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THIAMINE

(A Study of its Chemistry, Biochemistry
and Mechanism of Action)

by

A. J. KNELL

A dissertation submitted to the
UNIVERSITY OF WARWICK
for the degree of
DOCTOR OF PHILOSOPHY

November 1970

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PREFACE

The work described in this dissertation was done in the School of Molecular Sciences, University of Warwick, Coventry, between October 1966 and September 1969. It is the original work of the author, except where acknowledgement is made, and has not been submitted for a degree at any other university.

The author wishes to thank Professor V. M. Clark, who supervised this work, and Dr. D. W. Hutchinson for their interest and advice.

The award of a Junior Research Fellowship to the author by the Medical Research Council is acknowledged with gratitude.

A. J. Knell.

INDEX

INTRODUCTION

This thesis is in three parts. The first is an argument which is intended to establish four points :

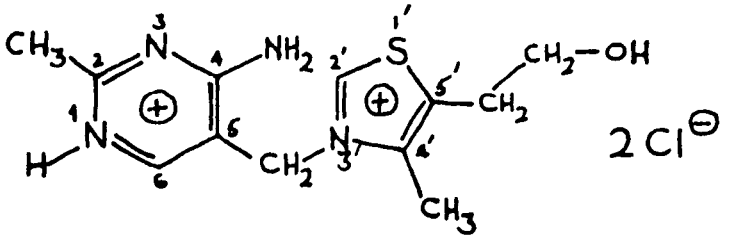
(a) that the mechanism of biological action of thiamine proposed by Breslow[6] is inadequate ;

(b) that the "yellow salt of thiamine", xantho-thiamine (5), is the form of thiamine most likely to be the enzyme cofactor ;

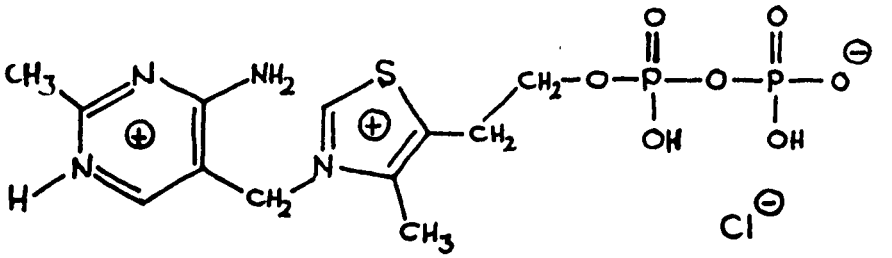
(c) that reasonable mechanisms explaining the biochemical functions of thiamine can be written if xantho-thiamine is the cofactor ; and

(d) that xantho-thiamine behaves chemically in the manner required by these mechanisms.

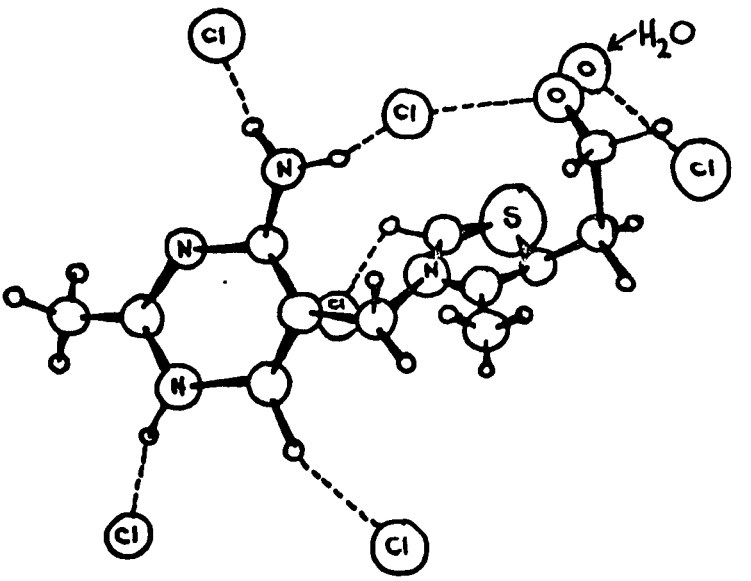
The second part is a commentary on the first, and is a survey of present knowledge of the chemistry and biochemistry of thiamine. New work is reported in context, leaving experimental details to the next section. The third part is a summary of experimental procedures used, with particular emphasis on the results of spectroscopic studies. It is concluded by a number of suggestions for future work.



THIAMINE bis-CHLORIDE (1).



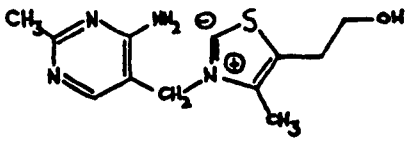
THIAMINE PYROPHOSPHATE CHLORIDE (2).



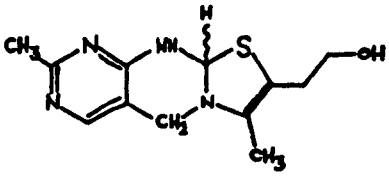
CRYSTAL STRUCTURE OF THIAMINE bis-CHLORIDE HYDRATE [50].

FIGURE 1: THIAMINE bis-CATIONS.

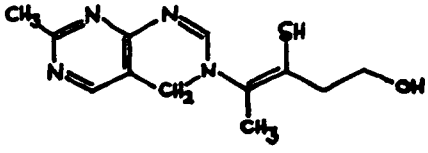
THIAMINE YLID (3)



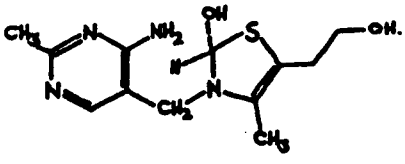
DIHYDRO-THIOCHROME (4)



XANTHO-THIAMINE (5)



THIAMINE PSEUDOBASE (6)



LEUCO-THIAMINE (7)

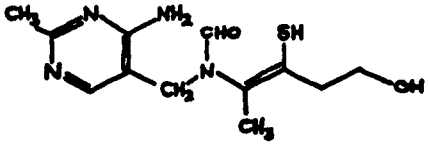


FIGURE 2: NEUTRAL FORMS OF THIAMINE.

Nomenclature

The I.U.P.A.C. Nomenclature Commission for Biological Chemistry[1] preferred the name "thiamine" to the older name "aneurine" proposed by Jansen.[2] An extension of the nomenclature is needed because various forms of thiamine have to be distinguished. The following system will be used in this thesis :

(a) bis-Cations $[C_{12}H_{18}N_4OS]^{2+}$ and mono-cations $[C_{12}H_{17}N_4OS]^{+}$ will be distinguished by the conjugate anion :

e.g. thiamine bis-chloride (1),
thiamine mono-nitrate.

Zwitterions of phosphate and sulphate esters are the only known mixed salts of the bis-cation :

e.g. thiamine pyrophosphate chloride (2).

(b) Neutral thiamine $[C_{12}H_{16}N_4OS]$ may exist as thiamine ylid (3), dihydrothiochrome (4), and xantho-thiamine (5).

(c) The hydrates of (3) and (5) $[C_{12}H_{18}N_4O_2S]$ are thiamine pseudobase (6) and leuco-thiamine (7).

(d) Anions $[C_{12}H_{15}N_4OS]^{-}$ or $[C_{12}H_{17}N_4O_2S]^{-}$ will be distinguished by the conjugate cation :

e.g. sodium xantho-thiamine.

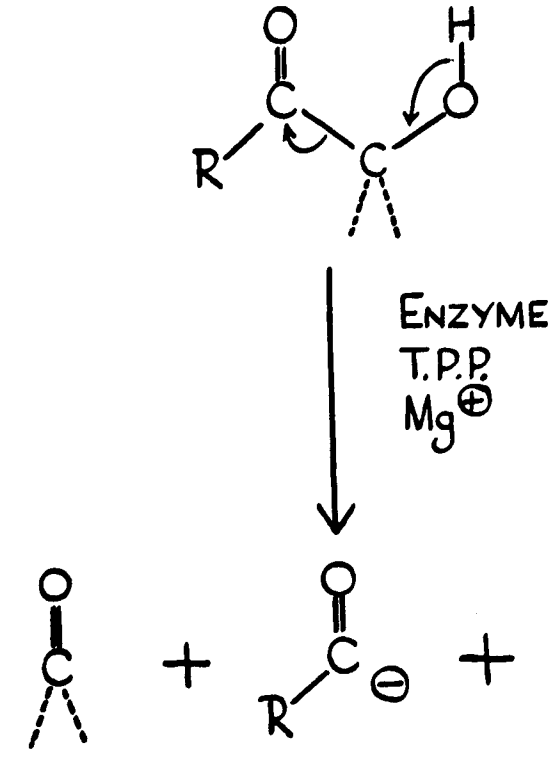
References from the argument to a section of the commentary will be enclosed in square brackets. References to the experiment section will have the prefix "exp" and be enclosed in square brackets. Other conventions and abbreviations are those recommended by the Chemical Society.[3]

ARGUMENT

"The remenant of the tale is long ynough"

Knight's Tale.

α-KETO ACID
KETOSE



CO_2
ALDOSE

ACYL ANION

FIGURE 3:

ARGUMENT

1. The classical determination of the structures of thiamine bis-chloride (1) and its pyrophosphate ester (2) [1] can be confirmed and extended by using spectroscopic methods [2]. The range of conformations of the molecule is restricted by interaction of the pyrimidine ring and the 4'-methyl group, which limits rotation around the bond between the methylene carbon and 3'-nitrogen atoms. An angle of about 90^{o} between the planes of the two rings gives least interaction. This effect can be demonstrated with space-filling molecular models and confirmed by X-ray crystallographic (figure 1) and N.M.R. studies [3]. These steric factors permit intramolecular reaction of the 2'-carbon atom with the 4-amino group, but hinder the approach of external reagents to the 2'-position. Thiamine cations in acid solution can be reduced, esterified, deaminated and cleaved [4].

2. The enzymes which require thiamine pyrophosphate (T.P.P.) and magnesium ions as cofactors [5] catalyse a specific reaction of α-keto acids and ketose sugars, which is heterolysis of the bond between an hydroxylated carbon atom and an α-carbonyl group (figure 3). The initial products are carbon dioxide or an aldose, and an "active aldehyde" [6], which is notionally an acyl anion. The acyl anion can react further in three ways (figure 4).

(a) Reductive acylation of a second substrate forms, for example, acyl-lipoate, a ketose or an acyloin.

(b) Oxidation by a second substrate allows subsequent acylation of a third, forming, for example, acyl-phosphate or a carboxylic acid.

(c) Protonation can lead to the release of free aldehyde.

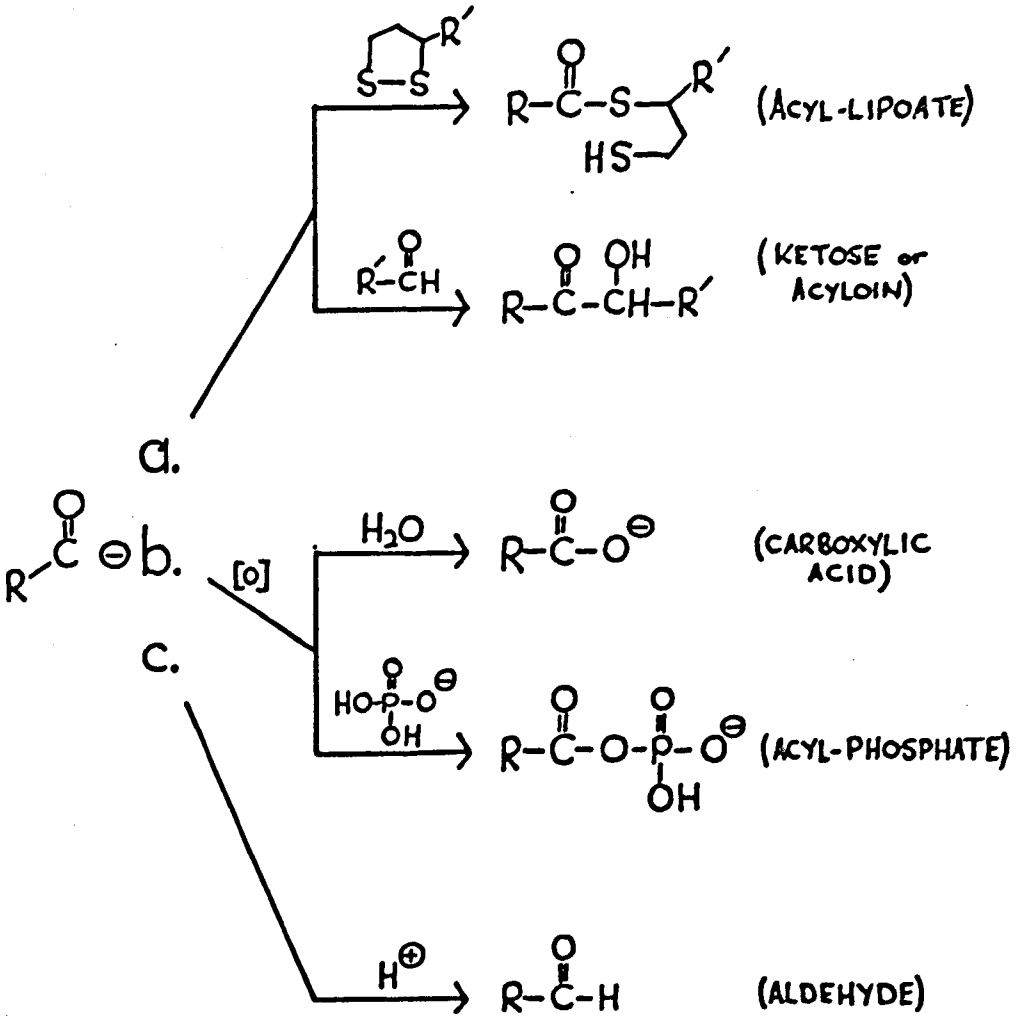

a.
b.
c.
[O]
H_2O
$H^{\oplus}$
(ACYL-LIPOATE)
(KETOSE or ACYLOIN)
(CARBOXYLIC ACID)
(ACYL-PHOSPHATE)
(ALDEHYDE)

FIGURE 4:

COENZYME ACTIVITY AND INHIBITOR ACTION OF TPP ANALOGUES

Coenzyme or analogue	Coenzyme activity (steady state value) (%)	Reduction of enzyme activity in simultaneous test (%)
Thiamine	100	-
4'-Hydroxy-4'-deamino-T	0	52 ± 7
N-Methyl-T	0	25 ± 3
N,N-Dimethyl-T	0	22 ± 3
Deamino-T	0	30 ± 5
2-Methyl-T	0	0
2-(1-Hydroxyethyl)-T	0	0
6'-Methyl-T	0	10 ± 3
6'-Methyl-4'-hydroxy-4'-deamino-T	0	2 ± 1
6'-Methyl-4-nor-T	22	-
4-Nor-T	24	25 ± 4
4-Ethyl-4-nor-T	32	4 ± 1
2-Methyl-4-ethyl-4-nor-T	0	18 ± 5
N-1-Pyridine analogue	13	11 ± 2
N-3-Pyridine analogue	0	0
Pyrithiamine	0	0
2'-Ethyl-2'-nor-T	48	11 ± 2
5-(3-Hydroxypropyl)-5-nor-T	0	22 ± 3

from : Schellenberger A.[4]
Angew. Chem. Internat. Edit. 6, 1030 (1967).

TABLE 1:

3. The catalysis of these reactions by thiamine may involve two steps. In the first, thiamine combines with the substrate in such a way that the heterolysis is facilitated. In the second, thiamine undergoes reversible reductive acylation, so obviating the acyl anion. This may imply that thiamine can react successively as a nucleophile and as an electrophile : as a nucleophile to add to the carbonyl group of the substrate ; as an electrophile to weaken the carbon-carbon bond of the substrate and to stabilise the negative charge produced when the bond is broken [7].

4. Schellenberger's studies[4] of the activity of analogues of thiamine as cofactors or inhibitors of yeast pyruvate decarboxylase (table 1) complement the results of previous biological studies.[5] The data allow an analysis of the relationship between the structure and function of thiamine, and the following structural features essential for function can be defined :

(a) the thiazolium ring, unsubstituted in the 2'-position ;

(b) the 4-aminopyrimidine system, with the amino group unsubstituted ;

(c) the spatial relation between (a) and (b) ;

(d) the ability of the molecule to adopt a planar configuration, approximating the 4-amino nitrogen and 2'-carbon atoms ;

(e) the 5'-(2"-hydroxy)ethyl pyrophosphate group.

5. Breslow's mechanism[6] explaining the biochemical function of thiamine is shown in figure 5. The theory is based on four observations :

(a) Simple thiazolium salts as well as thiamine will cause the production of acyloins from pyruvate in a model system [8].

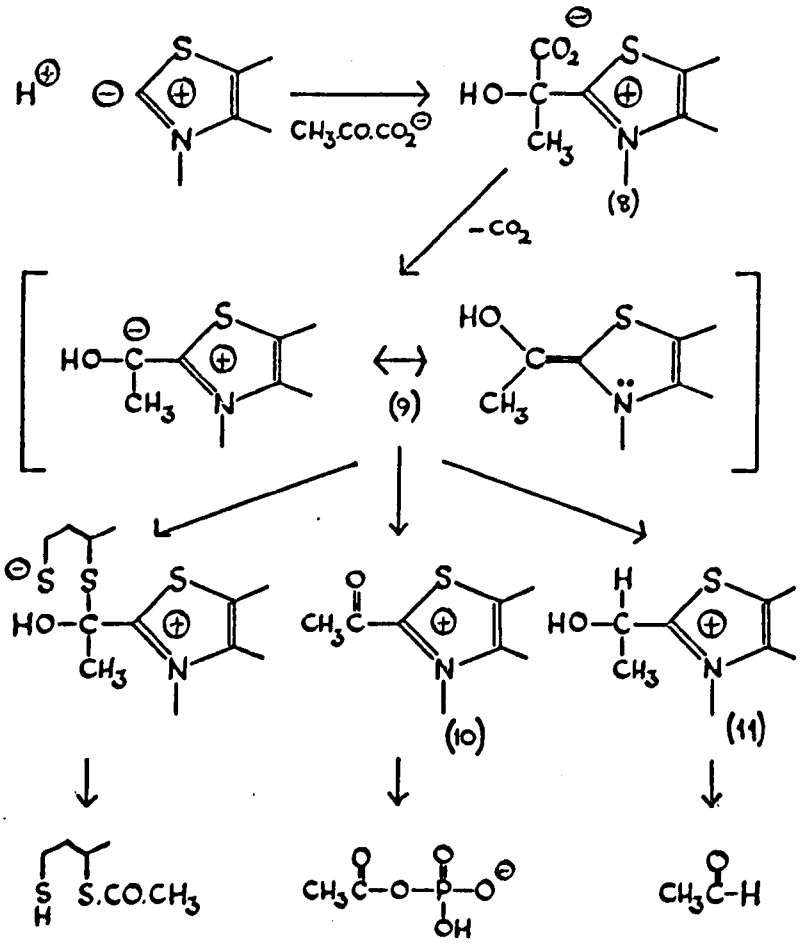


FIGURE 5: BRESLOW'S MECHANISM.

(b) The model reaction has a pronounced pH optimum at 8.8, which corresponds to the addition of two equivalents of base to thiamine bis-chloride.

(c) Substitution at the 2'-position inhibits the model reaction, and 2'-substituted thiamine analogues have no vitamin activity.

(d) Deuterium from D_2O exchanges into the thiazolium 2'-position. This implies that the ylid (3) has considerable stability [9]. Subsequent work has provided more evidence consistent with Breslow's theory [10], [11].

6. Breslow's theory is unsatisfactory for the following reasons.

(a) Intermolecular reaction at the 2'-position is sterically hindered, and it is difficult or impossible to build space-filling models of the proposed intermediates.

(b) It accounts for only the first of the structural features essential for activity [12].

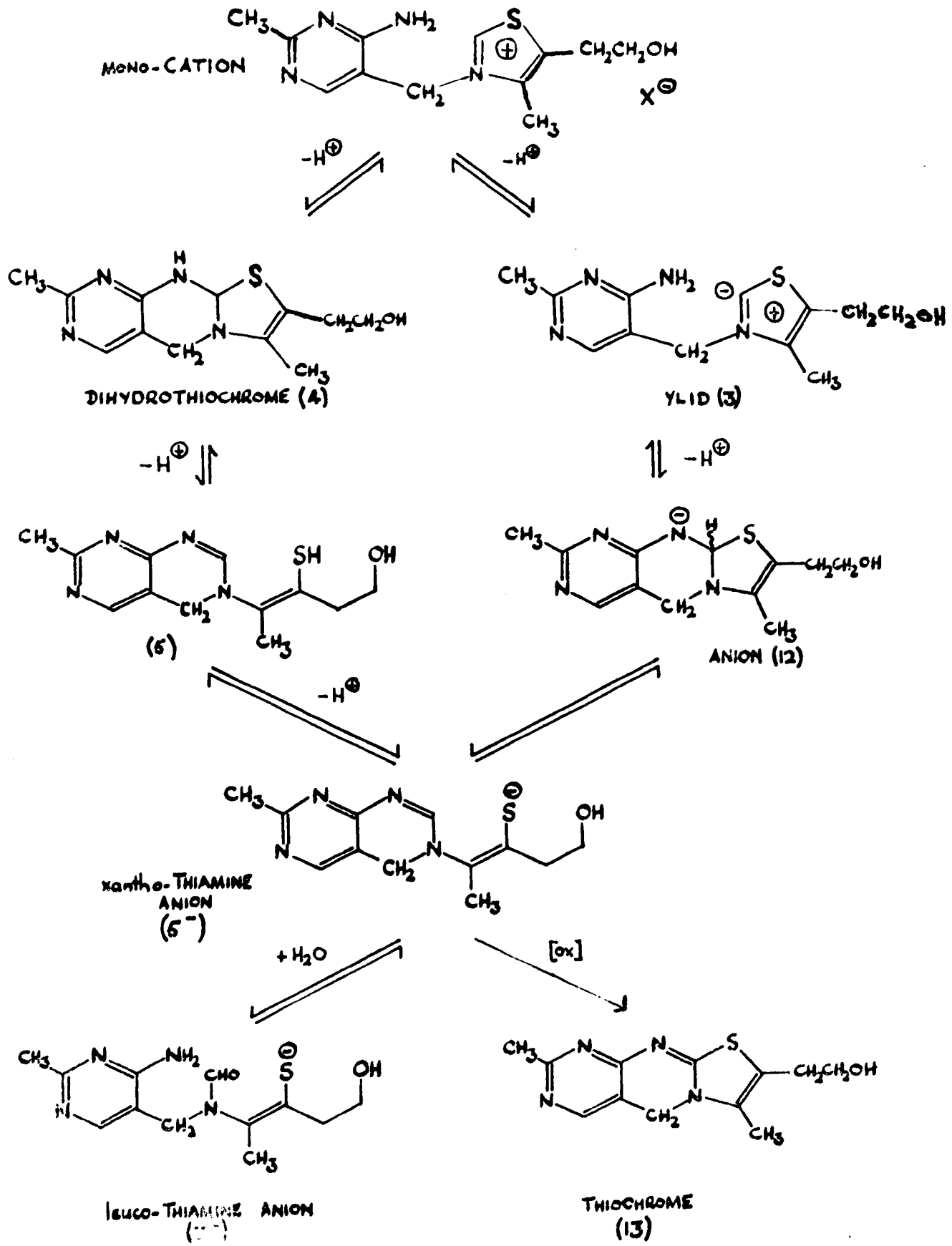
(c) It does not easily explain the oxidative functions of thiamine.

(d) 2'-(1"-Hydroxyethyl)thiamine pyrophosphate is not a cofactor for apo-pyruvate decarboxylase (table 1), and the 1"-proton exchanges slowly. 2'-Acetyl thiazolium salts are not acetylating agents in water.[147]

(e) It is difficult to show that thiamine acts catalytically in the model reaction, and the results obtained using thiamine analogues differ qualitatively and quantitatively from the results of enzyme studies.[4]

7. The evidence suggests that the biologically active form of thiamine is not the cation or the ylid. xantho-Thiamine (5) is a more likely candidate. This form of thiamine exists in basic anhydrous media. Its anion is the "yellow sodium salt" of Zima and Williams.[7] It is sensitive to water and to oxidising agents, forming in the first case leuco-thiamine (7),

FIGURE 6: FORMATION AND REACTIONS OF XANTHO-THIAMINE.



the "white salt" of Zima and Williams [13], and in the second, thiochrome (13) [14] (figure 6).

8. xantho-Thiamine anion ($5^{\ominus}$) is derived from thiamine monocation by the loss of two protons and rearrangement of two possible intermediates : dihydrothiochrome (4), formed by intramolecular addition of the 4-amino group [15], and a tricyclic anion (12), [16]. formed by a mechanism which may resemble that of the carbylamine reaction (figure 6).

9. The sulphur atom of xantho-thiamine is a nucleophilic centre near the electrophilic 7-carbon atom of the tetra-azanaphthalene nucleus. This system could stabilise an acyl anion by forming an intermediate such as (15) (figure 7). X might be magnesium ion [17].

10. An immediate difficulty is to explain why heterolysis of the carbon-carbon bond of the substrate should be facilitated by the formation of the initial adduct (14). The pyrophosphate group, and groups in the active centre of the enzyme might be involved at this point in the mechanism [18].

11. The advantages of this theory are as follows :

(a) There are no steric objections.

(b) It accounts for most of the observations relating to structure and function.

