STUDIES ON THE ANTITHIAMINE FACTOR PRESENT IN *Phaseolus radiatus*

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (SCIENCE)
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THIS WORK

IS

DEDICATED

TO MY

BELOVED WIFE

"GAYATRI"

FOREWORD

The work embodied in this thesis submitted independently for the degree of Doctor of Philosophy (Science) of the University of North Bengal relates to the studies of antithiamine factor present in *Phaseolus radiatus.*

While presenting the work, I would like to express my deepest gratitude to Dr. T. K. Chatterjee, Registrar, North Bengal University for his continuous inspiration in course of the investigation.

I would also like to express my gratitude to the Head, all teachers, research scholars and staff of the department of Chemistry, North Bengal University for their helpful suggestions and cooperations during the work.

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Lastly, I am deeply indebted to the management of **'THE BIOCHEM'** —a biochemical laboratory at Siliguri for rendering me the facilities of doing some experiments of the present investigation.

Prasanta Kumar Mitra

(PRASANTA KUMAR MITRA)

CONTENTS

INTRODUCTION

INTRODUCTION

ANTIVITAMIN-CONCEPT

The exact meaning of the term antivitamin is controversial. According to the classical definition of Woolley (1) and Shaw (2), an active agent should be considered an antivitamin only when the following criteria are fulfilled :

a. Similarity of chemical structure.

b. Similarity of symptoms produced by the antivitamin and by the lack of the corresponding vitamin.

c. Competitive in the effect with respect to the vitamin.

However, a number of compounds that are antivitamins do not fulfil one or more of these criteria. It seemed necessary to extend the definition of antivitamin without the extension of the concept, a great number of substances - nearly all naturally occurring vitamin antagonists - could not be considered antivitamins, e.g. avidin, the antithiamine factors of fern, carp, rice bran, mustard seed and certain antagonists of pyridoxin and vitamin D. Some of these were the very first active agents that were reported showing biological antagonism.

Somogyi (3) subsequently proposed to divide the antimetabolites and antivitamins into two groups : structurally similar compounds i.e. antivitamins as a specific type of antimetabolite, and structure modifying antivitamins i.e. substances, mainly of biological origin, that destroy or decrease the effect of a vitamin as by modifying the molecule itself or forming complexes with vitamin. This classification parallels that used for the inhibitors in enzymology. The structurally similar antivitamins

correspond to the competitive inhibitors, and the structure modifying antivitamins to the noncompetitive inhibitors.

Accordingly, an antivitamin may be defined as a compound that diminishes or abolishes the effect of a vitamin in a specific way.

Mellanby (4), in 1926, was the first to report an antivitamin action of certain cereals to be antagonistic to vitamin D. Similarly, avidin (present in egg white) was demonstrated having antagonistic action against biotin by several workers (5,6,7). Later on, the presence of antivitamin compounds was further established when Green (8) described the presence of antithiamine compound in the viseera of raw fish, and Woods (9) proposed the mechanism of action of the sulfonamides as antagonists of p-amino benzoic acid.

ANTAGONISTS OF THIAMINE

The conception of thiamine antagonists appeared in the literature as early as 1936 when Green (8,10) for the first time described the so-called 'Chastek paralysis' as a thiamine deficiency that appeared in silver foxes eating raw fish. As a continuation of this work Evans et al (11) then demonstrated that the so-called 'Chastek paralysis' of foxes could be prevented by administration of 10 mg of thiamine per day. Similar thiamine dificiency condition was also observed in chick when raw fish was given to them (12) — and even incubation of thiamine with raw carp fish intestine for fifteen minutes resulted in a lose of 50-100% of the biological activity of thiamine. Seelock and Goodland (13) confirmed the enzymatic nature of this toxic factor present in fish. The destructive nature of this factor was inhibited by a number of metal ions such as Cu^{++}, Zn^{++} etc. and certain organic compounds, as for example, iodoacetic acid, cysteine etc. According to Krampitz and Woolley (14) the isolated enzyme from the fish consisted of two parts-one was heat-stable, dialysable and the other was heat labile, non-dialysable. This enzyme was later termed as "Thiaminase" by them. Acute thiamine deficiency was induced in adult sheep by inclusion of low temperature dried, milled bracken rhizomes in diet which was a potent source of thiaminase (15). Autoclaving the rhizomes powder its avitaminosis B_1 capacity could be abolished.

Although antithiamine factors occur predominantly in the viscera of carp (cyprinidiae), these are also found in betel-nuts and in other fresh water and marine fish (16). Thus, antithiamine compounds were recognised by Deutsch and Hasler (17) in 15 of 21 species of fresh water fish (including 4 species of the carp family), by Neilands (18) in fish of the water of Nova Scotia and by Jacabson and Azevedo (19) in fish from the water of Portugal. Twenty species of fish available in Burma, two species of calms, crusteaceaus and their products were analysed for enzymic and thermostable antithiamine activity (20). Viscera had higher antithiamine activity than that of muscle of those fishes. Thiaminase was present in viscera of 4 species of fish tested whereas the heat stable thiamine inactivating factor was present in only two species. It was shown that the occurrence of heat labile thiaminase in fishes was much higher than that of heat stable antithiamine factors still not characterised. The thiamine activity of Crayfish (21) and Skip jack Tuna (22) was destroyed on cooking

and drying as well as on cold storage. Besides, the viscera of the carp fish, its spleen, liver, heart muscle and intestines also possess the antithiamine activity (23, 24).

Sure and Ford (25) detected the decomposition of thiamine by animal tissues as early as 1943. But, for many years there was a general feeling that no antithiamine compounds were present in the organ of warm blooded animals. According to Bojo (26), negative results were obtained from testing the tissues of pigeons, guinea-pigs and cattle. Somogyi (27) was able to recognize antithiamine activity in organs of rabbits and hens, particularly in spleen and heart muscle extracts. In contrast to carp, the intestinal extracts of warm blooded animals activated thiamine only to a very slight degree. Striated muscles from these animals also showed no antithiamine activity.

In an extensive study Bhagvat and Devi (28) detected the presence of thiamine-inactivating factors in different food-stuffs viz. Ragi *(Eleusine coracana)*, rice-polishing, a kind of bean *Phaseolus radiatus*, bazra *(Penniesteum typhodium)*, wheet germ, soyabean *(Glycine hispide)*, cowpea *(Vigna catiang)*, cotton seed *(Gossipium Sp.)*. mustard seed *(Brassica juncea)*, linceed etc. The activity was confirmed by thiochrome method as well as experiments (29) with mosquito larvae, rats and guinea pigs. The nonenzymatic nature of the thiamine inactivating factors present in these food-stuffs was also predicted by them. They showed that the extract obtained with 5% chloroform-water mixture from ragi was resolved into two components on dialysis - one was heat stable, dialysable and the other was heat labile, non-dialysable. Chaudhuri (30) also confirmed the presence of a heat stable thiamine inactivating factor in different varieties of rice and rice-bran.

Weswig and co-workers (31) produced experimental 'fern poisoning' by feeding bracken fern *(Pteridium equilinum)* to rats, just as it occurs in cattle and sheep. High thiamine doses cured the sick animals. Watanabe (32) and Parsons (33)separately demonstrated reduced thiamine excretion in human subjects given 15-20 g bracken fern per day. Haag et al (34) showed that the antithiamine factor of bracken fern was very stable to heat, it was water soluble but was not soluble in ether, petroleum ether, acetone or ethanol. Inactivation of thiamine by bracken fern extract produced rapidly (35). Further studies by various investigators showed that the bracken fern extract inactivates thiamine in vitro (36), that bracken fern has higher activity than other ferns (37-39) and that the thiamine antagonist of ferns is a small, thermostable molecule that, in electric field, migrates exclusively towards the anode (40).

Moore (41) observed the paralysis of legs of swine by feeding a diet containing mostly of rice- bran. Williams (42) studied the effect of commercial byproducts of rice-milling on pigeons and rats and found that rice-milling byproducts were harmful to pigeons and rats when fed a diet cor taining more than 65% of these products. He then predicted the presence of a thiamine-inactivating fac or in rice-milling byproduct. The existence of toxic substances in wheat-germ and other wheat products, which caused nervous disorders was recorded by McCollum (43) as well as Hart et al (44). Rommel and Vedder (45) also observed cotton seed poisoning in swine. A thiamine-inactivating effect was also detected in black berries, blue berries, black currants, red cicerone, red beets, brussel sprouts and red cabbage ; somewhat smaller amounts were found in watercress, green cicerone, kefen, spinach and black cherries (46). Hilker et al (47) made some extensive studies on thiamine-

inactivating factors present in various types of tea such as jasmine, colong, naganium, shui, black tea and instant tea. These workers demonstrated that thiamine-inactivating factors of these teas appeared to be related to the tanin content.

Although the presence of antithiamine compounds in different food-stuffs and other sources was established, the exact mechanism of inactivation of the vitamin is still not clear. The thiamine inactivating factors in the natural sources may also be called antithiamine compounds. According to Chaudhuri and his associates (48), the antithiamine compounds may be classified broadly into two categories, such as :

(a) Synthetic (structural analogues or antimetabolities)

(b) Natural (non-structural analogues and mostly present in different food-stuffs)

(a) Synthetic antithiamine compounds

Most of these antithiamine compounds in this category are structural analogues to the thiamine molecule (49,50). The mode of actions of these compounds towards the inactivation of thiamine are somewhat different from that of natural antithiamines (51, 52). Different types of structural analogues of thiamine molecule behaving as antithiamine compounds are given below.

Pyrithiamine type (53)

Replacement of the thiazole moiety in the thiamine molecule gives an important structurally analogue known as pyrithiamine in which a pyridine ring is attached to the pyrimidine through the methylene bridge. It was found that this compound played an important double inhibitory role on (i) thiamine absorption (54) as well as (ii) thiamine phosphorylation (55) *in vivo*. Several enzymes such as pyruvic decarboxylase from wheat germ (49) and yeast (56, 57) as well as acetoin synthetase (58) were inhibited by pyrithiamine pyrophosphate derivative. Pyrithiamine can also penetrate the brain tissue as a result of which there is the blocking of thiamine pyrophosphate biosynthesis.

In the cell free extract of *S. aureus*, thiaminokinase was strongly inhibited by pyrithaimine resulting in a deficiency of thiamine pyrophosphate in the system (59). Das and Chatterjee (60) prepared a pyrithiamine dependent mutant strain of *S. aureus* and its enzymatic pattern was also studied.

Oxythiamine type (61)

The replacement of amino group at the 6-position of thiamine molecule by a hydroxyl group gives rise to an active antagonist called oxythiamine, which gives deficiency symptoms in mice. Similar to oxythiamine its pyrophosphate derivative is also potent inhibitor of thiamine pyrophosphate requiring enzymes such as wheat-germ carboxylase and acetoin synthetase (62). The conversion of thiamine to thiamine pyrophosphate was less affected by oxythiamine (63). The growth of thiamine requiring bacterial species such as *Lactobacillus fermentii* and *Kloeckera brevis* was affected only at a high dose of oxythiamine (64, 65). Oxythiamine had a greater toxic effect to *S. aureus* in comparison to pyrithiamine (66).

Amproleum type

Several antithiamine compounds belonging to this group (67, 68) have been recorded. Amproleums have similar structure as pyrithiamine. Due to the absence of β-hydroxy chain in these compounds, they can't form their pyrophosphate derivatives. The absorption of thiamine was affected by amproleum in chick (69). In presence of amproleum, thiamine was unable to penetrate the cell membrane, consequently the phosphorylation of thiamine by thiamine kinase was inhibited resulting ultimately in the inactivation of thiamine biologically (70, 71).

Deoxy and ethyl deoxy thiamine

These compounds showed thiamine antagonistic activity (72). The biological activity of these compounds, which are structural analogues of thiamine, were tested on the growth of thiamine requiring L. *fermentii* and *Kloeckera apiculata.*

O-benzoyl thiamine and its derivatives

The antithiamine activity of the structural analogues such as 0-benzoyl thiamine (73), 0-S-dibenzoyl thiamine (74), O-p-nitro benzoyl thiamine and 0-p-methoxy benzoyl thiamine were studied extensively (75) using *L. fermentii* as experimental organism.

Butyl thiamine

If the methyl group is replaced in the pyrimidine ring by a butyl radical, an active antagonist is produced, capable of causing in rats signs of vitamin deficiency (76) which can be reversed by thiamine. This is an interesting phenomenon since inhibition is not caused by substitution with groups of less than 4 carbon atoms. Thus although ethyl homologue shows vitamin B_1 activity, the n-propyl homologue shows little activity either as a vitamin or as an antivitamin.

Phenyl triazinothiamine

The growth inhibition of *Kloeckera apiculata* by phenyl triazinothiamine was not recovered by addition of thiamine-hydrochloride (77). Phenyl triazinothiamine in the broth hydrolysed to pheyl hydrazine which inhibited the growth of the organism.

Imiodazole thiamine and benzoyl imidazole thiamine

The growth of *K. apiculata* in the broth containing thiamine and imidazole thiamine or benzoyl imidazole thiamine was restricted because of the strong inhibition of thiamine uptake by imidazole or benzoyl imidazole compound (78).

(b) Natural antithiamine compounds

Natural antithiamine compounds are further classified into two groups mainly,

1) Natural antithiamine compounds of large molecule

2) Natural antithiamine compounds of small molecule

1) Natural antithiamine compounds of large molecule

These antithiamine compounds are protein in nature (either enzyme-thiaminase or protein)and present in fishes, bacteria as well as in some seeds.

Two types of thiaminase were isolated from different sources, viz.,

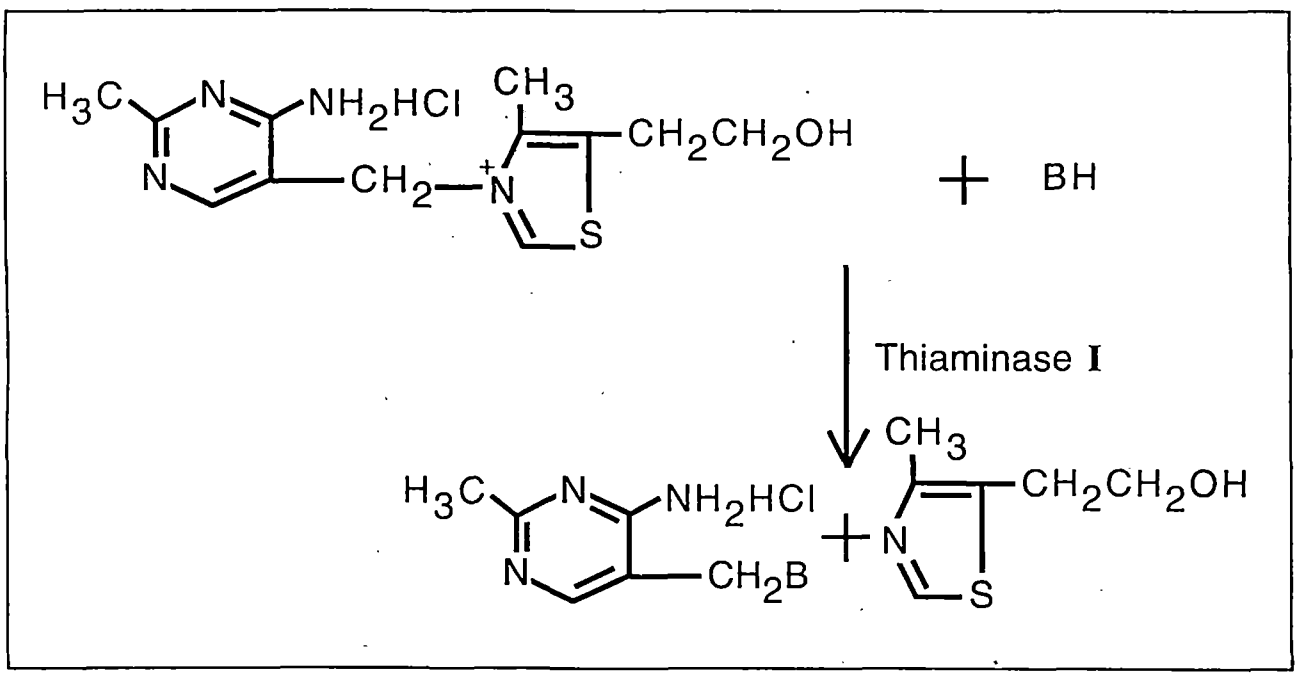
(i) Thiaminase I (79, 80)

(ii) Thiaminase II (81, 82)

(i) Thiaminase I

Matsukawa and Misawa (83,84) isolated one such organism from human feces and named it *Bacillus thiaminolyticus* - this was supposed to contain a 'thiaminase I'. Later, Kimura and Liao (85) discovered another agent in feces that produces ' thiaminase I' : *Clostridium thiaminolyticum*. This enzyme was also isolated from the viscera of fresh water fish, shell fish and bracken fern (86).

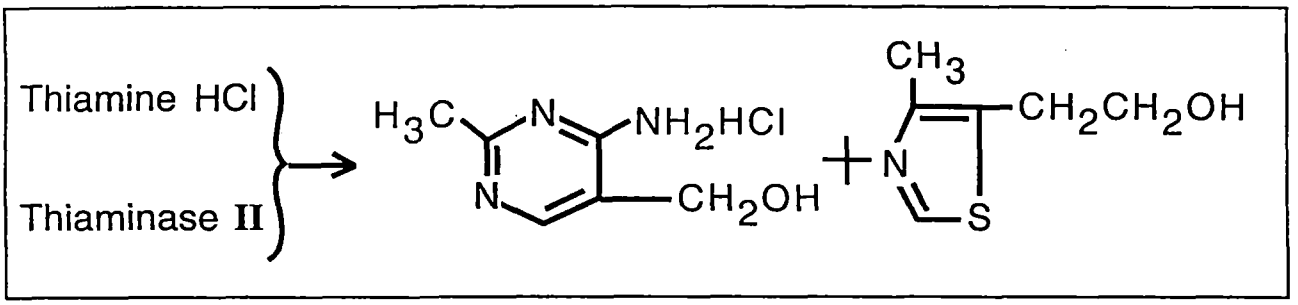
Thiaminase I catalyses a base-exchange reaction, whereby the thiazole moiety of thiamine is replaced by another base (e. g. an amine) and the vitamin activity is lost. The reaction mechanism is given below.



