Best Copy
Available

THE NON ENZYMATIC DESTRUCTION OF THIAMINE

By

WILLY LHOEST

PHARMACY LIBRARY
SCHOOL OF PHARMACY

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

MASTER OF SCIENCE

(Pharmacy)

at the

UNIVERSITY OF WISCONSIN

1957

ACKNOWLEDGMENT

The author wishes to express his gratitude and sincere appreciation to Dr. L. W. Busse for his guidance and interest during the course of this research and preparation of this thesis; and to Dr. C. A. Baumann for his helpful advice and the use of his laboratory facilities.

TABLE OF CONTENTS

INTRODUCTION

Since thiamine was discovered and isolated as a pure compound in 1934 people who had to work with it or to use it for therapeutic, nutritional, or pure research purposes encountered difficulties due to its instability.

Although these questions have been studied for more than 20 years, many aspects still remain unsolved and all that the literature provides us is partial information about some of the most important ways according to which thiamine can be destroyed. Especially the kind of information which we will find is concerned with reports of cases, of definite conditions in which thiamine was found to be unstable but unfortunately very few explanations of the process of destruction in itself.

In fact there are many possibilities of degradation of thiamine and many compounds which have been shown to enhance its destruction.

It appears that the most important factor affecting the stability of thiamine is the pH value of the medium in which it is dissolved. It was also one of the first degradation factors to be reported. K. H. T. Farrer[1] claims that aneurine is completely destroyed in fifteen minutes at 100° at pH 9, while at lower pH such as 8, 7, 6, 5, 4, and 3 the destroyed proportions within one hour are 100, 67.8, 53.4, 40.0, 20.3, and 16.0% respectively. No loss of activity occurs in 1% Hydrochloric acid solutions in 7 hours.

However the effect of pH is not solely to be considered in these mixtures but also the nature of the electrolytes regulating their pH is quite important. For instance a solution of thiamine heated for one hour at 100°C was completely destroyed with a borate buffer at pH 5.4 while at the same pH value and in the same experimental conditions, 10% were destroyed with an acetate buffer and only 3% in a phosphate buffer. These facts were established by B. W. Beadle, D. A. Greenwood and H. R. Kraybill[2] in 1943.

Working on diet mixtures and studying the effects of various salts, P. E. Waibel, H. R. Bird, and C. A. Baumann[3] showed the deleterious effect of K_2HPO_4, $CaCO_3$, and $MnSO_4$.

K. H. T. Farrer[4] in a further and very interesting work proved that a linear relationship existed between the reaction velocity of the destruction of thiamine and the hydrogen ion concentration for any given buffer in the pH range 3 to 8. For each buffer there was however a different relationship and the slope of the curves altered as the ionic constitution of the solution changed.

If thiamine is generally considered to be stable in acidic conditions, an exception must be made for solutions of pH range 4 to 7 containing sodium sulfite. This salt indeed is very well known to split quantitatively the vitamin into both of its constituent parts, the pyrimidine and the thiazol ring, and this property was largely used by R. R. Williams et al[5] in their work on the determination of the structure of this product.

Flavones and phenols may also be considered as dangerous products for the Vitamin B_1. E. Hasayawa et al[6] established that flavonoids are able to destroy the vitamin and that the most deleterious among these compounds are the derivatives possessing an o. diphenol group in the side chain.

Oxidation of course is another possibility and the irreversible transformation of thiamine to thiochrome by potassium ferricyanide, air, potassium permanganate and many other oxidative agents has been proven many years ago[7]. Generally this oxidation will not stop with the formation of thiochrome but a more complex reaction will occur giving rise to other degradation products that so far are still unknown.

Recently some publications have appeared showing the complex formation between thiamine and some heavy metals such as monovalent copper. We would refer here to the work of Etienne Gero[8] in France who was able to establish the existence of this complex which he labelled "Cuprothiamine". He also showed that the reaction is reversible and that the chlorine atom in the thiazol ring is necessary for its formation.

Finally two Dutch workers, P. de Lange and L. P. v. d. Mijl Dekker[9] described in 1954 a browning reaction between thiamine and glucose. Heating at 85°C mixtures in equal proportions of the vitamin and sugar, they could prove that after a few days the content in both of these compounds was considerably reduced. Further research showed that the rate of browning is dependent upon the

nature of the reacting aldose since the same experiment with a mixture of arabinose and Vitamin B_1 showed a greater browning effect under the same conditions. The reaction seems also to be limited to low moisture conditions since a solution of the mixture did not brown after heating at 85°C for ten days.

To complete this short and quite general review of the agents capable of influencing chemically the stability of thiamine, we should say a few words about the catalytic and protective actions which have been attributed to many agents. The catalytic role of metals and on the other hand the favorable action of ascorbic acid, hydroquinone, cysteine, sulfasuccidine, and especially glycerol on the stability of thiamine in typical diet mixtures was described by A. A. Kandutsch and C. A. Baumann[10]. The formation of a precipitate in aqueous solutions of Vitamin B_1 is also known to be prevented or retarded by additions of small amounts of thioglycolic or thiodibutyric acid[11].

Besides these many possibilities of chemical degradation, there is however another type of destruction the importance of which should not be neglected. We mean the important group of biological and microbiological degradations of this compound. However this aspect of the problem is beyond the subject of our study and shall not be discussed here.

In the following pages we will essentially consider two among the main aspects of the chemical degradation of thiamine: pH and reactions with sugars.

The effect of pH was studied because of the general role that this factor plays in most of the cases where the question of the stability of the vitamin arises. In addition, as previously mentioned, it seems to be the most important factor conditioning a good conservation of the vitaminic properties of the molecule.

The reactions of Thiamine with sugars was also of interest since this involves a new aspect of the question. Besides this reaction might occur frequently since thiamine is often mixed with sugars and heated in relatively dry state--for instance in the pastry and baking industries.

As far as the methods of study are concerned, we have been working especially with ultraviolet spectrophotometry and paper chromatography.

Ultraviolet spectrophotometry is a quite interesting method for studying the structure modifications of the molecule since a very small change in this structure generally induces large changes in its spectrophotometric properties. In addition confusion still existed in studies of Ultraviolet spectra of Thiamine especially in basic solution.

These experiments were run on a CARY recording spectrophotometer model 11.

On the other hand, paper chromatography provided an excellent method for following the successive steps of these reactions on account of its flexibility and its high sensitivity. It is well known that paper chromatography generally gives good results in

the separation of relatively low molecular weight products like those that we might expect in this study.

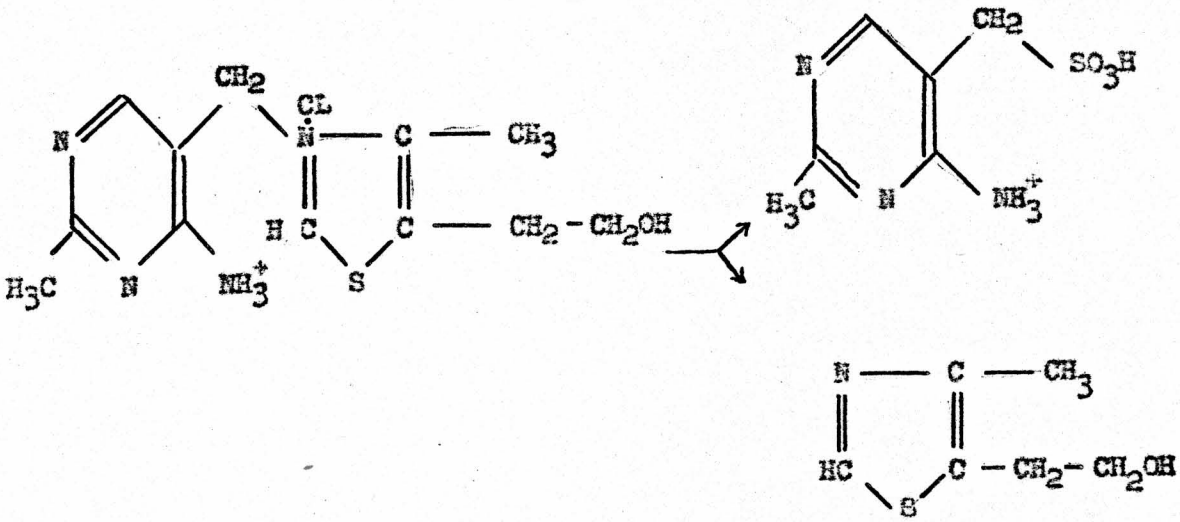
SECTION I. EFFECTS OF pH.

Part I. Spectrophotometric Study

We have described in a previous work[12] the spectrophotometric behaviour of Thiamine as a function of pH and as a function of time. Many absorption curves in the basic pH range however still remained partially unexplained, and consequently the first part of this study will deal with some more experiments providing a more satisfactory and more complete explanation of these curves.

1) Preparation of known cleavage products.

In the course of their work on Thiamine, Williams et al[5] developed a very simple method for splitting the vitamin into each of its constituent parts. This quantitative reaction occurs in the presence of sulfurous acid and according to the following scheme:



500 mg of anhydrous Thiamine Hydrochloride were dissolved in 7.5 ml of water in the presence of 1.228 g of anhydrous Sodium Sulfite. The resulting solution was brought to pH 4.8 by means of .6 ml of

concentrated Hydrochloric acid. Almost instantaneously the solution becomes milky due to the liberation of 2 methyl, 6 amino-pyrimidyl, 5 methylene sulfonic acid which is very sparingly soluble in water. This solution was kept at room temperature and in the absence of light for 12 hours in order to obtain a cleavage as quantitative as possible. After that, it was kept in a refrigerator at + 4°C for 8 hours in order to favor the precipitation of the sulfonic acid. This precipitate was separated by filtration on a Buchner funnel and generously washed by means of iced water until the filtrate was free of Chlorides. The filtrate and the first washings were kept for further extraction. The precipitate was recrystallised from boiling water, washed again in the same conditions and finally dried to constant weight in an exsiccator over Calcium Chloride.

The second fraction of the Thiamine molecule, the thiazol part, is very soluble in water and is contained in the filtrate of the preceding operations. This aqueous solution was consequently brought to pH 10 with 9 N Sodium Hydroxyde and extracted 6 times with 50 ml portions of Chloroform. These extracts were combined and their total volume reduced to about 50 ml by evaporation under vacuum at room temperature. We obtained in this way an impure solution of 4 methyl, 5 β hydroxyethyl thiazol. Further purification was accomplished by extracting the chloroform solution with 5 portions of 50 ml 2 N Hydrochloric Acid. After evaporation of this solution at room temperature and under vacuum, there remained an amorphous and very hygroscopic residue. Better desiccation could be obtained by

redissolving this residue in small fractions of absolute ethyl alcohol and evaporating to dryness again. The final residue was dissolved in a minimum amount of ethyl alcohol (3 ml) and an excess of dioxane (30 ml) was added. After 24 hours, this solution provides very nice crystals of 4 methyl, 5 β hydroxyethyl thiazol hydrochloride which can be separated, washed with dioxane, and dried under vacuum.

The absorption curves of 2 methyl, 4 amino pyrimidyl, 5 methylene sulfonic acid and of 4 methyl, 5 hydroxyethyl thiazol hydrochloride were studied as a function of pH. The curves of the equimolecular mixture of both of these compounds were studied as a function of pH and as a function of time. The results of these experiments are given in the following pages.

2) Spectrophotometric Data

a) Spectra of 2 methyl, 4 amino pyrimidyl, 5 methylene sulfonic acid.

The U.V. absorption curves of this product were run from 2200 to 3200 Å at the following pH: 1.85, 6.90, and 12.05. Since in our previous study the U.V. spectra of Thiamine had been run with concentrations of 20 mcg per ml, and we wanted to compare the present curves of the cleavage products with the previous curves of the whole molecule, we decided to use concentrations corresponding to 20 mcg of Thiamine Hydrochloride per ml. Consequently, since the ratio of the molecular weight of the pyrimidine fraction over the molecular weight of the vitamin is equal to 203.15/ 337.27, we had to use solutions containing 12 mcg of pyrimidine sulfonic acid per

ml. The pH of the pure aqueous solution was 6.90. The acidic solution was prepared by adding 0.1 ml of concentrated Hydrochloric Acid to 10 ml of a solution containing 120 mcg of the product per ml and diluting it to 100 ml. The basic solution was prepared in the same manner by addition of 0.1 ml of 9 N Sodium Hydroxyde. Blanks containing the same concentrations of acid and base were run concurrently.

