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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

PAUL NOEL SCOTT  
65121-10  
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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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ABBREVIATIONS

|          |   |  |
|----------|---|--|
| Thia     | = | Thiamine                                       |
| TMP      | = | Thiamine Monophosphate                         |
| TDP      | = | Thiamine Diphosphate                           |
| TTP      | = | Thiamine Triphosphate                          |
| A.O.A.C. | = | Association of Official Analytical<br>Chemists |

## CHAPTER 1

### INTRODUCTION

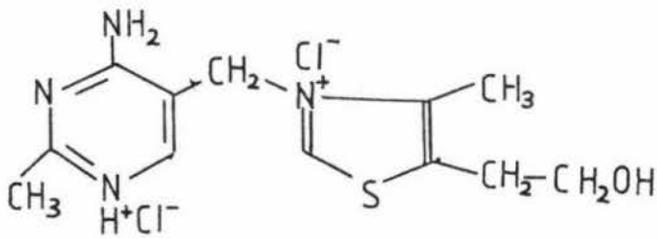
The link between dietary deficiency and disease was first recognised in 1897 by Eijkman and Grijns who observed that the disease beri-beri was due to the dietary absence of small quantities of a "protective substance". In 1911 Funk isolated from rice polishings a substance capable of curing beri-beri. The structure of this factor was finally established in 1936 by R.R. Williams and co-workers who showed it consisted of a pyrimidine moiety and a thiazole moiety linked together by a methyl bridge (Fig. 1). They named this compound thiamine.

#### 1.1 THE BIOSYNTHESIS AND DISTRIBUTION OF THIAMINE AND ITS PHOSPHATE ESTERS

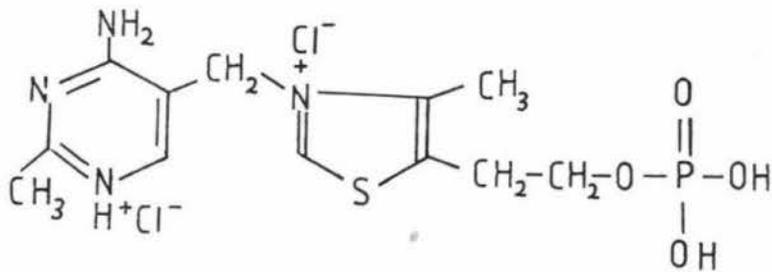
Thiamine biosynthesis occurs only in plants and some micro-organisms but the vitamin is essential to all forms of life. The initial product of thiamine biosynthesis is thiamine monophosphate (TMP) which is formed by combination of its thiazole and pyrimidine moieties (Brown, 1972). Free thiamine can be formed from this by the action of any one of several alkaline or acid phosphatases present in plants, animals and micro-organisms (Steyn-Parvé and Monfoort, 1963). In many plants this is the predominant form of the vitamin. Non-ruminant animals can neither synthesize nor store free thiamine and thus require a constant dietary intake. Ruminants however, are less dependant on diet as a large part of their thiamine requirement is supplied by the rumen flora (Robinson, 1966).

Thiamine diphosphate (TDP) is the predominant form of the vitamin in animals and many yeasts. This is produced, in all living organisms, by the transfer of a pyrophosphate group from adenosine triphosphate (ATP) to thiamine, catalysed by the enzyme thiaminokinase. TDP cannot pass through the animal cell membrane and thus must be formed inside the cell, *in situ* from thiamine.

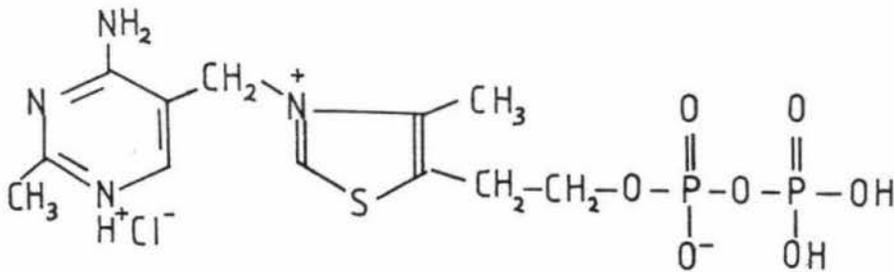
FIGURE 1: THE STRUCTURE OF THIAMINE, TMP AND TDP



Thiamine Synonyms: Vitamin B1, aneurin, antiberiberi factor, antineuritic factor, oryzamin



Thiamine monophosphate (TMP)



Thiamine Diphosphate (TDP)  
Synonym: Co-carboxylase

In addition to TDP, small quantities of thiamine, TMP and thiamine triphosphate (TTP) have been reported in many animal tissues (Pearson, 1967) (Table 1). TTP amounting to 5-9% of total thiamine content is present in most animal tissue (Barchi, 1976). Diphospho-thiamine disulfide also has been reported in bakers yeast, and has been claimed to be present in animal tissue (Stein-Parve and Monfoort, 1963).

## 1.2 THE CHEMISTRY OF THIAMINE AND ITS PHOSPHATE ESTERS

Thiamine hydrochloride exists as colourless, monoclinic crystals with a melting point of  $244^{\circ}$ . It is extremely soluble in water but less so in most alcohols. In water it forms a strongly acidic solution, the pH of a 5% solution being approximately 3.5.

In solutions with a pH of less than 5 thiamine is fairly stable to heat and oxidation but at a pH of 5 or higher it is destroyed by boiling or storing for long periods at room temperature. Its stability is not solely determined by the pH of the solution however, but depends on the nature of the buffer employed. Upon heating a solution of thiamine of pH 5.4 for 1 hour at  $100^{\circ}$ , 100% destruction occurs in borate buffer, 10% with an acetate buffer, 3% with a phosphate buffer and 57% with an unbuffered solution (Robinson, 1966).

Thiamine exhibits a number of reactions with alkali depending on how rapidly the pH of the solution is raised. It reacts slowly with alkali to form a colourless thiol form which can be oxidised to the dimer, thiamine disulphide (Fig. 2a). When thiamine is reacted rapidly with alkali it forms an unstable yellow thiol form which can either decompose to the colourless thiol form or be oxidised to the tricyclic compound, thiochrome (Fig. 2b). Thiamine, when titrated with alkali, displays a two step titration curve. The first step, with a midpoint of approximately pH 4.8, represents titration of the protonated amino-pyrimidine group (Fig. 2c). The structure of thiamine hydrochloride is commonly written as if

FIGURE 2a: REACTION OF THIAMINE WITH ALKALI (SLOW REACTION)

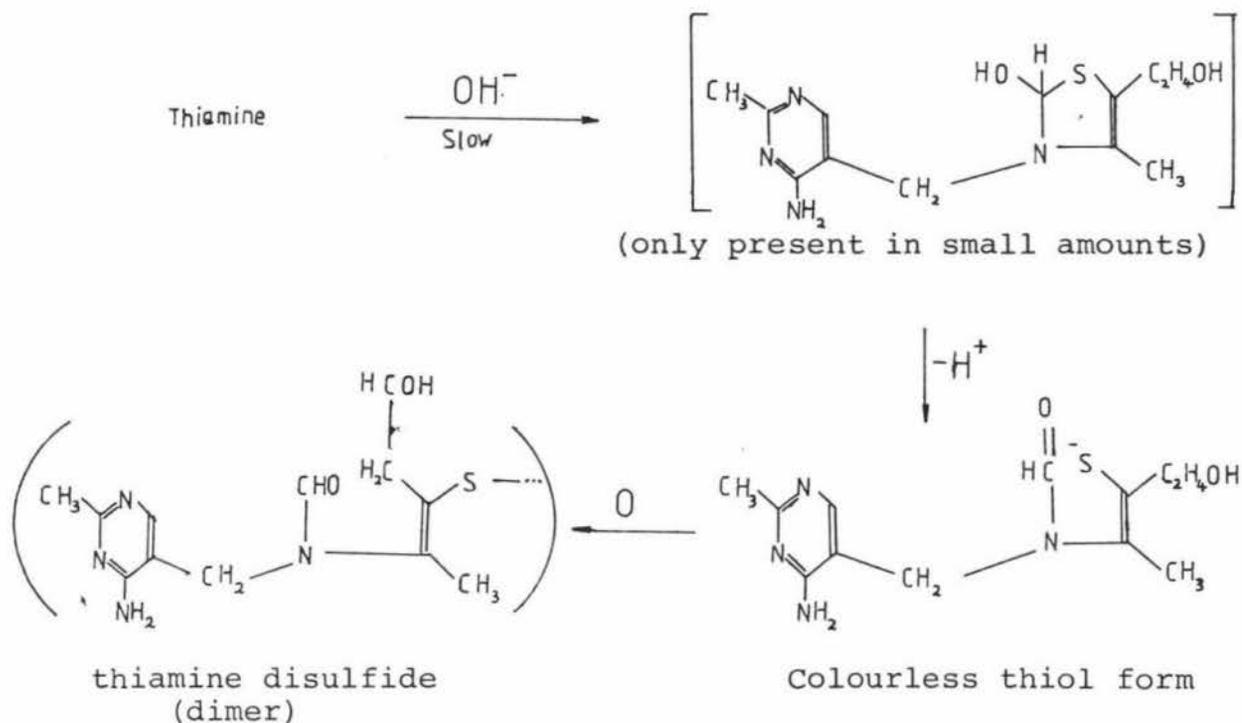


FIGURE 2b: REACTION OF THIAMINE WITH ALKALI (>pH 11) (FAST REACTION)

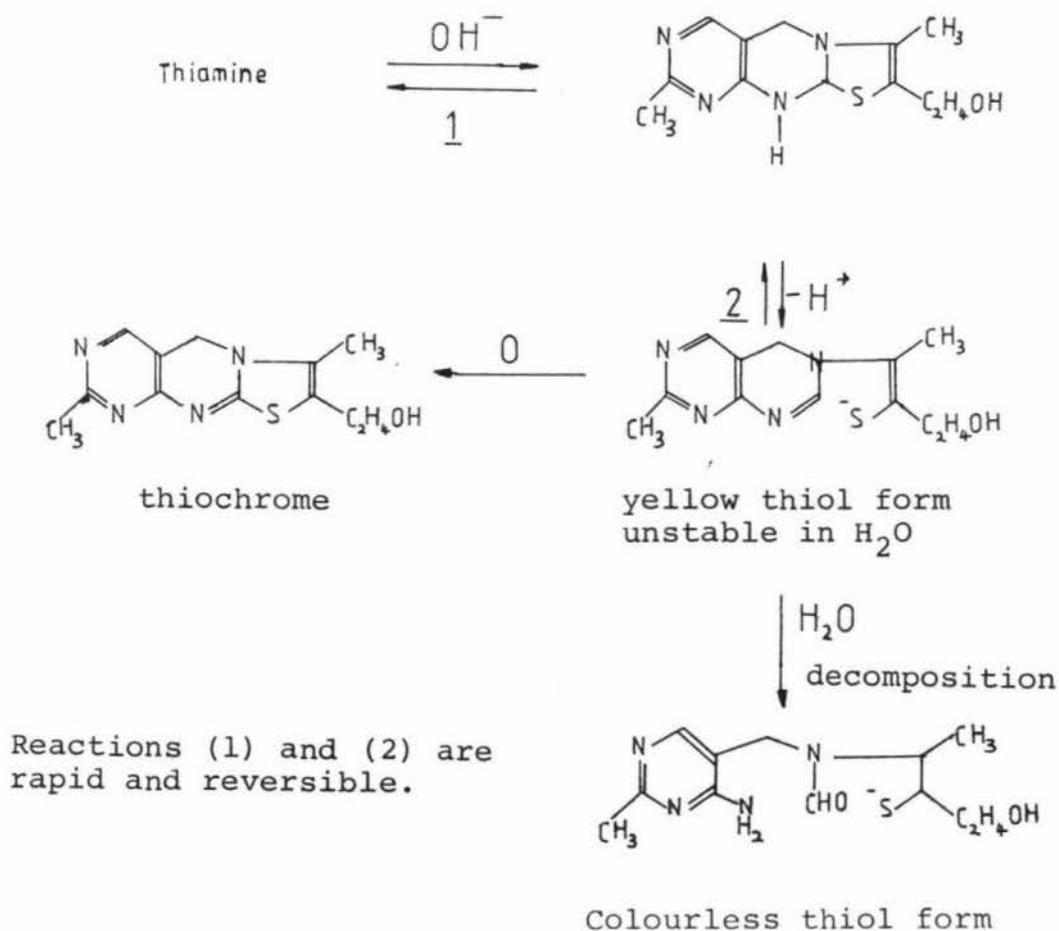


TABLE 1: THIAMINE AND THIAMINE PHOSPHATE ESTER CONTENT OF VARIOUS TISSUES  
 ( $\mu\text{g/g}$  tissue<sup>(1)</sup> or  $\mu\text{g/cm}^4$ <sup>(2)</sup>)

| Tissue   | Thiamine  | TMP       | TDP                  | TTP       | Total Thiamine |
|--|-----------|-----------|----------------------|-----------|----------------|
| Rat liver <sup>(1)</sup>   | 0.24-0.75 | 0.66-0.77 | 5.88-7.37            | 0.09-1.09 |                |
| Rat brain <sup>(1)</sup>   | 0.11      | 0.3       | 2.61                 | 0.19      |                |
| Rat heart <sup>(1)</sup>   | 0.16      | 0.41      | 7.44                 | 0.57      |                |
| Rat kidney <sup>(1)</sup>  | 0.22      | 0.35      | 3.47                 | 0.27      |                |
| Cow milk <sup>(2)</sup>  |           |           |                      |           | 0.38-0.51      |
| Human milk <sup>(2)</sup>  |           |           |                      |           | 0.16           |
| Human blood <sup>(2)</sup><br>plasma<br>corpuscles<br>leucocytes | 0.01      |           | 0.03-0.11<br>up to 1 |           |                |
| Human saliva <sup>(2)</sup>                                      |           |           |                      |           | 0-0.015        |
| Human urine <sup>(2)</sup>                                       |           |           |                      |           | 0.19           |

Rindi and De Giuseppe (1960)

Henssey and Cerecedo (1939)

the primary amino group were protonated, but the proton is more likely located on the pyrimidine ring nitrogens, probably at position 1 to the greatest extent (Metzler, 1960). The second titration step has a midpoint at pH 9.2 and represents the slow reaction of thiamine with two equivalents of base (Fig. 2c). Taking into account the pka's of the phosphate hydrogens this means that at pH 4.5 the net charge on thiamine, TMP and TDP will be +2, +1 and 0 respectively. Likewise at pH 6.0 thiamine, TMP and TDP will have net charges of +1, -1 and -2 (Gounaris and Schulman, 1980).

Thiamine exhibits a marked absorption of light in the ultra violet region of the spectrum, which is strongly dependent upon the pH of the solution. There are two absorption maxima in neutral solution (pH 7.0 phosphate buffer), at 232-233 nm and at 266 nm. In acidic solution (dilute hydrochloric acid, pH 2.0) the first maxima shifts to 246 nm and the other maxima becomes less pronounced.

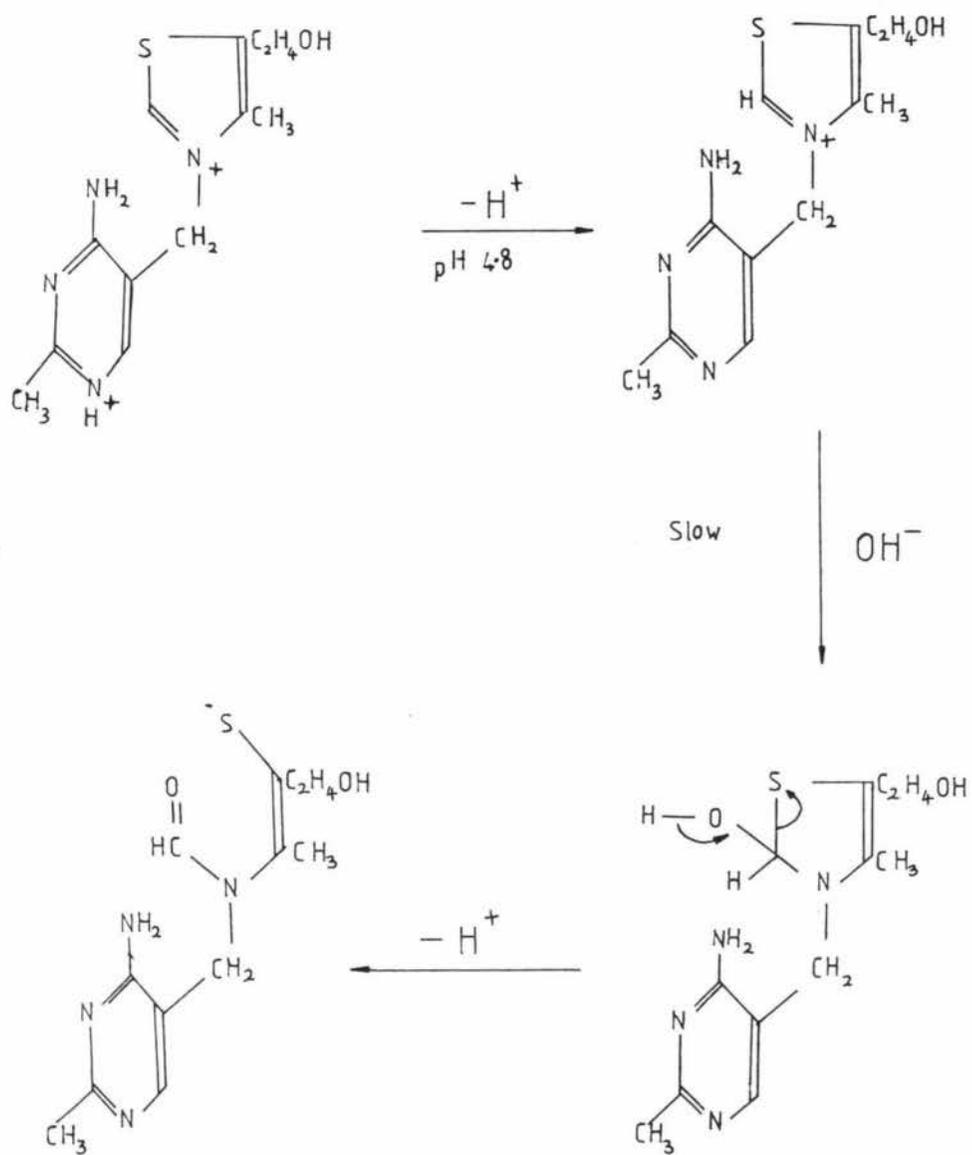
### 1.3 THE METABOLIC FUNCTION OF THIAMINE AND ITS PHOSPHATE ESTERS

Thiamine and its phosphate esters perform a number of functions in animal and plant metabolism, some of which have only recently been recognised. As TDP it is a co-factor in various reactions involving the transfer of an aldehyde group. In each case a bond is broken, which is immediately adjacent to a carbonyl group on one reacting species, with the production of an "active aldehyde-TDP complex" (Metzler, 1960). The "active aldehyde" is then transferred to any one of a number of acceptor molecules depending on the particular enzyme and substrates present. The types of reactions in which TDP is involved can be divided into 3 main categories (Fig. 3).

#### a) Non-oxidative decarboxylation

e.g. Conversion of pyruvate to acetaldehyde by pyruvate decarboxylase (yeast).

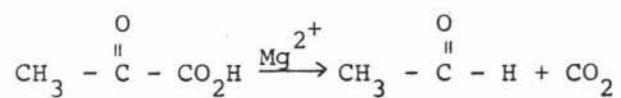
FIGURE 2c: TITRATION OF THIAMINE WITH ALKALI



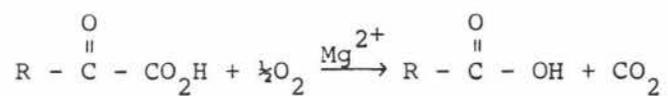
D.E. Metzler (1980)

FIGURE 3: GENERAL TYPES OF REACTIONS IN WHICH TDP ACTS AS A CO-FACTOR

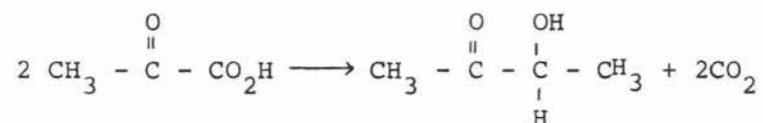
A. Non-Oxidative Decarboxylation



B. Oxidative Decarboxylation



C.  $\alpha$ -Ketol Formation



## b) Oxidative decarboxylation

- e.g. i) Conversion of pyruvate to acetyl CoA by the pyruvate dehydrogenase complex (animals, plants and micro-organisms).
- ii) Conversion of  $\alpha$ -ketoglutarate to succinyl CoA by the  $\alpha$ -ketoglutarate dehydrogenase complex (animals, plants and micro-organisms).
- iii) The phosphoroclastic cleavage of  $\alpha$ -keto acids to the corresponding acyl phosphate (bacteria and mammalian liver).

c)  $\alpha$ -ketol formation

- e.g. i) Formation of acetoin from two molecules of pyruvate (*Aerobacter aerogenes*).
- ii) Formation of acetoin from pyruvate and acetaldehyde (yeast and bacteria).
- iii) Transketolase reaction of the pentose phosphate pathway (e.g. animal liver, mammary gland, adipose tissue, adrenals etc and plants).

In addition to the role of TDP in intermediary metabolism, free thiamine, TMP and TTP probably have definite, though less clearly understood, roles in the body. At present an area of intensive study is the link between thiamine and nerve action. It has been recognised for many years that deficiencies of thiamine manifest themselves initially, in animals and humans, as nervous disorders. Since these symptoms usually occur before any alteration in intermediary metabolism can be detected, it is considered that thiamine has an additional role in nervous tissue, which is independent of its TDP co-factor function. Treatment of nerves with electrical stimulation and neuroactive reagents leads to the release of thiamine compounds, and in particular thiamine and TMP (Barchi, 1976). At present it is considered that TTP is likely to be the particular metabolite of thiamine which is important with regard to nervous tissue. This is because a characteristic of Subacute Necrotizing Encephalomyelopathy, a hereditary disease which resembles the encephalopathy of thiamine deficiency, is a lowered level of TTP in the brain

whereas, all other phosphate esters of thiamine are at their normal levels (Cooper and Pincus, 1976). Barchi (1976) has noted a correlation between thiamine triphosphatase activity and membranes active in transport processes in the kidney, brain and intestine and suggested a role for TTP in maintaining membrane negative surface charge density.

The presence of enzyme systems specific for the inter-conversion of thiamine and its phosphate esters suggests that the relative concentrations of thiamine metabolites may be important with respect to their physiological function. In addition to the enzymes already mentioned (section 1.1) the presence of TMP, TDP and TTP phosphatases with specific intracellular locations has been shown (Ogawa, Ago and Tanaka, 1976). Barchi (1976) has noted the presence of both soluble and membrane bound thiamine triphosphatases which are widely distributed throughout the body. Cooper and Pincus (1976) have shown a TDP-ATP phosphoryltransferase in brain, liver, kidney and heart. Work with *E. coli* mutants has also shown the presence of TMP pyrophosphorylase, thiamine monophosphokinase and TMP kinase (Kawasaki and Yamada, 1976).

#### 1.4 THIAMINE DEFICIENCY

Thiamine deficiency disease in humans (beri-beri) and animals is debilitating and, unless thiamine is administered, fatal. Beri-beri occurs in several forms and the pathological symptoms of these are well documented. Initial symptoms appear to be related to neuronal disfunction (Dreyfus, 1976) while later symptoms are due to impaired functioning of those enzymes for which TDP is a cofactor (Brubacher, 1979).

Beri-berih has been known in the Far East for centuries where it still occurs. Although rare in modern westernised countries, minor degrees of thiamine deficiency, which cause listlessness, apprehension and fatigue, are common. Brin (1976) has estimated that approximately 20% of the population in developing countries have a low thiamine intake. An average requirement of about 0.5 mg thiamine / 4.15 M J of dietary

intake is recommended although this varies depending on the age and health of the individual. During pregnancy increased tissue utilisation can cause a deficiency aggravated by loss of appetite and vomiting. The high energy intake of the chronic alcoholic serves to increase his thiamine requirement, whilst the poor diet often consumed decreases his intake. The popularity of processed foods has meant that even in industrialised countries, many individuals are getting barely sufficient thiamine from their diets. To combat this many countries are now enriching staple foods (e.g. bread) with thiamine and other vitamin supplements.

## 1.5 ESTIMATION OF THIAMINE AND ITS PHOSPHATE ESTERS

### ( i ) Animal Assays

Biological assay methods using animals were the first to be developed as they were the only practical assay methods before pure thiamine samples became available. Most were based on the curative effects of thiamine dosages after deficiency states had been induced in test animals. The rice bird and pigeon were used by early workers but were superseded by the rat in later years. Various parameters have been used to determine the curative effects of thiamine dosages. Sherman and Spohn (1923) used the weight gain of rats on a diet supplemented with thiamine. Other assays, which include the length of cure of bradycardia or polyneuritis, were used to measure the response following a single dose of thiamine (Pearson, 1967). Peters *et al* (1933) used oxygen uptake by avitaminous pigeon brain tissue before and after the addition of a test dose. Jukes and Heitman (1940) used the polyneuritic mortality index of chicks to test for thiamine.

Animal assay allows measurement of the physiological availability of a thiamine sample, most samples being able to be tested as they are. They do not however, distinguish between the various forms of thiamine. Results show wide variability, unless large numbers of animals are used, and

are often influenced by other substances in the diet which have a thiamine sparing effect (e.g. fats). This, in combination with more time, space and equipment required than for other assay methods, tends to make animal assays expensive and difficult to use for routine assay of large numbers of samples.

#### (ii) Microbiological Assay

Thiamine assay can be carried out with micro-organisms for which thiamine is an essential growth factor. Growth of the micro-organisms is usually measured in terms of turbidity of the culture on thiamine free nutrient media (Steyn-Parve and Monfoort, 1963) although other criteria have been used. For example Shultz *et al* (1939) measured carbon dioxide produced by fermentation of a sugar solution by *Saccharomyces cerevisiae* after addition of thiamine. Microbiological methods are very sensitive, fairly rapid and inexpensive and were, until quite recently, frequently used. However many of the micro-organisms used have a lack of specificity for thiamine as the separate thiazole and pyrimidine moieties both give a response (e.g. *Saccharomyces cerevisiae*, *Phycomyces blakesleeanus*). Other micro-organisms (e.g. *Lactobacillus fermenti*, *Lactobacillus viridescens*) which are specific for the intact thiamine molecule respond to TDP and thiamine to a different degree. Because of the popularity of chemical methods, microbiological methods are less commonly used nowadays. This is because chemical methods are more convenient for routine analysis of large numbers of samples, especially since they are easier to adapt for use in auto-analyser equipment than other analysis methods.

#### (iii) Chemical Estimation

Most chemical procedures have a number of steps in common. In most cases, before thiamine can be estimated, the vitamin must be extracted and the phosphate esters of thiamine hydrolysed. Substances which interfere with the assay procedure must be removed. This is usually done using

absorption and elution techniques. The vitamin solution can then be treated with reagents which convert thiamine to a coloured or fluorescent compound for measurement.

(a) Extraction of Thiamine and its Esters from Biological Material

Thiamine and its phosphate esters are usually protein bound in biological samples (Pearson, 1967). Hydrolysis of samples is therefore required before thiamine and/or its derivatives can be assayed. Mild acid hydrolysis is normally used because the vitamin is readily destroyed by heating in alkaline or neutral solutions (see section 1.2). The most common method used is to treat the sample with dilute solutions of boiling hydrochloric or sulphuric acid (Mickelson and Yamamoto, 1958). Pyke (1939) recommended the use of proteolytic enzymes (e.g. papain or pepsin) to avoid losses due to precipitation of thiamine with protein under acidic treatment. Mickelson and Yamamoto (1958) have commented however, that this procedure is of doubtful value as, with the exception of milk, acidic treatment appears to extract thiamine from protein sufficiently. Trichloroacetic acid has also been used to extract thiamine and its esters from blood as it also removes hematin which catalyses the destruction of thiamine. However Schultz and Natelson (1972) have cautioned against the use of this as it does not liberate TDP completely, some of it being precipitated with the protein.