The thiaminase I of *B. thiaminolyticus*, according to Douthit and Airth (87) is predominantly extracellular. The formation of thiaminase I (i. e. the increase of antithiamine activity) was inhibited by the addition of thiamine (88). Further purification and characterization of this thiaminase have been undertaken by Wittlife and Airth (89). These authors demonstrated a method for the determination of thiaminase I activity where aniline was used as a base. The formation of the product as an aniline derivative with pyrimidine moiety was measured spectrophotometrically by an increase of optical density at 248 nm.

(ii) Thiaminase II

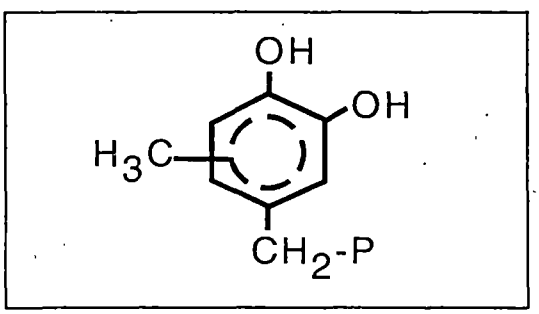
Kimura and Aoyama (90) isolated from hay and soil one type of thiamine degrading bacterium viz. *Bacillus aneurolyticus* which produces thiaminase II. It is also present in some yeasts, bacteria and fungi (91). Thiamine is hydrolysed in presence of thiaminase II without any base requirement according to the following reaction.



Somogyi et al (92, 93) isolated from carp viscera a protein other than the enzyme thiaminase with antithiamine activity and designated it as substance K having molecular weight in the range of 75,000 - 100,000. It contained a thermostable part similar to hemin.

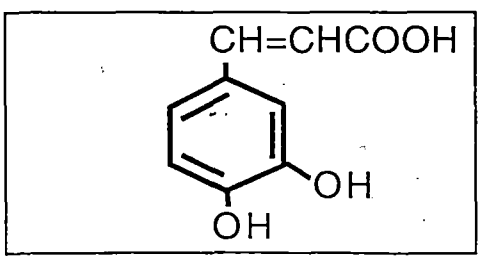
(2) Natural antithiamine compounds of small molecule

The existence of natural antithiamine compounds of small molecule was established in the literature when Chaudhuri (94), for the first time, isolated from rice-bran a heat stable simple organic molecule having antithiamine activity. De and Chaudhuri (95) identified it as a glucoside and named it 'compound X'. Subsequently the active principle of this glucoside (compound X) designated as 'Fraction A' which was separated and partly characterized as ortho dihydroxy phenolic compound (96) is represented below,

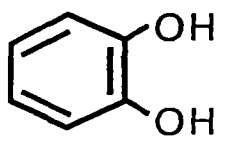


where P-an aliphatic side chain containing hydroxyl and carboxylic acid groups. The effect of fraction A and compound X on transketolase enzyme system and on the growth of *S. aureus* was also studied by these workers (loc. cit).

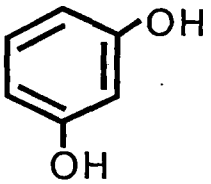
Later on, Somogyi and Beruter (97) isolated an antithiamine factor from fern *(Pteridicum aquilium)* and characterised it as caffeic acid, the structure of which is given below.



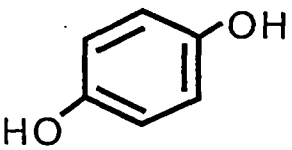
In the investigation of the mechanism of thiamine inactivation by caffeic acid, Davis and Somogyi (98) proposed that the reaction consists of two phases. The first phase was reversible, independent of pH and temperature whereas the second one was mostly an irreversible phenomenon. Somogyi and Bonicke (99) then carried out the experiments with several different phenolic compounds and postulated that ortho and pare dihydroxy phenolic compounds had maximum and moderate antithiamine activity respectively whereas meta dihydroxy phenolic compounds had no antithiamine activity as represented below.



Ortho—



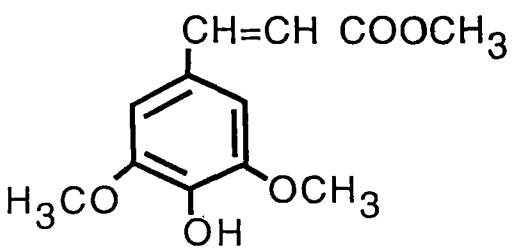
Meta—



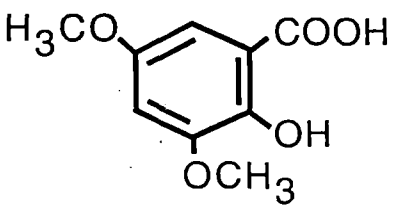
Para—

Hilker (100) also isolated caffeic acid from blue berries by a different method. Bonicke et al (101)showed that antithiamine compounds isolated from coffee were caffeic acid, chlorogenic acid and pyrocatechins.

Chaudhuri and his associates isolated two new antithiamine compounds characterised as methyl sinapate (102) and 3, 5 dimethoxy salicylic acid (103) from mustard seed *(Brassica Juncea)* and cotton seed *(Bombex melabericum)* respectively. The structure of these compounds is given below.



Methyl sinapate



3, 5 - dimethoxy salicylic acid

These workers also studied the effect of methyl sinapate (104) and 3, 5-dimethoxy salicylic acid (105, 106) on thiamine pyrophosphate requiring enzyme and on the growth of thiamine dependent bacteria.

Antithiamine activity of *Phaseolus radiatus* (a kind of bean) was reported in literature by Bhagvat and Devi (28). But attempts were not made to isolate and characterise the thiamine - inactivating factor(s) present in it. The present work was thus aimed to do isolation, characterisation and biochemical studies of the antithiamine factor(s) present in *Phaseolus radiatus*.

PLAN OF RESEARCH WORK

The work embodied in this thesis covers the studies of antithiamine factor(s) present in *Phaseolus radiatus*. An antithiamine compound was isolated and characterized. The chemical, physico-chemical, nutritional, enzymological and microbiological studies of antithiamine factor were carried out in details. The following scheme of work was followed in course of these studies.

Chapter - I : ***Isolation studies :***

(i) Isolation of the antithiamine factor from *Phaseolus radiatus*.

(ii) Determination of antithiamine activity of the isolated factor.

Chapter - II : ***Physico-chemical studies :***

(i) General, physical and chemical properties of the antithiamine factor.

(ii) Characterization of the antithiamine compound from its physico-chemical studies.

Chapter - III : ***Nutritional studies :***

(i) Effect of pure antithiamine factor on the growth of rats.

(ii) Estimation of plasma cholesterol (free) of rats treated with pure antithiamine factor.

(iii) Estimation of blood pyruvate of antithiamine treated rats.

Chapter - IV : ***Enzymological studies :***

(i) In Vivo effect of the antithiamine factor on TPP-TK system of rat

(a) Blood,

(b) Small intestine and

(c) Brain.

Chapter - V : ***Microbiological studies :***

(i) Effect of antithiamine factor on the growth of a thiamine requiring strain of S. aureus in thiamine supplemented broth.

(ii) Effect of thiamine on the growth of antithiamine treated S. aureus.

CHAPTER — I

ISOLATION OF THE ANTITHIAMINE FACTOR FROM *Phaseolus radiatus*

CHAPTER — 1

Bhagvat and Devi (28) detected the presence of toxic agents, responsible for the destruction of thiamine, in one type of bean *Phaseolus radiatus*.

Following the finding the existence of antithiamine factor was also demonstrated in *Phaseolus radiatus* by Mitra (107) who further observed that the antithiamine activity of *Phaseolus radiatus* was probably due to two components — one was heat-stable, dialysable and the other was heat-labile, non-dialysable. Subsequent attempts were made by the worker to isolate these factors by different techniques as a result of which one of the antithiamine factors was isolated in the pure state, the details of which are discussed in the present chapter.

EXPERIMENTAL

Method of estimation

The antithiamine activity was determined by estimating the residual thiamine present in a system containing thiamine hydrochloride and antithiamine factor either in pure or crude state as followed by Bhattacharya and Choudhuri (102), the main steps of which are described below.

An intimate mixture of thiamine hydrochloride and antithiamine factor either pure or crude state was incubated at 30°C for one hour in M/15 phosphate buffer at pH 6.5. An aliquot of this incubated mixture was taken and its residual thiamine hydrochloride was estimated by thiochrome method described by Harris and Wang (108). The intensity of fluorescence was then measured by Lumitron Photoelectric Fluorescence Meter (Model 402/EF) using the primary filter of 365-370 nm and two secondary filters 410-25 nm.

Standard curve using the above fluorometer was drawn with different concentrations of thiamine hydrochloride (D-10µg) which was given in Figure - 1.

Isolation of the antithiamine factor

Isolation of the antithiamine factor involves the following steps :

1. Solvent extraction
2. Acid hydrolysis
3. Column chromatography
4. Crystallisation
5. Preparative thin layer chromatography.

1. Solvent extraction

50 g of Phaseolus radiatus powder was extracted with 500 ml of 10% chloroform-water mixture for 1 hour. This was then centrifuged at 5,000 g for 10 min to remove the suspended material. The supernatant thus obtained was evaporated to dryness.

2. Acid hydrolysis

The brown mass was dissolved in 50 ml of 1 (N) hydrochloric acid and refluxed for 1 h on water bath. The refluxed solution was cooled and centrifuged at 5,000 g for 10 min to discard the suspended material. The supernatant was shaken with five times its volume of water saturated isobutanol. The isobutanol layer was washed several times with distilled water to free it from acid completely and then concentrated to dryness under reduced pressure on waterbath and kept in vacuum desiccator overnight.

3. Column chromatography

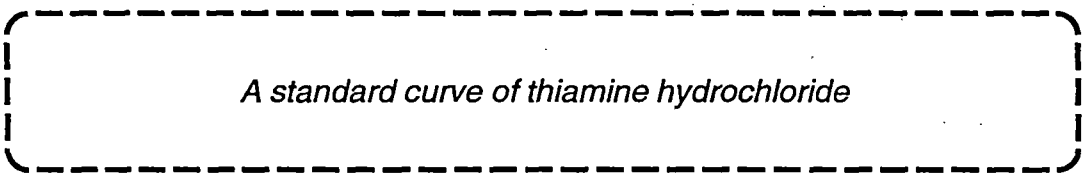
(i) Silica gel G column chromatography

10 g of silica gel G (E.Merck) was activated at 110° C for 18 h and cooled under desiccator. Silica gel G was first washed with methanol (A. R.). The slurry made with methanol was transferred to a column (44 X 2.5 cm). The dry mass as obtained after the step of acid hydrolysis was dissolved in methanol and charged to the column. Elution was done with 50% methanol-chloroform mixture when bands were separated out. The first band having the antithiamine activity was collected. This was then evaporated to dryness and from the dry brown mass, its ethyl acetate extract was collected for another column chromatography using polyamide as an adsorbent.

(ii) Polyamide column chromatography

Polyamide column was prepared in a similar manner as in case of silica gel G column, the difference was that in spite of methanol here ethyl acetate was used as washing and suspending agent of polyamide. The prepared ethyl acetate extract of the dry mass after silica gel G

FIGURE - 1



A standard curve of thiamine hydrochloride

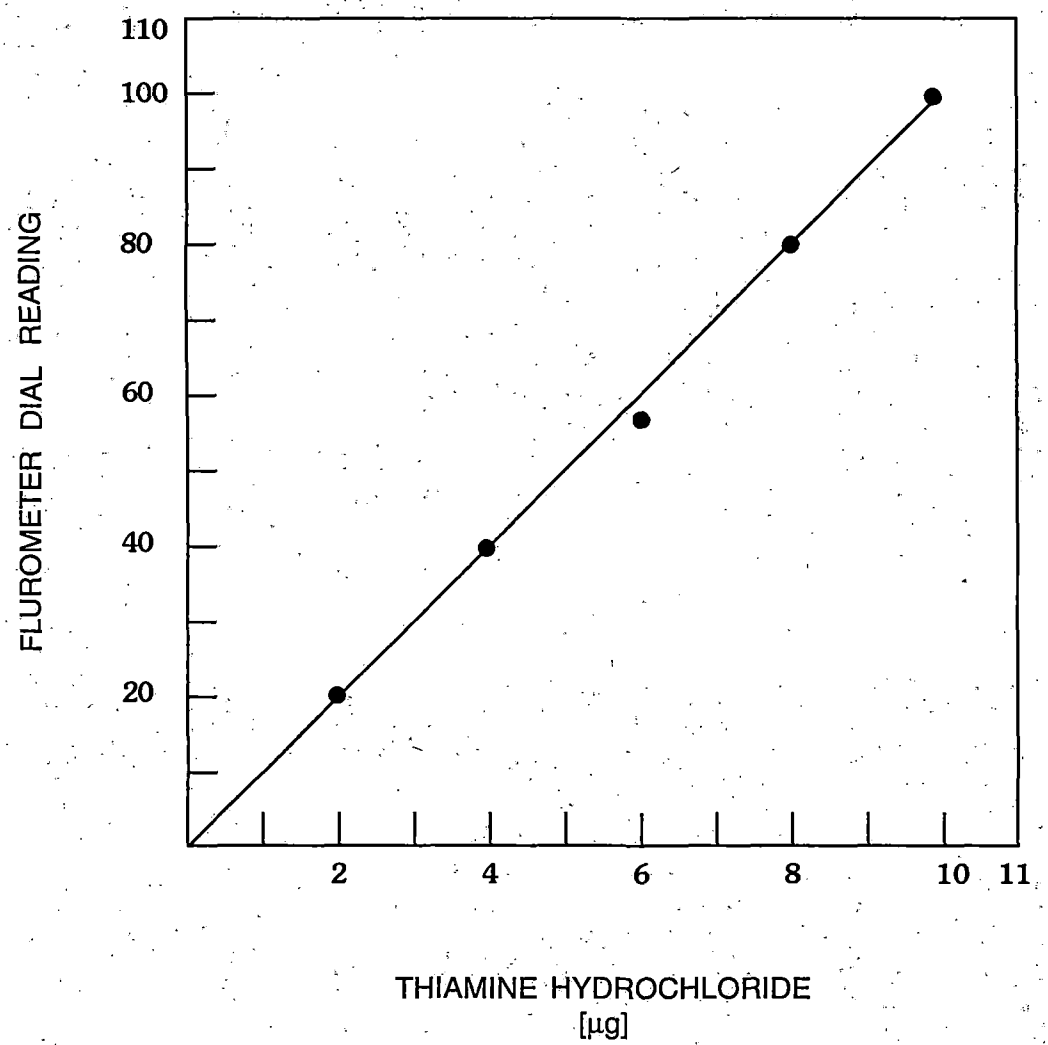

110
100
80
60
40
20
FLUROMETER DIAL READING
2
4
6
8
10
11
THIAMINE HYDROCHLORIDE
[µg]

FIGURE - 1

column was charged to the polyamide column and elution was started with ethyl formate ; formic acid mixture (100 : 5, v/v). The band thus separated was eluted.

4. Crystallisation

The eluant after polyamide column chromatography was evaporated to dryness. The antithiamine compound was obtained from this dry brown mass by repeated crystallisation from ethyl acetate and cyclohexane mixture. The active material thus obtained was a light yellow microcrystalline substance which was designated as compound 'G'

5. Preparative thin layer chromatography

The homogeneity of the isolated antithiamine compound was verified by subjecting it to preparative thin layer chromatographic separation using 22 X 10 cm silica gel G plates. The plates were activated at 110° C for 1 h. The following three developing solvent systems were used :

I. Acetone : methanol = 50 : 50 (v/v)

II. n-butanol : acetic acid : water = 80 : 10 : 10 (v/v/v) (Upper phase)

III. Chloroform : methanol : water = 60 : 20 : 20 (v/v/v).

Prior to the development of chromatogram saturation of the chamber for 3 h was essential. The spot was initially detected by exposing to iodine vapour followed by sulfuric acid charring test. A single and homogeneous spot was obtained in all the above three solvent systems having the R_f values 0.70 (I), 0.73 (II) and 0.91 (III) respectively (Figures - 2, 3, 4).

Determination of antithiamine activity of the isolated compound

Antithiamine activity was expressed as the amount of thiamine hydrochloride inactivated by 1 mg of antithiamine compound determined by the following method.

150 µg of thiamine hydrochloride was incubated with 1 mg of the isolated antithiamine compound for 1 h at 30° C and the total volume of the incubating mixture was made up to 5 ml. The residual thiamine-hydrochloride was estimated by the method described earlier (102). It was found that 1 mg of the antithiamine compound isolated from *Phaseolus radiatus* inhibited 135.0 µg of thiamine hydrochloride under these experimental conditions.

Effect of time on the inactivation of thiamine hydrochloride

Inactivation of thiamine hydrochloride with the antithiamine compound was studied with time. It was found that the maximum inactivation of thiamine hydrochloride was attained within 1 h of incubation at 30° C which was represented in Figure - 5.

FIGURE - 2

Silica gel G thin layer chromatography of the antithiamine factor (Compound 'G') using solvent system I, i.e., Acetone : methanol = 50 : 50 (v/v).

FIGURE - 3

Silica gel G thin layer chromatography of the antithiamine factor (Compound 'G') using solvent system II, i.e., n-butanol : acetic acid : water = 80 : 10 : 10 (v/v/v, upper phase).

FIGURE - 4

Silica gel G thin layer chromatography of the antithiamine factor (Compound 'G') using solvent system III, i.e., Chloroform : methanol : water = 60 : 20 : 20 (v/v/v).