Fig. 1 shows the typical absorption curves which are obtained in this way. We can see that at low pH values the spectrum of this product is characterised by only one maximum at the wavelength of 2476 Å. If we increase the pH to 6.90, the extinction coefficient decreases. At the same time the position of the maximum shifts to the left and there appears a shoulder at higher wavelengths. If we finally bring the solution to high pH values, the absorption spectrum will be characterised by two maxima at 2350 and 2775 Å respectively, and by one minimum at 2545 Å. It should be noted here that all these curves cross at two isobestic points I and I', the position of which is given by the following coordinates:

	λ (Å)	$E^{1cm}_{.6\%}$	$E^{1cm}_{1\%}$
I	2394	257	428
I'	2762	156	260

Tab. 1

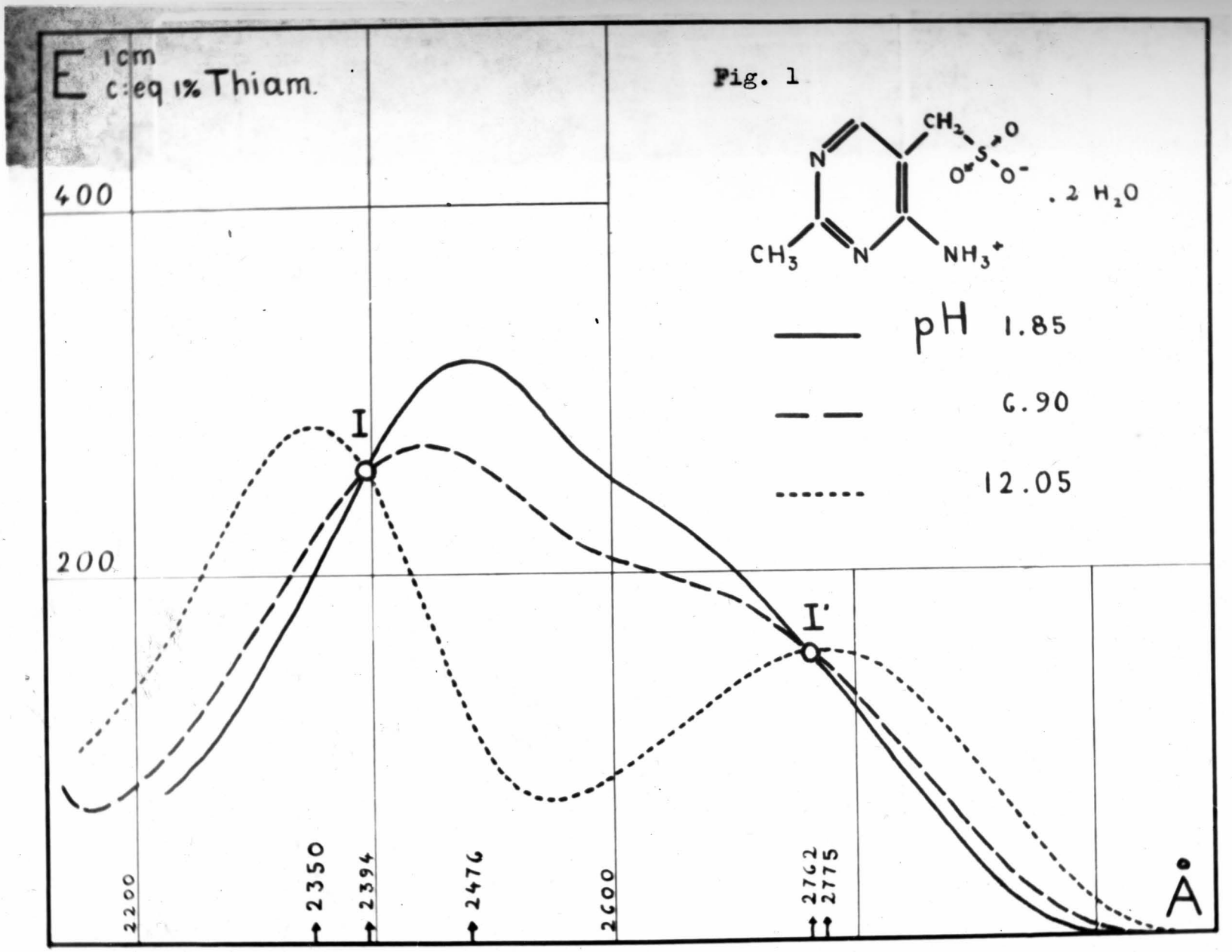

E 1cm
c: eq 1% Thiam.
400
200
I
I'
2200
2350
2394
2476
2600
2762
2775
Å
pH 1.85
6.90
12.05
CH2
O
S
O
O-
.2 H2O
CH3
N
N
NH3+

Fig. 1

It is obvious that the ratio of the extinctions at these wavelengths and at any pH is a constant and can be used for the identification of this compound.

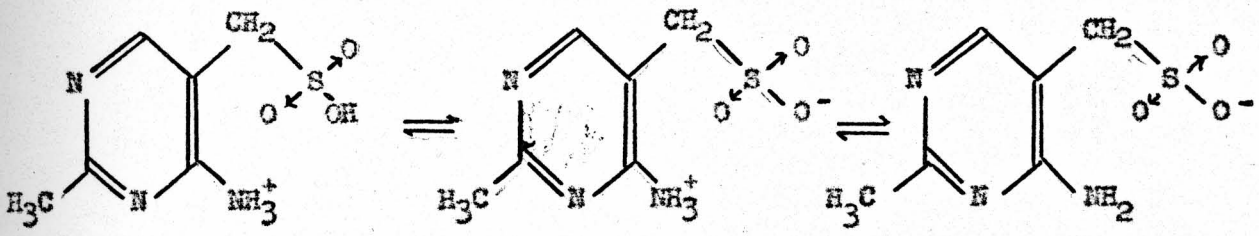
$$\frac{E_I}{E_{I'}} = \text{constant} = 1.65$$

It must be noticed here that these variations in the curves of absorption are perfectly reversible, even if we bring the molecule at high pH values. This fact seems to indicate a good stability for this compound and differentiates it from thiamine. The whole molecule of vitamin possesses indeed the same characteristic variations in its U.V. spectra, but these variations are only reversible below pH 7. We will describe later the effects of higher pH on the molecule, effects affecting this time the thiazole fraction.

b) Spectra of 4 methyl, 5 β hydroxyethyl thiazol.

As for the pyrimidine part of the molecule the U.V. absorption spectra of the thiazol fraction were run at 3 different pH values: 2.29, 6.63, and 11.50 respectively and at a concentration corresponding to 20 mcg of Thiamine Hydrochloride per ml. Therefore the concentration to be used in this case was 179.5 X 20 / 337.27 = 10.64 mcg /ml. Again the pH of 6.63 is that of the pure aqueous solution. The acidic and basic solutions were obtained by addition of .05 ml concentrated Hydrochloric Acid and .05 ml 9 N Sodium Hydroxyde respectively to two 20 ml fractions of a solution containing 53 mcg of product per ml and diluting them to 100 ml. Blanks were run with the same concentrations of acid and hydroxyde.

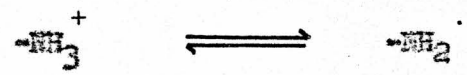
alkaline solution, we will thus have an equilibrium that we may represent in the following way:



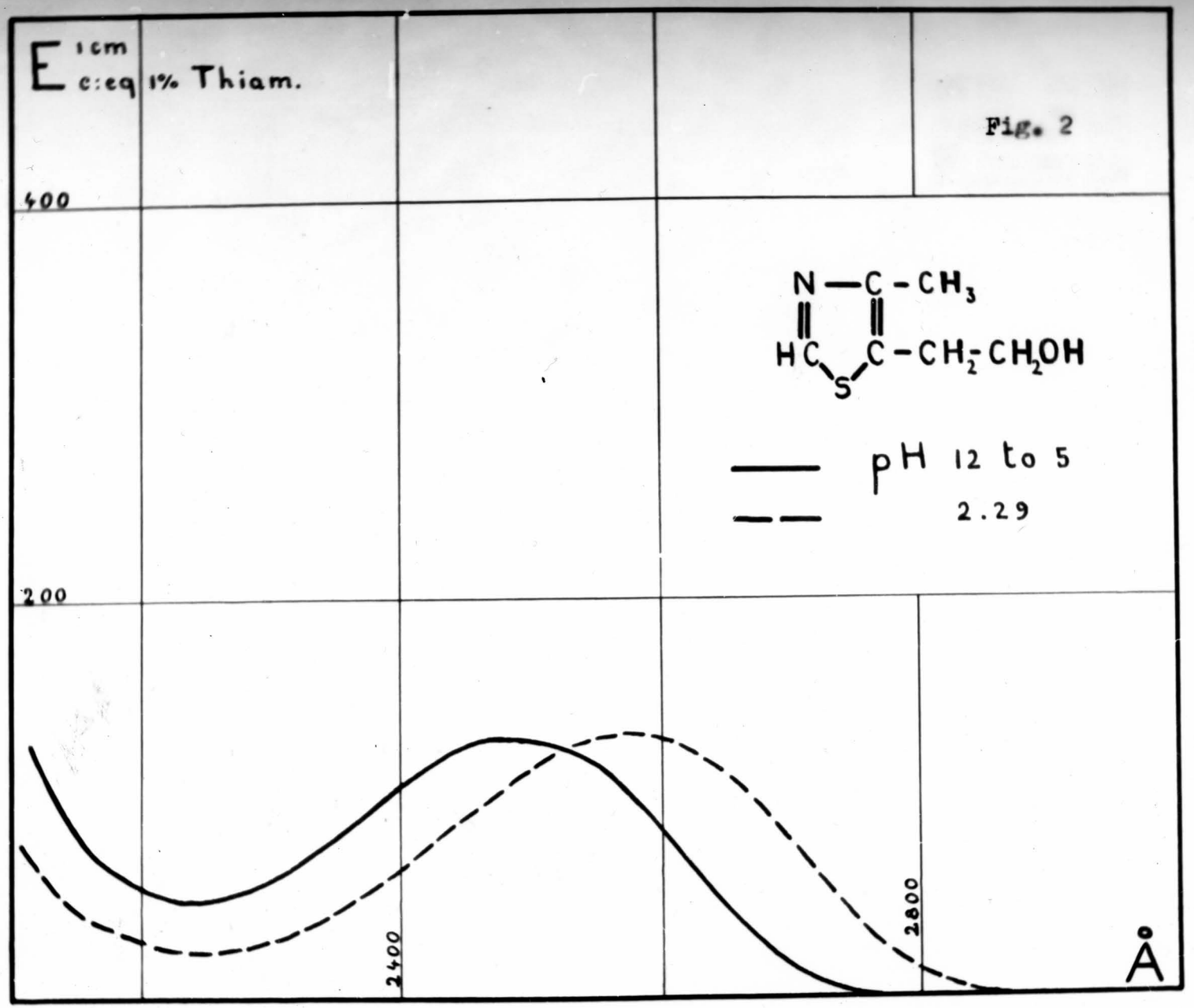
acidic form zwitterion basic form

The titration curve of this compound is represented in the Fig. 4. It shows the presence of a strongly acidic group, the pK of which is below 1, and a second group much less acidic characterised by a pK of 6.5. The pK_1 of course is to be attributed to the sulfonic group, while the pK_2 corresponds to the amino group of the molecule. Calculation of the equivalent weight for this product gave us a value of 242, showing that in the described conditions of purification, the product crystallises with 2 molecules of water.

It is interesting to notice on the Fig. 3 that all the variations in the shape of the absorption curves occur between pH 4.5 and 7.5. The titration curve shows us on the other hand that the neutralisation of the amino group occurs in this pH range.