(c) The oxidative function of thiamine is explained (figure 8). The intermediate (15) should be easily oxidised, by analogy with the oxidation of xantho-thiamine and dihydrothiochrome to thiochrome. The product is an S-acyl xantho-thiamine (16). Thiol esters are usually reactive, and S-acyl leuco-thiamine (17) will transfer the acyl group to the 5'-(2"-hydroxy)ethyl group or to solvent.[156]

(d) It is consistent with the results of studies of the active sites

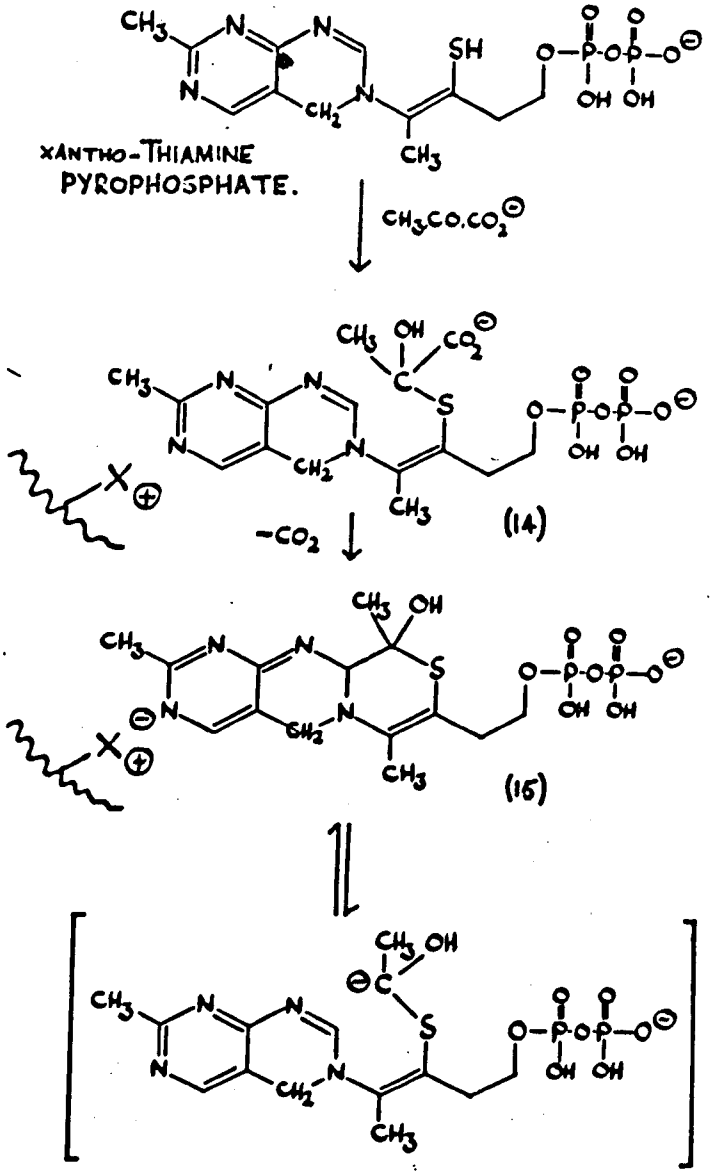


FIGURE 7:

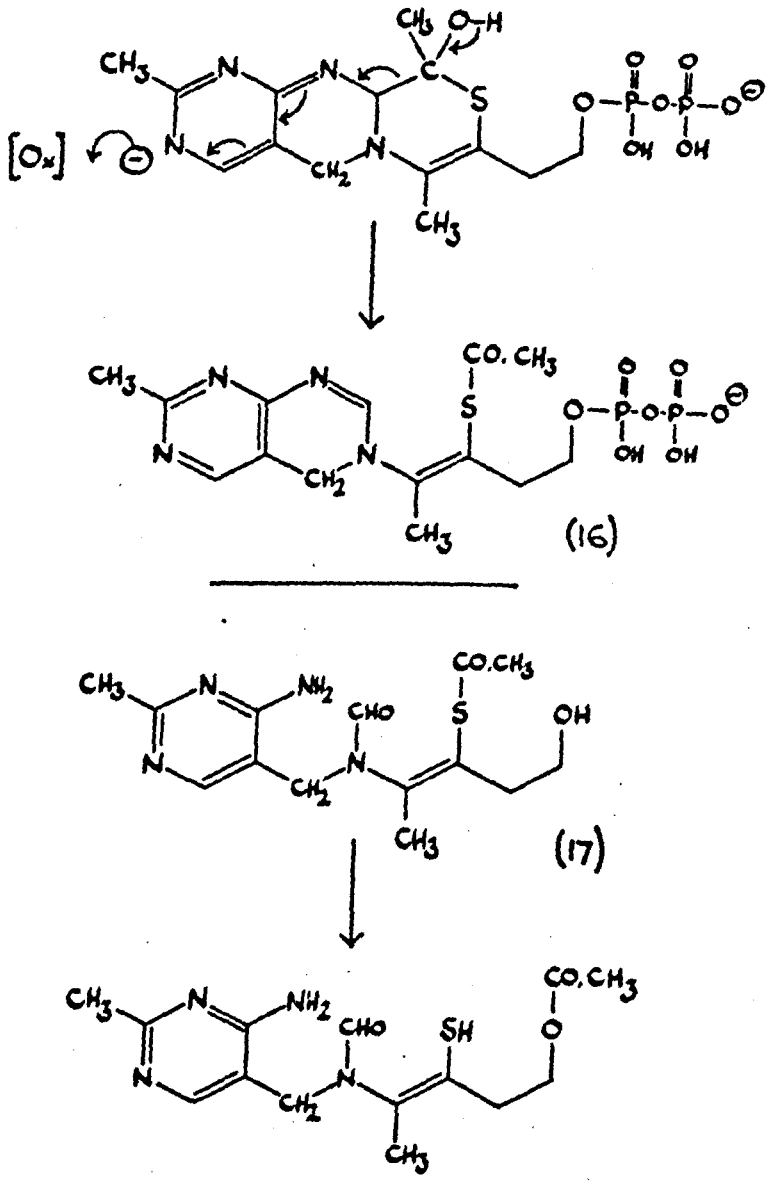
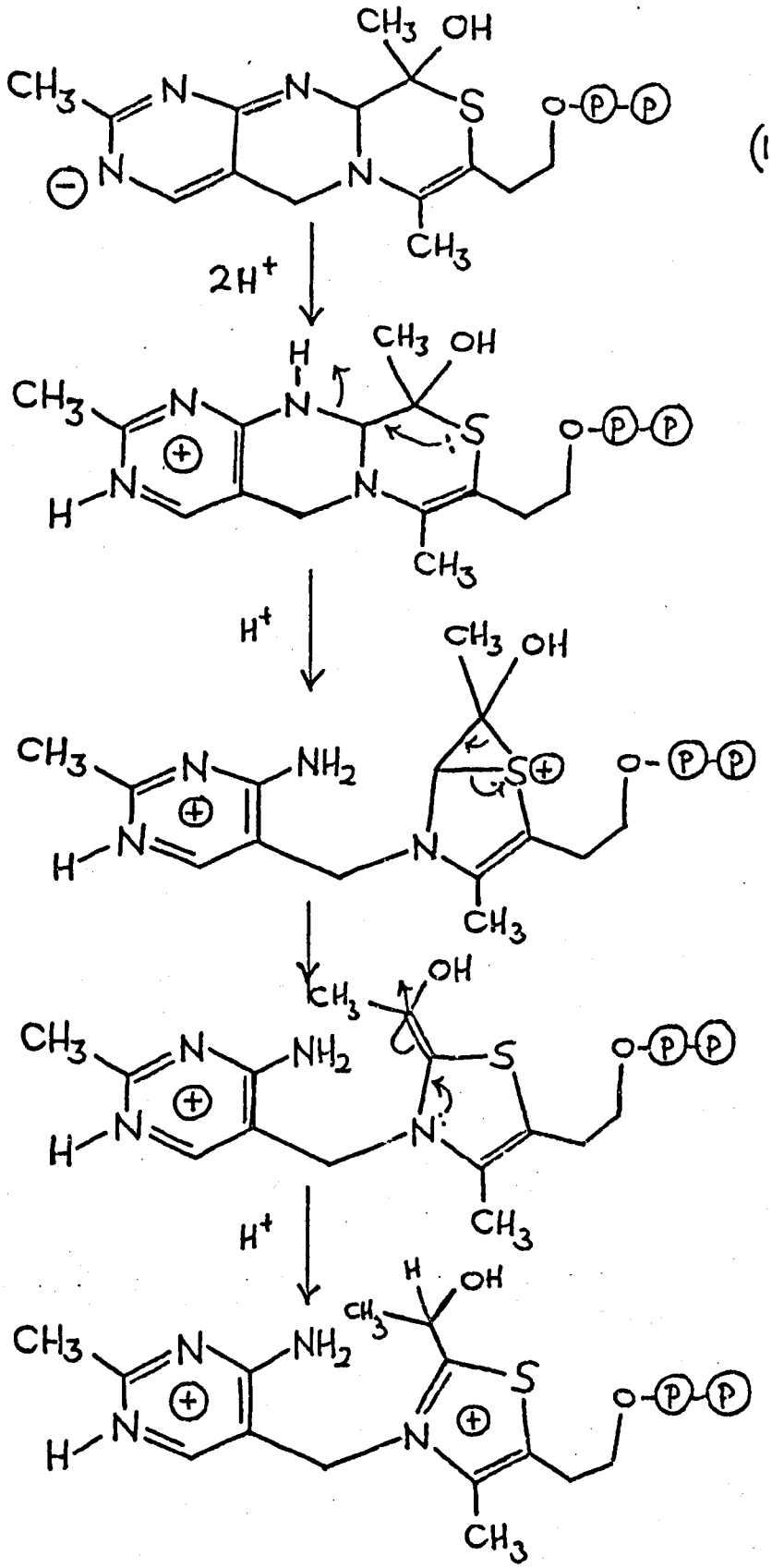


FIGURE 8:


CH3 OH
CH3
N
N
S
O-P-P
N
CH3
2H+
H
CH3 OH
CH3
N
N
S
O-P-P
H
N
N
CH3
H+
CH3 OH
CH3
N
NH2
S
O-P-P
H
N
N
CH3
CH3
OH
CH3
N
NH2
S
O-P-P
H
N
N
CH3
H+
H
OH
CH3
CH3
N
NH2
S
O-P-P
H
N
N
CH3

(15)

FIGURE 8A.

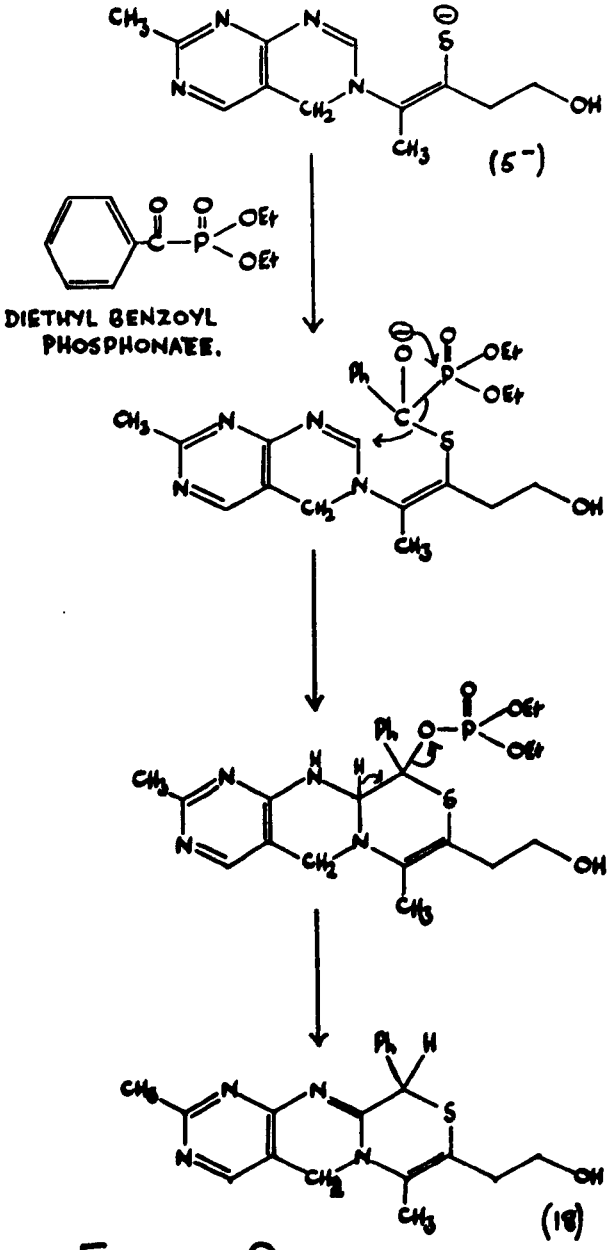


FIGURE 9:

of enzymes which require T.P.P., and allows an explanation of some unusual features [19].

12. 2'-(1"-Hydroxyalkyl)thiamine cations can arise by rearrangement of the intermediate (15) in acid solution. These compounds would therefore be inactive derivatives of the intermediate. Enzymic preparations of these compounds give greatest yields at low pH, where the enzyme is showing very little catalytic activity.

13. The reactions of xantho-thiamine with acyl phosphonates and α-haloketones are each examples of the property of xantho-thiamine to react consecutively as a nucleophile and as an electrophile, in the manner required by the mechanism. The acyl phosphonate reaction (Takamizawa) gives the crystalline product (18). Takamizawa proposed a mechanism involving the ylid: an alternative is shown in figure 9 [20].

α-Haloketones react with xantho-thiamine to give a blood-red compound (19), the colour of which is probably caused by intramolecular charge transfer. In acid the crystalline yellow compound (20) is formed [21]. A mechanism for this reaction is shown in figure 10.

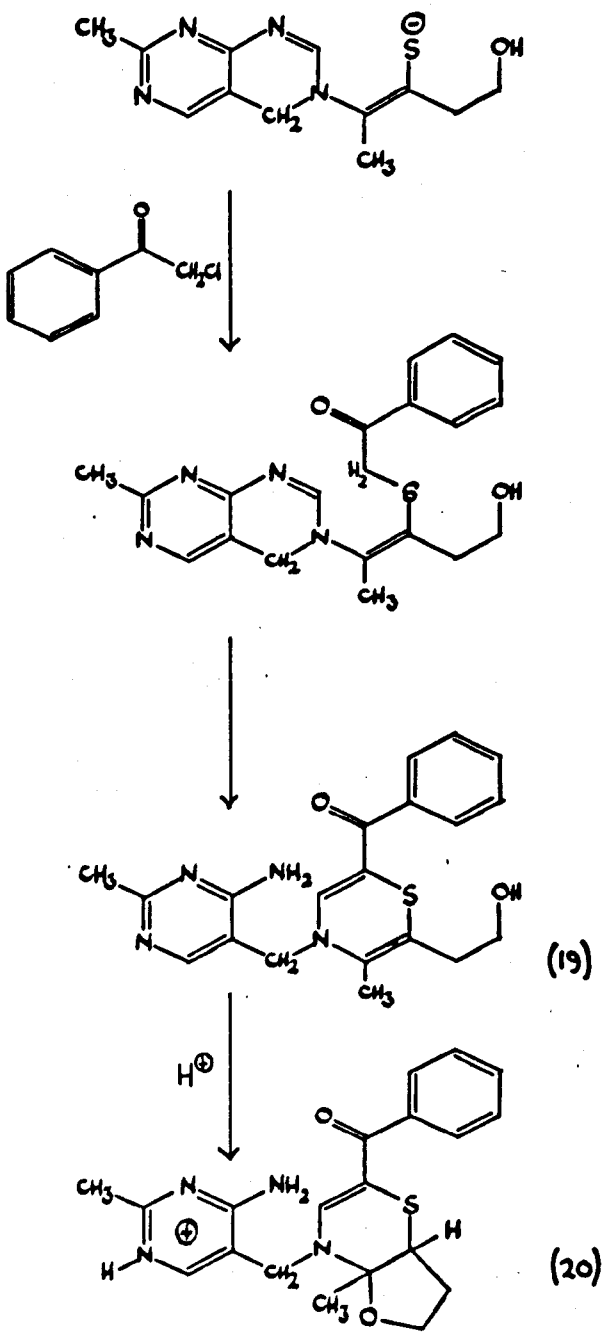

CH3
N
N
S
OH
CH2
N
CH3
O
CH2Cl
O
CH3
N
N
H2
S
OH
CH2
N
CH3
O
CH3
N
NH2
S
OH
CH2
N
CH3
(19)
H⊕
O
CH3
N
NH2
S
H
⊕
H
N
CH2
N
CH3
O
(20)

FIGURE 10:

COMMENTARY

"Lat every felawe telle his tale aboute,
And lat see now who shall the soper wynne."

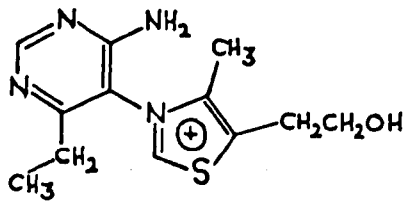
Knight's Tale.

[1] THIAMINE CATIONS : ISOLATION, STRUCTURE DETERMINATION AND SYNTHESIS[8]

The first crystalline preparations of thiamine bis-cation[9] gave inconsistent analyses,[10] but by 1935 the empirical formula of the "free base" was known.[11] Williams identified the base displaced by bisulphite from thiamine bis-chloride[12] as 5-(2'-hydroxyethyl)-4-methyl thiazole.[13] This was synthesised.[14] That thiamine bis-chloride contained quaternary nitrogen was attested by its solubility in water, the bisulphite reaction, the form of the titration curve,[15] and its decomposition by heat.[16]

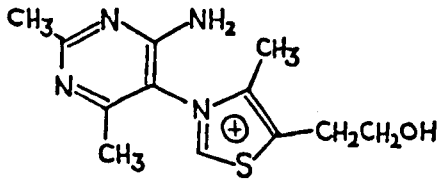
Williams provisionally identified the second product of the bisulphite reaction as 4-amino-6-ethyl-5-sulphopyrimidine, which implied structure (21) for thiamine.[17] Synthetic (21) was, however, biologically inactive.[18] Windaus oxidised thimaine sulphate with barium permanganate to obtain a base thought to be 4,5-diamino-2,6-dimethylpyrimidine, which implied structure (22) for thiamine.[19] Makino and Imai proposed structure (23), based on a faulty analysis of the ultraviolet spectrum.[20] Williams obtained the same base as Windaus by treating thiamine bis-chloride with liquid ammonia, and showed it to be 4-amino-5-aminomethyl-2-methylpyrimidine. Sodium in liquid ammonia reduced the aminosulphopyrimidine from the bisulphite reaction, yielding 4-amino-2,5-dimethylpyrimidine, identical with the synthesised compound.[21] Grewe showed that synthetic 4-amino-5-aminomethyl-2-methylpyrimidine was identical with Windaus' base.[22] These results established the structure of thiamine bis-chloride in 1936. Syntheses[23] and industrial production[24] were developed.

The high and low m.p. forms of thiamine bis-chloride[25] are the hydrated and anhydrous forms [exp. 1]. Thiamine bis-chloride hydrate occurs in at least three crystalline forms.[26] Thiamine can



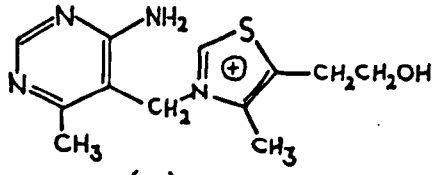
(21)

WILLIAMS (1935)[17]



(22)

WINDAUS et al. (1935)[19]



(23)

MAKINO and IMAI (1936)[20]

FIGURE 11:

be assayed by the thiochrome method.[27]

Auhagen (1932) discovered that carboxylase required a dialysable cofactor.[28] Lohmann and Schuster isolated thiamine pyrophosphate chloride from yeast in 1937, and established its structure.[29] Syntheses of thiamine pyrophosphate were developed.[30] It is hydrolysed to thiamine monophosphate by dilute acid, but this is not biologically active. Reports that thiamine triphosphate[31] is biologically active[32] have been shown to result from thiamine pyrophosphate and phosphatase contamination of the preparations.[33] The enzyme which produces thiamine pyrophosphate from thiamine and A.T.P. is a pyrophosphotransferase.[34] Thiamine pyrophosphate can be assayed enzymically.[35] It forms complexes with scandium and zirconium which are insoluble even in mineral acids.[36]

TABLE 2

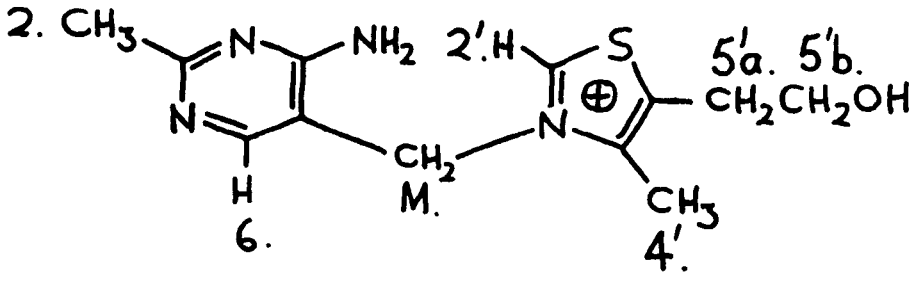
The Infra-red Spectrum of Crystalline Thiamine bis-Chloride Hydrate (H.C.B. and Nujol Mulls)

Origin		$\nu(cm^{-1})$	Character
4-amino ; N-H ; asymmetric stretch	:	3482	s, sh.
; symmetric stretch	:	3418	s, sh.
; internal deformation[40]	:	1664	s, sh.
N_1^+-H ; stretch	:	~2700	s, v.br.
5'-(2"-hydroxyethyl) ; C-O stretch	:	1048	s, sh.
5'-(2"-hydroxyethyl) ; O-H stretch	:	3182	s, br.
& Water O-H in plane	:	1237	s, sh.
deformations	:	1227	s, sh.
C_2'-H & $C_{2'}$-H ; stretch	:	3040	s, br.
Pyrimidine ring vibrations	:	1613	s, sh.
		1591	m, sh.
		1530	s, br.
		990	w, sh.
		790	s, sh.

(s = strong, m = medium, w = weak, sh = sharp
br = broad, v.br = very broad)

TABLE 3

N.M.R. Spectroscopic Data for Thiamine Cations



τ-Values in D_2O at 33°C, D.S.S. internal standard

Salt	2'	6	M	5'b	5'a	2	4'
bis-chloride	0.04	1.70	4.24	6.00	6.70	7.27	7.35
bis-tetrafluoroborate	0.14	1.76	4.29	5.97	6.71	7.28	7.37
pyrophosphate chloride[44]	0.22	1.92	4.35	5.70	6.60	7.31	7.39
oxythiamine bis-chloride	-0.07	1.54	4.34	6.08	6.78	7.16	7.38
thiamine mono-chloride	-	1.68	4.37	5.98	6.71	7.44	7.32

NOTES :

1) The 5'a and 5'b protons are seen as a doublet of triplets, J = 5.6 c/s.

2) At 84°C in D_2O long range coupling is resolved between 6 and M, J = 0.6 c/s. Second order splitting is also resolved in the 5'a and 5'b signals.

3) The identity of the two methyl group signals was established as follows :

   a) At high temperature (84°C) the 7.35 τ signal of thiamine bis-chloride is resolved into a triplet, J = 0.4 Hz, the 5'a proton signal being also broadened, with loss of resolution of the second order splitting which is clearly seen in the 5'b proton signal.

   b) The pyrimidine methyl protons undergo acid-catalysed deuterium exchange [Exp. 6].

   c) The assignation is consistent with the observed effects of pH and structure changes.

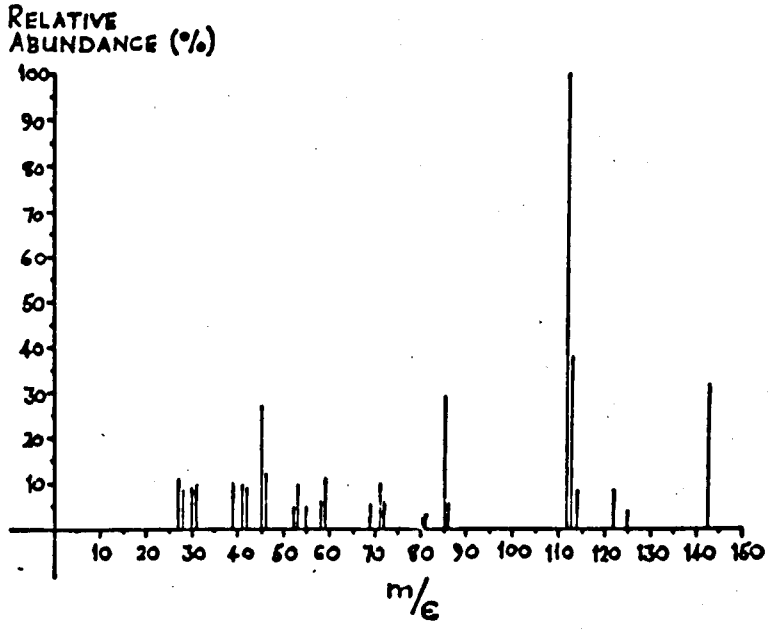
[2] SPECTROSCOPIC STUDIES OF THIAMINE CATIONS

The ultraviolet absorption spectra of thiamine cations[37] are dominated by the strong absorption of the 4-aminopyrimidine group.[38] The spectra of thiamine monocations show two bands : the band at about 267 mμ represents the $n \rightarrow \pi^*$ transition of the 4-aminopyrimidine, and the band at 233 mμ represents the $\pi \rightarrow \pi^*$ transition. On protonation the $n \rightarrow \pi^*$ transition is shifted to shorter wavelengths and appears as a shoulder, while the $\pi \rightarrow \pi^*$ transition is shifted to longer wavelengths (246 mμ). These are characteristic effects in the pyrimidine series.[39]

Only part of the infra-red spectrum of crystalline thiamine bis-chloride hydrate can be interpreted with reasonable certainty (Table 2). A sample recrystallised from D_2O showed a strong band at 2260 cm^{-1}, attributed to a C-D stretching vibration arising from deuterium which had exchanged into the 2'-position.[41] Hydrogen bonding of the 4-amino group in the crystal is nearly symmetrical[46] : the Bellamy-Williams relationship[42] is in error by only 20 cm^{-1}, but Mason's equations[43] give anomalous results for the angle H-N-H.

The proton magnetic resonance spectra of thiamine cations in D_2O are rather simple (Table 3).

The mass-spectrum of thiamine mono-nitrate [exp. 2] (figure 12) shows a small molecular ion at 264, corresponding to $[C_{12}H_{16}N_4OS]^{\cdot +}$, derived from neutral thiamine. Pyrimidine and



MOLECULAR ION: 264 (3·9%).

BASE PEAK: 112.

METASTABLE PEAKS: 89·4 (143 ⟶ 113)

64·6 (112 ⟶ 86)

FIGURE 12: MASS SPECTRUM OF THIAMINE MONO-NITRATE.

thiazole fragments give ions at 122 and 143 respectively. The pyrimidine fragment (24), which may undergo rearrangement to a diazepine structure (25), decomposes to acetonitrile, hydrogen cyanide, and acetylene (figure 13). The base peak at 112 is formed by a type of McLafferty rearrangement of the thiazole-5-β-hydroxyethyl system (figure 14). The product (26) or (27), decomposes to acetonitrile, hydrogen cyanide, acetylene, and a number of small sulphur-containing fragments (figure 14). The mass spectrum of thiamine bis-chloride is similar,[187] but higher temperatures are needed for vaporisation in the instrument, and the molecular ion is not seen [exp. 2].

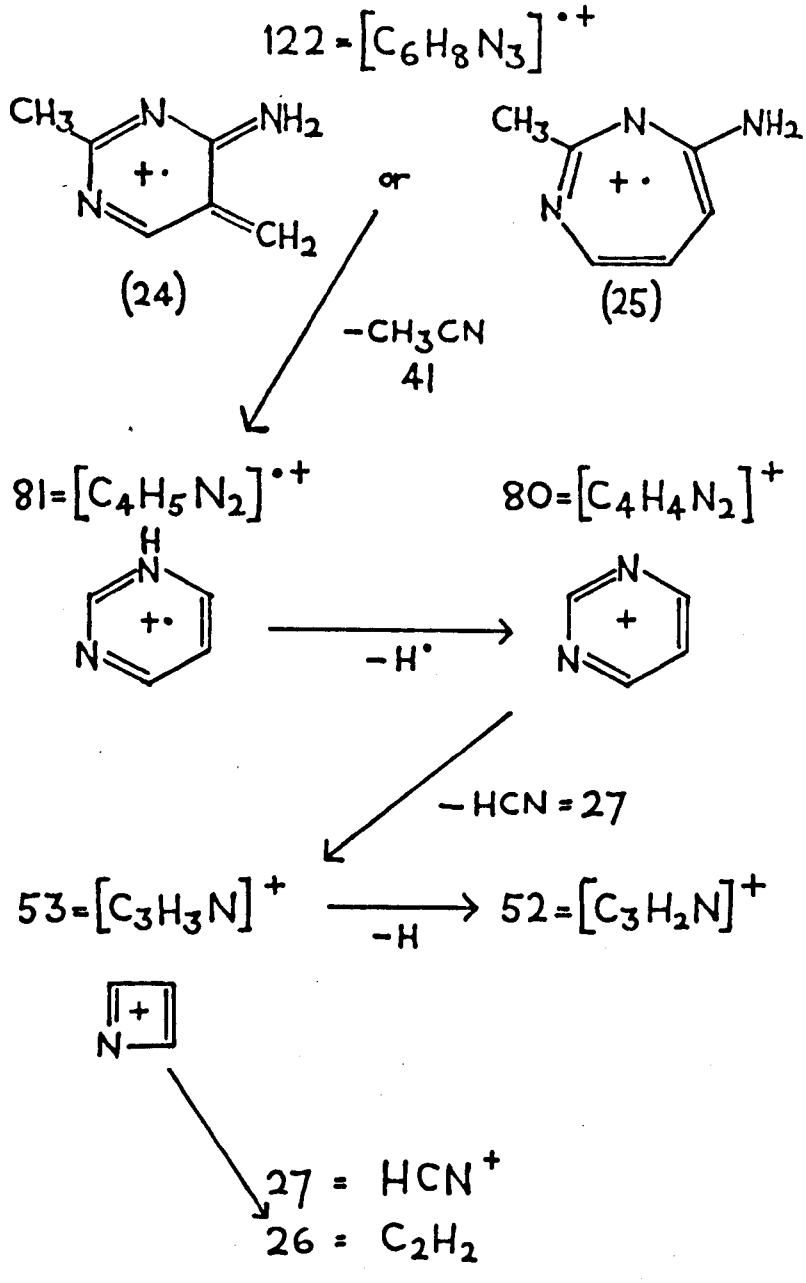


FIGURE 13: MASS SPECTRUM OF THIAMINE MONO-NITRATE:

a) DECOMPOSITION OF THE PYRIMIDINE FRAGMENT.