(b) Spectrophotometric Assay

Thiamine absorbs light in the ultra violet range of the spectrum. Doherty *et al* (1955) determined that thiamine has an isobestic point at 273 nm and used this to determine thiamine in pharmaceutical products. They noted that this method is of little use in estimating biological samples due to the presence of interfering substances.

A number of colorimetric assays for thiamine have been developed, most of which depend on the reaction with a diazotised amine. These include: -

- i ) precipitation with phosphotungstic acid and reduction of the resulting compound with zinc to form a brown colour (Spruyt, 1930).
- ii ) reaction with diazotised *p*-aminoacetophenone to form a pink colour which is extracted with xylene (Prebluda and McCollum, 1936).
- iii) reaction with other diazotised compounds such as dichlorobenzene-diazonium chloride, diazotised *p*-nitroaniline and diazotised *p*-aminobenzoate.

The spectrophotometric methods mentioned here are nowadays of historical interest only as they are all too insensitive to use for assay of biological material. The most widely used method, reaction with diazotised *p*-aminoacetophenone, can be used to estimate only 10-200  $\mu\text{g}$  of thiamine. Also most of these methods are subject to interference from other compounds present in biological samples. Many of these interfering compounds can be removed by precipitation or preliminary sample purification but this tends to make the procedures complex, laborious and time consuming.

A comparatively recent colorimetric method, the acid dye method, has been developed by Das Gupta and Cadwallader (1967). This method is based on the formation of a coloured salt upon reaction of thiamine with bromothymol blue at pH 6.6. The salt is extracted into chloroform, which acts to remove interfering substances. They reported no interference when the method was tested with certain other vitamins, minerals and hormones and that it followed Beer's law in the range 4-10  $\mu\text{g}/\text{cm}^3$  of thiamine.

(c) Fluorometric Analysis of Thiamine by the Thiochrome Assay

R.A. Peters (1935) showed that thiamine could be oxidised under alkaline conditions to yield the intensely blue fluorescent compound thiochrome (Fig. 2b). This reaction has been developed into the most widely used method for assay of thiamine. It has the sensitivity required for analysis of biological samples as it can be used to estimate thiamine in the range 0.5-2.5  $\mu\text{g}$ . With special precautions 0.1  $\mu\text{g}$  of thiamine can be measured with a 10% error (Mickelson and Yamamoto, 1958). Burch *et al* (1952) have adapted the method for microanalysis and have measured as little as 2-5 ng of thiamine. This was achieved by the use of an extremely sensitive fluorometer and correction for non-thiochrome fluorescence after destruction of thiochrome by ultra violet irradiation.

Various oxidising agents have been used to produce thiochrome. Alkaline ferricyanide is the most common oxidant used and most official methods of analysis are based on the use of this (A.O.A.C. Methods of Analysis handbook, 1975). A variety of concentrations of potassium ferricyanide have been used, the most common of which is a 0.04% solution in 30% sodium hydroxide. Some workers claim that potassium ferricyanide should be added to the thiamine solution before the sodium hydroxide while others add both together (Mickelson and Yamamoto, 1958). The wide variety of concentrations and conditions used suggest that, within limits, the conditions used to form thiochrome with potassium ferricyanide are not exacting. Using alkaline ferricyanide total conversion of thiamine to thiochrome does not occur but, as a constant proportion is converted over the concentration range encountered in biological samples, this oxidising agent can be used in quantitative determinations. Most investigators agree that approximately 67% of thiamine is converted to thiochrome in the range 0.2-2  $\mu\text{g}$ . Excess ferricyanide has been reported to destroy thiochrome but as thiochrome is usually extracted into isobutanol immediately, this should

not affect the quantitative reliability of the assay.

Oxidants that are gaining a wider acceptance now are mercury (II) chloride and cyanogen bromide. Results obtained using these two oxidants are virtually identical but are slightly higher than those obtained using alkaline ferricyanide (Edwin *et al*, 1975). Mercury (II) chloride is to be preferred because cyanogen bromide must be freshly prepared for each estimation which increases handling of this poisonous chemical. In addition cyanogen bromide reacts only with low concentrations of thiamine to produce thiochrome. When thiamine is present in high concentrations it reacts to form a coloured compound which is not estimated by the thiochrome method. (Mickelson and Yamamoto, 1958). Thus the conditions required for formation of thiochrome with cyanogen bromide are more exacting than with alkaline ferricyanide.

Virtually all workers extract the thiochrome formed with isobutanol before determination. This acts as a purification step, to remove interfering impurities, and to increase the sensitivity of the assay as thiochrome is more strongly fluorescent in isobutanol than in aqueous solutions. In addition, since the phosphate esters of thiochrome are insoluble in isobutanol, the extraction allows discrimination between thiamine and its phosphate esters. After extraction the isobutanol must be dried to eliminate cloudiness. Sodium sulphate has been used for this but a wide variation in blank values with different batches of the chemical have been reported (Mickelson and Yamamoto, 1958). Watson (1946) has recommended the use of ethanol to clarify the isobutanol. The most common method used is low speed centrifugation.

The thiochrome assay must be performed in low light conditions as thiochrome is rapidly degraded by ultra violet light. The rate of destruction of thiochrome, while being determined in the fluorometer, is insufficient to cause serious error in reading but high enough to require constant reading time (Mickelson and Yamamoto, 1958). Strict attention to detail is important during the thiochrome assay because of its

extreme sensitivity to interference from other fluorescent, and quenching substances. For example when potassium ferricyanide is used to oxidise urine extracts fluorescent impurities are produced with N-methyl nicotinamide (Edwin *et al*, 1975). Because such impurities are common in biological material, purification of samples prior to assay is usually performed. This is usually done by absorption and elution of samples from columns of absorbents or ion exchange resins (see Section 1.5 viii). Leveille (1972) has claimed that this is unnecessary if background fluorescence of samples is determined after destruction of thiamine by benzene sulphonyl chloride. He reported good agreement with assays performed using other methods but Shultz and Natelson (1972) have cautioned against the use of methods which do not include sample purification prior to assay. They found that unless interfering substances were removed "significant decreases in fluorescence due to the absorption of the exciting light by the coloured impurities" occurred. Since the thiochrome procedure is rapid, reproducible, sensitive and specific, large numbers of determinations can be achieved in a single day, making it the best choice for routine analysis of large numbers of samples.

#### (d) Hydrolysis of Thiamine Phosphate Esters

In many chemical methods for the estimation of thiamine the coloured or fluorescent compound produced is extracted into an organic solvent before estimation. As those compounds produced by the phosphate esters of thiamine often are not able to be extracted into the organic solvent used, they must be hydrolysed before they can be determined. Amylolytic enzyme preparations (e.g. Takadiastase, Diastase, Clarase) have been widely used for this purpose due to their residual phosphatase activity (Schultz and Natelson, 1972). A solution of the particular ester to be digested is usually made 0.1 - 1% with respect to the enzyme and digested at 37° overnight or 45° for 3-4 hours (A.O.A.C. Methods of Analysis handbook, 1975).

Schultz and Natelson (1972) compared the action of several hydrolytic enzymes and concluded that wheat germ acid phosphatase was the best of the commercially available enzymes. They noted that complete hydrolysis, occurred at levels of 3 mg of this enzyme per cm<sup>3</sup> of the reaction mixture after digestion for 1 hour at 37<sup>o</sup>. They also cautioned against the use of diastase enzyme preparations as "some diastases available today are free of acid phosphatase activity".

Hydrolysis of samples has been widely used to differentiate between the levels of thiamine and thiamine phosphate esters present. This is accomplished by determination of samples before and after phosphatase treatment (Koike *et al*, 1967).

(e) Purification of Thiamine

Because of the presence of impurities in biological extracts that can complicate the estimation of thiamine by most methods, a preliminary purification of thiamine is usually performed. The most common method of achieving this is by column chromatography using an absorbent which binds thiamine phosphate esters and/or thiamine. Interfering impurities can then be washed through the absorbent before thiamine (phosphate esters) are eluted. Early workers used base exchange silicates for this purpose (Mickelson and Yamamoto, 1958). This was usually done following hydrolysis of samples with phosphatase, to assay for total thiamine content, as the thiamine phosphate esters were not absorbed. Hennessey and Cerecedo (1939) absorbed thiamine with Decalso (a synthetic zeolite) and eluted with boiling acidic potassium chloride solution. This method has been incorporated into the standard thiochrome procedure in the A.O.A.C. Methods of Analysis handbook (1975) and is still used extensively. Problems with this method include, incomplete absorption and elution of thiamine, particularly when urine samples are estimated (Bessey *et al*, 1951), and crystallisation of the potassium chloride whilst eluting the sample. Edwin *et al* (1975) improved recoveries (98-109%) from Decalso by

converting thiamine to thiochrome while it was still bound to the Decalso and eluting with isobutanol.

Rindi and De Giuseppe (1960) used a column of activated charcoal to purify thiamine. This absorbed thiamine and its phosphate esters making treatment of the sample with phosphatase before absorption unnecessary. Elution was with 10% propan-1-ol and they reported recoveries of up to 95% of the sample applied to the column (i.e. thiamine and thiamine phosphate esters). Shultz and Natelson (1972) used Amberlite GC50 to purify a sample containing thiamine after phosphatase treatment. They eluted with 1M sulphuric acid and reported recoveries of 94%.

#### (iv) Gas Chromatographic Estimation of Thiamine

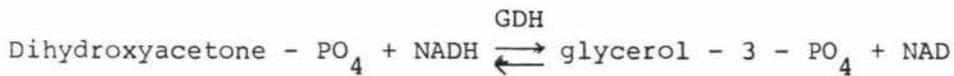
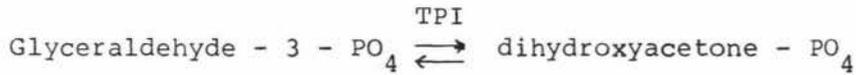
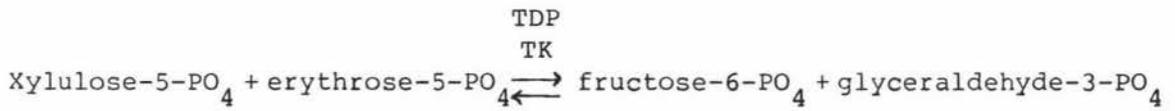
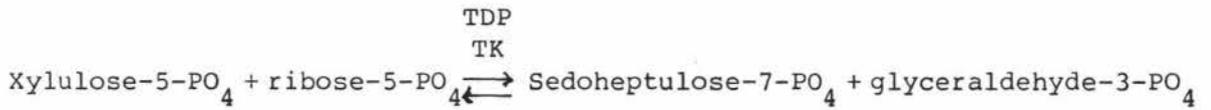
Several workers (Dwivedi and Arnold, 1972; Seifert and Miller, 1973) have used gas chromatography after sulphate cleavage for estimation of thiamine. Analysis was carried out on the thiazole product and recoveries ranged from 83-97%.

#### (v) Enzymatic Assay Methods

The activity of transketolase can be used to measure the amount of TDP in a sample. This is usually done by coupling this enzyme with triose phosphate isomerase and glycerophosphate dehydrogenase (Fig. 4). The reaction can be followed by monitoring the appearance of hexoses, disappearance of pentoses or oxidation of NADH. Transketolase can be used to assay for TDP by two methods.

- a) Transketolase activity is progressively depressed in animals as thiamine deficiency becomes more severe. The measurement of erythrocyte transketolase activity and specifically the relative enhancement of transketolase activity after saturation with TDP (the so called thiamine pyrophosphate effect) *in vitro* is a sensitive index for detection and evaluation of thiamine deficiency (Vo-Khactu *et al*, 1974).

FIGURE 4: THE USE OF THE TRANSKETOLASE REACTION TO MONITOR LEVELS OF TDP



TK = Transketolase

TPI = Triose-phosphate isomerase

GDH = Glycerophosphate dehydrogenase

after Vo-Khactu *et al* (1974)

- b) Assay of an unknown quantity of TDP can be performed by addition of it to a purified apotransketolase (e.g. from yeast) preparation and measuring the rate of reaction. The rate is proportional to the amount of TDP available to combine with the apoenzyme and form transketolase (Gubler *et al*, 1970). This method will only determine the amount of TDP present in a sample as neither thiamine nor TMP will act as co-factors for transketolase.

## 1.6 SEPARATION OF THIAMINE AND ITS PHOSPHATE ESTERS

Estimation of the relative quantities of thiamine and of each of its phosphate esters in biological material is of considerable interest. Separation of each ester must be carried out to determine each independently. A wide variety of techniques have been used for this purpose.

### ( i ) Paper and Thin Layer Chromatography

Separations using a wide variety of solvents with paper and thin layer chromatographic systems have been reported (Siliprandi and Siliprandi, 1954; Pearson, 1967; Strohecker and Henning, 1965). Strohecker and Henning note that separations achieved using paper chromatography are sharper than those using thin layer chromatography but, as the latter technique requires less time, it is preferable for qualitative purposes. To obtain sufficient thiamine and/or its phosphate esters from biological material for good separation, considerable concentration of samples is required prior to chromatography. This means that, unless samples are desalted before use, separations tend to be indistinct (Mickelson and Yamamoto, 1958). After chromatography, thiamine and its esters are usually visualised under ultra violet light (366 nm) after the chromatograms have been sprayed with a dilute alkaline ferricyanide solution. As little as 5 ng of thiamine can be detected by this method and even lower amounts (down to  $5 \times 10^{-4}$   $\mu$ g) can be detected by bioautographic techniques (Stejn-Parve and Monfoort, 1963).

Quantitative evaluation of samples can be performed after elution of the samples from the chromatograms or directly on the paper using the apparatus developed by Esselborn and Klaus (Strohecker and Henning, 1965). Siliprandi and Siliprandi (1954) reported that a sample containing 10 - 30  $\mu\text{g}$  of each compound could be quantitatively estimated after paper chromatography and elution of the thiochrome derivatives. Losses of sample during elution are said to be more marked with thin layer systems making this technique unsuitable for quantitative estimation (Strohecker and Henning, 1965).

(ii) Paper Electrophoresis

Itokawa and Cooper (1970) used paper electrophoresis in acetate buffer (pH 3.8) to separate thiamine and its phosphate esters. They eluted thiamine and its esters by soaking the strips of paper containing sample in curvettes containing alkaline ferricyanide, and estimated these by the thiochrome method. A linear relationship between fluorescent units and amount of TTP electrophoresed was reported up to at least 200 ng. Penttinen (1978) noted that this method gave recoveries of only 66%, when compared against non-electrophoresed standards, making electrophoresis and elution of both samples and standards necessary. Penttinen (1978) also observed that many substances (e.g. salts, buffers and organic bases) reduced the separation of thiamine and its esters in the method of Itokawa and Cooper (1970) and that good separation was only achieved when the thiamine compounds were dissolved in water. He recommended the use of electrophoresis on paper saturated with citrate buffer followed by elution with 50% ethanol and reported recoveries of better than 96% when compared with non-electrophoresed thiamine standards.

(iii) Ion Exchange Chromatography

A variety of ion exchange resins have been used in attempts to separate thiamine and its derivatives. However, until recently all methods reported showed incomplete separations

unless two superimposed columns containing the same or different resins were used. For example Siliprandi and Siliprandi (1954) used a single column of Amberlite IRC 50 resin and found that although TTP and TDP were slightly separated, thiamine and TMP were eluted together. Rossi-Fanelli *et al* (1960) used this resin in two superimposed columns. The upper column (sodium form) bound thiamine and the lower column (hydrogen form) bound TMP. TDP and TTP were washed through both columns with water and the bound compounds were eluted with 0.1M hydrochloric acid. They also used Dowex 50 resin (hydrogen form) to separate TTP from thiamine and its other phosphate esters. TTP was washed through the column with water while thiamine, TMP and TDP were bound. No attempt to elute these compounds was reported. Rindi and De Giuseppe (1958) also used two superimposed columns of Dowex 1-X8. They found that a mixture containing thiamine, TMP, TDP and TTP could be separated using various elutants. Thiamine bound to neither column while TMP bound to the lower column (borate form) and TDP and TTP were separated by elution from the upper column (acetate form). They used a single column of Dowex 1-X8 (acetate form) to assay thiamine and its phosphate esters in various biological extracts but, although they found that TTP and TDP could be separated, they failed to achieve separation between thiamine and TMP. These had to be differentiated by estimation before and after hydrolysis (Rindi and De Giuseppe, 1960). Koike *et al* (1970) have claimed that the separation between TTP and TDP is improved if Dowex 1-X4 is used instead of Dowex 1-X8 (of the same mesh size) in the above procedure but they also failed to separate thiamine and TMP. Recently Gounaris and Schulman (1980) have reported a single column method using Dowex 1-X8 which separates thiamine and its phosphate esters. However they also found that, depending on the eluting buffers used, an artifact of TDP was produced or TDP was apparently hydrolysed to TMP (see section 5.7.2).

The identity of eluted fractions was determined, in the separations described, by various methods. Rindi and De Giuseppe (1958) used paper chromatography. The paper

published by Siliprandi and Siliprandi (1954) also contained a number of other techniques for the separation of thiamine and its phosphate esters and presumably one of these was used to test the identity of the peaks separated on Ambelite IRC 50 although this was not mentioned. Koike *et al* (1970) looked for an increased absorbance of light (270 nm) in each peak after either thiamine or one of its phosphate esters was added to a mixed sample of known composition and eluted from Dowex 1-X4. Gounaris and Schulman (1980) used phosphate analysis and the ultra violet spectrum of each peak. The techniques used to identify the peaks in some experiments seem slightly suspect as they do not test adequately for sample breakdown on the resin. Breakdown could result in each peak having several components. Without the use of paper chromatography or electrophoresis this would be undetected. Recoveries were determined either spectrophotometrically at 270 nm or by the thiochrome method. In all separations reported recoveries were claimed to be adequate, being 90% or higher.

#### (iv) Gel Chromatography

Gel chromatography has also been used to separate thiamine and its derivatives. Davidek *et al* (1977) used Sephadex G10 with an elution gradient of 0-0.01M hydrochloric acid. TDP was eluted with water but the separation between TMP and thiamine was poor. Nishimune *et al* (1972) also used Sephadex G10 to separate an oxidised sample consisting of the thiochrome derivatives of thiamine and its phosphate esters. They obtained a good separation but found that thiochrome eluted in two peaks which had the same positions on re-chromatography. Recoveries of 94-100% were obtained. Sephadex G25 cation exchanger was used by Parkomenko *et al* (1979) but thiamine and TMP were unseparated and had to be determined by difference after phosphatase treatment. TTP obtained after separation was contaminated, presumably with impurities from the sample, and required additional purification by ion exchange. They claimed recoveries of 98-100%.

( v ) High Pressure Liquid Chromatography (HPLC)

High pressure liquid chromatography has been used for separation and determination of thiamine and its phosphate esters in pharmaceutical materials (Gubler and Hemming, 1972). This technique could be expected to provide a very versatile and rapid method for estimation of thiamine and its derivatives but few reports are yet available on its suitability for biological samples.

1.7 CONCLUSION

The aim of this thesis was to determine methods suitable for the separation and estimation of thiamine and its derivatives in biological tissues. Of the many methods available for estimation of thiamine the fluorometric estimation by the thiochrome method appears to be best suited for biological material extracts as it is the most sensitive of the chemical methods. The susceptibility of this assay to interference and the large number of variations to the method used by previous workers meant however, that it was necessary to examine the effect of various procedures on results. Since the thiochrome method requires extraction of thiochrome with isobutanol, and can thus only estimate thiochrome, methods for hydrolysis of thiamine phosphate esters were also investigated. The only other method which appeared promising, the acid dye method of Das Gupta and Cadwallader (1967), was also investigated. This method has reasonable sensitivity for the estimation of thiamine ( $0-10 \mu\text{g}/\text{cm}^3$ ) but the effect of thiamine phosphate esters and amino acids (which are likely to be present in biological samples) do not appear to have been investigated.

A large number of methods have also been used for the separation of thiamine and its derivatives. Some of these methods (thin layer and paper chromatography, high voltage electrophoresis) seem to be suitable for the separation and detection of thiamine and related compounds but appear to be difficult to use quantitatively. Ion exchange chromatography

appears to offer the best method for quantitative separation and estimation of thiamine and its phosphate esters. Such methods also provide a means of concentration of samples, which is desirable, since thiamine and its derivations are only present in small amounts in tissues. However at the time of this work all separations using ion exchange resins were unsatisfactory either because separation of thiamine and its phosphate esters were incomplete or because the procedure involved the use of two columns. Therefore, in an attempt to develop a satisfactory method of separation, alternative ion exchange resins and procedures were investigated. The single column method reported by Gounaris and Schulman (1980) was not published until most of this work had been completed. The separation of thiamine and its esters by gel chromatography was also investigated even though this would probably have limited application since this procedure does not allow concentration of samples. It was however, at the commencement of this work, the only reported method for complete separation of thiamine and its esters on a single column.

This thesis is divided into two parts. Part 1 is concerned with the estimation of thiamine and part 2 with the separation of thiamine and its esters.

PART 1

ESTIMATION OF THIAMINE, THIAMINE MONOPHOSPHATE  
AND THIAMINE DIPHOSPHATE

## CHAPTER 2

## MATERIALS

(Materials in this section refer to parts 1 and 2 of this thesis).

(i) Thiamine and its Phosphate Esters

- (a) Thiamine hydrochloride was obtained from the Sigma Chemical Company and shown to be pure (i.e. contain no traces of either thiamine mono or diphosphate) by high voltage electrophoresis.
- (b) Thiamine monophosphate used in experiments to test the acid dye method was prepared according to the procedure of Matsukawa *et al* (1970). The TMP prepared was shown to contain traces of thiamine and thiamine diphosphate by chromatography of a concentrated sample on paper using propanol/H<sub>2</sub>O/acetate buffer (7:2:1) solvent.
- (c) Thiamine monophosphate hydrochloride used in all other experiments was obtained from the Sigma Chemical Company and was shown to be pure by high voltage electrophoresis.
- (d) Thiamine diphosphate chloride (A grade) was supplied by Calbiochem. This, when examined by high voltage electrophoresis, was shown to contain traces of thiamine and TMP. In most experiments the TDP sample used was not purified before use, but to obtain a TDP solution for use in estimating recoveries after elution from a column of Dowex 1-X8 the thiamine and TMP traces were removed. This was achieved by chromatography of the TDP solution on a column of Amberlite GC50 resin (hydrogen form) prior to application to the Dowex 1-X8 resin. TDP was washed through the column of Amberlite GC50 with 0.05M sodium formate buffer (pH 5.5) while

thiamine and TMP were retained by the resin. That the sample contained only TDP after this procedure was verified by high voltage electrophoresis.

- (e) Thiamine, TMP and TDP powders and solutions were stored in a dark refrigerator. Before use the powders were desiccated under vacuum over phosphorous pentoxide, for several days in a refrigerator.

(ii) Other Chemicals

Unless otherwise stated all chemicals were of laboratory grade. Potassium dihydrogen phosphate (Analar grade), anhydrous sodium acetate, acetic acid (Aristar grade), magnesium chloride, ammonia and urea were obtained from British Drug Houses Limited. Bromothymol blue, sodium hydroxide (analytical grade), potassium ferricyanide, ammonium acetate (analytical grade), anhydrous sodium formate and pyridine were obtained from May and Baker. Chloroform and formic acid (95-100% pure) were obtained from Riedel-de Haen. Ammonium formate (analytical grade) and propanol were purchased from Ajax Chemicals Limited. Hydrochloric acid (analytical grade) was supplied by Berk-Spencer Limited. Anhydrous sodium acetate was supplied by Sigma Chemical Company. Ethanol (95%) was obtained from the Colonial Refining Company (Aust). Methanol was obtained from Mobil. Amino Acids used (Tyrosine, Tryptophan, Alanine, Glutamine, Cysteine) were analytical (Sigma) grade and supplied by the Sigma Chemical Company.

Water used throughout the experiments was glass distilled and deionised using AG 501-X8 mixed bed resin supplied by Bio Rad Laboratories. Isobutanol (supplied by British Drug Houses Limited) was fractionally distilled in all glass apparatus before use; only that which distilled between 105-110° was used. Formic acid used in the experiments with Dowex 50 resin was also distilled in glass apparatus.

(iii) Acid Phosphatase

Wheat germ acid phosphatase (EC 3.1.3.1) was supplied by the Sigma Chemical Company. This was stored desiccated in refrigerator. Fresh solutions were prepared for each digestion.

(iv) Ion Exchange Resins, and Gels

Silica gel was obtained from Riedel-de Haen. Amberlite GC50 resin (hydrogen form) (type 1, 100-200 mesh) was obtained from British Drug Houses Limited and Dowex 1-X8 (200-400 mesh, chloride form) was purchased from Bio Rad Laboratories Limited. Sephadex G10 and Dowex 50W-X8 (hydrogen form, 100-200 mesh) were supplied by the Sigma Chemical Company. All resins, when not in use, were stored in a 0.02% sodium azide solution and were thoroughly washed with distilled-deionised water before use.

## CHAPTER 3

### METHODS

#### 3.1 THE ACID DYE METHOD

The method of Das Gupta and Cadwallader (1967) was followed. Samples ( $10 \text{ cm}^3$ ) of thiamine ( $0-10 \mu\text{g}/\text{cm}^3$ ) in  $0.2\text{M}$  phosphate buffer ( $\text{pH}6.6$ ) were pipetted into clean, dry separating flasks. These were extracted, by shaking by hand for one minute, with  $10 \text{ cm}^3$  of a  $1 \times 10^{-4}\text{M}$  solution of bromothymol blue in chloroform. After a further extraction with  $10 \text{ cm}^3$  of the chloroform, the extracts were combined and evaporated at  $50^\circ$  to a volume of less than  $10 \text{ cm}^3$ . The extracts were made up to  $10 \text{ cm}^3$  with chloroform and centrifuged for 5 minutes at  $755 \text{ xg}$ . The absorbance of the clarified solutions was determined at  $420 \text{ nm}$  in a Hitachi Spectrophotometer.

The ability of various thiamine phosphate esters and amino acids to form dye salts was examined by performing the acid dye procedure on separate solutions of each substance in  $10 \text{ cm}^3$  of  $0.2\text{M}$  phosphate buffer.