Consequently we may say that these modifications in the resonance of the pyrimidine fraction are induced only by changing the charge of

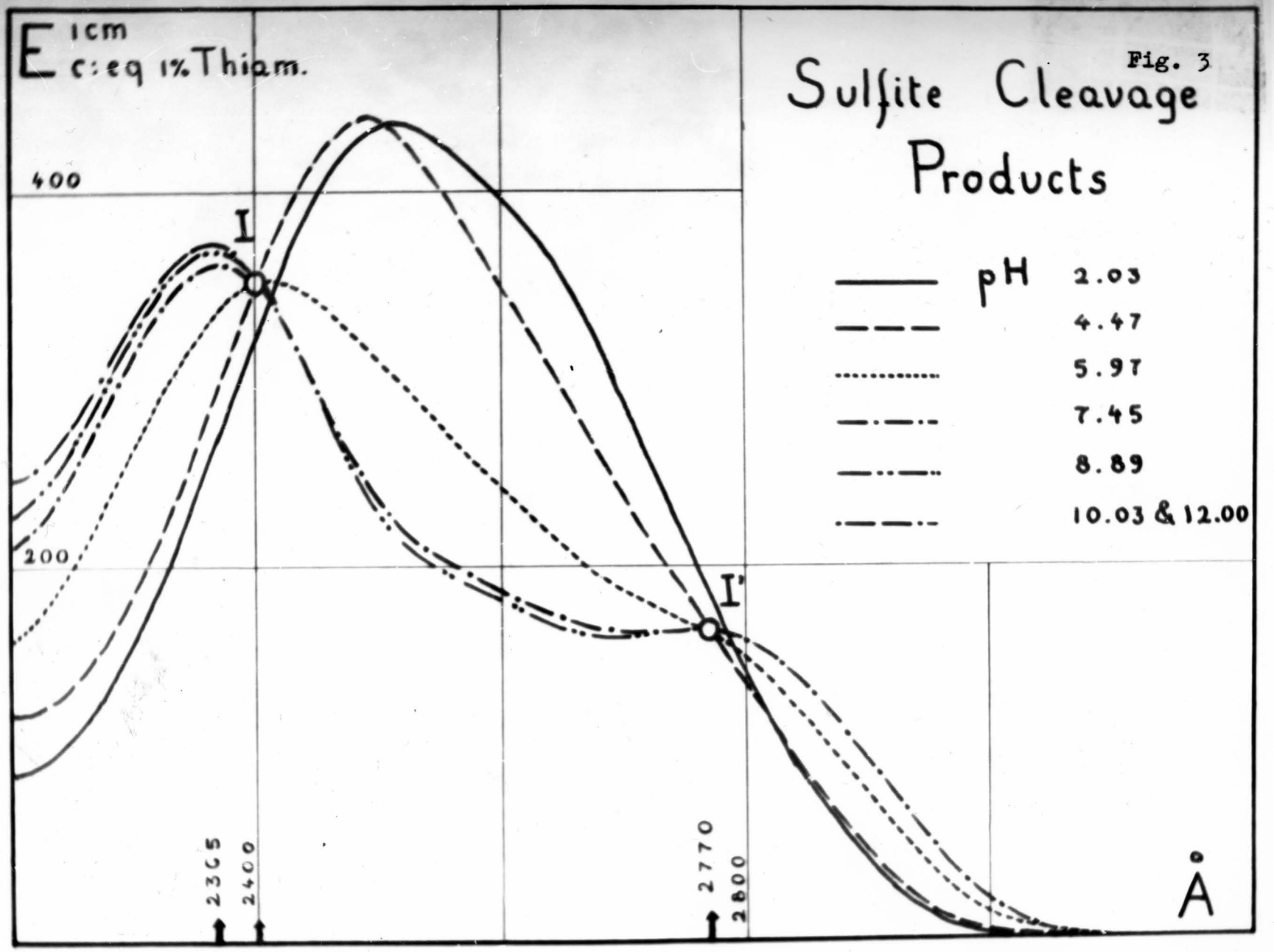

E 1cm c:eq 1% Thiam.
Fig. 2
400
200
N—C-CH3
HC C-CH2-CH2OH
S
pH 12 to 5
2.29
2400
2800
Å

2.03, 4.47, 5.97, 7.45, 8.89, 10.03, and 12.00. Great care must be taken in the preparation of the blanks for these solutions because they must contain sodium sulfite, and this compound also gives some variations in its absorption spectra when the hydrogen ion concentration of its solutions are changed. Therefore the pH of the blanks has to be adjusted as accurately as possible to the same value as the studied solution.

The absorption curves of these solutions are reproduced on Fig. 3. If we consider the curves which are obtained from pH 12.00 to pH 4.47, we notice that they are subject exactly to the same kind of variations as these which we have seen before, in the study of the pyrimidine ring. At low pH we find one maximum at the same wavelength, two maxima at high pH, and two isobestic points at 2400 and 2770 Å. As far as the extinction of these curves is concerned, each curve of the mixture, at any given pH, is equal to the sum of the curves of the separate fractions at the same pH value. Comparing the curve of the mixture at pH 4.47 with the curve at pH 2.03, we notice a shift to the right when the acidity is increased. This obviously reflects the shift of the thiazol fraction in this pH range.

d) Absorption curves of the equimolecular mixture of the cleavage products as a function of time.

We have shown previously[12] that the spectrophotometric characteristics of a basic solution of thiamine are subject to significant variations as a function of time. We were wondering whether


E 1cm
c: eq 1% Thiam.
Fig. 3
Sulfite Cleavage Products
pH 2.03
4.47
5.97
7.45
8.89
10.03 & 12.00
400
200
I
I'
2365
2400
2770
2800
Å

the cleavage products would also give similar variations, and therefore the absorption spectra of the equimolecular mixture were determined after increasing periods of time. As in the previous study, these solutions were kept at 0°C and in the darkness. The absorption curves were run after 2, 48, and 168 hours.

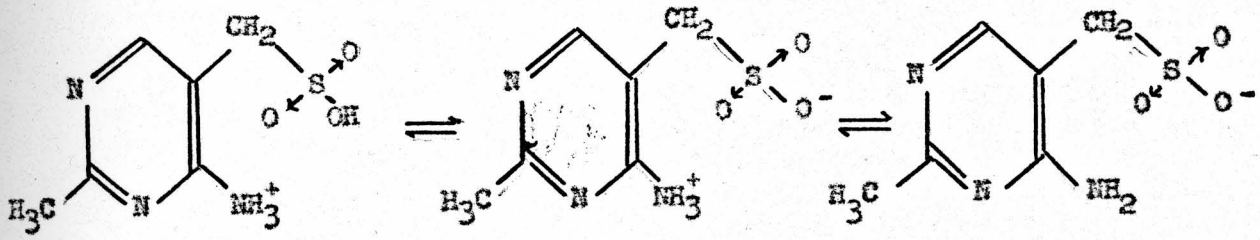
In no case were any variations observed and we may conclude that in the described experimental conditions the pyrimidine and the thiazol fractions of thiamine are perfectly stable. These curves will not be reproduced here since they are exactly similar to the curves of Fig. 3.

3) Interpretation- Application to the absorption curves of thiamine in acidic solution.

The existence of two isobestic points (Fig. 1) where all the curves of absorption of the 2 methyl, 4 amino-pyrimidyl, 5 methylene sulfonic acid cross, whatever the pH, suggests that the change in the absorption spectrum is due to an alteration in the position of equilibrium between two forms: an acidic form of the molecule, characterised with an absorption at 2476 Å and an alkaline form with two maxima and one minimum, respectively at 2350, 2775, and 2545 Å.

From the point of view of resonance the molecule in acidic solution appears to behave as one entity, while in basic solution, there are two centers of resonance corresponding to both maxima observed in these conditions. Between the acidic form which is found at low pH values and the basic form which is present in strongly

alkaline solution, we will thus have an equilibrium that we may represent in the following way:



acidic form zwitterion basic form

The titration curve of this compound is represented in the Fig. 4. It shows the presence of a strongly acidic group, the pK of which is below 1, and a second group much less acidic characterised by a pK of 6.5. The pK_1 of course is to be attributed to the sulfonic group, while the pK_2 corresponds to the amino group of the molecule. Calculation of the equivalent weight for this product gave us a value of 242, showing that in the described conditions of purification, the product crystallises with 2 molecules of water.

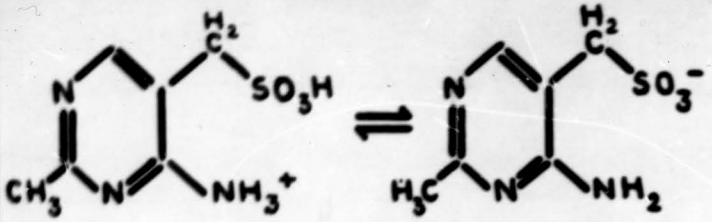
It is interesting to notice on the Fig. 3 that all the variations in the shape of the absorption curves occur between pH 4.5 and 7.5. The titration curve shows us on the other hand that the neutralisation of the amino group occurs in this pH range.

$$-NH_3^+ \rightleftharpoons -NH_2$$

Consequently we may say that these modifications in the resonance of the pyrimidine fraction are induced only by changing the charge of

Fig. 4

Titration Curve



$pK_1 < 1$

$pK_2 = 6.5$

the amino group in position 4, while the sulfonic group does not seem to have an influence.

This conclusion can be extended to the whole molecule of thiamine itself. It possesses indeed the same characteristic variations in the U.V. spectra (Fig. 5). As shown before[12], these modifications occur in the same conditions, in the same pH range and are also reversible. It can be said consequently that these reversible changes in the absorption curves of thiamine below pH 7, are a function of the charge of the amino group in the pyrimidine ring. This conclusion of course applies only to a solution of pure thiamine. As we will see in the next pages, an increase of the pH value above 7, leads to degradation of the thiazole ring, with subsequent modifications in the U.V. spectra.

4) Absorption curves of thiamine in basic solution.

It would be necessary before going on with further considerations, to refer to the previous study[12] and to summarize the aspects of the spectra of solutions of thiamine where the pH is raised above 7. The curves can be classed in two main groups:

a) The curves obtained from pH 7 to 9,

b) The curves obtained above pH 9.

In each of these groups, we should also consider the time effects, since above pH 7, the absorption curves of thiamine are subject to modifications, even when kept at a temperature very close to their freezing point.

a) In the pH range 7 to 9.