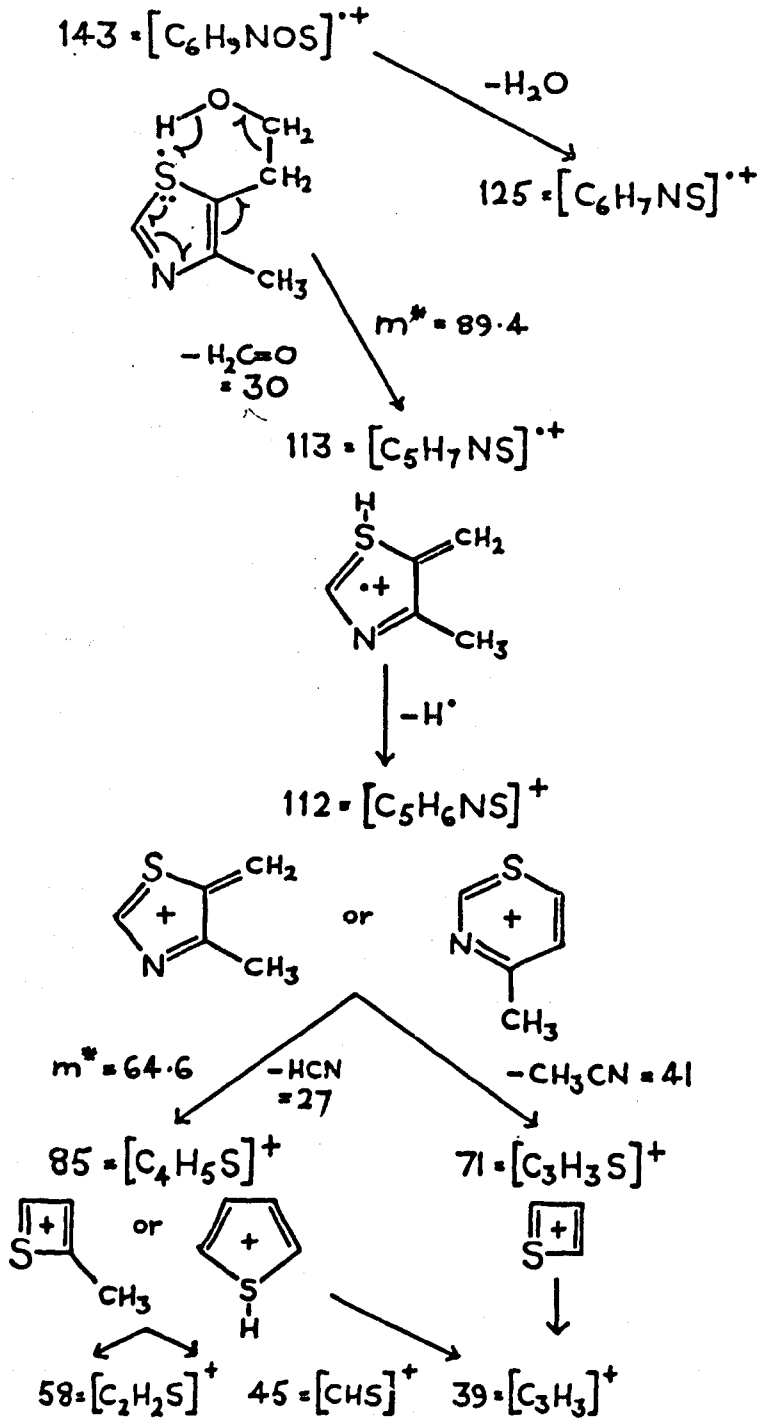


FIGURE 14: MASS SPECTRUM OF THIAMINE MONO-NITRATE:
b) DECOMPOSITION OF THE THIAZOLE FRAGMENT.

[3] THE MOLECULAR CONFORMATION OF THIAMINE CATIONS

X-ray diffraction analyses are available for the crystals of the following thiamine cations :

(a) monoclinic thiamine bis-chloride hydrate[45] ;

(b) triclinic thiamine monophosphate phosphate trihydrate ;

(c) monoclinic thiamine pyrophosphate chloride hemihydrate ;

(d) triclinic thiamine pyrophosphate dihydrate.

Details of the analyses are given in Table 4 :

TABLE 4

Cation	Space group	Reflections measured	Reliability factor %	Dihedral angle	Ref.
(a)	$P2_1/C$	3039	8.0	76^o	(46)
(b)	$P\bar{1}$	2398	13.0	90^o	(47)
(c)	$P2_1/C$	-	12	84^o	(48)
(d)	-	4920	27.9	70^o, 85^o	(49)

The dihedral angles found between the planes of the pyrimidine and thiazolium rings are 90^o or less, turned to approximate the 4-amino-nitrogen and 2'-carbon atoms (figure 1). Examination of molecular models shows that a dihedral angle of between 70^o and 90^o gives least interaction.

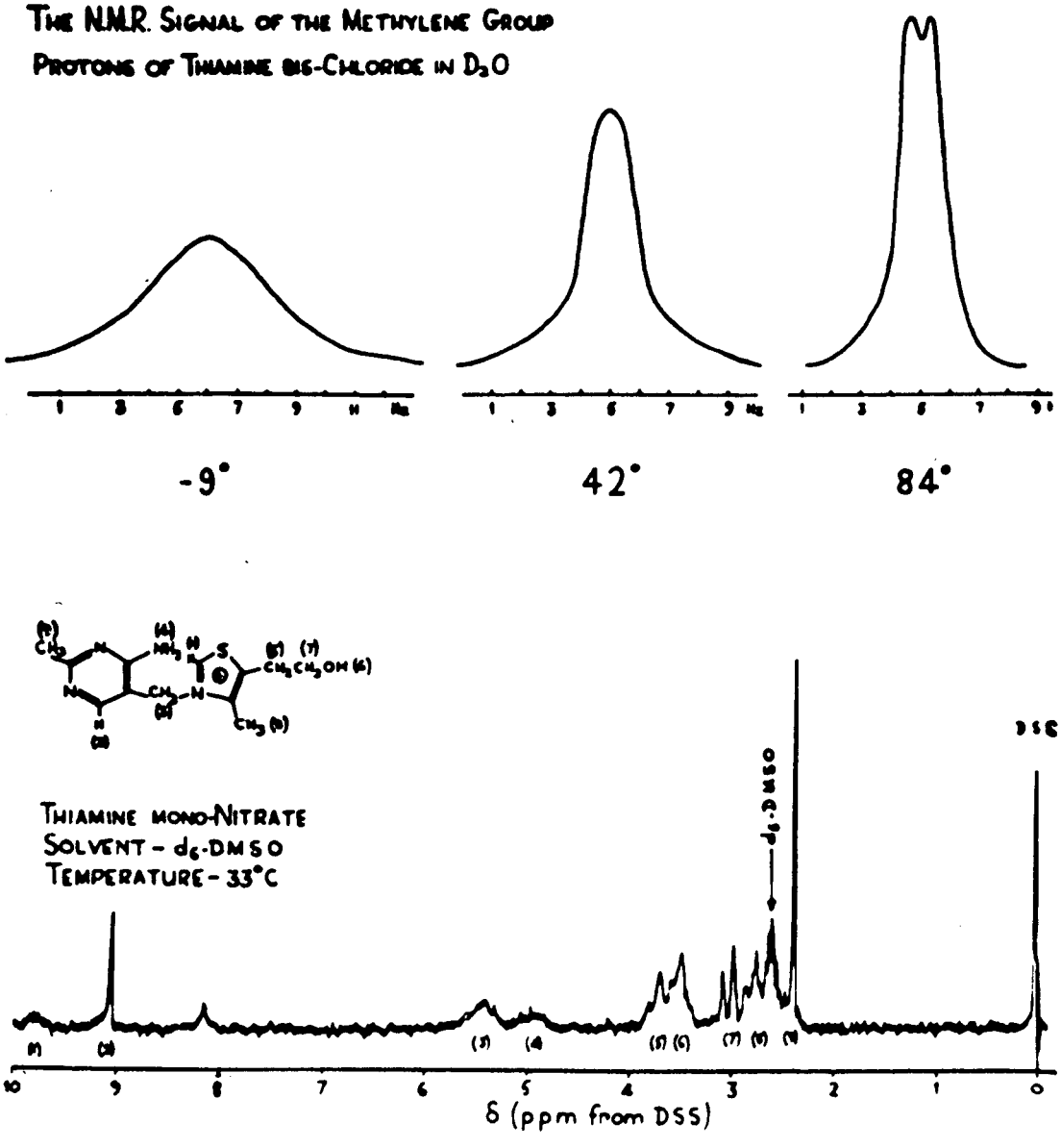

THE N.M.R. SIGNAL OF THE METHYLENE GROUP
PROTONS OF THIAMINE BIS-CHLORIDE IN D_2O
-9°
42°
84°
THIAMINE MONO-NITRATE
SOLVENT - d_6-DMSO
TEMPERATURE - 33°C
d_6-DMSO
DSS
δ (ppm from DSS)

FIGURE 15:

The H-N-H angle of the 4-amino group of thiamine bis-chloride hydrate is $118.4 \pm 10.1^{\circ}$ [46], which agrees with the angle 119.3° calculated from infra-red data for 4-aminopyrimidine in solution.[43] The 4-amino nitrogen atom is therefore probably sp^2 hybridised. Protonation of the 4-aminopyrimidine system occurs on the 1-nitrogen atom.[40,46]

The restricted rotation around the bond between the methylene-carbon and 3'-nitrogen atoms can also be demonstrated by N.M.R. (table 5). The spectrum of thiamine bis-chloride in D_2O shows progressive broadening and loss of resolution as the temperature is lowered [exp. 3].

TABLE 5

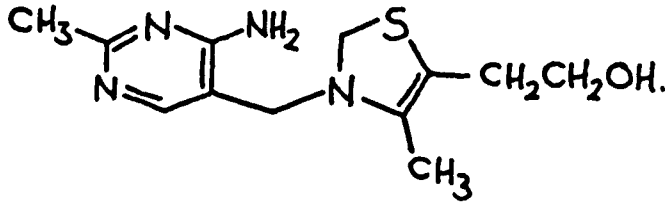
Variable temperature N.M.R. study of thiamine bis-chloride hydrate in D_2O (Varian A.60)

	Bandwidth (c/s)	
Temperature $^{\circ}C$	2-methyl protons	methylene protons
84	0.9	1.8 (d, J = 0.6 c/s)
42	1.0	2.1
-9	2.4	5.0

The spectra of thiamine mono-cations in d_6-D.M.S.O. at 33° show marked broadening of the signals from the 2'-proton, the methylene bridge protons, the 4-amino-protons, and the 4'-methyl-protons (figure 15). The peaks sharpen at higher temperatures, but decomposition occurs before the temperature is high enough to give a fully resolved spectrum ($>100^{\circ}$). This effect is unique to thiamine mono-cations in D.M.S.O. : it is much less marked in thiamine bis-tetrafluoroborate in D.M.S.O., and in thiamine mono-chloride in water.

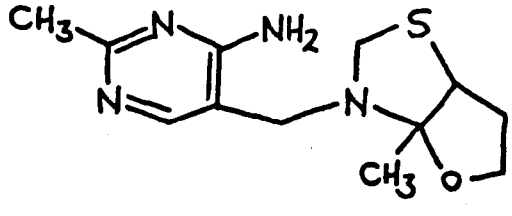
[4] THE REACTIONS OF THIAMINE CATIONS

Lithium aluminium hydride[50] and sodium trimethoxyborohydride[51] reduce thiamine bis-chloride to the 2'-thiazoline, dihydrothiamine, (28), m.p. 151°.



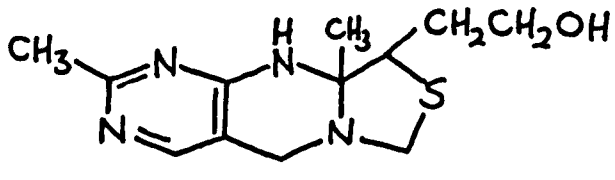
(28)

This forms the isomeric perhydrofurano[2,3d]thiazole derivative, (29), m.p. 175°, in dilute acid or hot water.[51,52]



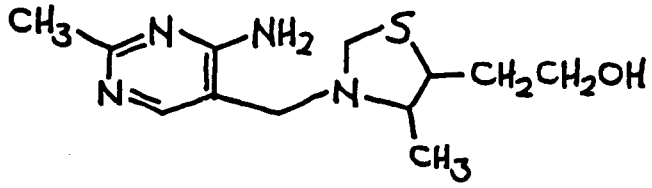
(29)

A third isomer (30) has been reported.[52]



(30)

Sodium borohydride reduces thiamine bis-chloride[51] and dihydrothiamine[53] to the thiazolidine, tetrahydrothiamine (31), m.p. 129 - 131°.



(31)

The N.M.R. spectrum of this material shows that two diastereoisomers are present, one of which can be purified by repeated recrystallisation to a final m.p. of 150 - 150.5°.[54] The relatively simple mass spectrum of tetrahydrothiamine is discussed in [exp.4].

The 5'-2"-hydroxyethyl group can be esterified in strongly acid solution. Thus, hydrobromic acid in glacial acetic acid gives the 5'-2"-bromoethyl bis-bromide, m.p. 234°[30c]; sulphuric acid gives the sulphate ester chloride, m.p. 259-9°[30e]; and phosphoric acid gives a number of phosphate esters.[31] Hydrochloric acid at 150° gives chloro-oxythiamine bis-chloride, dec. 150°.[55] Formic acid yields an O-formyl derivative. Other organic esters of thiamine have been prepared by condensing the appropriate thiazole ester with 4-amino-5-bromomethyl-2-methylpyrimidine.[56] Thiamine bis-chloride is slowly deaminated by nitrous acid.[30c]

Thiamine mono-chloride is not methylated by methyl iodide, instead the insoluble thiamine mono-iodide is formed [exp.5].

Pyridine and other organic bases accelerate the displacement of 5-(2'-hydroxyethyl)-4-methylthiazole from thiamine bis-chloride by bisulphite.[57] The base-exchanged product, N-(4-amino-2-methyl-5-pyrimidinyl)methylpyridine is reconverted to thiamine by incubating

with 5-(2'-hydroxyethyl)-4-methylthiazole and bisulphite. Base exchange reactions with primary and secondary amines, such as aniline, p-aminobenzoic acid, and indole, are not reversible. Similar base-exchange reactions are catalysed by thiaminase enzymes.[58] Thiamine bis-chloride is reductively cleaved by sodium hyposulphite,[59] and is hydrolysed in hot water, yielding 4-amino-5-hydroxymethyl-2-methyl-pyrimidine.[60] An enzyme from bacillus aneurinolyticus catalyses a similar reaction.[58a]

TABLE 6

Enzymes which require Thiamine Pyrophosphate as a Cofactor

(A.1.)	Aldehyde transferases (non-oxidative)	
1.	2-Oxoacid carboxylyase	4.1.1.1.
2.	Benzoylformate carboxylyase	4.1.1.7.
3.	Oxalyl-coenzyme A carboxylyase	4.1.1.8.
4.	Glyoxylate carboxylyase	
(A.2.)	Aldehyde transferases (oxidative)	
1.	Pyruvate : cytochrome-b_1 oxidoreductase	1.2.2.2.
2.	Pyruvate : oxygen oxidoreductase (phosphate acetylating)	1.2.3.3.
3.	Pyruvate : lipoate oxidoreductase (lipoate acetylating)	1.2.4.1.
4.	2-Oxoglutarate : lipoate oxidoreductase (lipoate acetylating)	1.2.4.2.
(B.1.)	1-Glycolaldehyde transferase (non-oxidative)	
1.	D-Sedoheptulose-7-phosphate : D-glyceraldehyde-3-phosphate glycolaldehyde transferase	2.2.1.1.
(B.2)	1-Glycolaldehyde transferase (oxidative)	
1.	D-Xylulose-5-phosphate : D-glyceraldehyde-3-phosphate lyase (phosphate acetylating)	4.1.2.9.

[5] THE ENZYMES WHICH REQUIRE THIAMINE PYROPHOSPHATE AS A COFACTOR (TABLE 6)

2-Oxoacid carboxy-lyase[4,61] (carboxylase, pyruvic decarboxylase) was reported in yeast in 1911,[62] and is widely distributed in plants. It has been purified from yeast[63] or wheat germ.[64] The yeast enzyme has a molecular weight of about 170,000, and consists of two probably identical polypeptide chains of molecular weight 90,000 each (an $\alpha\alpha$ structure).[65] The protein is approximately spherical, with an α-helical content of 20 - 30%. Its amino-acid analysis shows a high content of hydrophobic residues, and low cysteine, proline, serine and threonine values.[66] The enzyme rapidly dissociates into apo-enzyme and cofactors at pH 8, but re-synthesis at the stability pH-optimum of 6.8 requires a large excess of thiamine pyrophosphate and is slow.[4,67] It catalyses the decarboxylation of pyruvate to acetaldehyde, and acetoin is produced if excess acetaldehyde is present.[68] The initial reaction product is carbon dioxide, not carbonic acid or bicarbonate.[69] It will produce acetate by the oxidative decarboxylation of pyruvate if an oxidant such as 2,6-dichlorophenolindophenol is present.[68a] The possible reversibility of the decarboxylation reaction has not been studied. The enzyme is strongly inhibited by heavy metals and sulphydryl reagents.[70] A "two-centre" mechanism has been proposed to explain kinetic and other experiments.[68a,71]

The bacterial enzyme benzoylformate carboxy-lyase decarboxylates benzoylformate and benzaldehyde is released.[72] Oxalyl-coenzyme A carboxy-lyase from a bacterium species[73] or from pseudomonas oxaliticus[74] produces formyl-coenzyme A from oxalyl-coenzyme A. Radioactivity from $^{14}CO_2$ is not incorporated into substrate. Cells of E.Coli[75] or pseudomonas[76] grown on substrates metabolised via glyoxylate contain glyoxylate carboxy-lyase, which produces tartronic semialdehyde from

glyoxylate :

$$2\,CHO\cdot CO_2^{\ominus} + H^{\oplus} \rightarrow CHO\cdot CHOH\cdot CO_2^{\ominus} + CO_2$$

This enzyme contains flavin-adenine-dinucleotide (F.A.D.). Activity is lost if the flavin is reduced, and restored on oxidation in air. It is inhibited by sulphydryl reagents. The initial reaction product is carbon dioxide.[77]

Cells of aerobacter contain an enzyme which catalyses the formation of acetolactate from pyruvate. It has a molecular weight of about 200,000, it is rich in hydrophobic amino-acids, contains four sulphydryl groups per molecule, and binds three molecules of T.P.P. per molecule.[78]

A crystalline flavoprotein from E.Coli catalyses the oxidative decarboxylation of pyruvate to acetate. The enzyme contains dissociable F.A.D., requires thiamine pyrophosphate and magnesium cofactors, and is reoxidised by potassium ferricyanide or a membrane bound cytochrome-b_1 fraction.[79] Another flavoprotein from lactobacillus delbrückii catalyses the production of acetyl-phosphate from pyruvate using oxygen, methylene blue or potassium ferricyanide as oxidants :

$$2\,CH_3COCO_2H + 2\,HOPO_3^{2-} + O_2 \rightarrow 2\,CH_3CO\cdot OPO_3^{2-} + 2\,CO_2 + 2\,H_2O$$

The enzyme does not catalyse exchange of radioactive inorganic phosphate into acetyl-phosphate.[80] A bacterial preparation[81] catalyses the reaction :

$$CH_3COCO_2^{\ominus} + HOPO_3^{2-} \overset{T.P.P.}{\rightleftharpoons} CH_3CO\cdot OPO_3^{2-} + HCO_2^{\ominus} + H^{\oplus}$$

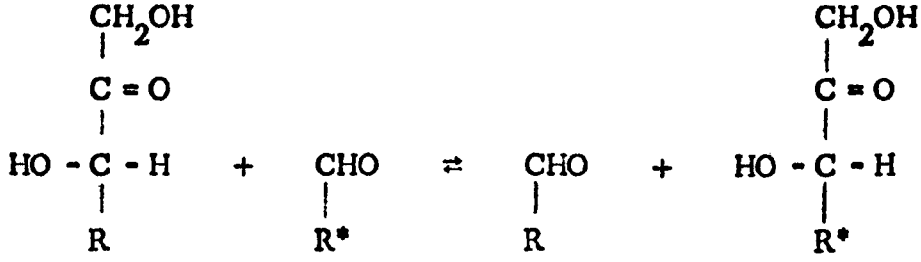
Another, from Clostridium butylicum,[82] catalyses the reaction :

$$CH_3COCO_2^{\ominus} + HOPO_3^{2-} + H^{\oplus} \overset{T.P.P.}{\rightarrow} CH_3CO\cdot OPO_3^{2-} + H_2 + CO_2$$

Pyruvate :lipoate oxidoreductase is a component of the pyruvate dehydrogenase complexes isolated from bacterial or animal tissues.[83] The isolated enzyme from E.Coli has a molecular weight of 183,000 and

can be dissociated into two pairs of chains (an $\alpha\alpha\beta\beta$ structure).[83b] At pH 9.5 a marked conformational change accompanies dimerisation of the entire enzyme.[83] Thiamine pyrophosphate and magnesium ions are the only cofactors. It catalyses the reductive acylation of lipoate,[85] the oxidation of pyruvate to acetate by potassium ferricyanide, and the exchange of radioactivity from $^{14}CO_2$ into pyruvate.[86] 2-Oxoglutarate : lipoate oxidoreductase is the analogous enzyme from the 2-oxoglutarate dehydrogenation complex. It also will catalyse the oxidation of substrate by ferricyanide.[87] The enzymes catalysing the oxidative decarboxylation of the branched chain oxo-acids formed during catabolism of valine, leucine and isoleucine are probably similar.[88]

Transketolase[89] is an enzyme found in all cells examined, which catalyses a glycolaldehyde transfer reaction :



Donor Acceptor

It has been crystallised.[90] All donors have the C_3 and C_4 hydroxyl groups in the trans-configuration. The enzyme requires thiamine pyrophosphate and magnesium ions, and is not inhibited by sulphydryl reagents. The apo-enzyme recombines with thiamine pyrophosphate alone to form an inactive binary complex, which does not easily form the active ternary complex when magnesium is added. Magnesium must be added before or at the same time as thiamine pyrophosphate to restore activity.[91]

The bacterial enzyme phosphoketolase[92] catalyses the oxidative phosphorolysis of xylulose-5-phosphate, fructose-6-phosphate, glycolaldehyde

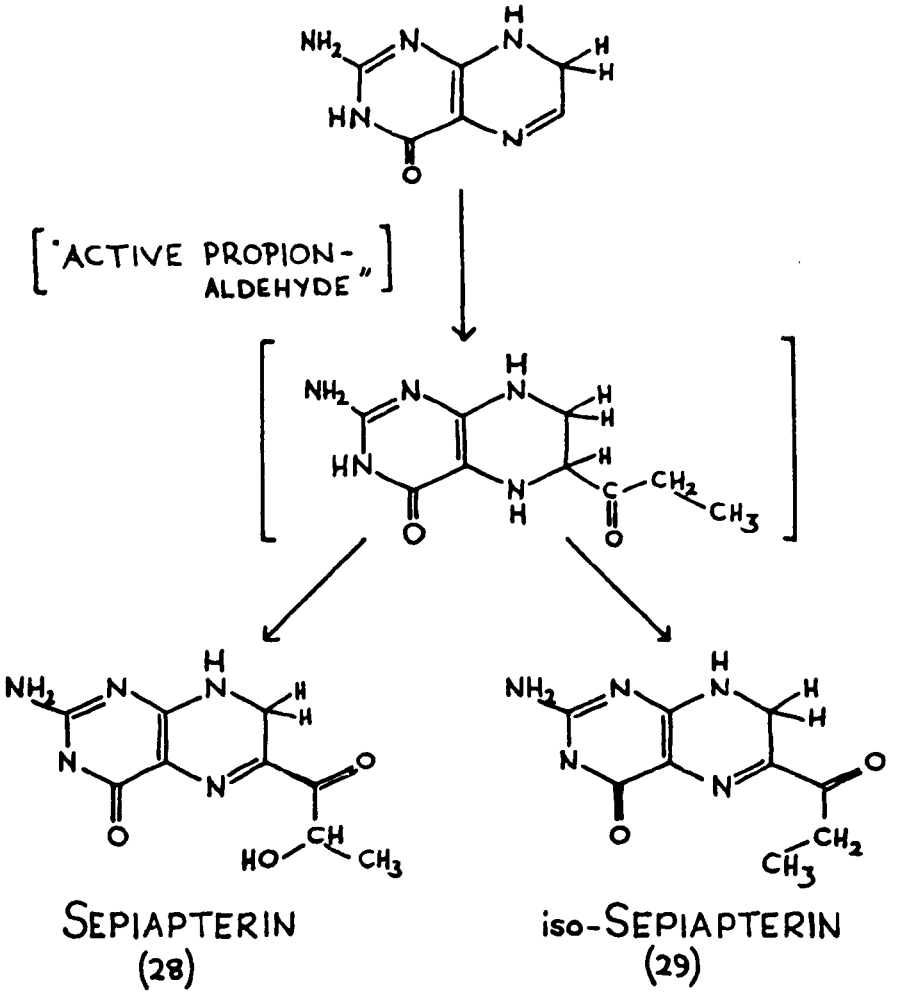
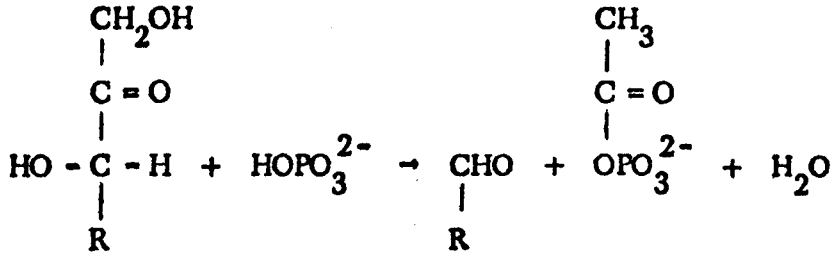


FIGURE 16: THE BIOSYNTHESIS OF SEPIAPTERIN AND iso-SEPIAPTERIN [94].

and hydroxypyruvate. The product is acetyl phosphate :

e.g.



The enzyme requires thiamine pyrophosphate and magnesium ions, and is inhibited by sulphydryl reagents. The reaction is irreversible.

The claim that thiamine pyrophosphate is a cofactor for the dimerisation of farnesyl pyrophosphate requires substantiation.[93]

Thiamine pyrophosphate may be a cofactor for the synthesis of the yellow pigments sepiapterin (28) and iso-sepiapterin (29) found in Drosophila and the blue green alga anacystis indulans (Figure 16).[94] Thiamine pyrophosphate may prove to be a cofactor for the enzymes catalysing the loss of the 17β-side chain from 17α-hydroxyprogesterone, a step in the biosynthesis of androgens and oestrogens.

[6] THE CONCEPT OF "ACTIVE ALDEHYDES"[95]

Three sets of observations led to the suggestions that "active aldehydes" occur as intermediates in metabolism, and that these are aldehyde-thiamine pyrophosphate compounds.

(a) Preparations from E. Coli and pigeon muscle catalysed an exchange reaction between $^{14}CO_2$ and pyruvate, which was dependent on thiamine pyrophosphate, independent of lipoic acid,[96] and insensitive to arsenite.[86] Preparations from pig heart gave similar results for 2-oxoglutarate.[96a]

(b) Preparations requiring thiamine pyrophosphate were obtained from mammalian tissue or E. Coli, which catalysed the production of acyloins from pyruvate.[97] Acyloin synthesis does not require lipoate.[98]

(c) Similar preparations catalysed the oxidative decarboxylation of pyruvate to acetate, using potassium ferricyanide as oxidant.[97,99]

[7] THE REDUCTIVE ACYLATION OF COENZYMES

A coenzyme which acts as a catalyst by undergoing reversible reductive acylation facilitates two biochemical processes, namely oxidation-reduction and group transfer. Lipoic acid may catalyse either or both of these processes, but in the case of thiamine, it is probable that neither process can be catalysed alone. Other coenzymes which have been thought to have only an oxidation-reduction function, may, in addition, catalyse group transfer. 5-Formyl- and 5-acetyl-leuco-flavins have been synthesised,[100] and recently a number of biochemical reactions have been shown to involve 5-acyl or 5-alkyl leuco-flavins.[190] Flavin-adenine-dinucleotide may have a similar function in the glyoxyate carboligase reaction (see [5]), and it is possible that quinones may have a similar rôle in oxidative phosphorylation.[102] Another similar reaction is the formation of addition products by nicotinamide coenzymes.[101]

TABLE 7

Compound	Catalytic Activity in the Model Reaction. % Acetoin Formation from		Coenzyme Activity of the Pyrophosphate
	Acetaldehyde	Pyruvate	(%)
Thiamine (T)	100	100	100
N-methyl-T	165	96	0
N, N-dimethyl-T	281	74	0
4-hydroxy-4-desamino-T	65	15	0
6-methyl-T	-	18	0
2'-methyl-T	0	0	0

[8] MODEL REACTIONS CATALYSED BY THIAMINE

Mizuhara[103] extended Ugai's earlier discovery[104] that thiamine in mildly basic solution catalysed the condensation of furfural to furoin, and showed that a similar system produced acetoin from pyruvate-acetaldehyde and biacetyl-acetaldehyde mixtures. Yields of acyloins were greatest when two equivalents of base per molecule of thiamine bis-chloride were added. Pyruvate and formate gave only acetoin, but pyruvate and formaldehyde gave acetoin and monohydroxyacetone. In the model system, thiamine did not catalyse the exchange of radio-activity from $^{14}CO_2$ into pyruvate.[105]

Metzler found that pyruvate incubated with thiamine at pH 8.9 gave acetolactate, which decarboxylates easily, forming acetoin. After 40 hours at 40°C about one-fifth of the pyruvate added was recovered as acetoin, although nearly all the pyruvate was destroyed. Three-quarters of the thiamine added was recovered. Metal salts had no effect on the reaction. Special care was required to show that the thiamine was acting catalytically: under favourable conditions up to three molecules of acetoin were formed per molecule of thiamine;[106] usually the yield is less than 10% relative to catalyst.