#### 3.2 THE THIOCHROME METHOD

The method described in Methods of Analysis of the A.O.A.C. (1975) was followed. Aliquots containing  $0.4 \mu\text{g}$  ( $0-12 \text{ n moles}$ ) of thiamine in  $4 \text{ cm}^3$  of  $1\text{M}$  sodium acetate buffer ( $\text{pH} 4.8$ ) were placed in light proofed (tin foil),  $50 \text{ cm}^3$  glass stoppered centrifuge tubes. A  $3 \text{ cm}^3$  aliquot of a solution of potassium ferricyanide ( $0.01\%-0.6\%$ ) in  $15\%$  sodium hydroxide was added to these tubes. The thiochrome produced was immediately extracted with  $13 \text{ cm}^3$  of isobutanol or a combination of  $11 \text{ cm}^3$  of isobutanol and  $2 \text{ cm}^3$  of  $95\%$  ethanol, by shaking at maximum speed on a Gallenkamp flask shaker for two minutes. The alcohol extracts were clarified by low speed centrifugation in a Gallenkamp bench centrifuge. The fluorescence of the extracts was determined in a Turner 320 spectrofluorometer with an excitation wavelength of  $378 \text{ nm}$ , an emission wavelength of  $428 \text{ nm}$  (Fig 5) and a band width of  $15 \text{ nm}$ . Glassware used

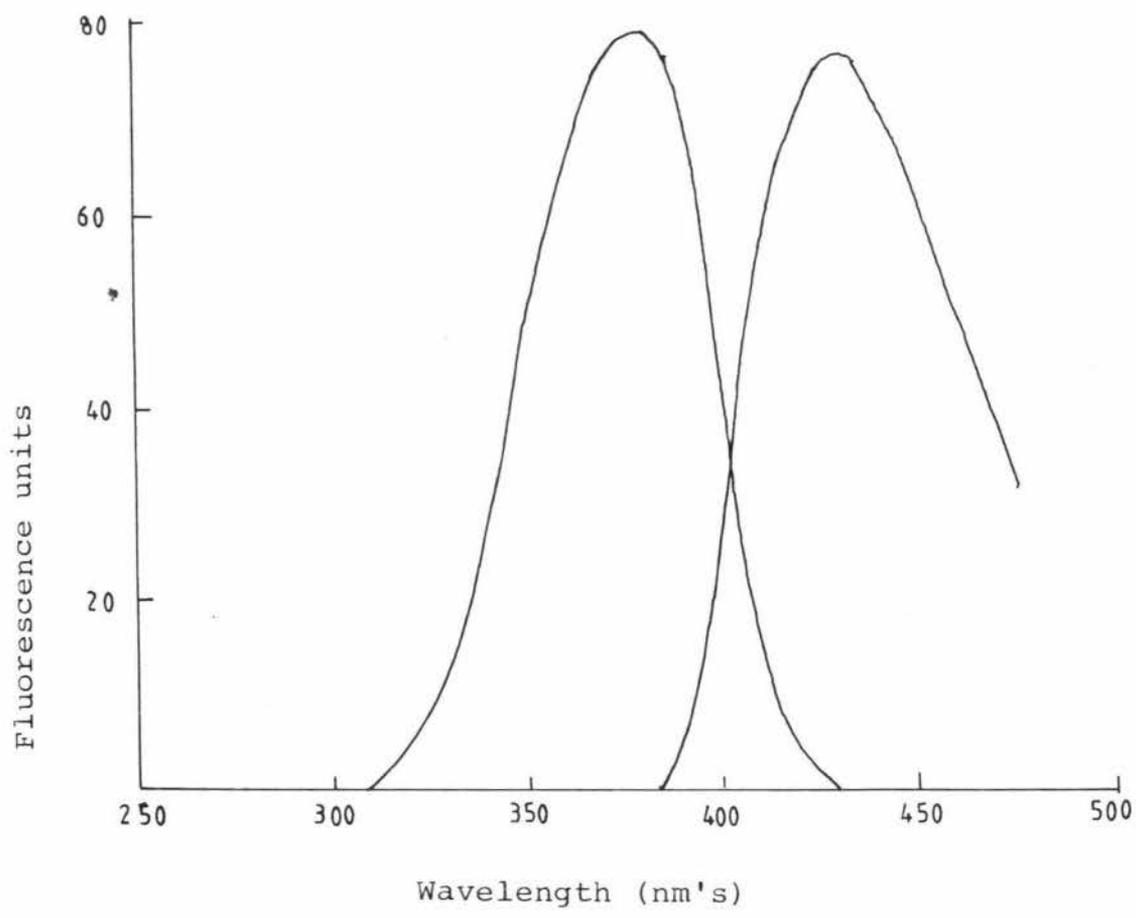


FIGURE 5: FLUORESCENT SPECTRUM OF THIAMINE  
(Thiochrome in isobutanol)

throughout the procedure was acid washed in 50% nitric acid.

### 3.3 HYDROLYSIS OF THIAMINE PHOSPHATE ESTERS

The procedure of Shultz and Natelson (1972) using wheat germ acid phosphatase was followed but the effect of different buffers, enzyme concentrations, addition of magnesium ions and variation in hydrolysis time was investigated. As hydrolysis of thiamine phosphate esters was to be performed directly before thiochrome estimation, the effect of phosphatase concentration on extraction of thiochrome by isobutanol was also investigated. Aliquots containing  $2 \mu\text{g}$  of thiamine, TMP and/or TDP in  $2 \text{ cm}^3$  of pH 4.8 buffer (0.01-2M sodium acetate, sodium formate or ammonium acetate) were placed in glass stoppered, centrifuge tubes which were light proofed with tin foil. The effect of adding  $1 \text{ cm}^3$  of phosphatase (0.05-10mg) in various buffers (pH4.8) and  $1 \text{ cm}^3$  of aqueous magnesium chloride (10-800nm) was investigated. After mixing, the reaction mixture was incubated at  $37^\circ$  for 1-45 hours. The amount of free thiamine present after incubation was estimated by the thiochrome procedure.

## CHAPTER 4

### RESULTS AND DISCUSSIONS

#### 4.1 THE ACID DYE METHOD

A linear standard curve passing through zero indicated that, in the range tested, this method follows Beer's law (Fig. 6). Good reproducibility was indicated by close agreement between absorbance readings produced by samples containing equal quantities of thiamine (Table 2). Even at high concentrations, TMP and TDP did not produce extractable dye salts (Table 3a). The small absorbance reading (6% of the reading observed for a thiamine solution of comparable concentration) observed when a TMP solution of high concentration was tested was probably due to a small quantity of thiamine which was present in the TMP samples used (see Chapter 2) and not to formation of an extractable dye salt with TMP. TDP had also been shown, by high voltage electrophoresis, to contain traces of thiamine contaminant and the fact that this was not detected by these tests suggests that the acid dye method is less sensitive than the thiochrome method. When thiamine phosphate esters were tested at lower concentrations ( $8-12\mu\text{g}/\text{cm}^3$ ), slightly negative absorbance readings were observed (Table 3b).

Amino acids, when tested at concentrations expected to occur in human blood, showed little extractable absorbance (Table 3c). Most gave slightly negative absorbance readings. Whether these negative absorbances constitute serious interference with this assay method is unknown but, as the acid dye method relies on a simple acid-base reaction, it is likely that amino acids and low levels of thiamine phosphate esters form a dye salt with bromothymol blue. If unextractable dye salt was formed with amino acids or thiamine phosphate esters the concentration of unreacted bromothymol blue in the organic phase would be expected to decrease. Das Gupta and Cadwallader (1967) have shown that unreacted bromothymol blue exhibits considerable absorption of light at the wavelength used to estimate the dye salt. Thus amino acids and thiamine phosphate

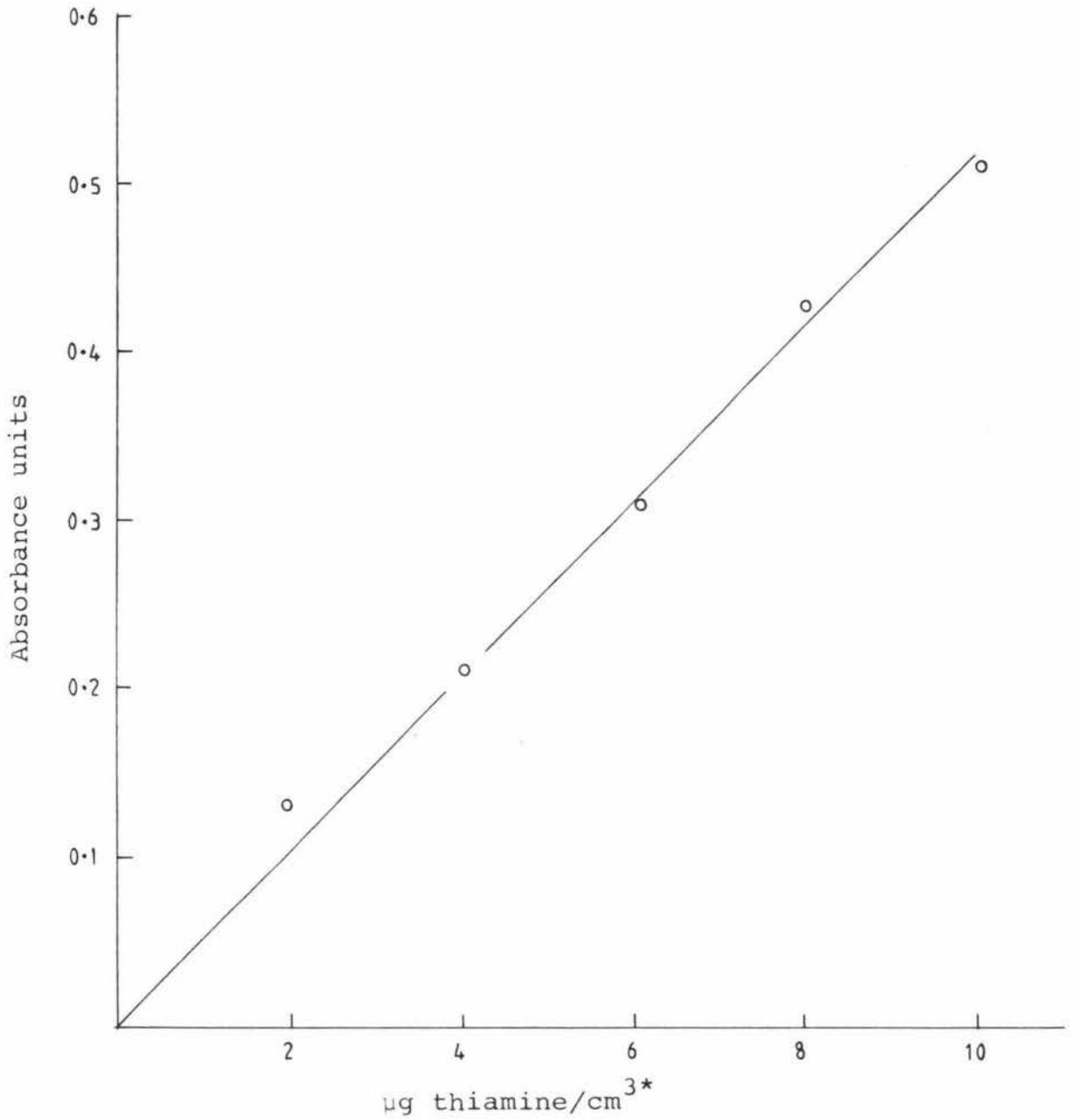


FIGURE 6: A THIAMINE STANDARD CURVE PREPARED USING THE ACID DYE METHOD

\*  $10\text{cm}^3$  of thiamine solution (of stated concentration) in phosphate buffer (pH 6.6) was estimated.

TABLE 2: TRIPLICATE DETERMINATION OF A SINGLE THIAMINE SOLUTION ESTIMATED BY THE ACID DYE METHOD

| Thiamine Concentration*<br>(mg/cm <sup>3</sup> ) | Absorbance Reading |
|--|--------------------|
| 4.06   | 0.208              |
| 4.06   | 0.214              |
| 4.06   | 0.213              |

TABLE 3a: ABSORBANCE OF THIAMINE AND THIAMINE PHOSPHATE ESTERS ESTIMATED BY THE ACID DYE METHOD

|          | Concentration*<br>(mg/cm <sup>3</sup> ) | Absorbance Reading | Corresponding quantity of thiamine <sup>+</sup><br>(mg/cm <sup>3</sup> ) |
|----------|---|--------------------|--|
| Thiamine | 0.385                                   | 1.49               | 0.385  |
| TMP      | 0.506                                   | 0.093              | 0.024  |
| TDP      | 0.550                                   | 0                  | 0  |

\* A 10cm<sup>3</sup> solution of thiamine or the thiamine phosphate ester of the stated concentration was estimated.

+ The concentration of a 10cm<sup>3</sup> solution of thiamine which would correspond to the absorbance reading obtained.

TABLE 3b: ABSORBANCE OF THIAMINE AND THIAMINE PHOSPHATE ESTERS ESTIMATED BY THE ACID DYE METHOD

|          | Concentration*<br>(mg/cm <sup>3</sup> ) | Absorbance<br>Reading |
|----------|---|-----------------------|
| Thiamine | 8                                       | 0.369                 |
| TMP      | 10                                      | -0.11                 |
| TDP      | 12                                      | -0.11                 |

TABLE 3c: ABSORBANCE OF AMINO ACIDS ESTIMATED BY THE ACID DYE METHOD

| Amino Acid | Concentration*<br>(mg/100cm <sup>3</sup> ) | Absorbance<br>Reading |
|------------|--|-----------------------|
| Tyrosine   | 2.2  | -0.02                 |
| Tryptophan | 3.6  | -0.02                 |
| Alanine    | 7.4  | -0.03                 |
| Glutamine  | 9.2  | +0.03                 |
| Cysteine   | 5.2  | -0.03                 |

\* A solution (10cm<sup>3</sup>) of the compound of stated concentration was estimated.

esters present in the aqueous phase could cause a decrease in the absorption of light by the organic phase, when compared with the organic phase of a blank in which amino acids or thiamine phosphate esters were not present. Park (1975) has noted that interference, observed as an increase or decrease in the absorption intensity, occurs when various multi-vitamin preparations are estimated by the acid dye method. He suggested that this was due to the nature of the compositional constituents of these preparations and that the acid dye method was not highly specific for thiamine. Das Gupta and Cadwallader (1967) tested various diverse substances and none were recorded as giving negative absorbance readings.

#### 4.2 ESTIMATION OF THIAMINE BY THE THIOCHROME METHOD

##### 4.2.1 Variation of the Ferricyanide Concentration

Samples containing 2 $\mu$ g of thiamine were estimated as this approximates the quantity of thiamine present in 100 cm<sup>3</sup> of human blood (Table 1). Maximum fluorescence was obtained when 0.01-0.04% potassium-ferricyanide in 15% sodium hydroxide was used to oxidise 2 $\mu$ g of thiamine (Fig. 7). When a concentration of greater than 0.1% ferricyanide was used to produce thiochrome, the quantity of extractable fluorescence decreased. This was presumably due to destruction of thiochrome by oxidation with the excess ferricyanide present (Yamamoto and Mickelson, 1958). It was considered that, within the narrow range of ferricyanide concentrations in which apparently insignificant destruction of thiochrome occurred, it would be an advantage to use as high a ferricyanide concentration as possible. This would ensure complete oxidation within the range of thiamine concentrations likely to be encountered in biological extracts. Therefore a 0.04% ferricyanide solution was used in all subsequent determinations. All samples estimated in subsequent work were diluted so as to contain 0.5-3 $\mu$ g of thiamine.

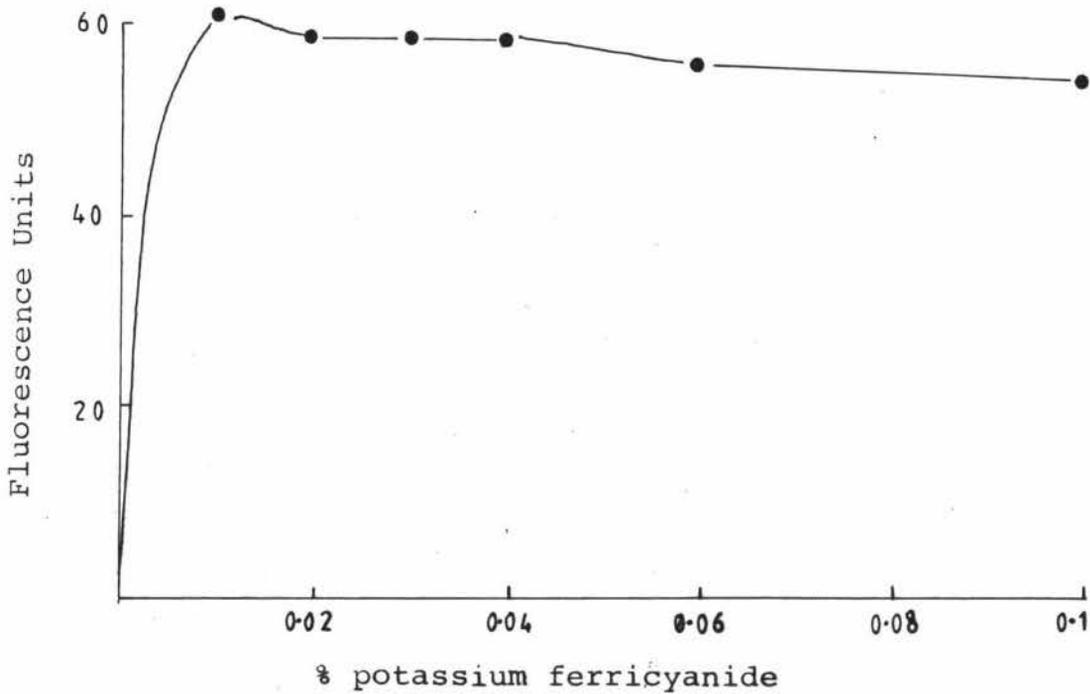
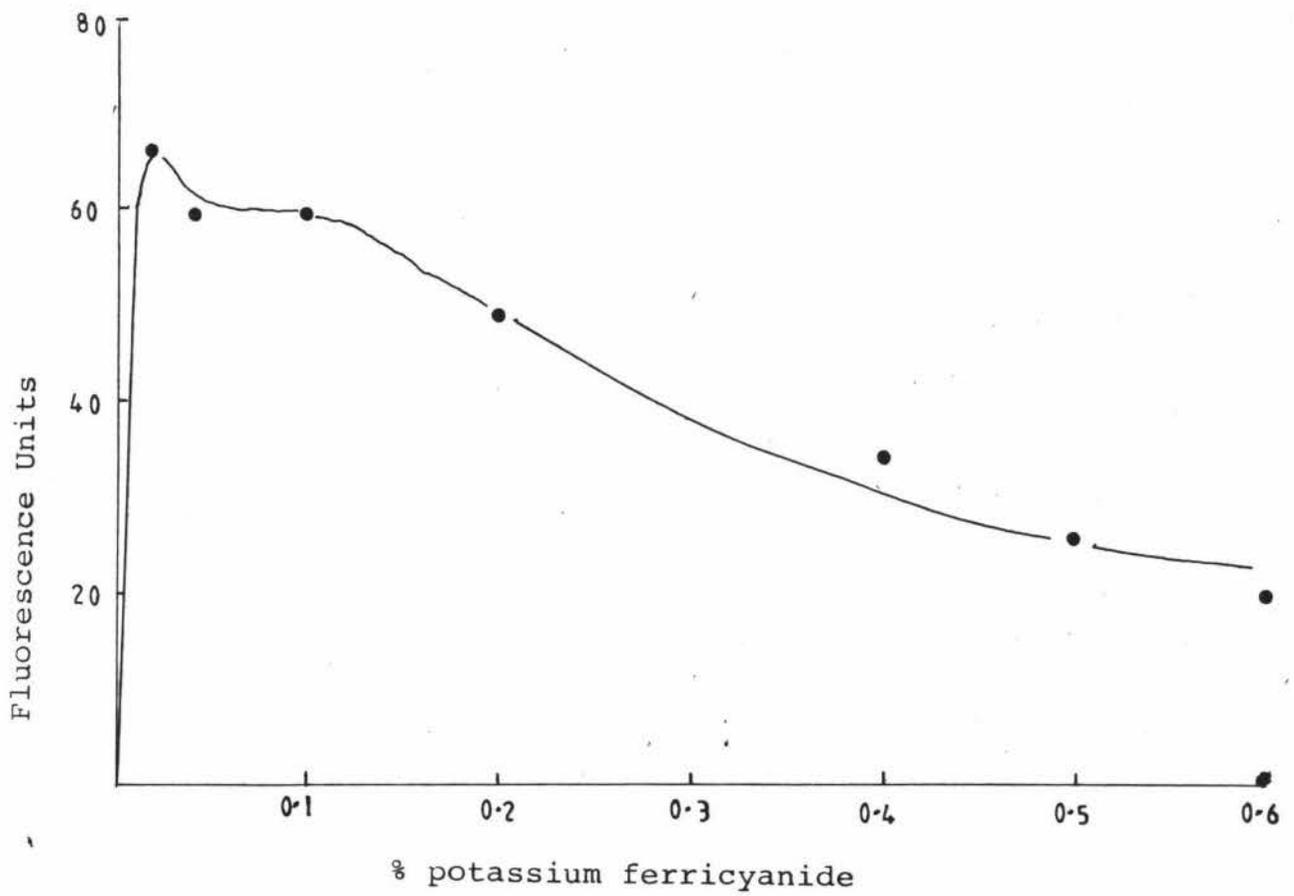


FIGURE 7: THE EFFECT OF VARIATION OF THE CONCENTRATION OF THE POTASSIUM FERRICYANIDE SOLUTION USED TO OXIDISE THIAMINE.

Samples of thiamine ( $2 \mu\text{g}$ ) were oxidised with  $3\text{cm}^3$  of varying alkaline ferricyanide solutions (concentrations stated above as % w/v). The thiochrome produced was immediately extracted with  $13\text{cm}^3$  of isobutanol and determined in a fluorometer.

#### 4.2.2 Extraction of Thiochrome by Isobutanol

A single extraction of thiochrome with isobutanol was normally used as it was found that extracting twice with isobutanol resulted in a slightly lower fluorescence recovery (Table 4). This was presumably due to losses incurred with increased handling (two centrifugations were necessary).

The inclusion of a small quantity of ethanol with the isobutanol (2cm<sup>3</sup> of ethanol with 11cm<sup>3</sup> isobutanol) was suggested by Watson (1946) as an aid for clarification of the extracts. This was found to slightly increase the recorded fluorescence (Table 5). Since the increase was very small only isobutanol was used to extract thiochrome in all subsequent work.

#### 4.2.3 Standard Curve Preparation

In the range 0-2µg of thiamine a linear standard curve was obtained (Fig. 8). Reproducibility, judged on seven determinations performed on identical thiamine samples, was good (Table 6). Due to the rapid degradation of thiochrome by the high intensity ultra violet light of the fluorometer (Mickelson and Yamamoto, 1958) rapid and accurate recording of fluorescence was imperative.

#### 4.2.4 Summary - Thiochrome Method

The conditions used in all subsequent determinations were similar to those outlined in the A.O.A.C. methods of analysis handbook (1975). Thiamine was oxidised with 3cm<sup>3</sup> of 0.04% ferricyanide in 15% sodium hydroxide followed by immediate extraction with a single quantity of 13cm<sup>3</sup> of isobutanol. Instead of using a quinine sulphate standard to calibrate the fluorometer a new standard curve was prepared for each assay. This was considered a better method as production of a standard curve would serve as a check on procedure.

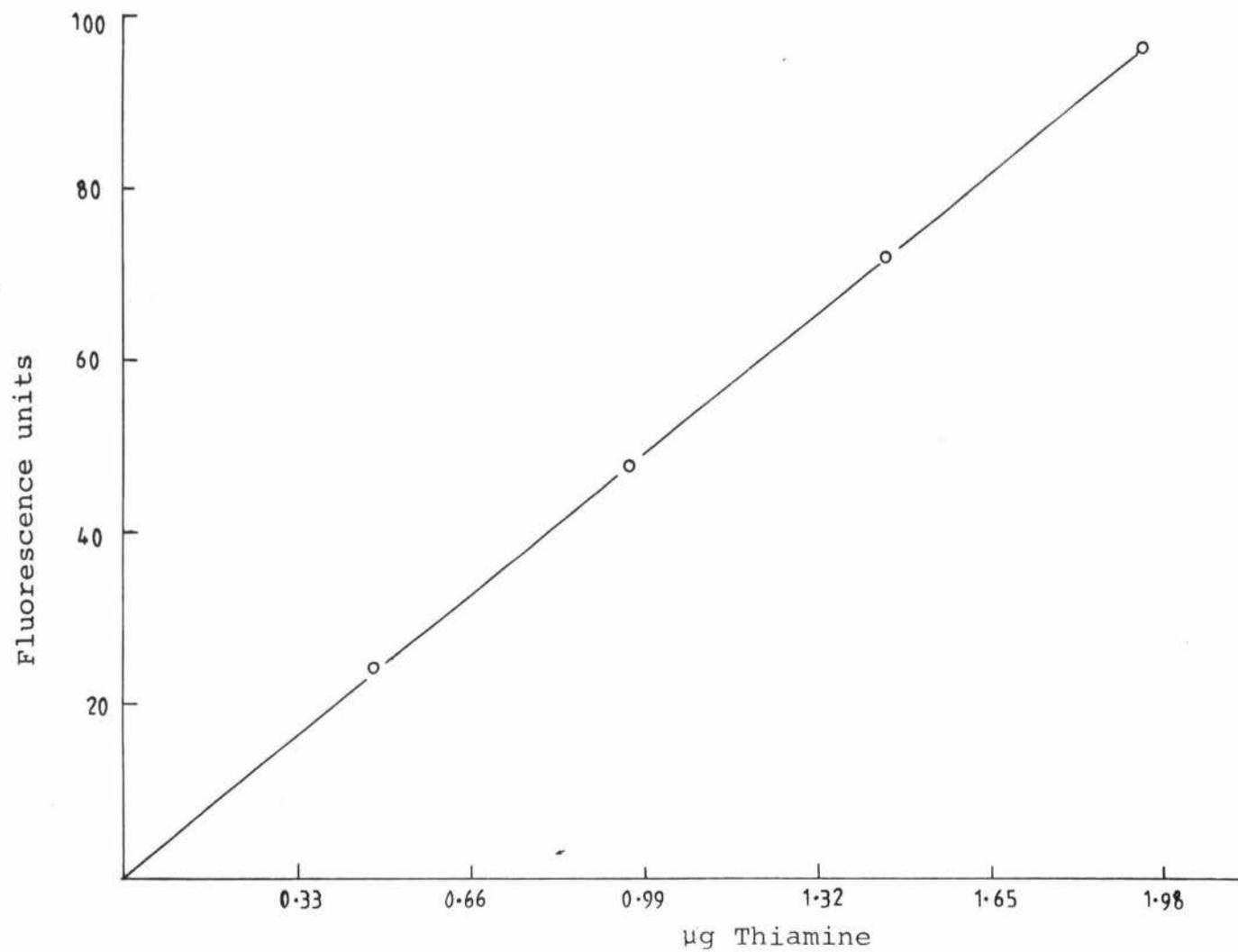


FIGURE 8: A TYPICAL THIAMINE STANDARD CURVE PREPARED BY THE THIOCHROME METHOD (see text for method)

TABLE 4: THE EFFECT OF MULTIPLE ISOBUTANOL EXTRACTION ON THE RECOVERY OF FLUORESCENCE FROM A THIAMINE SOLUTION\*

| Number of extractions | Fluorescence Reading |
|-----------------------|----------------------|
| 1                     | 63.5                 |
| 1                     | 60                   |
| 2                     | 59                   |
| 2                     | 59.5                 |
| 2                     | 61                   |

\* 2  $\mu\text{g}$  of thiamine in  $4\text{cm}^3$  of 1M sodium acetate buffer (pH 4.8) was given either a  $1 \times 12\text{cm}^3$  extraction or  $2 \times 6\text{cm}^3$  extractions with isobutanol.

TABLE 5: THE EFFECT OF ADDITION OF 95% ETHANOL TO THE ISOBUTANOL USED TO EXTRACT THIOCHROME ON RECOVERY OF FLUORESCENCE

| Organic phase composition    |                               | Absorbance Reading |
|------------------------------|-------------------------------|--------------------|
| Isobutanol ( $\text{cm}^3$ ) | 95% Ethanol ( $\text{cm}^3$ ) |                    |
| 13                           | -                             | 49.50              |
| 11                           | 2                             | 51.75              |

\* 2  $\mu\text{g}$  of thiamine in  $3\text{cm}^3$  of 1M sodium acetate buffer (pH 4.8) was extracted once with an organic phase prepared as above.