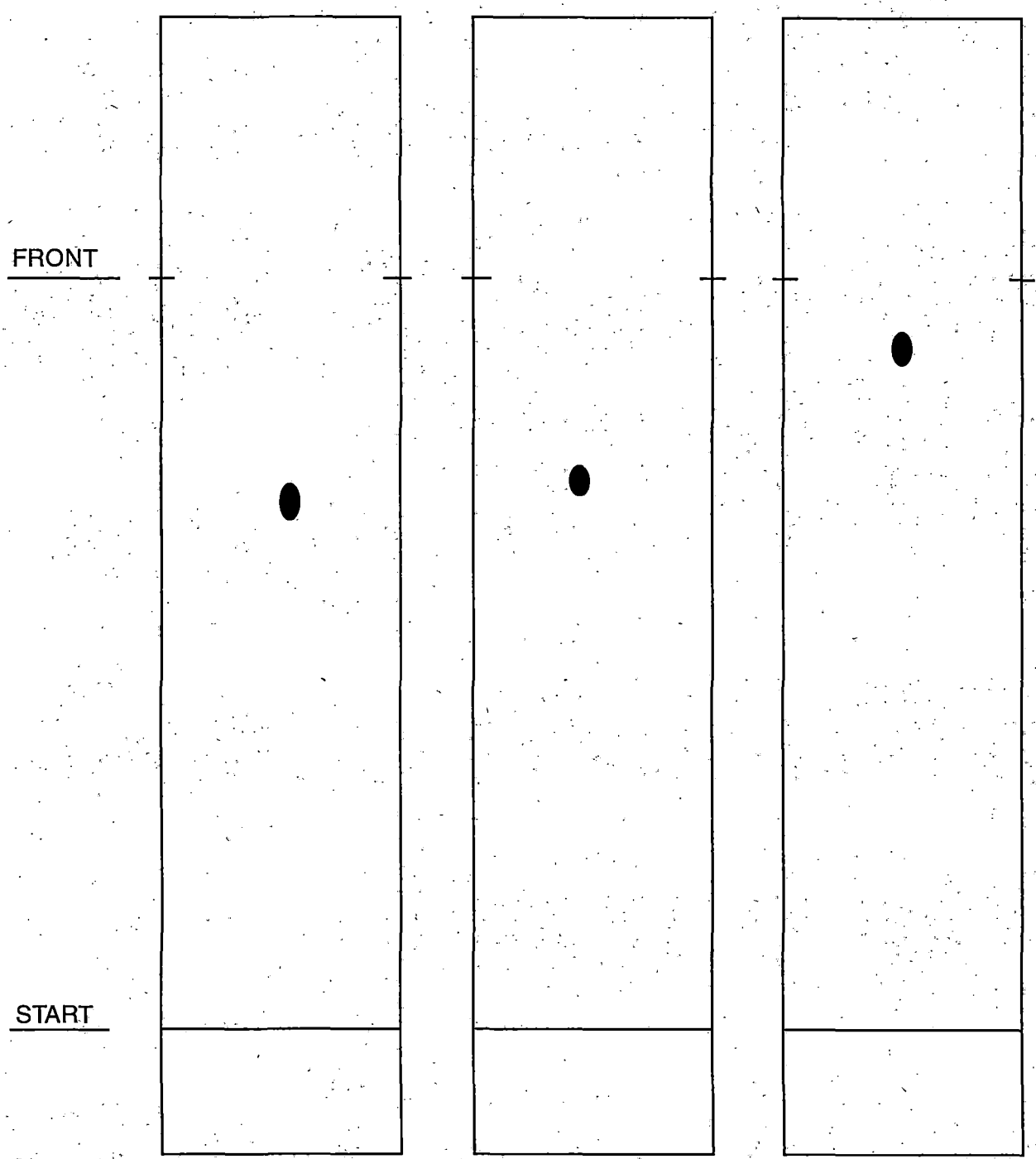
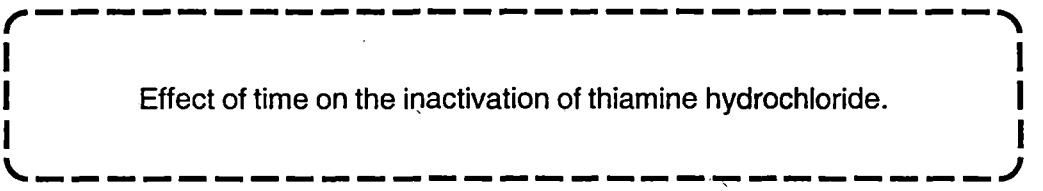


FIGURE - 2

FIGURE - 3

FIGURE - 4

FIGURE - 5



Effect of time on the inactivation of thiamine hydrochloride.

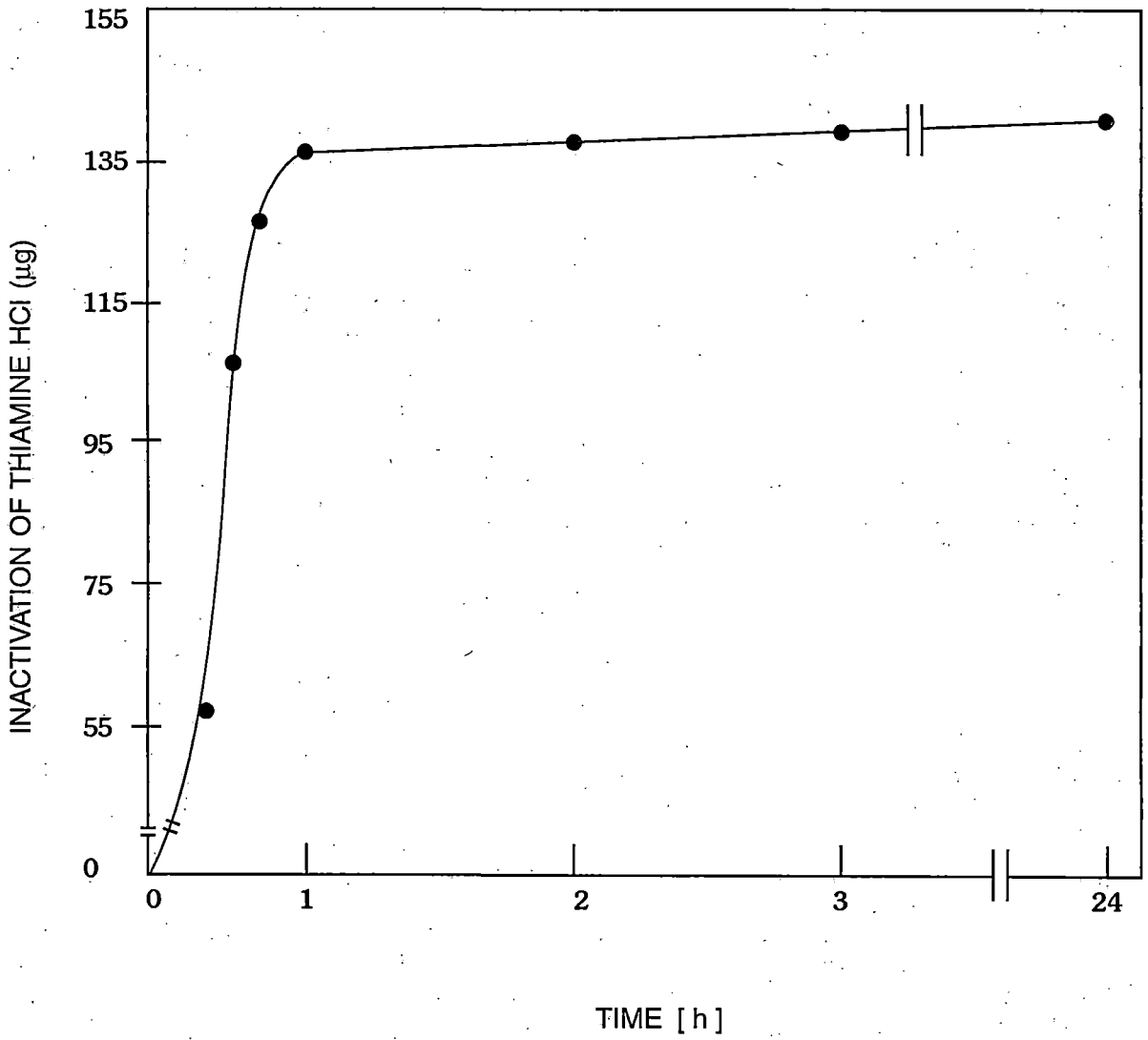

155
135
115
95
75
55
0
INACTIVATION OF THIAMINE HCl (µg)
0
1
2
3
24
TIME [h]

FIGURE - 5

Diagramatic scheme for the isolation of the antithiamine factor from *Phaseolus radiatus* (Details given in the method).

50 g of *Phaseolus radiatus* (Powder)

Extracted with 500 ml of 10% chloroform-water mixture

Precipitate (Discarded)

Supernatant

Evaporated to dryness.

Dry brown mass

Extracted with 1 (N) hydrochloric acid.

Acid extract

Refluxed for 1 h on waterbath, cooled and centrifuged.

Precipitate (Discarded)

Supernatant

Extracted with 5 volumes of water saturated isobutanol.

Water layer (Discarded)

Isobutanol layer

Completely freed from acid, evaporated to dryness and kept overnight in vacuum desiccator.

Dry brown mass

Dissolved in methanol.

Silica gel G column chromatography

Eluted with 50% methanol-chloroform mixture.

Fraction

Evaporated to dryness, extracted with ethyl acetate.

Column chromatography on polyamide

Eluted with ethyl formate : formic acid mixture (100:5, v/v).

Fraction

Repeated crystallisation from ethyl acetate-cyclohexane mixture.

Antithiamine factor (Compound 'G') (Roughly, 2 mg)

CHAPTER — II

CHARACTERISATION OF THE ANTITHIAMINE FACTOR ISOLATED FROM *Phaseolus radiatus*

CHAPTER — II

Physical and chemical properties of the antithiamine factor isolated from *Phaseolus radiatus* designated as compound 'G' were studied and summarised in Tables - 1 and 2.

Table - 1

PHYSICAL PROPERTIES OF COMPOUND 'G'

Properties	Observation
1. Colour	Light yellow
2. Texture	Micro crystalline substance
3. Solubility	Soluble in water, ether and easily soluble in ethanol, acetone and ethyl acetate
4. Stability	Heat-stable
5. Dialysis	Dialysable
6. Behaviour to litmus paper	Acidic
7. UV absorption maxima	300 nm

Table - 2

CHEMICAL PROPERTIES OF COMPOUND 'G'

Experiment	Observation	Inference
1. αα'-dipyridyl ferric chloride solution	Rapid formation of red colour	Presence of reducing group
2. Bromine in chloroform treatment	Slow decolorisation of colour	Presence of unsaturation
3. Treatment with sodium hydroxide	Intense yellow colour	May be due to presence of phenolic hydroxyl group

From the above physical and chemical properties of compound 'G', the presence of unsaturation and phenolic hydroxyl group can be predicted.

Detection of elements

The compound 'G' was shown not to contain nitrogen, sulphur, halogen and phosphorus.

Micro-analysis

Micro-analysis of compound 'G' showed the following percentage of the constituents : Carbon - 60.83%, Hydrogen - 4.57% and Oxygen - 34.6%.

Comparative study of different antithiamine compounds

The antithiamine activity of different compounds so far isolated from different sources as determined by the method as mentioned earlier (102) is given in Table - 3.

Table - 3

Name of the compound	Reference	Antithiamine activity (µg of thiamine hydrochloride inactivated by 1 mg of the compound)
Compound 'G'	—	135.0
Compound 'X'	(96)	3.5
Fraction 'A'	(96)	26.5
Methyl sinapate	(102)	45.0
3,4-dihydroxy cinnamic acid	(102)	135.0
3,5 dimethoxy salicylic acid	(103)	20.5

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From the table, it appeared that antithiamine activity of the isolated compound 'G' was identical with that of 3, 4-dihydroxy cinnamic acid.

To demonstrate further similarity, if any, of the compound 'G' with 3, 4-dihydroxy cinnamic acid, various physico-chemical properties of compound 'G' and 3, 4-dihydroxy cinnamic acid were studied further under different conditions which are summarised below.

Melting point

Melting point of compound 'G' was found to be 191° C which was essentially identical with that of known standard 3, 4-dihydroxy cinnamic acid (97). Mixed melting point of the compound 'G' and that of known 3, 4-dihydroxy cinnamic acid mixture was also noted to be 192° C.

Thin layer chromatography

The identical nature of compound 'G' and 3, 4-dihydroxy cinnamic acid was verified by silica gel G thin layer chromatography using the following solvent systems:

I. Acetone : methanol =50 : 50 (v/v)

II. n-butanol : acetic acid : water = 80 : 10 : 10 (v/v/v) (Upper phase)

III. Chloroform : methanol : water = 60 : 20 : 20 (v/v/v).

After exposing the plates to iodine vapour followed by sulfuric acid charring test, spot for compound 'G' was found at a distance from base line which was essentially identical not only with known 3, 4-dihydroxy cinnamic acid but a mixture of compound 'G' and 3, 4-dihydroxy cinnamic acid in case of every solvent system. Thus, R_f values of compound 'G', 3, 4-dihydroxy cinnamic acid and a mixture of compound 'G' with 3, 4-dihydroxy cinnamic acid were found to be 0.70 in case of solvent system I, 0.73 in case of solvent system II and 0.91 in case of solvent system III. The chromatograms were shown in Figures - 6, 7 and 8. From this experiment it can be suggested that the isolated compound 'G' might be identical with 3, 4-dihydroxy cinnamic acid.

UV absorption spectral study

The UV absorption spectrum of compound 'G' was practically the same as that of 3, 4-dihydroxy cinnamic acid. Absorption maxima for compound 'G' was found to be at 300 nm which was identical to that of commercial 3, 4-dihydroxy cinnamic acid.

Infra-red absorption spectral study

To prove conclusively that the isolated compound 'G' was nothing but 3, 4-dihydroxy cinnamic acid, infra-red spectra of both compound 'G' and 3, 4-dihydroxy cinnamic acid in mujol were studied in the same paper using Parkin Elmer (model-137) infrared spectrometer. The profile was shown in Figure - 9. The IR spectra showed that curves for 3, 4-dihydroxy cinnamic acid and that of isolated compound 'G' were superimposeable with each other. Both the two compounds showed strong peaks at 3420 cm^{-1} (for phenolic hydroxyl group), 3250 cm^{-1} (for hydrogen bonded hydroxyl group), 1650 cm^{-1} (for carbonyl of carboxylic acid group), 1630 cm^{-1} (for aromatic and conjugated double bond).

FIGURE - 6

Silica gel G thin layer chromatographic experiment using solvent system I, i. e. Acetone : methanol = 50 : 50 (v/v)

FIGURE - 7

Silica gel G thin layer chromatographic experiment using solvent system II,
i. e. n-butanol : acetic acid : water = 80 : 10 : 10 (v/v/v, upper phase)

FIGURE - 8

Silica gel G thin layer chromatographic experiment using solvent system III, i. e.Chloroform : methanol : water = 60 : 20 : 20 (v/v/v).