Schellenberger compared the activities of thiamine analogues as catalysts in the model system and as cofactors of apo-pyruvate decarboxylase. There was very little correlation (Table 7).[4]

The model reaction is not catalysed by oxazolium salts, and no interaction of benzaldehyde and oxazolium salts is detectable spectrophotometrically.[107] N,N'-diphenylimidazolium ion is also a poor catalyst, producing the 2-benzoyl derivative with benzaldehyde.[108]

[9] THE EXCHANGE REACTION

The 2'-proton of thiamine and other thiazolium salts undergoes rapid, base-catalysed exchange in D_2O (Breslow,[109] see also Hamill (1937)[110]). The rate is a linear inverse function of the proton concentration, showing no change as the pH of the solution approaches 4.85, the pKa of the 4-aminopyrimidine system. The rate is not increased by yeast pyruvate decarboxylase.[111] Oxythiamine does not show this exchange, but the protons of the pyrimidine 2-methyl group exchange [exp. 6].

The ionisation of hydrogen bound to carbon in such compounds is facilitated by four factors[112] :

(a) high s-character of the C - H bond;

(b) the inductive effect of electronegative atoms linked to the carbon atom;

(c) stabilisation of the carbanion by delocalisation;

(d) a special effect of sulphur linked to the carbon atom.

Overlap of a hydrogen s-orbital with a hybrid carbon orbital which has much s-character produces a bond which is relatively short and which transmits nuclear spin effects efficiently. A short bond implies a high stretching force constant and a high vibration frequency. The C - H stretching frequencies of thiazole and oxazole in the vapour phase are 3140 and 3168 cm^{-1} respectively,[113] and of imidazole in carbon tetrachloride solution is 3120 cm^{-1}.[114] These are high values, intermediate between the frequencies observed for aromatic nitrogen heterocycles and alkynes.

The coupling constant, J (^{13}C - H) is a measure of the degree of

s-character in the C-H bond in the ground state, although the correlation is not exact.[115] The values of $J(^{13}C-2H)$ for 3,4-dimethyloxazolium, 3,4-dimethyl thiazolium, and 1,3,4-trimethylimidazolium iodides in D_2O are 246, 216 and 220 Hz respectively. These are unusually high values : the value for the oxazolium ion is in the range expected for alkynes. However, high s-character as measured by coupling constants is a poor index of acidity. Thus, the exchange of the thiazolium 2-proton is 3000 times faster than the exchange of the imidazolium 2-proton, although the coupling constants are similar. 1,2,3-Thiadiazole shows exchange of the 5-proton but not of the 4-proton, although the coupling constants are almost the same.[116] Exchange of the 5-protons of the three compounds mentioned above is very slow, although the $J(^{13}C-5H)$ values are 224, 202 and 201 Hz respectively. The following observations demonstrate the importance of inductive effects in the mechanism producing the acidity of these systems.[116]

(a) Thiazolium, isothiazolium, oxazolium, thiadiazolium and tetrazolium cations exchange $10^5 - 10^{10}$ times faster than the free bases.

(b) The exchange in phenyl substituted isothiazolium salts is greater than in the methyl substituted analogues.

The chemistry of such systems also shows the importance of inductive effects. Thus, the basicities of imidazoles, thiazoles and oxazoles differ by factors which are about the same as the difference factors for the exchange rates.[117] N-methyl quinaldinic acid betaine decarboxylates much more easily than quinaldinic acid.[118] That other effects are important is shown by the very slow exchange of chloroform[119] and methyl orthoformate,[120] and the lack of exchange of tetramethyl ammonium iodide compared with the relatively rapid base-catalysed

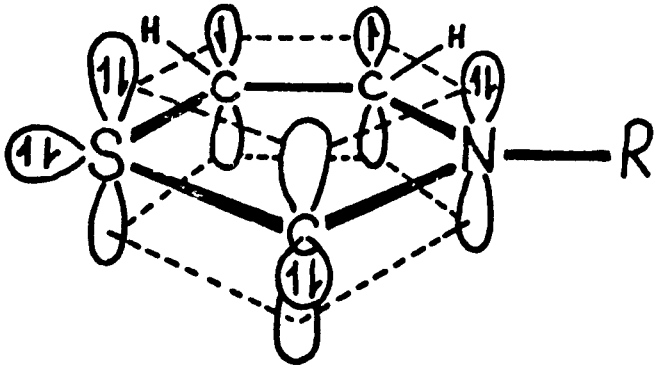
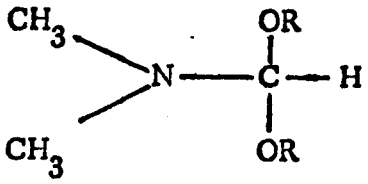


FIGURE 17:

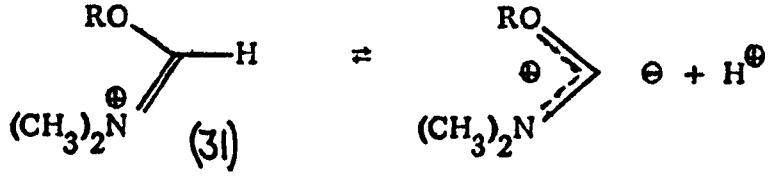
exchange of trimethylsulphonium and tetramethylphosphonium iodides.[121]

Carbon atoms which are doubly bonded to one positively charged heteroatom and singly bonded to a second heteroatom tend to be acidic. Thus, acetals of dimethyl formamide (30) show very rapid exchange



(30)

of the methine proton[120] ($t_{\frac{1}{2}}$ is about 98 minutes in perdeuteromethanol at 0°C). Furthermore, exchange of the alkoxide residues occurs some 175 times faster,[122] and the species which exchanges the methine proton is probably (31).



(31)

A feature common to this and the heterocycles under consideration is the possibility that the carbon atom is hybridised as an sp^2 carbene, with a vacant p_z orbital into which lone pair electrons in p_z orbitals of the heteroatoms can be delocalised. Such an electronic configuration gives a π-system containing 6 electrons in the case of the heterocyclic compounds (figure 17). The suggestion[123] that a true carbene form (32) makes an important contribution to the resonance of these systems

(32)

has been criticised repeatedly,[115] but the formulation given above is not

a true carbene, and accords with the nucleophilic and electrophilic properties of these systems.[124]

The special effect of sulphur in promoting the acidity of an α-carbon atom is attributed to delocalisation of the charge produced by ionisation into 3d orbitals of the sulphur atom.[109,115,125] This idea has been criticised.[109] The rapid exchange of the α-protons of bicyclo[2.2.1.]heptane-1-sulphonium iodide[121] is possibly an illustration that such delocalisation is feasible. The diameter of the sulphur d-orbitals may be reduced by the positive charge of thiazolium systems,[116] which in turn may facilitate the postulated delocalisation.

[10] THE SYNTHESIS, STRUCTURE, AND PROPERTIES OF 2-α-HYDROXYALKYLTHIAZOLIUM SALTS

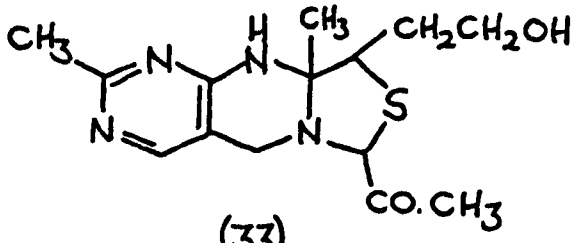
Synthetic 2-α-hydroxyethyl derivatives of 3,4-dimethylthiazolium iodide and 3-benzyl-4-methylthiazolium bromide are more active than the 2-unsubstituted thiazolium salts in catalysing the acetoin condensation. The 3-benzyl salt is more active than the 3-methyl salt but it is less active than thiamine.[126]

2'-α-Hydroxyethylthiamine bis-chloride has been synthesised in three ways :

(a) by the condensation of 2-(α-benzoyloxyethyl)-5-(β-hydroxyethyl)-4-methylthiazole and 4-amino-5-bromomethyl-2-methylpyrimidine ;[127]

(b) from thiamine, by the direct addition of acetaldehyde ;[128]

(c) by the condensation of 4-amino-5-aminomethyl-2-methylpyrimidine, 3-acetyl-3-mercapto-1-propanol and pyruvic aldehyde to give (33) which undergoes



(33)

rearrangement in ethanolic hydrochloric acid to give hydroxyethylthiamine.[129] (-)-2'-α-hydroxyethylthiamine pyrophosphate can be prepared enzymically, or by incubating thiamine pyrophosphate with acetaldehyde at pH 8.8.[130]

The ultraviolet spectra of hydroxyethylthiamine,[127] its pyrophosphate,[130] and thiamine itself are similar. The infrared[129] and N.M.R.[44,130] spectra of hydroxyethylthiamine and its pyrophosphate are consistent with the structure. The 2'-α-proton exchanges in

deuterium oxide, but very slowly when compared with the exchange of the 2'-proton of thiamine,[44] and the exchange is not catalysed by pig heart pyruvate dehydrogenase.[130]

Hydroxyethylthiamine reacts with acetaldehyde at pH 8.8. to give acetoin.[130b] Racemic hydroxyethylthiamine and an A.T.P. generating system restores pyruvate decarboxylating activity to alkali washed yeast as efficiently as thiamine. A soluble carboxylase preparation from yeast (presumably contaminated with thiamine kinase) liberates acetaldehyde from hydroxyethylthiamine.[127]

Hydroxyethylthiamine pyrophosphate is dephosphorylated by prostatic acid phosphatase.[130b] It is cleaved by bisulphite,[130b] and is oxidised by alkaline potassium ferricyanide to give a blue fluorescent material, probably thiochrome pyrophosphate.[131] It is oxidised slowly by 2,6-dichlorophenolindophenol at pH 6.0.[136] It liberates acetaldehyde when incubated with a wheat germ carboxylase, gives acetoin with an enzyme from aerobacter aerogenes, and acetate with the same enzyme and potassium ferricyanide.[132] A crystalline pyruvate oxidase reacts with hydroxyethylthiamine pyrophosphate ; under anaerobic conditions the flavin prosthetic group is reduced.[133] All these enzyme-catalysed reactions can only be demonstrated using substrate quantities of enzyme, perhaps because thiamine pyrophosphate is very tightly bound in the active site. The acetoin formed by the aerobacter enzyme from ^{14}C-hydroxyethylthiamine pyrophosphate is labelled in the acetyl group.[132] A system composed of yeast pyruvate oxidase, liver arylamine acetylase, N.A.D. and coenzyme A produces acetyl-p-nitroaniline from p-nitroaniline and hydroxyethylthiamine pyrophosphate.[134]

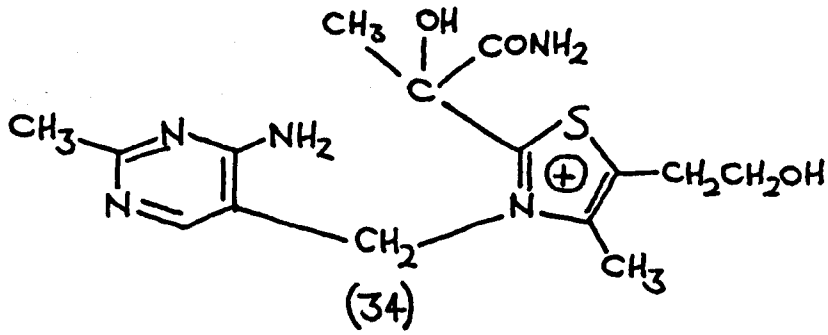
2'-α-Hydroxymethylthiamine pyrophosphate can be prepared by incubating thiamine pyrophosphate with formaldehyde at pH 5 - 6,[135] or from incubation mixtures of pig heart pyruvate oxidase, thiamine pyrophosphate, and glyoxylate.[136] It is cleaved by sulphite, and

reacts with hydroxylamine to give glycolic hydroxamate.[136] It is oxidised in alkali to thiochrome in 15% yield, and at pH 6.0 by 2,6-dichlorophenolindophenol to formate, a reaction which is not accelerated by pyruvate oxidase or glyoxylate carboligase.[136] Glycerate is formed by a system containing hydroxymethylthiamine pyrophosphate, glyoxylate carboligase, glyoxylate, and tartronic semialdehyde reductase.

Incubation of thiamine or thiamine pyrophosphate with formaldehyde at pH 8.8 gives 2-α,β-dihydroxyethylthiamine or its pyrophosphate (glycolaldehyde and hydroxypyruvate do not react).[128,132] Dihydroxyethylthiamine pyrophosphate can be detected in incubation mixtures of transketolase and fructose-6-phosphate,[137] and pig heart pyruvate oxidase and 3-hydroxypyruvate.[138] It is cleaved by sulphite, and reacts with periodate to give formaldehyde.[137] Radioactive sedoheptulose-7-phosphate can be detected if ^{14}C-dihydroxyethylthiamine pyrophosphate is incubated with transketolase and ribose-5-phosphate.[132,137] It is oxidised by pyruvate oxidase and 2,6-dichlorophenolindophenol to give glycolic acid.[138] With substrate quantities of phosphoketolase acetate is formed from dihydroxyethylthiamine pyrophosphate.[139]

2-α-Hydroxybenzylthiamine is prepared by the condensation of thiamine with benzaldehyde at pH 8.8, or by the reaction of 4-amino-5-aminomethyl-2-methylpyrimidine with 3-benzoyl-3-mercapto-1-propanol and pyruvic aldehyde.[140] The 2'-α-proton exchanges in deuterium oxide faster than the 2'-α-proton of hydroxyethylthiamine.[141] The pKa of the first step in the titration curve is greater than the pKa of thiamine bis-chloride.[141] It reacts with acetaldehyde to give benzoyl methyl carbinol.[141]

The amide of the 2'-adduct of pyruvate with thiamine (34) has



(34)

been synthesised, but attempts to prepare the pyruvate adduct itself were unsuccessful.[142]

[11] THE PROPERTIES OF 2-ACYLTHIAZOLIUM SALTS

2-Acylthiamine derivatives are unknown. 2-(1-hydroxyethyl)-3,4-dimethylthiazolium iodide is oxidised to 2-acetyl-3,4-dimethylthiazolium iodide by t-butyl hypochlorite in nitromethane.[143] The ultraviolet and infrared absorption bands arising from the carbonyl group disappear when the compound is dissolved in methanol.[143] 2-Benzoyl-3,4-dimethylthiazolium iodide undergoes rapid hydrolysis and methanolysis.[144] 2-Acetyl-3,4-dimethylthiazolium iodide undergoes nucleophilic attack by water to give acetic acid, by hydroxylamine to give acetylhydroxamic acid, and by n-butyl mercapton and D,L-dihydrolipoamide to give thiol esters.[145] The free energy changes for the methanolysis and hydrolysis of 2-benzoyl-3,4-dimethylthiazolium iodide are approximately -15 k.cal/mole and -22 k.cal/mole respectively.[146]

2-Acetyl-3,4-dimethylthiazolium iodide is in equilibrium with its hydrate, 2-(1,1-dihydroxyethyl)-3,4-dimethylthiazolium iodide, in neutral or acid aqueous solution. It is hydrolysed to acetate and 3,4-dimethylthiazolium iodide in alkali, a reaction specifically catalysed by hydroxide ion. The ratio of the rate-constant of the hydroxide-ion catalysed dehydration of the hydrate to the rate constant of the carbon-carbon bond cleavage reaction is about 10. This compares with a value of 1.4×10^{-3} for the same ratio in the case of diethyl acetylmalonate hydrate. It will not acetylate phosphate, thiols or imidazole in water, although phosphate dianion and thiols do form carbonyl adducts.[147] This work makes the interpretation of some previous studies difficult.[148]

[12] EXTENSIONS OF BRESLOW'S THEORY

The strict requirement for the 4-aminopyrimidine system if thiamine analogues are to be active has been explained in three ways. The first proposal is that the amino group catalyses the exchange reaction.[6] This is unlikely, because

(a) the amino group is not the basic centre of an aminopyrimidine, and

(b) the inverse linear relation of the exchange rate and proton concentration is unchanged as the pH of the solution passes through the pKa of the 4-aminopyrimidine.[111]

The second proposal is that the inductive effect of the 4-aminopyrimidine on the thiazolium ring is an optimum.[126] This is difficult to evaluate, but it might be expected that 3-p-nitrobenzylthiazolium ions would be better catalysts of the model reaction than 3-benzylthiazolium ions. The reverse is found.[106]

The third proposal is that nucleophilic attack by the 4-amino-nitrogen atom on the 2'-α-hydroxyl proton or on the 2'-α-carbon atom facilitates release of product. Hydrogen bonding of the amino group to a group in the active site of an enzyme has been suggested to circumvent objections arising from the low basicity of the 4-amino nitrogen atom.[4] However, the steric possibility of the reaction is doubtful, and the model experiments on which it is based are unconvincing.

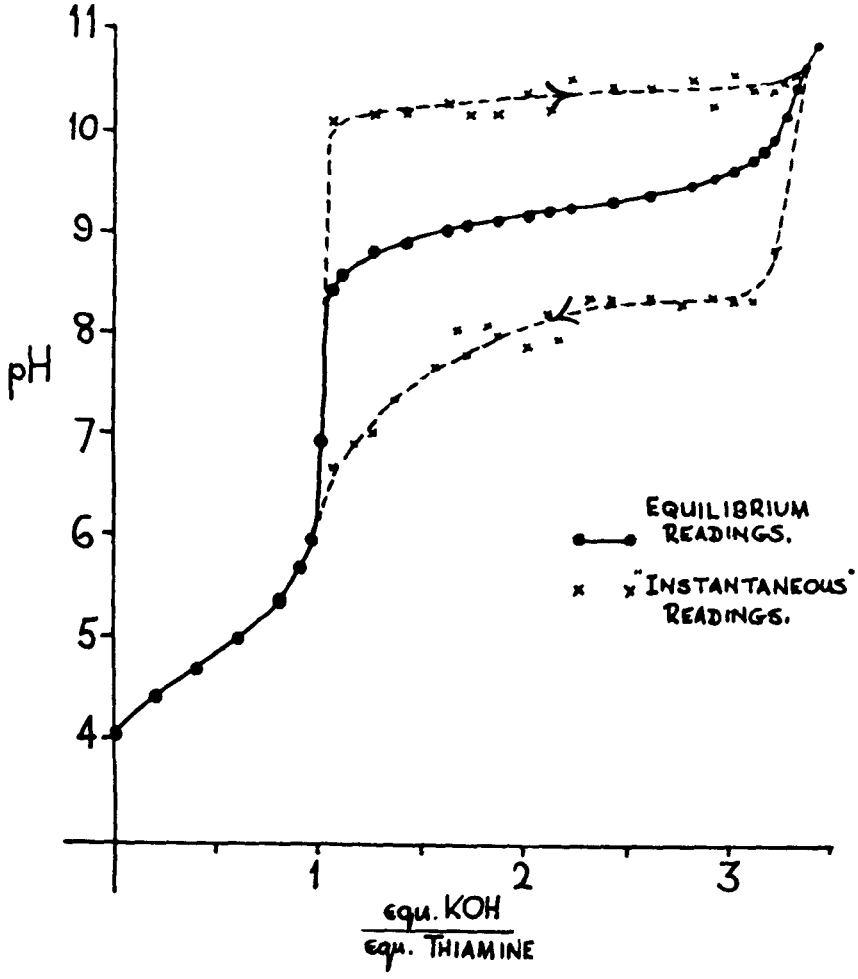


FIGURE 18: TITRATION OF A 0·01 MOLAR THIAMINE bis-CHLORIDE SOLUTION.

[13] THE FORMATION, STRUCTURE AND REACTIONS OF leuco-THIAMINE

The titration curve of thiamine bis-chloride[15] is shown in figure 18. The first, one equivalent, step is the neutralisation of the aminopyrimidine cation. The second, two equivalent, step takes about 30 minutes to reach equilibrium at room temperature. Each addition of alkali produces a yellow solution of high pH, which slowly decays to a colourless solution of lower pH. No colour appears during the back-titration, but a similar pH drift in the reverse direction occurs. These observations are attributed to the initial formation of xantho-thiamine anion, and its subsequent hydration to give leuco-thiamine anion (figure 6). xantho-Thiamine anion is reasonably stable in dry alcoholic solution, but rapid hydration follows the addition of traces of water (figure 19).[149]

leuco-Thiamine was first prepared as a "white sodium salt" by Zima and Williams.[7] The ultraviolet absorption spectrum of leuco-thiamine anion is similar to that of thiamine mono-cation, except that the extinction coefficients are greater, especially in the region of 250 mμ (Table 6), probably because leuco-thiamine has the additional $N-C=C-S^{\ominus}$ chromophore.

TABLE 8

		λ (mμ)	log ε
1. Thiamine chloride :	peak	234.5	4.11
	peak	267	3.94
		250	3.86
2. Sodium leuco-thiamine :	peak	237	4.23
	shoulder	267	4.08
		250	4.14

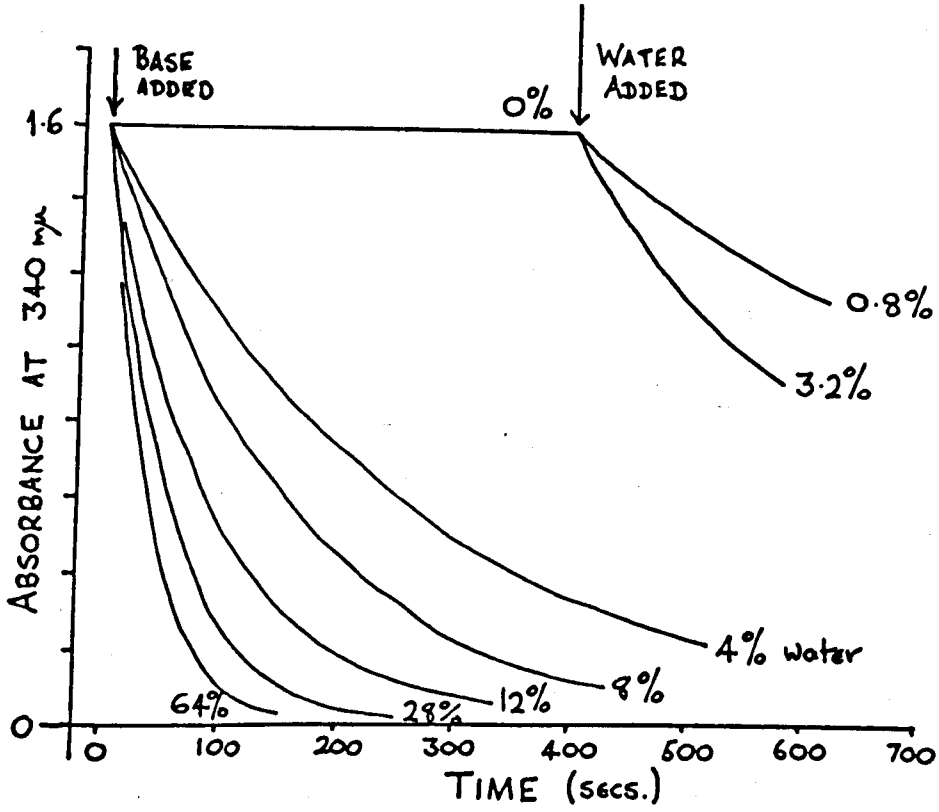


FIGURE 19: THE HYDRATION OF xantho-THIAMINE ANION.

A drop of sodium ethoxide solution was added to a 3 ml. aliquot of thiamine bis-chloride solution in dry ethanol, or in ethanol-water mixtures.

TABLE 9

The Infra-red Spectrum of Sodium leuco-Thiamine (Nujol Mull)

Origin		ν (cm^{-1})	Character
4-amino ; N-H ; asymmetric stretch	:	3420	w.
; symmetric stretch	:	3260	s, sh.
; internal deformation	:	1632	s, sh.
N-CHO ; C=O strech	:	1678	s, sh.
$-CH_2OH$; C-O stretch	:	1037	s, sh.
Pyrimidine ring vibrations	:	~1610	m.
	:	1560	s, sh.
	:	981	m, sh.

(s = strong, m = medium, w = weak, sh = sharp)

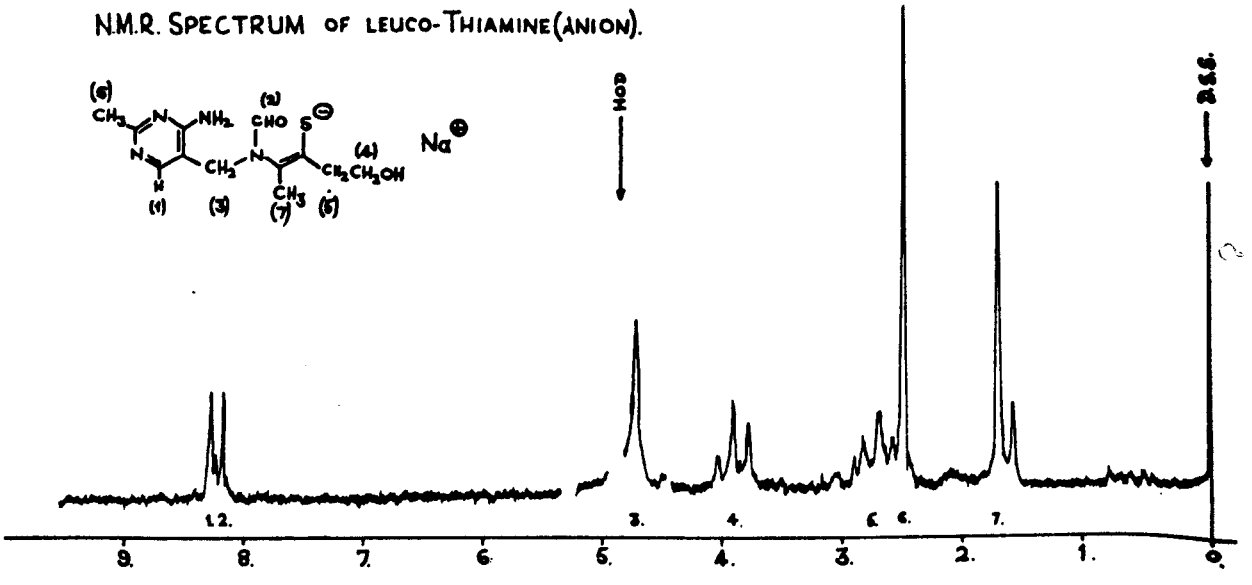

N.M.R. SPECTRUM OF LEUCO-THIAMINE(ANION).
HOD
D.S.S.
1.2.
3.
4.
5.
6.
7.
9.
8.
7.
6.
5.
4.
3.
2.
1.
0.

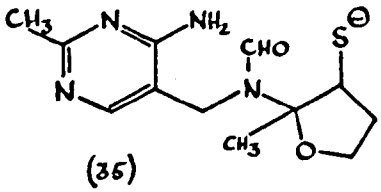
FIGURE 20.

A partial interpretation of the infra-red spectrum of sodium leuco-thiamine is given in table 9. The N.M.R. spectrum of sodium leuco-thiamine (figure 20) shows a constant small peak at 8.43 τ, with some loss of resolution of the signals from the β-hydroxyethyl group. This suggests that in solution sodium leuco-thiamine is in equilibrium with the cyclised tautomer (35). The mass spectrum of sodium leuco-thiamine is discussed in [exp.7].

leuco-Thiamine anion is easily oxidised to thiamine disulphide (36).[7] This gives thiamine thiazolone (37) and thiochrome if heated to reflux in a high boiling solvent, probably by a free radical mechanism.[150] Free radicals may also be intermediates of the reaction of sodium leuco-thiamine with 2,6-di-t-butyl-4-methylphenol and 4-oxo-2,2,6,6-tetra-methylpiperidine-1-oxide in the presence of ferricyanide. The crystalline products (38) and (39) are obtained.[151] The X-ray crystallographic analysis of (39) confirms the structure, which shows two unusual features.[152] The N-S bond is very short (1.715 Å), and the sulphur atom is only 2.90 Å from the N-formyl group, compared with a theoretical minimum distance of 3.5 Å. This is attributed to overlap of the sulphur d-orbitals and the N-formyl π-orbitals.