#### 4.3 HYDROLYSIS OF THIAMINE MONOPHOSPHATE AND THIAMINE DIPHOSPHATE WITH WHEAT GERM ACID PHOSPHATASE

##### 4.3.1 The Effect of Phosphatase Concentration, Magnesium Ion Concentration and Time on the Hydrolysis of Thiamine Diphosphate

Interference to the alcoholic extraction of thiochrome by phosphatase was examined by adding various amounts of phosphatase to a sample containing 2 $\mu$ g of thiamine only in 1M sodium acetate buffer (pH 4.8). This was immediately oxidised with alkaline ferricyanide and extracted with 13cm<sup>3</sup> of isobutanol. High concentrations of phosphatase inhibited the extraction of thiochrome (Fig. 9). Only 72% of the extractable fluorescence of a thiamine solution (compared to that with no phosphatase present) was extracted when 10mg of phosphatase, the maximum concentration tested, was included. Thus digestion of TDP must be achieved using a low enough concentration of phosphatase to avoid interference to extraction of thiochrome.

Since magnesium ions had been shown to increase the activity of phosphatase preparations (Appaji Rao and Vaidyanathan, 1966) the effect of magnesium chloride on a 24 hour hydrolysis of TDP (2 $\mu$ g) in 3cm<sup>3</sup> of 1M sodium acetate buffer (pH 4.8) by phosphatase (1mg) was investigated. The production of free thiamine (as indicated by the thiochrome method), when 1cm<sup>3</sup> of aqueous 200-400mM magnesium chloride was added to the digestion mixture, was 1½ times greater than that of a digestion mixture containing no magnesium chloride. Above a magnesium chloride concentration of 400mM the production of extractable fluorescence decreased (Fig. 10).

Experiments were next performed to determine the optimum quantity of phosphatase, and time to achieve digestion of 2 $\mu$ g of TDP when 1cm<sup>3</sup> of 400mM magnesium chloride was added to the digestion mixture (4cm<sup>3</sup>). Even though a previous experiment had shown that 1mg of phosphatase per digestion tube interfered with the extraction of thiochrome by isobutanol (Fig. 9) complete hydrolysis of TDP and recovery of

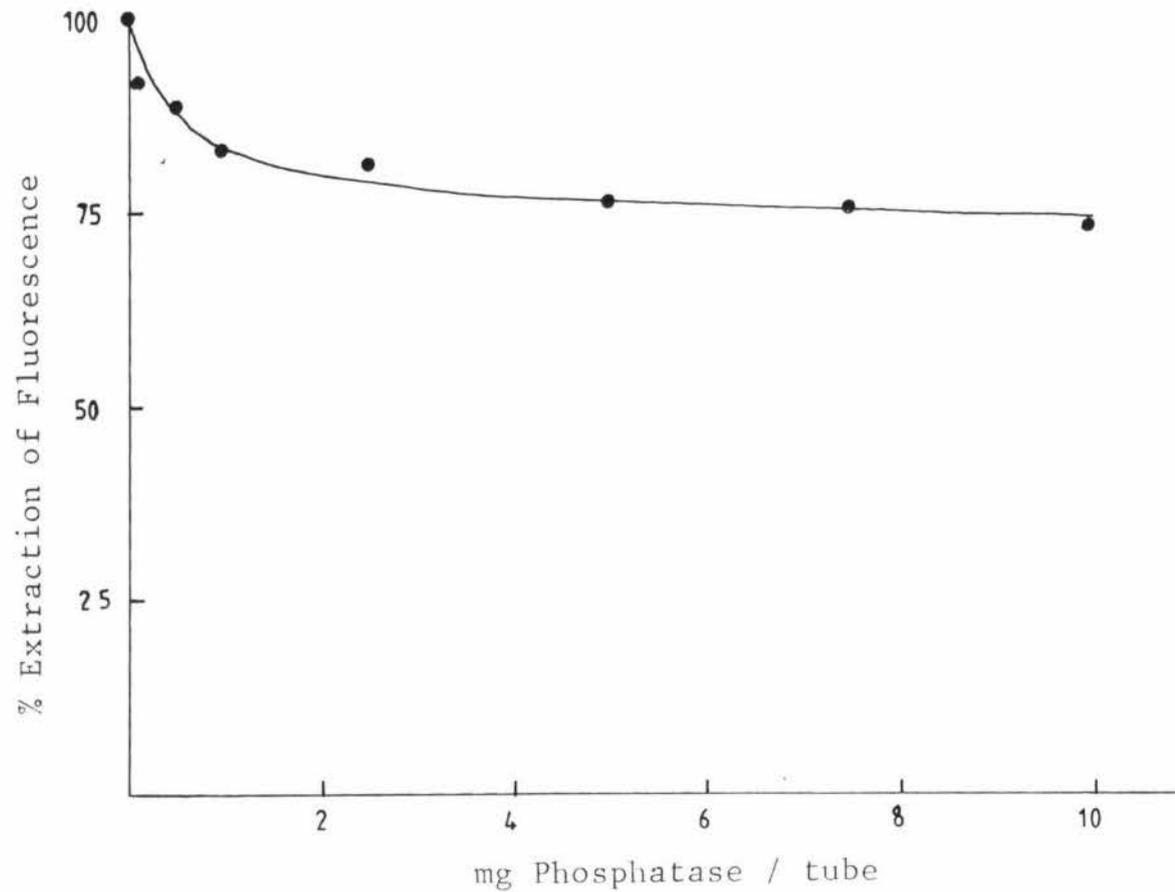


FIGURE 9: EFFECT OF VARYING PHOSPHATASE CONCENTRATION ON THE EXTRACTION OF FLUORESCENCE BY ISOBUTANOL.

2  $\mu$ g samples of thiamine were mixed with varying amounts of phosphatase and determined by the thiochrome method.

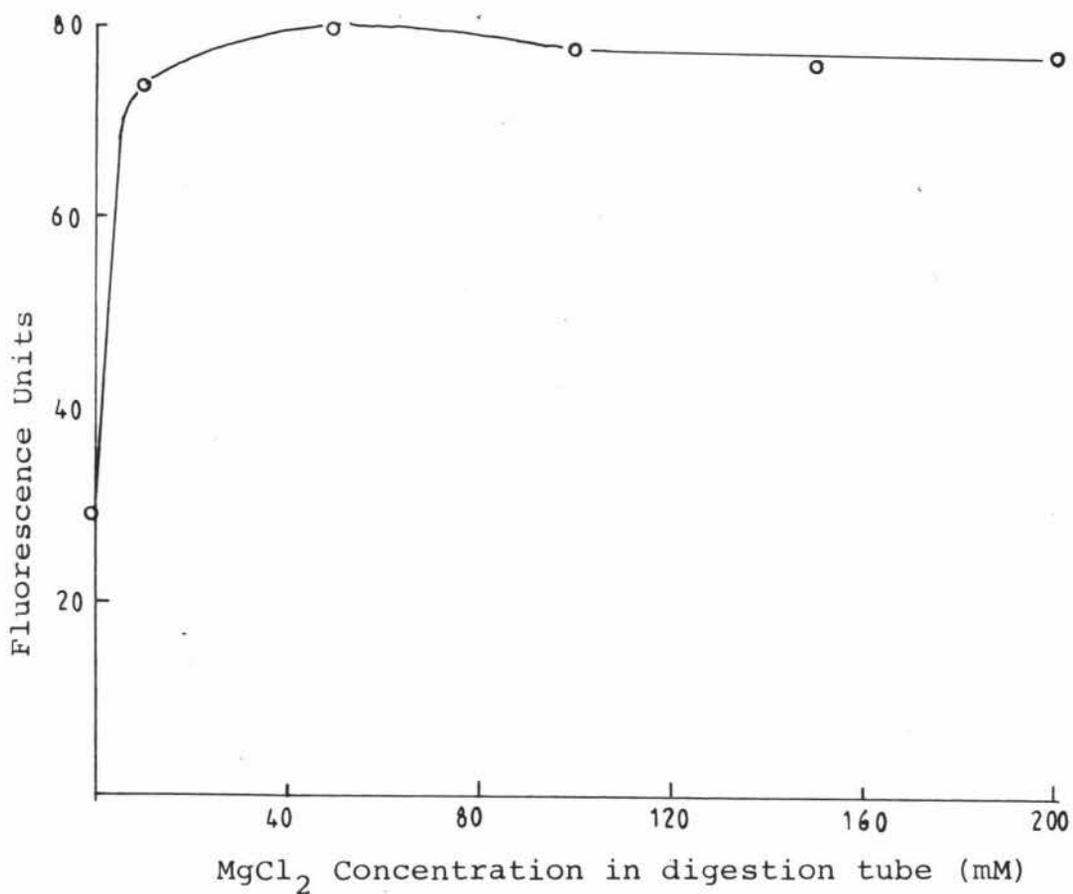
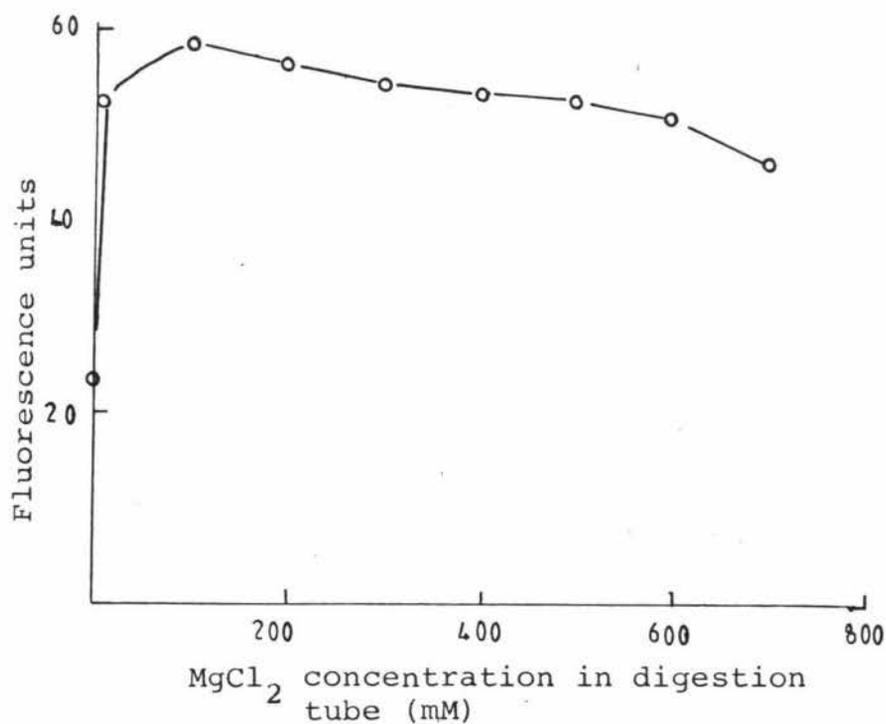


FIGURE 10: STIMULATION OF PHOSPHATE ACTIVITY BY MgCl<sub>2</sub>

2  $\mu\text{g}$  samples of TDP were digested with 1mg of phosphate in 3cm<sup>3</sup> of 1M sodium acetate buffer (pH 4.8). To these 1cm<sup>3</sup> samples of varying concentration aqueous MgCl<sub>2</sub> solutions were added to give the MgCl<sub>2</sub> concentrations in the digestion mixture shown above. The digestion mixtures were estimated by the thiochrome method.

TABLE 6: REPRODUCIBILITY OF THE THIOCHROME ASSAY\*

| Determination | Fluorescence Reading |
|---------------|----------------------|
| 1             | 64.0                 |
| 2             | 65.5                 |
| 3             | 65.0                 |
| 4             | 64.0                 |
| 5             | 66.5                 |
| 6             | 66.0                 |
| 7             | 65.0                 |

\* 7 solutions containing 1.3  $\mu\text{g}$  of thiamine each were determined by the thiochrome method.

TABLE 7: THE EFFECT OF PHOSPHATASE CONCENTRATION ON HYDROLYSIS OF TDP\*

| Phosphatase concentration (mg/digestion tube) | Fluorescence Reading | % Recovery |
|---|----------------------|------------|
| 0   | 0                    | 0          |
| 0.05  | 29                   | 42         |
| 0.25  | 67                   | 97         |
| 1.0   | 70.5                 | 101        |
| 10  | 56                   | 81         |

\* 2  $\mu\text{g}$  of TDP was digested with varying quantities of phosphatase in 3  $\text{cm}^3$  of 1M sodium acetate buffer (pH 4.8). 1  $\text{cm}^3$  of aqueous 400mM  $\text{MgCl}_2$  was present in each digestion tube. Recoveries were estimated by reference to a thiamine standard curve by the thiochrome method.

thiochrome was obtained using 0.25-lmg of phosphatase in the digestion mixture when digestion was performed at 37° for 48 hours (Table 7). This apparent discrepancy could be possibly due to the incubation of the assay mixture in this experiment, whereas in the previous experiment the assay mixture was extracted with isobutanol immediately after preparation. At lower enzyme concentrations digestion of TDP was incomplete and at higher enzyme concentrations the recovery of fluorescence from the digestion mixture, after oxidation of the free thiamine by the thiochrome method, was incomplete.

Since 1mg of phosphatase gave complete digestion of TDP in 48 hours, the effect of reducing the time of digestion was investigated (Fig. 11). Hydrolysis of TDP required a minimum of 15 hours. No increase in the quantity of TDP hydrolysed occurred from 15 hours to 45 hours (the maximum digestion time tested) and a maximum recovery of 97% was obtained.

#### 4.3.2 The Effect of Buffer Ionic Strength and Composition on Digestion of TDP

TDP was digested in buffers of various ionic strength and composition in the absence of magnesium ions. Since magnesium ions were not included in the digestion mixture a higher phosphatase concentration (10mg/digestion tube) was used to ensure as complete a digestion as possible, even though this would decrease the quantity of fluorescence extractable by isobutanol.

##### i) Ionic Strength

When the digestion of TDP was performed in 1M sodium acetate buffer (pH 4.8) total fluorescence could not be recovered (Table 8a). This was probably due to interference with the extraction of thiochrome by high concentrations of phosphate as the recovery obtained (73%) is comparable with that obtained in Section 4.3.1. However, this effect did not occur when 2M sodium acetate buffer (pH 4.8) was used as the complete recovery of fluorescence was observed when this buffer was used.

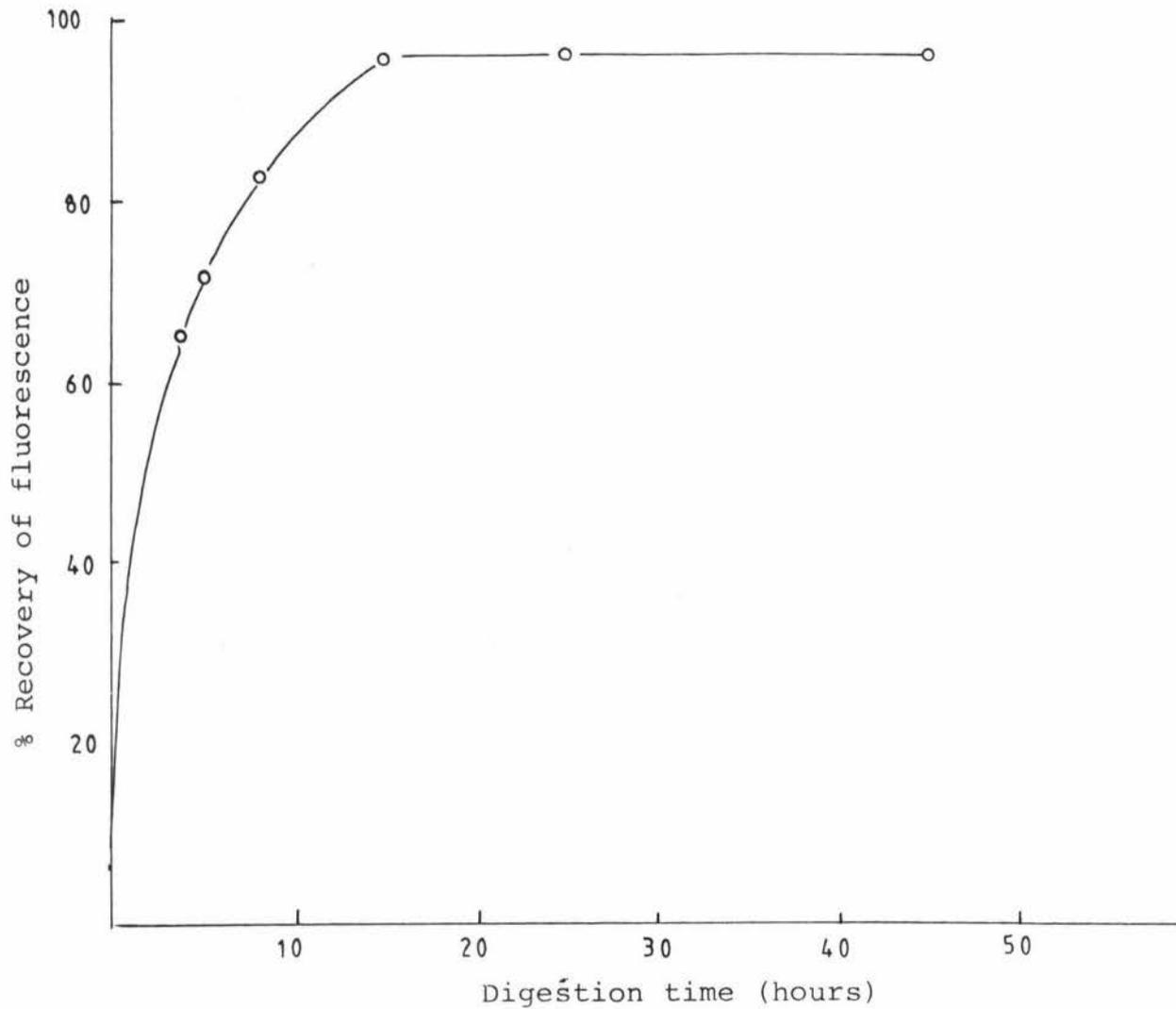


FIGURE 11: % DIGESTION OF TDP AFTER VARIOUS DIGESTION TIMES

\* 2 g of TDP was digested with 1mg Pase in 3cm<sup>3</sup> of 1M sodium acetate buffer (4.8 lcm<sup>3</sup> of aqueous 400mM MgCl<sub>2</sub> was present in each digestion tube.

Thus it would appear that buffers of high ionic strength assist the extraction of thiochrome, from the digestion mixture, by isobutanol. This may be due to high ionic strength solutions facilitating the release of thiochrome from phosphatase.

ii) Buffer Composition

Digestion of TDP in 2.0M buffers other than sodium acetate buffer (pH 4.8) resulted in less than total recovery of fluorescence (Table 8a). Even when small quantities of formate buffer were combined with 2M sodium acetate buffer (pH 4.8), and used as the digestion buffer, recoveries were drastically reduced. This was not due to variation in the pH of the digestion mixture as this was shown to be unchanged after digestion. It therefore appears that either;

- a) the formate buffers used contained an impurity which interfered with the digestion of TDP, or
- b) the formate buffers partially converted TDP to a form which could not be hydrolysed by phosphatase and/or estimated by the thiochrome method. This is a possibility in view of the work of Gounaris and Schulman (1980) (not published until after these experiments had been completed) which showed that TDP forms an artifact in the presence of formate ions.

Since ammonium formate buffers were to be used to separate thiamine and its esters on ion exchange resin, obtaining a digestion mixture which contained only sodium acetate buffer would be impossible. However when magnesium chloride was included in the digestion mixture to stimulate the activity of the enzyme (which could then be used at the lower level of 1mg/digestion tube) the buffer type (with the exception of phosphate buffer) had no effect on the digestion of TDP obtained (Table 8b). This was confirmed during the stability experiments on TDP (see Section 4.4) when the initial determinations of TDP in various buffers, after digestion with 1mg of phosphatase in the presence of magnesium ions,

TABLE 8a: RECOVERIES OF TDP AFTER HYDROLYSIS WITH PHOSPHATASE IN VARIOUS BUFFERS\* (Magnesium ions absent)

| Buffer   | % Recovery |
|--|------------|
| 1M sodium acetate (pH 4.8)   | 73         |
| 2M sodium acetate (pH 4.8)   | 95-100     |
| 2M ammonium formate (pH 4.8)   | 45- 50     |
| 2M sodium formate (pH 4.8)   | 60- 70     |
| 1cm <sup>3</sup> of TDP in 0.0075M sodium formate buffer (pH 4.5) made up to 100cm <sup>3</sup> with 2M sodium acetate buffer (pH 4.8) | 70         |

\* 2 µg of TDP in 4cm<sup>3</sup> of the stated buffer was digested with 10mg of phosphatase at 37° for 24 hours. Magnesium ions were absent from the digestion mixture. The digestion mixtures were estimated by the thiochrome method and recoveries were obtained by reference to a thiamine standard curve.

TABLE 8b: RECOVERIES OF TDP AFTER HYDROLYSIS WITH PHOSPHATASE IN VARIOUS BUFFERS\* (Magnesium ions present)

| Buffer                          | % Recovery |
|---------------------------------|------------|
| 1M sodium acetate (pH 4.8)      | 96         |
| 1M sodium formate (pH 4.8)      | 99         |
| 0.25M ammonium acetate (pH 4.8) | 103        |

\* 2 µg of TDP in 3cm<sup>3</sup> of the stated buffer was digested with 1mg of phosphatase at 37° for 24 hours, 1cm<sup>3</sup> of aqueous 400mM MgCl<sub>2</sub> was present in each digestion mixture. Estimation was by the thiochrome method.

gave recoveries of greater than 90%.

#### 4.3.3. Summary - Digestion of Thiamine Phosphate Esters

Complete digestion of TDP/TMP can be obtained in a minimum of 15 hours at 37° using 1mg of wheat germ acid phosphatase and 1cm<sup>3</sup> of aqueous 400mM magnesium chloride made up to a total volume of 4cm<sup>3</sup> with 1M sodium acetate buffer (pH 4.8). The inclusion of magnesium ions in the digestion mixture has not been used in previously reported work and allows the use of a lower phosphatase concentration (0.25 mg/cm<sup>3</sup> of digestion mixture) to achieve complete hydrolysis. This was necessary because high phosphatase concentrations interfered with the extraction of thiochrome by isobutanol. In many previously published methods (Schultz and Natelson, 1972; Hennessy and Cerecedo, 1939; A.O.A.C. Methods of Analysis hand book, 1975) which use the thiochrome procedure, samples are subjected to column chromatography after hydrolysis which probably prevents interference by phosphatase. This would explain the use of high phosphatase concentrations (e.g. Schultz and Nateslon recommended a phosphatase concentration of 3mg/cm<sup>3</sup> of digestion mixture) without interference to the thiochrome assay. In the present work the aim was to separate thiamine and its esters first and then estimate their concentration by hydrolysis and subsequent determination of thiochrome. Thus it was important to ensure that phosphatase would not interfere with the thiochrome procedure. The inclusion of magnesium ions ensured that complete hydrolysis could be obtained with phosphatase concentrations which were low enough not to affect the extraction of thiochrome.

#### 4.4 THE STABILITY OF THIAMINE AND TDP IN SOLUTIONS OF VARIOUS PH

One cm<sup>3</sup> of aqueous thiamine or TDP (0.01 mg/cm<sup>3</sup>) was made up to 100 cm<sup>3</sup> with buffers of various pH's (given below). The solutions were stored in the light at room temperature and samples were removed for assay at various known times.

Estimation was performed, directly on the thiamine samples and on the TDP after hydrolysis with phosphatase, by the thiochrome method.

i ) Thiamine

The buffers used were 0.2M acetate (pH 4.0; 5.0), 0.2M phosphate (pH 6.0; 7.0; 8.0), and 0.2M carbonate-bicarbonate (pH 10.0). Thiamine solutions showed little degradation at less than pH7 even after 2 weeks storage (Fig 12). Solutions at pH8 showed some degradation and at pH10 were quickly degraded.

ii ) Thiamine Diphosphate

The buffers used were 1M acetate buffer (pH 4.8), 0.2M malate buffer (pH 6.0), 0.2M barbital buffer (pH 8.0; 7.0) and 0.2M glycine-sodium hydroxide buffer (pH 9.0). Solutions of TDP showed less stability than thiamine solutions although stability was good at less than pH 5.0 even after 5 days storage (Fig. 13).

iii) Stability after Rotary Evaporation

Thiamine and TDP showed good stability after rotary evaporation in acidified aqueous solutions (pH < 3, temp < 40<sup>o</sup>) when compared with non-evaporated control solutions (Table 9).

These results suggest that thiamine and TDP are fairly stable at all pH's above neutral. However the thiochrome method only measures the fluorescence produced by a sample after oxidation and provides no information on the possible partial degradation of thiamine or its esters. For example hydrolysis of TDP to TMP and/or thiamine could have occurred during storage without change in the fluorescence observed after the formation of thiochrome.

