) and high NaCl concentrations, can be considered as the hitherto most simple process that could have led to the evolution of peptides under primitive earth conditions (Schwendinger, 1989; Rode 1989; Schwendinger, 1991; Schwendinger, 1992). The easy availability of the inorganic condensation reagents according to geological data (Cloud, 1973; Hay, 1984) and the relatively good yields of this reaction, but also its applicability to different amino acids as glycine, alanine, valine and aspartic acid are in favour of this hypothesis. The selectivity of the reaction to utilize α-amino acids for the peptide synthesis more than their β-analogues (Schwendinger, submitted) is another indication for this hypothesis' credibility.

With a few exceptions, the salt-induced peptide synthesis has been monitored so far under experimental conditions with constant volume and temperature and with high concentrations of salts and amino acids, as such conditions allowed also electrochemical monitoring (Eder, 1992) and investigations on the mechanism of the reaction, which seems to be satisfactorily explained so far (Rode, 1990; Tauler, 1990; Schwendinger, 1992). When discussing possible prebiotic scenarios, however, constancy of reaction volume and continuous high concentration of the reactants seem to be less 'realistic' than a periodically changing 'environment' and initially low concentrations. One of the main aspects of the present work has been, therefore, the investigation of the reaction under such conditions, which were established by starting from low reactant concentrations and the use of open systems, allowing evaporation within a period of 24 hours, and subsequent redissolution in water. This evaporation cycle process should, after several repetitions, allow one to conclude,

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whether peptides are formed in similar yields as under constant laboratory conditions.

Another important aspect for the credibility of the possible prebiotic relevance of the reaction is its universal applicability to a wide variety of amino acids. For this reason, two more amino acids were included in the investigation, namely glutamic acid and proline, and studied by both of the aforementioned types of experiments.

In order to investigate sequence preferences in analogy to previous works (Schwendinger, 1991; Schwendinger, 1992; Eder, 1992), also mixtures of all amino acids with glycine have been studied.

Finally, interest was also focused to the question of racemization in the course of the reaction. A larger number of experiments has been carried out therefore with the enantiomers of alanine in order to obtain data for this aspect, which seems also of importance with respect to the reactions' prebiotic significance.

2. Experimental

2.1. Reaction system

The experiments were run with L-amino acids only, except for alanine. Amino acids, $CuCl_2$ and NaCl were purchased in analytical grade quality from Senn Chemicals and Fluka Co., respectively.

For the evaporation experiments, 300 ml of each solution were prepared, with initial amino acid, copper(II) and NaCl concentrations of 0.04 M, 0.04 M, and 0.5 M, respectively. For the additional experiments with constant volume performed with glutamic acid and proline, 100 ml solution with the concentrations 0.4 M/ 0.4 M/5 M were used.

Temperature was adjusted between 80 °C and 95 °C (sandbath), which led to complete evaporation of the solvent within 20–24 hours, after which the next cycle was started by adding 300 ml of distilled water.

The experiments with constant volume were performed under identical conditions as in previous works (Rode, 1990).

2.2. Analytical methods

For analysis, the solid residue was dissolved in 300 ml of distilled water from which 80 μl sample were taken, which were diluted by 500 μl water before injection of the sample into the HPLC apparatus.

Reference peptides were purchased in p.a. quality from Senn and Sigma Co. as far as commercially available. All aspartyl-aspartic acid dipeptides had to be synthesized by ourselves according to standard procedures (Houben-Weyl, 1974).

Analysis was carried out by HPLC on a HP 1090M LC system using different methods for the various combinations of amino acids. On the one hand, a Shannon Hypersil column (ODS 5μm/200*2.1 mm) was used with 50 mM KH_2PO_4/7.2 mM $C_6H_{13}SO_3Na$, pH 2.5 adjusted by H_3PO_4, as mobile phase for the separation of glycine and its oligomers (Rode, 1989). For valine, 5% of acetonitrile were added to the mobile phase (Eder, 1992). In the case of proline and glycine/proline the

TABLE Ia

Evaporation experiments of: a) 0.08 M glycine and 0.04 M Cu(II) in 0.5 M NaCl solution (b) 0.16 M glycine and 0.08 M Cu(II) in 1.0 M NaCl solution. All results are percent of initial amino acid concentration