Mixed disulphides are obtained by oxidising sodium leuco-thiamine in the presence of thiols.[153] Such mixed disulphides undergo exchange reactions if incubated with other thiols.[154]

Sodium leuco-thiamine reacts with benzoyl chloride or acetic anhydride to give O,S-dibenzoyl-leuco-thiamine and O,S-diacetyl-leuco-thiamine respectively. Under carefully controlled conditions, S-acyl derivatives can be prepared.[155] These will transfer the acyl group to solvent or to the β-hydroxyethyl group (figure 8).[156] A number of S-alkyl leuco-thiamine derivatives are known.[153] Cyanogen bromide gives cyanthiamine (40).[157] 4-Nitroquinoline-N-oxide reacts to give



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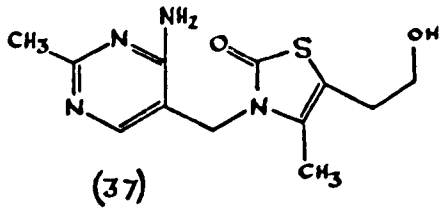

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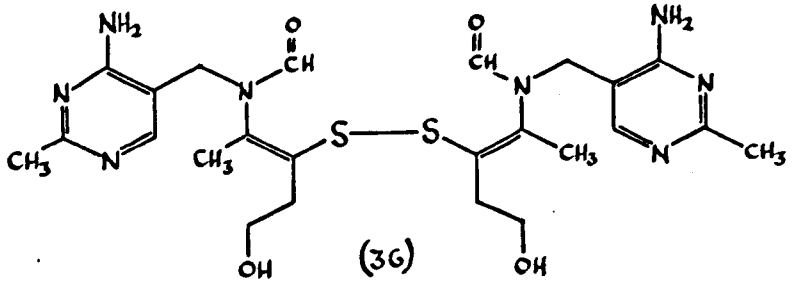

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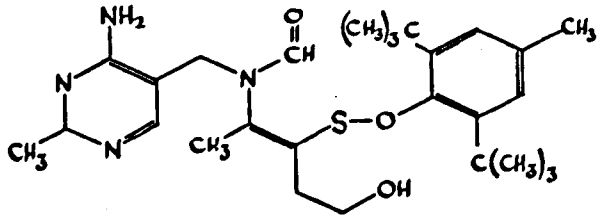

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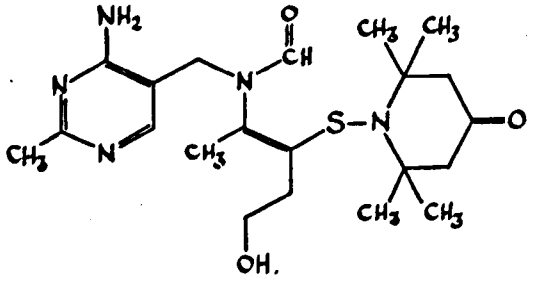
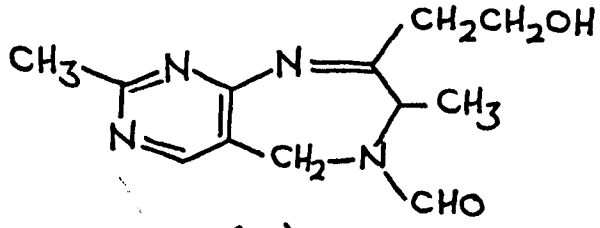


(39)

FIGURE 21.

an adduct formulated as (41).[158]

Solutions of thiamine bis-chloride treated with two equivalents of alkali and then with reagents which react with ketones yield crystalline derivatives of the thione tautomer of leuco-thiamine,[159] such as the oxime (42), the thiosemicarbazone (43), and the azine derivatives (44) and (45). Ammonia and amines react to give the imidazole (46) and imidazolium (47) products respectively [exp. 8]. A similar intramolecular process in strongly alkaline solution may lead to the formation of the 1,4-diazepine derivative (48).



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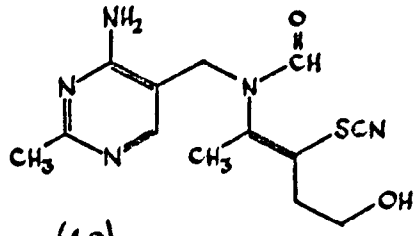

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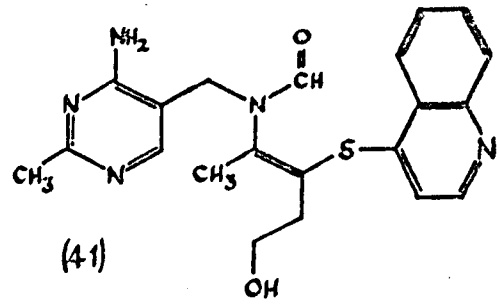

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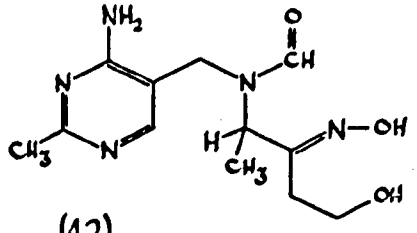

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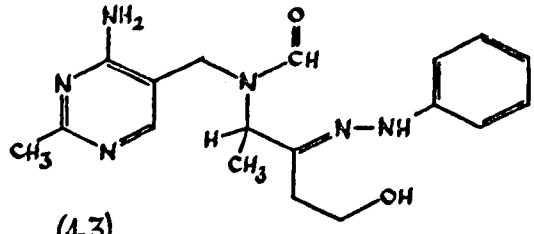

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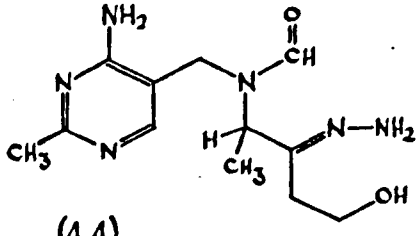

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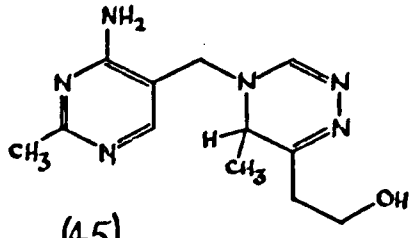

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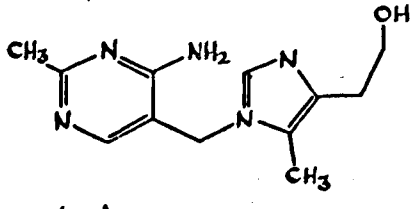

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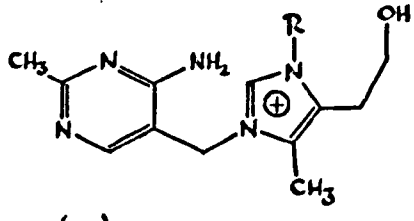


(47)

FIGURE 22.

TABLE 10

The ultraviolet absorption spectra of thiochrome, thiochrome cation, and xantho-thiamine anion

Substance	Solvent	λ (mμ)	ε	Assignment
a) Thiochrome	MeOH	206	6.83 x 10^3	$\pi - \pi^*$
		368	7.00 x 10^3	$n - \pi^*$
b) Thiochrome Cation	MeOH - HCl	205.5	6.70 x 10^3	$\pi - \pi^*$
		350	6.11 x 10^3	$n - \pi^*$
c) xantho-Thiamine Anion	MeOH - KOH	226	12.1 x 10^3	$\pi - \pi^*$
		250	10.4 x 10^3	$N-C=C-S^{\ominus}$
		333	6.07 x 10^3	$n - \pi^*$

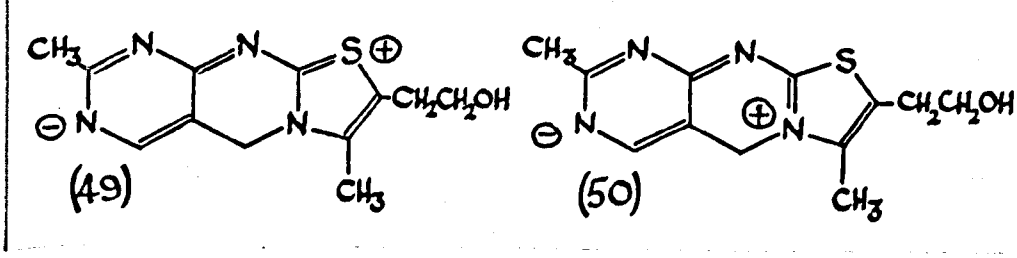
[14] THIOCHROME

Compounds which have a blue fluorescence in ultraviolet light are produced by the oxidation of thiamine in alkaline solution.[160] A yellow coloured, blue fluorescent pigment from yeast, molecular formula $C_{12}H_{14}N_4OS$, was named thiochrome.[161] The same compound was prepared by oxidising thiamine bis-chloride with alkaline ferricyanide. It lacked both a quaternary nitrogen atom and an amino group.[162] The elucidation of the structure of thiamine bis-cation suggested a possible structure for thiochrome, which was proven by synthesis.[163] Thiochrome has no biological activity.

Thiochrome is the oxidation product of xantho-thiamine. It is formed when leuco-thiamine disulphide is heated to reflux in high boiling solvents. Thiamine 2'-thiazolone is a second product : this will not undergo intramolecular condensation to form thiochrome.[150]

Thiochrome is a stronger base than thiamine mono-cation, its pKa is 6.2.[164]

Table 10 gives details of the ultraviolet absorption spectra of thiochrome, thiochrome cation, and xantho-thiamine anion. The band attributed to an $n \rightarrow \pi^*$ transition of thiochrome is asymmetrical, tailing towards shorter wavelengths. Two structures, (49) and (50), may be important in the excited state.



Protonation causes a hypsochromic shift of this transition, but does not affect the band attributed to a $\pi \rightarrow \pi^*$ transition. The spectrum of xantho-thiamine anion differs in three ways :

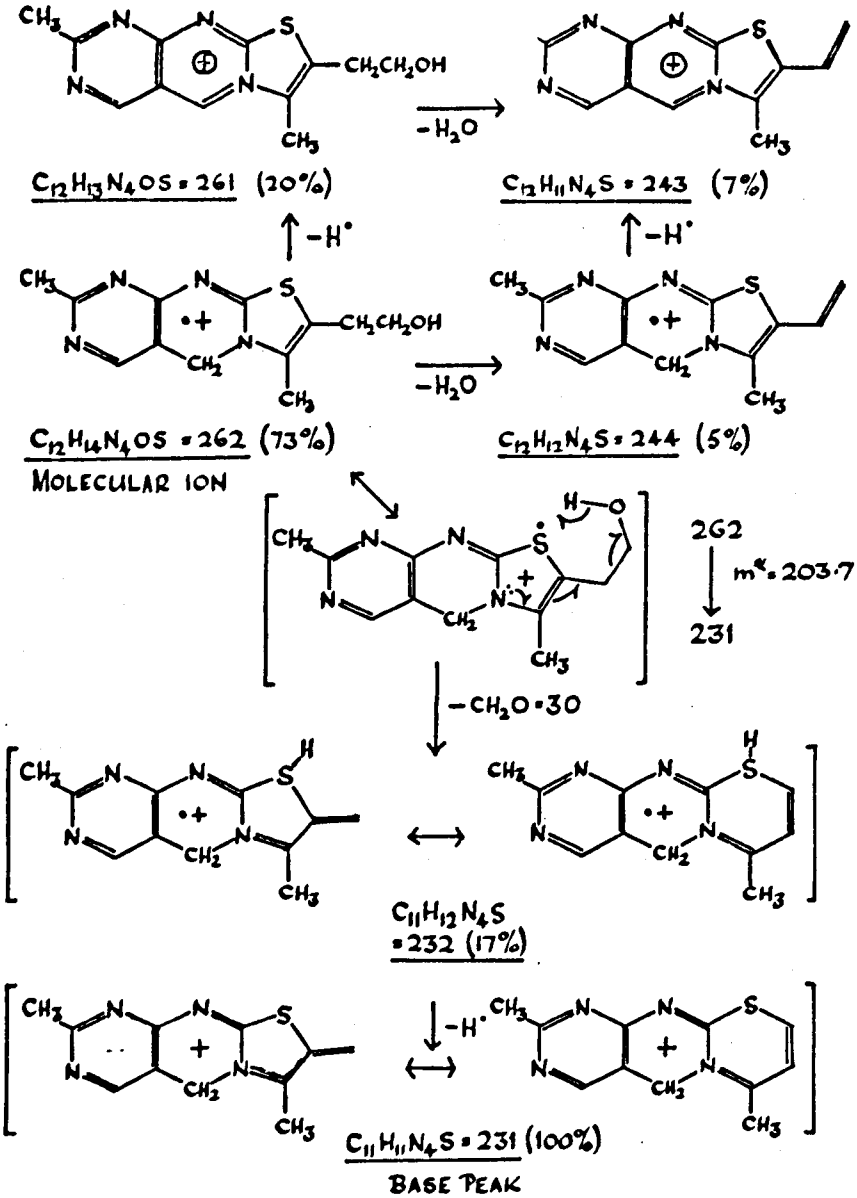
(a) the $n \rightarrow \pi^*$ band is at a shorter wavelength, and is symmetrical ;

(b) the $\pi \rightarrow \pi^*$ band is at a longer wavelength ;

(c) there is an additional band seen as a shoulder at about 250 mμ, attributed to the $N-C=C-S^{\ominus}$ chromophore.

The planar, rigid, tricyclic thiochrome molecule is fluorescent, the bicyclic xantho-thiamine anion is not. Thiochrome fluorescence is increased in n-butanol solution.: this is presumably a viscosity effect.

An interpretation of the mass spectrum of thiochrome is shown in figure 23 [exp.9].



204 = $C_{10}H_{10}N_3S$ (5%)
($m^* = 190.1$)
190 = $C_9H_8N_3S$
173 = $C_9H_9N_4$
147 = $C_7H_7N_4$

FIGURE 23:
THE MASS SPECTRUM
OF
THIOCHROME

[15] DIHYDROTHIOCHROME (4)

Thiochrome can be reduced catalytically, or with sodium hydrosulphide, but the products have not been isolated.[164] Dihydrothiochrome is probably oxidised rapidly in air.[150] The reported preparation of dihydrothiochrome by treating thiamine bis-chloride with two equivalents of sodium ethoxide in ethanol needs confirmation. This product could be a thiamine mono-cation : leuco-thiamine anion salt.[165]

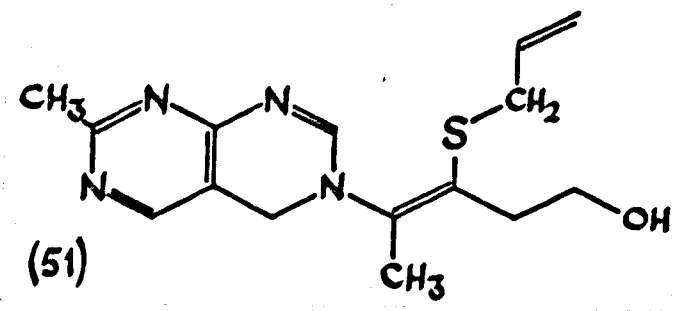
[16] THE STRUCTURE OF xantho-THIAMINE ANION

xantho-Thiamine anion could exist in a stabilised tricyclic form (12) (Figure 6), similar to the known anions of pteridines.[166] However, the bicyclic structure ($5^{\ominus}$) is more likely, because :

(a) the ultraviolet absorption spectrum of the anion shows strong absorption near 250 mμ, attributed to the N-C=C-$S^{\ominus}$ chromophoric system (Table 10);

(b) this band is much diminished on alkylation, and the spectrum of the non-fluorescent product resembles that of thiochrome;

(c) the spectroscopic properties of S-allyl xantho-thiamine are consistent with the structure (51) [exp.10].



(51)

In the pteridine series, a sulphur anion is similarly expelled following the formation of a nuclear anion.[166]

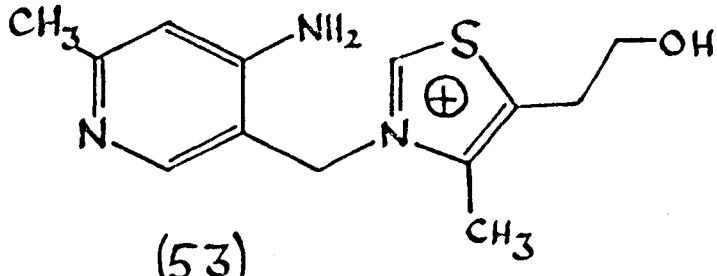
[17] THE ASSOCIATION OF T.P.P., apo-ENZYME, AND MAGNESIUM IONS

The following observations suggest that the magnesium ion is enclosed by thiamine pyrophosphate and groups in the active site of the enzyme.

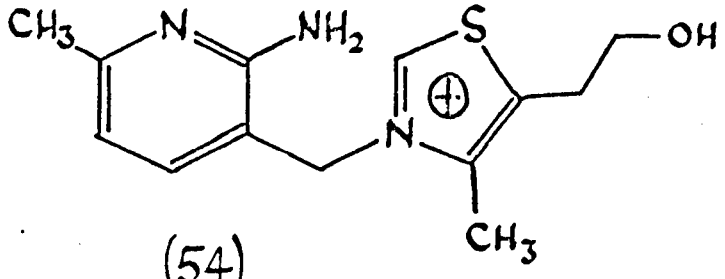
1) Stable, active holo-pyruvate decarboxylase from yeast is only formed if all three components are present (apo-enzyme, T.P.P. and magnesium). The holo-enzyme will not dissociate if treated with E.D.T.A.[167]

2) apo-Transketolase and T.P.P. combine, but the binary complex will not subsequently bind magnesium to form active holo-transketolase. Magnesium must be added before or with T.P.P. if active holo-enzyme is to be formed.[168]

The N - 1 pyridine analogue of thiamine (52) forms an active tertiary complex with apo-pyruvate decarboxylase, but the N - 3 analogue (53) does not.[169] Therefore, it is possible that the pyrimidine 1-nitrogen atom of thiamine participates in the binding of magnesium. Other divalent metal ions will form tertiary complexes with T.P.P. and apo-pyruvate-decarboxylase. These have the same steady state activity as the magnesium complex, but are less stable.[170] Kinetic experiments suggest that the pyrophosphate group of T.P.P. is bound to apo-pyruvate-decarboxylase by an ionic mechanism which does not involve metal ions.[167]



(53)



(54)

FIGURE 24.

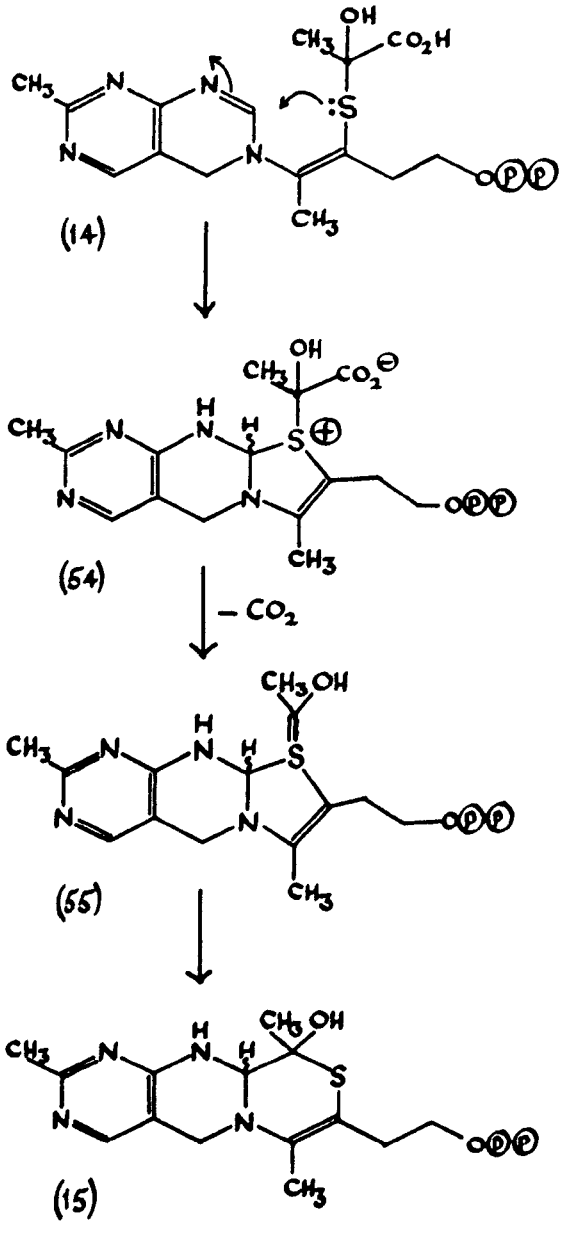


FIGURE 25.

[18] THE DECARBOXYLATION OF PYRUVIC ACID

The non-oxidative decarboxylation of pyruvic acid to give acetaldehyde can be effected by the use of 10% sulphuric acid at 150°,[171] or by finely powdered osmium, palladium or rhuthenium at 100°.[172] Ultraviolet light causes the production of acetoin from pyruvic acid.[173] Acetic acid is produced by the oxidative decarboxylation of pyruvic acid by air or oxygen at room temperature, and the reaction is catalysed by activated charcoal or copper powder.[174] Hydrogen peroxide reacts directly.[175] The mechanisms of these reactions are not known in detail, and no indications of a possible mechanism of biochemical decarboxylation emerge from a survey of the literature on this subject.

In general, the presence of a group which can accept electrons in a β-position relative to a carboxyl group facilitates decarboxylation. Thus, carbon dioxide is easily obtained from β-unsaturated and β-keto acids, from pyridine 2-carboxylic acids at neutral pH, and from the N-alkyl betaines of pyridine and quinoline 2-carboxylic acids.[176]

In figure 25 is shown a possible mechanism for the decarboxylation of an initial addition product of pyruvic acid and xantho-thiamine (14). The formation of a sulphonium system β to the carboxyl group (54) facilitates decarboxylation, with subsequent expansion of the valency electron shell of the sulphur atom (55).[177] Rearrangement of (55) gives the proposed intermediate (15).

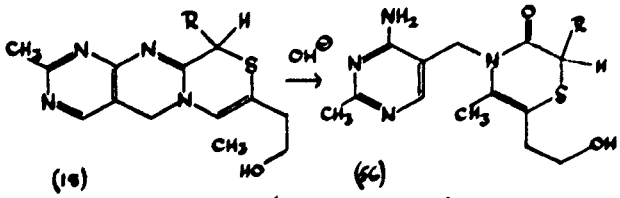
[19] THE ACTIVE SITE OF YEAST PYRUVATE DECARBOXYLASE

The active site is a hydrophobic, slit-like cavity containing tryptophan, as attested by the following observations :

a) The fluorescence of tryptophan residues in the apo-enzyme is diminished when T.P.P. is bound.[178] Indole derivatives, including tryptophan, form 1 :1 complexes with thiamine mono-cation.[179]

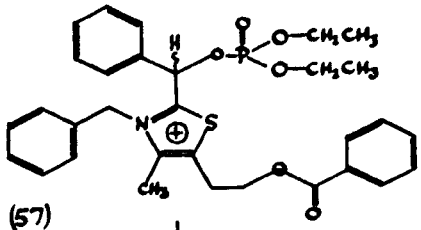
b) The active site binds 2-p-toluidinylnaphthalene-6-sulphonate, which is displaced by a mixture of T.P.P. and magnesium ions. The fluorescence of the bound dye indicates that it is in a hydrophobic environment.[178]

c) The apo-enzyme binds thiochrome pyrophosphate in the presence of magnesium ions. The thiochrome fluorescence increases, in a manner resembling the change of thiochrome fluorescence in non-polar solvents. Thiochrome pyrophosphate is a competitive inhibitor with T.P.P. in this system.[180]

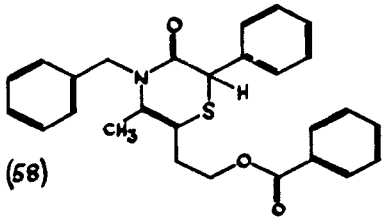


(56)

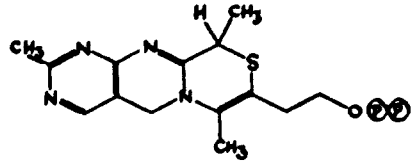
(R = C_6H_6, CH_3, etc.)



(57)



(58)



(59)

FIGURE 26.

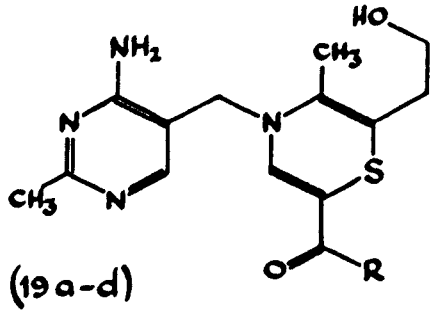
[20] THE REACTION OF THIAMINE WITH ACYL PHOSPHONATES

Thiamine bis-chloride hydrate suspended in dry D.M.F. is first treated with three equivalents of triethylamine, so xantho-thiamine anion is likely to be the species of thiamine which reacts with the acyl-diethyl-phosphonate subsequently added. The crystalline products were identified by analytical and spectroscopic means as 1-alkyl or 1-phenyl derivatives of 4,9-dimethyl-3-(2'-hydroxyethyl)-1,6-dihydropyrimido [4,5-4',5']-pyrimido [2,3-c]-1,4-thiazine (18).[181] The mass spectrum of the 1-phenyl derivative is consistent with this structure [exp. 11]. In aqueous alkali these compounds are hydrolysed to derivatives of 2-keto-1,4-thiazine (56).[181]

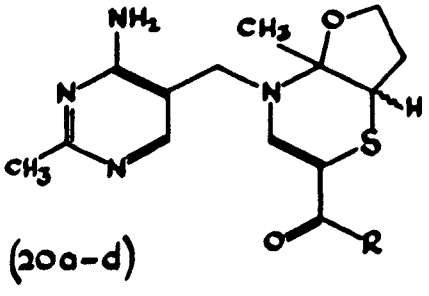
Benzoyldiethylphosphonate reacts with 3-benzyl-4-methyl-5-(2'-benzoyloxyethyl) thiazolium halides in the presence of two equivalents of triethylamine. The elemental analysis and N.M.R. spectrum of the product is consistent with the structure proposed (57). Sodium ethoxide reacts with this compound to give 3-benzyl-4-methyl-5-(2'-benzoyloxyethyl)-1,4-thiazin-2-one (58).[182] The yields are poor, however, and chromatography on alumina has to be used to purify the product. This work needs confirmation.

It is claimed that deuteroamino-O-acetyl-thiamine bis-chloride reacts with benzoyl diethyl phosphate to give a product containing deuterium in the 1-position.[183]

A consequence of the proposed mechanism of action of thiamine is that the pyrophosphate of the 1-methyl compound (59) should be a powerful competitive inhibitor of pyruvate decarboxylase.



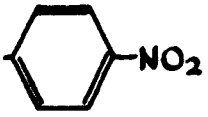
(19a-d)



(20a-d)

a: R = $-CH_3$

b: R = 

c: R = 

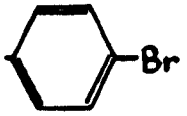
d: R = 

FIGURE 27.

[21] THE REACTION OF THIAMINE WITH α-HALOKETONES

The reaction of thiamine in basic alcohol solution with haloketones of the general type $R.CO.CH_2X$ produces a blood-red colour. Oxythiamine and haloketones which are further substituted on the α-carbon atom do not react in this manner, but haloacetic esters and bromopyruvic acid react slowly [exp. 12]. A number of the red products have been purified by column chromatography and isolated as amorphous powders of indefinite melting point [exp. 12]. If the red solution is made acid the colour becomes yellow in about 30 minutes and crystalline products can be isolated [exp. 13].

The spectroscopic studies which follow suggest that the red compounds are 2-acyl derivatives of 4-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl-6-(2'-hydroxy)ethyl-5-methyl-1,4-thiazine (19), which undergo cyclisation in acid to become 2-acyl derivatives of the acid salt of 4-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl-6H, 7H, 8H-9-methylfuro [3,2b]-1,4-thiazine (20). The conclusions of an earlier study of this reaction[184] are probably incorrect.

The ultraviolet spectra of the products of the thiamine-chloroacetone reaction are shown in figure 28. The red compound gives peaks at 231 mμ and 276 mμ, which change immediately (on adding acid) to a peak at 244 mμ and a shoulder at 265 mμ. This suggests that the 4-aminopyrimidine group is still present. The colour of the red compound is attributed to the very broad absorption peak, of low extinction coefficient, centred on 445 mμ. This peak is thought to arise from a process of intramolecular charge transfer between the 1,4-thiazine as donor and the 4-aminopyrimidine as acceptor, by analogy with other systems which have donor and acceptor groups joined by a methylene bridge.[185] The spectroscopic parameters of this peak are not significantly altered by concentration changes. It

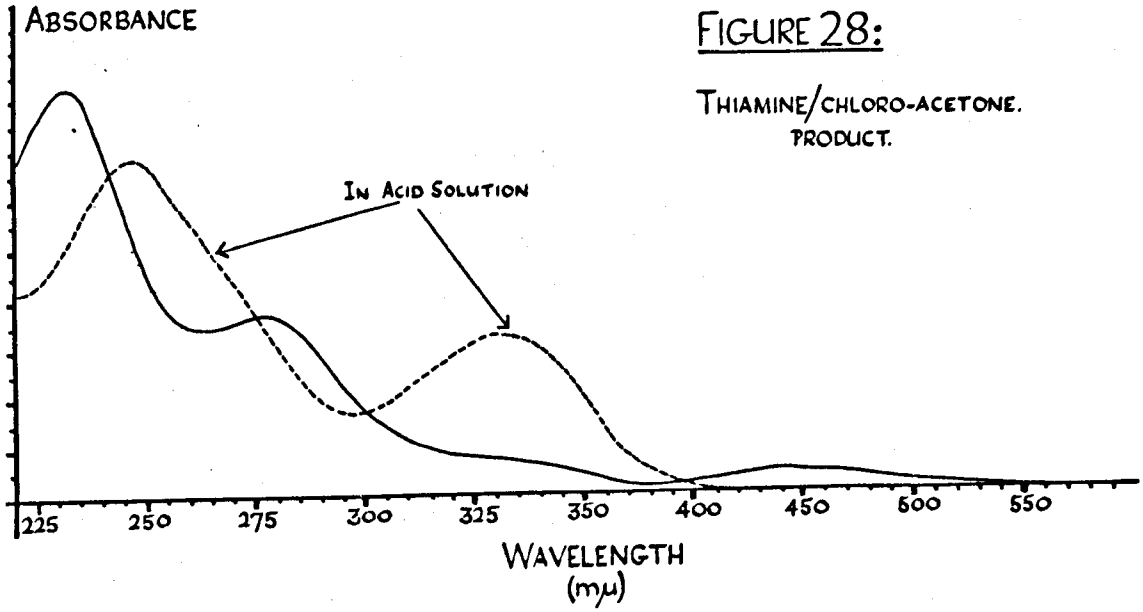
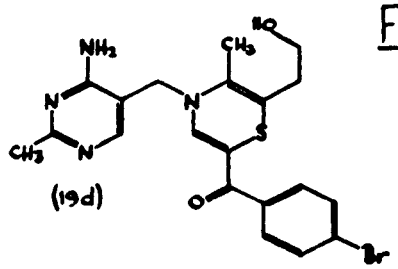


FIGURE 28:

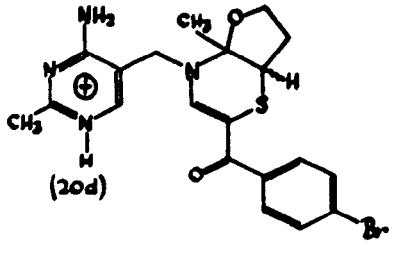
THIAMINE/CHLORO-ACETONE. PRODUCT.

