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

A THESIS PRESENTED IN PARTIAL FULFILMENT
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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.

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CONTENTS

	PAGE
TITLE PAGE	
ABSTRACT	ii
ACKNOWLEDGEMENTS	v
CONTENTS	vi
LIST OF FIGURES	ix
LIST OF TABLES	xii
ABBREVIATIONS	xiv
CHAPTER 1: INTRODUCTION	1
Section 1.1 The Biosynthesis and Distribution of Thiamine and its Phosphate Esters	1
1.2 The Chemistry of Thiamine and its Phosphate Esters	2
1.3 The Metabolic Function of Thiamine and its Phosphate Esters	3
1.4 Thiamine Deficiency	5
1.5 Estimation of Thiamine and its Phosphate Esters	6
(i) Animal Assays	6
(ii) Microbiological Assay	7
(iii) Chemical Estimation	7
(a) Extraction of Thiamine and its Esters from Biological Material	8
(b) Spectrophotometric Assay	8
(c) Fluorometric Analysis of Thiamine by the Thiochrome Assay	10
(d) Hydrolysis of Thiamine Phosphate Esters	12
(e) Purification of Thiamine	13
(iv) Gas Chromatographic Estimation of Thiamine	14
(v) Enzymatic Assay Methods	14
1.6 Separation of Thiamine and its Phosphate Esters	15
(i) Paper and Thin Layer Chromatography	15
(ii) Paper Electrophoresis	16
(iii) Ion Exchange Chromatography	16
(iv) Gel Chromatography	18
(v) High Pressure Liquid Chromatography	19
1.7 Conclusion	19

TABLE OF CONTENTS CONTINUED

	PAGE
PART 1: ESTIMATION OF THIAMINE, THIAMINE MONOPHOSPHATE AND THIAMINE DIPHOSPHATE	
CHAPTER 2: MATERIALS	21
CHAPTER 3: METHODS	24
Section 3.1 The Acid Dye Method	24
3.2 The Thiochrome Method	24
3.3 Hydrolysis of Thiamine Phosphate Esters	25
CHAPTER 4: RESULTS AND DISCUSSION	26
Section 4.1 The Acid Dye Method	26
4.2 Estimation of Thiamine by the Thiochrome Method	27
4.2.1 Variation of the Ferricyanide Concentration	27
4.2.2 Extraction of Thiochrome by Isobutanol	28
4.2.3 Standard Curve Preparation	28
4.2.4 Summary - Thiochrome Method	28
4.3 Hydrolysis of Thiamine Monophosphate and Thiamine Diphosphate with Wheat Germ Acid Phosphatase	29
4.3.1 The Effect of Phosphatase Concentration, Magnesium Ion Concentration and Time on the Hydrolysis of Thiamine Diphosphate	29
4.3.2 The Effect of Buffer Ionic Strength and Composition on Digestion of TDP	30
4.3.3 Summary - Digestion of Thiamine Phosphate Esters	32
4.4 The Stability of Thiamine and TDP in Solutions of Various pH	32
PART 2: SEPARATION OF THIAMINE AND ITS PHOSPHATE ESTERS	
CHAPTER 5: METHODS	34
Section 5.1 Paper Chromatography	34
5.2 Thin Layer Chromatography	34
5.3 High Voltage Paper Electrophoresis	34

TABLE OF CONTENTS CONTINUED

	PAGE
CHAPTER 5: CONTINUED	
Section 5.4 Detection of Sample after Separation by Chromatography or Electrophoresis	34
5.5 Gel Filtration	35
5.6 Ion Exchange Chromatography	35
5.6.1 General Procedure	
5.6.2 Detection of Thiamine and its Phosphate Esters in Fractions Eluted from the Columns	37
CHAPTER 6: RESULTS AND DISCUSSION	
Section 6.1 Paper Chromatography	38
6.2 Thin Layer Chromatography	38
6.3 High Voltage Paper Electrophoresis	38
6.4 Recovery from Paper after Electrophoresis	38
6.5 Summary	39
6.6 Sephadex G10	40
6.7 Ion Exchange Chromatography	41
6.7.1 Amberlite GC50	41
6.7.2 Dowex 1-X8	43
6.7.3 Dowex 50W-X8	48
CHAPTER 7: CONCLUSIONS	56
Section 7.1 Estimation Methods	56
7.2 Separation Methods	57
REFERENCES	62

