ASPECTS OF THE QUANTITATIVE SEPARATION AND ESTIMATION OF THIAMINE AND ITS PHOSPHATE ESTERS

A THESIS PRESENTED IN PARTIAL FULFILMENT

OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE IN BIOCHEMISTRY

AT MASSEY UNIVERSITY

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1981

ABSTRACT

Methods for the separation and estimation of thiamine, thiamine monophosphate and thiamine diphosphate which would be applicable to biological extracts were investigated. Two methods for the estimation of thiamine were compared, the acid dye method and the thiochrome method. The thiochrome method was preferred as the acid dye method was more difficult to perform and some interference by certain amino acids was indicated.

As both methods only estimate free thiamine, the optimum conditions for hydrolysis of thiamine phosphate esters by wheat germ acid phosphatase were also investigated. High phosphatase concentrations in the digestion mixture interfered with the extraction of thiochrome, by isobutanol, after oxidation of the free thiamine produced. Variation of the buffer in which the digestion was performed also affected the recoveries obtained. The inclusion of magnesium ions in the digestion mixture increased the activity of the enzyme so that it was possible to use an amount of phosphatase which was low enough to avoid interference with the extraction of thiochrome but which was sufficient to completely hydrolyse thiamine phosphate esters. The presence of magnesium ions also prevented the interference observed when formate rather than acetate buffers were used in the digestion mixture.

A variety of separation techniques were investigated. Compared to paper and thin layer chromatography, high voltage paper electrophoresis (at 3kV in pH 3.5 buffer) gave the best and quickest separations. However only a 60% recovery was obtained after samples were eluted from the paper with 0.1M hydrochloric acid.

Separation was achieved by elution of the thiochrome derivatives of thiamine, TMP and TDP from Sephadex G10 gel. Recoveries, estimated spectrophotometrically, indicated that this method could be used for the quantitative separation

of thiamine and its phosphate esters. However since the method does not allow concentration of samples, it would be unsuitable for the estimation of biological extracts.

Separation of thiamine and its esters using three ion exchange resins was also investigated. Partial separation of thiamine and its phosphate esters was obtained with Amberlite GC50 resin, the separation being determined by the form of the resin used. The hydrogen form of the resin allowed separation between TDP and thiamine-TMP while the sodium form separated thiamine from TMP-TDP. Neither form of the resin bound TDP firmly even when water was used as the eluent, so that separation of TDP and TTP would not be possible.

Separation was attempted by eluting samples from Dowex 1-X8 resin with formate buffers of increasing ionic strength or pH. While the separation of thiamine, TMP and TDP appeared to be complete, by the elution profile, it was found that sample breakdown occurred. Electrophoresis of the eluted samples showed that the only peak which contained a single component was that corresponding to thiamine. Sample breakdown was further indicated by a low recovery obtained when a sample containing only TDP was eluted. Identification of the peak contaminants was attempted using high voltage electrophoresis but proved difficult due to salt retardation affecting the positions of the peak components after electrophoresis.

With Dowex 50 resin TDP and TMP were easily separated and eluted with ammonium acetate buffer of varying pH and ionic strength but the elution of thiamine required high pH or ionic strength solutions. Sample breakdown also appeared to occur on elution of samples from the resin. When TMP and TDP were eluted, separation appeared to be complete but a recovery of greater than 100% was obtained for TMP and both eluted compounds exhibited a progressive breakdown after elution. Sample breakdown was particularly notable when thiamine alone was eluted as 2 peaks were eluted and, after oxidation, yellow fluorescent material as well as the usual

blue (characteristic of thiochrome) was observed. Characterisation of the yellow fluorescent compound(s) was attempted using electrophoresis, ultra-violet spectra and fluorescent spectra and it was found to be similar, but not identical, to thiamine.

ACKNOWLEDGEMENTS

I am extremely grateful to my supervisor Dr M.N. Wilson for her helpful advice and encouragement during the course of this thesis. I would also like to thank Dr G. Midwinter for assistance in obtaining the fraction collector and my fellow laboratory students, Mr D. Fenemor, Mr D. Colls and Mr P. Morris, for their helpful discussion.

Thanks is also due to Mrs V. Lobb for her excellent typing and Miss N. Ranginui for her encouragement and help throughout this work.

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ABBREVIATIONS

Thia	=	Thiamine
TMP	=	Thiamine Monophosphate
TDP	=	Thiamine Diphosphate
TTP	=	Thiamine Triphosphate
A.O.A.C.	=	Association of Official Analytical Chemists