FIGURE 29



(19d)

SOLVENT:
d_6-D.M.S.O.
containing a
little D_2O.



(20d)

SOLVENT:
d_6-D.M.S.O
containing a
little D_2O and
trifluoracetic acid.

slowly disappears on adding acid, while a shoulder at 325 mμ increases to become a peak at 331 mμ. This peak is attributed to the -N-C=C-C=O system in the molecule, by analogy with the spectra of β-amino-αβ-unsaturated ketones.[186] In the red compound the properties of this chromophore are thought to be perturbed by the process of charge transfer.

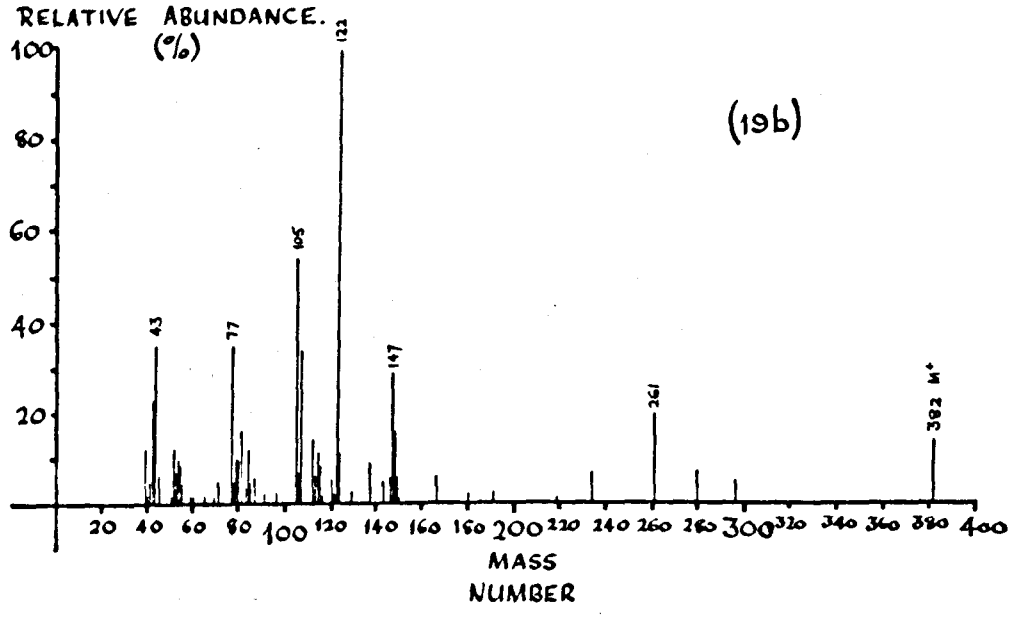
The infrared spectra of these compounds are complex, but all show bands in the regions 3540 - 3300 cm^{-1} and 1700 - 1650 cm^{-1} indicating that the 4-aminopyrimidine group is intact. The carbonyl absorption band is not easily defined, but a sample of (20b) recrystallised from D_2O had a sharp band at 1655 cm^{-1}, and bands in this region can be seen in most of the other compounds. The red compounds all show a strong band at about 1060 cm^{-1}, attributed to the C-O stretching vibration of the hydroxyethyl side chain. In the yellow compounds this is shifted to about 1100 cm^{-1}.

The red compounds generally gave poorly resolved N.M.R. spectra, but (19d) was an exception. The spectrum is shown in figure 29, and the spectrum 30 minutes after adding a little trifluoracetic acid is shown underneath. Of particular significance is the change in the signals from the hydroxyethyl group. These appear as a doublet of triplets in the red compound, and as a poorly resolved doublet of multiplets in the yellow compound. The upfield shift of the singlet arising from the 5-methyl group is also significant.

High resolution mass spectrometry confirms the molecular formulae of these compounds. The mass spectra (figure 30) are consistent with the structures proposed. The spectra of (19b) and (20b) are interpreted in figures 31 and 32.

Some attempts were made to acylate the red compounds [exp. 12]. The products were oils, but their infrared spectra showed additional bands in the 1750 - 1700 cm^{-1} region, suggesting that esterification had occurred.

(20b) did not form a crystalline oxime, phenylhydrazone or semicarbazone, nor was it reduced at room temperature by sodium borohydride.


RELATIVE ABUNDANCE.
(%)
100
80
60
40
20
(19b)
43
77
105
122
147
261
382 M+
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400
MASS
NUMBER

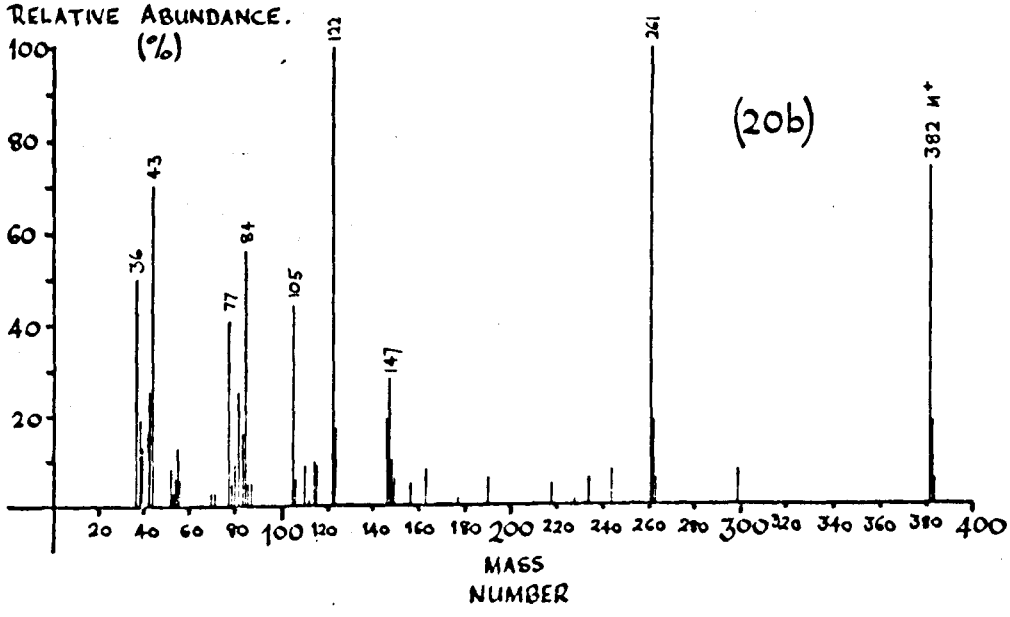
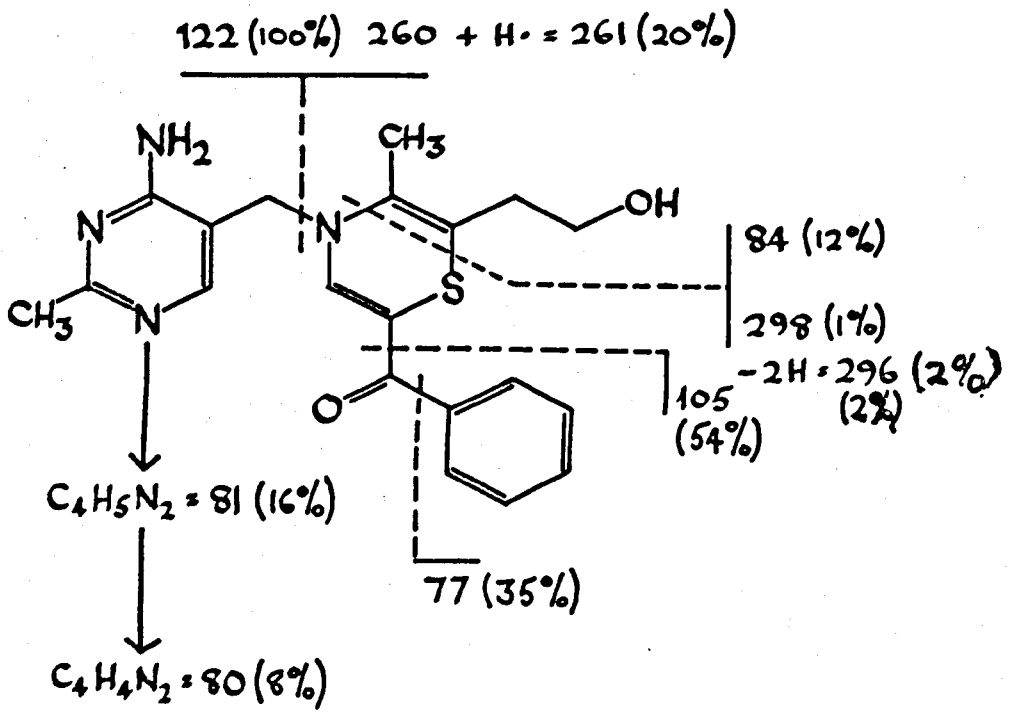
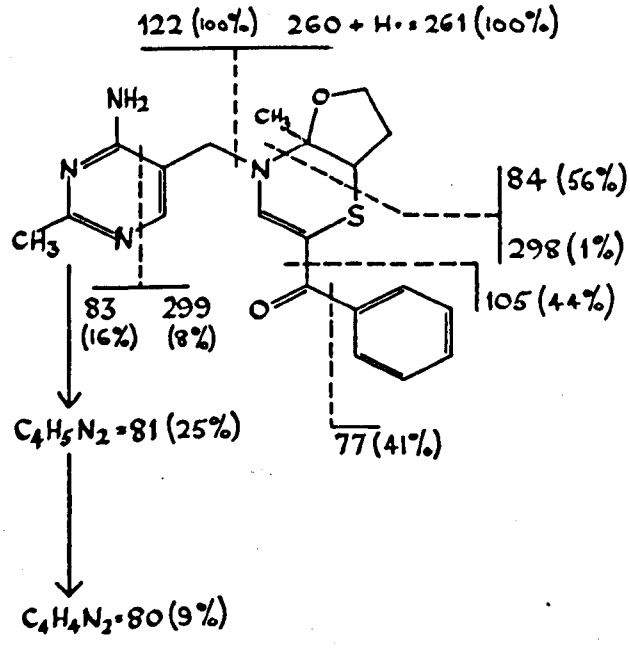

RELATIVE ABUNDANCE.
(%)
100
80
60
40
20
(20b)
36
43
77
84
105
122
147
261
382 M+
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400
MASS
NUMBER

FIGURE 30.



TRANSITION	METASTABLE PEAK
382 — 261	178·3
122 — 81	53·8
105 — 77	56·5

FIGURE 31.



TRANSITION	METASTABLE PEAK
382 — 261	178·3
299 — 261	128·0
122 — 81	53·8
105 — 77	56·5

FIGURE 32.

EXPERIMENTS

Instrumentation :

Ultraviolet spectrometer	:	Unicam SP 800.
Infrared spectrometer	:	Perkin-Elmer 337 or 457.
Nuclear magnetic resonance spectrometer	:	Perkin-Elmer R 10 except where otherwise stated.

Mass spectrometry was done as a service on the A.E.I. MS 902 instrument at the University of Hull.

Elemental analyses were done by several commercial laboratories.

Melting points were determined on a Köffler heated stage assembly. Many were recorded in the following way :

1) temperature at which birefringence of the crystals is lost ;

2) temperature at which melting of the crystals is complete.

EXP.1 THE HIGH AND LOW MELTING POINT FORMS OF THIAMINE bis-CHLORIDE[50]

A saturated solution of thiamine bis-chloride hydrate in methanol was recrystallised by isothermal distillation of dry dioxan, yielding moderately birefringent, apparently rectangular crystals, m.p. 1) 232 - 233°, 2) 234 - 235°.

Analysis : $C_{12}H_{18}Cl_2N_4OS$ requires C, 42.7%; H, 5.34%; N, 16.7%
found C, 42.5%; H, 5.20%; N, 16.8%

A saturated aqueous solution of thiamine bis-chloride hydrate was similarly recrystallised by isothermal distillation of ethanol, yielding strongly birefringent needles, m.p. 1) 246°, 2) 249 - 249.5°.

EXP.2 MASS SPECTROMETRY OF THIAMINE CATIONS

a) Thiamine mono-nitrate (ionizing potential 70 eV, source pressure 3×10^{-6} m.m. mercury, direct insertion, ion chamber temperature 200°).

b) Thiamine bis-chloride[187] (ionizing potential 70 eV, source pressure 4×10^{-6} m.m. mercury, direct insertion, ion chamber temperature 260°).

The interpretation shown in figure 14 is supported by the following high resolution measurements :

113 : Calculated for C_5H_7NS - 113.0299
found - 113.0302

112 : Calculated for C_5H_6NS - 112.0221
found - 112.0227

EXP.3 VARIABLE TEMPERATURE N.M.R. STUDIES OF A SOLUTION OF THIAMINE bis-CHORIDE IN D_2O

I thank Dr. P. J. Q. English of Courtaulds Limited, Coventry who performed these studies for me on the Varian A-60 machine.

EXP.4 MASS SPECTROMETY OF TETRAHYDROTHIAMINE (31)

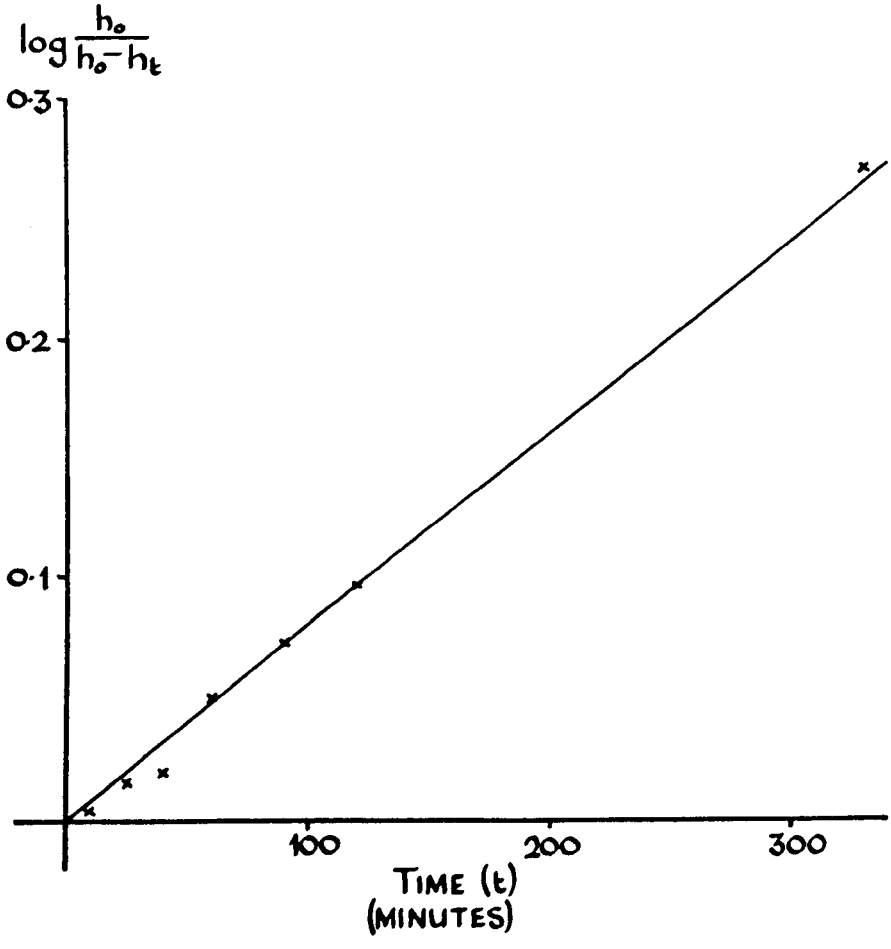
The material was prepared by the method of Clarke and Sykes.[54]

Instrument conditions : ionizing potential 70eV
source pressure 4×10^{-7} m.m. mercury
direct insertion, ion chamber temperature 140°C

Spectrum :

m/e	Relative Abundance (%)	Comment
268	3.3	Molecular ion = $C_{12}H_{20}N_4OS$
178	9.3	
163	7.2	
151	8.6	
149	8.7	
146	51	Thiazolidine fragment = $C_6H_{12}NOS$
123	56	
122	100	Base peak, pyrimidine fragment = $C_6H_8N_3$
81	21	$C_4H_5N_2$
80	8.7	$C_4H_4N_2$
56	56	
55	9.5	
54	12	
53	6.3	C_3H_3N

Metastable peak : 53.7 (122 → 81)



(h_o : initial height of peak,
h_t : height at time t.)

FIGURE 33.

EXP.5 THE REACTION OF THIAMINE CHLORIDE WITH METHYL IODIDE

To a suspension of thiamine bis-chloride hydrate in dry methanol was added 1 gm. - equivalent of potassium hydroxide dissolved in methanol, and the suspension filtered. The filtrate was mixed with a moderate excess of methyl iodide and heated to reflux temperature on a water bath for 5 minutes. The crystals which separated on cooling were recrystallised from water, yielding flat crystals, m.p. 1) 169°, 2) 170°.

U.V. (9.2×10^{-5}M. in methanol)

λ_{max} 224 mμ, $\epsilon = 20,200$,

λ_{max} 266 mμ, $\epsilon = 8,500$.

(8.75×10^{-5}M. in methanol made 0.05N in hydrochloric acid)

λ_{max} 221 mμ, $\epsilon = 18,300$,

λ_{max} 246 mμ, $\epsilon = 15,800$.

shoulder at 260 mμ, $\epsilon = 13,500$.

I.R.) identical with the spectra of thiamine mono-nitrate.
N.M.R.)

EXP.6 DEUTERIUM EXCHANGE OF THE PYRIMIDINE METHYL PROTONS OF OXYTHIAMINE bis-CHLORIDE AND THIAMINE bis-CHLORIDE

Oxythiamine bis-chloride (50 mg.) was dissolved in 1 ml. of deuterium oxide, giving a solution which had a "pD" of about 2. The disappearance of the peak at 7.16 τ in the proton magnetic resonance spectrum of this solution was followed at 33°C. It was assumed that the reaction obeys first-order kinetics (figure 33). The calculated first-order rate constant was 1.8×10^{-3} min^{-1}, and the calculated half life was about 6 hours.

The peak at 7.16 τ gave a three proton signal on integration. It

was at lower field than the corresponding signal in the spectrum of thiamine bis-chloride, and was slightly sharper than the thiazolium methyl proton signal at 7.38 τ. A similar solution (50 mg. in 1 ml. D_2O) was left for three days at room temperature, then lyophilised. The solids were redissolved in water, left for a few minutes, then lyophilised. This process was then repeated. Mass spectrometry of the product showed no incorporation of deuterium in the fragments attributed to decomposition products of the thiazolium group (m/e = 143, 113, 112, 85).

The much slower exchange of the pyrimidine methyl protons of thiamine bis-chloride in 6N. DCl/D_2O was then studied. The half life in this case is several days. A specimen was prepared for mass spectrometry in a fashion similar to that described for oxythiamine, allowing an appropriately longer time for the exchange. Again no deuterium was incorporated into the thiazole decomposition products, but in this case the pyrimidine fragment could be clearly identified, shifted from m/e = 122 in the starting material to m/e = 125 in the deuterated product.[188]

This exchange permits an unequivocal assignation of the two methyl group signals in the proton magnetic resonance spectra of thiamine and oxythiamine bis-cations (Table 3). The results contradict the assignations made by Krampitz and his colleagues.[48,179]

EXP.7 SPECTROSCOPIC STUDIES OF leuco-THIAMINE AND S-SUBSTITUTED leuco-THIAMINE DERIVATIVES

7a) Preparation of Sodium leuco-Thiamine (modified from Zima and Williams[7])

To a stirred solution under nitrogen of thiamine bis-chloride hydrate (10 gm., 30 mMole) in 50 ml. water was added slowly sodium

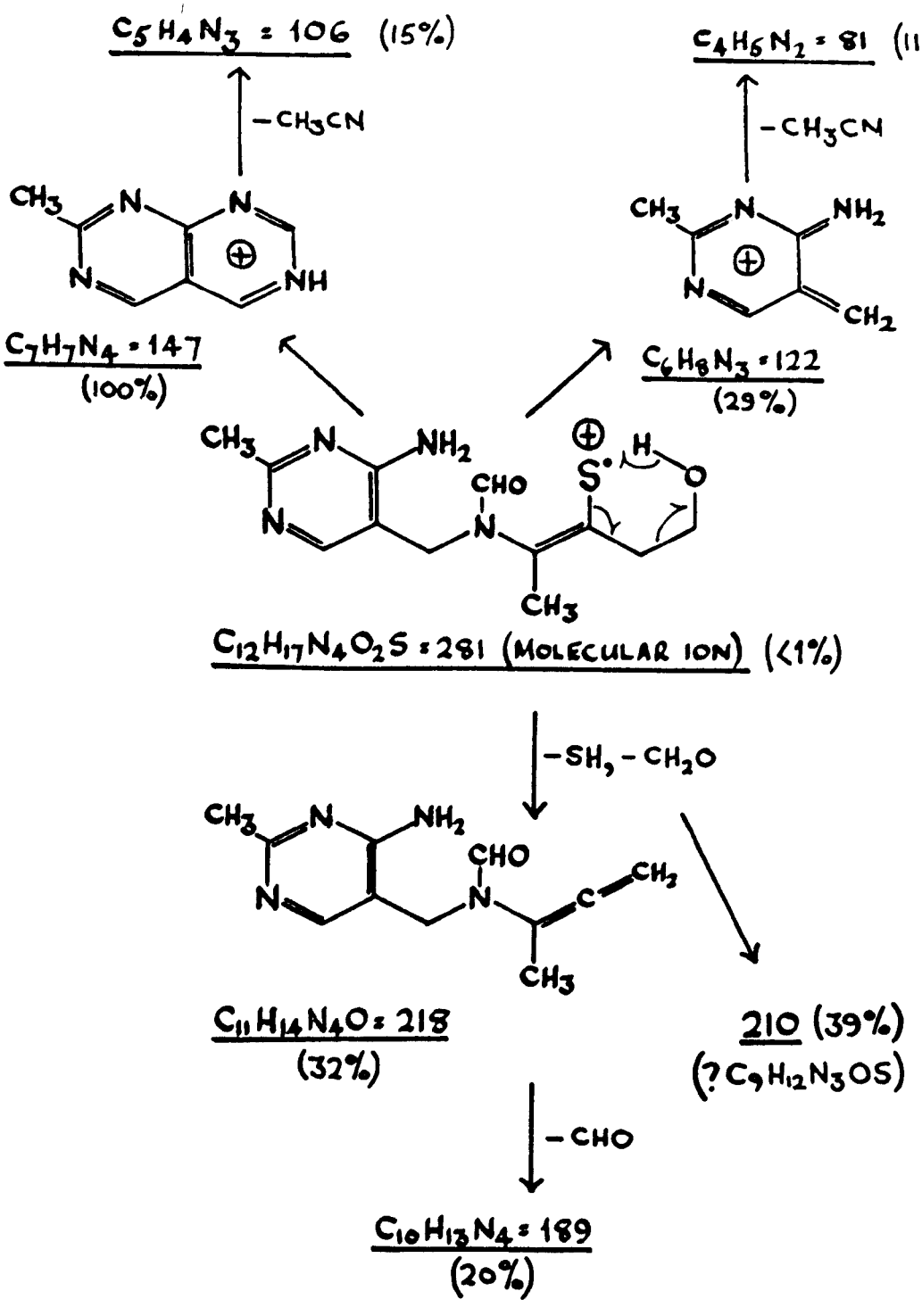


FIGURE 34: MASS SPECTRUM OF leuco-THIAMINE (sodium salt).

hydroxide solution (10% w/v., 36 ml., 90 mMole). The yellow colour produced by each addition of base was allowed to fade before more base was added. The solution was stirred for 2 hours after all the base had been added, and was then lyophilised. The solids were extracted several times with isopropanol, to a final volume of 150 ml., and the solution was then shaken with charcoal and filtered through a Celite bed with suction. Addition of 500 ml. of ether to the filtrate produced a white crystalline precipitate of sodium leuco-thiamine, 6.8 gm., 79%, of m.p. 169 - 170°C (with decomposition).

M.S. (ionizing potential 70 eV, source pressure 4×10^{-7} m.m. mercury, direction insertion, ion chamber temperature 210°C).

Molecular ion : 281 (very small) ($C_{12}H_{17}N_4O_2S$).

Base peak : 147 ($C_7H_7N_4$)

A general interpretation of the spectrum is shown in figure 34. A prominent peak at mass number 210 is not explained in this scheme. This might arise from the molecular ion by the concerted loss of fragments of mass 29 ($CHO^{\oplus}$) and 42 ($CH_3C \equiv NH^{\oplus}$), but the nature of the process is uncertain.

7b) Preparation and spectroscopic properties of S-p-Nitrobenzyl-leuco-Thiamine (2-N-[(4'-amino-2'-methyl-5-pyrimidinyl) methyl]-N-formyl-5-hydroxy-4-(S-p-nitrobenzyl) thio-pent-2-ene) (60). Preparation modified from Sykes and Todd.[150]

To a solution in 10 ml. water of thiamine bis-chloride hydrate (1.05 gm., 3.2 mMole) was added slowly a 10% w/v. solution of sodium hydroxide (3.75 ml., 9.7 mMole). The solution was left at room temperature for 1 hour and then lyophilised. The solids were extracted with 25 ml. of methanol, the suspension filtered through a Celite bed, and the filtrate added to a solution of p-nitrobenzyl chloride (0.55 gm., 3.2 mMole) in 25 ml. methanol. After 3 days the crystals were filtered off and recrystallised from acetone. Final yield 0.56 gm. (44%), m.p. 94 - 95°. (lit. 203° : of anhydrous form).

Analysis : $C_{19}H_{23}N_5O_4S \cdot H_2O$ requires C, 52.6%; H, 5.79%

found C, 52.81%; H, 6.11%

U.V. (9.2×10^{-5}M in methanol).

λ_{max} = 218 mμ, ε = 14,000,

λ_{max} = 240 mμ, ε = 14,600,

λ_{max} = 266 mμ, ε = 16,400.

(3 ml. of 9.2×10^{-5}M solution in methanol, plus a trace of 36% w/v. hydrochloric acid)

λ_{max} = 215 mμ, ε = 13,200,

λ_{max} = 258 mμ, ε = 20,000.

I.R. (Nujol Mull)

$-NH_2$ vibrations : 3440 cm^{-1} (shoulder), 3400 cm^{-1} (sharp) 1658 cm^{-1} (sharp).

C=O vibrations : 1644 cm^{-1} (sharp), 1270 cm^{-1} (sharp).

C-O vibration : 1061 cm^{-1} (sharp)

$-NO_2$ vibrations : 1520 cm^{-1} (sharp), 1347 cm^{-1} (sharp).

p-substituted benzene ring, C-H vibration 856 cm^{-1} (sharp)

N.M.R. (d_6-D.M.S.O.)

2.02δ (3H, s, 1-CH_3); 2.38δ (3H, s, 2'-CH_3); 2.62δ (2H, t, J = 6 Hz, 4-CH_2-); 3.92δ (2H, s, benzyl-CH_2-); 4.42δ (2H, s, N-CH_2-); 4.86δ (1H, broad s, 5-OH); 6.98δ (2H, s, $-NH_2$); 7.58, 7.73, 8.34, 8.49δ (4H, AB system, aryl-H); 7.96δ (1H, s, -CHO); 8.08δ (1H, s, 6'-H). The triplet arising from the 5-CH_2- protons was probably centred at 3.65δ, but was not identified with certainty. A small peak at 1.65δ attested the presence of a small proportion of the cyclised tautomeric form.