The typical curve obtained for instance at pH 7.9 is represented

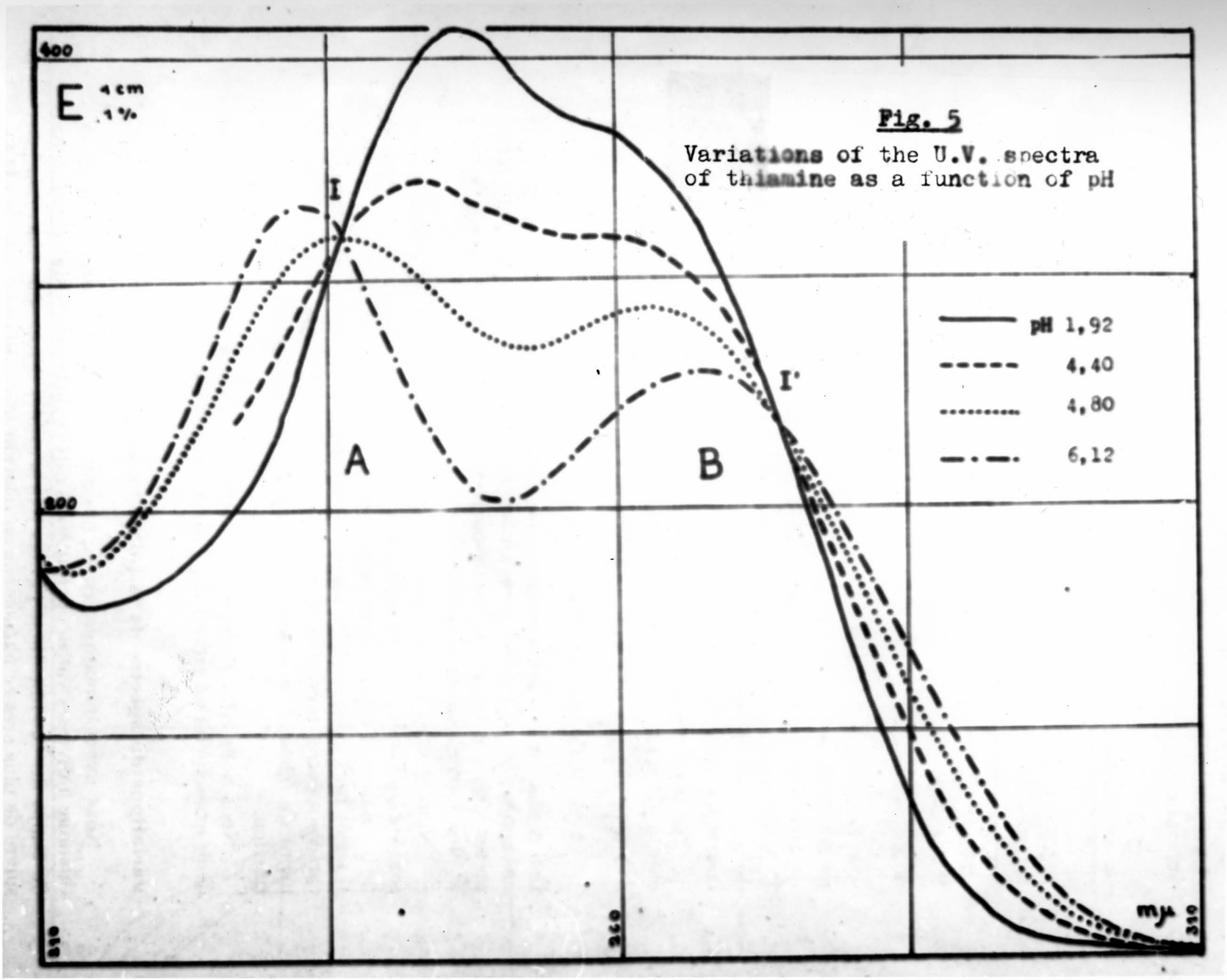


Fig. 5

Variations of the U.V. spectra of thiamine as a function of pH

in Fig. 6. If we increase the pH of such a solution, the maximum (A) shown to the left will remain unchanged, while the second peak (B) will decrease slowly. In this pH range the time effect is of the same nature. Such a solution, kept at 0°C for a few days, does not show any change in the maximum at 233 mμ, while the maximum at 268 mμ slowly decreases and tends to reach a more or less stable state which is represented by the lower curve.

b) Above pH 9.

Quite different modifications occur when thiamine solutions are brought above pH 9. Such solutions indeed show an immediate and very large increase of the extinction at 235 mμ. This increase however is very transient. It starts flattening rapidly within the first hours following the alcalinisation, more slowly afterwards, and finally the whole curve reaches the stabilisation after one month at 0°C. This is shown on Fig. 7.

5) Interpretation of these results

a) In the pH range 7 to 9.

It has been shown that these spectra represent the changes induced by the substitution of the Chlorine atom in the thiazole ring, a process which can be represented as follows:

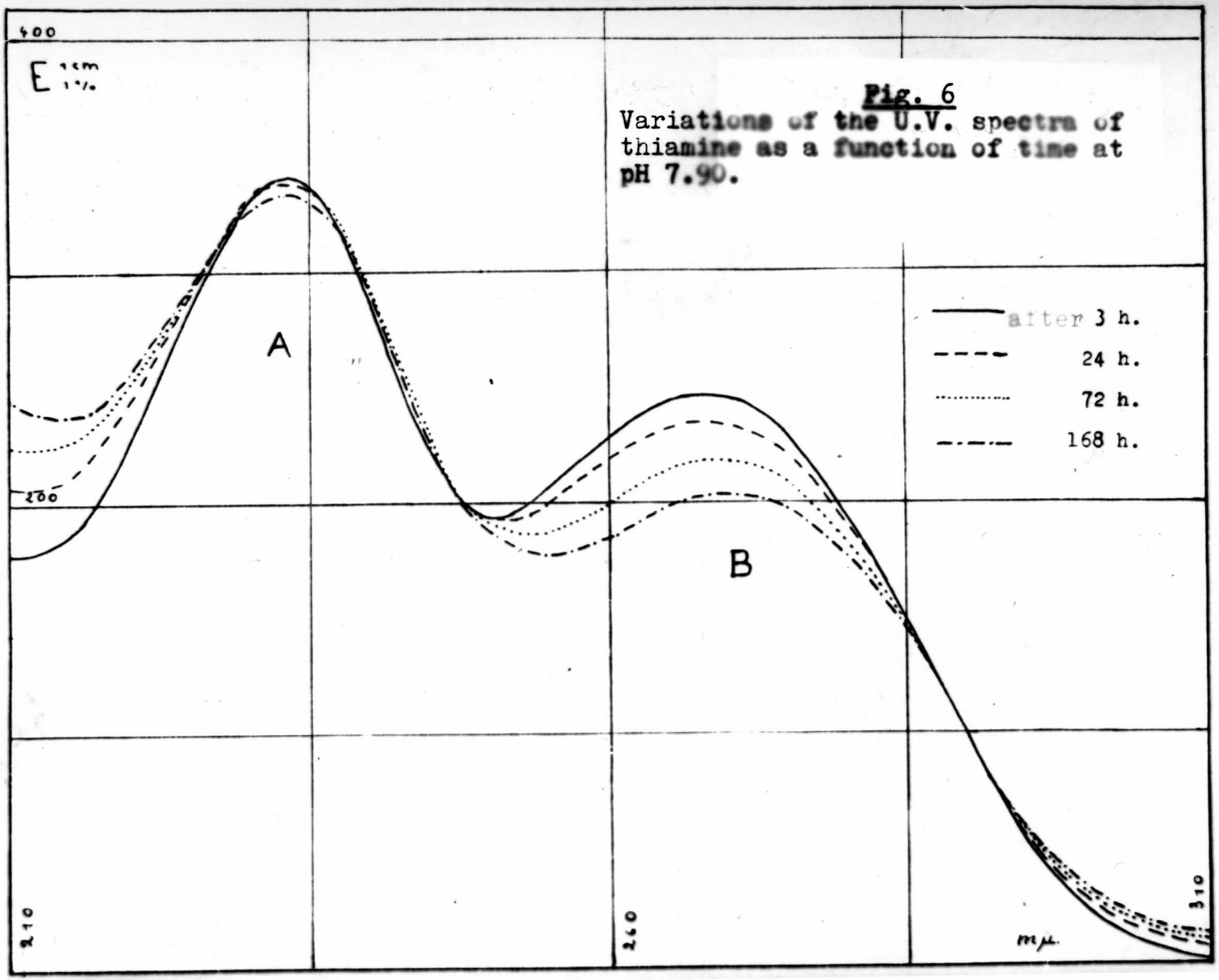


Fig. 6

Variations of the U.V. spectra of thiamine as a function of time at pH 7.90.

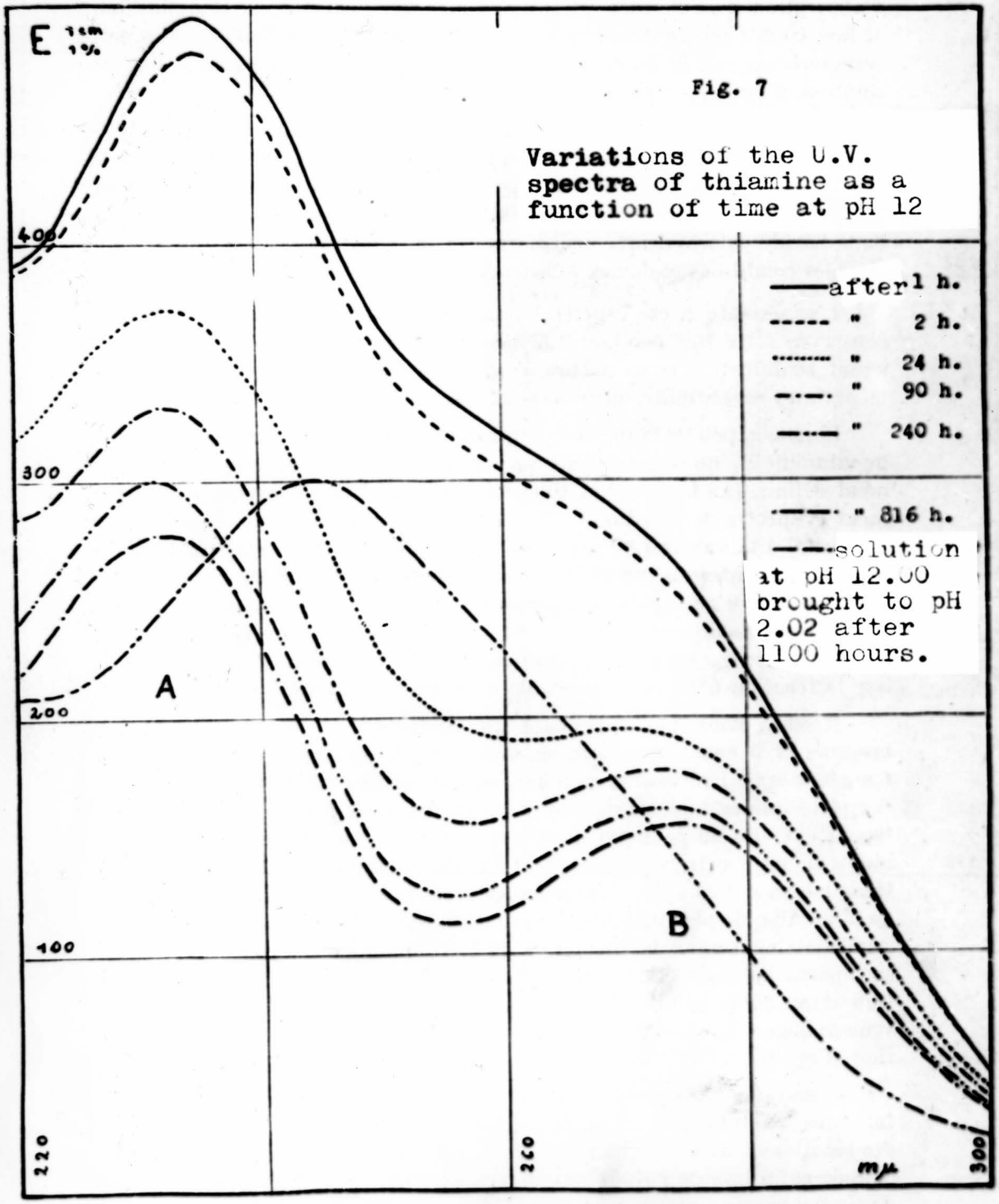
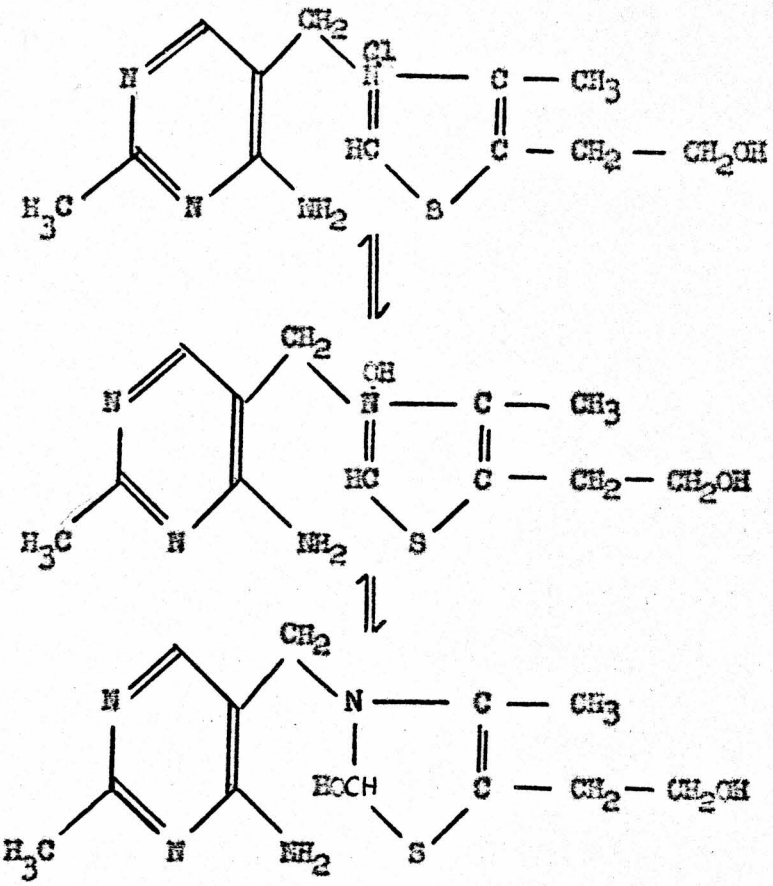


Fig. 7

Variations of the U.V. spectra of thiamine as a function of time at pH 12



b) Curves obtained above pH 9.

