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# Polymerization of Glycine and L-Phenylalanine to Short Homooligopeptides by Nitrobiuret Under Aqueous Conditions

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## ABSTRACT

*The reactions between nitrobiuret and the amino acids glycine and L-phenylalanine at pH 10, have been examined by mass spectrometry. Initially, the expected amino acyl biurets are formed, however these may react with a further nitrobiuret molecule yielding the amino acyl biuret N-carboxyanhydride. The anhydride then N-acylates a second amino acid molecule to afford a dipeptide derivative. The process is repeated and peptides up to glycine5, glycine5-biuret, phenylalanine4 and phenylalanine5-biuret have been detected. In addition the unstable N-carboxyanhydrides may also be converted to their respective ureides with loss of CO2. These reactions are of particular interest to the area of pre-biotic chemistry.*

**Introduction**

In previous studies on the binding of N-acylurea proteins to DNA (Huckett, Gordhan, Hawtrey, Moodley, Ariatti and Hawtrey, 1986; Gordhan, Ariatti and Hawtrey, 1986; Ariatti and Hawtrey, 1987; Huckett, Ariatti, and Hawtrey, 1990), in the area of targeted non-viral gene delivery, it was observed that the binding interaction consisted of electrostatic and non-electrostatic components (Figure 1a). To determine the relative importance of the non-electrostatic component we prepared, from nitrobiuret, a number of N-substituted biurets by the method of Dunnigan and Close (1953), for competition assays (Hawtrey, Ariatti and Hawtrey, 1992) (Figure 1b).

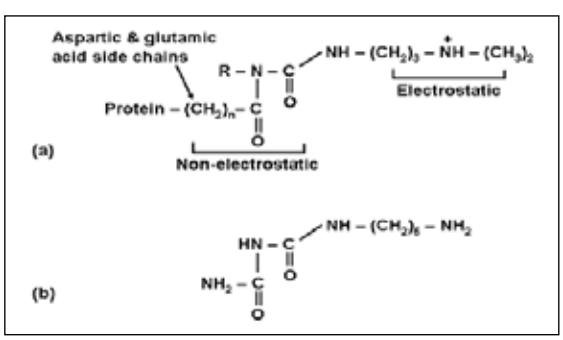


Fig. 1: (a) Structure of N-acylurea protein showing both electrostatic and non-electrostatic areas of the molecule associated with binding of DNA (R = ethyl). (b) Structure of N-substituted biuret.

Nitrobiuret (NH2-CO-NH-CO-NH-NO2) reacts with primary and secondary amines under mild conditions to give N-substituted biurets (Dunnigan and Close, 1953). The reactive species implicated here is the biuret isocyanate, a dearrangement product of nitrobiuret (Equations 1 and 2, and Figure 2).

NH2-CO-NH-CO-NH-NO2 → NH2-CO-N=C=O + N2O + H2O (1)
NH2-CO-N=C=O + R-NH2 → NH2-CO-NH-CO-NHR (2)

Results revealed that N,N'-1,6-diaminohexane dibiuret inhibited plasmid DNA-N-acylurea transferrin association by 48% at 10-4 M while the monobiuret achieved 41% inhibition (Hawtrey, Strydom, van der Merwe, Hawtrey and Ariatti, 1992). The scope of this study was to be broadened by the preparation of amino acyl biurets. Hence in the present communication we report on the reactions between nitrobiuret and two separate monobasic, monocarboxylic amino acids under aqueous conditions at pH 10. Thus glycine, which

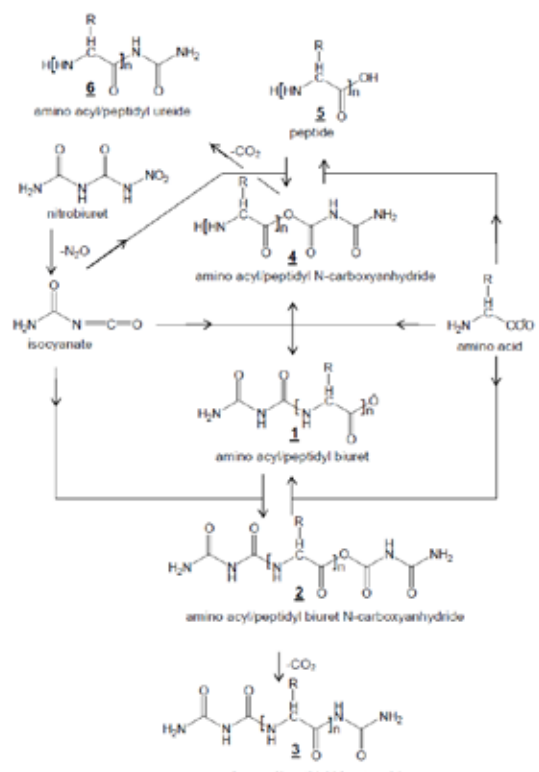


Fig. 2: Reaction products identified in the reaction of nitrobiuret with glycine and L-phenylalanine. (a), R = H; (b), R = C6H5CH2.

is achiral and has a small polar R group (H), and the L- isomer of phenylalanine with a larger non-polar R group (C6H5CH2) were selected, as two neutral amino acids with widely differing hydrophobicity (pI = 6 and 5.5 respectively), for the study. Under these conditions (pH > pKa α-amino) the unprotonated amino acid α-amino group was anticipated to be reactive to the nitrobiuret. The anticipated biurets were obtained, however several unexpected peptide products were detected. A novel, α-carboxyl group activation by nitrobiuret is implicated in this polymerization process.