M.S. (ionizing potential 70eV, source pressure 4×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 200°C).

Molecular ion : $C_{19}H_{23}N_5O_4S$ requires 417.1470

found 417.1460

Base peak : 122 ($C_6H_8N_3$)

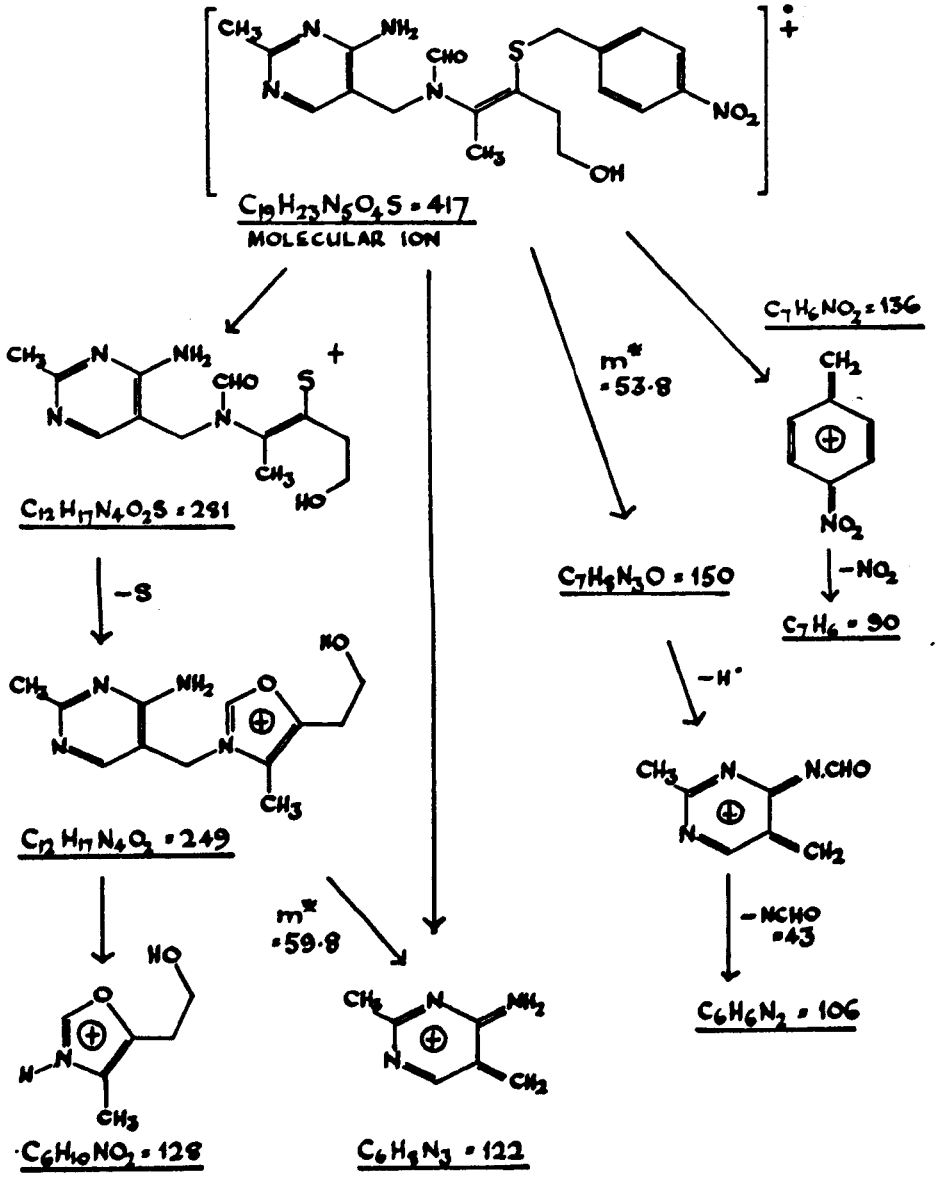


FIGURE 35: THE MASS SPECTRUM OF S-p-NITROBENZYL leuco-THIAMINE.

The fragmentation pattern was different from that of leuco-thiamine (figure 35). A strong peak at mass number 249 is attributed to the oxazolium compound (61). This did not readily undergo γ-hydrogen rearrangement, eliminating formaldehyde from the 2'-hydroxyethyl group. Other studies have shown that the oxazole ring is very stable in the mass spectrometer.[191] A strong peak at mass number 149 is derived from the molecular ion by a concerted fragmentation involving transfer of the formyl group to the pyrimidine amino group.

EXP.8 THE REACTION OF leuco-THIAMINE WITH AMMONIA AND WITH HYDROXYLAMINE (REF. MASUDA[109])

8a) Preparation and Spectroscopic Properties of 1-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl-3-(2'-hydroxy)ethyl-2-methyl-imidazole (46).

To a solution of thiamine bis-chloride hydrate (5 gm., 14 mMole) in 5 ml. of water was added a 10% w/v. solution of sodium hydroxide (12 ml., 30 mMole) and 3 ml. of 33% ammonia solution. The mixture was left for 7 days at room temperature, and then the crystalline product was filtered, washed briefly with water, and dried over P_2O_5 in vacuo. Yield 2.2 gm. (63%). A sample recrystallised from ethanol had m.p. (1,2) 116 - 117° (lit. 215°).

Analysis : $C_{12}H_{17}N_5O \cdot H_2O$ requires C, 54.5%; H, 7.17%; N, 26.4%
found C, 54.65%; H, 7.74%; N, 25.3%.

N.M.R. (d_6-D.M.S.O.)

2.10δ (3H, s, 3-CH_3); 2.39δ (3H, s, 2'-CH_3); 2.67δ (2H, t, J = 7 Hz, -$\underline{CH_2}$-CH_2OH); 3.70δ (2H, t, J = 7 Hz, -$\underline{CH_2}OH$); 5.05δ (2H, s, methylene bridge protons); 7.10δ (2H, broad, s, -NH_2); 7.74δ (1H, s, 6'-H); 7.78δ (1H, s, 5-H).

The peak at 2.39δ exchanges in 6N. DCl/D_2O, with a half life of about 2 days.[188]

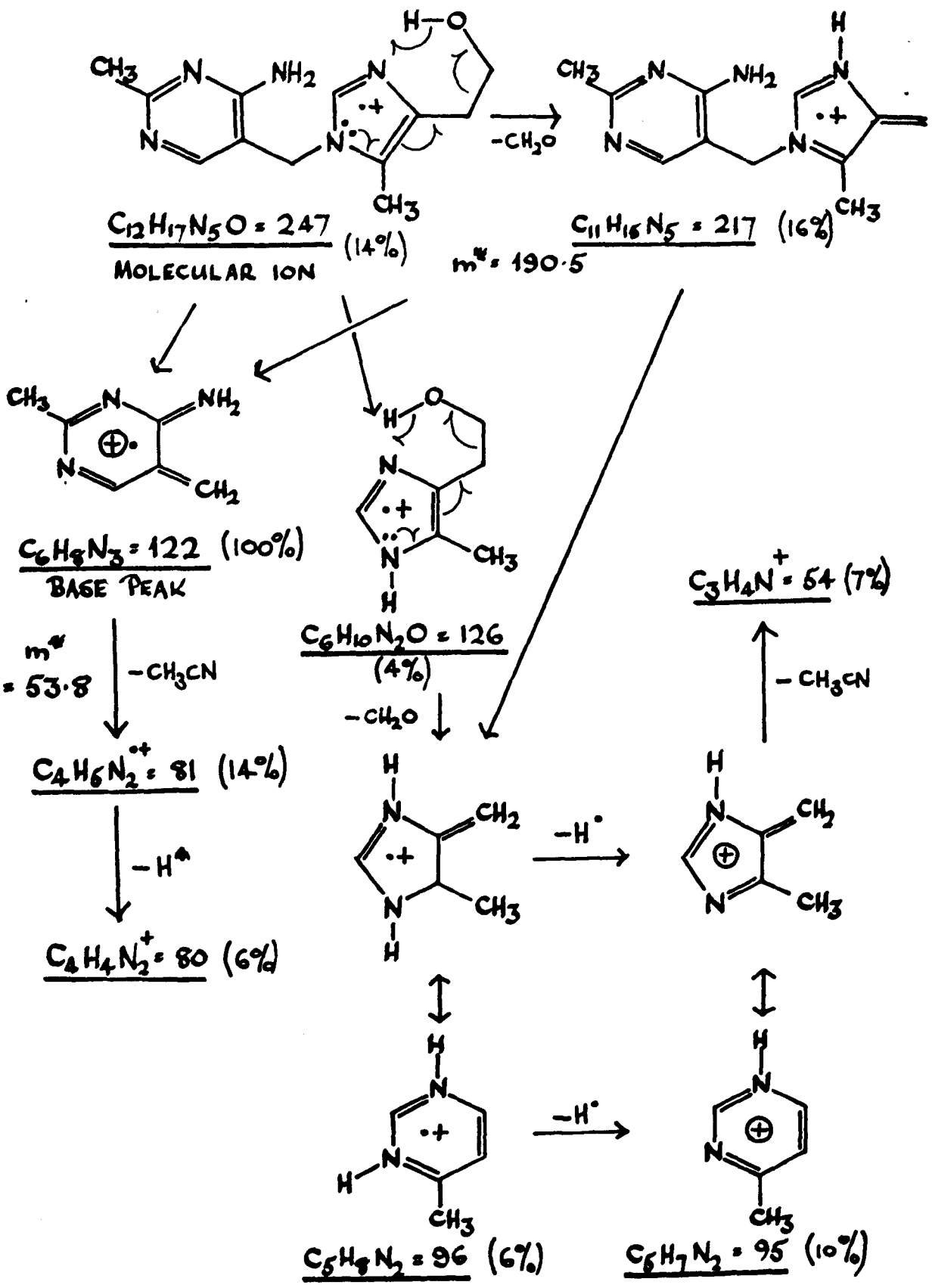

$C_{12}H_{17}N_5O = 247$
MOLECULAR ION
(14%)
$-CH_2O$
$C_{11}H_{16}N_5 = 217$ (16%)
$m^* = 190.5$
$C_6H_8N_3 = 122$ (100%)
BASE PEAK
$m^* = 53.8$
$-CH_3CN$
$C_4H_6N_2^{\cdot+} = 81$ (14%)
$-H^{\cdot}$
$C_4H_4N_2^+ = 80$ (6%)
$C_6H_{10}N_2O = 126$
(4%)
$-CH_2O$
$-H^{\cdot}$
$C_3H_4N^+ = 54$ (7%)
$-CH_3CN$
$-H^{\cdot}$
$C_5H_8N_2 = 96$ (6%)
$C_5H_7N_2 = 95$ (10%)

FIGURE 36.

M.S. (ionizing potential 70eV, source pressure 6×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 120°C).

Molecular ion : calculated for $C_{12}H_{17}N_5O$ - 247.1433,
found - 247.1428.

The spectrum was consistent with the interpretation shown in figure 36.

8b) Preparation and Spectroscopic Properties of 2-[N-formyl-N-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl]-amino-5-hydroxypentan-3-one oxime (42).

This was prepared in a similar manner to (46), replacing the ammonia solution by hydroxylamine solution (1.1 gm. hydroxylamine hydrochloride (16 mMole) dissolved in 12 ml. 5% w/v sodium hydroxide). Yield 3.2 gm. (81%). A sample recrystallised from water had m.p. (1,2) 203° (lit. 204°).

Analysis : $C_{12}H_{19}N_5O_3$ requires C, 51.3%; H, 6.77%; N, 24.96%
found C, 51.9%; H, 7.28%; N, 25.16%

N.M.R. (d_6-D.M.S.O.)

1.06δ (3H, d, J = 7Hz, 1-CH_3); 1.83δ (2H, t, J = 7Hz, 4-CH_2-); 2.03δ (3H, s, 2'-CH_3); 3.42δ (2H, t, J = 7Hz, 5-CH_2-); 3.98δ (2H, δ, J = 3Hz, N-methylene protons); 4.13δ (1H, q, J = 7Hz, 2-H); 6.50δ (2H, broad s, -NH_2); 7.54δ (1H, s, -CHO); 8.09δ (1H, s, 6'-H).

M.S. (ionizing potential 70eV, source pressure 6×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 210°C).

Molecular ion : calculated for $C_{12}H_{19}N_5O_3$ - 281.1487,
found - 281.1494.

The fragmentation pattern showed no evidence for γ-hydrogen rearrangement processes.

m/e	Relative Abundance (%)	Comment
281	10	Molecular ion
264	22	$M^+ - OH$
165	43	$C_7H_9N_4O$
149	9	
147	10	$C_7H_7N_4$
137	60	$C_6H_9N_4$
122	100	Base peak, $C_6H_8N_3$
99	16	
96	18	
81	34	$C_4H_5N_2$

EXP.9 THE PROTON MAGNETIC RESONANCE AND MASS SPECTRA OF THIOCHROME

Thiochrome was prepared from thiamine bis-chloride by the method of Barger, Bergel and Todd.[162] A sample recrystallised from chloroform had m.p. (1,2) 228° (lit. 228°). Free sublimation was observed at temperatures greater than 190°C.

N.M.R. (CF_3CO_2H)

2.59 δ (3H, s, 7-CH_3); 2.99 δ (3H, s, 2-CH_3); 3.26 δ (2H, t, J = 7 Hz, -CH_2-CH_2OH); 4.23 δ (2H, t, J = 7 Hz, CH_2OH); 5.93 δ (2H, s, 5-CH_2); 9.01 δ (1H, s, 4-H).

M.S. (ionizing potential 70 eV, source pressure 2×10^{-7} m.m. mercury, direction insertion, ion chamber temperature 195°C).

The interpretation of the spectrum shown in figure 23 is supported by the following high resolution measurement :

231 : calculated for $C_{11}H_{11}N_4S$ - 231.0704
found - 231.0714

EXP.10 PREPARATION AND PROPERTIES OF S-ALLYL-xantho-THIAMINE (5-Hydroxy-2-[6'N-(2'-methyl-5'H, 6'H,-1',3',6',8'-tetra-azanaphthalenyl)]-3-(2'-propenyl) thio pent-2-ene) (51)

To a stirred ice-cold suspension under nitrogen of thiamine bis-chloride hydrate (3.4 gm., 10 mMole) and molecular sieve (4A grade, 20 gm.) in dry isopropanol (50 ml.) was added potassium hydroxide (1.8 gm., 31 mMole) dissolved in dry methanol (25 ml.). Allyl bromide (5 ml.) was added and after five minutes the mixture was filtered with suction through a Celite bed. The solids were washed with isopropanol (10 ml.) and the combined filtrates were reduced to about 10 ml. on a rotary evaporator, then added with stirring to ether (sodium dried, 500 ml.). After 24 hours at 0°C the crystals were collected, washed with ether and dried. Yield 2.0 gm. (66%). A sample recrystallised from acetone had m.p. 123 - 125°. The crystals became brown on exposure to air for long periods.

U.V. (1.8×10^{-4}M. in methanol)

λ_{max} = 224 mμ, ε = 10,600;
shoulder at 250 mμ, ε = 4,900;
λ_{max} = 335 mμ, ε = 9,500.

(1.71×10^{-4}M in methanol made 0.05N in HCl)

The spectrum showed no change after 30 minutes.

λ_{max} = 212 mμ, ε = 7,200;
λ_{max} = 245 mμ, ε = 2,200;
λ_{max} = 298 mμ, ε = 2,300.

I.R. (Nujol Mull)

-OH vibration : 3180 cm^{-1} (broad)
C-O vibration : 1068 cm^{-1} (sharp)

There were no bands between 3,550 and 3300 cm^{-1}, or between 1700 and 1600 cm^{-1}.

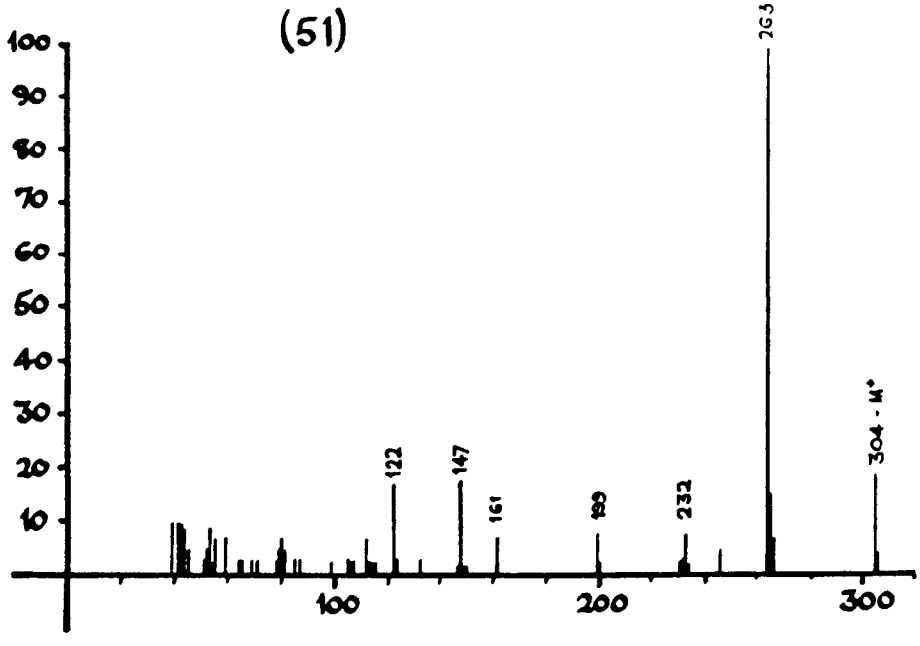

(51)
100
90
80
70
60
50
40
30
20
10
122
147
161
189
232
263
304 - M+
100
200
300

FIGURE 37.

N.M.R. ($CDCl_3$)

2.12δ (3H, s, 1-CH_3); 2.65δ (3H, s, 2'-CH_3); 2.70δ (2H, t, J = 7Hz, 4-CH_2-); 3.34, 3.45δ (2H, d, J = 7Hz, 3-1'-CH_2-); 3.97δ (2H, t, J = 7Hz, 5-CH_2-); 4.53δ (2H, broad s, removed by adding a drop of D_2O, 5-OH); 4.80δ (2H, s, 5'-CH_2-); 5.00 - 6.25δ (3H, complex m, 3-1', 3'-CH=CH_2); 7.43δ (1H, s, 7'-H); 8.29δ (1H, s, 4'-H).

M.S. (ionizing potential 70eV, source pressure 2.2×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 145°C). (figure 37)

Molecular ion : calculated for $C_{15}H_{20}N_4OS$ - 304.1357
found - 304.1351.

Base peak : calculated for $C_{12}H_{15}N_4OS$ - 263.09665
found - 263.09652

An interpretation of the mass spectrum is shown in figure 38. It is supported by the following high resolution measurements :

232 : calculated for $C_{11}H_{12}N_4S$ - 232.07826
found - 232.07818

199 : calculated for $C_{11}H_{11}N_4$ - 199.09837
found - 199.09729

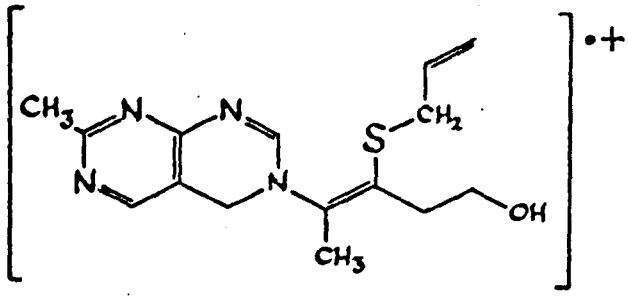
161 : calculated for $C_9H_{11}N_3$ - 161.09529
found - 161.09504

147 : calculated for $C_7H_7N_4$ - 147.06706
found - 147.06721.

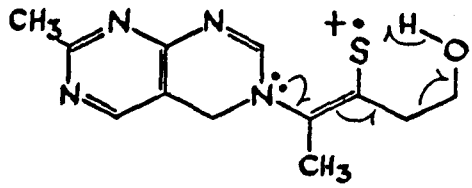
10b) The Reaction of S-Allyl-xantho-Thiamine with Malononitrile

S-allyl-xantho-thiamine did not react with water at room temperature, and a mixture of products was obtained from acid solution.

S-allyl-xantho-thiamine (288 mg., 0.95mMole) was dissolved in dry pyridine (2 ml.) containing malononitrile (1 ml.). The yellow solution

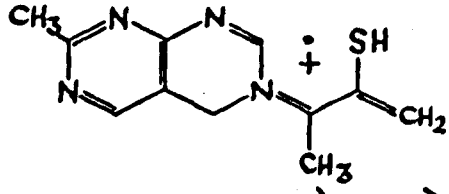


$C_{15}H_{20}N_4OS = 304.$ (21%) MOLECULAR ION.



$C_{12}H_{15}N_4OS = 263.$ BASE PEAK.

$-CH_2O$

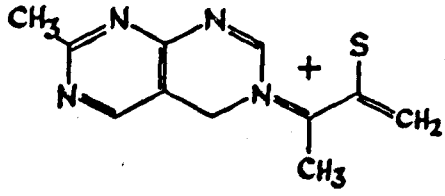


$C_{11}H_{13}N_4S = 233.$ (3%)

$-H^{\bullet}$

$C_{11}H_{11}N_4 = 199.$ (6%)

$C_7H_7N_4 = 147.$ (17%)



$C_{11}H_{12}N_4S = 232.$ (7%)

METASTABLE PEAK : 204.5 (263 → 232)

FIGURE 38.

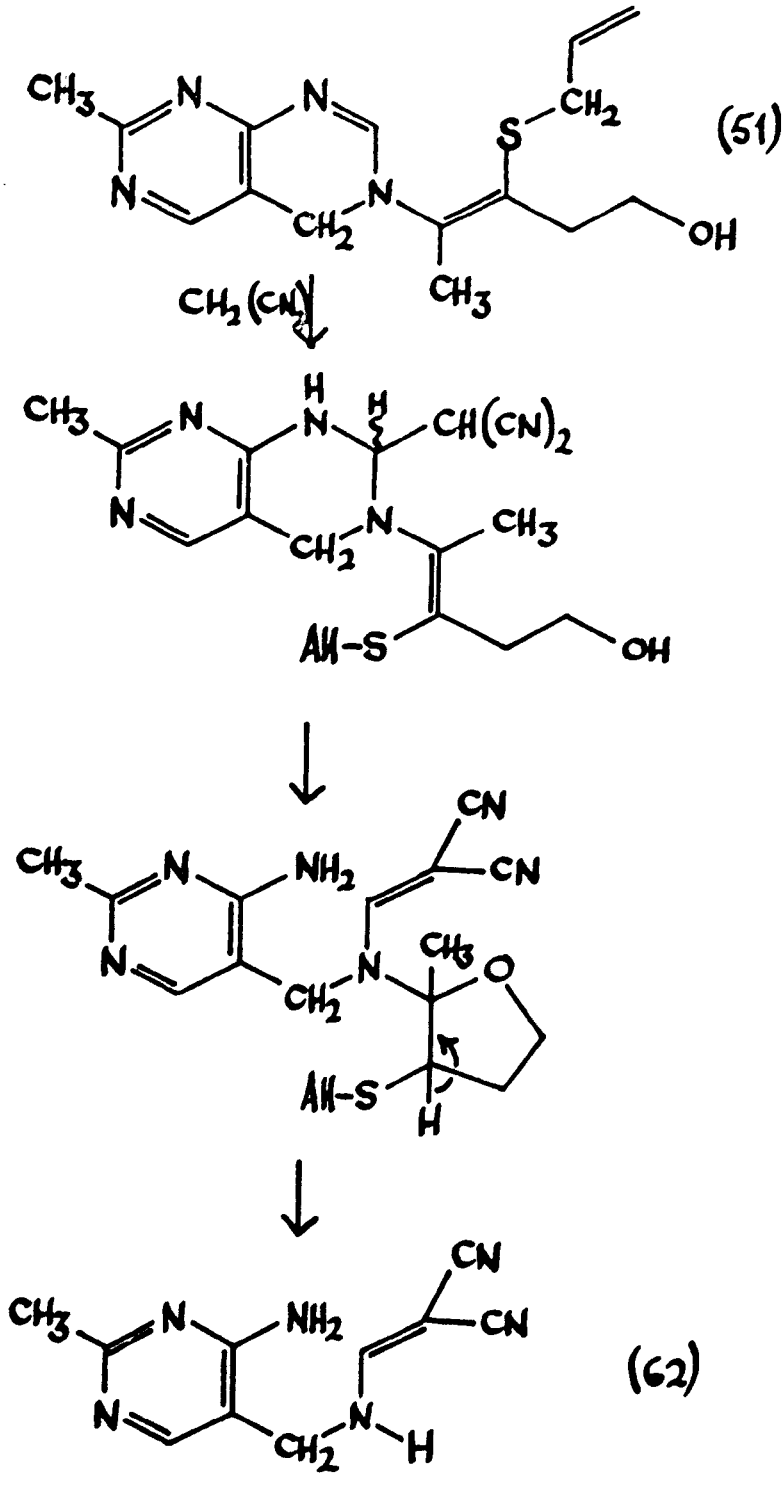


FIGURE 39.

became brown and crystals began to form after a few minutes. After 3 hours ether (4 ml.) was added and the mixture stood for 1 hour more. The crystals were separated by centrifugation, washed with ether, and dried. Yield 182 mg., of cubic crystals m.p. 217°. The product was identified by the following spectroscopic studies as 4-amino-5-(N-2',2'-dicyanethenyl)aminomethyl-2-methylpyrimidine (62). This product may be formed by the mechanism shown in figure 39.

I.R. (Nujol Mull)

-NH_2 vibrations : 3445 cm^{-1} (sharp), 3335 cm^{-1} (sharp) 1642 cm^{-1} (strong).

-NH vibration : 3160 cm^{-1} (broad).

-CN vibrations : 2220 cm^{-1}, 2207 cm^{-1} (sharp), 2175 cm^{-1} (weak, sharp).
The low value of these nitrile frequencies suggests that the groups are further conjugated.

N.M.R. (d_6-D.M.S.O.)

2.38δ (3H, s, 2-CH_3); 4.38δ (2H, s, 5-CH_2-); 6.98δ (2H, broad s, disappears if a little D_2O added, 4-NH_2); 8.23δ (1H, s, ethenyl-H); 8.27δ (1H, s, 6-H); about 9.4δ (1H, very broad s, disappears if a little D_2O is added, 5-CH_2N-H).

M.S. (ionizing potential 70eV, source pressure 4 x 10^{-7} m.m. mercury, direct insertion, ion chamber temperature 200°C).