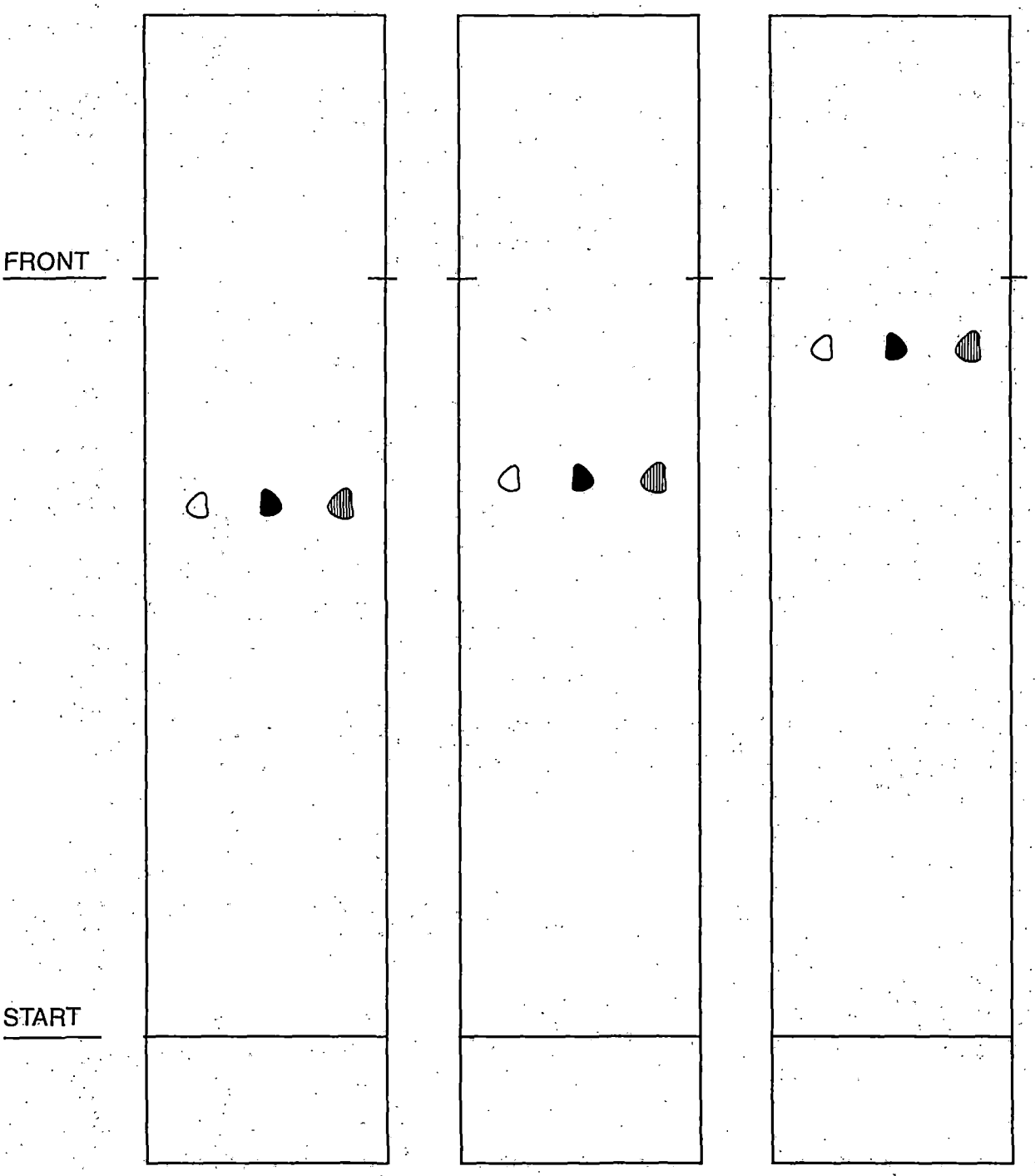


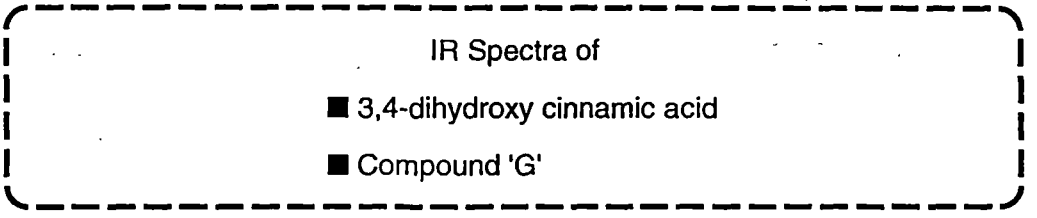
FIGURE - 6 FIGURE - 7 FIGURE - 8

COMPOUND 'G' —

3,4-DIHYDROXY CINNAMIC ACID —

MIXTURE OF COMPOUND 'G' & 3,4-DIHYDROXY CINNAMIC ACID —

FIGURE - 9



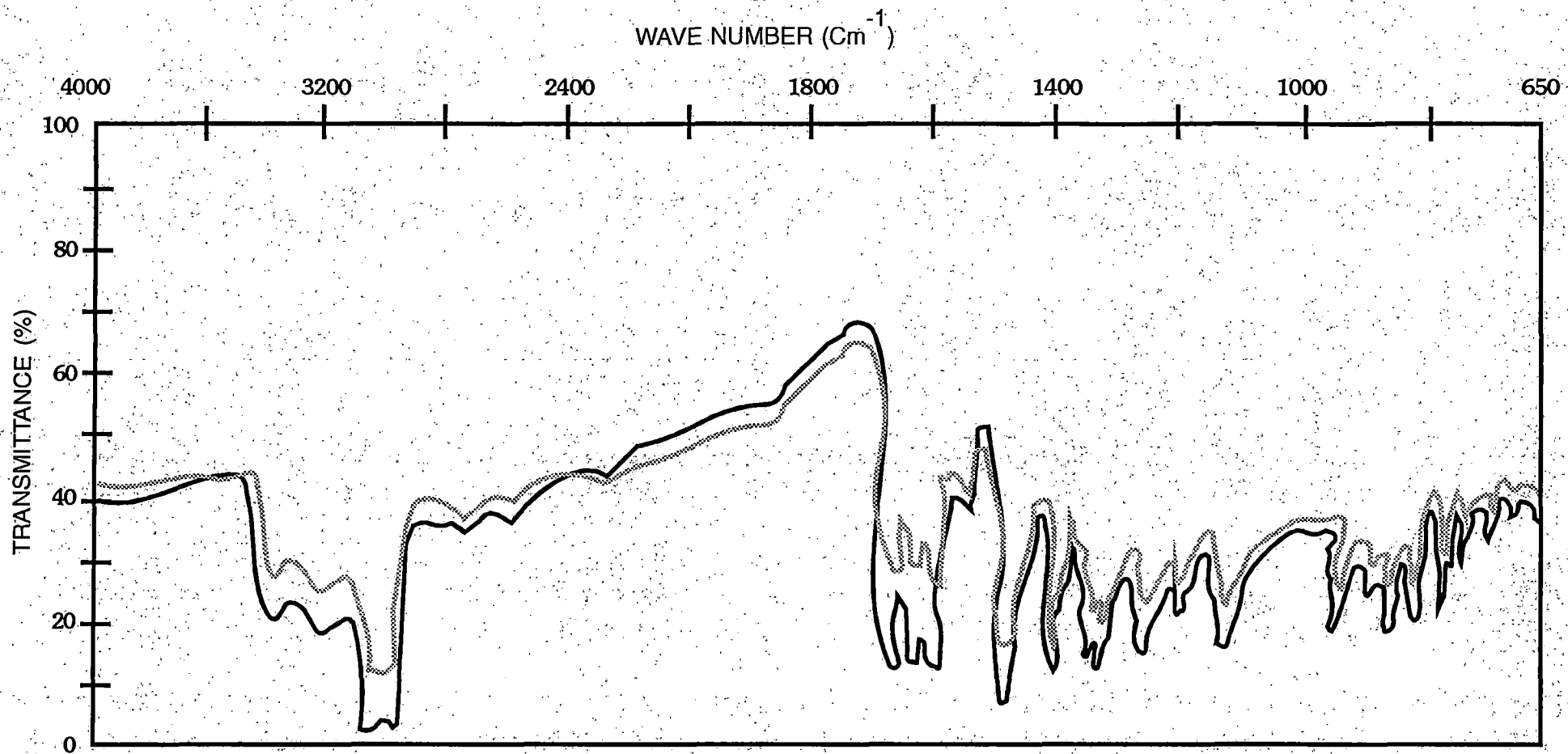
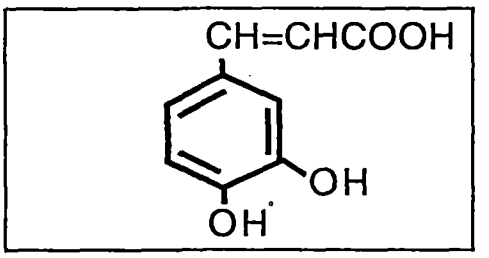

WAVE NUMBER (Cm -1)
4000
3200
2400
1800
1400
1000
650
100
80
60
40
20
0
TRANSMITTANCE (%)
: 3,4- DIHYDROXY CINNAMIC ACID
: COMPOUND 'G'

FIGURE - 9

Thus, the antithiamine factor isolated from *Phaseolus radiatus* and designated as compound 'G' possessing high antithiamine activity was found to be 3, 4-dihydroxy cinnamic acid.



3, 4-dihydroxy cinnamic acid.

(Compound 'G'.)

CHAPTER — III

NUTRITIONAL STUDIES OF THE ANTITHIAMINE FACTOR ISOLATED FROM *Phaseolus radiatus*

CHAPTER -III

Suzuki *et al.* (109) observed head retraction syndrome called opisthotonus in thiamine deficient condition in birds. Other significant symptoms in thiamine deficiency are cardiac abnormalities, necrosis of the heart muscle, nerve depression (110), paralysis and polyneuritis in animals (111). Safry (112) reported that adrenaline content of adrenals and blood was affected in thiamine deficient pigeons. These animals also showed an increased level of lactic acid in the brain (113) indicating defects in certain stages of carbohydrate metabolism. Similar observation in other tissues was made by Fisher (114) who suggested that there was a block in the oxidative utilization of pyruvate resulting in the accumulation of pyruvate in the blood. Thompson and Johnson (115) found an appreciable amount of blood pyruvate accumulation in thiamine deficient pigeons. In man, a considerable elevation of blood pyruvate level due to inactivation of the enzyme pyruvic oxidase which needed thiamine pyrophosphate as co-enzyme (116), also occurred in the advanced stages of beri-beri (117, 118).

A significant decrease in plasma and liver cholesterol levels in rats was also associated with thiamine deficient condition (119). This was due to the inhibition of the formation of acetyl coenzyme A, since thiamine pyrophosphate was required in the oxidative decarboxylation of pyruvate to acetyl coenzyme A, which is known to serve as the precursor for the biosynthesis of fatty acids and cholesterol (120, 121). As the naturally occurring thiamine antagonists do not have the structural similarities, it may be of interest to evaluate the effect of these compounds on the growth, as well as on blood pyruvate and cholesterol levels in rats.

The following experiment has, therefore, been planned to find out the effect of the antithamine factor (Compound 'G'), as isolated from *Phaseolus radiatus*, on rats.

EXPERIMENTAL

Preparation of thiamine deficient diet

Thiamine free basal diet was prepared according to Gubler (119). 5 kg of basal diet consisted of the following ingradients :

Vitamin free casein	1,100 g
Sucrose	3,400 g
Choline chloride	20 g
Corn oil	250 g
Vitamin mixture	11.1 g
Salt mixture	200 g

VITAMIN MIXTURE

Inositol	10 g
p-amino benzoic acid	0.5 g
Calcium pantothanate	0.3 g
Nicotinic acid	0.2 g
Pyridoxin	0.03 g
Riboflavin	0.06 g
Biotin	0.001 g
Folic acid	0.0025 g
2-methyl 1, 4 napthaquinone	0.02 g

SALT MIXTURE

$CaCO_3$	60 g
K_2HPO_4	64.5 g
$CaHPO_4$, 2 H_2O	15 g
$MgSO_4$, 7 H_2O	20.4 g
NaCl	33.5 g
Fe-citrate	5.5g
KI	0.16 g
$MnSO_4$, H_2O	0.76 g
$ZnCl_2$	0.05 g
$CusO_4$, $5H_2O$	0.06 g

Male albino rats weighing between 40—55 g were used for this experiment. They were first acclimatised with laboratory condition by feeding them laboratory diet for 2-3 days and then were divided into three groups and given diet according to Table-4.

Table—4

EXPERIMENTAL PLAN

Group No.	No.of rats	Diet	Supplements
I. Normal	50	Thiamine deficient	10 µg of thiamine-HCl in 0.2 ml of N Saline/100 g body weight/day.
II. Thiamine deprived	50	"	0.2 ml N saline/100 g body weight/ day
III. Isolated antithiamine factor (Compound 'G')	50	"	10 µg thiamine-HCl + 100 µg of antithiamine factor in 0.2 ml of N saline/100 g body weight/day. This was administered after preincubation of thiamine-HCl and antithiamine mixture for 1h at 30°C.

Rats from the different groups were injected respective supplements subcutaneously daily and the following parameters were measured.

a) Weight gain by the rats of above three groups at the interval of every five days for 40 days.

b) Plasma cholesterol (free) level at an interval of every five days for 40 days.

c) Blood pyruvate level at the terminal stage of thiamine deficiency.

Symptoms developed in rats during experimental period

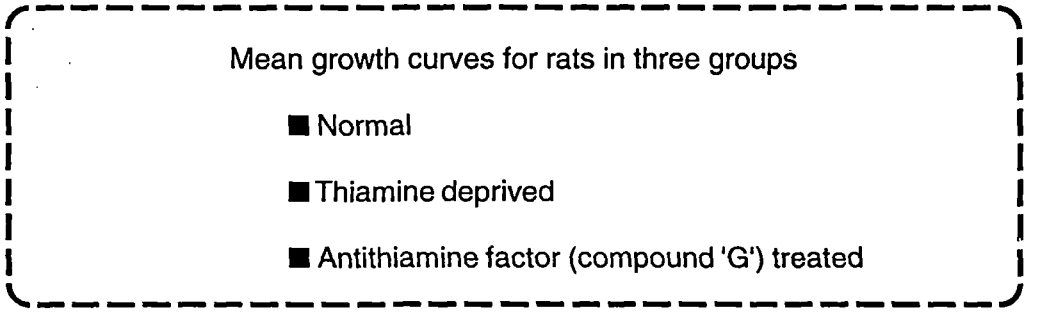
Group I rats showed gradual increase of their body weight throughout the experimental period. The increase was about 1 g per day for the first fifteen days and about 2 g per day during the remaining period.

Rats of group II showed an increase in weight for first fifteen days but thenafter began to lose their weight. They developed the symptoms of anorexia and became weak. Hair from neck and tail began to fall off fifteen days after the start of experiment. Mortality rate was found to be 5% after fifteen days of experiment.

Group III rats also showed symptoms similar to that of group II but the fall in body weights of rats of this group was started after 10 days of experiment. Anorexia, fallen of hair from neck and tail of rats were also observed. Two rats of this group died on fifteenth day after the start of experiment.

All these results were represented graphically in Figure-10.

FIGURE - 10


Mean growth curves for rats in three groups
Normal
Thiamine deprived
Antithiamine factor (compound 'G') treated

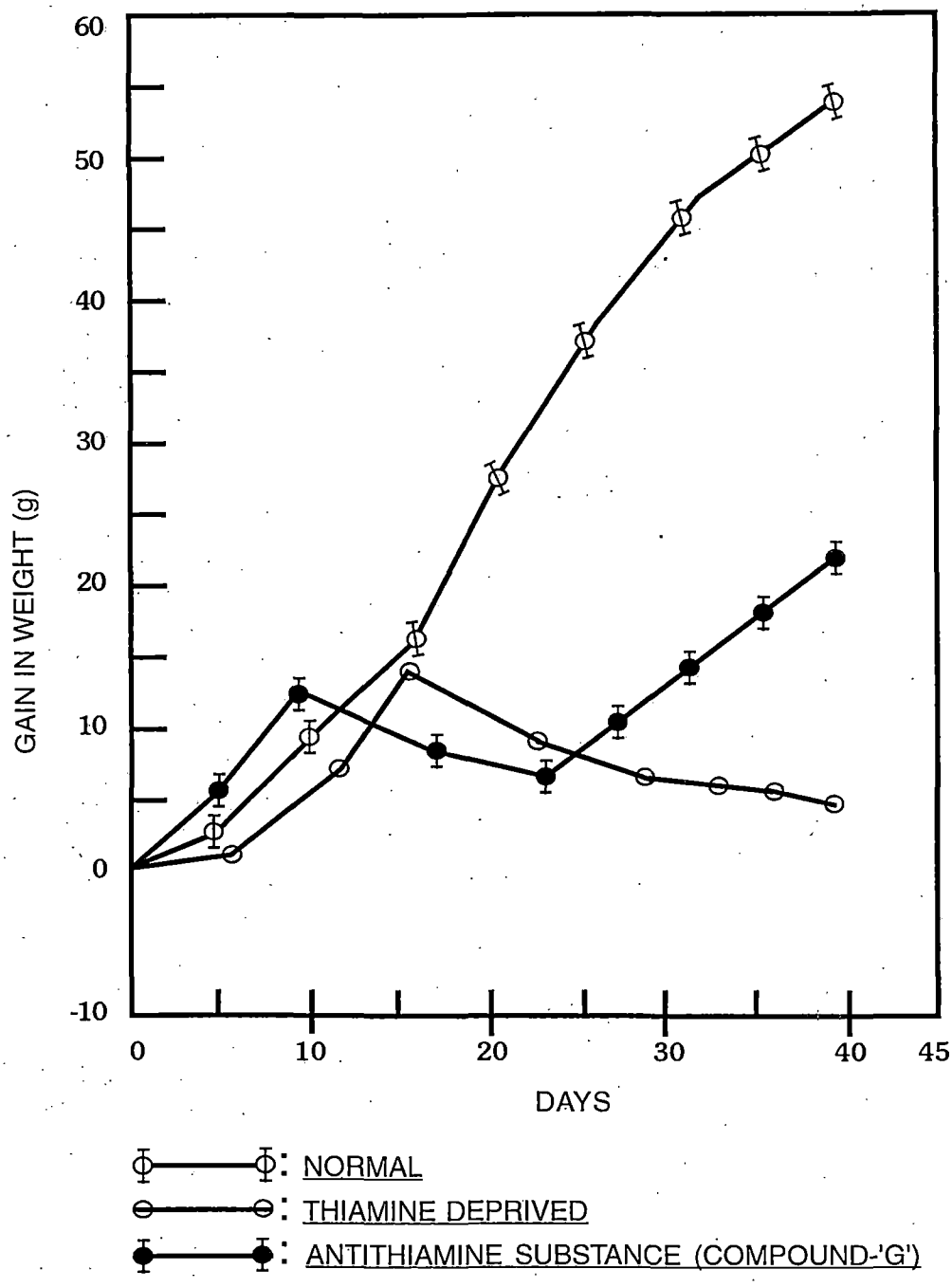

60
50
40
30
20
10
0
-10
GAIN IN WEIGHT (g)
0
10
20
30
40
45
DAYS
: NORMAL
: THIAMINE DEPRIVED
: ANTITHIAMINE SUBSTANCE (COMPOUND-'G')

FIGURE - 10

Supplementation of thiamine hydrochloride to rats suffering from thiamine deficiency

After twenty days of thiamine depletion period 20 μg of thiamine hydrochloride in 0.2 ml of N saline per 100 g body weight per day was injected subcutaneously to rats of group III. It was observed that the thiamine deficient symptoms disappeared within 4-5 days (Figure-10) and the animals started taking food as that of normal rats of group I.

Estimation of plasma cholesterol (free) and blood pyruvate

Five rats from each group (I, II and III) were killed on the day when thiamine deficient diet and suppliments were given to rats (marked '0') as well as on 5th, 10th, 15th, 20th, 25th, 30th, 35th and 40th day from the start of experiment. Blood of each rat was separately collected by decapitation in centrifuge tubes containing heparin and centrifuged in the cold. The plasma separated by suction was kept at 0°C for cholesterol assay and the packed cells stored in the cold for pyruvate estimation.

Estimation of plasma cholesterol (free)

The free cholesterol of plasma sample was estimated by the method described by Liebermann - Burchard (122) with slight modifications.

0.2 ml of plasma was taken for each sample and extracted with 10 ml of ethyl alcohol : ether mixture (4:1) with vigorous shaking for 30 min and then centrifuged. The clear supernatant thus obtained was evaporated to dryness and extracted with chloroform (thrice) and the final volume made upto 5 ml with chloroform. Protocol for estimation of free cholesterol is given in Table - 5.

Table — 5

PROTOCOL FOR ESTIMATION OF CHOLESTEROL

Group no.	Chloroform extract (ml)	Chloroform (ml)	Acetic anhydride (ml)	Conc. H_2SO_4 (ml)	Incubation at 25°C (min)
I	5	—	2	0.1	15
II	5	—	2	0.1	15
III	5	—	2	0.1	15
Reagent blank	—	5	2	0.1	15

The bluish green colour was recorded at 625 nm within 15—20 min by Bausch and

Lomb (sepectronic 20). A standard curve using several concentrations (100 to 500 µg) of cholesterol was drawn and represented in Figure -11.

