# The Occurrence of Reducible Compounds in an Invertebrate Structure Protein of *Buccinum undatum* (L.)

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*Summary.* Reducible compounds, probably similar to lysine-derived cross-links found in collagen and elastin, have been detected in an invertebrate scleroprotein, the egg case of *Buccinum undatum* (L.)

The egg capsules of the gastropod mollusc *Buccinum undatum* (L.) consist of an unusual structural protein, whose mode of cross linking is not understood[2]. We report that our preliminary findings suggest that this scleroprotein is at least partially stabilized by lysine-derived compounds, similar to those found to occur in vertebrate collagens and elastins[3]. Certain of these compounds contain aldehyde functional groups, and the capsule material is rich in free aldehydes as evidenced by the strong reaction with the methylbenzothiazolone hydrazone reagent of SAWICKI[4].

*Materials and methods.* Egg capsules of *B. undatum* were collected from the sea shore near Lancaster, and thoroughly cleaned before use[2]. Tritiated sodium borohydride was obtained from The Radiochemical Centre, Amersham, England. 6 mg of egg case material was reduced for 3 h with 10 μCi of tritiated borohydride at pH 7. The reduced material was thoroughly washed and then hydrolyzed in 5 ml 6 *N* HCl, at 105 °C for 24 h under nitrogen. After removal of acid in vacuo the residue was dissolved in 1.0 *N* sodium citrate buffer, pH 2.2 for amino acid analysis.

Lysinonorleucine, N-(5-amino-5-carboxypentyl) lysine, was synthesized according to FRANZBLAU et al.[5]. Amino acid analyses were carried out on a Locarte Automatic Amino Acid Analyser, fractions were collected and assayed for radioactivity in BRAY's liquid[6] using a Hewlett-Packard Tri Carb Liquid Scintillation spectrometer.

*Results.* The Figure A shows the amino acid chromatogram of tritiated egg case, together with the elution position of the synthesized Lysinonorleucine (L*). The analysis shows an abundance of acidic residues (aspartic acid 11%, glutamic acid 20%), and relatively high proportions of lysine (9%). Sulphur-containing amino acids are totally absent, ruling out disulphide linkages as a means of covalent stabilization. Figure B shows the tritium profile, indication incorporation into 5 compounds. The most basic of these (No. 5) elutes in a position identical with that of synthesized lysinonorleucine. The remaining isotopic derivatives elute pre- and post-aspartic acid (Nos. 1 and 2), pre-glycine (No. 3) and pre-tyrosine (No. 4).

*Discussion.* *B. undatum* egg capsule protein has remarkable physiochemical properties. The abundance of acidic residues and of lysine, together with the low content of glycine (10%), and absence of both proline and hydroxyproline, distinguish the capsule protein from the collagens (Gly 33%, Pro 10%, Hypro 10%, Lys 2%), and from elastin (Gly 30%, Pro 10%, Lys 0.5%). Two of the main features of the capsule material are the presence of, as yet unidentified fluorescent chromophores[2], and a high content of aldehyde functional groups.

All of the collagens and elastins so far studied contain α-amino adipic-δ-semialdehyde, a compound derived from

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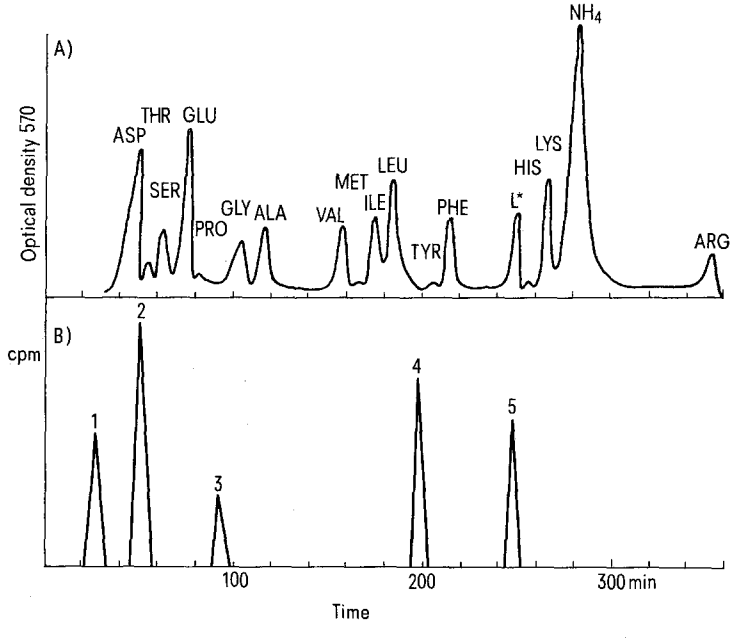


Amino acid analysis of tritiated egg case. A) Ninhydrin profile showing elution of lysinonorleucine (L*). B) Radioactivity profile.

lysine and the common intermediate in the synthesis of a range of cross linking compounds, of which lysinonorleucine is one.