Reaction cycle	Product amounts	
	$(Gly)_2$	$(Gly)_3$
1	2.76	0.20
2	3.92	0.26
3	6.20	0.43
4	6.57	0.57

TABLE Ib

Evaporation experiments of: a) 0.08 M glycine and 0.04 M Cu(II) in 0.5 M NaCl solution (b) 0.16 M glycine and 0.08 M Cu(II) in 1.0 M NaCl solution. All results are percent of initial amino acid concentration

Reaction cycle	Product amounts	
	$(Gly)_2$	$(Gly)_3$
1	3.44	0.23
2	5.66	0.36
3	7.71	0.43
4	8.56	0.43

pH of the mobile phase was adjusted to 2.0. On the other hand, pre-column derivatization with o-phthalaldehyde (OPA)/3-mercaptopropionic acid (MPA) (Schuster, 1988) and separation of derivatized amino acids and peptides with an HP Amino-Acid-Column 200*2.1 was performed for all other systems. In the case of aspartic acid, glutamic acid and their mixed systems with glycine, mobile phase and gradient had to be changed as in previous works (Eder, 1992).

Only all possible dimers were monitored, as no reference substances for higher peptides were available at this time. It is also likely that the separation and detection of tri- and higher peptides can be achieved only after having performed another separation step by gel permeation chromatography (GPC).

3. Results and Discussion

3.1. Single amino acids

3.1.1. *Glycine*

Although evaporation experiments for glycine have been reported previously, showing the formation on oligopeptides up to the hexamer (Rode, 1990), control experiments have been performed for comparison, focussing to the yield of dimer and trimer only. The results are shown in Tables Ia and Ib. The yields are high, and it is interesting to observe that the percentage of the trimer is higher when the initial amino acid and salt concentrations are lower.

3.1.2. *Alanine*

For this amino acid, L-Ala and D-Ala were used as starting materials, in order to observe racemization processes carefully. For this reason, and because it has been observed already in constant-volume experiments that ala dimerization can sometimes be inhibited and thus lead to strongly varying yields (Schwendinger, 1991), each experiment was repeated 5–7 times instead of the usual 2–3 parallel experiments, so that statistical averages could be given in the corresponding Tables IIa and IIb.

TABLE IIa

Evaporation experiments of 0.16 M L-alanine and 0.08 M Cu(II) in 1.0 M NaCl solution: (a) L-Ala (b) D-Ala

Reaction cycle	Product amounts		Ratio
	$(ala)_2^{+/-}$	$(ala)_2^{+/+}$	x
1	0.04±0.03	0.09±0.07	0.68
2	0.86±0.20	0.98±0.22	0.53
3	1.33±0.26	1.38±0.20	0.51
4	1.45±0.25	1.53±0.28	0.51
5	1.76±0.21	1.86±0.28	0.51
6	1.97±0.25	2.07±0.32	0.51

$(ala)^{2++}$ denotes L-Ala – L-Ala plus D-Ala – D-Ala
$(ala)^{2+-}$ denotes L-Ala – D-Ala plus D-Ala – L-Ala
$x = \%\ (Ala)_2^{+/+} / \%\ (Ala)_2^{+/-} + (ala)_2^{+/+}$

TABLE IIb

Evaporation experiments of 0.16 M L-alanine and 0.08 M Cu(II) in 1.0 M NaCl solution: (a) L-Ala (b) D-Ala

Reaction cycle	Product amounts		Ratio
	$(ala)_2^{+/-}$	$(ala)_2^{+/+}$	x
1	0.07±0.06	0.17±0.09	0.70
2	0.69±0.32	0.81±0.31	0.54
3	1.10±0.40	1.22±0.38	0.52
4	1.35±0.39	1.46±0.41	0.52
5	1.39±0.44	1.50±0.49	0.52
6	1.65±0.53	1.86±0.69	0.53

$(ala)^{2++}$ denotes L-Ala – L-Ala plus D-Ala – D-Ala
$(ala)^{2+-}$ denotes L-Ala – D-Ala plus D-Ala – L-Ala
$x = \%\ (Ala)_2^{+/+} / \%\ (Ala)_2^{+/-} + (ala)_2^{+/+}$

The results show that starting from optically pure amino acids, a slight excess of the corresponding $(L\text{-}Ala)_2$ or $(D\text{-}Ala)_2$ is observed even after 6 cycles. The fact that no complete racemization process occurs, despite of considerable changes in pH during the reaction (increasing acidity during evaporation due to Cu(II) until pH~2), seems to be an important aspect for the prebiotic significance of the salt-induced peptide formation reaction. Tables IIa and IIb also seem to indicate that the peptide formation reaction gives better yields with L-Ala than D-Ala.