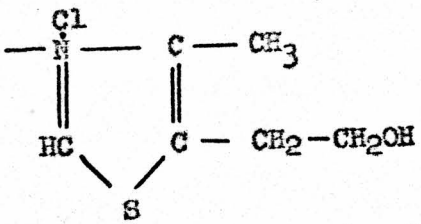
It is very interesting to compare the spectrum of the 2 methyl, 4 amino pyrimidyl, 5 methylene sulfonic acid at high pH, with that of an altered basic solution of thiamine. As we saw in the preceding paragraph, such a solution reaches, after a few weeks, a stabilisation state which is represented by the lower curve on the Fig. 7. This last curve presents two maxima and one minimum at the same position as the curve of the pyrimidine sulfonic acid at pH 12.05 (Fig. 1). Extinction values are also identical. By lowering the pH to 2.02 the altered solution of thiamine shows an absorption curve characterised by only one maximum at 247 mμ, and this curve again can practically be superimposed on the spectrum of the

pyrimidine sulfonic acid at low pH. Finally we must notice that both isobestic points can be found at the same wavelength and with the same extinction in both cases.

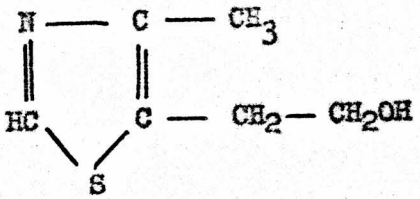
This leads us to the following conclusion: Above pH 9, a solution of thiamine which is kept for a sufficiently long time, will have its thiazol moiety quantitatively destroyed or at least quantitatively converted to products devoid of U.V. absorption. On the other hand, the pyrimidine fraction remains perfectly unaltered and can be found quantitatively in the degradated solution. Isolation of this product, for instance by paper chromatography, would probably allow us to determine whether or not there is some residue of the methylene bridge or of the thiazole ring linked to the carbon 5 of the pyrimidine fraction. In any case the previous experiments show that if it exists, it does not have any influence on the absorption properties of the pyrimidine ring. On the basis of these facts, it is theoretically possible to determine the initial concentration of thiamine in a basic solution where this vitamin has been destroyed. Indeed if we make sure that the pH is above 9, and that the spectra are stabilized, - i.e. that the thiazole fraction has been completely destroyed - a simple measurement of the extinction at 2394 or at 2762 Å will give us the concentration of the pyrimidine fraction and will allow us to calculate the original concentration of pure thiamine.

The perfect stability of a mixture of the cleavage products indicates the stability of its constituents and particularly that of the 4 methyl, 5 hydroxyethyl thiazole, even in a basic solution.

On the other hand if we keep thiamine exactly in the same conditions, its thiazole moiety will be quantitatively destroyed, while the pyrimidine fraction remains unaltered, as we have seen before. A simple comparison of the thiazole ring such as it is found in the vitamin with the 4 methyl 5 β hydroxyethyl thiazole indicates that the only difference lies in the nitrogen in the position 3.



Thiazole fraction as found in Thiamine
Unstable



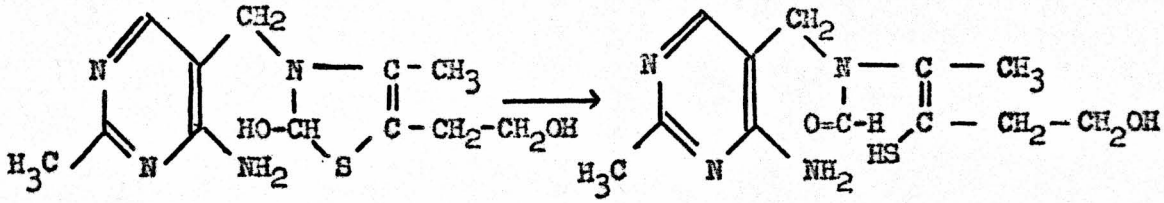
Thiazole fraction obtained by sulfite cleavage
Very Stable

In thiamine this Nitrogen is indeed a quaternary ammonium, while in the cleavage product, we find it as a normal trivalent Nitrogen. This difference makes the molecule of thiamine unstable and we would say that this Nitrogen in the Thiazole ring, may be considered to be the "Center of Instability" of the vitamin.

From the spectrophotometric point of view, a comparison of the absorption curves of thiamine (Fig. 5) with those of an equimolar mixture of the sulfite cleavage products (Fig. 3) indicates that this transition from N^{+5} to N^{+3} is made evident by an important decrease in the extinction values in the region of 260-270 mμ.

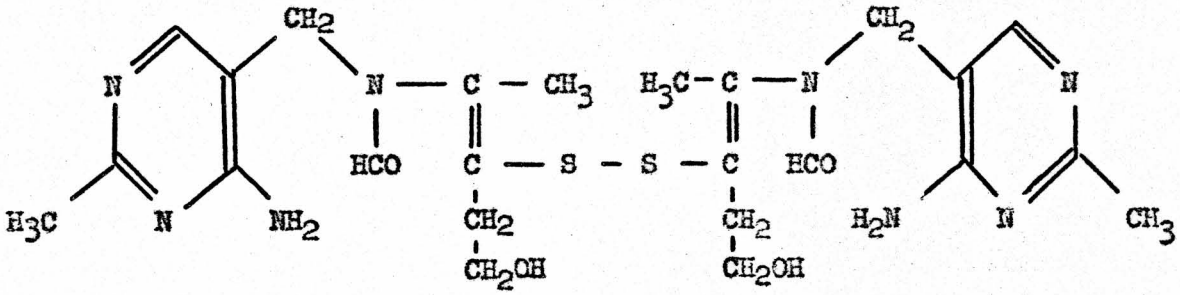
As far as the large increase at 235 mμ is concerned, we should remember that in a solution of thiamine at high pH (above 9), the thiazole ring is opened, and an aldehyde-sulfhydryl form is produced.

The reaction may be represented as follows:



Carbinol form — Aldehyde-Sulfhydryl form

On the other hand it is well known that -SH groups absorb in the region of 235 mμ[13, 14]. Consequently, the appearance of this high peak when the pH is increased is probably due to the formation of the -SH group, while the progressive flattening of the curve could be explained, for instance, by a subsequent oxidation giving rise to thiamine disulfide and then to other decomposition products.

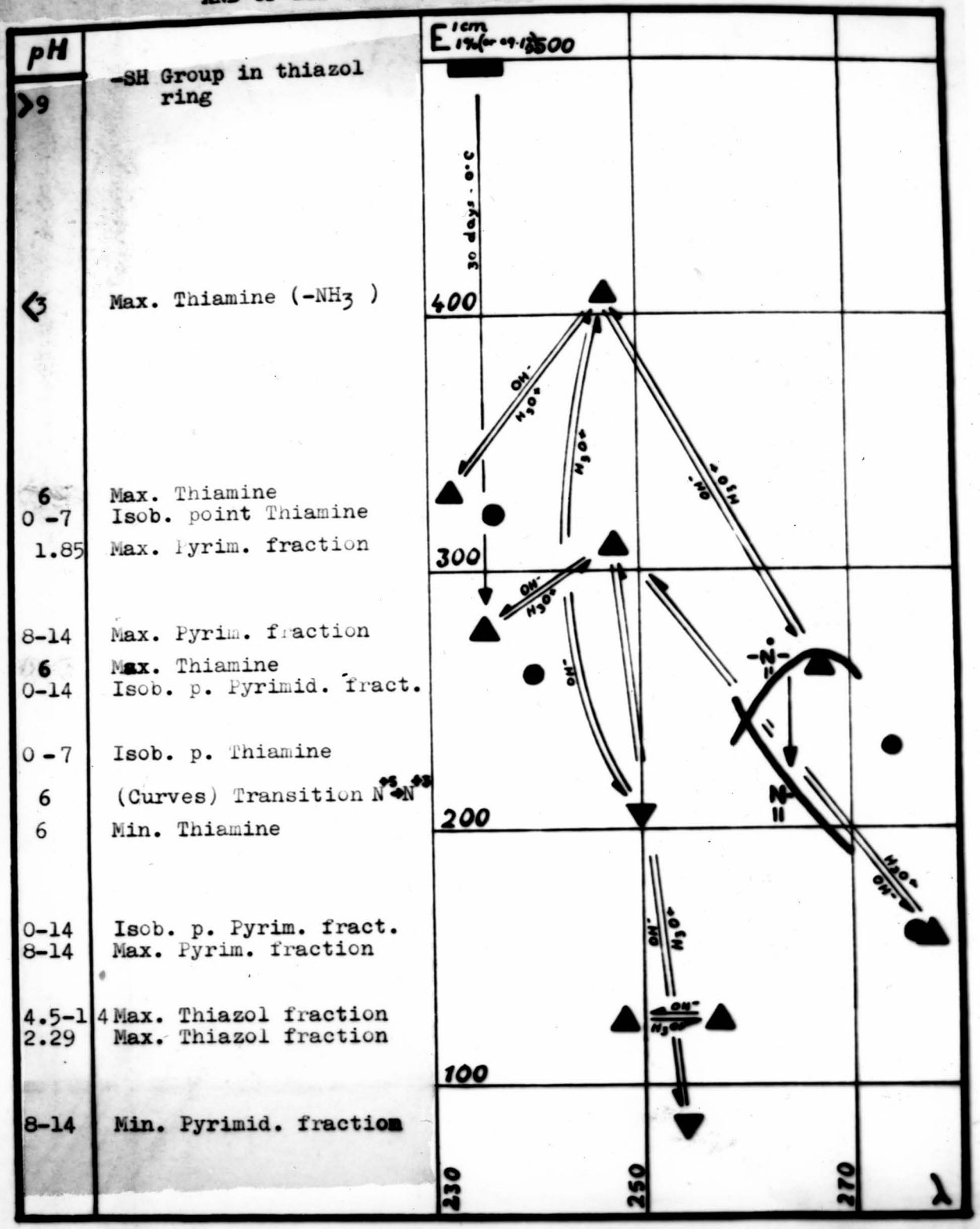


Thiamine Disulfide

Further evidence for this explanation will be given in the chromatographic part of this study.

The general scheme of the following page summarizes the significant points of the U.V. spectra of thiamine and of its sulfite cleavage products, the arrows indicating the interrelationships between all these points (Fig. 8).

SIGNIFICANT POINTS IN THE ULTRA-VIOLET SPECTRA OF THIAMINE AND OF ITS CLEAVAGE PRODUCTS



● Isobestic points ▲ Maxima ▼ Minima

FIG. 8

Part II. Chromatographic Study

The U.V. Spectrophotometry gives us useful information on the degradation process of thiamine, however many aspects are still to be studied.

We have seen that the degradation products originating from the thiazole ring seem to be devoid of U.V. absorption.

We do not know how many degradation products are formed at the various pH's.