Results (average of five) of this experiment, shown in Figure-12, indicated that the free plasma cholesterol level in both the experimental group of rats (group II and group III) were not affected upto at least 5 days in comparison to that of the normal level (group I). From 10 days onwards there was a tendency of decrease in free cholesterol level which was more significant on 20th day after the start of experiment (terminal stage of thiamine deficiency) in experimental animals of group II and also of group III. Free cholesterol level, however, came back to normal level (group I) after 35 days of experiment when thiamine hydrochloride was administered exogenously to antithiamine (compound 'G') treated rats (group III).

Estimation of erythrocyte pyruvate level

The erythrocyte was washed several times with isotonic saline to remove the traces of adhering materials of cell. The cell was then haemolized with equal volume of cold distilled water with alternate freezing and thawing to enhance the hemolysis. The homolysate thus obtained by centrifugation at 5,000 r.p.m. in the cold was deproteinized with 15% PCA (Perchloric acid). The clear deproteinized filtrate was used for pyruvate estimation by the method of Straub (123). The pyruvate content was estimated only at the terminal stage of thiamine deficiency i.e. after 20 days of thiamine depletion and on 25th day after the administration of 20 µg of thiamine hydrochloride / 100 g body weight / day to group III rats.

The protocol for pyruvate estimation was given in Table-6.

Table — 6

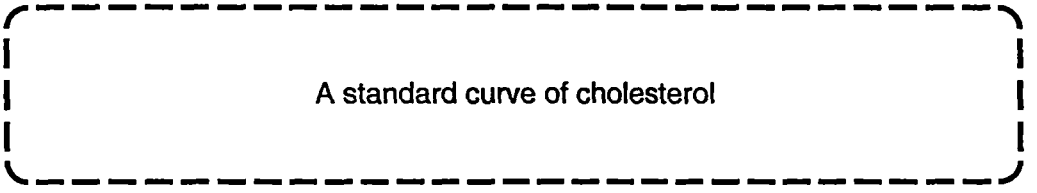
PROTOCOL FOR PYRUVATE ESTIMATION

Group no.	Deproteinized filtrate (ml)	15% PCA (ml)	Reagent A* (ml)	Reagent B** (ml)	Incubation at 37°C (min)
I	1	—	1	0.5	10
II	1	—	1	0.5	10
III	1	—	1	0.5	10
Reagent blank	—	1	1	0.5	10

* Reagent A— A mixture of 100 g of potassium hydroxide and 60 ml of water.

** Reagent B — A 2% (v/v) solution of salicyldehyde in 96% ethanol.

FIGURE - 11



A standard curve of cholesterol

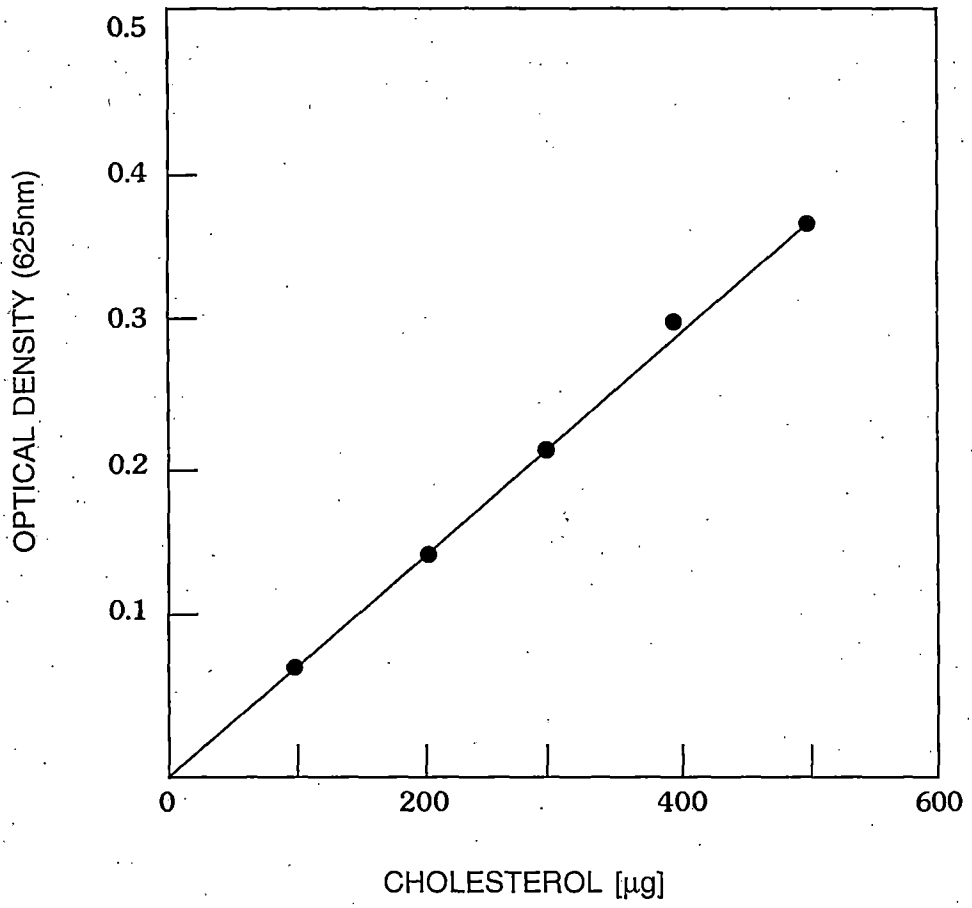
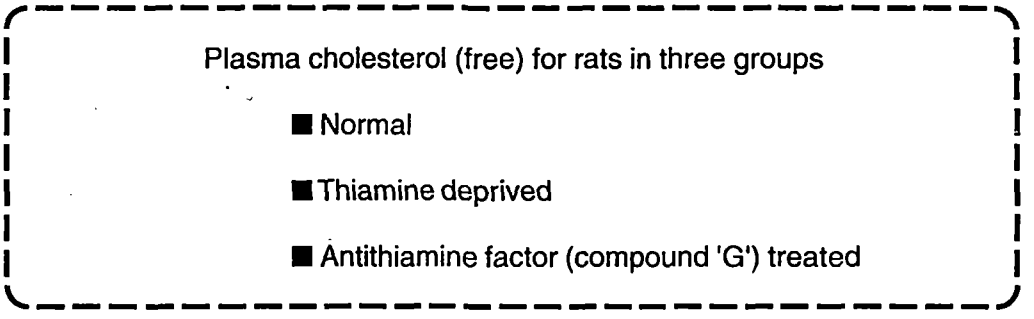

0.5
0.4
0.3
0.2
0.1
OPTICAL DENSITY (625nm)
0
200
400
600
CHOLESTEROL [µg]

FIGURE - 11

FIGURE - 12


Plasma cholesterol (free) for rats in three groups
Normal
Thiamine deprived
Antithiamine factor (compound 'G') treated

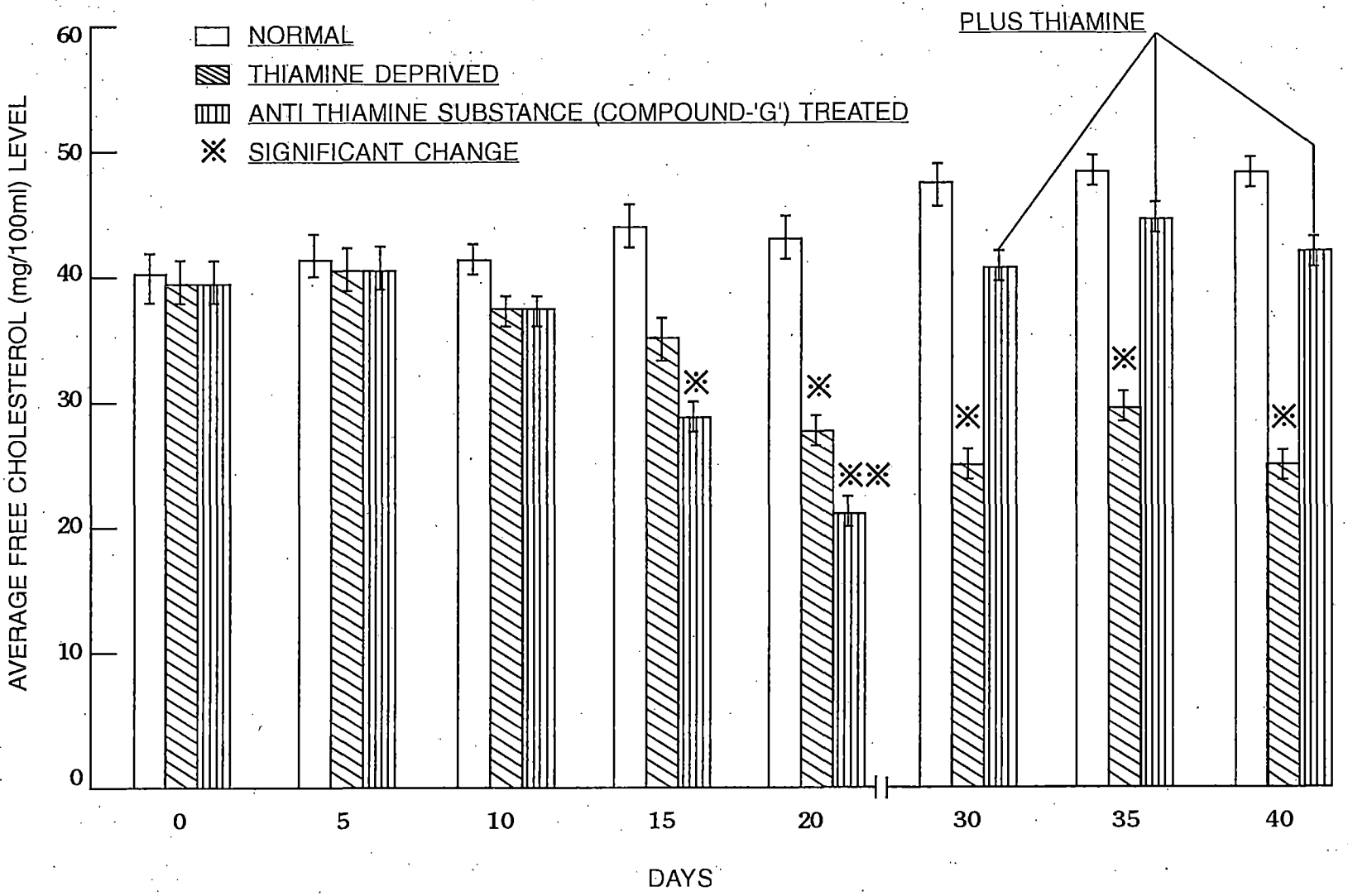

NORMAL
THIAMINE DEPRIVED
ANTI THIAMINE SUBSTANCE (COMPOUND-'G') TREATED
※ SIGNIFICANT CHANGE
PLUS THIAMINE
AVERAGE FREE CHOLESTEROL (mg/100ml) LEVEL
60
50
40
30
20
10
0
0
5
10
15
20
30
35
40
DAYS

FIGURE - 12

With the help of this method, using different concentrations of pyruvate (0 to 20 µg), a standard curve was drawn which was shown in Figure-13. The colour was read at 470 nm by Bausch and Lomb (Spectronic 20) setting the reagent blank at '0'. The amount of pyruvate content of the blood of experimental rats in three groups (average of 5) was calculated from the standard curve. The data thus obtained were given in Table-7.

Table — 7

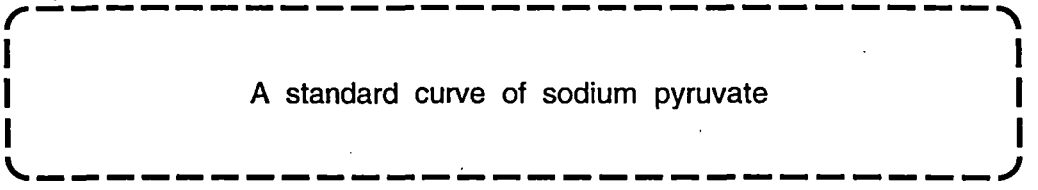
PYRUVATE CONTENT PER 100 ml BLOOD

Group no.	Blood pyruvate level in mg/100 ml of blood	
	20th day	25th day
I	1.34 ± 0.25	1.28 ± 0.19
II	4.0 ± 0.28 ($p < 0.001$)	4.2 ± 0.31 ($p < 0.001$)
III	4.35 ± 0.31 ($p < 0.001$)	1.42 ± 0.21*

* Thiamine was administered from the 20th day of the experiment to group III.

Table showed that blood pyruvate level of thiamine deficient rats was increased significantly to that of normal rats. This high value of blood pyruvate reduced to normal level after the administration of thiamine to group III rats.

FIGURE - 13



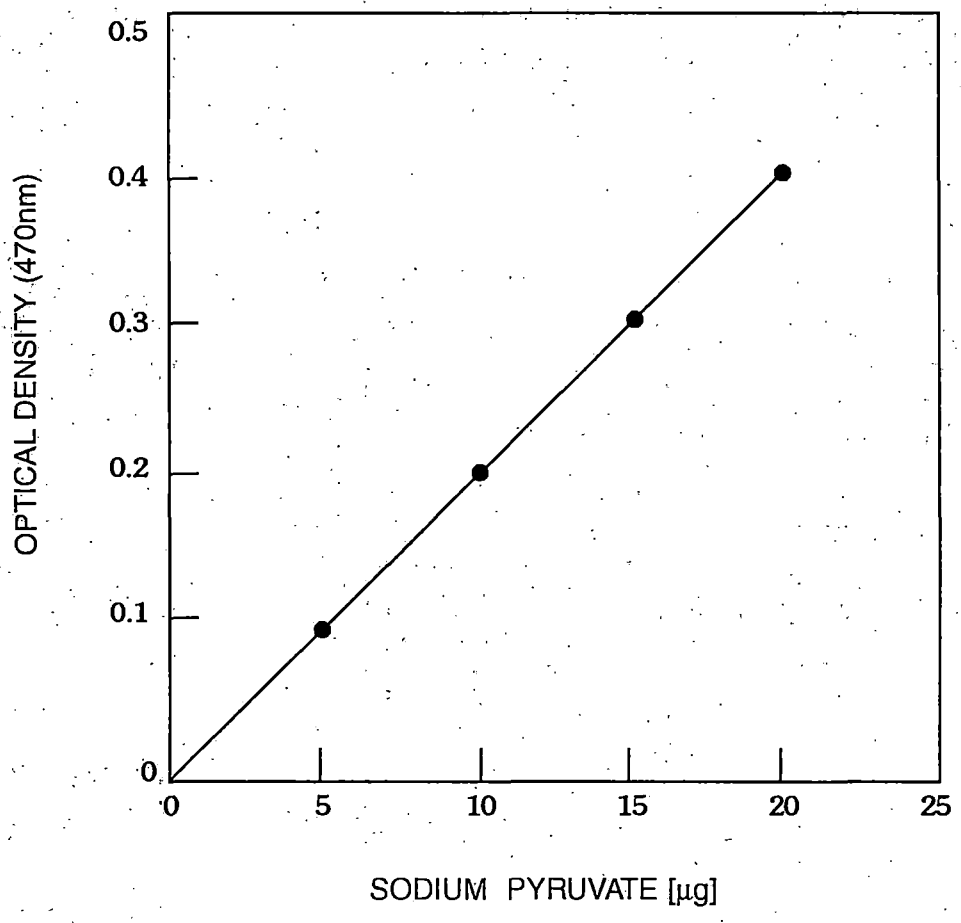

0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY (470nm)
0
5
10
15
20
25
SODIUM PYRUVATE [µg]

FIGURE - 13

CHAPTER — IV

ENZYMOLOGICAL STUDIES OF THE ANTITHIAMINE FACTOR ISOLATED FROM *Phaseolus radiatus*

CHAPTER — IV

In various enzyme systems, viz. pyruvate dehydrogenase, phosphoketolase, oxalogluterate dehydrogenase and transketolase, thiamine pyrophosphate acts as an essential co-enzyme (124—129). The activity of transketolase which played an important role in the utilization of pentose sugar (130, 131) was highly affected in thiamine depletion state *in vivo* (132, 133). The activity of this enzyme of hemolysate was very much sensitive to thiamine deficiency (134, 135) but with the addition of thiamine pyrophosphate to hemolysate, the enzyme activity was quickly restored to normalcy (136).

To study the binding of thiamine pyrophosphate with the different coenzyme analogues, various techniques (137) were developed. A number of workers (138, 139) have isolated the enzyme transketolase and found to be heterogeneous in nature. Morey et al (140) recently observed the non-dissociating phenomenon of thiamine pyrophosphate-transketolase (TPP-TK) system. It was shown by Schellenberger *et al* (141, 142) that thiamine pyrophosphate and Mg^{++} ions were bound at different sites of apopyruvate decarboxylase and studied the different equillibrium constants for the coenzyme (TPP) and its analogues.

In thiamine deficient conditions, the transketolase activity was markedly depressed not only in intact red cells of rats (143) and man (144), but also in intact erythrocytes obtained from rats treated with oxythiamine (145). Pearl Bai (146) studied the effect of oxythiamine and pyrithiamine on pyruvic dehydrogenase and transketolase of intestinal mucosa of rats *in vivo*. Brin (147) extended the study of transketolase activity in eight different tissues of rat. Unlike the synthetic antithiamines (structural analogues) the natural antithiamines did not act as competetive inhibitors (104). With this

object, therefore, studies to evaluate the effect of the isolated antithiamine factor (Compound 'G') from (*Phaseolus radiatus*) on TPP-TK system were undertaken and the results obtained are enumerated below.

The transketolase activity, developed by Dutta and Racker (148), was further modified by Gubler (149, 150). Gubler's method consisted of a coupling of the transdehydrogenase—DPNH system. A comparative simple method to assay the transketolase activity was developed by Brin (151) which was followed in the present study.