Although this would be a most intriguing result, the standard deviations do not let it appear significant. It would also be difficult to find a reasonable argument for this preference of the L-stereomer (hinting towards the exclusive use of L-amino acids in natural proteins); the only possibility would be an assymmetric centre in the chlorocuprate – amino acid complexes responsible for the peptide formation reaction. This matter maybe worthwile further investigations, however.

TABLE III

Evaporation experiment of 0.08 M aspartic acid and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction cycle	Product amounts	
	β-asp-asp	L-Asp – L-Asp
1	1.73	0.38
2	1.29	0.33
3	1.23	0.23
4	1.57	0.32

3.1.3. *Aspartic Acid*

Condensation to peptides takes also place readily with L-aspartic acid (cf. Table III). In the case of L-Asp, a preference is found for the formation of the β-asp-asp dimer, the second observed peptide is optically pure L-Asp – L-Asp, whereas the racemic forms D-Asp – L-Asp and L-Asp – D-Asp cannot be detected within the observed 4 cycles. This appears to be another interesting aspect, as racemization does not develop to a large extent during peptide formation with aspartic acid.

3.1.4. *Valine*

As in all previous experiments with constant reaction volume (Eder, 1992), divaline cannot be found also in the evaporation experiments. Val peptides are formed, however, with other amino acids (Schwendinger, 1992; see also section 2).

3.1.5. *Glutamic Acid*

This essential amino acid has not been investigated before within the salt-induced peptide formation framework, and thus both types of experiments were performed, in the form of evaporation cycles and with constant volume as in previous investigations. The results are collected in Tables IVa and b. With constant reaction volume, the two main products are γ-glu_2 and α-glu_2. The latter forms more readily and in larger amounts, but is decomposed (oxidized) after 2 weeks of reaction time. In the evaporation experiments, the 'nonbiological' dimer is formed faster and in higher yield, but the important α-dimer seems to be stable over all observed cycles; the equilibrium concentrations of both peptides are reached already after the second cycle.

3.1.6. *Proline*

Proline is the first nonaliphatic amino acid studied for its ability to deliver peptides under these reaction conditions. Therefore both types of experiments were carried out, as in the previous case. No formation of diproline was observed in either of the experiments, apparently for steric reasons, but the reaction of proline with another amino acid to peptides will be reported in the following section.

TABLE IVa

(a) Evaporation experiment of 0.08 M L-glutamic acid and 0.04 M Cu(II) in 0.5 M NaCl solution.
(b) Constant volume experiment of 0.6 M L-glu and 0.4 M Cu(II) in 5.0 M NaCl solution

Reaction cycle	Product amounts	
	γ-glu$_2$	α-glu$_2$
1	1.03	0.48
2	1.08	0.63
3	1.04	0.63
4	0.92	0.52
5	1.16	0.79
6	1.08	0.63

TABLE IVb

(a) Evaporation experiment of 0.08 M L-glutamic acid and 0.04 M Cu(II) in 0.5 M NaCl solution.
(b) Constant volume experiment of 0.6 M L-glu and 0.4 M Cu(II) in 5.0 M NaCl solution

Reaction time days	Product amounts	
	γ-glu$_2$	α-glu$_2$
1	tr	1.34
2	1.03	1.92
3	1.12	2.20
5	1.44	1.70
7	1.06	1.93
10	0.89	0.96
15	0.53	0
18	0.66	0

tr = traces

3.2. Mixtures of amino acids

In order to study the ability of the amino acids investigated up to now to form hetero-peptide linkages, mixtures of these amino acids with the simplest and prebiotically surely most available amino acid glycine were subjected to the same evaporation reaction conditions. For the two amino acids that have not been investigated by constant volume experiments before, Glu and Pro, also these experiments were performed. In addition to the Gly mixtures, also a mixture of the apparently less reactive acids Asp and Val was studied.

Among the glycine oligomers observed in these experiments, only the dimer has been recorded as reference substance for the comparison of yields.

