1948

The Chastek paralysis or thiamine destroying enzyme of fish tissues

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NOTE TO USERS

THE CHASTEK PARALYSIS OR THIAMINE DESTROYING ENZYME OF FISH TISSUES

by

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A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Physiological and Nutritional Chemistry

Approved:

Signature was redacted for privacy.

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Dean of Graduate College

Iowa State College
1948

ACKNOWLEDGMENT

The writer wishes to express her real appreciation to Dr. R. R. Sealock for his interest and help during the course of this work.

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I. INTRODUCTION

As far as is known all living things require thiamine for good growth. The higher animals are heterotrophic with respect to this vitamin, since they are unable to synthesize thiamine and therefore require an external supply. Plants and microorganisms differ in that a number of them can synthesize vitamin B_1 in whole or in part. A deficiency of thiamine in man is manifested by both "beri-beri" and "Wernicke's disease", while the disease in foxes known as Chastek paralysis was found to be a counterpart of these, and thus due to a deficiency of the vitamin. Although it was at first believed that Chastek paralysis was produced merely as the result of an inadequate intake of thiamine, it was later found that certain species of fishes, when included without previous cooking in the rations of foxes, actually destroyed dietary thiamine.

Thus, in addition to the attention given to the nutritional problems associated with this paralysis, attention was also focused upon the mechanism by which fish destroyed thiamine, and it was found that the destruction was enzymatic. Although the practical nutritional problems connected with the feeding of fish to foxes have been solved, at present the more strictly chemical problems of the nature and mechanism of action of the thiamine-destroying enzyme have not been sufficiently elucidated.

Therefore, in this laboratory the Chastek paralysis problem

has been approached from the view-point of characterization of the fundamental nature of the enzyme responsible for the paralysis. Studies were directed towards an understanding of its mechanism, with emphasis placed on the equilibrium of the reaction and on the possibility of reversing the enzymatic destruction of thiamine. Although the question of the physiological function of this enzyme was not of primary importance, results obtained in these studies would be expected to clarify the role played by this thiamine-destroying enzyme in certain fish, which certainly must require the vitamin.

A further manifestation of thiamine deficiency was found in the so-called "fern-poisoning" of cattle. This deficiency is similar to that observed in Chastek paralysis, in that it appears to result from the destruction of dietary thiamine by a factor present in bracken fern. Therefore, because of the possibility that this inactivation of thiamine is also enzymatic, concurrent investigations regarding the nature of the "fern factor" were initiated.

II. HISTORICAL

A. Chastek Paralysis Factor of Certain Fish

In the early 1930's several acute outbreaks of a new disease were observed on fox ranches in the midwestern part of the United States. R. G. Green (1), professor of bacteriology at the University of Minnesota, was the first to report on the nature of this disease. He began his studies in 1932 when asked to investigate an outbreak which occurred on the J. S. Chastek Ranch, Glencoe, Minnesota. Because this outbreak on the Chastek Ranch was the first to be recorded, the disease has since been known as Chastek paralysis.

Chastek paralysis in foxes (1-3) was characterized first by a loss of appetite. Within a few days there developed a marked stiffness and a lack of coordination as evidenced by an abnormal gait and by the bizarre activities exhibited by the animals. Progress of the disease was very rapid after the appearance of these initial nervous disorders and finally a complete spasticity developed, the animals becoming so paralyzed that they could not rise or stand. Convulsions often occurred before death, which usually came within one to four days after the onset of neurologic disturbances.

Necropsy (2-4) of animals dying from Chastek paralysis showed that pathological changes had occurred throughout the

body. There was fatty degeneration of the liver, acute congestion and inflammation of the intestine, and ulcers were often found in the stomach. The heart was swollen and was described as having the appearance of being cooked. Although small hemorrhages were found in many parts of the body, those found in the brain were particularly characteristic of Chastek paralysis. Here the fundamental change was in the capillaries and other small vessels which were very prominent as a result of a marked overgrowth of the cells of the lining membranes. When these vessels ruptured characteristic bilaterally symmetrical lesions were produced.

The work of Green and his associates, W. E. Carlson and C. A. Evans, (1,2,5) to determine the etiology of the disease indicated that it was not of bacterial origin, since it was not transmissable from one animal to another, although the disease appeared to be epizootic in nature. Furthermore, the possibility that the paralysis was due to food poisoning was eliminated by the fact that the disease did not occur in a large number of animals at one time, even though all had been receiving the same diet.

In a series of publications Green and his co-workers (1-8) described several outbreaks of Chastek paralysis, most of which occurred during the first three months of the year. In the winter months the fox farmers made a practice of using frozen raw fish as a cheap source of protein in the diets, and at the

same time, since fresh food was not as readily obtainable, the rations became more restricted. In general the outbreaks were observed from three to six weeks after the inclusion of the frozen raw fish, usually carp, as ten per cent or more of the diet. However, a severe outbreak was observed on the Howland-Daly Ranch where fish comprised only 5.4 per cent of the diet, but here an unusually large amount of cod liver oil had been included in the ration.