LIST OF FIGURES

Figure		After Page
1	The structure of thiamine, TMP and TDP.	1
2a & b	Reaction of thiamine with alkali	2
2c	Titration of thiamine with alkali	3
3	General types of reactions in which TDP acts as a co-factor.	3
4	The use of the transketolase reaction to monitor levels of TDP	14
5	The fluorescent spectrum of thiamine (thiochrome)	24
6	A thiamine standard curve prepared using the acid dye method	26
7	The effect of variation of the concentration of the potassium ferricyanide solution used to oxidise thiamine.	27
8	A typical thiamine standard curve prepared by the thiochrome method.	28
9	The effect of varying phosphatase concentration on the extraction of fluorescence by isobutanol	29
10	Stimulation of phosphatase activity by $MgCl_2$.	29
11	% Digestion of TDP after various digestion times	30
12	The stability of thiamine in solutions of various pH stored for various times.	33

LIST OF FIGURES CONTINUED

FIGURE		After Page
13	The stability of TDP in solutions of various pH	33
14	Separation of thiamine and its esters by high voltage electrophoresis at 3kV	38
15	Retardation of samples on electrophoresis by salts	38
16	Chromatography of thiochrome and thiochrome phosphate esters on Sephadex G10	40
17	Elution of TMP and TDP from Amberlite GC50 (Na ⁺ form) as monitored by spotting	43
18	Elution of thiamine and its phosphate esters from Dowex 1-X8 at pH 6.0	44
19	Separation of thiamine and its esters on Dowex 1-X8 eluted at pH 4.5	44
20	Elution of TDP from Dowex 1-X8	45
21	Chromatography of TDP and TMP on Dowex 50 resin	49
22	Elution of thiamine from Dowex 50 resin using ammonium acetate buffers	51
23	The ultra violet spectra of peak 1, peak 2, and thiamine	52

LIST OF FIGURES CONTINUED

FIGURE		After Page
24	The ultra violet spectra of thiamine in 1M ammonium acetate buffer (pH 6.0) and 2M ammonium acetate buffer (pH 5.2)	52
25	The ultra violet spectra of the oxidation products of peak 1, peak 2 and thiamine	52
26	The fluorescent spectra of peak 1, peak 2, and thiamine	53
27	Elution of thiamine from Dowex 50 using 0.5M ammonia.	53

LIST OF TABLES

Table	After Page
1. Thiamine and thiamine phosphate ester content of various tissues.	2
2. Triplicate determination of a single thiamine solution estimated by the acid dye method.	26
3a. Absorbance of thiamine and thiamine phosphate esters estimated by the acid dye method.	26
3b. Absorbance of thiamine and thiamine phosphate esters estimated by the acid dye method.	26
3c. Absorbance of amino acids estimated by the acid dye method.	26
4. The effect of multiple isobutanol extraction on the recovery of fluorescence from a thiamine solution.	28
5. The effect of addition of 95% ethanol to the isobutanol used to extract thiochrome on recovery of fluorescence.	28
6. Reproducibility of the thiochrome assay.	29
7. The effect of phosphate concentration on hydrolysis of TDP.	29
8a Recoveries of TDP after hydrolysis with & b. phosphatase in various buffer.	31
9. The stability of thiamine and TDP after rotary evaporation.	33

LIST OF TABLES CONTINUED

Table		After Page
10 & 11	Paper chromatography of thiamine, TMP and TDP.	38
12	Thin layer chromatography of thiamine and TDP using silica gel G.	38

ABBREVIATIONS

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