3.2.1. *L-Alanine + Glycine*

3 experiments were performed, the values in Table V are the averages of them. A most interesting result is the yield of (L-Ala)$_3$, which even exceeds that of (ala)$_2$ from the first cycle (Only (L-Ala)$_3$ + (D-Ala)$_3$ could be monitored, due to the lack of available heterochiral trialanine reference substances; it can be expected therefore that the actual peptide yields are even higher, due to racemization of the initially used L-ala). As in the constant volume experiments, gly-ala is initially the prefered product, but – probably due to inversion processes involving mixed cyclic anhydrides – ala-gly dominates from the second cycle on. Equilibrium amounts are already reached after the third cycle.

3.2.2. *L-Aspartic Acid + Glycine*

When glycine is present, formation of mixed peptides seems to be favoured, as indicated by the decreasing amount of asp-asp peptides (cf. Table VI). β-asp-Gly is obtained in best yield from the second cycle on, whereas α-asp-Gly (preferentially

TABLE V

Evaporation experiment of mixed glycine and L-alanine: 0.04 M Gly, 0.04 M Asp and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction cycle	Product amounts				
	$(Gly)_2$	$(ala)_2$	$(L\text{-}ala)_3$	(Gly)-ala	ala-(Gly)
1	1.25	0.39	0.48	0.69	0.42
2	2.13	1.01	1.32	0.84	0.95
3	2.82	1.27	1.58	0.91	1.20

TABLE VI

Evaporation experiment of mixed glycine and L-aspartic acid: 0.04 M Gly, 0.04 M Asp and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction cycle	Product amounts					
	β-asp-asp	L-Asp – L-Asp	$(Gly)_2$	β-asp-Gly	asp-Gly	(Gly)-asp
1	1.63	0.49	tr	0.65	0.73	0.08
2	1.45	0.49	2.50	0.87	0.57	0.24
3	1.36	tr	3.84	1.03	0.48	0.17

tr = traces

formed in the first cycle) slightly decreases. Gly-asp is also formed, but the yield is less than half of α-asp-Gly.

3.2.3. *L-Valine + Glycine*

Divaline is not observed also in this system, but valine forms both mixed peptides with glycine (cf. Table VII). Gly-val is the prefered sequence and remains it also when equilibrium yields are reached after the fourth cycle.

3.2.4. *L-Glutamic Acid + Glycine*

Comparing the results of experiments at constant reaction volume and with evaporation cycles (Tables VIIIa and VIIIb), the advantage of the evaporation cycles for a mixed-peptide synthesis is evident. At constant volume, only γ-glu_2 and γ-glu-Gly are formed in quantitatively determinable amounts. On the other hand, when evaporation cycles are performed, the main product is Gly-glu, closely followed by the γ-glu-Gly and the biologically relevant α-glu-Gly, which is produced in equivalent amounts to γ-Gly-glu after 3 cycles. In the evaporation experiments, no homo-dimers of glu are observed; $(Gly)_2$ forms, but has not been monitored in this case for technical reasons.

3.2.5. *L-Proline + Glycine*

The results of these experiments are listed in Tables IXa and IXb. A good yield of

TABLE VII

Evaporation experiment of mixed glycine and val: 0.04 M Gly, 0.04 M L-Val and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction cycle	Product amounts		
	$(Gly)_2$	Gly-val	val-Gly
1	0.92	0.90	0.13
2	1.98	1.22	0.42
3	2.62	1.51	0.78
4	3.31	2.05	0.89

TABLE VIIIa

(a) Evaporation experiment of mixed glycine and L-glutamic acid: 0.04 M Gly, 0.04 M L-Glu and 0.04 M Cu(II) in 0.5 M NaCl solution. (b) Constant volume experiment of Gly and L-Glu: 0.4 M L-Pro and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction cycle	Product amounts		
	γ-glu-Gly	glu-Gly	Gly-glu
1	0.14	0.02	0.31
2	0.32	0.10	0.49
3	0.29	0.26	0.51
4	0.28	0.28	0.48
5	0.38	0.30	0.46
6	0.33	0.35	0.42