EXPERIMENTAL

Male albino rats weighing between 45-55 g were used for this study. On arrival the rats were fed on laboratory diet for 2 days to get them acclimatised and then they were caged into three groups as given in Table-8.

Thiamine free basal diet was prepared exactly in the same way as described in Chapter-III.

Table—8

EXPERIMENTAL PLAN

Group No.	No. of rats	Diet	Supplements
I. Normal	50	Thiamine deficient	10 µg of thiamine-HCl in 0.2 ml of N Saline/100 g body weight/day.
II. Thiamine deprived	52	"	0.2 ml N saline/100 g body weight/day
III. Isolated antithiamine factor (Compound 'G')	52	"	10 µg of thiamine-HCl + 100 µg of antithiamine factor in 0.2 ml of N saline/100 g body weight/day. This was administered afterpreincubation of thiamine-HCl and antithiamine mixture for 1h at 30°C.

All supplements were injected subcutaneously daily. Transketolase activity of

(i) intestinal mucosa
(ii) blood erythrocyte and
(iii) brain

was determined at an interval of 5 days of the three groups of rats shown in Table-8.

Symptoms developed during thiamine depletion period

The fall of body weight of rats of group II and group III with respect to the normal was consistent with that described in the chapter III. While group II rats showed the symptoms of anorexia and fall of hair after 15 days of experiment, group III rats suffered from the symptoms after 10 days of experiment.

Supplementation of thiamine-hydrochloride

After 20 days of experiment when the thiamine deficient symptoms were pronounced, group III rats were subcutaneously injected by 20 µg of thiamine-hydrochloride daily in 0.2 ml N saline/100 g body weight / day which was continued upto 45 days. It was observed that thiamine deficient symptoms disappeared within 4-5 days as described earlier in chapter III.

Assay of transketolase activity

Transketolase activity in intestinal mucosa, blood erythrocytes and brain of five rats from each group (I, II and III) was determined on the day when thiamine deficient diet and the supplements were given to rats ('O' day) as well as on 5th, 10th, 15th, 20th, 25th, 30th, 35th, 40th and 45th day from the start of the experiment.

Reagents required for transketolase assay

a. **Phosphate buffer— pH 7.4**

i)	0.9% NaCl	—	40 ml
ii)	1.13% KCl	—	1030 ml
iii)	1.75% K_2HPO_4	—	200 ml (17.5 g, 18-20 ml of (N) HCl per 1000 ml to adjust pH 7.4)
iv)	3.82% $MgSO_4$, $7H_2O$	—	10 ml

b. **Tris-HCl buffer — pH 7.3**

i)	0.15 M KCl solution	—	2.79 g of KCl dissolved in 250 ml of water
ii)	0.2 mM Tris buffer	—	60.57 mg of Tris buffer dissolved in 250 ml of water

c. **Normal saline**

9.0 g sodium chloride was dissolved in 1000 ml of water

d. **Thiamine pyrophosphate solution**

A stock solution of thiamine pyrophosphate 1 mg/ml was prepared and stored in refrigerator. This was diluted by mixing 1 mg of the stock solution with 8 ml of phosphate buffer for daily use.

e. **Ribose-5-phosphate solution**

A solution of ribose-5-phosphate (sodium salt) 2 mg/ml was prepared in distilled water and stored in freeze.

f. **D-Ribose solution**

A stock solution of D-ribose 1 mg/ml was prepared with distilled water and its dilute solutions were made with 7.5% TCA.

g. **Trichloro acetic acid solution (TCA)**

7.5% of TCA solution was prepared by dissolving 226 g of it in 3 litre of distilled water.

h. **Orcinol reagent**

0.4 g of orcinol and 0.02 g of ferric chloride were dissolved in 10 ml of distilled water and the final volume was made upto 200 ml with 30% HCl.

Collection of blood, intestine and brain and preparation of their homogenates

BLOOD

Blood from each rat was separately collected by decapitation in centrifuge tubes containing heparin and centrifuged in the cold at 4,000 r.p.m. for 10 min. The plasma and buffy coats were removed by suction. The packed cell was washed several times with cold saline to remove the traces of adhering materials with cell. The cells were then lysed with an equal volume of cold water with respect to that of packed cell. Freeze and thawing technique was adopted for complete lysis of the cell. The stroma was then removed by centrifugation at 5,000 r.p.m. for 15 min. The clear hemolysate was stored below 0°C for estimation of transketolase activity in terms of pentose utilisation by the method as developed by Brin (151).

INTESTINE

After collection of blood, the small intestine was rapidly removed and mucosal enzyme was prepared by the modifed method of Husbscher (152) and Robinson (153). Immediately after the removal, the entire small intestine was washed by blowing ice-chilled saline through pipette to remove food and other loss materials. Ice-chilled saline was preferred for washing to 0.25 M sucrose as Pearl Bai (146) used, because fructose derived from sucrose by the invertase activity in the mucosa, interferes with the assay of transketolase activity. The washed intestine was then placed on a cold glass plate, cut longitudinally to expose the mucosa and washed again with cold saline. The mucosa was then collected by gentle scrapping with a spatula. The scrapped mucosa was weighed and homogenized with 5 volumes (w/v) of 0.2 mM Tris-HCl buffer in 0.15 M KCl solution of pH 7.3 for 1 min in ice bath and centrifuged at 23,000 g for 10 min. The supernatant was stored below 0°C.

BRAIN

The intact brain was collected, blotted with absorbent paper and weighed. It was then homogenized with 9 volumes (w/v) of cold saline in ice-bath and centrifuged at 8,000 g for 15 min. The brain homogenate was preserved below 0°C for the transketolase assay.

Different incubation systems

Four incubation systems marked as 1, 2, 3 and 4 were set for each group of three tissues (i.e. blood, intestine and brain).

System 1 — Hemolysate or homogenate (Control, deprived and treated) without exogenous TPP.

System 2 — Hemolysate or homogenate (Control, deprived and treated) with exogenous TPP.

System 3 — Enzyme control to determine endogenous pentose.

System 4 — Substrate control to determine the amount of substrate added to each of the above two systems (1 and 2).

Incubation was done in centrifuge tubes using 0.5 ml of hemolysate or homogenate. The protocol of different incubation systems was given in Table-9.

Table—9

PROTOCOL FOR DIFFERENT INCUBATION SYSTEMS

System no.	Hemolysate or homogenate (ml)	Buffer (ml)	TPP solution (0.11 mg/ml) (ml)	Incubation at 38°C (min)	R-5-P (2 mg/ml) (ml)	Incubation at 38°C (min)	TCA 7.5% (ml)
1	0.5	0.85	—	30	0.15	60	6
2	0.5	0.40	0.45	30	0.15	60	6
3	0.5	1.00	—	—	—	—	6
4	0.5	0.85	—	—	0.15	—	6

Total volume — 7.5 ml

System 2 was incubated at 38°C for 30 min to allow the enzyme to combine with the coenzyme TPP (thiamine pyrophosphate) before the addition of the substrate. Then to each of the system, but the system 3, 0.15 ml of R-5-P (ribose-5-phosphate) was added noting the time and order of addition. These systems were then mixed well on vortex mixture and further incubated at 38°C for 1 h exactly. The reaction was then terminated with 6 ml of 7.5% TCA (trichloro acetic acid) solution, using the same tube order and time interval as with the substrate addition. It was then shaken vigorously in a vortex mixture and centrifuged at 5,000 r.p.m. for 10 min. 0.5 ml of the supernatant from system 1, 2 and 3 and 0.2 ml from system 4 were taken for determination of the residual pentose according to the following protocol given in Table-10. Standards of two different concentrations of D-ribose were always run side by side.

Table—10

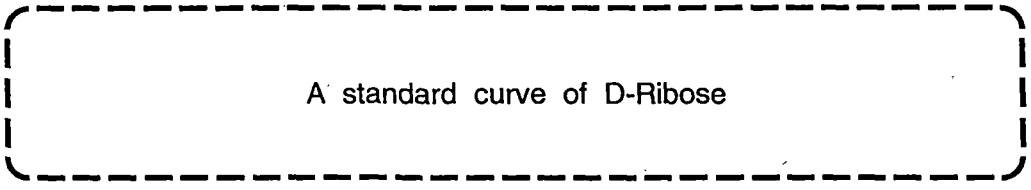
PROTOCOL FOR DETERMINATION OF RESIDUAL PENTOSE

Systems	Filtrate (ml)	Pentose solution (0.1 mg/ ml) (ml)	Distilled water (ml)	Orcinol reagent (ml)	Boiling water bath (min)	Cold water bath (min)
1	0.5	—	1.0	4.5	20	5
2	0.5	—	1.0	4.5	20	5
3	0.5	—	1.0	4.5	20	5
4	0.2	—	1.3	4.5	20	5
Standard (10 µg)	—	0.1	1.4	4.5	20	5
Standard (20 µg)	—	0.2	1.3	4.5	20	5
Reagent blank	—	—	1.5	4.5	20	5

The colour developed by the orcinol reagent was recorded at 670 nm in a Bausch and Lomb spectronic '20' setting the instrument "0" with reagent blank. A standard curve for D-ribose was drawn and represented in Figure-14.

The transketolase activity was estimated in terms of pentose utilization, which in turn was expressed as TPP effect (%). The increase in enzyme activity (due to incubating the homolysate with TPP before substrate addition) was expressed as a percentage of the activity of the

FIGURE - 14



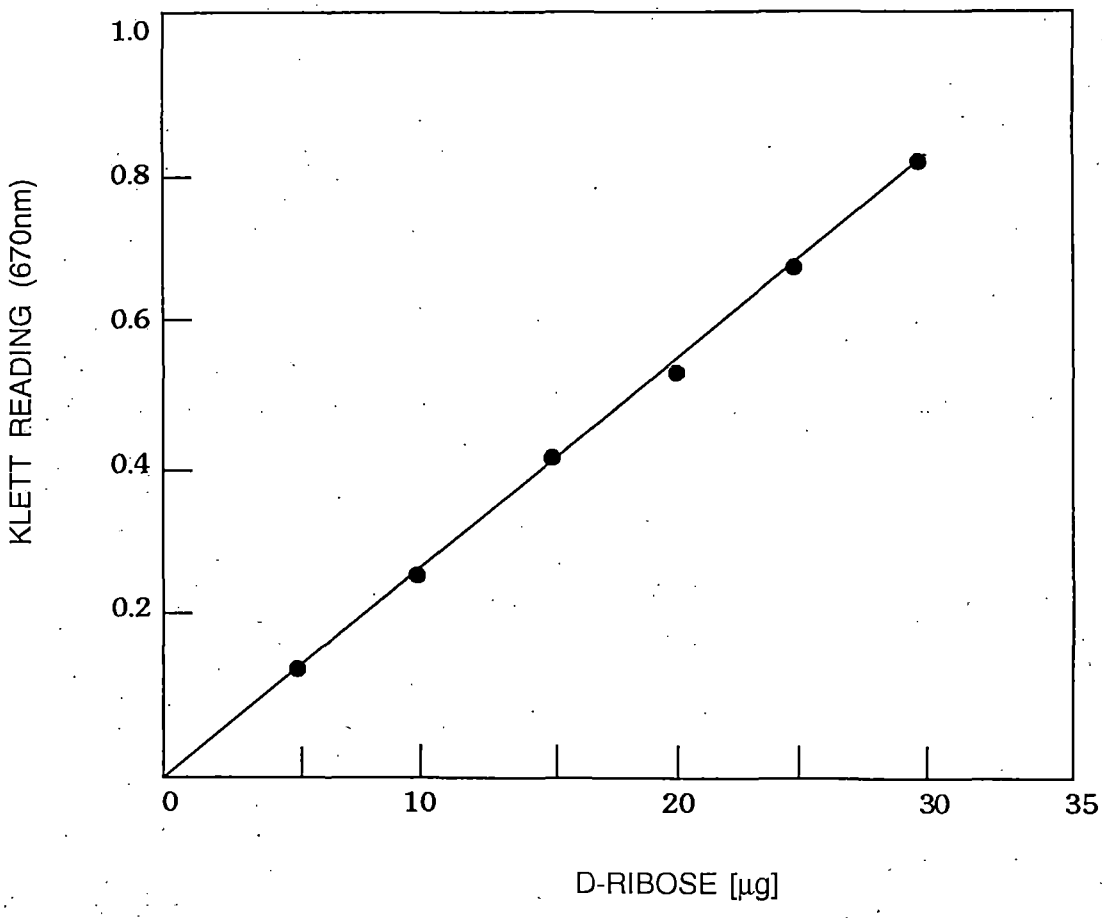


FIGURE - 14

sample which was not treated with TPP. This % was referred to as 'TPP effect'.

The 'TPP effect' (%) was calculated in the following manner.

Pentose utilized in micrograms per ml of hemolysate or homogenates per hour for the incubation without TPP which may be referred to TP_1.

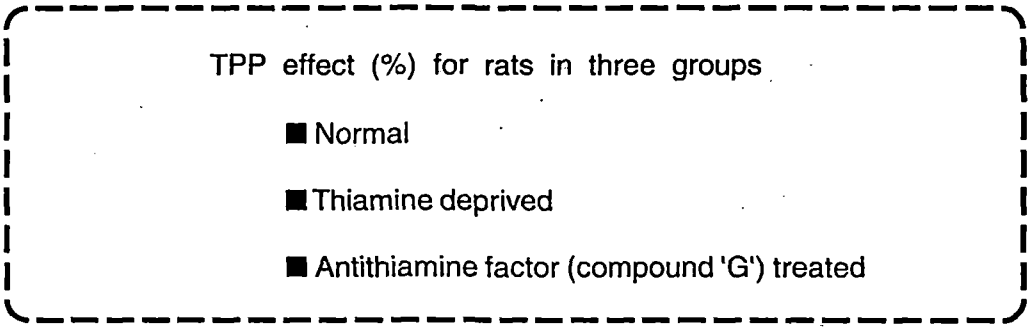
Pentose utilization in micrograms per ml of hemolysate or homogenates per hour for the incubation with TPP which may be called TP_2.

$$\text{TPP effect (\%)} = \frac{TP_2 - TP_1}{TP_1} \times 100$$

The results thus obtained for transketolase activity of blood, intestine and brain were shown graphically in Figures-15 (a,b.c) under three different conditions as described above.

Results showed that TPP effect of hemolysate of normal rats (Figure-15a) was about 10% which in case of thiamine deficient rats (Groups II and III) increased to more than 35%. This again came to normal level in group III animals after 20 days of experiment when thiamine hydrochloride was exogenously supplied to them. TPP effect of small intestine also increased with the thiamine deficient condition (Figure-15b) and followed the same pattern as in case of TPP effect of hemolysate. But no significant increase of TPP effect was observed (Figure-15c) in the case of brain of thiamine deficient rats.

FIGURE - 15


TPP effect (%) for rats in three groups
Normal
Thiamine deprived
Antithiamine factor (compound 'G') treated

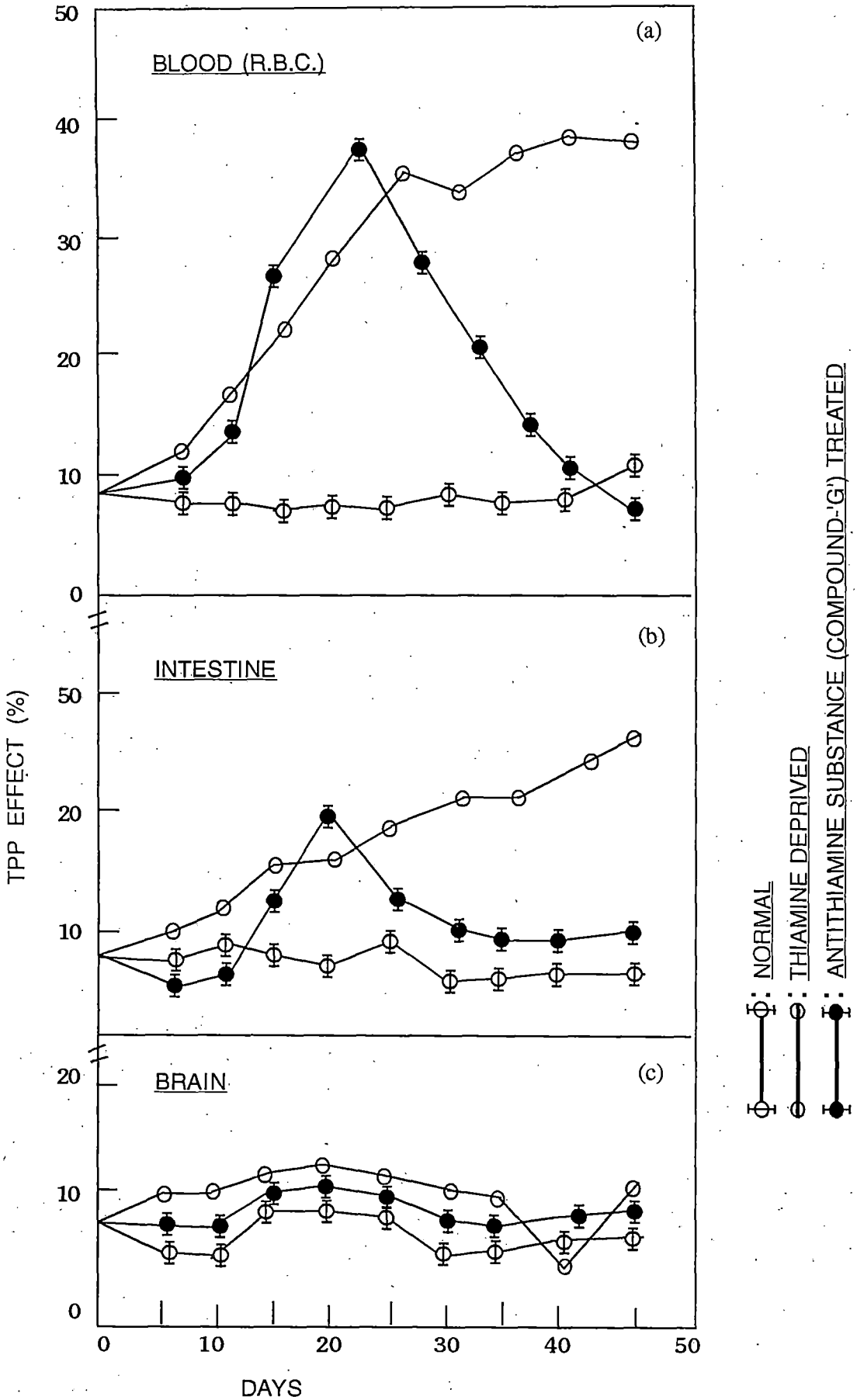

(a)
BLOOD (R.B.C.)
(b)
INTESTINE
(c)
BRAIN
TPP EFFECT (%)
DAYS
0
10
20
30
40
50
: NORMAL
: THIAMINE DEPRIVED
: ANTITHIAMINE SUBSTANCE (COMPOUND-'G') TREATED

FIGURE - 15

CHAPTER — V

MICROBIOLOGICAL STUDIES OF THE ANTITHIAMINE FACTOR ISOLATED FROM *Phaseolus radiatus*

CHAPTER - V

To maintain growth and various biochemical functions, micro organisms require essential nutrients in minute quantity. Among the water soluble B-vitamins, thiamine occupies an important position for the growth and maintenance of various microbes viz. bacteria, mold, fungi including higher fungi and yeasts. *Staphylococcus aureus*, a pathogenic bacterium required the whole thiamine molecule for its growth instead of its fragments namely pyrimidine or thiazole (154), whereas *Polytomells caces* (155) could grow with thiamine or its two components added together. Structural analogues of thiamine viz. pyrithiamine and oxythiamine etc. are now well established as competitive inhibitors in utilizing thiamine molecule for the growth of *S. aureus* (156). The growth of *Lactobacillus fermentii*, another thiamine requiring bacterium was inhibited by 6-amino pyrimidine and its derivatives (157). Some fluoromethyl derivatives of pyrimidine were potent growth inhibitor of *Bacillus subtilis* (158) and certain other micro organisms (159). Several thiol derivatives had bacteriostatic effect but the inhibition was not reversed on addition of thiamine (160).