Molecular ion : 214 ($C_{10}H_{11}N_6$)

Base peak : 147 ($C_7H_7N_4$)

There were prominent peaks at mass number 122 ($C_6H_8N_3$), 106 ($C_5H_4N_3$), 94 ($C_4H_4N_3$), 81 ($C_4H_5N_2$), 80 ($C_4H_4N_2$), and 66 ($C_3H_2N_2$). The following metastable peaks were seen :

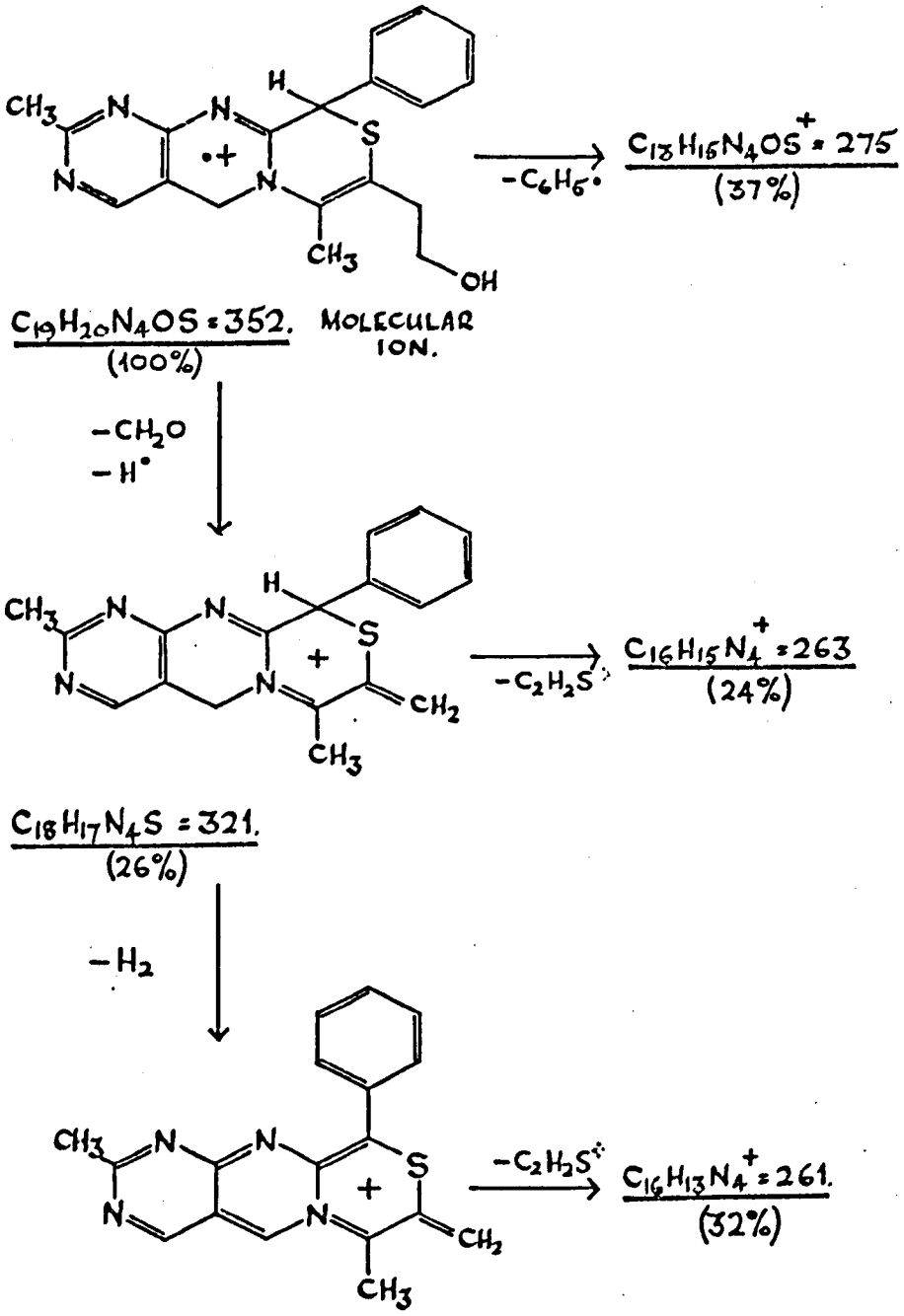

$C_{19}H_{20}N_4OS = 352.$ (100%) MOLECULAR ION.
$-CH_2O$
$-H^\bullet$
$C_{18}H_{17}N_4S = 321.$ (26%)
$-H_2$
$C_{18}H_{15}N_4S = 319.$ (32%)
$-C_6H_6^\bullet$
$C_{13}H_{15}N_4OS^+ = 275$ (37%)
$-C_2H_2S^{\bullet}$
$C_{16}H_{15}N_4^+ = 263$ (24%)
$-C_2H_2S^{\bullet}$
$C_{16}H_{13}N_4^+ = 261.$ (32%)

FIGURE 40.

76.4 (147 → 106)

69.6 (214 → 122)

53.7 (122 → 81)

There were impurity peaks at 291 and 411.

EXP.11 THE MASS SPECTRUM OF 1-Phenyl-4,9-dimethyl-3-(2'-hydroxyethyl)-1,6-dihydropyrimido[4',5'-4,5]pyrimido[2,3c]-1,4-thiazine

The material was prepared by the procedure of Takamizawa et al.[181] The ultra-violet, infrared and N.M.R. spectra of the product were the same as those published, and the m.p. was 206° (lit. 207°).

Instrument conditions : ionizing potential 70eV
source pressure 3×10^{-7} m.m.
direct insertion, ion chamber
temperature 185°C.

Molecular ion ($C_{19}H_{20}N_4OS$) = 352.

The remainder of the spectrum was consistent with the interpretation shown in figure 40.

EXP.12 THE REACTION BETWEEN xantho-THIAMINE AND α-HALOKETONES : 1) INITIAL PRODUCTS

12a) Preliminary experiments.

A solution of xantho-thiamine became deep red after the addition of each of the following compounds : chloroacetone, phenacyl chloride, p-nitrophenacyl bromide, p-bromophenacyl bromide, bromopyruvic acid. Ethyl chloroacetate gave a pink colour. A red colour was not given by chloroacetal, desyl chloride, chloral, chloracetonitrile, allyl bromide. A solution of sodium leuco-thiamine and phenacyl chloride in methanol became deep red over a period of about 6 hours. Oxythiamine bis-chloride gave only a faint pink colour.

12b) Preparation of 2-acetyl-4-(4'-amino-2'-methyl-5'-pyrimidinyl) methyl-6-(2'-hydroxy)ethyl-5-methyl-1,4-thiazine (19a).

An ice-cold solution of potassium hydroxide (A.R. grade, 3.1 gm., 45 mMole) in 75 ml. of dry ethanol was added slowly with stirring to a mixture of thiamine bis-chloride hydrate (5.0 gm., 14.8 mMole) and chloroacetone (2 ml., about 25 mMole). The mixture was stirred for 10 minutes, then 100 ml. of chloroform was added and the mixture filtered with suction through a Celite bed. The solids were washed with two 10 ml. volumes of chloroform and the combined filtrates were then reduced to dryness on a rotary evaporator (water bath temperature 50°C or less). The red solid was taken up in 25 ml. of chloroform and applied to a "Florisil" column 50 cm x 2 cm made up in methylene chloride. Washing with gradually increasing proportions of methanol in methylene chloride resulted in elution of the red material by 50% (v/v) methanol : methylene dichloride. The solution was reduced to dryness on the rotary evaporator, then dried over phosphorus pentoxide under water-pump vacuum. Yield 2.5 gm. (53%) of an amorphous powder of indefinite m.p. Thin-layer chromatography on active silica gel plates developed with chloroform : methanol, (3 : 1 by volume) showed only one component, R_F = 0.60 located under U.V. lamp or with iodine vapour.

U.V. (10^{-4} Molar solution in methanol) :

λ_{max} = 231.5 mμ, ε = 16,400;

λ_{max} = 276 mμ, ε = 7,400;

λ_{max} = 450 mμ, ε = 900.

(9.5×10^{-5} Molar solution in methanol made about 0.2N in hydrochloric acid); the spectrum showed no further change after 40 minutes :

λ_{max} = 248 mμ, ε = 14,300;

λ_{max} = 331 mμ, ε = 6,700.

I.R. (Nujol Mull)

$-NH_2$ vibrations : 3325 cm^{-1} (broad), 1656 cm^{-1} (sharp).

C=O vibrations : possibly about 1630 cm^{-1}, 1212 cm^{-1} (sharp).

C-O vibration : 1048 cm^{-1} (sharp).

N.M.R. (d_6-D.M.S.O. containing about 50% $CDCl_3$)

1.80δ (3H, s, 5-CH_3); 2.15δ (3H, s, CO-CH_3); 2.46δ (3H, s, 2'-CH_3); 3.67δ (2H, t, J = 7Hz, -CH_2OH); 4.49δ (2H, broad s, 4-CH_2-); 6.43δ (2H, broad s, -NH_2); 7.39δ (1H, s, 3-H); 8.02δ (1H, broad s, -OH); 8.53δ (1H, s, 6'-H).

The 1'-protons of the 6-(2'-hydroxy)ethyl group probably gave a triplet centred on 2.44δ, but this could not be identified with certainty.

M.S. (ionizing potential 70eV, source pressure 1 x 10^{-7} m.m. mercury, direct insertion, ion chamber temperature 170°C).

Molecular ion	:	calculated for $C_{15}H_{20}N_4O_2S$	- 320.1307
		found	- 320.1307
Base peak	:	calculated for $C_6H_8N_3$	- 122.0718
		found	- 122.0723
Peak at mass	:	calculated for $C_9H_{12}NO_2S$	- 198.0588
number 198		found	- 198.0598

The spectrum was consistent to the proposed structure, and similar to the spectrum of the 2-benzoyl compound (see 12b), with appropriate variations caused by the substitution of an acetyl for a benzoyl group.

To a solution of 19a (320 mg., 1 mMole) in dry pyridine (5 ml.) was added acetic anhydride (2 ml.) slowly, with stirring. The solution was left for 12 hours in the refigerator, and then reduced in volume as much as possible on the rotary evaporator. The resulting oil was frozen in dry ice/acetone and dried at 0.1 mm. pressure for 7 days. The waxy residue showed a strong band in the infrared at 1725 cm^{-1}, consistent with the formation of an acetate ester. The red colour of the solution

was stable in acid.

12c) Preparation of 4-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl-2-benzoyl-6-(2'-hydroxy)ethyl-5-methyl-1,4-thiazine (19b).

This was done by the same procedure as 12a, substituting phenacyl chloride (2.5 gm., 16 mMole) for chloroacetone. The red material was eluted from the 'Florisil' column by a 25% v/v methanol : dichloromethane mixture. Thin-layer chromatography (as in 12a) : 1 red spot, R_F = 0.85.

N.M.R. (d_6-D.M.S.O. containing about 50% $CDCl_3$)

1.95 δ (3H, s, 5-CH_3); 2.54 δ (3H, s, 2'-CH_3); 3.82 δ (2H, t, J = 7 Hz, -CH_2OH); 4.53 δ (2H, broad s, 4-CH_2-); 6.72 δ (2H, broad s, -NH_2); 7.08 δ (1H, s, 3-H); 7.63 δ (5H, s, aryl-H); 8.14 δ (1H, s, -OH); 8.54 δ (1H, s, 6'-H). The 1'-protons of the 6-(2'-hydroxy)ethyl group probably gave a triplet centred on 2.41 δ, but this could not be identified with certainty.

M.S. (ionizing potential 70 eV, source pressure 3×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 190°C).

Molecular ion : 382 ($C_{20}H_{22}N_4O_2S$)

Base peak : 122 ($C_6H_6N_3$)

There were prominent peaks at mass numbers 261 ($C_{14}H_{15}NO_2S$) and 105 (C_7H_5O). An interpretation of the spectrum is shown in figure 31.

To a solution of (19b) (about 1 mMole = 0.38 gm.) in 10 ml. chloroform was added benzoyl chloride (0.13 ml.) and triethylamine (1 ml.). After one week at 0°C red crystals had formed and were collected. They proved to be soft with an indefinite m.p. The infrared spectrum showed a strong band at 1715 cm^{-1}, and another at 712 cm^{-1}, consistent with the formation of a benzoyl ester. However, the material was impure and other spectroscopic data were not obtained.

A similar solution of (19b) in chloroform was treated with 1-isocyanotonaphthalene (0.169 gm.). After 3 days at 0°C the solvent was removed and the material dried in a high vacuum. This yielded 0.52 gm.

(94%) of a red powder, which again did not give good spectroscopic data.

12d) Preparation of 4-(4'-amino-2'-methyl-5'-pyrimidyl)methyl-2-(p-bromo)benzoyl-6-(2'-hydroxy)ethyl-5-methyl-1,4-thiazine (19c).

This was done by the same procedure as 12a, with appropriate modifications. The red material was eluted from the 'Florisil" column by a 20% methanol : dichloromethane mixture. The product was an amorphous brown powder of indefinite m.p., but easier to handle than (19a) or (19b). Thin-layer chromatography (as in 12a) : 1 red spot, $R_F = 0.88$.

U.V. (6.52×10^{-5} Molar solution in methanol).

λ_{max} = 233.5 mμ, ϵ = 26,500;
λ_{max} = about 480 mμ, ϵ = 2,600;
shoulder at about 255 mμ, ϵ = 18,000;
shoulder at about 325 mμ, ϵ = 2,900.

(6.2×10^{-5} Molar solution in methanol made about 0.2N in hydrochloric acid); the spectrum showed no further change after 40 minutes.

λ_{max} = 248 mμ, ϵ = 28,200;
λ_{max} = 348 mμ, ϵ = 11,800.

I.R. (Nujol Mull)

$-NH_2$ vibrations : 3430 cm^{-1} (sharp), 3310 cm^{-1} (broader), 1655 cm^{-1} (sharp).
-OH vibration : 3100 cm^{-1} (very broad)
C=O vibration : ?1230 cm^{-1} (sharp)
C-O vibration : 1070 cm^{-1} (sharp).
p-disubstituted benzene ring, C-H vibration, ? 832 cm^{-1} (sharp)

N.M.R. (d_6-D.M.S.O. + 2 drops D_2O)

1.85δ (3H, s, 5-CH_3); 2.30δ (2H, t (partly obscured), J = 6Hz, 6-CH_2-); 2.42δ (3H, s, 2'-CH_3); 3.64δ (2H, t, J = 6Hz, -CH_2OH); 4.49δ (2H, s, 4-CH_2-); 7.04δ (1H, s, 3-H); 7.42, 7.56, 7.70, 7.84δ

(4H, AB system, aryl-H); 8.35 δ (1H, s, 6'-H). In d_6-D.M.S.O. alone there were also broad peaks at 4.08 δ (about 1H, s, -OH) and 6.94 δ (2H, s, $-NH_2$).

To this solution was then added 2 drops of trifluoroacetic acid, and the spectrum was taken again after 20 minutes : 1.63 δ (3H, s, 5-CH_3); 2.62 δ (3H, s, 2'-CH_3); 4.53 δ (2H, s, 4-CH_2-); 7.46 δ (1H, s, 3-H); 7.49, 7.63, 7.72, 7.86 δ (4H, AB system, aryl-H); 8.08 δ (1H, s, 6'-H). The triplets seen in the spectrum before acidification at 2.30 δ and 3.64 δ had become broad, poorly resolved multiplets centred on 2.27 δ and 3.95 δ.

M.S. (ionizing potential 70 eV, source pressure 7×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 140°C).

Molecular ion : calculated for $C_{20}H_{21}N_4O_2S^{79}Br$ - 460.0571
found - 460.0573.

Base peak : 122 ($C_6H_8N_3$)

There were prominent peaks at 339, 341 ($C_{14}H_{14}NO_2SBr$), 312, 314 ($C_{13}H_{13}O_2SBr$), and 183, 185 (C_7H_4OBr).

EXP.13 THE REACTION BETWEEN xantho-THIAMINE AND α-HALOKETONES : 2) PRODUCTS FROM ACIDIFIED SOLUTION

13a) Preparation of 4-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl-2-benzoyl-6H, 7H, 8H-9-methylfuro[3,2-b]-1,4-thiazine (20b)

To a stirred ice-cold suspension under nitrogen of thiamine bis-chloride hydrate (5.0 gm., 14 mMole) in 25 ml. of dry isopropanol containing phenacyl chloride (3.0 gm., 20 mMole) was added slowly 23.6 ml. of a 10% w/v (calculated) methanolic potassium hydroxide solution (42 mMole). The deep red solution was stirred at 0°C for 30 minutes, then filtered with suction through a Celite bed. The filtered solids were washed with two 10 ml. volumes of isopropanol. To the ice-cold combined filtrates was

added, with stirring, an ice-cold solution of 5 ml. of 36% w/v hydrochloric acid in 20 ml. of isopropanol. The mixture was left until the colour was orange (about 1 hour) and then refrigerated for 3 days. The mother-liquors were then decanted, and the crystals washed with isopropanol, water, dioxan and ether, and then dried in vacuo over phosphorus pentoxide. Yield 4.15 gm. (70%). A sample recrystallised three times from water had m.p. 1) 165.5° 2) 166°.

Analysis : calculated for $C_{20}H_{22}N_4O_2S \cdot HCl \cdot H_2O$ = 436.96;
C, 55.45%; H, 5.77%; N, 12.82%; S, 7.35%
found C, 55.52%; H, 5.86%; N, 13.17%; S, 7.64%

U.V. (5.57×10^{-5} M solution in methanol made approximately 10^{-3} N with respect to HCl) :

λ_{max} = 246 mμ, ε = 21,700;
λ_{max} = 345 mμ, ε = 12,400.

(5.31×10^{-5} M solution in 0.05 N methanolic potassium hydroxide) :

λ_{max} = 236.5 mμ, ε = 21,500
λ_{max} = 346 mμ, ε = 13,900
shoulder = 270 mμ, ε = 8,900

I.R. (Nujol mull)

$-NH_2$ vibrations : 3510 cm^{-1}, 3380 cm^{-1} (broad bands)
1696 cm^{-1}, 1670 cm^{-1} (sharp bands).
These bands were all absent from the spectrum of a sample recrystallised twice from D_2O.

C=O vibration : 1651 cm^{-1} (sharp)

C-O vibration : 1096 cm^{-1} (sharp)

Phenyl group vibrations : 1602 cm^{-1}, 721 cm^{-1}, 700 cm^{-1} (sharp bands)

N.M.R. (d_6-D.M.S.O.)

1.64δ (3H, s, 9-CH_3); 2.64δ (3H, s, 2'-CH_3); approximately

1.95 - 2.5 δ and approximately 3.4 - 4.2 δ (probably 5H, doublet of multiplets, 6,7,8-H); 4.57 δ (2H, broad s, 4-CH_2-); 7.49 δ (1H, s, 3-H); 7.71 δ (5H, s, phenyl-H); 8.17 δ (1H, s, 6'-H); 9.00 δ (2H, broad s, -NH_2, absent in sample recrystallised from D_2O).

M.S. (ionizing potential 70 eV, source pressure 4×10^{-7} m.m. mercury, direction insertion, ion chamber temperature 200°C).

Molecular ion :	calculated for $C_{20}H_{22}N_4O_2S$	- 382.1463
	found	- 382.1467
Base peak :	calculated for $C_{14}H_{15}NO_2S$	- 261.0830
	found	- 261.0830

An interpretation of the mass spectrum is shown in figure 32, and is supported by the following high resolution measurements :

Peak	Formula	Mass calculated	Mass found
298	$C_{15}H_{14}N_4OS$	298.0888	298.0892
122	$C_6H_8N_3$	122.0718	122.0723

13b) Preparation of 4-(4'-amino-2'-methyl-5'-pyrimidinyl)methyl-2-p-nitrobenzoyl-6H, 7H, 8H-9-methylfuro[3,2-b]-1,4-thiazine (20δ)

p-Nitrophenacyl bromide was prepared from p-nitroacetophenone by the method of Engler and Zielke[189], m.p. (1,2) 98°C = lit.

To a stirred ice-cold suspension under nitrogen of thiamine bis-chloride hydrate (3.4 gm., 10 mMole) in 50 ml. of dry isopropanol containing p-nitrophenacyl bromide (2.5 gm., 10.25 mMole) was added slowly a solution of potassium hydroxide (A.R. grade, 1.7 gm., 30 mMole) in 25 ml. of dry methanol. The purple solution was stirred for 30 minutes at 0°C, then filtered with suction through a Celite bed. The filtered solids were washed with two 10 ml. volumes of isopropanol. To the ice-cold combined filtrates was added slowly with stirring, 5 ml. of 36% w/v hydrochloric acid. The mixture was left at room temperature overnight, then refrigerated for 24 hours. The orange crystals were then filtered, washed with ether,

and dried over phosphorus pentoxide in vacuo. Yield 3.8 gm. (96%). A sample recrystallised from N/10 HCl had m.p. 155°C (with decomposition). The crystals were hygroscopic.

Analysis : calculated for $C_{20}H_{21}N_5O_4S \cdot HCl \cdot 3H_2O$ = 517.99;
C, 46.85%; H, 5.46%; N, 13.52%
found C, 46.85%; H, 5.48%; N, 13.19%.

U.V. (6.4×10^{-5}M solution in methanol made approximately 10^{-3}N with respect to HCl) :

λ_{max} = 252 mμ, ε = 22,800;
λ_{max} = 318 mμ, ε = 8,100;
shoulder at about 350 mμ, ε = 6,200.

I.R. (Nujol Mull)

$-NH_2$ vibrations : 3510 cm^{-1}, 3400 cm^{-1} (broad)
1696 cm^{-1} (sharp)
C=O vibration : 1657 cm^{-1} (sharp)
C-O vibrations : 1092 cm^{-1}, 1104 cm^{-1} (sharp)
$-NO_2$ vibrations : 1510 cm^{-1} (broad), 1325 cm^{-1} (sharp)
p-disubstituted benzene ring, C-H vibration : 832 cm^{-1} (sharp).

N.M.R. (d_6-D.M.S.O)

1.65δ (3H, s, 9-CH_3); 2.64δ (3H, s, 2'-CH_3); approximately 1.95 - 2.5δ and approximately 3.4 - 4.2δ (probably 5H, doublet of multiplets, 6,7,8-H); 4.61δ (2H, broad s, 4-CH_2-); 7.55δ (1H, s, 3-H); 7.93, 8.09, 8.48, 8.63δ (4H, AB system, aryl-H); 8.23δ (1H, s, 6'-H); 9.10δ (2H, broad s, $-NH_2$).

M.S. (ionizing potential 70 eV, source pressure 4×10^{-7} m.m. mercury, direction insertion, ion chamber temperature 150°C).

Molecular ion : calculated for $C_{20}H_{21}N_5O_4S$ - 427.1310
found - 427.1306

Fragment of mass 306 : calculated for $C_{14}H_{14}N_2O_4S$ - 306.0674
found - 306.066

There was a prominent peak of mass number 150 ($C_7H_4NO_3$) and the base peak had mass number 122 ($C_6H_8N_3$). The spectrum was consistent with the proposed structure, and similar to the spectrum of the 2-benzoyl (see 13a) compound (20b) with appropriate variations caused by the additional nitro group.

13c) Attempts to prepare the 2-acetyl compound (~~64a~~ 20a) gave only oils. The reaction with p-bromophenacyl bromide gave a 25% yield of (~~64d~~ 20d), m.p. 250 - 252°C (with decomposition).

M.S. (ionizing potential 70 eV, source pressure 5×10^{-7} m.m. mercury, direct insertion, ion chamber temperature 270°C).

Molecular ion : calculated for $C_{20}H_{21}N_4O_2S^{79}Br$ - 460.0571
found - 460.0573

There were strong peaks at mass numbers 143, 113 and 112, in addition to the expected peaks. The material was therefore contaminated with thiamine bis-chloride.

13d) Neither (20b) or (20d) gave a crystalline oxime, phenylhydrazone, 2,4-dinitrophenylhydrazone, or semicarbazone. (20b) in methanol solution was not reduced by sodium borohydride. A crystalline product, m.p. (1,2) 216°C, was identified by its U.V. spectrum as the free base of (20b).

EXP.14 TOPICS WHICH NEED FURTHER INVESTIGATION

1. Synthesis of compound (59) (figure 26). If the proposed mechanism of action of thiamine is correct, then this should be a competitive inhibitor of thiamine-requiring enzymes. One enantiomer should bind to the active site of the enzyme more strongly than the other.

2. xantho-Thiamine anion reacts rapidly with water: S-alkyl-xantho-thiamines react slowly, if they react at all. What is the reason for this difference?

3. Can S-acyl-xantho-thiamines be prepared, and what properties do they have?

4. Can the 7,8-double bond of xantho-thiamine derivatives be reduced, and does re-oxidation occur easily?

5. Will other reagents react with xantho-thiamine by addition to the 7,8-double bond?

EXP.15 THE ATTEMPTED PREPARATION OF ADDUCTS OF xantho-THIAMINE AND PYRUVATE OR ACETOIN

15a) Experiments with Sodium Pyruvate

To a suspension in sodium-dried ethanol (50 ml), under a dry nitrogen stream, of thiamine bis-chloride hydrate (3.30 gm, 9.3 mMole) and sodium pyruvate (5.50 gm, 55 mMole), was added, drop by drop with stirring and cooling in ice, a solution of sodium metal (0.689 gm, 30 mMole) in dry ethanol (150 ml). A control experiment using sodium acetate (4.00 gm, 50 mMole) in place of sodium pyruvate was run simultaneously. Thin layer chromatography of the reaction mixtures on silica developed with a chloroform-methanol mixture (1 :1) showed a number of components, but no difference between the two. Attempts to work up the reaction mixtures yielded no defined products.

The experiments were repeated using redistilled dimethylformamide as solvent and triethylamine as base. Again no reaction products were defined.

To a solution of pyruvic acid (1.6 ml, 22.6 mMole) in sodium-dried ethanol (50 ml) was added thiamine bis-chloride hydrate (5 gm, 14.7 mMole). To the stirred suspension under nitrogen was added drop by drop a solution of sodium (1.00 gm, 43 mMole) in dry ethanol (150 ml). The mixture was stirred for 5 minutes and then a solution of potassium ferricyanide (10 gm, 30 mMole) in distilled water (25 ml) was added rapidly. A few minutes later the solution was filtered through a Celite bed with suction. Examination of the blue-fluorescent solution by thin layer and paper chromatography showed that thiochrome was the major component, with traces of several other non-fluorescent compounds.

15b) Experiments with Acetoin

Thiamine bis-chloride hydrate (9.8 gm, 29.1 mMole) was suspended in redistilled dimethylformamide under a dry nitrogen stream, and redistilled triethylamine (12 ml, 87 mMole) was added slowly with ice-cooling and stirring until the mixture became solid. Acetoin (2.52 ml, 29.1 mMole) dissolved in dimethylformamide (25 ml) was then added and the slurry stirred for 5 hours, while the effluent nitrogen stream was bubbled through a solution of phenylhydrazine (5 ml) in 2N hydrochloric acid (30 ml). No acetaldehyde was trapped in this way.

The reaction mixture was then oxidised with hydrogen peroxide (100 vol, 10 ml) dissolved in dimethylformamide (25 ml). The solution developed a bright blue fluorescence, but attempts to work up the mixture failed.

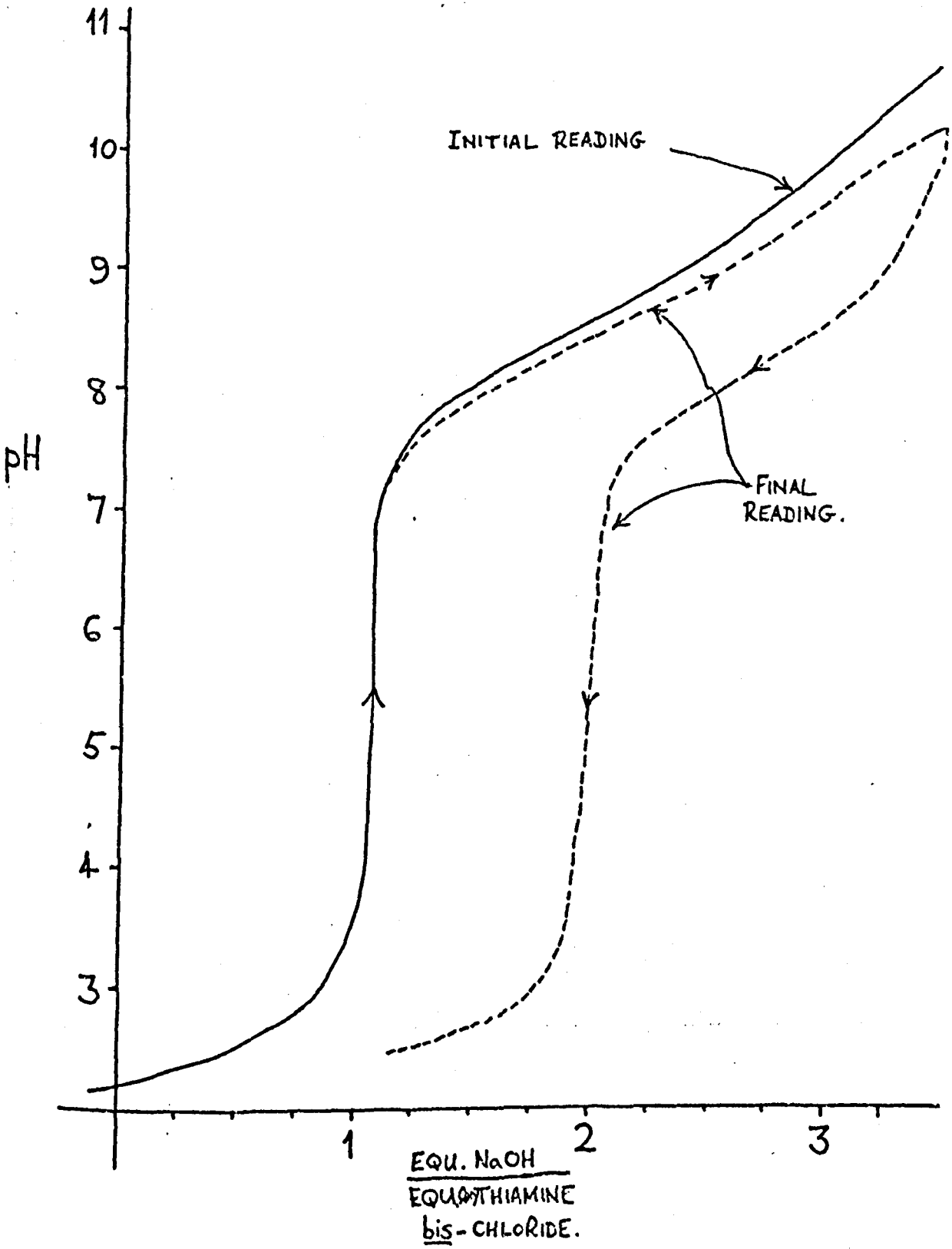


FIGURE 41. TITRATION OF OXYTHIAMINE.

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