We do not know if all these fragments are produced together or if they are formed successively in a sequence of reactions.

Do different basic salts give different kinds of degradations or is their pernicious action due only to their alkalinity?

How can a basic degradation process compare with a sulfite cleavage reaction?

These are all questions that arose and we thought that a good method of isolation of all these destruction compounds would be a great help in the further study.

1) Method.

Paper chromatography seemed a priori to be able to give some results. If one assumes that no polymerisation arises in the course of these reactions, one might expect to find products of relatively low molecular weight, and we know that paper chromatography is generally successful in the separation of these products. It possesses a very high sensitivity and allows us to run further chemical tests, either on the paper or on the eluted sample. The

ascending method was used since it generally provides less streaking than the descending one. The chambers used in these experiments were cabinets fabricated of air tight plastic bags (polyvinyl chloride), supported by an adequate glass frame. The paper used was Whatman No. 1.

a) Detection methods.

In order to detect the colorless spots, we needed appropriate reagents, and since most of the compounds we had to locate were unknown, we selected two kinds of reagents:

I/ Quite general reagents such as Tollens reagent or Potassium Permanganate which are able to reveal almost any organic compound.

II/ More specific tests providing some information on the presence of definite groups in the detected substances.

The various reagents which were used are listed in the following table.

Reagent	Detected Products	Method of Application
Potassium Permanganate[15]	Almost any organic compound	Spraying
Tollens Reagent	Reducing substances and a great deal of organic compounds	Spraying Drying at room T° or in an oven at 80°C
Bromephenol Blue/ Silver Nitrate[16]	Halides (pink) Purines and Pyrimidines (blue)	Spraying, drying at room T° and washing by immersion in dist. water.

Aniline Phthalate[17]	Glucosamines Reducing compounds Furfural Uronic acids	Spraying Heating at 110°C
Ehrlich[18]	Glucosamines	Spraying Heating at 30 - 40°C
2-4 dinitrophenyl Hydrazine[19]	Furfurals	Spraying Heating at 37°C for 2 h
Potassium Ferricyanide[20]	Thiamine	Spraying Observation in U.V. light
Tillmans Reagent[21]	Glucosamines Reducing Compounds	Spraying
Diazoreaction[22]	Thiamine	Spraying
N-Ethyl Maleinimide[23]	-SH Groups	Spraying with soln I Drying in air for 15 min. Spraying with soln II
Ninhydrin [24]	$-NH_2$ Groups in aliphatic chains	Spraying Drying in darkness for 24 hours
Triphenyl Tetrazolium Chloride[25]	Reducing Sugars	Spraying and keeping in moist atmosphere at 40°C for 2 hours Washing with water
Sodium Nitroprusside/ Sodium Cyanide [26]	-SH groups -S-S- groups	Spraying with soln I (-SH) Dipping in Soln II (-S-S-)

Table 2

In addition, we should say that a direct examination of a chromatogram in U.V. light at 366 mμ (Mineralight Long Wave U.V.- Model SL 3660) generally gives interesting information and sometimes allows us to immediately locate 4 or 5 spots.

b) Solvent systems.

The second point was to develop a solvent system which would allow us to fractionate as many products of degradation as possible.

Therefore, a 10% solution of thiamine in N KOH was boiled for 30 min. Under these drastic conditions, thiamine is transformed to an extremely complex mixture of degradation products. This mixture was used, because of its complexity, to find out a solvent system which would provide satisfactory separations, and would be used consequently in the study of degradation processes under much milder conditions. The various types of solvents which were used are given in the next table:

Solvent systems used for chromatography					
N Butanol	2	Ethyl Alcohol	1	Water	1 part (Vol.)
"	1	"	2	"	4
"	1	"	1	"	1
"	4	Glac. Acet. Acid	1	"	5
Iscamyl Alcohol	5	Pyridine	5	"	3
Phenol 80 gr.				" q.s. ad ml	100

Table 3

Most of these solvents are simple mixtures. Some of them however produce two different layers saturated with each other. In

this case the organic phase was always used for the chromatography itself, while the aqueous layer was reserved for saturating the chamber.

The mixtures N Butanol/Acetic acid/Water, 4/1/5, and N Butanol/Ethyl alcohol/Water, 2/1/1, have provided the most satisfactory results. We did not observe any tailing effect and in both cases the diffusion of the spots is negligible. The solvent Isoamyl alcohol/pyridine/water, 5/5/3, gave practically the same results as did N Butanol/Acetic acid/Water, 2/1/1.

We found that a combination of N Butanol/Acetic Acid/Water, 4/1/5, and N Butanol/Ethyl Alcohol/Water, 2/1/1, in a two dimensional chromatography was so far the best method for separating a highly complex mixture of degradation products of thiamine.

2) Effects of pH on the chromatographic behaviour of thiamine.

A 10% aqueous solution of thiamine hydrochloride (pH 2.4) was divided in 12 fractions. Increasing amounts of 9 N Sodium Hydroxide were added to these solutions, and the following series of pH values was obtained: 2.4, 4.1, 4.9, 6.4, 7.8, 8.5, 9.0, 9.5, 10.0, 11.2, 12.0, 13.0. Immediately after their preparation, 0.1 ml samples of each of these solutions were put on six different papers and run concurrently for 15 hours with the solvent N Butanol/Ethyl alcohol/water, 2/1/1. After drying, each of these chromatograms was observed in U.V. light and sprayed with different reagents. Parallely we ran Sodium Hydroxide, Sodium Chloride, Thiamine (free base), and Thiochrome as known reference compounds.

The results of these experiments are shown on Fig. 9. Direct examination of the dried chromatogram under U.V. light shows a row of bright blue fluorescent spots, all having exactly the same R_F (0.58) as Thiochrome. Repeated chromatographies with different solvent systems confirmed this fact. Under the described conditions, thiochrome was formed in very small amounts at pH 7.8, reached a maximum of intensity at pH 8.5 and 9.0, started to decrease progressively from pH 9.5 to reach finally the pH 13.0 where the spot is scarcely visible.

If such a paper is sprayed with a basic solution of Potassium Ferricyanide, the Thiamine molecule will be transformed into Thiochrome. It is interesting to note that this procedure reveals two different rows of spots, one in the acidic region at R_F 0.40 (B_1) and another in the basic region with R_F value of 0.46 (B_1'). Spraying another paper with Tollens reagent produced a brown coloration with all the spots having an R_F of 0.40 while the row of spots at R_F 0.46 remains uncolored.

Furthermore we see that at pH 8.5 the spots B_1' start appearing while the intensity of B_1 decreases slightly. At pH 9.0, both spots are approximately of the same size and at pH 9.5 all B_1 seems to be converted to B_1'. If we take a look at the titration curve of thiamine[12], it will indicate to us that the reaction occurring at this last pH is the conversion of the free thiamine to the carbinol form. This is an acceptable explanation, since the carbinol form can give the thiochrome reaction. Moreover, since its Chlorine atom

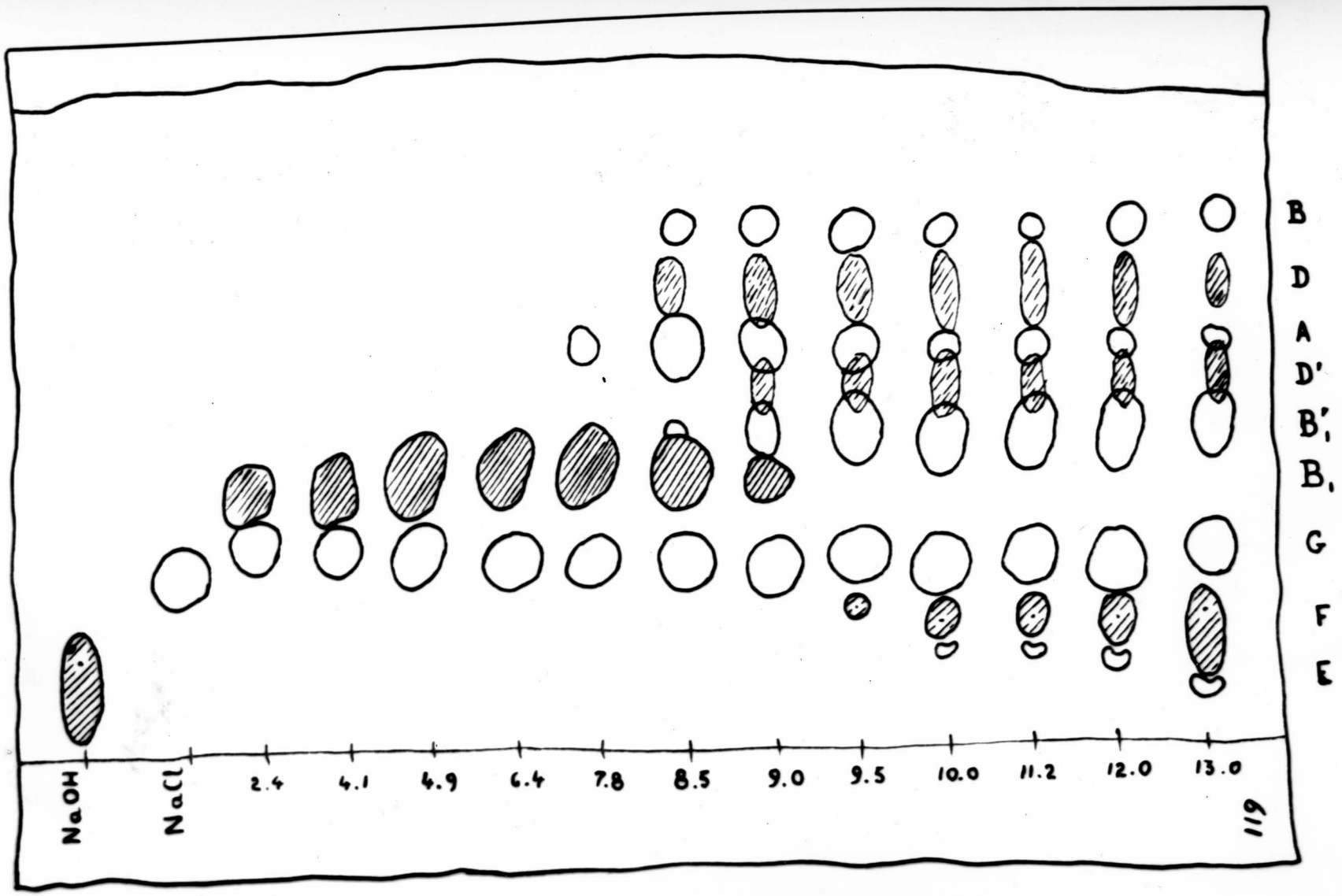


FIG. 9

Effects of pH on the chromatographic behaviour of thiamine.(Solvent system: N-Butanol,Ethyl alcohol, Water.-2,1,1.)

in the thiazol ring has been replaced by a hydroxyl group, this compound would not be expected to react with silver nitrate (Tollens), which was observed previously.

It is also interesting to note that thiamine hydrochloride is ionized in such a chromatographic system and that both fractions, the free base of thiamine and hydrochloric acid, more or less neutralised, move separately. Above pH 7.8, the whole row of spots "C" appears to be sodium chloride, as shown by its R_F (0.28) and its violet coloration with Tollens reagent. Below pH 7.8, all these spots still give a violet coloration showing the presence of the chlorine atom. In addition, as the pH is lowered, the intensity of the yellow coloration produced by spraying the paper with an acid-base indicator such as Brome-phenol blue, is increased. This allows us to conclude that at pH 2.4 (pure aqueous solution of thiamine hydrochloride) this spot "C" is hydrochloric acid and consequently that the upper spot giving the thiochrome reaction must be the free base of thiamine. It also appears that before the neutralisation occurs, both fractions have a tendency to stick together, what is due to their electrostatic attraction, while at higher pH's they can move quite independantly.

The spots "F", present in increasing concentrations from pH 9.5 to 13.0, appeared to be sodium hydroxide. This was shown by comparison with a standard and by spraying with acid-base indicators. The presence of sodium hydroxide above pH 9.5 is also in good agreement with the characteristics of the titration curve.