It is of interest to note that a mutant strain of *S. aureus* could grow in presence of pyrithiamine prepared by Das and Chatterjee (161, 162). These workers also studied the intermediary carbohydrate metabolism of this pyrithiamine requiring *S. aureus*.

Somogyi (99) studied the effect of different phenolic compounds on *L. fermentii* in presence of thiamine to find out their antithiamine activity. Choudhuri and his associates (96, 104) studied thoroughly the requirement of thiamine and the role of two isolated antithiamines on the growth of *S. aureus*. In the present study the effect of compound 'G' (antithiamine factor) as isolated from *Phaseolus radiatus* on the growth of *S. aureus* was made according to the following scheme of work.

Scheme of work

a) Determination of the growth kinetics of *S. aureus* (wild type).

b) Effect of thiamine hydrochloride on the growth of *S. aureus*.

c) Effect of graded concentrations of compound 'G' (antithiamine factor) on the growth of *S. aureus*, medium of which was supplemented with a definite amount of thiamine hydrochloride.

d) Effect of graded concentrations of thiamine hydrochloride on the growth of *S. aureus*, medium of which was supplemented with compound 'G' (antithiamine factor).

e) Effect of thiamine hydrochloride on the growth of *S. aureus* which was pretreated with compound 'G' (antithiamine factor).

Materials

1) Organism

A thiamine dependent strain of *Staphylococcus aureus* (wild type) used in these studies was maintained on nutrient agar slants.

2) Preparation of inoculum

Inoculum of this organism was prepared in nutrient broth containing glucose 10%, peptone 0.5% and beef extract 0.3%. The pH of the medium was adjusted to 7.0.

3) Synthetic medium

The chemically defined synthetic medium selected for studying the effect of compound 'G' (isolated antithiamine factor) on the growth of *S. aureus* consisted of the followings.

Vitamin free casein hydrolysate	— 4 g
Glucose	— 3 g
K_2HPO_4	— 5 g
Cysteine hydrochloride	— 20 mg
Nicotinic acid	— 20 mg
Water	— 1000 ml

The pH of the medium was adjusted to 7.

4) Bacterial cell suspension

One loopful of the organism from the nutrient agar slants (2-3 weeks old) was inoculated to 5 ml of sterile nutrient broth and incubated at 37° C for 20 h. It was then centrifuged and washed repeatedly with sterile saline to remove adhering materials in asceptical condition. A uniform cell suspension was then prepared by suspending the cells in 5 ml of sterile saline.

5) Thiamine hydrochloride: stock solution

A stock solution of thiamine hydrochloride was prepared by dissolving 3.017 mg of thiamine hydrochloride in 10 ml of water and kept in refrigerator. The working solution of thiamine was prepared by diluting this stock solution.

6) Compound 'G' (isolated antithiamine factor): stock solution

A stock solution of compound 'G' was prepared by dissolving 0.5 mg of compound 'G' in 10 ml of ethyl alcohol. The working solution of compound 'G' was prepared by diluting stock solution with normal saline.

7) Sterilization of the solution

The nutrient and synthetic broth were sterilized by autoclaving at 15 lb pressure at 120° C for 20 min. Sterile solution of thiamine and compound 'G' were prepared by passing through the Jena glass bacteria filter (porosity G 5) which was previously sterilized by autoclaving at 15 lb pressure for 20 min.

EXPERIMENTAL

DETERMINATION OF THE GROWTH OF S. aureus TURBIDOMETRICALLY

The growth of microorganism was measured turbidometrically. After necessary supplementation of the ingrediants in the medium the organism was inoculated and incubated at 37° C. After incubation the growth of the organism was stopped by steaming for 5 min and growth was measured in Klett-Summerson Colorimeter using 660 nm.

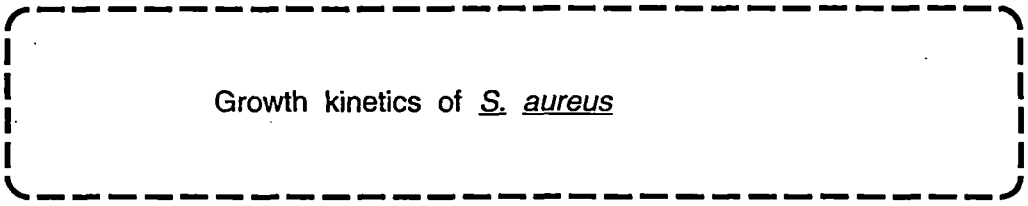
a) Determination of the growth kinetics of *S. aureus* (wild type)

5 ml of sterile double strength synthetic medium containing 150 mμg of thiamine was taken in Erlenmyer flask (100ml) and was inoculated with 0.1 ml of thoroughly washed bacterial cell suspension. Total volume of the medium was made upto 10 ml with sterile distilled water.

The flask was than incubated at 37° C and the growth of the organism measured turbidometrically at an interval of 1 h. The growth was plotted against the time of incubation, which was represented in Figure - 16.

It was observed from figure -16 that lag phase, early log phase, log phase and

FIGURE - 16



Growth kinetics of *S. aureus*

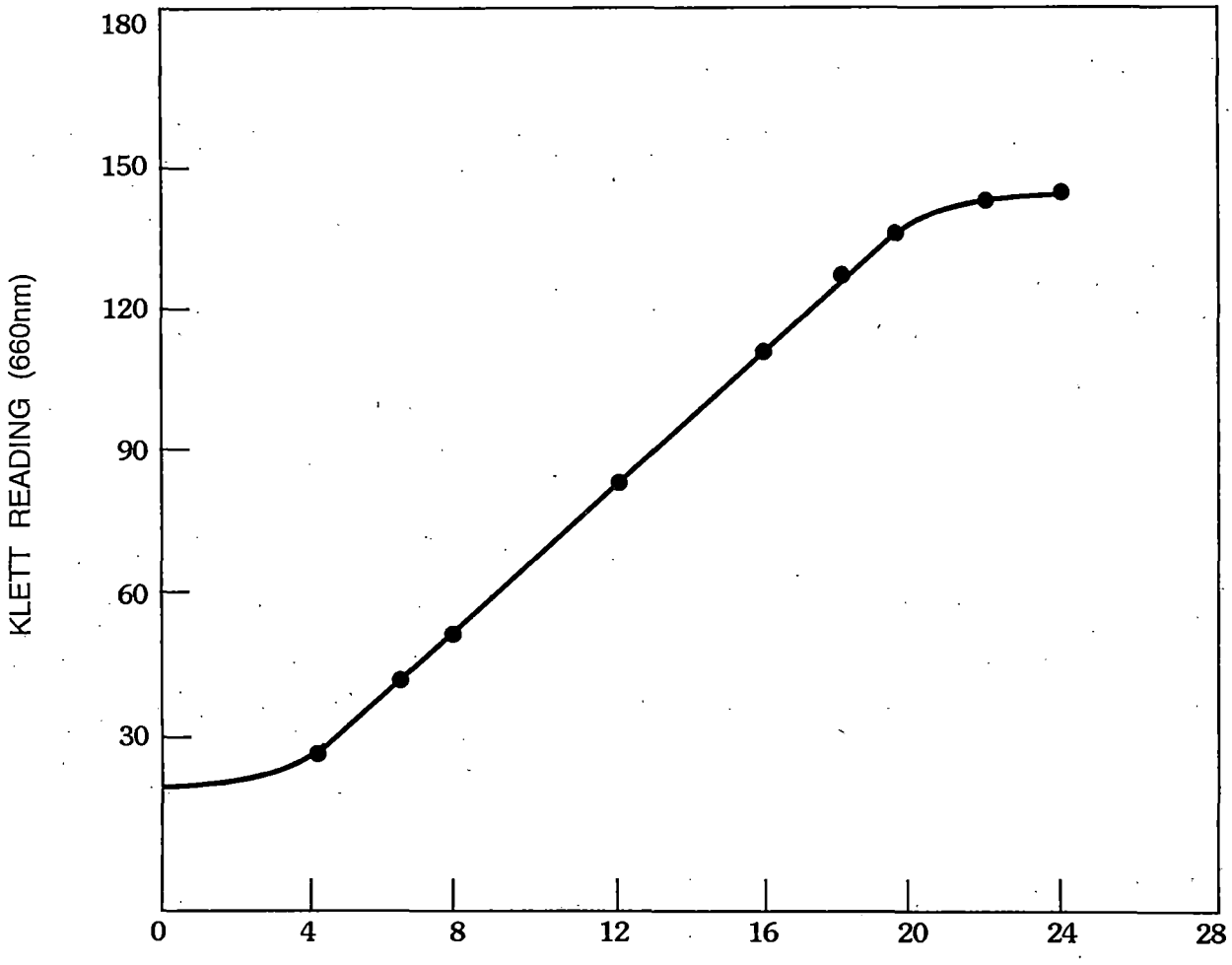

180
150
120
90
60
30
KLETT READING (660nm)
0
4
8
12
16
20
24
28
HOURS OF INCUBATION

FIGURE - 16

stationary phase of the growth of *S. aureus* were attained between 0-1 h, 1-4 h, 4-19 h and 19-24 h respectively and the death phase after 24 h.

b) Effect of graded concentrations of thiamine hydrochloride on the growth of *S. aureus*

To study the growth of *S. aureus* in presence of graded concentrations of thiamine hydrochloride, following experiment was performed.

5 ml of the double strength synthetic medium was taken in several Erlenmyer flasks (100 ml) marked as 1,2,3 .....etc. The flasks were given to 0-150 mμg of thiamine and incubated with 0.1 ml of bacterial cell suspension according to the following Table-11. The volume of the medium was made upto 10 ml.

Table—11

PROTOCOL FOR DIFFERENT INCUBATION SYSTEMS

Flask no.	Double strength synthetic medium (ml)	Thiamine solution (150 mμg/ml) (ml)	Distilled water (ml)	Bacterial suspension (ml)
1.	5	—	4.9	0.1
2.	5	0.1	4.8	0.1
3.	5	0.2	4.7	0.1
4.	5	0.3	4.6	0.1
5.	5	0.4	4.5	0.1
6.	5	0.5	4.4	0.1
7.	5	0.6	4.3	0.1
8.	5	0.7	4.2	0.1
9.	5	0.8	4.1	0.1
10.	5	1.0	3.9	0.1

Flasks were then incubated at 37° C for 20 h and the growth rate determined turbidometrically at 660 nm. The growth of *S. aureus* thus obtained was represented graphically in Figure - 17a. It may appear from the graph that the maximum growth was attained with the thiamine concentration of 150 mμg/ml.

C) Effect of graded concentrations of compound 'G' (antithiamine factor) on the growth of *S. aureus*, medium of which was supplemented with a definite amount of thiamine hydrochloride

The following experiment was performed to study the effect of compound 'G' (antithiamine factor) on the growth of *S. aureus* in the medium containing 150 mμg of thiamine

hydrochloride. Several Erlenmyer flasks marked 1,2,3 ..... etc. containing 150 mμg of thiamine hydrochloride in 5 ml of double strength synthetic medium were supplemented with 0 to 1.35 μg of compound 'G'. The medium was inoculated with 0.1 ml of bacterial cell suspension and the total volume made upto 10 ml with sterile distilled water as illustrated in Table -12.

Table—12

PROTOCOL FOR DIFFERENT INCUBATION SYSTEMS

Flask no.	Double strength synthetic medium (ml)	Compound 'G' (antithiamine factor) (2.25 μg/ml) (ml)	Thiamine solution (150 mμg/ml) (ml)	Distilled water (ml)	Bacterial suspension (ml)
1.	5	—	0.1	4.80	0.1
2.	5	0.06	0.1	4.74	0.1
3.	5	0.08	0.1	4.72	0.1
4.	5	0.1	0.1	4.70	0.1
5.	5	0.2	0.1	4.60	0.1
6.	5	0.3	0.1	4.50	0.1
7.	5	0.4	0.1	4.40	0.1
8.	5	0.5	0.1	4.30	0.1
9.	5	0.6	0.1	4.20	0.1

The growth rate of the microorganism in each flask was then determined turbidometrically at 660 nm after 20 h of incubation at 37° C. The results were shown graphically in Figure - 17b. It may appear from the figure - 17b, that there was a decrease in growth until the concentration of compound 'G' (isolated antithiamine factor) was reached at 1.35μg/ 10 ml.

d) Effect of graded concentrations of thiamine hydrochloride on the growth of *S. aureus*, medium of which was supplemented with compound 'G' (antithiamine factor).

Effect of thiamine hydrochloride on the growth of *S. aureus* in the medium containing excess of compound 'G' (1.50μg/ml) was studied as described below.

Erlenmyer flasks (100 ml) containing 1.50μg of compound 'G' in 5 ml of double strength synthetic medium marked as 1,2,3 ..... etc. were supplemented with 0 to 180 mμg of thiamine hydrochloride and inoculated with 0.1 ml of cell suspension according to the Table - 13. The total volume of the medium was made upto 10 ml with sterile distilled water.

FIGURE - 17

Effect of antithiamine factor (Compound 'G') on the growth of *S. aureus*

a) Effect of thiamine on the growth of *S. aureus*

b) Effect of antithiamine factor (Compound 'G') on the growth of *S. aureus* in the medium supplemented with thiamine.

c) Effect of thiamine on the growth of *S. aureus* in the medium supplemented with antithiamine factor (Compound 'G')

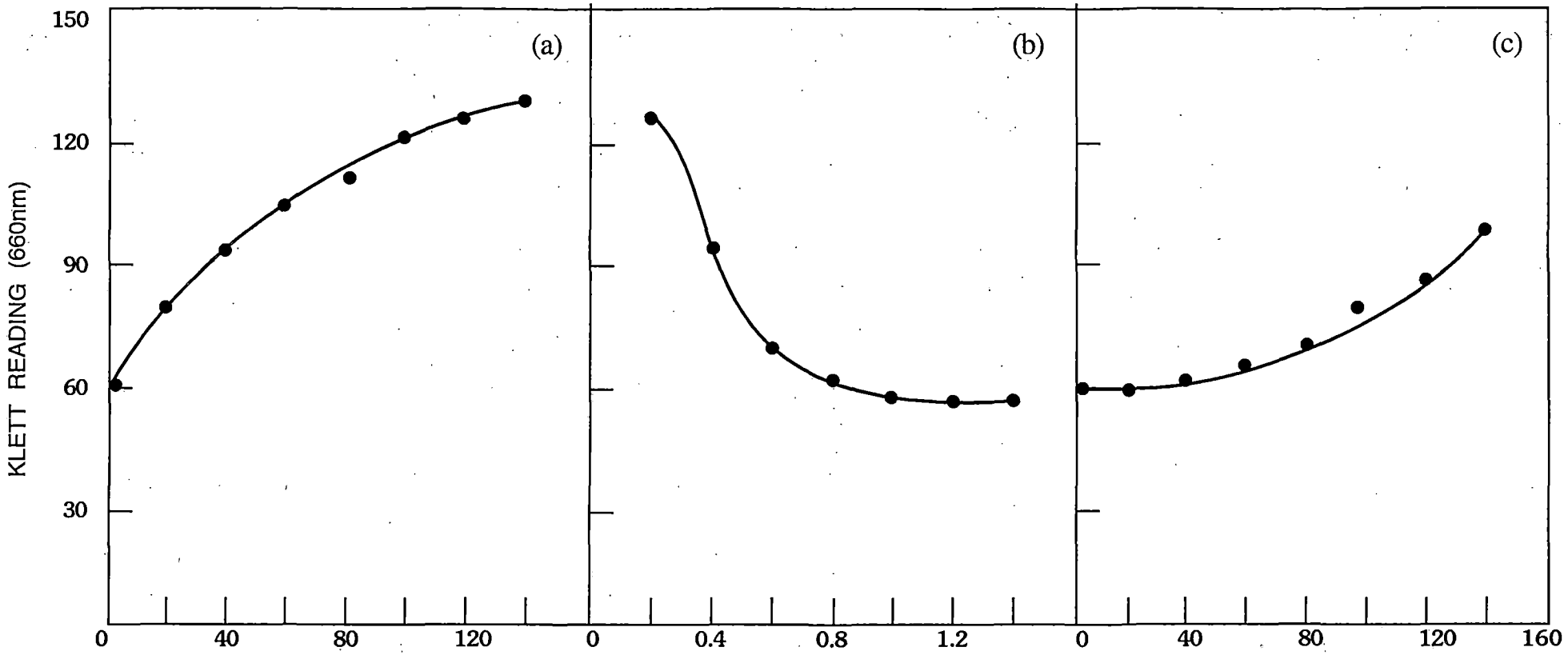

KLETT READING (660nm)
150
120
90
60
30
(a)
(b)
(c)
0
40
80
120
0
0.4
0.8
1.2
0
40
80
120
160
THIAMINE HCl (mμg/10ml)
ANTI THIAMINE FACTOR
(COMPOUND 'G') (μg/10ml)
THIAMINE - HCl (mμg/10ml)

FIGURE - 17

Table - 13

PROTOCOL FOR DIFFERENT INCUBATION SYSTEMS

Flask no.	Double strength synthetic medium (ml)	Compound 'G' (antithiamine factor) (1.50µg/ml) (ml)	Thiamine solution (150 mµg/ml) (ml)	Distilled water (ml)	Bacterial suspension (ml)
1.	5	1	—	3.9	0.1
2.	5	1	0.2	3.7	0.1
3.	5	1	0.4	3.5	0.1
4.	5	1	0.5	3.4	0.1
5.	5	1	0.6	3.3	0.1
6.	5	1	0.7	3.2	0.1
7.	5	1	0.8	3.1	0.1
8.	5	1	1.0	2.9	0.1
9.	5	1	1.2	2.7	0.1

The growth was measured turbidometrically after incubation at 37° C for 20 h. The results were graphically represented in Figure - 17c. From the figure - 17c, it may be observed that the growth inhibition was reversed when thiamine hydrochloride concentration reached at 150 mµg/ml.

e) Effect of thiamine hydrochloride on the growth of *S. aureus* treated with compound 'G' (antithiamine factor)

The growth of compound 'G' treated *S. aureus* in thiamine supplemented medium was studied as given below.