**Experimental**

Biuret, L-phenylalanine and glycine were purchased from Sig-

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ma-Aldrich (St Louis, MI). Ultrapure 18 MOhm water was used throughout. Mass spectrometry was performed on an API 2000 Triple Quadrupole (Applied Biosystems) instrument equipped with a turbulon atmospheric ionization chamber. Small quantities of the reaction mixtures were taken up in methanol (HPLC grade) containing 0.1% formic acid and subjected to mass spectrometry in positive mode.

Thus L-phenylalanine (65 mg, 0.37 mmol) was dissolved in 1.2 ml H2O and 0.37 ml I N KOH. The solution (pH 10) was then added to crystalline nitrobiuret (45 mg, 0.34 mmol). Complete dissolution was achieved on refluxing for 20 minutes. The solution was then sampled for mass spectrometry.

In a similar reaction, glycine (6.8 mg, 0.09 mmol) was dissolved in 1.5 ml of H2O and 0.1 ml KOH. Following mixing the pH of the clear solution was found to be 9.5. This solution was then added to solid nitrobiuret (11.2 mg, 0.09 mmol). The mixture was stirred at 18 oC and gave a clear solution after 30 minutes (pH 7.5). After a further 20 hours at this temperature the solution was heated under reflux for 20 minutes (pH 7.5), cooled and sampled for mass spectrometry as described above.

**Results and discussion**

Analysis of reaction mixtures, described above, by mass spectrometry (Figure 2a and b) revealed that several other products of higher molecular weight had been generated in addition to the expected biuret adducts in each case. This was due to oligomerization of the amino acids to afford homopeptides and homopeptide derivatives. The reaction of biuret with the α-amino groups of glycine and L-phenylalanine results in the formation of the amino acyl biuret (NH2-CO-NH-CO-NH-CH-R1R2 [R1 = H or C6H5CH2] R2 = COO-), which serves as a primer for the addition of a further amino acid. More particularly the carboxylate of the aminoacyl biuret (Figure 2, 1) appears to be activated by nitrobiuret to give an N-carboxyanhydride (NCA) (Figure 2, 2), which reacts with the α-amino group of a free amino acid. The process is repeated several times with chain extension in the N→C direction, as is the case in protein biosynthesis, and oligomeric products up to the pentapeptide level are discernible with their corresponding biuret N-carboxyanhydrides (Figure 2, Table 1). Interestingly, Taillades, Collet, Garrel, BeuZelin, Boiteau, Choukroun and Commeyras, (1999) have demonstrated that α-amino acid internal cyclic N-carboxyanhydrides may have been good candidates for prebiotic peptide synthesis. Under aqueous conditions these compounds readily hydrolyse or polymerize to form oligopeptides. In a more recent study, this group has determined the kinetic constant of the coupling reaction of the NCA of valine with an oligovaline as a function of its degree of

Table 1: Molecular weights of products from the reaction of biuret with glycine (a); or L-phenylalanine (b).

| No. | Molecular weight[c] | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 1a/4a[a] | 161(160) | 218(220) | 275(275) | 332(331) | 389(391) |
| 1b/4b[b] | 251(252) | 398(402) | 545(547) | - | 839(842) |
| 2a | 247(245) | 304(302) | 361(363) | 418(417) | - |
| 2b | 337(336) | 484(483) | 631(629) | - | - |
| 3a | 203(201) | 260(261) | 317(315) | 374(373) | 431(429) |
| 3b | 294(295) | 440(439) | 587(585) | - | - |
| 5a | - | 132(133) | 189(189) | 246(245) | 303(302) |
| 5b | 165(166) | 312(312) | - | - | - |
| 6a | 117(117) | 174(176) | 231(232) | 288(289) | 345(345) |
| 6b | 208(206) | - | 501(500) | - | - |

a 1a and 4a have the same molecular formula (fragmentation may differ). b 1b and 4b have the same molecular formula (fragmentation may differ). c Calculated molecular weights and observed m/z values (in brackets) are to nearest whole numbers. M-1 peaks result from β-elimination of hydrogen radical. M-2 arise from loss of dihydrogen. M, M+1 and M+2 signals are also prominent.

polymerization (Plasson, Biron, Collet, Commeyras and Taillades, 2002). In the present study, formation of homopeptides (Figure 1, 5) is believed to proceed by the normal amine mechanism (NAM) in which the open N-caboxyanhydride undergoes a nucleophilic attack from the unprotected and unprotonated α-amino group of the incoming amino acid (Peggion, Terbojevich, Cosani and Colombini, 1966). Apart from molecular ion peaks, prominent M-18 peaks were observed for gly2, gly3 and gly4 (114, 171 and 227 respectively) and for phe2 and phe4 (296 and 589 respectively). Unlike the mechanism outlined for the polymerization of internal N-carboxyanhydrides by Habraken, Wilsens, Koning and Heise (2011), carbamic acid intermediates here are by-products of the polymerization and not intermediate forms of the growing peptide. N-carboxyanhydrides are unstable and may also undergo loss of CO2 to give ureides. Evidence for the formation of aminoacyl/peptidyl biuret ureides (Figure 2, 3) and aminoacyl/peptidyl ureides (Figure 2, 6) may also be found in the mass spectra of reaction mixtures (spectra available on request). The molecular weights of products identified in mass spectra and corresponding m/z values are given in Table 1.

**Conclusion**

To the best of our knowledge this is the first report of the activation of the α-carboxylate of an amino acid (or peptide) by nitrobiuret to afford NCA derivatives which lead to homopeptides upon reaction with the α-amino group of amino acids under basic conditions. The reactions observed here are of relevance to the theme of prebiotic peptide synthesis and more particularly the role of isocyanates in the oligomerization of amino acids. Further studies to explore the possible chiral preference of these oligomerization reactions are underway.

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