Chastek paralysis, both that which occurred naturally and that which was produced experimentally during the summer months upon feeding of frozen raw fish, could be controlled or stopped by removing fish from the diet. Green therefore concluded that Chastek paralysis resulted from the feeding of raw fish in concentrations of 10 per cent or more of the diet. Several species of fishes, including carp, quillbacks, mullets, suckers, northern pike, canned Pacific Coast mackerel and Atlantic whiting, from such varied sources as the Atlantic Ocean, fresh water lakes in Utah, Minnesota and Canada, and the Cedar River in Iowa, were found to produce Chastek paralysis, when included in fox rations.

Although the withdrawal of fish from the ration prevented the occurrence of new outbreaks, the effect upon animals already showing symptoms of the disease was dependent upon the type of diet then given. If a diet consisting of two-thirds horse meat and one-third poor grade cereal was given, deaths continued for two or more weeks; but if a large proportion of liver,

milk and eggs were included and if the diet was otherwise balanced, there was a more rapid recovery. In contrast to the effect observed upon raw-fish feeding, canned fish, fish meal or previously cooked fish were fed without producing an outbreak of Chastek paralysis. A ration containing raw fish could be fed with no ill effects if it was alternated with a non-fish or a cooked fish ration, and in addition, Chastek paralysis was not produced if the fish ration was fed in the morning and the remaining diet constituents in the evening.

Although the feeding of fish had been definitely established as the causative factor in Chastek paralysis, the reason for this was not immediately apparent. In 1937 Green (4) reported that this disease was actually due to a deficiency of vitamin B. Although no particular member of the B complex was at first specified, later observation (2,6) of the similarity between the brain lesions characteristic of Chastek paralysis and those found in B_1-deficient dogs, suggested that the deficiency was that of the antineuritic factor of the complex. That the symptoms were suggestive of a thiamine deficiency gave at least a clue to the action of the fish in producing the disease.

The fact that substitution of the usual basic diet, upon withdrawal of fish from the diet of foxes after symptoms of paralysis had appeared, was not completely effective in promoting rapid recovery indicated that this basic diet was almost deficient in the vitamin. The addition of fish, which had a

low content of the vitamin, not only decreased the total vitamin concentration, but in addition increased the vitamin requirement due to the increased intake of fats and oils coincident with the introd ction of fish into the diet. If this were the case, the inclusion of any type of fish in such a diet should produce a vitamin B_1 deficiency, but later work has shown that not all species of fishes are capable of producing Chastek paralysis. Furthermore, since instances of outbreaks of the disease were observed when the vitamin intake was adequate, and since cooked fish did not produce Chastek paralysis, it appeared that the deficiency was not due merely to an insufficient intake of vitamin B_1.

Green and his co-workers (2, 5-8) described several instances in which Chastek paralysis was controlled or prevented by the administration of yeast and other materials high in vitamin B content, lending support to the idea that the disease was the result of a vitamin deficiency.

In 1937 Carlstrom and Jonsson (9) reported studies of a disease occurring among foxes in Sweden, which was characterized by loss of appetite, emaciation, ataxia and convulsions, symptoms which were similar to those found in rats receiving a thiamine deficient diet. Therefore, when a fox showing severe symptoms was brought to them for treatment the only therapy employed was the intravenous injection of 1 mg. of thiamine. On the following day the animal was completely recovered. Similar responses were obtained upon thiamine administration to

other animals with the disease, and upon inclusion of yeast in the fox ration, no further outbreaks were observed.

The similarity of the symptoms of the disease and of the diet, which contained fish ("stromming" and other small herring), to those associated with Chastek paralysis, made it appear that the Swedish workers were studying this type of paralysis, although no mention was made of the possible relationship. Carlstrom and Jonnson considered that the deficiency characterizing the disease resulted from an inadequate intake of thiamine, since the fish had a low content of the vitamin and since the cereals comprising the remainder of the diet had been previously cooked, thereby possibly inactivating thiamine.

In 1939 Enderog and Helgebostad (10) described a paralysis occurring among silver foxes in Norway, which appeared to be similar to that characteristic of beri-beri in man. The successful therapeutic effect of thiamine administration to these paralytic animals, and the fact that an identical paralysis was produced experimentally by maintaining foxes on a thiamine deficient diet, indicated that the condition was a manifestation of thiamine deficiency. It would appear that the "beri-beri in foxes" of Norway was a further example of the disease which was known in this country as Chastek paralysis, although Enderog and Helgebostad did not mention such a relationship nor did they associate the occurrence of the disease with the feeding of fish.