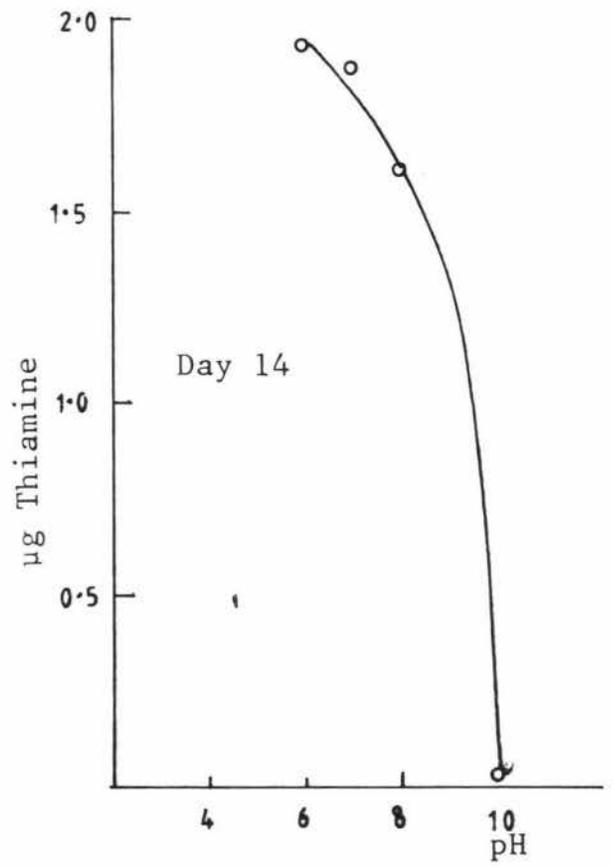
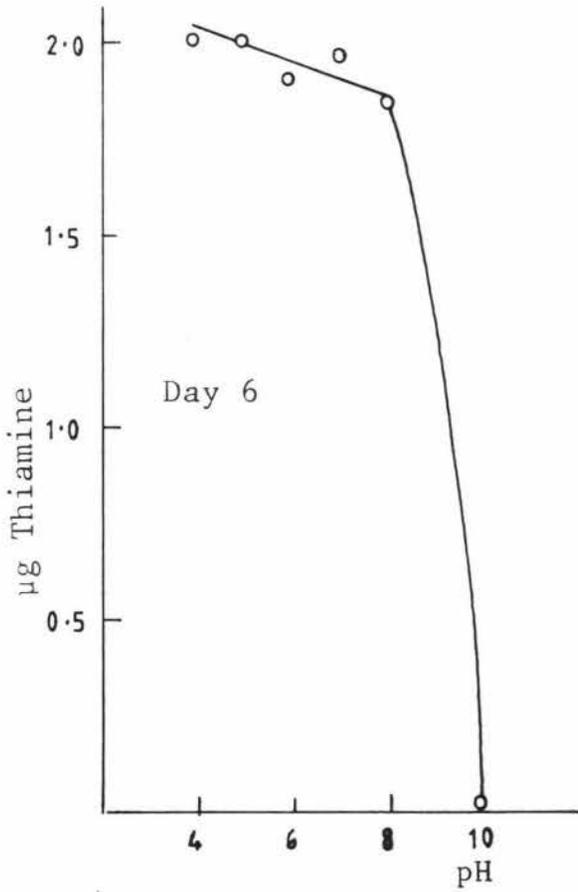
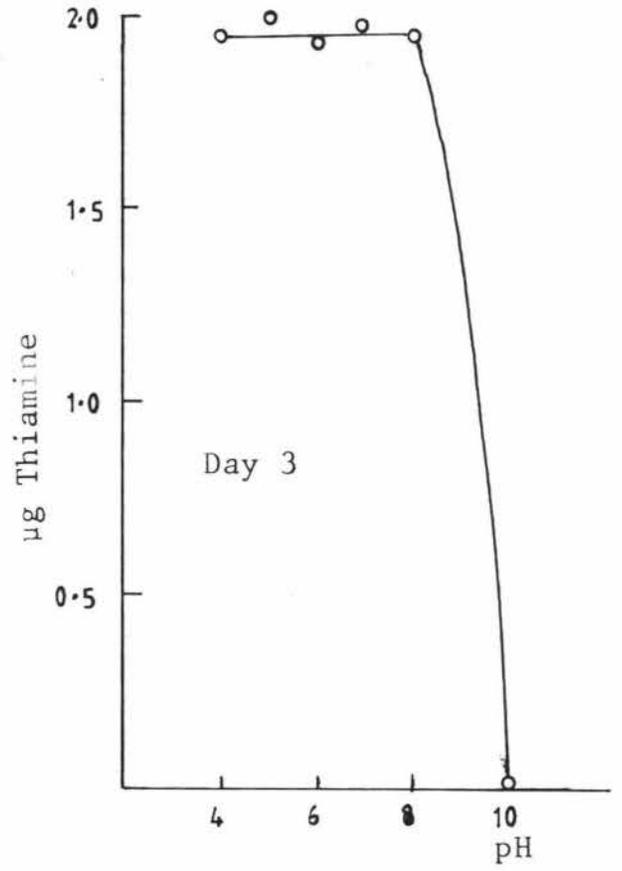
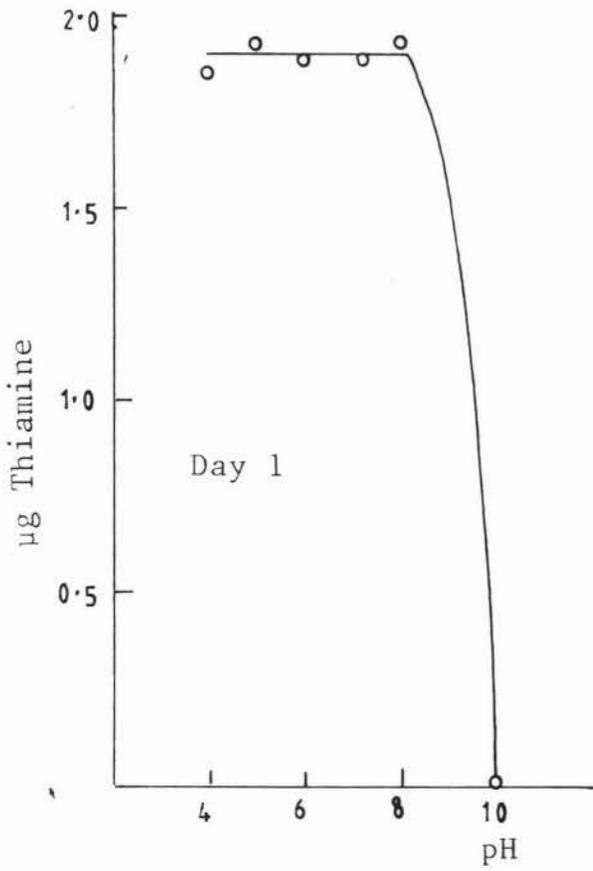


FIGURE 12: THE STABILITY OF THIAMINE IN SOLUTIONS OF VARIOUS pH STORED FOR VARIOUS PERIODS

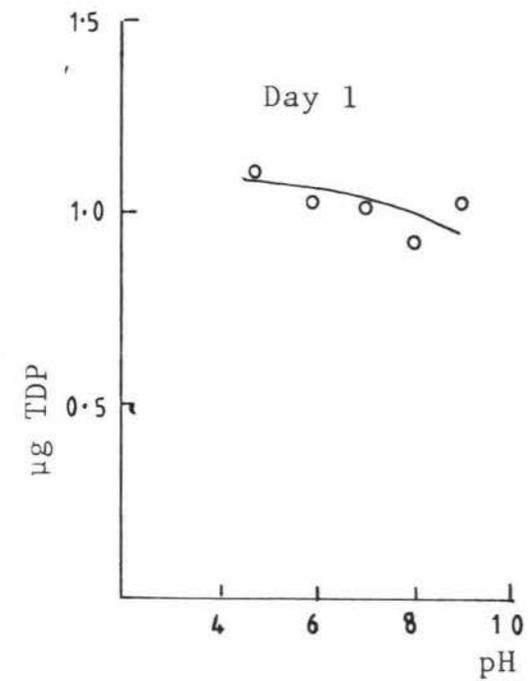


FIGURE 13: THE STABILITY OF TDP IN SOLUTION OF VARIOUS pH

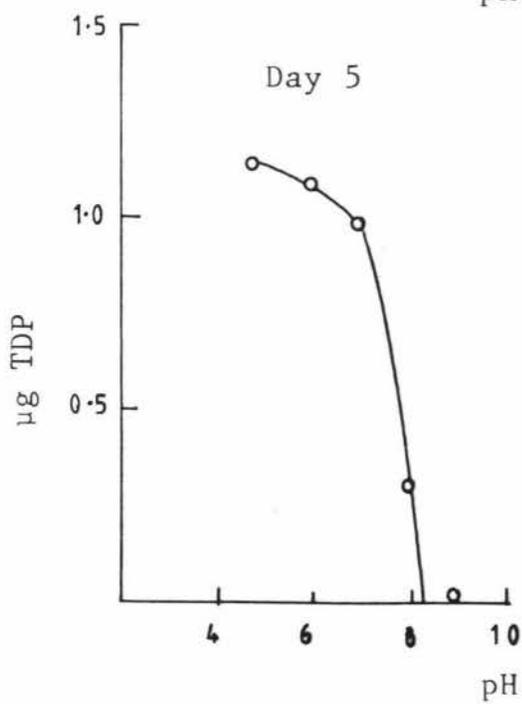
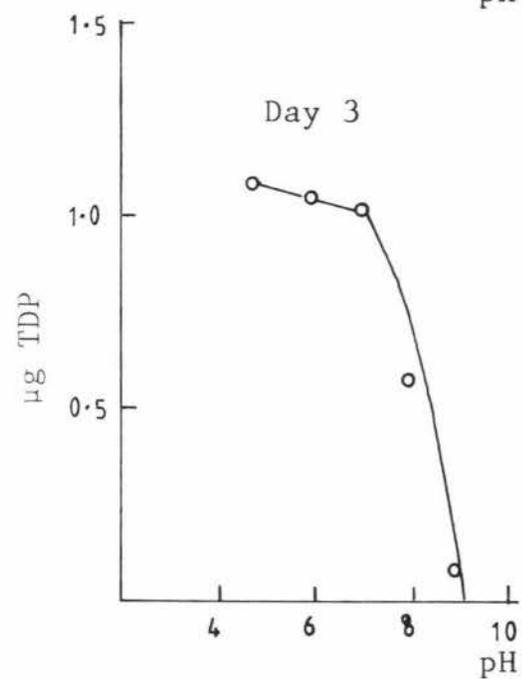


TABLE 9: THE STABILITY OF THIAMINE AND TDP AFTER ROTARY EVAPORATION.\*

|                    | Fluorescence<br>Reading |
|--------------------|-------------------------|
| Thiamine           |                         |
| before evaporation | 80.5                    |
| after evaporation  | 81                      |
| TDP                |                         |
| before evaporation | 89                      |
| after evaporation  | 93                      |

\* 10cm<sup>3</sup> of aqueous solutions containing 1µg/cm<sup>3</sup> of thiamine or TDP (made to pH 3.0 with diluted acetic acid) were evaporated to near dryness in a rotary evaporator at less than 40°. The evaporated solutions were made up to 10cm<sup>3</sup> with 1M sodium acetate buffer (pH 4.8) and 1cm<sup>3</sup> aliquots of each solution were estimated by the thiochrome method (after digestion for TDP).

PART 2

THE SEPARATION OF THIAMINE  
AND ITS PHOSPHATE ESTERS

## CHAPTER 5

### METHODS

#### 5.1 PAPER CHROMATOGRAPHY

Aliquots containing 0.5 - 5  $\mu\text{g}$  of thiamine and its phosphate esters were spotted on the baseline of sheets (130mm x 130mm; 180mm x 180mm) of Whatman No 1 chromatography paper. These were fastened into tubes and developed in various solvents in sealed jars. The chromatograms were developed at room temperature in the dark.

#### 5.2 THIN LAYER CHROMATOGRAPHY

Chromatographic glass plates (20cm x 20cm) were prepared with silica gel G (8gm/plate) and developed in Whatman chromatography tanks. Each tank had paper wicks inserted to ensure the air was saturated with solvent. Aqueous solutions containing 10  $\mu\text{g}/\text{cm}^3$  of thiamine and TDP were spotted onto the plates with capillary pipettes and developed in various solvents.

#### 5.3 HIGH VOLTAGE PAPER ELECTROPHORESIS

Samples of thiamine and its phosphate esters in 0.2M formic acid were streaked as 1cm bands onto 57cm long strips of Whatman 3mm chromatography paper. The loaded strips were wetted with buffer and excess buffer was blotted off. The papers were developed vertically at 3kV for 30-60 minutes in pyridine-acetate buffer pH2.1, 3.5 or 6.5.

#### 5.4 DETECTION OF SAMPLE AFTER SEPARATION BY CHROMATOGRAPHY OR ELECTROPHORESIS

The plates and papers were dried in a darkened fume hood. They were then sprayed with a 0.02% alkaline ferricyanide solution to produce thiochrome. This was observed as a blue fluorescence under ultra violet light (350nm).

## 5.5 GEL FILTRATION

Sephadex G10 was soaked in eluting solvent (either 5% acetic acid or 0.1M phosphate buffer pH6.6) to allow swelling of the gel. After decantation of the fines, the gel was degassed under vacuum and carefully poured into glass columns (approx. 1.5 cm x 150 cm) ensuring no bubbles formed in the gel bed. The samples were applied and developed with the appropriate solvent, using a gravity feed system, at a flow rate of 14 cm<sup>3</sup>/hr. Experiments were performed at room temperature in the light. Fractions were collected in an LKB rotary fraction collector.

## 5.6 ION EXCHANGE CHROMATOGRAPHY

A weak cation exchange resin, Amberlite GC50; a strongly acidic cation exchange resin; Dowex 50W-X8; and a strongly basic anion exchange resin Dowex 1-X8 were used to investigate the separation of a mixture of thiamine, TMP and TDP.

### 5.6.1 General Procedure

The resins were cleaned under vacuum with several alternating washings of 1M formic acid and 1M sodium hydroxide. Between each washing 1 litre of distilled-deionised water was passed through the resin. The resins were then soaked in water and the fines were decanted off several times. After the resins were converted to their appropriate forms, they were equilibrated with the initial eluting buffer. The resins were degassed under vacuum and packed into glass columns (approx 10mm x 100mm). Samples of thiamine, TMP and TDP were dissolved in the same buffer used to equilibrate the resin and the pH adjusted, where necessary, with dilute ammonia or sodium hydroxide. Aliquots of these (0.5 cm<sup>3</sup> samples, containing 0.5mg of thiamine, TMP and/or TDP) were applied to the columns and washed in with the initial eluting buffer. The columns were developed under gravity feed and fractions were collected in an LKB rotary fraction collector.

i) Amberlite GC50

Both the hydrogen and sodium forms of the resin were used. The hydrogen form was prepared by treatment with 1M formic acid and the sodium form by treatment with 10% sodium hydroxide. After washing, the resin was placed in 1M ammonium formate and the pH was adjusted to 4.5 with 1M formic acid. After a further washing with distilled-deionised water, the resin was equilibrated with 0.3M ammonium formate buffer (pH4.5). The columns were developed with distilled-deionised water initially and then with 1M hydrochloric acid.

ii) Dowex 1-X8

The resin was converted to its formate form by treatment with 1M formic acid. Excess formic acid was removed by washing with distilled-deionised water and the resin was then washed with either 1M ammonium formate or 1M sodium formate. The resin was then thoroughly washed with distilled-deionised water until the effluent pH=5.0-6.0. Two systems for separation of thiamine and its esters were investigated.

- a) The column was developed with distilled-deionised water followed by 0.4M ammonium formate buffer (pH 6.0) and then 2M formic acid.
- b) The column was developed with distilled-deionised water followed by 0.0075M sodium formate buffer (pH4.5) and then 1M formic acid.

iii) Dowex 50W-X8

The resin was converted to its hydrogen form by treatment with 1M formic acid and then thoroughly washed with distilled-deionised water and 1M ammonium acetate buffer (pH4.8). The resin was then equilibrated with 0.1M formic acid in the column until the effluent pH=2.4. The sample was placed on the column and developed with 0.25M ammonium acetate buffer (pH 4.5) followed by 0.25M ammonium acetate buffer (pH 5.2) and 0.5M ammonia solution.

### 5.6.2 Detection of Thiamine and its Phosphate Esters in Fractions Eluted from the Columns

Two methods were used to identify the fractions containing thiamine or its esters. After chromatography with Amberlite GC50 small aliquots of each fraction were spotted on Whatman No 1 chromatography paper, sprayed with 0.02% alkaline ferricyanide solution and examined under ultra violet light (350 nm).

After separation on Dowex 1-X8, Dowex 50 and Sephadex G10 the absorbance of the fractions at 265nm was recorded. Fractions identified as containing thiamine or its esters were then combined, acidified, and concentrated (if necessary) by rotary evaporation. Those fractions which were investigated by high voltage electrophoresis were developed at 3kV in pH 3.5 buffer (see Section 5.3). After electrophoresis the paper was dried, sprayed with 0.02% alkaline ferricyanide solution and examined under ultra violet light (350 nm).

## CHAPTER 6

### RESULTS AND DISCUSSIONS

#### 6.1 PAPER CHROMATOGRAPHY

Trials performed using two different sized papers are summarized in tables 10 and 11. Adequate separation was achieved with both propanol/HCl and propanol/water/acetate buffer solvent systems.

#### 6.2 THIN LAYER CHROMATOGRAPHY (TLC)

Separations between thiamine and TDP were poor using TLC with all solvent systems tested (Table 12). In many cases there was little movement of the compounds and when movement did occur it was accompanied by extensive tailing making the separations unsatisfactory.

#### 6.3 HIGH VOLTAGE PAPER ELECTROPHORESIS

Separation was good in all buffers tested (Fig. 14). The pH 3.5 buffer was considered best for routine use. This was because it was less volatile than the pH 6.5 buffer and so was easier to handle, and less tailing of spots was observed at pH 3.5 than at pH 2.1. The salt concentration of the solution in which the samples were applied to the paper had a marked effect on the distances moved by samples in a particular time. At high salt concentrations (1.0M solution) considerable retardation of sample was observed (Fig. 15). In later work this effect of salt retardation often made it difficult to identify components of fractions if these had been concentrated by rotary evaporation before electrophoresis.

#### 6.4 RECOVERY FROM PAPER AFTER ELECTROPHORESIS

A 5  $\mu$ l aliquot containing 2  $\mu$ g of thiamine in 0.1M hydrochloric acid was streaked onto the center of the baseline on 3mm

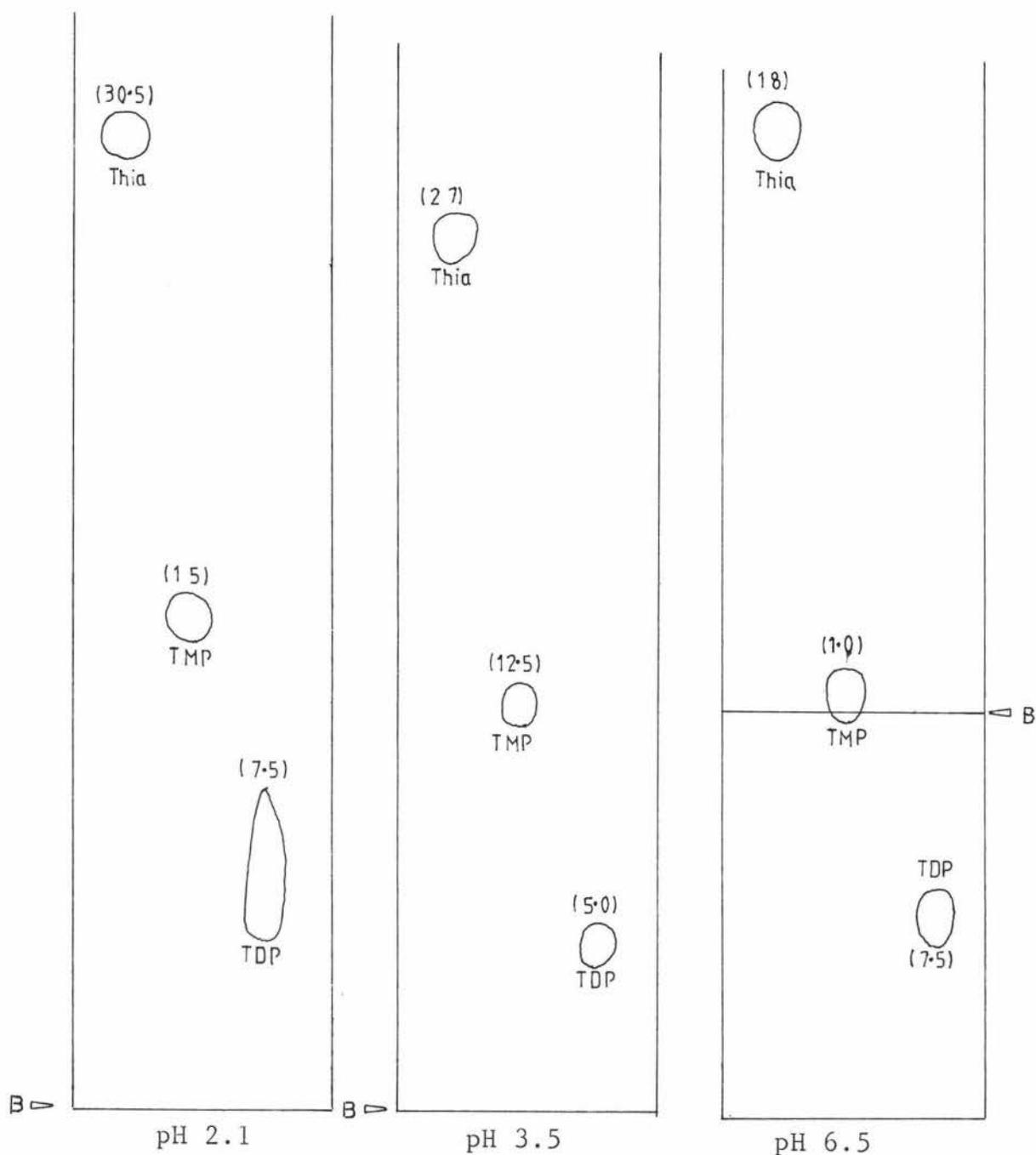


FIGURE 14: SEPARATION OF THIAMINE AND ITS ESTERS BY HIGH VOLTAGE PAPER ELECTROPHORESIS AT 3 kV

Developing buffers:

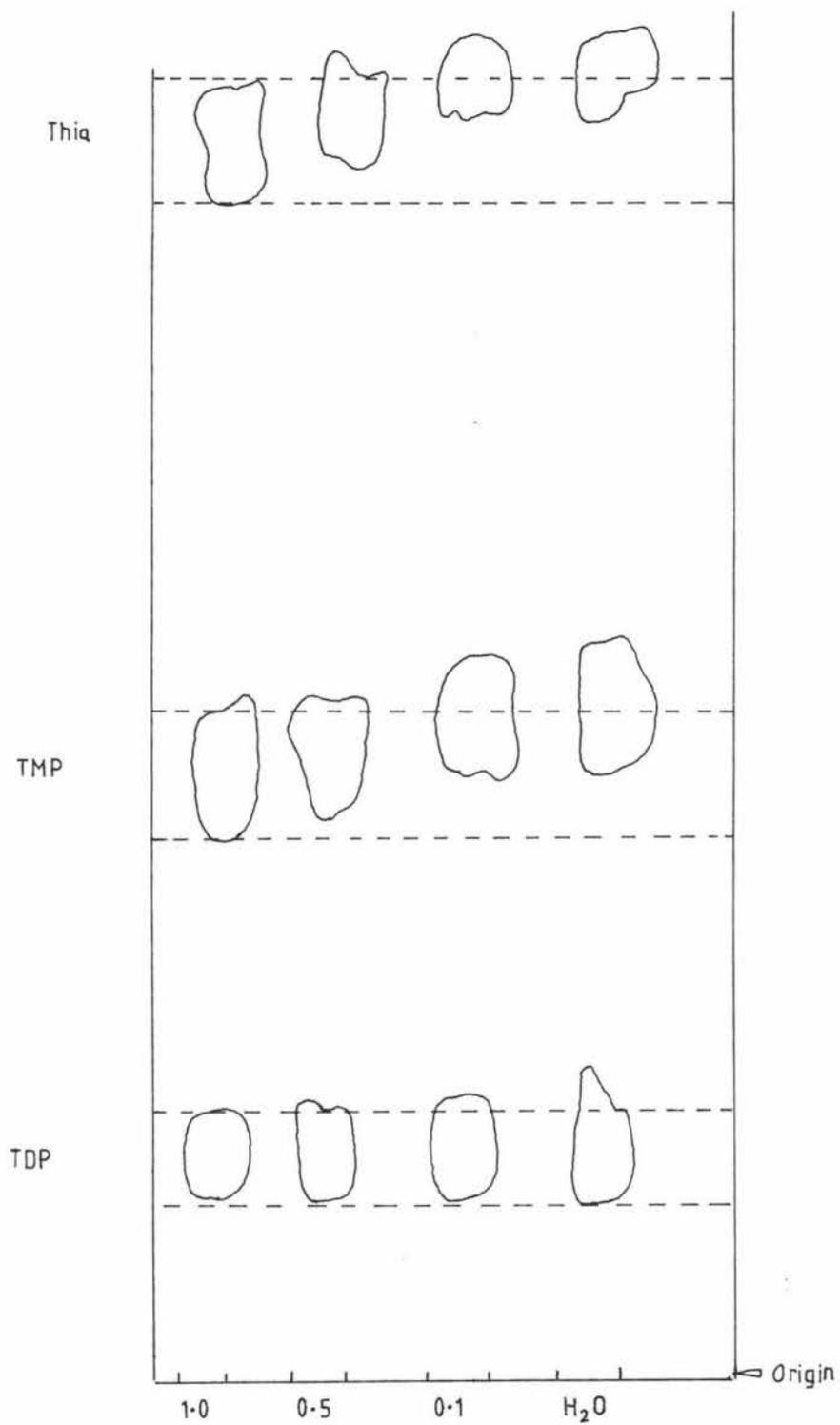
pH 2.1 = 100cm<sup>3</sup> formic acid; 400cm<sup>3</sup> acetic acid;  
4.5l water

pH 3.5 = 25cm<sup>3</sup> pyridine; 250cm<sup>3</sup> glacial acetic acid;  
4.6l water

pH 6.5 = 1000cm<sup>3</sup> pyridine; 40cm<sup>3</sup> glacial acetic acid;  
made up to 10l with water

B▷ = baseline (origin)

Numbers in brackets = distance moved from baseline(cm)



Molarity of salt solution in which samples were prepared (sodium acetate buffer pH 4.8).

FIGURE 15: RETARDATION OF SAMPLES ON ELECTROPHORESIS BY SALTS

(Drawn to scale)

TABLE 10: PAPER CHROMATOGRAPHY OF THIAMINE, TMP, TDP  
(Short Chromatograms; 130mm x 130mm)

| Solvent  | Rf values |      |      |
|--|-----------|------|------|
|  | Thiamine  | TMP  | TDP  |
| Propanol/H <sub>2</sub> O/acetate buffer<br>(7 : 2 : 1)        | 0.59      | 0.23 | 0.6  |
| Urea/HCOOH/Propanol<br>(1 : 2 : 3)                             | 0.69      | 0.55 | 0.4  |
| Propanol/HCl<br>(67 : 33)                                      | 0.61      | 0.23 | 0.1  |
| Pyridine/Acetic acid/H <sub>2</sub> O)<br>(10 : 40 : 1)        | 0.9       | 0.95 | 0.95 |
| Propanol/10% ammonia<br>(40 : 10)                              | 0.31      | 0    | 0    |
| Methanol/NH <sub>3</sub> /CHCl <sub>3</sub><br>(35 : 3.5 : 65) | 0.38      | 0    | 0    |

TABLE 11: PAPER CHROMATOGRAPHY OF THIAMINE, TMP, TDP  
(Long Chromatograms; 180mm x 180mm)

| Solvent  | Rf values |      |      |
|--|-----------|------|------|
|  | Thiamine  | TMP  | TDP  |
| Propanol/HCl<br>(67 : 33)                        | 0.59      | 0.26 | 0.11 |
| Propanol/H <sub>2</sub> O/acetate<br>(7 : 2 : 1) | 0.54      | 0.19 | 0.07 |
| Urea/HCOOH/Propanol<br>(1 : 2 : 3)               | 0.66      | 0.50 | 0.37 |

TABLE 12: THIN LAYER CHRONATOGRAPHY OF THIAMINE AND TDP USING SILICA GEL G (PLATES 20cm x 20cm).

| Solvent   | Thiamine RF           | TDP RE             |
|---|-----------------------|--------------------|
| Propanol/HCl<br>(67:33)   | 0.06                  | 0                  |
| Propanol/10% ammonia  | NO MOVEMENT           |                    |
| Propanol/H <sub>2</sub> O/acetate<br>buffer<br>(7:2:1)                          | 0.07                  | 0                  |
| Pyridine/H <sub>2</sub> O/acetic acid<br>(10:40:1)                              | 0.7                   | 0.5                |
| Methanol/10% NH <sub>3</sub><br>(45:5)  | NO MOVEMENT           |                    |
| Propanol/isoamyl alcohol/<br>isobutyric acid/NH <sub>3</sub><br>(72/2.5/7.5/12) | 0.57<br>Solvent front | 0.445<br>fluoresed |
| Propanol/NH <sub>4</sub> COOH+/H <sub>2</sub> O                                 | 0.12                  | 0.02               |

Whatman chromatography paper. A small marker spot of thiamine prepared in the same solvent was also applied to the baseline 2cm from the edge of the paper. These were developed by high voltage electrophoresis for 30 minutes (3kV, pH 3.5). After drying, a 3cm wide longitudinal strip containing the marker thiamine spot was removed from the edge of the paper and sprayed with alkaline ferricyanide to indicate the position of the thiamine sample. A 4cm wide strip containing the thiamine sample was then removed from the unsprayed portion of the paper. A strip was also removed above this position to act as a blank. These strips were eluted with 10-15cm<sup>3</sup> of 0.1M hydrochloric acid and the sample was collected in 50cm<sup>3</sup> glass centrifuge tubes. These were assayed by the thiochrome method and determined by comparison with thiamine prepared in a volume of 0.1m hydrochloric acid equal to that in which the samples were eluted from the paper. Light was excluded as far as possible at all stages. A recovery of 60% was obtained.