Isotope peak 5 has an elution volume identical to lysinonorleucine and indicates that this compound is participating in the interchain stabilization of this unique α-protein. The elution positions of isotope peaks 3 and 4 closely resemble those of hydroxynorleucine and chloronorleucine, 2 further compounds detectable in proteins cross-linked by lysine derivatives[7]. Peaks 1 and 2 are harder to speculate upon but may be yet further Schiff bases such as dihydroxynorleucine[8] or hydrolysis artefacts[7].

The cross-linking of collagen and elastin has been shown to depend largely on the modification of lysine and hydroxylysine residues in the polypeptide chain. Oxidative 'maturation' reactions give rise to a family of unique cross links, some aldehydic in nature, and all having free amino groups (2). Thus in theory these compounds should be detectable by amino acid analysis and their lack of appearance on a ninhydrin trace is explained by the fact that less than 1 residue of cross link per 1000 amino acid residues is enough to stabilize long stretches of polypeptide, but insufficient to be detected by amino acid analysis.

The capsule protein of *B. undatum* is neither a collagen or an elastin, though it bears resemblances to both. It seems likely however that its interchain cross links may be the same as those found almost exclusively in vertebrate connective tissue and further work is being carried out to confirm this.

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## Stimulation of Ribonuclease Activity and its Isoenzymes in Germinating Seeds of Cowpea (*Vigna sinensis*) by Gibberellic Acid and Adenosine-3′,5′-Cyclic Monophosphate

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*Summary.* Application of $GA_3$ and cyclic AMP to cowpea seedlings caused a 2–3 fold stimulation of RNAase activity, together with the augmentation of RNAase isoenzymes. Inhibitor studies indicated the requirement of fresh RNA and protein synthesis for enzyme stimulation.

In recent years, controversial views have been expressed concerning the physiological and biochemical role of cyclic AMP in higher plants. Some workers have claimed the natural occurrence of cyclic AMP in plant tissues[1–3], while others were unable to demonstrate its presence[4–6]. Cyclic AMP has been regarded as a mediator of plant growth substances[7–9]. This view has been questioned[10–12], and therefore the precise relationship between plant hormone and cyclic AMP needs further investigation[13,14]. Nevertheless, exogenous application of cyclic AMP is reported to trigger the activity of several hydrolytic enzymes, such as α-amylase[15–17], protease, acid phosphatase[7], ATPase[9], isocitrate lyase[18] and tryptophan oxygenase[19]. In addition, cyclic AMP has also been implicated in RNA synthesis[20,21]. In the present communication, we report the stimulation of RNAase activity and its isoenzymes in seedlings of cowpea by the exogenous application of $GA_3$ and cyclic AMP.

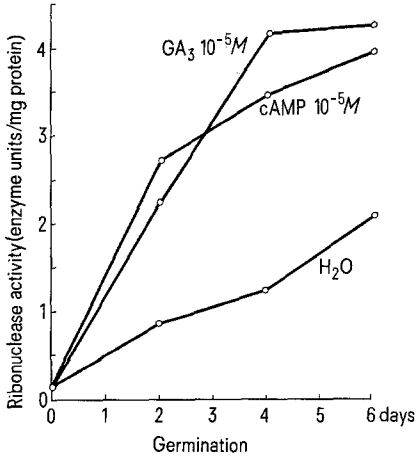


Fig. 1. Time course studies showing the enhancement of RNAase activity in cowpea seedlings in presence of $GA_3$ and cyclic AMP. The seedlings were grown at 35 ± 2°C in dark.

*Materials and methods.* The seeds of cowpea (*Vigna sinensis*) were surface sterilized with 0.1% solution of $HgCl_2$ for 5 min and germinated in dark at 35° ± 2°C. Chloramphenicol (20 μg/ml) was added to prevent bacterial contamination. Seedlings (10 g) were homogenized in 20 ml of 0.05 *M* phosphate buffer, pH 6.5 at 4°C. The homogenate was centrifuged in Sorvall at 10,000 *g* for 20 min in cold. The supernatant (crude extract) was employed for measuring RNAase activity, according to the procedure of KALNITSKY et al.[22]. Protein was estimated by the procedure of LOWRY et al.[23]. The crude extract was fractionated on acrylamide gel electrophoresis, using the technique of DAVIS[24]. The method of WILSON[25] was adopted for developing the isoenzymes of RNAase on acrylamide gels.

*Results and discussion.* Both $GA_3$ and cyclic AMP promoted 2-3-fold stimulation of RNAase activity in 96 h old seedlings of cowpea (Figure 1). Relatively high concentration of cyclic AMP ($10^{-5}$ *M*) was required to achieve stimulation of RNAase activity comparable to that observed with $GA_3$, $10^{-7}$ *M* (Table I). Addition of structural analogues of adenine (e.g., adenosine, AMP, ADP, ATP) showed only 20–70% stimulation of RNAase activity over the controls. These values were relatively less effective than cyclic AMP which gave 165% enhanced enzyme activity (Table II). Seedlings raised in presence of $GA_3$ $10^{-8}$ *M* + theophylline $10^{-5}$ *M* showed no additive effect on RNAase activity. Similarly, there was no significant additive increase in RNAase activity at optimum