TABLE VIIIb

(a) Evaporation experiment of mixed glycine and L-glutamic acid: 0.04 M Gly, 0.04 M L-Glu and 0.04 M Cu(II) in 0.5 M NaCl solution. (b) Constant volume experiment of Gly and L-Glu: 0.4 M L-Pro and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction time, days	Product amounts		
	γ-glu_2	α-glu_2	γ-glu-Gly
1	0.63	0	0
2	0.77	tr	0
3	0.87	tr	0.14
5	1.09	tr	0.19
7	1.16	tr	0.25
10	1.17	0	0.35
15	0.89	0	0.30
18	0.12	0	0

tr = traces

TABLE IXa

(a) Evaporation experiment of mixed glycine and L-proline: 0.04 M Gly, 0.04 M L-Pro and 0.04 M Cu(II) in 0.5 M NaCl solution. (b) Constant volume experiment of mixed glycine and L-proline: 0.4 M Gly, 0.4 M L-Pro and 0.4 M Cu(II) in 5.0 M NaCl solution

Reaction cycle	Product amounts		
	$(Gly)_2$	Gly-pro	pro-Gly
1	1.91	0.77	0
2	3.08	1.02	0.46
3	3.40	1.09	0.71

TABLE IXb

(a) Evaporation experiment of mixed glycine and L-proline: 0.04 M Gly, 0.04 M L-Pro and 0.04 M Cu(II) in 0.5 M NaCl solution. (b) Constant volume experiment of mixed glycine and L-proline: 0.4 M Gly, 0.4 M L-Pro and 0.4 M Cu(II) in 5.0 M NaCl solution

Reaction time, days	Product amounts		
	$(Gly)_2$	Gly-pro	pro-Gly
1	0.91	0.29	0
2	1.04	0.30	0.25
5	1.49	0.40	0.44
8	2.63	0.85	1.05

TABLE X

Evaporation experiment of mixed L-valine and L-aspartic acid: 0.04 M L-Val, 0.04 M L-Asp and 0.04 M Cu(II) in 0.5 M NaCl solution

Reaction cycle	Product amounts		
	α-asp_2	L-asp–L-val	L-val–L-asp
1	0	0.65	0
2	tr	0.88	0.18
3	tr	0.58	0.25

tr = traces

Gly-pro and pro-Gly is found under both laboratory conditions, besides $(Gly)_2$. Again no evidence is seen for the formation of $(pro)_2$. At constant volume, gly-pro and pro-gly are formed in similar amounts, whereas the evaporation cycles clearly favour gly-pro.

3.2.6. *L-Valine + L-Aspartic Acid*

This mixture was subjected to evaporation experiments, in order to obtain some preliminary information, whether the salt-induced peptide formation reaction can also lead to mixed-peptide linkages between amino acids without the presence of glycine, which is known to be the 'easiest' reactant. The results, collected in Table X, show that no homo-dimers are formed, but that both val-asp and α-asp-val are readily produced, the latter in more than twice the amount of the former, in accordance with the proposed influence of nucleophilicity of the amino acids on the product formation (Schwendinger, 1992). Only all-L-reference substances were available; the given yields refer to these substances therefore.

4. Conclusions

1. The performance of the salt-induced peptide formation reaction in the form of evaporation cycles starting from relatively low educt concentrations leads to peptides within 1–3 days (cycles). Valine and proline do not dimerize themselves, but form peptides when other amino acids are present. Since the conditions of the evaporation experiments approximate a more 'realistic' scenario for prebiotic processes, these results seem to give further credibility to the significance of this peptide formation reaction on the primordial earth. As no attempts have been made so far to optimize reaction conditions, the relatively low yields do not seem to contradict this assumption, especially as these yields are obtained within a very short time.
2. The conservation of optical purity over several days and/or cycles, some indications of slight advantages for L-amino acids in the reaction, as observed in the cases of alanine, and the always observed production of the biologically relevant peptides are other arguments for the prebiotic relevance of the reaction. Side-chain branched peptides are also formed, but as preliminary investigations (Rode, 1992) have shown, they are mostly formed in secondary reactions (e.g. β-asp-Gly from asp-Gly) and their amounts are much lower, when glycine is present in excess, as can be expected for any prebiotic scenario. Some of these aspects will have to be studied therefore in more detail.
3. Among 5 aliphatic and 1 nonaliphatic amino acids studied so far within the framework of the copper(II)/NaCl – induced peptide formation reaction, no non-reactive amino acid has been found. This seems a good indication towards a general applicability of the reaction mechanism to widely varying types of amino acids.

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