#### 6.5 SUMMARY

Paper chromatography was used initially for qualitative evaluation of samples obtained using Sephadex G10 but as this technique required at least 4 hours to achieve adequate separation it was replaced by high voltage paper electrophoresis at pH 3.5. This provided the quickest (1 hr) and best separations of thiamine and its esters and was used to identify components of fractions in other experiments.

A low recovery of sample after electrophoresis and elution from the paper was obtained using the elution system described. The recovery obtained (60%) was similar to that of the widely used method of Itokawa and Cooper (1970) (see Section 1.6.ii). Penttinen (1978) has observed, using the method of Itokawa and Cooper, that salts reduced the separations obtained and this was also observed in this experiment. In view of these results, and a basic similarity between the method used here and that of Itokawa and Cooper (1970), the criticisms of their method would also probably be applicable to the method described here.

Several workers have used high voltage electrophoresis for the separation and estimation of thiamine and its esters (Hashitani and Cooper 1972; Itokawa, Schulz and Cooper, 1972) but, as recoveries by this method have been shown to be incomplete when compared with non-electrophoresed standards (Penttinen 1978), either substantial correction factors or concurrent electrophoresis of the standards and unknowns would be required. It seems that this technique can be used satisfactorily to compare relative amounts of thiamine and its esters but the quantitative estimation of these by high voltage electrophoresis appears to be difficult.

#### 6.6 SEPHADEX G10

Since Nishimune *et al* (1972) found that the thiochrome derivatives of thiamine and its esters could be separated by elution from Sephadex G10 using phosphate buffer (pH6.6) this method was investigated. The thiochrome derivatives of thiamine, TMP and TDP were produced by treating the samples with alkaline ferricyanide (as used for the thiochrome procedure) and this solution was neutralised by addition of dilute hydrochloric acid. Complete separation was achieved by elution of the sample with phosphate buffer (pH 6.6) (Fig. 16). Paper chromatography of aliquots from each peak with propanol/HCl showed no cross contamination between peaks. Thiochrome was eluted as a single peak in this experiment while in the experiments reported by Nishimune *et al* (1972) it was eluted in two peaks. The reason for this is unknown but is presumably due to the use of a different oxidising agent (Nishimune *et al* (1972) used cyanogen bromide whereas potassium ferricyanide was used in the present work) to prepare the thiochrome derivatives as all other factors were approximately the same in both experiments. An approximate estimate of recovery of total sample applied was obtained spectrophotometrically by comparing the absorbance at 370nm of all three peaks combined, with that of the sample solution applied to the column. This showed a combined recovery of 93% for all three derivatives.

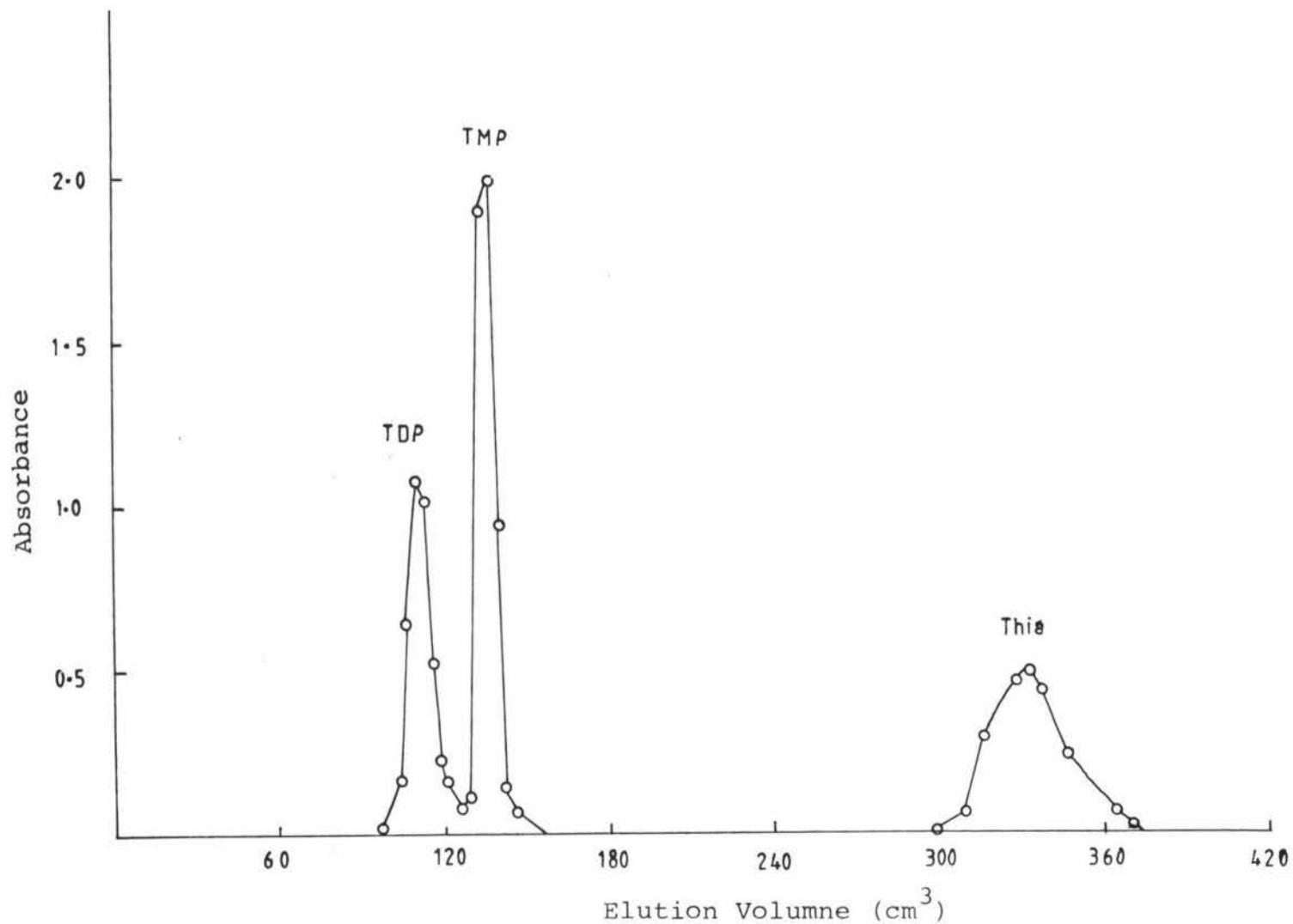


FIGURE 16: CHROMATOGRAPHY OF THIOCHROME AND THIOCHROME PHOSPHATE ESTERS ON SEPHADEX G10.

Column dimensions, 143 x 1.46cm; Fraction size, 3cm<sup>3</sup>; Flow rate, 14cm<sup>3</sup>/hr; Sample, 1.5mg each of the thiochrome derivatives of thiamine, TMP and TDP neutralised with dilute HCl; Eluent, 0.1M potassium phosphate buffer (pH6.6).

This slightly low recovery is probably due to the column being developed in the light at room temperature as Doherty *et al* (1955) have indicated that thiamine (and presumably thiochrome) undergoes some destructive action by light. Therefore with this procedure the thiochrome derivatives of thiamine, TMP and TDP can be completely separated and, within the accuracy limits imposed by the use of less sensitive spectrophotometric assay techniques, apparently quantitatively recovered.

Since it was considered an advantage to use volatile eluents, and phosphate buffer complicates hydrolysis of thiochrome phosphate esters (phosphate inhibits acid phosphatase), the use of 5% acetic acid instead of phosphate buffer as the eluent was also investigated. Separations were attempted using either oxidised or unoxidised samples of thiamine and its esters on the same gel column (140 x 1.4cm) used in experiments with phosphate buffer. However acetic acid (5%) was unsuccessful in achieving separation and all 3 compounds (oxidised or unoxidised) eluted together.

As the sample does not bind to the gel, the procedure of Nishimune *et al* (1972) would seem to be limited to the separation of fairly concentrated solutions of thiamine and its esters. Extracts from biological material therefore would require extensive concentration and purification before this method could be used. Nishimune *et al* (1972) recognised this when they commented that, when samples were applied at levels occurring in biological systems, "accurate quantitative determination was difficult".

## 6.7 ION EXCHANGE CHROMATOGRAPHY

### 6.7.1 Amberlite GC50

The method of Rossi-Fanelli *et al* (1960) for separation of thiamine, TMP and TDP was investigated but the resin used was Amberlite GC50 rather than Amberlite IRC50. The results obtained by Rossi-Fanelli *et al* (1960) indicated that single columns of this resin would only achieve partial separations

and that the use of both forms (sodium and hydrogen) of the resin in superimposed columns would be required to achieve complete separation of thiamine, TMP and TDP. While the main purpose of this project was to achieve a single column separation, it was of interest to see if the use of a chromatographic grade resin, with higher surface area and well defined particle size, would improve on the separations obtained by Rossi-Fanelli *et al* (1960).

Both the hydrogen and sodium forms of the resin were used. A sample containing thiamine, TMP and TDP (0.5mg of each) neutralised with dilute ammonia, was used and the column (75mm x 10mm) was developed at a flow rate of 60 cm<sup>3</sup>/hr. It should be noted that in this experiment thiamine and its phosphate esters were detected by spotting aliquots of fractions on paper and spraying with 0.02% alkaline ferricyanide solution (see Section 5.6.2).

i ) Hydrogen Form

TDP was eluted with distilled-deionised water and thiamine and TMP were both eluted together with 1.0M hydrochloric acid. TDP was only slightly retarded by the resin as it eluted in a volume just greater than the bed volume of the resin. High voltage electrophoresis of the eluted fractions containing maximum fluorescence showed then to contain only those compounds expected (i.e. the fractions containing TDP contained only TDP and those containing thia and TMP contained only those compounds).

ii ) Sodium Form

Both TDP and TMP were eluted with distilled-deionised water and thiamine was eluted with 1.0M hydrochloric acid. Partial separation between TDP and TMP occurred, TMP being retarded by the resin slightly more than TDP. The elution volumes of TDP and TMP appeared to be 13cm<sup>3</sup> and 33cm<sup>3</sup> respectively but as the fractions were not assayed spectrophotometrically, these volumes are approximate only. Fractions shown to contain maximum

fluorescence (by spotting) were examined by high voltage electrophoresis and contained only TDP or TMP. However some overlap was indicated as fluorescence was detected in all fractions between those containing just TDP or TMP (Fig. 17). As these fractions were not examined further by high voltage electrophoresis, the extent of this overlap is unknown.

### iii) Discussion

The results obtained here, with both forms of the resin, were essentially the same as those reported by Rossi-Farelli *et al* (1960) using Amberlite IRC50. The use of chromatographic grade resin did not appear to greatly improve separations although the results obtained do indicate that a separation between thiamine, TMP and TDP using a single column could be possible. The use of the hydrogen form of the resin with different buffers may permit the separation of thiamine and TMP and a longer column of the sodium form of the resin would probably permit separation of TDP and TMP. Two superimposed columns, one of each form of Amberlite GC50 (i.e. in a similar manner to that used by Rossi-Farelli *et al* with Amberlite IRC50) would certainly achieve a separation of thiamine, TMP and TDP. However, the use of this resin was not investigated further because, since neither form of the resin retarded TDP, a separation between TDP and TTP would not be possible. Likewise the removal of interfering impurities, present in biological extracts, from TDP/TMP would also be difficult. Furthermore the separation of thiamine and its esters in samples of biological extracts would require extensive sample desalting prior to development on this resin as the presence of salt would probably adversely affect the separations obtained.

#### 6.7.2 Dowex 1-X8

Rindi and De Giuseppe (1960) achieved a partial separation of thiamine and its phosphate esters on a column of Dowex 1-X8 resin by elution with sodium acetate buffers. In the

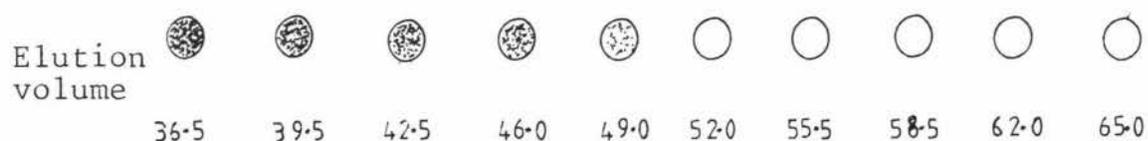
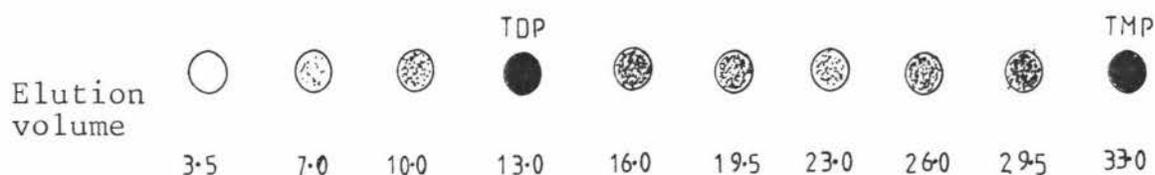


FIGURE 17: ELUTION OF TMP AND TDP FROM AMBERLITE GC50 (Na<sup>+</sup> FORM) AS MONITORED BY SPOTTING\* (see text).

Column dimensions, 100 x 10mm; Initial column pH, 4.5 in 0.03M formate buffer; Flow rate, 1cm<sup>3</sup>/min; Elution, all fractions above eluted with distilled deionised water and thiamine eluted after with 1M HCl; Sample, 0.5cm<sup>3</sup> of a solution of thiamine, TMP and TDP (0.5mg/cm<sup>3</sup> each) in 0.03M formate buffer (pH 6.0).

\* The intensity of fluorescence is indicated by shading.

present work the separation of these compounds was investigated using more volatile ammonium formate buffers (pH 6.0 and pH 4.5).

i ) Separation at pH 6.0

Initial experiments showed thiamine to be eluted with distilled-deionised water, TMP to be eluted with  $> 0.01M$  ammonium formate buffer (pH 6.0) and TDP to be eluted with  $> 0.5M$  formic acid. The elution profile using distilled-deionised water,  $0.04M$  ammonium formate buffer (pH 6.0) and  $2.0M$  formic acid is shown in Fig. 18. This shows three well separated peaks and suggests that the sample had been separated into its three components, thiamine, TMP and TDP. However, when the fractions corresponding to these peaks were examined by electrophoresis it was found that only the first peak contained a single component, namely thiamine. The second peak, eluted with  $0.04M$  formate buffer (pH 6.0), contained mainly TMP but also showed a faint trace of thiamine. Similarly the third peak, eluted with  $2.0M$  formic acid, contained mainly TDP but also showed faint traces of TMP and thiamine.

ii) Separation at pH 4.5

The elution profile using distilled-deionised water,  $0.0075M$  sodium formate buffer (pH 4.5) and  $1.0M$  formic acid is shown in Fig. 19. Electrophoresis showed the first peak, eluted with water in the void volume, to contain only thiamine. The second peak contained mainly TMP but also thiamine in trace amounts. Likewise the third peak, eluted with  $1.0M$  formic acid, contained mainly TDP but also had trace amounts of thiamine and TMP present.

iii) Recoveries from the Column

The recoveries of thiamine and TDP were estimated when these were applied separately to a column of Dowex 1-X3 resin and eluted, in the dark at room temperature, with distilled-deionised water or distilled-deionised water followed by  $1.0M$  formic acid.

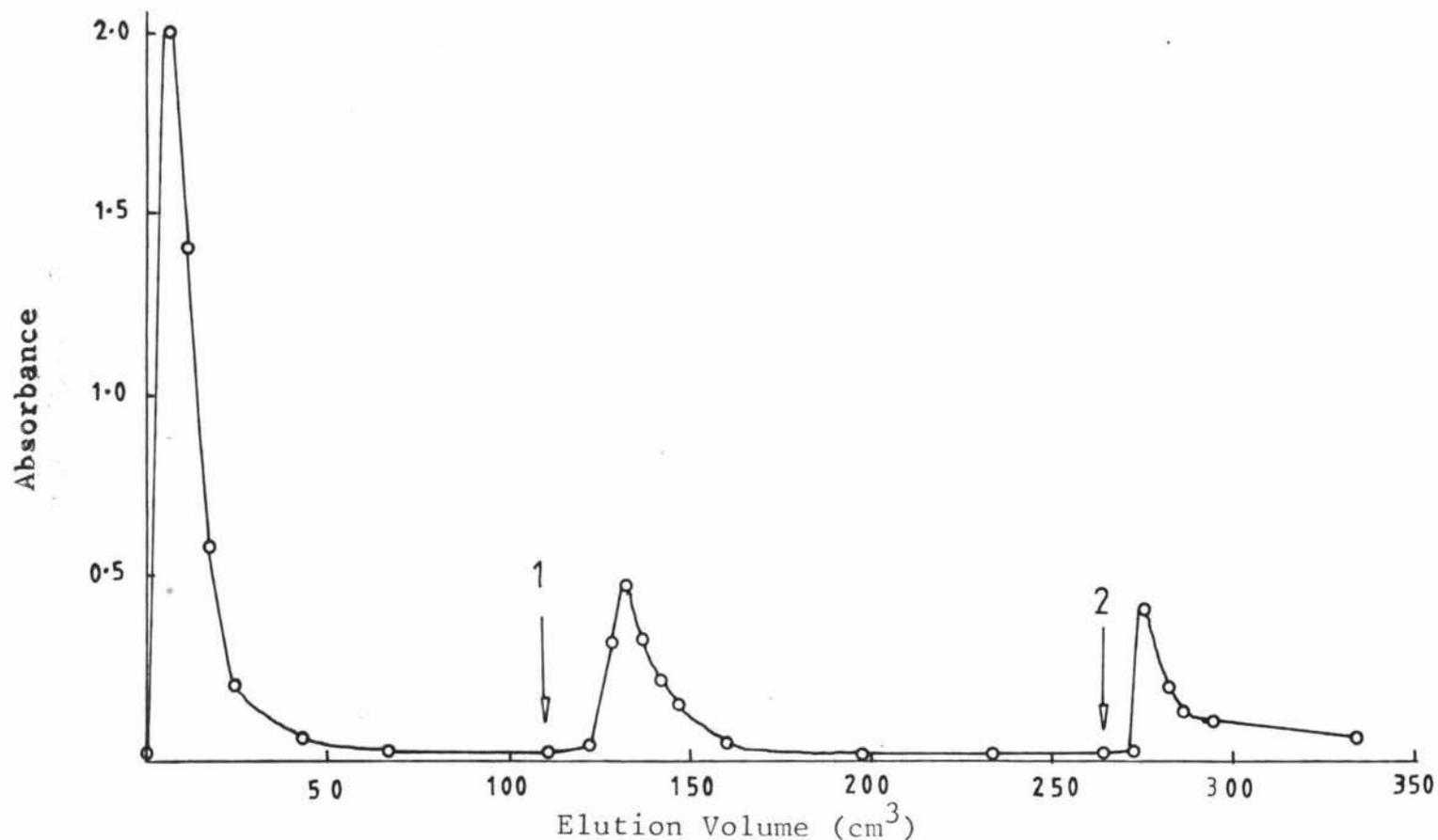


FIGURE 18: ELUTION OF THIAMINE AND ITS PHOSPHATE ESTERS FROM DOWEX 1-X8 AT pH 6.0

Column dimensions, 100 x 10mm; Resin, Dowex 1-X8 (formate form) 400 mesh; Initial pH, 5.0 - 6.0 with water; Flow rate, 1cm<sup>3</sup>/min; Fraction size, 2.5cm<sup>3</sup>; Sample, 0.5cm<sup>3</sup> of sample containing 0.5mg each of thiamine, TMP and TDP in water brought to pH 6.0 with dilute NaOH. Eluents, Initially eluted with distilled-deionised water then at 1 changed to 0.04M formate buffer pH 6.0 and at 2 changed to 2M HCOOH.

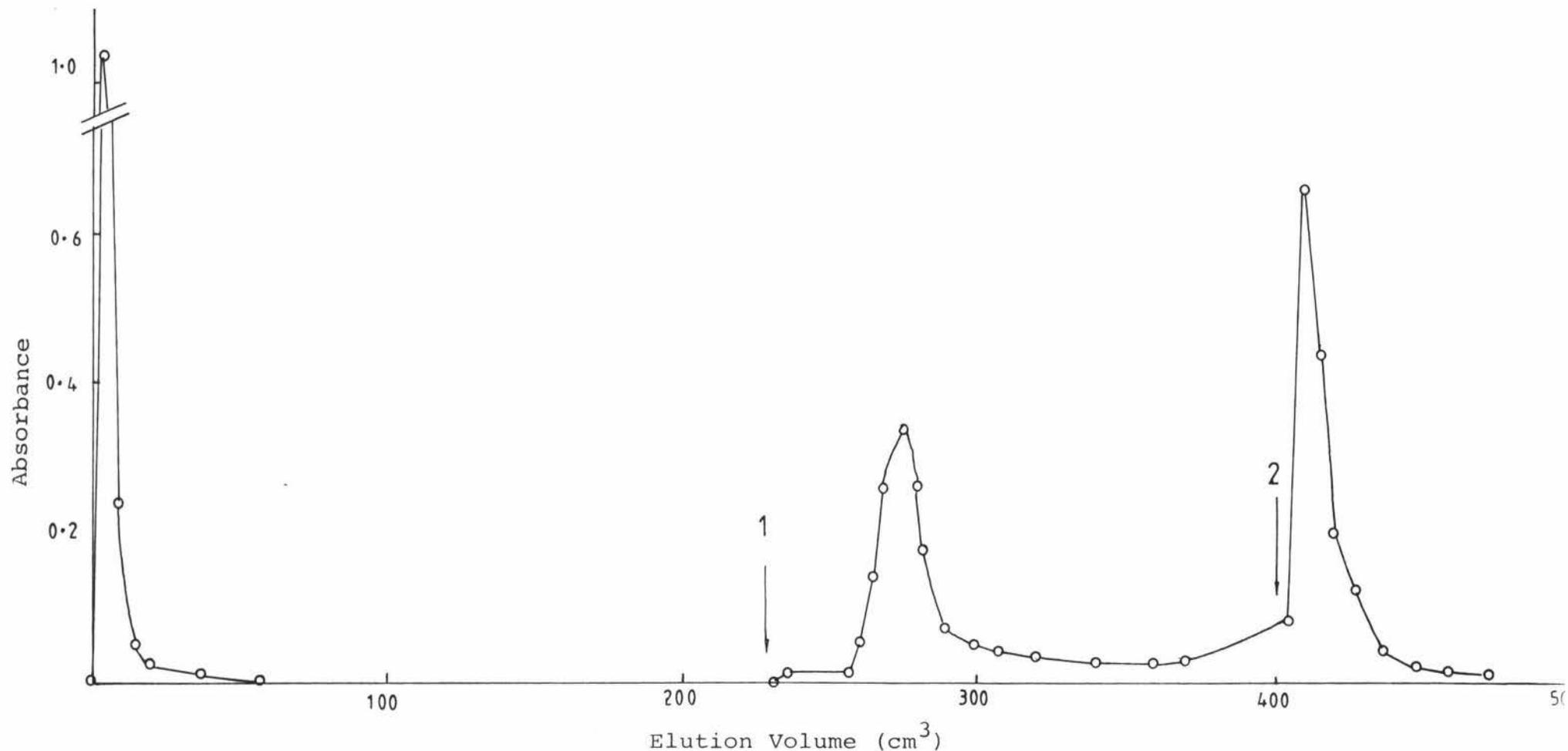


FIGURE 19: SEPARATION OF THIAMINE AND ITS ESTERS ON DOWEX 1-X8 ELUTED AT pH 4.5

Column dimensions, 100 x 10mm; Resin; Dowex 1-X8 (400 mesh) formate form equilibrated with water (pH 6.0); Flow rate, 4cm<sup>3</sup>/min; Fraction size, 3.5cm<sup>3</sup>; Sample, 0.5cm<sup>3</sup> of sample containing 0.5mg each of thiamine, TMP and TDP in water made to pH 6.0 with dilute Na OH; Eluents, initially eluted with distilled-deionised water and at 1 changed to 0.0075M sodium formate buffer (pH 4.5) and at 2 changed to 1M HCOOH.

## a) Thiamine

A neutralised sample containing 0.5mg of thiamine was estimated after elution from Dowex 1-X8 with distilled-deionised water. Estimation was by the thiochrome method and fractions before and after the thiamine peak were assayed as control blanks. A recovery of 100% was obtained.

## b) Thiamine Diphosphate

A sample of TDP was purified, by elution from a column of Amberlite GC50 resin with 0.05M sodium formate buffer (pH 5.5), to remove traces of thiamine and TMP (see Chapter 2 i(d) ). A 1cm<sup>3</sup> aliquot of this sample containing 60 µg of TDP in 0.05M formate buffer was used to examine recoveries of TDP on elution from Dowex 1-X8 resin. The sample was eluted from Dowex 1-X8 with distilled-deionised water followed by 1.0M formic acid. Two peaks were eluted, a small one with water and a much larger one with 1.0M formic acid (Fig. 20). Each peak was assayed by the thiochrome method after appropriate dilution, pH adjustment (to pH 4.8) and digestion with phosphatase. The first peak, eluted with distilled-deionised water, was estimated to have the equivalent of 8% of the TDP applied to the column and the second peak, eluted with 1M formic acid, 72%.

## iv) Discussion

TMP and TDP appear to undergo some form of degradation on elution from Dowex 1-X8 resin with formate buffers. This was indicated by;

- a) electrophoretic evidence showing several components present in TDP and TMP peaks eluted from the resin when a mixed sample was applied and
- b) the appearance of 2 peaks and low recoveries obtained when a sample containing TDP only was eluted from the resin.

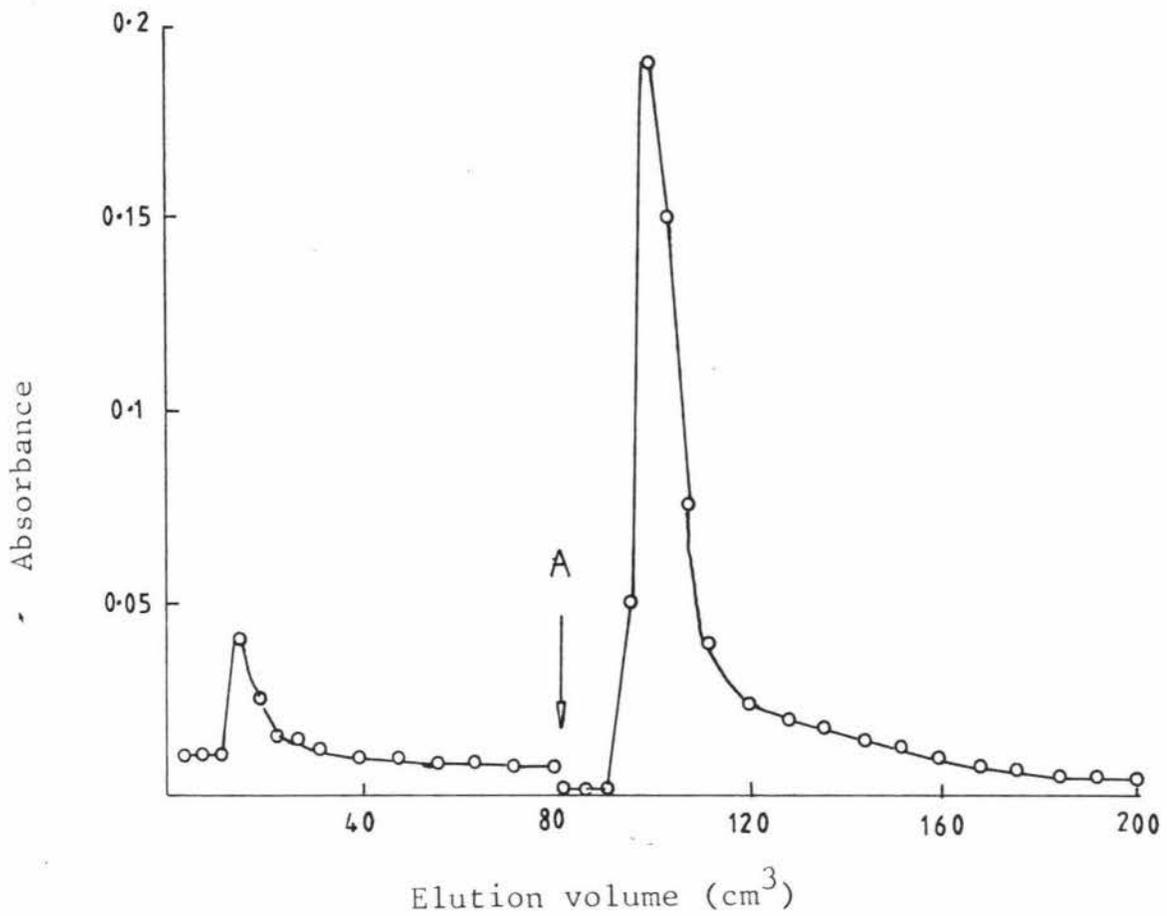


FIGURE 20: ELUTION OF TDP FROM DOWEX 1-X8

Column dimensions, 100 x 10mm; Resin, Dowex 1-X8 (formate form) 400 mesh; Flow rate, 4cm<sup>3</sup>/min; Fraction size, 4cm<sup>3</sup>; Sample, 1cm<sup>3</sup> of TDP solution (60µg) in 0.05M sodium formate buffer (pH 5.5); Eluents, initially eluted with distilled deionised water then at A changed to 1M HCOOH.

