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Note

Determination of adenine, adenosine and related nucleotides at the low picomole level by reversed-phase high-performance liquid chromatography with fluorescence detection

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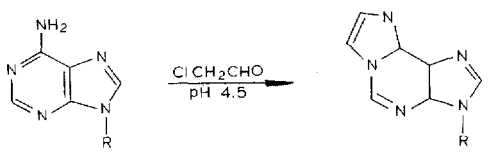
The determination of individual nucleotides and nucleotide phosphates at the low picomole level is not particularly straightforward. Conventional chromatographic techniques such as ion-exchange chromatography coupled with UV detection [1—4] are not always sufficiently sensitive, whilst enzymatic techniques, such as the bioluminescent lucerferin—luciferase reaction with adenosine 5'-triphosphate (ATP), are highly sensitive and specific but require specialist equipment and expertise and may not always be suitable for routine use.

The reaction of adenine and its analogues with chloroacetaldehyde to form the highly fluorescent 1,N^6-etheno derivatives has been described [5—8] and utilised in a number of analytical procedures [9, 10], however it has not previously been possible to separate the more polar components (i.e. the nucleotide phosphates) without resorting to ion-exchange chromatography [10]. Recently, it has been shown that reversed-phase high-performance liquid chromatography (HPLC) can be utilised to separate nucleotide phosphates [11, 12] and it was therefore decided to determine whether the 1,N^6-etheno derivatives of the adenine nucleotides could be similarly separated.



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EXPERIMENTAL

Materials

ATP, adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), cyclic AMP (cAMP), adenosine (ADO) and adenine (ADE) were purchased from Sigma (London, Great Britain). Standard solutions were made up in borate buffer (0.05 *M*, pH 10.0) and were stable at room temperature.

Chloroacetaldehyde (chloroethanol) was prepared by distillation from a chloroacetaldehyde dimethyl acetal (Aldrich, Gillingham, Great Britain)—5% sulphuric acid (1:5, v/v) mixture. The distillate (b.p. 85—86°C) was diluted with doubly distilled water to give a 4 *M* solution which was stable at room temperature.

Phosphate (0.05 *M*, pH 7.0) and acetate (1.0 *M*, pH 4.5) buffers were prepared from analytical-grade reagents. The phosphate buffer was made up in HPLC-grade water (Rathburn Chemicals, Walkerburn, Great Britain) and was subsequently filtered (0.5 μm) and further purified before use by passage through a pellicular reversed-phase column (Whatman Co:Pell ODS).

Methods

Aliquots of the nucleotide solution, acetate buffer and chloroacetaldehyde (50:10:1, v/v/v) were mixed together in glass stoppered test tubes and then incubated for a minimum of 30 min in a boiling water bath. The resultant solutions were then injected directly into the chromatograph.

Chromatography

A high-performance liquid chromatograph consisting of two Waters Assoc. 6000A pumps, a Model 660 solvent programmer and a U6K injector coupled to a LDC Fluoromonitor III fluorescence detector (mercury lamp, 254-nm interference exciting filter and 410—700 nm emission filter) were used throughout.

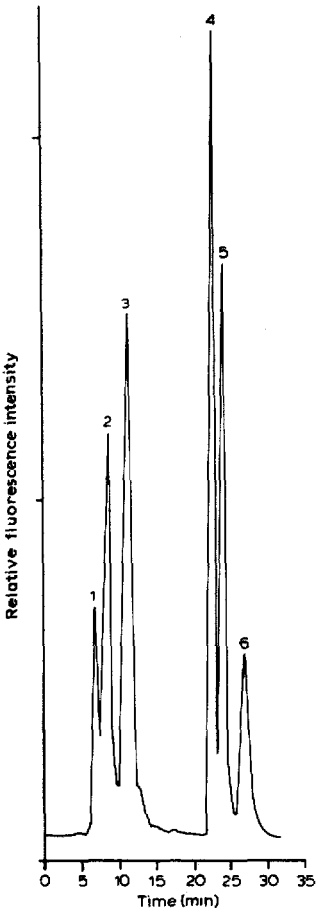
Elution was performed by programming (Curve 8, 15 min) from 97% phosphate buffer (0.05 *M*, pH 7.0), 3% methanol solution (Solution A) to a 75% phosphate buffer, 25% methanol solution (Solution B).

The chromatographic separation was performed on a 30-cm column packed with Whatman ODS-2 (10 μm) reversed-phase packing. The analytical column was protected by a 5-cm guard column packed with Whatman Co:Pell ODS medium. A flow-rate of 1 ml min^{-1} was used throughout.

RESULTS

A typical chromatogram of a six-component nucleotide mixture (derivatised as described under *Methods*) is shown in Fig. 1. Improved separation of the ATP/ADP pair can be obtained by reducing the amount of organic modifier in Solution A, but only at the expense of some peak broadening which may detract slightly from the overall sensitivity.

The effects of incubation time on the formation of the various derivatives are shown in Fig. 2. It is clear from this that reaction is essentially complete after 30 min in all cases and this time was therefore used in all subsequent work.