The upper spot "B" (R_F : 0.76) and the lower one "E" (R_F : 0.14) are still unknown. Spot "E" gives a blue fluorescence under U.V. light and a brown coloration with Tollens reagent. It is always present in the pH range 8 - 14. On the other hand, "E", which appears from pH 10 to 13, might bear some relation to the opening of the thiazol ring, which occurs as we saw previously above pH 9.

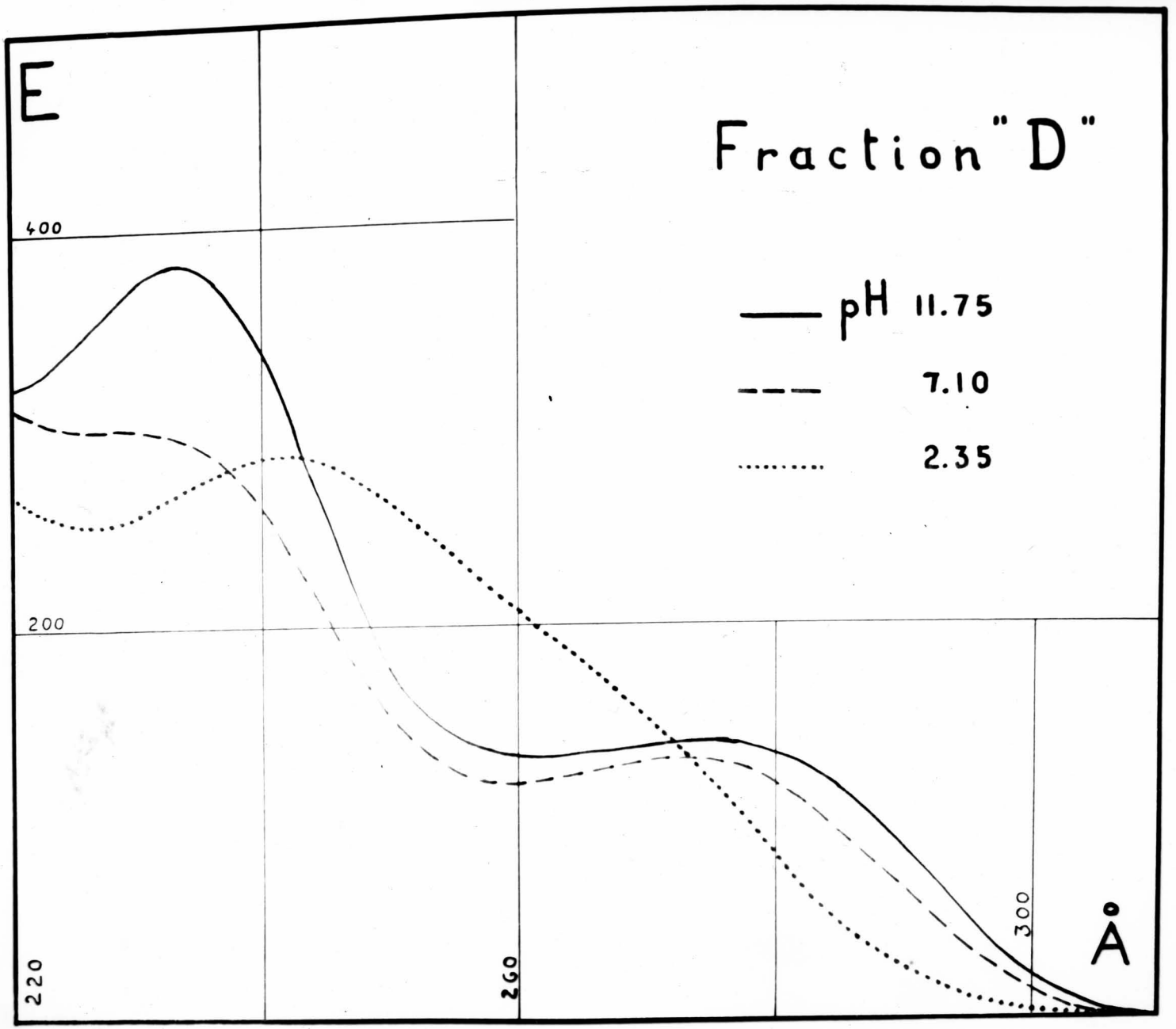
In the next experiment we sprayed the chromatogram with the reagent brome-phenol blue/silver nitrate which has been described as a reagent for purines and pyrimidines. After generous washing by dipping in distilled water, there remain on the chromatogram two rows of intense blue spots which did not appear before. We designated them D and D'. Their respective R_F's are 0.64 and 0.52, and they are separated by the spot of thiochrome. Spot "D'" partially interferes with the spot of the carbinol form and that of thiochrome, the color reactions however allow us to differentiate them immediately. Further information on the nature of "D" was obtained by spraying the paper with reagents for -SH and -S-S- groups. Copious wetting of the paper with sodium nitroprusside reagent does not give any evidence of -SH groups. However if we spray afterwards with the solution of sodium cyanide, we obtain a very bright red coloration of the spot "D", suggesting the presence of an -S-S- bridge.

Knowing the chromatographic behaviour of this spot, we were able to locate it by its R_F on a chromatogram that had not been sprayed. We cut the strip of paper corresponding to "D" and by

elution we obtained an aqueous solution of this compound. Three fractions of this solution were adjusted to pH 2.35, 7.10 and 11.75 respectively, and run on the spectrophotometer. The absorption curves (Fig. 10) suggest undoubtedly the presence of the pyrimidine ring in this molecule, as is shown by two maxima at 235 and 276 mμ in basic solution, and only one at 242 mμ in acidic media (We found the values 235, 277, and 247 for the pure pyrimidine fraction). Although the curves at pH 2.35 and 7.10 cross at two points very close to the values which we have established for the isobestic points of the pyrimidine fraction, the third curve at pH 11.75 does not pass through these points. This abnormal behaviour might be due either to an impurity or to a modification in the side chain, leading to spectrophotometric variations at high pH's.

On the basis of these results, the compound "D" appears to contain the pyrimidine ring and an -S-S- bridge. This suggests a substance such as thiamine disulfide which has already been mentioned in the spectrophotometric section. We would say however, that since the nitroprusside-cyanide reaction is not quite specific for -S-S- bridges, the above facts are not definite enough to confirm the presence of thiamine disulfide without any restriction. Further study and especially synthesis of this compound for comparison of the chromatographic behaviour would provide the final proof.

To sum up these experiments, we could say consequently that there is no appearance of thiamine destruction in the acidic range,


E
Fraction "D"
400
200
pH 11.75
7.10
2.35
220
260
300
Å

when the chromatography is run immediately after the preparation of the solution.

From pH 7 to 9, the chromatography as well as the titration curve, shows the transformation of the free base of thiamine to its carbinol form. In addition however, the chromatography indicates a partial transformation to thiochrome, especially at pH 8.5 and also to a very small amount of a pyrimidine derivative (D).

Above pH 9, we get destruction of thiamine. Under these conditions, there appear the carbinol form of thiamine, thiochrome, a pyrimidine derivative which might be thiamine disulfide, another purine or pyrimidine derivative and two unknown products. Their location on the chromatogram may be represented as follows:

R_F	Designation	Compound
0.76	B	Unknown
0.64	D	Pyrimidine derivative. May be thiamine disulfide
0.58	A	Thiochrome
0.52	D'	Purine or pyrimidine derivative
0.46	B_1'	Thiamine as carbinol form
0.40	B_1	Thiamine as a free base
0.28	C	Sodium Chloride
0.19	F	Sodium Hydroxide
0.14	E	Unknown

Table 4

3) Effects of time on the chromatographic behaviour of thiamine.

In the next experiment we decided to prepare solutions of thiamine at different pH's and to follow by chromatography the modifications of the vitamin as a function of time. Three 10% solutions were prepared and adjusted respectively to pH 6.54, 8.72, and 12.34 which represent characteristic points on the titration curve of vitamin B_1. These solutions were kept in an incubator at 37°C and chromatograms were run after 1, 2, 5, and 11 days. As usual we used the system N Butanol/Ethyl alcohol/Water, 2/1/1. Table 5 summarizes the results and shows the different products which were formed or destroyed, at the three selected pH values, as a function of time.

At pH 6.54, we found the spots corresponding to thiamine as a free base, sodium chloride and thiochrome. Over the 11 days period no change was observed at this pH value.

At pH 8.72, we did not notice any difference from the preceding case, except the normal equilibrium between the free base and the carbinol form of thiamine. After one day at 37°C, two unknown products, B and E were formed. After 5 days, we noticed the appearance of both pyrimidine derivatives and a new blue fluorescence at R_F 0.86 (G).

Finally, at pH 12.34, the decomposition rate was much higher. We found five products of decomposition immediately after the preparation of the solution, a new one after one day, and a second one after 5 days at 37°C. The carbinol form of thiamine was destroyed

Days (37°C)	Formed or destroyed ( ) products at pH		
	6.54	8.72	12.34
0	B_1	B_1	B_1'
		E_1'	
	A	A	A
	C	C	C
			B
			D
			D'
			F
1		B	
		E	E
2		D	(B_1')
		D'	
		G	
5			G
11			(A)
			(G)

A, Thiochrome (R_F: 0.58); B, Unknown (R_F: 0.76); B_1, Thiamine as a free base (R_F: 0.40); B_1', Thiamine as carbinol form (R_F: 0.46); C, Sodium Chloride (R_F: 0.28); D, Thiamine disulfide ? (R_F: 0.64); D', Purine or Pyrimidine derivative (R_F: 0.52); E, Unknown (R_F: 0.14); F, Sodium hydroxide (R_F: 0.19); G, Unknown (R_F: 0.86).

Table 5

after two days in these conditions and the same happened to thiochrome after eleven days.

Thiochrome appeared almost in all the experiments but while we found it in highest amounts at pH 8.5 and 9.0, it was progressively destroyed when the pH was increased.

Under very drastic conditions (Boiling a 10% solution in N Potassium Hydroxide for 30 min.), we could separate in a two dimensional chromatography 15 different spots. Since two of them must be Sodium hydroxide and Sodium chloride, there appear to be at least 13 different products of degradation. Five of these destruction products give the reactions of purines and pyrimidines.

This provides us a general view of the reactions of destruction of thiamine in basic solution. It is evident however that further identification of the still unknown products, determination of the relationships between these products, and quantitative study would be necessary to give a satisfactory explanation of these very intricate processes.

4) Mode of action of Dipotassium Phosphate.

Since Waibel, Bird and Beumann[3] have reported the deleterious action of dipotassium phosphate on thiamine, we wondered whether or not this reaction could be compared with the kind of degradation described for sodium hydroxide. Consequently we ran paper chromatograms with 10% solutions of thiamine adjusted to pH 6.54 and 8.13 by means of dipotassium phosphate. Concurrently we ran on the same paper thiamine solutions at the same concentration and

adjusted at the same pH, but this time with sodium hydroxide. The solutions were kept at 37°C and again chromatograms were run after 0, 1, 2, 5, and 11 days.

In no case were we able to show any difference between the degradation products obtained from dipotassium phosphate and those given by sodium hydroxide. Maybe there was some slight variations in terms of the amount of the degradation products produced by one method or by the other, but anyway, it was not perceptible.

Consequently, we may say that at least qualitatively, the effect of dipotassium phosphate is identical to that of sodium hydroxide at the same pH value.

SECTION II. REACTIONS OF THIAMINE WITH GLUCOSE.

Our attention was directed towards this question by the recent study of P. de Lange and L. P. v d Mijl Dekker[9] in Holland. These workers were able to show that by heating mixtures of thiamine and sugars, the content of both of these products was considerably reduced. They also noticed that there occurred an intense browning of the mixtures and that the pH of their aqueous solutions was progressively lowered. Concurrently the sample developed an intense fluorescence. Similar results were obtained with arabinose, but much more intensively.

We were interested to know whether or not this was a Maillard type reaction like those occurring between amino-acids and sugars[27].