These 2 peaks were unfortunately not examined by high voltage electrophoresis but it seems likely that breakdown of TDP had occurred on the column. It would be unlikely that the TDP sample would have been contaminated to this extent (8%) and for this not to have been detected by high voltage electrophoresis performed on the sample before application to the column.

Thiamine itself was the only compound which did not appear to undergo degradation. This was indicated by the estimates of recovery and examination by electrophoresis of the eluted fractions containing thiamine. The fact that thiamine was apparently not degraded whereas TDP was maybe due to thiamine not being bound by the resin.

The apparent presence of thiamine in the fractions containing mainly TMP, and of both thiamine and TMP in those fractions containing TDP, was deduced from results obtained by high voltage electrophoresis. However, as the spots due to the contaminants did not co-electrophoresis exactly next to the marker compounds used, the identification of the contaminants as thiamine and TMP was only tentative. It was originally thought that the difference in positions, between the electrophoresed samples and markers, was due to salt retardation, since the fractions were concentrated by rotary evaporation before electrophoresis, but it is possible that the peak contaminants were different compounds altogether.

That this is possible is suggested by the work of Gounaris and Schulman (1980) in a paper published after the completion of the present work. They found two types of sample degradation occurred when thiamine and its esters were eluted from Dowex 1-X8. When formate buffers were used as eluents they found that TDP formed an artifact. On the basis of results obtained from nuclear magnetic resonance, they suggested this was due to an interaction between TDP and formate ions resulting in the substitution of the C<sub>2</sub> hydrogen of the thiazole ring of TDP. This effect was not

observed when acetate buffers were used as eluents but they found that some of the TDP eluted was hydrolysed to TMP. The amount of TMP eluted increased by a similar amount to that apparently missing from the TDP fractions.

Either or both processes may have occurred in the present work.

- (a) The formation of a  $C_2$  derivative from TMP would explain the contaminant observed after electrophoresis of the TMP containing fractions eluted from the resin when a mixed sample was applied. Gounaris and Schulman (1980) did not observe a  $C_2$  derivative of TMP but, in their experiment, TMP did not bind to the resin and was eluted in the void volume. Thus a  $C_2$  derivative of TMP may not have been produced in their work or, if it was produced, would not have separated from the bulk of the TMP and not have been detected. As TMP did bind to the resin in the present experiments it is possible that the contaminant observed was the  $C_2$  derivative of TMP.

The  $C_2$  derivative of TDP could also have been formed. In the present work the  $C_2$  derivatives of TDP (and TMP) would not be expected to separate from the main peak(s), as observed by Gounaris and Schulman (1980), due to the use of a shorter column (10 cm vs the 30 cm column used by Gounaris and Schulman) and a batchwise elution system as opposed to the elution gradient used by Gounaris and Schulman (1980). However the formation of a  $C_2$  derivative could only explain the presence of one of the two contaminants in the peak corresponding to TDP, observed by electrophoresis.

In the recovery experiment performed with TDP the  $C_2$  derivative of TDP could have been separated from TDP as the sample was applied in 0.05M ammonium formate buffer (see Chapter 2) and this may have been sufficiently strong to cause its elution. This would explain the elution of two peaks when TDP only was applied.

- b) Hydrolysis of TDP to TMP may have occurred and this also would explain the elution of two peaks in the recovery experiments performed with TDP. That this could be occurring is supported by the proportion of TDP eluted in the first peak (8%) being similar to the quantity of TDP hydrolysed to TMP (10.8%) observed by Gounaris and Schulman (1980). The 0.05M formate buffer in which the sample was applied to the column could also have caused the elution of TMP as the separation experiments performed suggest that TMP could be eluted using a very weak buffer (0.0075M sodium formate).

It appears therefore that the results obtained in the present work can be rationalised by reference to the work of Gounaris and Schulman (1980). The unfortunate choice of formate buffers means that the samples applied to the resin were probably extensively degraded. It is likely that the C<sub>2</sub> derivative of TDP and of TMP were produced and that TDP was also hydrolysed to TMP. The results obtained seem to exclude the use of Dowex 1-X8 resin with formate buffers for the quantitative separation of thiamine and its esters.

### 6.7.3 Dowex 50W-X8

The results obtained with a weakly acidic cation exchange resin (Amberlite GC50) indicated that the separation of thiamine and its esters on a strongly acidic cation exchange resin (Dowex 50) should be investigated. The only reported use of this resin was by Ross-Fanelli *et al* (1960). They observed that thiamine, TMP and TDP were all bound by this resin, but did not attempt a separation by elution of these compounds.

Initial trials with Dowex 50 resin confirmed that thiamine, TMP and TDP were all bound by the resin but that, while TMP and TDP could easily be eluted, the elution of thiamine was difficult. For this reason the elution of thiamine was treated separately.

i ) Elution of TMP and TDP

The elution profiles of TDP and TMP were initially established separately. TDP was found to be eluted with pH 4.5 buffer (0.25M ammonium acetate) as a single peak, and TMP was eluted with pH 5.2 buffer (0.25M ammonium acetate) again as a single peak. High voltage electrophoresis of samples from the combined fractions of each peak showed only the presence of the compound applied to the column.

A mixture of TDP and TMP was also shown to be separated by eluting with the above buffers batch wise (Fig. 21) and it was assumed that the two peaks would be pure TDP (peak 1) and TMP (peak 2) respectively, that is, the components of the two peaks were not characterised by high voltage electrophoresis. This was an unfortunate omission as estimates of the recovery of TDP and TMP, after separation of a mixed sample, were found to be 92% and 137% respectively. It was initially thought that TDP may have been hydrolysed to TMP, although this could not completely explain the recoveries, but upon re-estimation of the samples (which had been stored in a refrigerator) two days later values of 84% and 116% were obtained for the two peaks respectively. When recoveries were again checked, after a further two days of storage, the values were found to have decreased still further (76%, 104%). These results were found to be reproducible in a subsequent repeat experiment although the actual recoveries obtained varied from the above (peak 1 initially indicated a 108% recovery which decreased to 78% after 5 days storage; peak 2 initially indicated a 150% recovery which decreased to 95%).

Since the stability of TDP had not been checked in 0.25M ammonium acetate buffer this was performed using the previously described procedure (see Section 4.4). A pH 4.8 buffer was used as this was approximately half way between the pH's of the actual eluting buffers (pH 4.5 and 5.2). After two weeks storage in the light

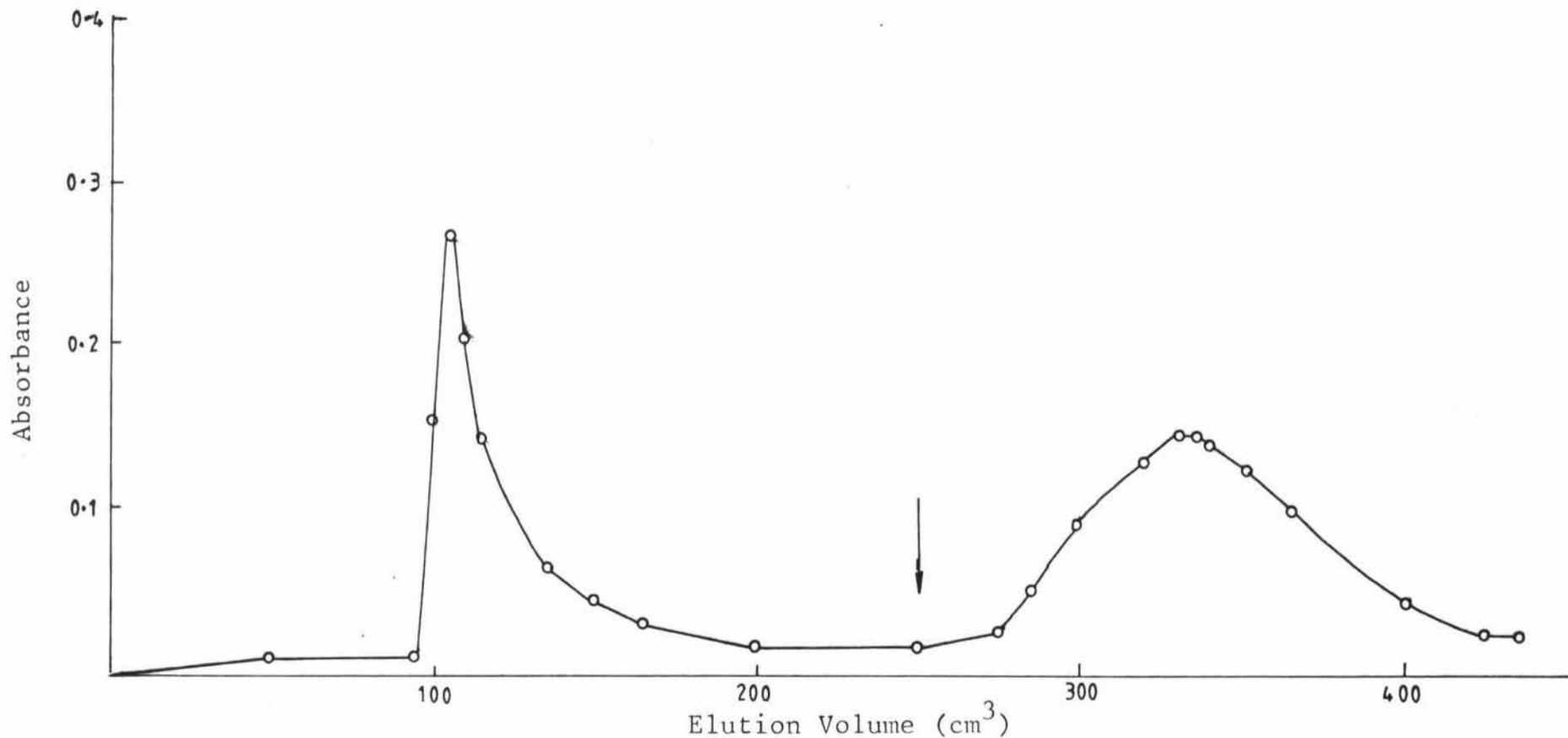


FIGURE 21: CHROMATOGRAPHY OF TDP AND TMP ON DOWEX 50 RESIN

Column dimensions; 100 x 10mm; Resin, Dowex 50W-X8 (200-400) formate form equilibrated with 0.1M formic acid. Flow rate, 10cm<sup>3</sup>/hr; Fraction size, 5cm<sup>3</sup>; Sample, 0.5cm<sup>3</sup> containing 0.5mg of TDP and TMP in 0.1M HCl, Eluents, initially 0.25M ammonium acetate buffer (pH 4.5) then 0.25M ammonium acetate buffer (pH 5.2) (arrow indicates point when buffer was changed).

at room temperature no change in fluorescence of a TDP sample was found to have occurred when samples were tested by the thiochrome method. However, as samples were not examined by high voltage electrophoresis, some change in TDP could have occurred and been undetected by the thiochrome method. Even so, the fact that the fluorescence recorded for the TDP sample was constant for two weeks contrasts with the decrease in fluorescence observed for the samples eluted from Dowex 50. It therefore appears that either;

- a) Dowex 50 resin or the eluting buffers contribute a fluorescent compound which breaks down on storage and which exhibits considerable fluorescence at the excitation wavelength used for the estimation of thiochrome. However this is unlikely as the resin was cleaned with 1.0M ammonium formate buffer (pH 4.8) and the 1.0M formic acid, used to convert the resin to the formate form, was glass distilled before use.

and/or

- b) Dowex 50 resin catalyses a conversion of TDP and/or TMP to products which are unstable and which on oxidation form highly fluorescent derivatives which are included in the thiochrome estimation. Such compounds presumably must move electrophoretically in a similar manner to TMP and TDP since the initial experiment showed that TMP and TDP could be eluted from Dowex 50 without any apparent breakdown according to the results obtained by electrophoresis.

Either possibility could explain the high initial recoveries obtained (especially for the fractions thought to contain TMP) but the subsequent continuous decrease in recovery for both peaks suggests a loss of TMP and TDP.

ii) Elution of Thiamine with Ammonium Acetate buffer

The above observations were further complicated by results obtained when attempts were made to elute thiamine from Dowex 50 resins. Initially 1.0M ammonium acetate buffer (pH 5.2) was used to elute thiamine. This appeared to be successful as only one peak was observed to be eluted when the fractions were monitored spectrophotometrically at 265nm. However high voltage electrophoresis of samples from the combined fractions containing this peak, followed by oxidation with 0.04% alkaline ferricyanide, yielded only a single yellow fluorescing spot in ultra violet light (360nm) whereas thiochrome exhibits a blue fluorescence. This yellow fluorescent compound or compounds (since electrophoresis was only performed at one pH, 3.5) moved close to TDP on high voltage electrophoresis.

This process was repeated with 1.0M ammonium acetate buffer (pH 5.2) as the initial eluent but followed by 2.0M ammonium acetate buffer (pH 6.0) (Fig. 22). Examination of the fractions by high voltage electrophoresis followed by oxidation with alkaline ferricyanide showed a yellow fluorescent compound(s) was present in both peaks and that the first peak appeared to consist solely of this compound(s). The yellow compound(s) in both peaks moved in the same position on electrophoresis (near TDP). In addition to the yellow compound(s) the major portion of the second peak consisted of a blue fluorescent compound which moved near TMP on electrophoresis. Since the buffers used were of a high concentration, and this was increased by rotary evaporation of the combined fractions, it seems likely that the blue fluorescent compound is indeed thiamine, or a closely related derivative of it, and that its movement on electrophoresis was retarded by the presence of salt.

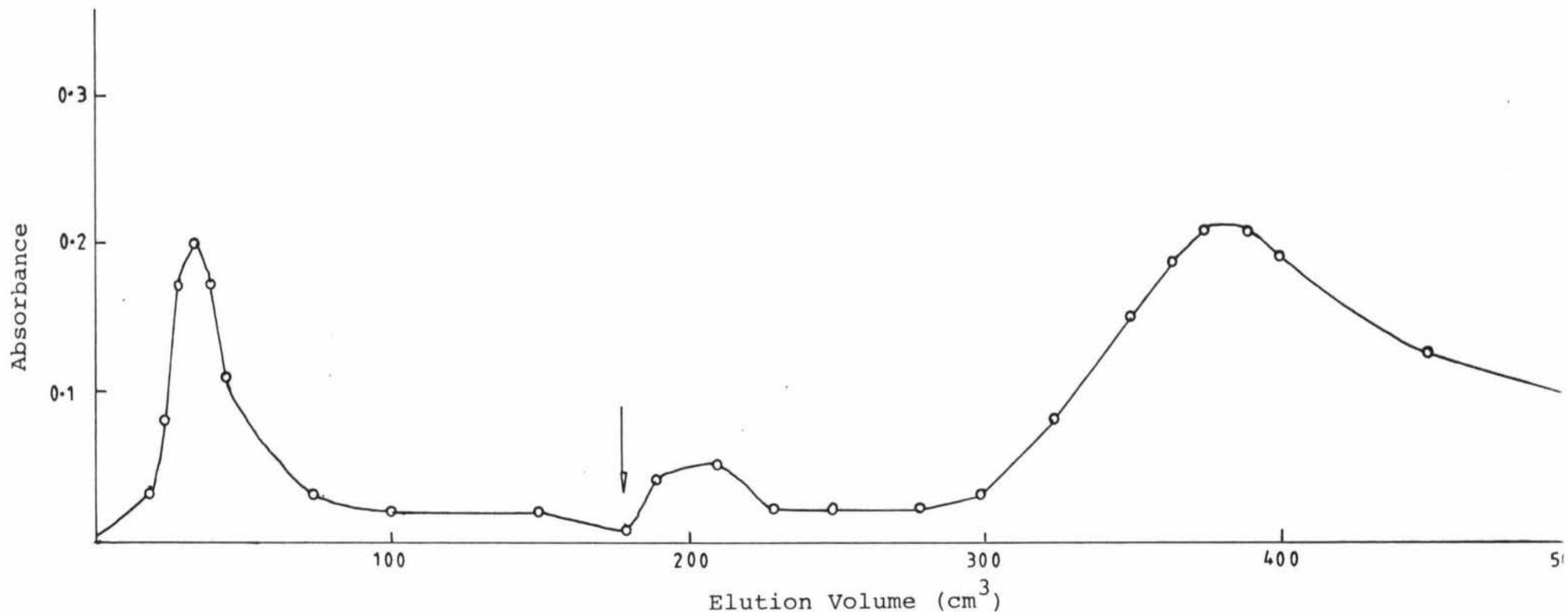


FIGURE 22: ELUTION OF THIAMINE FROM DOWEX 50 RESIN USING AMMONIUM ACETATE BUFFERS.

Column dimensions, 100 x 10mm; Resin, Dowex 50W-X8 (200-400 mesh) formate form; Flow rate, 12cm<sup>3</sup>/hr; Fraction size, 5 cm<sup>3</sup>; Sample, 0.5cm<sup>3</sup> containing 0.5 mg of thiamine in 0.1M HCl; Eluents, initially with 1M ammonium acetate buffer (pH 5.2) then 2M ammonium acetate buffer (pH 6.0) (arrow indicates point at which buffers were changed).

The ultra violet spectra of both peaks were recorded, after they had been concentrated by rotary evaporation, in an attempt to characterise the yellow fluorescent compound(s). Both peaks had similar but not identical ultra violet spectra which were also similar to that of thiamine (Fig. 23). This was unexpected as the electrophoretic mobility and fluorescent colour of the yellow fluorescent compound(s) indicated that they were not thiamine (thiochrome). However these apparently conflicting observations may possibly be explained in view of;

- a) the observation of Doherty *et al* (1955) that breakdown products of thiamine (e.g. the pyrimidine and thiazole components), when mixed together and examined spectrophotometrically, have an ultra violet spectrum which closely resembles that of the unaltered vitamin, and
- b) results obtained in the present work indicating that the ionic strength (in addition to pH) of the buffers in which the spectra were recorded has an affect on the spectra observed (Fig. 24). This made it difficult to say whether the differences in ultra violet spectra observed for the two peaks was due to a difference in the compounds present or the different buffers in which their spectra was recorded.

In an attempt to characterise the peaks by a method which would not be susceptible to these difficulties the ultra violet spectra of both peaks was examined after the aliquots were oxidised with 0.04% alkaline ferricyanide. The presence of thiamine in either peak would then be indicated by the appearance of a peak at 369nm, characteristic of thiochrome. This peak was present in fractions collected which contained the second peak but not those which contained the first peak (Fig. 25). Thus it would appear that thiamine was only present in the second peak.

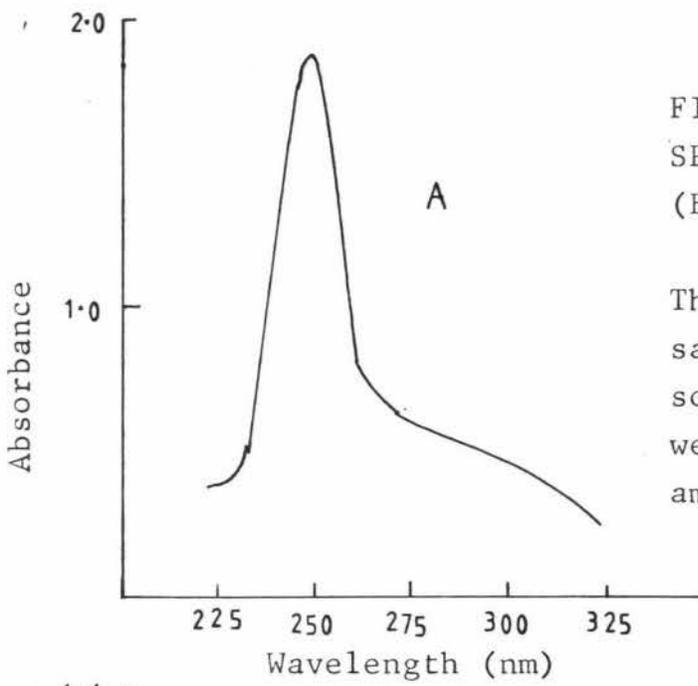
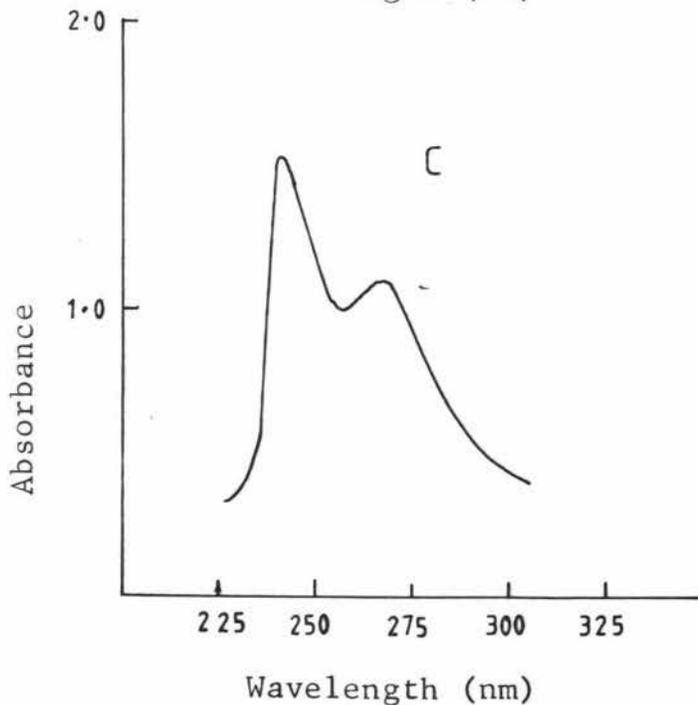
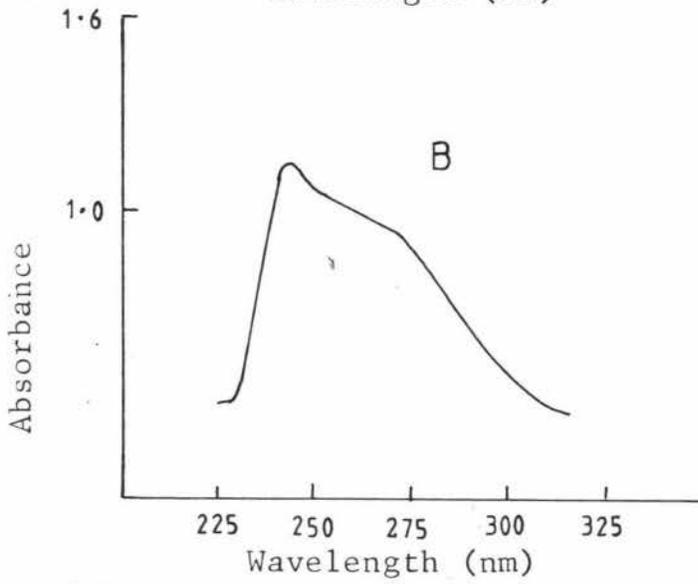


FIGURE 23: THE ULTRA VIOLET SPECTRA OF PEAK 1(A), PEAK 2 (B) AND THIAMINE (C).

Thiamine was prepared in saturated ammonium acetate solution and all solutions were blanked against 1M ammonium acetate buffer (pH5.2)



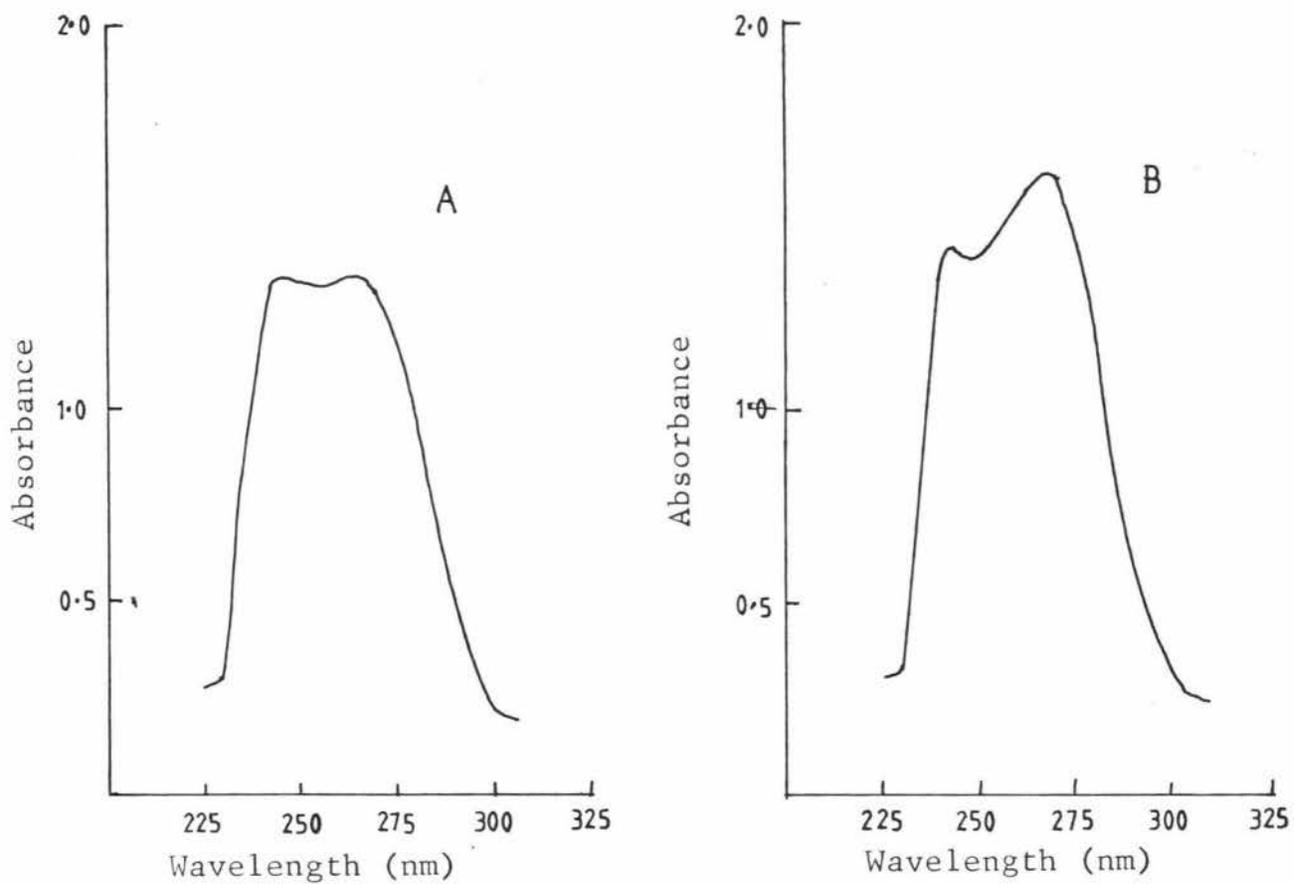


FIGURE 24: THE ULTRA VIOLET SPECTRA OF THIAMINE IN (A) 1M AMMONIUM ACETATE BUFFER (pH 5.2) AND (B) 2M AMMONIUM ACETATE BUFFER (pH 5.2).