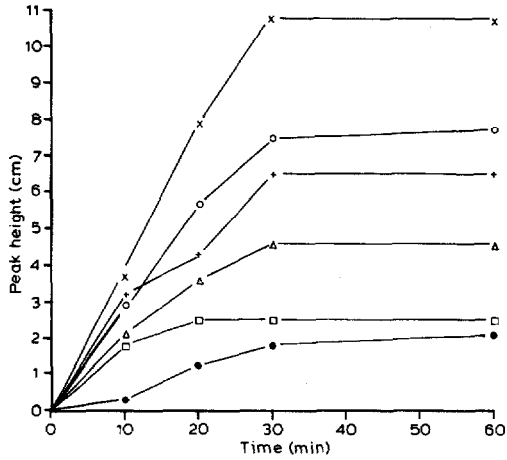


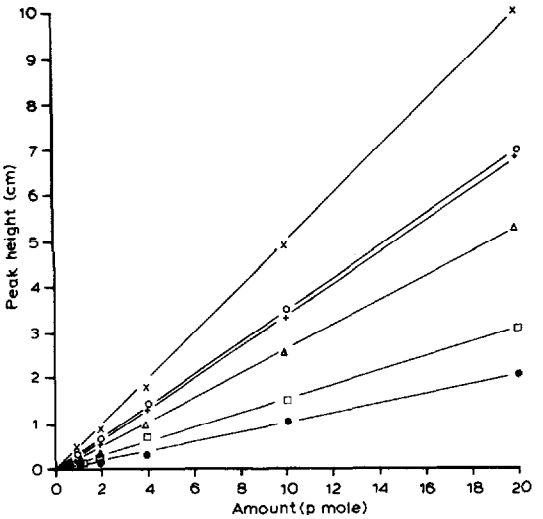
Fig. 1. A typical chromatogram of the derivatives of a six-component mixture prepared as described in the text. Peaks: 1 = ATP; 2 = ADP; 3 = AMP; 4 = cAMP; 5 = ADO; 6 = ADE.

Fig. 2. The effect of incubation time on the derivative formation. Mixtures prepared as described in the text were incubated for various periods of time, cooled and subsequently injected into the chromatograph. □ = ATP; △ = ADP; + = AMP; × = cAMP; ○ = ADO; • = ADE.

Calibration curves for each of the components are shown in Fig. 3. Linearity is preserved up to some hundreds of picomoles and the curves are independent of injection volume of at least 500 μl. With a detection limit of 0.5—1 pmol for each compound, solutions with concentrations as low as 2 pmol ml^{-1} may be satisfactorily analysed.

The reagent blank is very low and is negligible over the portions of the chromatogram of interest. No significant interferences with the overall procedure have been detected. Cytidine, which does form a weakly fluorescent derivative, has its emission maximum at 347 nm compared with 410 nm for the adenine derivatives and its contribution to fluorescence is removed by the emission filter. The technique is therefore specific to adenine and related compounds.

The reproducibility of the technique was assessed by derivatising five identical aliquots of a standard mixture of the six compounds (1 nmol ml^{-1} of



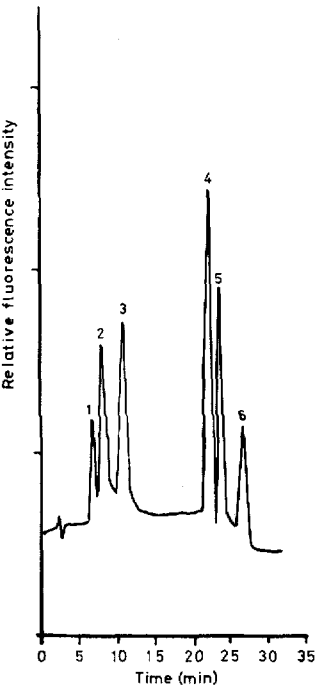


Fig. 3. Calibration curves for each of the six adenine compounds examined. □ = ATP; △ = ADP; + = AMP; × = cAMP; ○ = ADO; • = ADE.

Fig. 4. A typical chromatogram of the chloroacetaldehyde derivatised extract of phytoplankton collected from Irish Sea water (10.0 μl injection). Peaks as in Fig. 1.

each component) and injecting 20 μl (20 pmol). Coefficients of variation of 3.75, 1.78, 2.99, 1.83, 1.69 and 3.18% were found for ATP, ADP, AMP, cAMP, ADO and ADE, respectively. Once prepared the stability of the derivatives is excellent and they may be safely stored at room temperature for at least a week without measurable change.

A further interesting feature of this technique is that if it is only required to analyse for the nucleotide phosphates, then this may be achieved by isocratic elution with solution A in approximately 10 min. The derivatives of the less polar components are retained on a guard column which may be cleared periodically by elution with solution B.

The method has been successfully utilised by the author for the analysis of adenine related compounds in marine phytoplankton. The plankton cells were concentrated from sea water by filtration and the adenine compounds were extracted with boiling borate buffer and subsequently derivatised in the manner described previously. An example of the type of chromatogram obtained is shown in Fig. 4 from which it can be seen that there is no evidence of interference from other co-extracted compounds. This technique is at present under investigation with a view to using it to obtain improved standing crop estimates.

The high specificity of the chloroacetaldehyde reaction with adenine related compounds to form a fluorescent derivative has made it a useful tool in the

study of enzyme systems, RNA/DNA and biological fluids in general (for review see ref. 6). The reaction combined with the simple HPLC technique described in this paper is likely to find a considerable number of applications in future biological studies.

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