A graded concentrations of compound 'G' (0, 0.15, 0.45 and 1.35µg) were incubated with washed cell suspension of *S. aureus* (0.1 ml) in 2.0 ml of sterile 0.2 N phosphate buffer, for 1h at 37° C. The cells were then centrifuged, thoroughly washed with sterile buffer. These cells were then inoculated in the medium containing 300 mµg of thiamine hydrochloride marked as 0, A, B and C and incubated at 37° C. The growth of *S. aureus* was then measured turbidometrically at different time interval. The growth curve of compound 'G' treated *S. aureus* was represented in Figure - 18.

It may be seen from figure - 18 that growth of compound 'G' treated *S. aureus* was almost normal after 2 h of incubation. This indicated that compound 'G' had practically no inhibitory effect on the growth of *S. aureus* after a time lag.

FIGURE - 18

Effect of thiamine on the growth of *S. aureus* previously treated with antithiamine factor (Compound 'G')

O	0.00	µg	ANTI THIAMINE SUBSTANCE (COMPOUND'G')					
A	0.15	"	"	"	(	"	)	
B	0.45	"	"	"	(	"	)	
C	1.35	"	"	"	(	"	)	

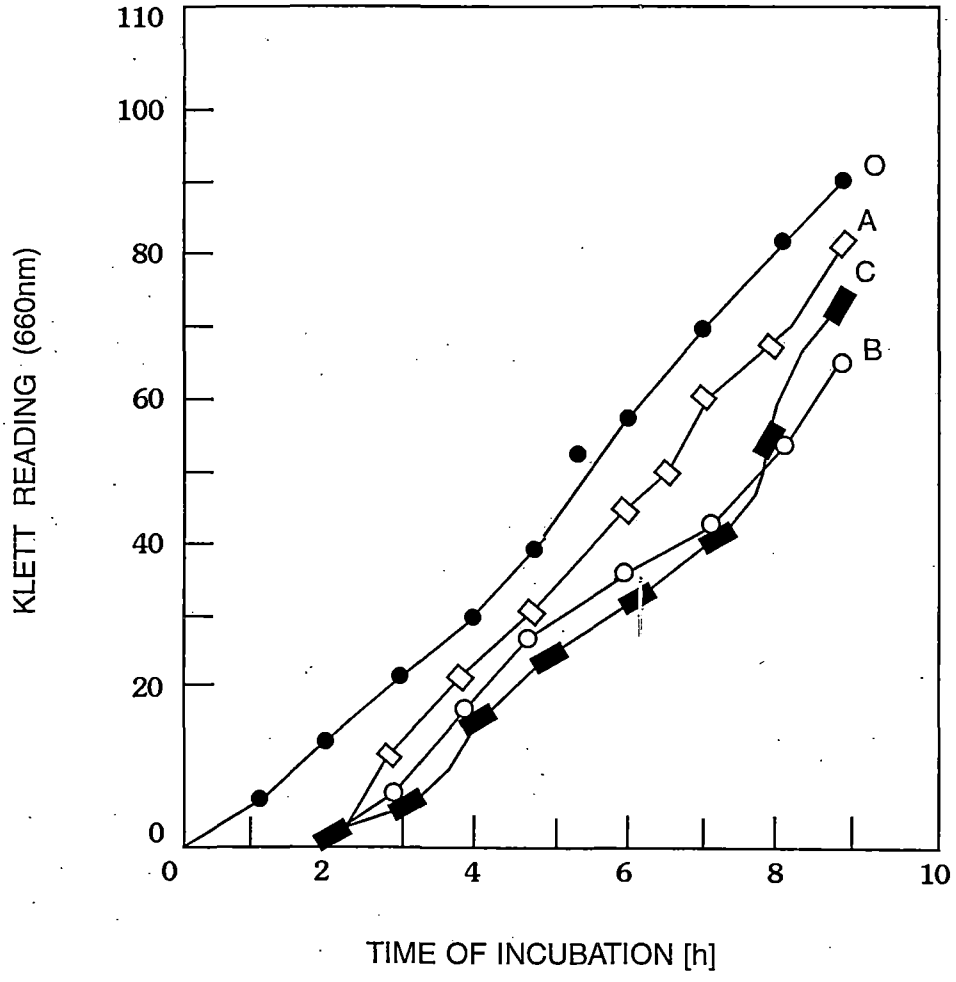


FIGURE - 18

DISCUSSION

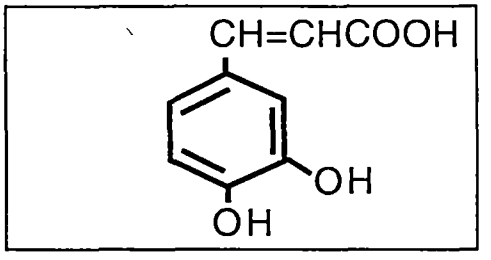
DISCUSSION

The existence of low molecular weight antithiamine factors (not structural analogues) in different food-stuffs throws new light on the dietary source and availability of thiamine. The natural antithiamines isolated till date from different sources have diverse structures although their mode of action may not so much different towards thiamine. All these antithiamines so far isolated from different food-stuffs viz. caffeic acid from fern (97), chlorogenic acid and pyrochatechins from coffee (163), fraction A from rice bran (95), methyl sinapate from mustard seed (102) and 3,5 dimethoxy salicylic acid (103) are of phenolic nature having one or more than one phenolic hydroxyl groups. The number and position of the hydroxyl groups are of primary importance for the antithiamine activity. Molecules with ortho- hydroxyl groups showed (99) a marked antithiamine effect, those with hydroxyl in para- position a medium one and diphenols with hydroxyl groups in meta- position are inactive. Further, the antithiamine activity does not depend upon the side chain of the phenolic compounds and a significant amount of antithiamine activity is reduced when phenolic hydroxyl group is substituted (99). Thus, compound 'G' characterized as 3,4 dihydroxy cinnamic acid, as isolated from *Phaseolus radiatus*, with two hydroxyl groups in the orthoposition and a side chain possesses a marked antithiamine activity.

Preliminary extraction of the antithiamine factor from *Phaseolus radiatus* with 10% chloroform-water mixture indicated that the compound was likely to be polar. Before acid hydrolysis the dry mass of the centrifuged chloroform- water extract of *Phaseolus radiatus* was not soluble in pure methanol indicating the polar nature of the active compound in the impure state and suggesting that it might exist in a bound form. On acid hydrolysis the active principle could be extracted with isobutanol. In the process of purification in silica gel G column chromatography, the active principle was obtained only by eluting with 50% methanol-chloroform mixture.

The active antithiamine factor isolated from *Phaseolus radiatus* was found to be a pure compound as tested on thin layer chromatography using three different solvent systems in which this compound gave a single spot. Various colour reactions of this isolated factor indicated the presence of phenolic hydroxyl group(s). Acidic property of this antithiamine factor was supported by its high solubility in alkaline solution and its reaction towards litmus paper.

That the isolated active component was 3,4 dihydroxy cinnamic acid was first suspected from the similarity of its antithiamine activity with that of known 3,4 dihydroxy cinnamic acid. This was first confirmed by its various physico-chemical properties viz. colour, texture, solubility, stability, dialysis, different chemical reactions, detection of elements, micro analysis and melting point. Further, the isolated compound gave the identical spots when tested on thin layer chromatography using three different solvent systems, with the corresponding R_f values which were identical to that of commercial 3,4 dihydroxy cinnamic acid (Figures - 6,7,8). Lastly, conclusive proof about the identity of the isolated compound as 3,4 dihydroxy cinnamic acid was obtained from spectral studies. The ultraviolate absorption maxima of the isolated compound was similar to that of standard 3,4 dihydroxy cinnamic acid. Moreover, infrared absorption studies of the isolated compound gave a superimposeable curve with that of known 3,4 dihydroxy cinnamic acid (Figure - 9). Thus, the antithiamine compound isolated from *Phaseolus radiatus* was 3, 4 dihydroxy cinnamic acid as represented below.



3, 4-dihydroxy cinnamic acid.

The antithiamine activity of the phenolic compounds was generally determined on the weight basis by thiochrome method (102). Accordingly, 1 mg of 3,4 dihydroxy cinnamic acid (isolated antithiamine factor) inactivated 135.0µg of thiamine hydrochloride, whereas fraction A (95), methyl sinapate (102), 3,5 dimethoxy salicylic acid (103) and caffeic acid (102) inactivated 26.5µg, 45.0µg, 20.5µg and 135.0µg of thiamine hydrochloride respectively.

As far as could be ascertained from the available literature, no study had yet been conducted to evaluate the nutritional, enzymological and microbiological (using *S. aureus*) status of 3,4 dihydroxy cinnamic acid as an antithiamine compound although several studies had already been conducted to explore different properties of 3,4 dihydroxy cinnamic acid, (164-176). The *in vivo* effect of 3,4 dihydroxy cinnamic acid (antithiamine factor as isolated from *Phaseolus radiatus*) on growth of rats was thus studied. It was observed that on administration of preincubated mixture of thiamine hydrochloride and 3,4 dihydroxy cinnamic acid to the rats caused acute thiamine deficiency such as anorexia, loss in weight, fall of hair etc. as in case of thiamine deprived rats. Thiamine deficient symptoms

of 3,4 dihydroxy cinnamic acid treated rats disappeared within 2-3 days after administration of thiamine hydrochloride.

The plasma cholesterol (free) level of the thiamine deficient rats was reduced to about half in comparison to that of normal rats. It is therefore presumed that the isolated antithiamine compound i. e. 3,4 dihydroxy cinnamic acid had an inhibitory effect on pyruvate oxidase (thiamine pyrophosphate requiring enzyme) system as a result of which formation of acetyl Co A, the precursor for cholesterol biosynthesis was considerably inhibited. The accumulation of high level of pyruvate in blood also supported this fact that there was a block in the oxidative removal of pyruvate.

Inactivation study between thiamine hydrochloride and the isolated antithiamine factor (3,4 dihydroxy cinnamic acid) was extended at the enzymic level. A number of workers (145) studied the effect of structural analogues of thiamine which caused the change in thiamine pyrophosphate dependend enzyme activity. But no extensive studies on the enzyme activity had yet been made by administering the natural antithiamines to the animal system. The *in vivo* studies on the inactivation of transketolase enzyme by natural antithiamines were carried out by Chaudhuri and his associates (96, 104, 105, 106). It is known that transketolase enzyme catalyses the conversion of ribose-5-phosphate to sedoheptulose-7-phosphate in pentose phosphate pathway requiring thiamine pyrophosphate as co-enzyme. It was found that on administration (by injection) of 3,4 dihydroxy cinnamic acid (a preincubated mixture of thiamine hydrochloride and 3,4 dihydroxy cinnamic acid) the transketolase activity of hemolysate and intestinal mucosa was markedly decreased as shown by increase in TPP effect in comparison to that of normal rats. This depressed enzyme activity was found to be restored on further administration of thiamine hydrochloride (by injection).

The loss of enzyme activity was probably due to a non-competitive inhibition, because only the structural analogues can compete with thiamine pyrophosphate to combine with the apo-transketolase protein. The restoration of the enzyme activity with the administration of thiamine hydrochloride led to the conclusion that 3,4 dihydroxy cinnamic acid had no inhibitory effect on transketolase enzyme but only on the thiamine pyrophosphate.

The TPP effect of brain was not much elevated in the case of 3,4 dihydroxy cinnamic acid (the isolated antithiamine factor) treated rats as compared with that of normal rats indicating that transketolase activity of brain was least affected in the case of thiamine deprived rats as well as 3,4 dihydroxy cinnamic acid treated rats during the forty- five days of experiment. It might be of interest to add that Salcedo and co-workers (177) showed that brain could retain its thiamine for about three weeks in thiamine deficient rats.

The inhibitory study of thiamine hydrochloride in presence of the isolated antithiamine factor from *Phaseolus radiatus* i. e. 3,4 dihydroxy cinnamic acid as determined by thiochrome method as well as on thiamine pyrophoshate-transketolase (TPP-TK) system was further extended to the microbiological level. The growth of *Staphylococcus aureus*, a thiamine dependent strain was retared when 3,4 dihydroxy cinnamic acid was added to the medium

supplemented with thiamine hydrochloride. In presence of 3,4 dihydroxy cinnamic acid thiamine was inactivated resulting in the block of carbohydrate metabolism which might ultimately be responsible for the inhibition of growth of the organism. The growth of *S. aureus* in the medium containing 3,4 dihydroxy cinnamic acid was restored to the normal level with the further addition of thiamine hydrochloride to the medium. This evidence also supported the fact that 3,4 dihydroxy cinnamic acid had only inhibitory effect on thiamine. When thiamine hydrochloride was added to the medium of 3,4 dihydroxy cinnamic acid treated *S. aureus* cell, the growth of the organism became normal after one hour, suggesting that 3,4 dihydroxy cinnamic acid had no toxic effect on *S. aureus*.

In conclusion, it may be stated that the antithiamine factor isolated from *Phaseolus radiatus* was found to be chemically analogous to 3,4 dihydroxy cinnamic acid. It possesses marked antithiamine activity. Its antithiamine property was initially estimated by thiochrome method and finally confirmed by nutritional, enzymological and microbiological studies.

Lastly, it can be added that in some preliminary chromatographic and absorption studies, it was observed that the interaction between thiamine and 3, 4 dihydroxy cinnamic acid (antithiamine) was probably a reversible process involving the formation of a complex. Further study on the exact mechanism involved in this interaction is now in progress.

SUMMARY

SUMMARY

Isolation and characterization of an antithiamine compound from a kind of bean *(Phaseolus radiatus)* and its biochemical and nutritional studies were described.

1. The procedure for isolation of antithiamine factor from *Phaseolus radiatus* consisted of solvent extraction, acid hydrolysis, column chromatography using different adsorbents, repeated crystallisation etc. The isolated antithiamine factor was a light yellow amorphous substance and proved to be a single compound on thin layer chromatography using three different solvent systems.

a) acetone : methanol (1:1, v/v)

$R_f = 0.70$

b) n-butanol : acetic acid : water (upper phase 8:1:1, v/v/v)

$R_f = 0.73$

c) chloroform : methanol : water (3:1:1, v/v/v)

$R_f = 0.91$

2. A simple method to measure the antithiamine activity was developed in which the residual thiamine was estimated by the modified thiochrome method. The antithiamine activity of the isolated factor was measured by this method and it was found that 1 mg of this compound inactivated 135.0μg of thiamine hydrochloride.

3. From different chromatographic studies, spectral (IR) and micro-analytical data

as well as from the standpoint of biological activity, the antithiamine factor was characterized as 3, 4 dihydroxy cinnamic acid.

4. Effect of the antithiamine factor (3, 4 dihydroxy cinnamic acid) was studied on the growth of rats (nutritional). 3, 4 dihydroxy cinnamic acid when administered to rats under *in vivo* condition, animals developed symptoms of thiamine deficiency which completely disappeared by further administration of thiamine.

5. Estimation of free plasma cholesterol of antithiamine factor (3, 4 dihydroxy cinnamic acid) treated rats showed about half value in comparison with that of normal rats.

6. The blood pyruvate level of the antithiamine factor (3, 4 dihydroxy cinnamic acid) treated rats was found to be increased about 4 times its normal value.

7. Effect of the antithiamine factor (3, 4 dihydroxy cinnamic acid) on transketolase activity in blood, brain and small intestine of rats, *in vivo*, was studied in terms of TPP effect (%). While in case of 3, 4 dihydroxy cinnamic acid treated rats, the TPP effect was found to have increased in blood and intestine but not significantly in brain and this increased TPP effect became normal with further administration of thiamine hydrochloride.

8. In a broth containing thiamine the growth of *S. aureus*, a thiamine requiring strain, was inhibited in presence of the antithiamine factor (3, 4 dihydroxy cinnamic acid). After administration of excess thiamine into the broth, this retarded growth of *S. aureus* was found to have reached normal level.

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