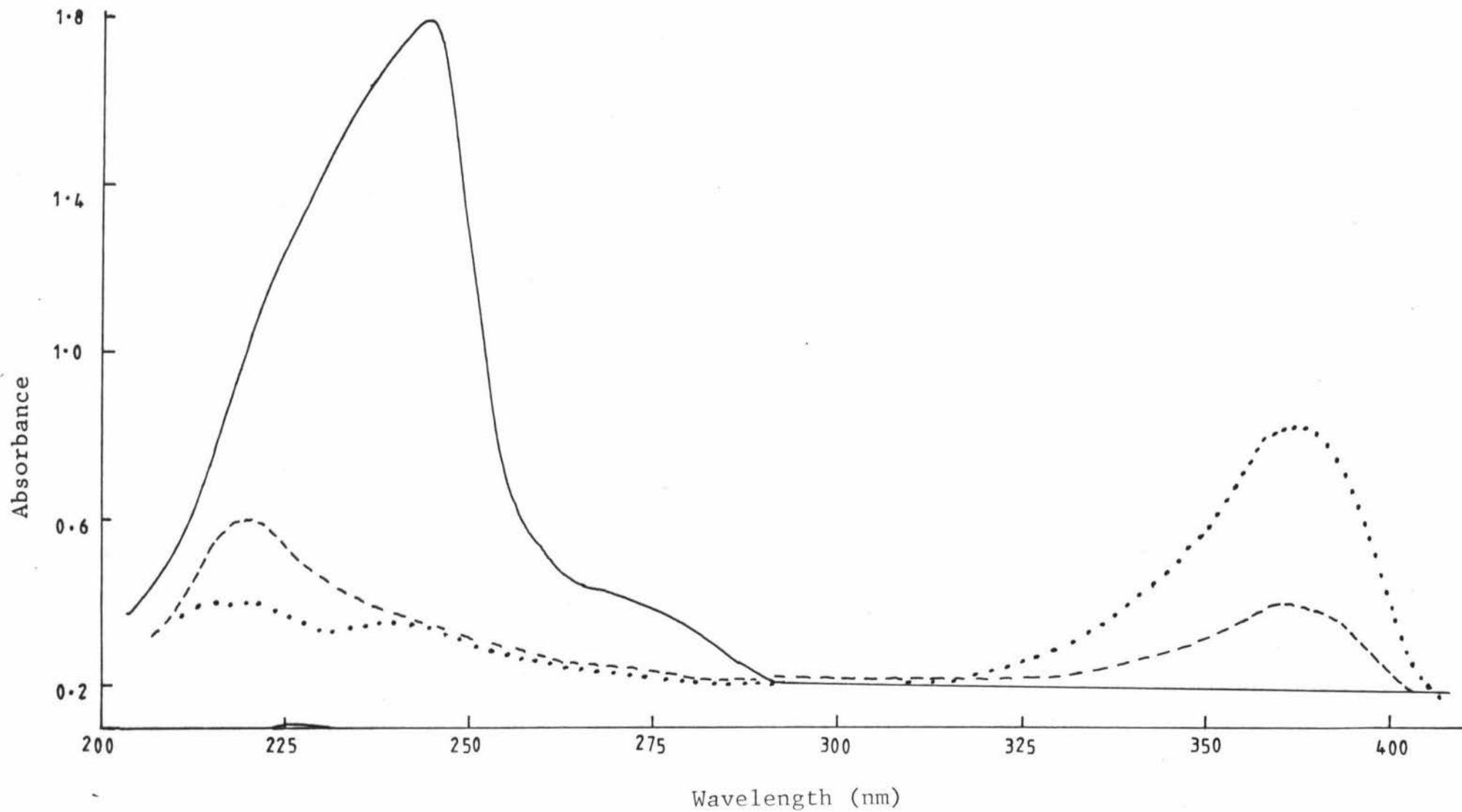


FIGURE 25: THE ULTRA VIOLET SPECTRA OF THE OXIDATION PRODUCTS OF PEAK 1 ( — ), PEAK 2 ( ---- ), AND THIAMINE ( ···· ).

However in contrast, the fluorescent spectra, also determined after oxidation with 0.04% alkaline ferricyanide, of isobutanol extracts of both peaks were very similar to that of thiochrome (Fig. 26). This suggests that the yellow compound(s), observed in both peaks, are derivatives of thiamine which on oxidation form compound(s) similar to thiochrome. It is uncertain whether the yellow compound(s) eluted in both peaks were the same compound(s) although this was suggested by their similar electrophoretic mobility and ultra violet spectra. However if both compounds were the same it is difficult to explain why a portion should be eluted from Dowex 50 with 1.0M ammonium acetate buffer (pH 5.2) and portion with 2.0M ammonium acetate buffer (pH 6.0).

Since the resin was thoroughly washed with 1.0M ammonium acetate buffer (pH 4.8) during its preparation it is unlikely that the yellow compounds were contaminants present in the resin. Thus it would seem that some conversion of thiamine is occurring during its elution from the resin. This is further indicated by similarities between the ultra violet and fluorescent spectra of the yellow compound(s) and thiamine (thiochrome) which would not be expected if the yellow compound(s) represented contaminants of the resin or buffers.

### iii) Elution of thiamine with 0.5M Ammonia

In an attempt to improve the elution of thiamine and the identification of the fractions, on high voltage electrophoresis, by reducing the salt concentration, 0.5M ammonia was used (instead of 1.0M or 2.0M ammonium acetate) to elute thiamine in a further experiment. The resin was eluted initially with 0.2M ammonium acetate buffer (pH 5.2) and as soon as the pH reached 5.2 the eluent was changed to 0.5M ammonia. Fractions were acidified immediately upon collection. Two peaks were eluted (Fig. 27). Samples of the first peak, when spotted on paper and sprayed with alkaline ferricyanide, gave a strong yellow fluorescence.

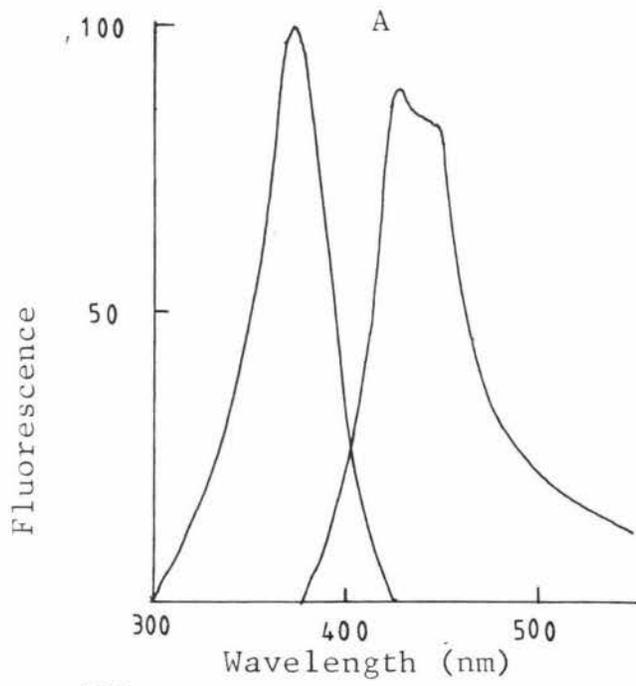
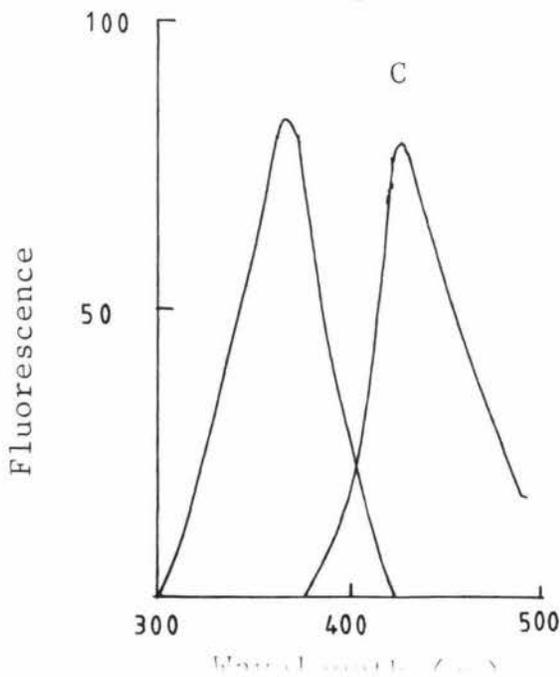
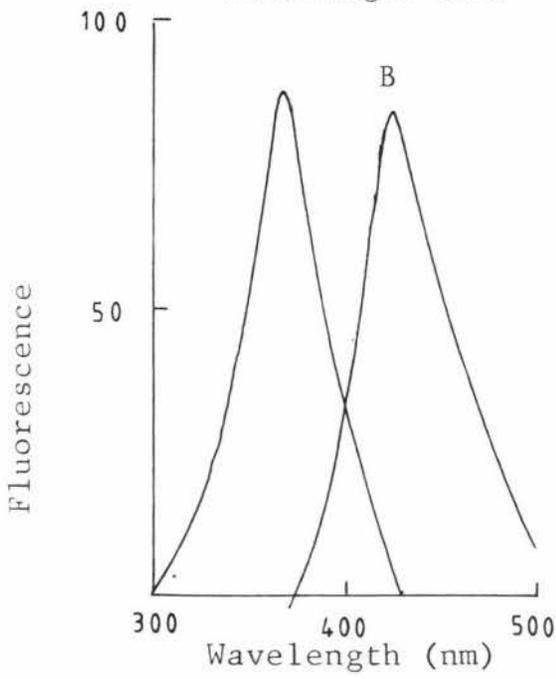


FIGURE 26: THE FLUORESCENT SPECTRA OF: -

- A) Peak 1 (the yellow compound)
- B) Peak 2
- C) Thiamine



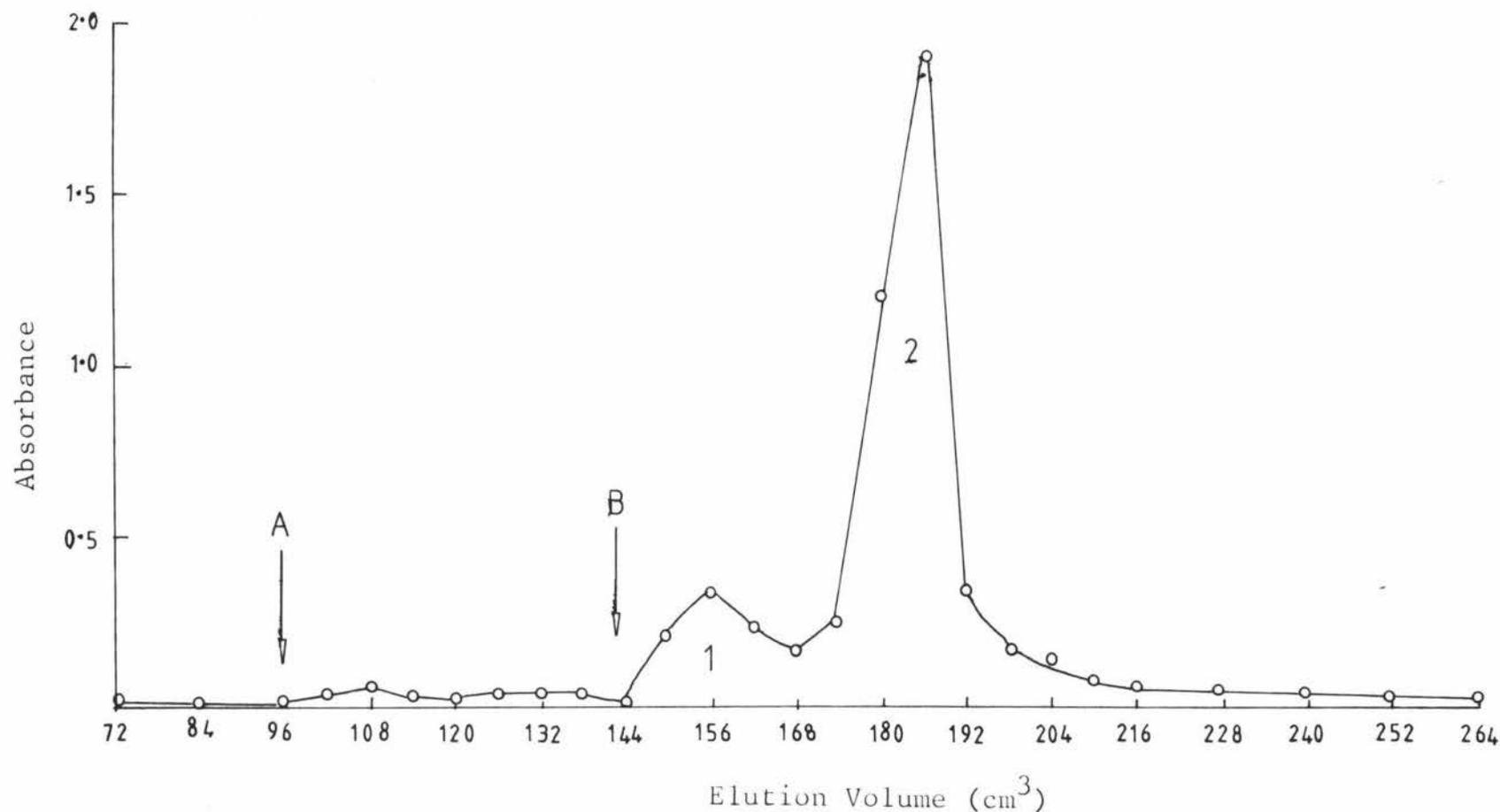


FIGURE 27: ELUTION OF THIAMINE FROM DOWEX 50 RESIN USING 0.5M AMMONIA

Column Dimensions, 100 x 10mm; Resin, Dowex 50 (200-400 mesh) equilibrated with 0.1M HCOOH; Flow rate, 10cm<sup>3</sup>/hr; Fraction size, 6cm<sup>3</sup>; Sample, 0.5cm<sup>3</sup> containing 0.5mg thiamine in 0.1M HCl; Eluents; Initially eluted with distilled-deionised water and at A changed to 0.2M ammonium acetate buffer (pH 5.2) then at B changed to 0.5M Ammonia.

When subjected to high voltage electrophoresis, after concentration by rotary evaporation, the compound(s) was identified as a single yellow fluorescent spot which moved between thiamine and TMP. The compound(s) eluted in the second peak, when treated in a similar manner, gave a blue fluorescence which moved on high voltage electrophoresis with thiamine. It seems probable that the yellow compound(s) observed in this experiment was the same as that eluted with 1.0M and 2.0M ammonium acetate buffer in the previous experiment. The different position on high voltage electrophoresis could be due to the lower ionic strength of the eluting solvent. The blue fluorescent compound(s) eluted also ran further after high voltage electrophoresis (i.e. close to thiamine) than that eluted with 2.0M ammonium acetate buffer (pH 5.2).

#### iv) Summary and Discussion

The results obtained suggest that considerable degradation of thiamine, TMP and TDP occurs when they are eluted from Dowex 50 resin. The breakdown product(s) of thiamine appear to be different to those obtained when TMP and TDP were eluted from Dowex 50 and this may be due to the harsher conditions required to elute thiamine (high ionic strength or pH) causing a different type of breakdown to that observed with TDP and TMP. The latter compounds formed unstable derivatives which, after oxidation, exhibited a blue fluorescence in contrast to the yellow fluorescent compound(s) derived from thiamine.

The type of breakdown product(s) that are formed after elution of thiamine, TMP and TDP from Dowex 50 is unknown but the fact that the resin was equilibrated with 1.0M formic acid during its preparation may be significant in view of Gounaris and Schulman's (1980) finding that TDP forms artifacts in the presence of formate ions. Comparison of the breakdown product(s) of thiamine with those of TDP and TMP was not pursued but it is possible that the yellow compound(s) formed from thiamine may also

have been produced from TDP and TMP during the observed progressive breakdown of these compounds. This is unknown because the product(s) of TDP and TMP were not subjected to electrophoresis after the observed progressive breakdown. In addition no information on the stability of the yellow compound(s) was obtained.

It is known that thiamine will form a transient yellow thiol form in strongly alkaline solutions such as the 0.5M ammonia used to elute thiamine (Metzler, 1960). Doherty *et al* (1955) have noted the appearance of a yellow colour in thiamine solutions (pH 3.7) incubated at 70° or left exposed to sunlight, which was apparently quite stable as they did not observe the colour to fade. They noted that this was suggestive of thiamine breakdown, but that the yellow compound could be estimated by fluorometric and spectrophotometric means in the same way as thiamine (thiochrome). Therefore there are similarities between the observations of Doherty *et al* (1955) and those made in the present work which show that the yellow fluorescent compound(s) had ultra violet and fluorescent spectra similar to that of thiamine (thiochrome). However it is unlikely that the yellow compound observed by Doherty *et al* (1955) is the same as the yellow fluorescent compound(s) observed in the present work as the compound(s) eluted from Dowex 50 required oxidation before they exhibited a yellow colour.

Since the results obtained in the present work indicated that the quantitative separation of thiamine and its esters would be difficult if not impossible with Dowex 50, the nature of the degradation products was not pursued further.

## CHAPTER 7

### CONCLUSIONS

#### 7.1 ESTIMATION METHODS

Two methods for the estimation of thiamine were investigated, the colorimetric, acid dye method of Das Gupta and Cadwallader (1967) and the fluorometric thiochrome method. Both followed Beer's law and exhibited good reproducibility in the respective thiamine concentration ranges tested. Also both methods required a similar time to perform although greater attention to detail and preparation was required for the thiochrome method. This was in part due to the low light conditions and freshly prepared solutions necessary for the thiochrome method and, in part due to its greater sensitivity to both thiamine and potential interference from other substances. All glass-ware used during the thiochrome procedure had to be acid washed and standard solutions, due to their low concentrations, required very careful preparation. In contrast to the thiochrome method, the acid dye method has been reported to be fairly insensitive to interference (Das Gupta and Cadwallader 1967). Tests on this aspect of the acid dye method were extended, in the present work, to include amino acids and thiamine phosphate esters but it is uncertain if the slightly negative absorbance readings observed constitute interference with this method. Extraction with chloroform in the acid dye method was difficult to perform as, due to the volatility of the solvent, leaks in the extraction flask were common. Thus the thiochrome method was judged easier to perform than the acid dye procedure. The thiochrome procedure has also been extensively used by other workers for the estimation of thiamine in biological extracts and is thus well documented.

Both methods require the substance assayed, thiochrome or the acid dye salt, to be extracted by an organic solvent before estimation. The non-extractability of the thiochrome derivatives of TMP and TDP is well documented (Mickelson and

Yamamoto 1950) and tests performed in the present work likewise showed that the acid dye salts of TMP and TDP were not extracted by chloroform. This meant that, whichever estimation procedure was used, the thiamine phosphate esters required hydrolysis before estimation. Thus the presence of high wheat germ acid phosphatase concentrations in the solution estimated by the thiochrome method was investigated. It was found that, using the high phosphatase concentrations recommended by Shultz and Natelson (1972) for hydrolysis of thiamine phosphate esters, thiamine was not quantitatively recovered. High concentrations of the enzyme interfered with the thiochrome assay, presumably by absorbing thiochrome thus making it unavailable for extraction into isobutanol. Quantitative recoveries could only be obtained at low enzyme concentrations. Incomplete digestions were also obtained when some types of buffers were used in the digestion mixture. The only buffer which allowed complete recovery of TDP digested was 2.0M sodium acetate buffer (pH 4.8) and the inclusion of even small quantities of formate buffers in the digest drastically reduced recoveries. It was found however that magnesium ions stimulated the activity of wheat germ acid phosphatase making the type of buffer in which digestion was performed unimportant. The presence of magnesium ions also allowed the use of lower enzyme concentrations to achieve complete digestion and so avoid any problems, caused by high enzyme concentrations, in the extraction of thiochrome. Even so, at 37<sup>o</sup>, overnight digestion was required.

## 7.2 SEPARATION METHODS

High voltage electrophoresis proved to be the best method for qualitative separation of thiamine and its phosphate esters. Using this technique good separations could be achieved more quickly than by any other method. It also allowed convenient separation of samples as the apparatus normally used for peptide separation could be used. By application of buffer from each end of the paper, after the sample had been applied to the base line, it was possible to concentrate the sample

into a very narrow band. This was useful when large amounts of sample required electrophoresis. However, difficulties were encountered in quantitative elution of samples from the paper after electrophoresis. Low recoveries were observed and considerable retardation of samples by salt made it difficult to estimate the positions of samples after electrophoresis by reference to marker standards. This technique would therefore probably be adequate for estimation of the relative quantities of thiamine and its esters in biological extracts but estimation of absolute quantities present would be difficult.

Separation of thiamine and its phosphate esters was also good when they were applied to a column of Sephadex G10, in the form of their thiochrome derivatives. Elution of sample from the column appeared to be quantitative when determined by spectrophotometric methods. However, the extreme length (140 cm) of the column required to achieve this separation made this technique time consuming and meant that samples were eluted over a large number of fractions. This presented no problem when large quantities of thiamine and its esters were applied to the column but difficulties in detection would probably occur when the sample contained only small amounts of thiamine and its esters. Thus extensive sample concentration before and after development of the column would probably be required as, as with all non-ion exchange gels, samples could not be concentrated on the column.

For quantitative separation of thiamine and its esters in biological extracts, ion exchange techniques were considered the most potentially useful. Three types of resin were investigated, Amberlite GC50, Dowex 1-X8, and Dowex 50W-X8. Difficulties were encountered with the stability of samples on all except Amberlite GC50 resin. This was the only resin in which sample breakdown was not indicated by more than one component in each peak eluted when tested by high voltage electrophoresis. The use of both the sodium and hydrogen forms of Amberlite GC50 resin was investigated and the results

were essentially the same as those reported by Rossi-Fanelli *et al* (1960) using Amberlite IRC 50 resin. The sodium form of the resin bound thiamine while the hydrogen form bound both thiamine and TMP. Separation between TDP and TMP, when eluted from the sodium form of the resin, was incomplete but could probably be improved by the use of a longer column or the two forms of the resin superimposed. The method was not pursued any further however as, since TDP did not bind to the resin, a separation between TDP and TTP could not be achieved on this resin. Combined TDP and TTP eluted from biological extracts would probably also contain impurities which could be a possible source of error in their estimation by the thiochrome method.

- While both Dowex 50 and Dowex 1-X8 resin appeared to give good separation (as judged by the elution profiles) quantitative estimation of samples was complicated by breakdown of samples during elution. When working with Dowex 1-X8, it was initially thought that this was due to incomplete separation of samples by this resin as, high voltage electrophoresis of the eluted peaks showed that the peak contaminants moved near to thiamine or thiamine phosphate markers. However in a recent paper Gounaris and Schulman (1980) observed a similar sample breakdown on elution of thiamine and its esters from Dowex 1-X8 which they attributed to the development of an artifact caused by the eluting formate buffers. They recommended the use of acetate buffers but noted that, with these buffers some hydrolysis of TDP to TMP apparently occurred. Thus the use of formate and acetate buffers with Dowex 1-X8 seems to be unadvisable but it is possible that with other buffer systems separation could be achieved in which sample breakdown does not occur.

Dowex 50 was the only resin used in which tightly bound thiamine, TMP and TDP but breakdown of samples also occurred on elution from this resin. TDP and TMP were apparently eluted easily with 0.25M ammonium acetate buffer (pH 4.5 and 5.2) but the eluted material exhibited a ridiculously high recovery (especially TMP) and progressive breakdown on

storage. As this breakdown did not occur in solutions of TDP prepared in the eluting buffer it was presumed that TDP and TMP underwent a transformation to unstable form(s) when eluted from Dowex 50 resin. These unstable form(s) were presumably structurally quite closely related to thiamine as, upon oxidation, they exhibited considerable fluorescence at the wavelength used to estimate thiochrome.

The elution of thiamine from Dowex 50 resin proved difficult and required strong salt or high pH solutions. The eluted fractions were shown, on oxidation, to contain a yellow fluorescent compound(s) as well as the usual blue fluorescent compound (thiochrome). As the resin was thoroughly washed before use with the eluting buffer and chemicals used were of high quality or glass distilled, it is unlikely that the yellow fluorescent compound(s) represented a contaminant. This was further indicated by a similarity between their ultra violet spectra and that of thiamine and a close resemblance between the fluorescent spectra of the oxidised yellow compound(s) and thiochrome. That it was a different compound(s) from thiamine was indicated by its inability to absorb light at 369nm after oxidation (characteristic of thiochrome), its yellow fluorescent colour, and its movement on high voltage electrophoresis which was different to that of thiamine.

Although the exact nature of the interaction between thiamine and its phosphate esters and ion exchange resins is uncertain, it is clear that some form of breakdown of sample does occur on certain resins. This is re-enforced by the observation that reasonable recoveries were obtained on Sephadex G10 gel, even when the column was eluted in the light, while on ion exchange resins recoveries of those compounds which were tightly bound by the resins were generally poor, even when elution was performed in low light condition. The breakdown was especially noticable on Dowex 50 resin as the breakdown products had properties which allowed them to be easily differentiated from thiamine and its esters. Previously published papers which have used ion exchange resins have

not mentioned this effect (with the exception of the recent paper of Gounaris and Schulman, 1980). This may be due to the use of acetate buffers as eluents in many of these experiments (e.g. Rindi and De Giuseppe, 1958), whereas formate buffers were mainly used throughout this work. Further information as to the nature of the breakdown products from Dowex 1-X8 and Dowex 50 (e.g. by mass spectrometry and nuclear magnetic resonance) would be of interest. It is uncertain whether the breakdown products of TMP and TDP were different on the two resins. However this is suggested by the fact that the products formed from TMP and TDP on Dowex 1-X8 could be separated by high voltage electrophoresis while those formed on Dowex 50 could not be observed as distinctly different from TMP and TDP after electrophoresis.

In contrast the breakdown product(s) of thiamine eluted from Dowex 50 were extremely distinctive as they had a different fluorescent colour and moved in a different position on electrophoresis to the breakdown products of TDP and TMP eluted from either Dowex 1-X8 or Dowex 50 resin. It is possible however, that the type of breakdown exhibited by thiamine on Dowex 50 was different to that exhibited by TDP and TMP on either resin. The harsh conditions required to elute thiamine may have been responsible for its degradation whereas the breakdown of TDP and TMP appears to require chromatography by ion exchange resins.

Therefore this work has shown that there are serious problems in using some ion exchange resins with thiamine and its esters. Of the resins investigated Dowex 1-X8 appears to be the most promising for future study as sample breakdown on this resin appears to be related to the type of buffers used as eluents (Gounaris and Schulman, 1980). Thus it may be possible to select buffers which cause little or no breakdown of sample on this resin. Some of the other ion exchange resins and gels available today may also be worth investigating. In addition future development of new techniques (e.g. high pressure liquid chromatography) may provide useful methods for the quantitative separation of thiamine and its esters.

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