We were also wondering if the described reaction could not be the result of an interaction of the hydrochloric acid of thiamine hydrochloride with glucose and subsequent destruction of thiamine by the formed products.

In order to investigate these questions, we decided to perform analogous experiments and to see what kind of information paper chromatography would provide.

Equal part mixtures of the following products were prepared and heated in sealed test tubes in an oven at 85°C for 13 days:

1) Thiamine Hydrochloride + Glucose
2) " " + Sucrose .
3) " as a free base + Glucose
4) " Mononitrate + Glucose
5) Diaminotoluene Dihydrochloride + Glucose

Samples were removed successively after 1, 2, 4, 9, and 13 days.

Thiamine as a free base was obtained by lyophilisation of a 10% solution of thiamine hydrochloride brought to pH 6.54 with 9N sodium hydroxide.

After 13 days of heating, the sample of the free base of thiamine / Glucose turned to a very dark black coloration. Thiamine hydrochloride / Glucose became brown, Thiamine mononitrate gave a light brown colour, while Thiamine hydrochloride / Sucrose gave no reaction at all. Diaminotoluene plus Glucose also took a darker coloration. Whenever these browning reactions occurred, they were accompanied by a noticeable hardening of the whole mass.

The fact that thiamine does not react with sucrose seems to indicate that the free aldehyde group would be required in the sugar. On the other hand, the large increase in the intensity of the interaction when the amino group of thiamine is free and the fact that diaminotoluene heated with glucose in the same conditions gave the same kind of reaction, suggest very markedly the participation of this amino group in the reaction.

It was specially interesting to follow by paper chromatography the reaction occurring between the free base of thiamine and glucose.

The products of reaction being perfectly soluble in water, 10% aqueous solutions were prepared with fresh mixtures, with mixtures having been heated for 2 days, and with samples heated for 13 days. Fractions of 0.1 ml of each solution were placed on Whatman No. 1

chromatographic papers, and the operation run for 15 hours with N Butanol/Ethyl alcohol/Water, 2/1/1, as a solvent. Six papers were run together in order to develop them with various spray reagents.

The pool of spots obtained is represented on the Fig. 11.

It appears from this chromatogram, that the concentration of thiamine is considerably reduced in the course of the heating period. The same is true with glucose. At the base of the paper a new compound, very soluble in water and giving some tailing effect is formed concurrently. This compound which possesses a very bright yellow fluorescence results from the reaction of thiamine with glucose as can be shown in the following manner.

We have seen in the preceding section that thiamine gives by destruction purine or pyrimidine derivatives characterised by a typical and very intense blue coloration when the paper is treated by the mixture brome-phenol blue/ silver nitrate and washed afterwards. Paper chromatograms prepared in the above described way were kept at room temperature for one month in order to allow thiamine to be progressively destroyed. After that period, the papers were sprayed with the brome-phenol blue / silver nitrate reagent, washed and dried. All the spots of thiamine appeared with an intense blue coloration, test wich was also given and with an equal high intensity, by the new lower spot (R_F: 0). This reaction shows that at least a large fraction of the thiamine molecule is combined in this new compound. Its R_F on the other hand is quite different from those of the purine or pyrimidine derivatives

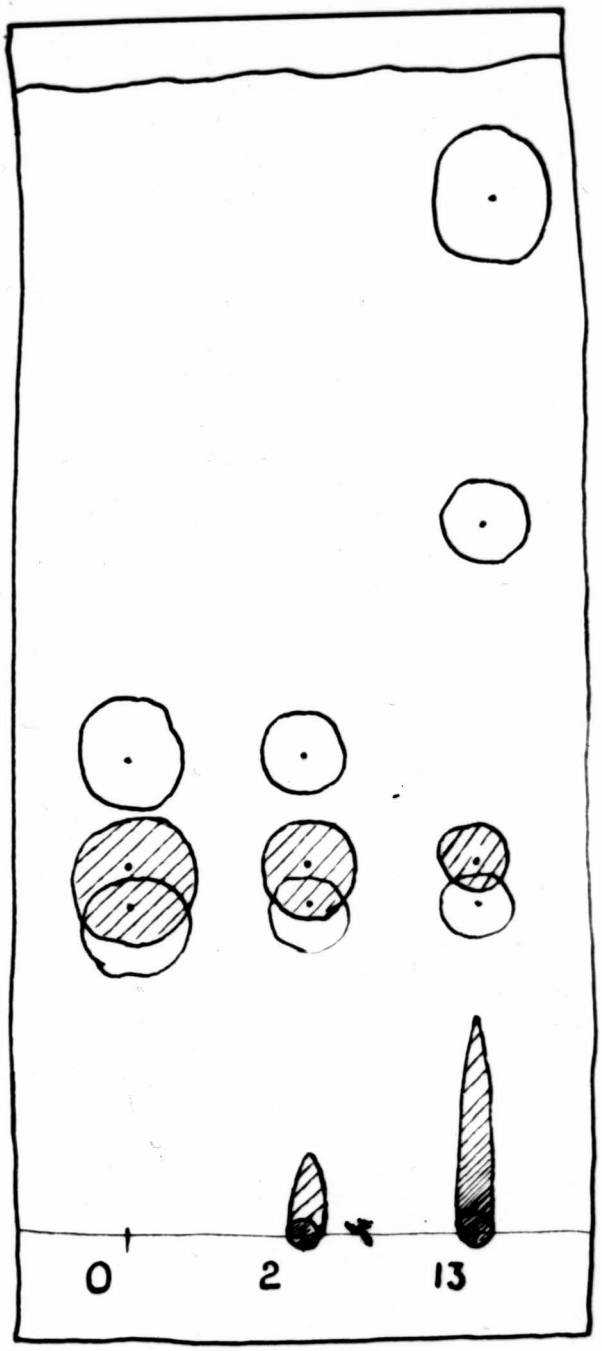


FIG. 11

Chromatogram showing the reaction Thiamine-Glucose
(Solvent System: N-Butanol, Ethyl alcohol, water, 2,1,1)

(0.52 and 0.64).

Secondary reactions are also involved in the whole process, at least two of which should be mentioned: Oxidation of minute amounts of thiamine to thiochrome and the formation of a new blue fluorescent product (R_F: 0.85). This new compound seems to be the same as the unknown substance "G" (R_F: 0.86) found in the basic degradation products.

Similar results are obtained by chromatography of the heated mixture of glucose with thiamine hydrochloride and thiamine mononitrate. Although the reaction is not as strong as in the preceding case, they show formation of high amounts of glucothiamine and parallel decrease on the concentrations of thiamine and glucose in the sample.

It is interesting to note that in 1934, some workers[28] had reported the stabilizing action of amino-acids on solutions containing thiamine. They claimed that the effect was lost when the amino group was acetylated but not when the carboxyl group was converted into an amino group. Such a statement seems now rather easy to explain since amino-acids would compete with thiamine in the Maillard reaction.

As a conclusion we would say that definitely there is a reaction between thiamine and glucose and that all the actual data indicate a Maillard type reaction.

SUMMARY

The present studies on the nonenzymatic destruction of thiamine can be summarized as follows:

A) SPECTROPHOTOMETRIC STUDIES.

1) The U.V. spectrophotometric properties of thiamine in the pH range 7 to 14 were discussed.

2) The influence of time on the absorption characteristics of these solutions was described.

3) In the same way were established the spectrophotometric properties of 2 methyl, 4 amino-pyrimidyl, 5 methylene sulfonic acid and of 4 methyl, 5 β hydroxyethyl thiazol.

4) It was shown that the particular properties of the isobestic points encountered in these curves can be applied to the identification and assay of these compounds.

5) In some cases, the absorption curves of a degradated solution of thiamine could be used to determine the concentration of the vitamin in the original solution.

6) The stability of the respective rings was discussed and the great influence of the quaternary ammonium on the stability of the thiamine molecule was established.

B) CHROMATOGRAPHIC STUDIES.

1) A paper chromatographic method for separating the destruction products of thiamine was developed.

2) This method was applied to the study of the effects of pH and time on the successive degradation steps of the vitamin.

3) Studies on freshly prepared basic solutions of thiamine have shown the existence of its carbinol form, thiochrome, a pyrimidine derivative which might be thiamine disulfide, another purine or pyrimidine derivative and two still unknown products.

4) Under more severe conditions the reaction continues and the number of destruction products increases to 13.

5) The mode of action of dipotassium phosphate is identical to that of sodium hydroxide at the same pH.

6) The chromatographic method can be used to prepare any of the degradation products of thiamine and to quantitatively determine the amount of vitamin B_1 in complex mixtures. (Pharmaceuticals....)

C) REACTION BETWEEN THIAMINE AND SUGARS.

1) There is a strong reaction between thiamine and glucose.

2) A brown compound, having a bright yellow fluorescence is produced. It can be isolated by paper chromatography. (R_F: 0 in the described conditions)

3) The data indicate a Maillard type reaction between the amino group of thiamine and the aldehyde group of the sugar.

LITERATURE CITED

1) Farrer, K. H. T. - J. Proc. Austral. Chem. Inst. 1941, 8, 113.

2) Beadle, B. W., Greenwood, D. A., and Kraybill, H. R. - J. Biol. Chem. 1943, 149, 339, 349.

3) Waibel, P. E., Bird, H. R., and Baumann, C. A. - J. Nutrition 1954, 52, 273.

4) Farrer, K. H. T. - Biochem. J. 1945, 39, 128.

5) Williams, R. R., Waterman, R. E., Kereszteszy, J. C., and Buchman, E. R. - J. Am. Chem. Soc. 1935, 57, 536.

6) Haseyawa, E., Sakamoto, S., Nagayama, K., and Fujita, A. - J. Vitaminol. 1956, 2, 31.

7) Kuhn, R. and Vetter, H. - Ber. deut. chem. Ges. 1935, 68, 2375.

8) Gero, E. - Bull. soc. chim. biol. 1954, 36, 1003.

9) De Langa, P. and v d Mijl Dekker, L. P. - Nature 1954, 173, 1040.

10) Kandutsch, A. A., and Baumann, C. A. - J. Nutrition 1953, 49, 209.

11) Philips Gloeilampenfabrieken - Dutch, 1955, 79, 470.

12) Lhoest, W. - Journ. pharm. Belg. 1956, 11-12, 495.

13) Noda, L. N., Kuby, S. A., and Lardy, H. A. - J. Am. Chem. Soc., 1953, 75, 913.

14) Oster, G. and Pollister, A. W. - Physical Techniques in Biol. Research. (Academic Press) 1955, Vol. I, 139.

15) Dagliesh, C. E. Nature. 1951, 166, 1076.

16) Wood, T. - Nature. 1955, 176, 175.

17) Baar, S. - Biochem. J. 1954, 58, 175.

18) Lea, C. H. and Hannan, R. S. - Nature. 1950, 165, 438.

19) Brady, O. L. and Elsmie, G. V. - Analyst. 1926, 51, 77.

20) Heyndrickx, A. - J. Am. Pharm. Assoc. - Sci. Ed. 1953, 42, 315.

21) Bessey, A. O. - J. Assoc. Offic. Agr. Chemists. 1944, 27, 537.

22) Smith, F. and Spriestersbach. - Nature, 1954, 174, 466.

23) Benesch, R., Benesch, R. E., Gutcho, M., and Laufer, L. - Science, 1956, 123, 981.

24) Moore, S. and Stein, W. H. - J. Biol. Chem. 1954, 211, 907.

25) Block, R. J., Durrum, E. L., and Zweig, G. - A Manual of paper chromatography and paper electrophoresis. (Academic Press) 1955, 133.

26) Aronoff, S. - Techniques of radiobiochemistry (The Iowa State College Press) 1956, 178.

27) Hodge, J. E. - Agric. Food Chemistry. 1953, 15, 928.

28) McIntire, F. C. and Frost, D. V. - J. Am. Chem. Soc. 1944, 66, 1317.

APPROVED _____

Dr. L. W. Busse
Associate Dean of Pharmacy

_____

Dr. C. A. Baumann
Professor of Biochemistry

DATE June 7, 1957