By 1939 the feeding of fish had been established as the cause of Chastek paralysis and the evidence indicated that the fish in some manner was producing a thiamine deficiency. Although at this time opinion favored the hypothesis that the deficiency was nutritional, resulting from an inadequate intake of thiamine, Gulbrand Lunde, Director of the Research Laboratory of the Norwegian Canning Industries,(11), presented evidence in opposition to this idea. By calculating the thiamine contents of the diets described by Green, he found that a diet containing 18.5 per cent fresh fish, the feeding of which had produced an outbreak of Chastek paralysis, had a higher thiamine content than a 7 per cent fish diet which had been fed with no ill effects. Furthermore, since canned mackerel had been fed to silver foxes for a period of six months with no incidence of Chastek paralysis, it appeared that the feeding of fish did not result in a requirement for thiamine in an amount greater than that found in the normal diet constituents. Lunde therefore concluded that the most logical explanation for the production of the paralysis by feeding fish was that the fish used were spoiled or contaminated. However, the evidence presented by Green and his co-workers denied this possibility, since all fish fed to foxes was strictly fresh or had been kept in a frozen state until used.

At this time the first of several publications from the University of Wisconsin College of Agriculture appeared.

Coombes (12-13) reported that the feeding of fish was potentially dangerous to both foxes and minks, but that the latter animals were less susceptible to the fish-induced paralysis. Chastek paralysis was produced experimentally by feeding a diet containing 25 per cent raw smelt, but it could not be produced by feeding the same amount of previously cooked fish. Since the administration of thiamine in amounts up to 2,500 International Units was not effective in combating the paralysis, and since the feeding of fish after autoclaving, a procedure used in the preparation of thiamine-deficient diets, did not result in the production of the disease, Coombes concluded that a thiamine deficiency was not the cause of Chastek paralysis, but that the fish contained some factor of a toxic nature.

In spite of the contradictory results obtained by Carlstrom and Jonsson and by Coombes with respect to thiamine therapy in Chastek paralysis, Green in 1939 (8) suggested that the crystalline vitamin as well as yeast or liver could be used to prevent outbreaks of the disease among foxes.

In 1940 Green and Evans (5) described the pathology of the brain characteristic of these paralytic animals and showed Chastek paralysis to be the counterpart of Wernicke's disease in man. In the same year Alexander (14) had shown that Wernicke's disease or acute alcoholism in man, was identical with the experimentally produced B_1-avitaminosis in pigeons

with respect to the nature of the brain lesions. The relationship of Chastek paralysis to Wernicke's disease, together with the findings by Carlstrom and Jonsson (9) and by Green and Evans (5) that a similar paralysis could be produced on a thiamine deficient diet, gave additional support to Green's earlier suggestion that Chastek paralysis was the result of a thiamine deficiency.

Further confirmation came in 1941 and 1942 when Green, Carlson and Evans (15-16) reported the experimental production of Chastek paralysis in foxes receiving a diet containing 20 per cent whole carp, 45 per cent horse meat, 9 per cent cereal and 0.5 per cent cod liver oil. Another group of foxes receiving this basal diet plus 20 mg. of nicotinic acid per day also developed the disease, while a group maintained on the basal diet plus 25 mg. of thiamine per day showed no symptoms of paralysis. At this time, since diets containing a moderate excess of thiamine were capable of producing a vitamin deficiency, when fresh fish comprised 10 per cent or more of the ration, the question of how the deficiency was produced presented itself. Green, Carlson and Evans suggested three possible answers: the fish might (1) increase the thiamine requirement, (2) prevent the absorption of thiamine, or (3) inactivate or destroy the vitamin.

In 1941 Alexander, Green, Evans and Wolf (17) published a joint paper in which the clinical and pathological identities

of alcoholic encephalopathy in man (Wernicke's Disease), Chastek paralysis of foxes and Wolf's disease in fish were demonstrated. Wolf (18) by feeding raw fish had been able to produce a disease in trout which was prevented by the addition of extra thiamine to the diet.

Characteristics of the fish-diet disease of trout as described by Wolf included loss of balance, anorexia, excitability, deflated swim bladder, muscular atony of stomach and intestinal walls, keratitis and paralysis. Diets containing buckeye shiners (Notropus atherinoides) were most active in producing the disease; herring (Clupea harengus) were not as active, but were still injurious; while diets containing smelt (Osmerus mordax) did not cause the paralysis to appear. Two groups of rainbow trout fingerlings, 100 fish in each group, were placed on diets consisting of 50 per cent sheep plucks and pork spleen and 50 per cent herring. In the group receiving this diet containing raw herring symptoms of the disease developed quickly, while after 12 weeks no symptoms of the fish-diet disease appeared in the group receiving the diet containing previously cooked herring.

Marked improvement was observed in fish displaying symptoms of the disease upon insertion into the stomach of tablets containing 166 or 333 international units of thiamine. From this evidence Wolf concluded that the so-called fish-diet disease was a B_1-avitaminosis produced by the feeding of certain species

of fishes. Since feeding mixtures of raw fish and other foods immediately after mixing produced no injurious effect, while such a mixture which had been allowed to stand for any considerable time prior to feeding was capable of producing the disease, it appeared that the deficiency resulted from the destruction of the dietary thiamine by the fish.

Further reports in 1941 by Coombes (19,20) and by Spitzer, Coombes, Elvehjem and Wisnicky (21) established the fact that raw carp actually inactivated thiamine. Initial *in vivo* experiments were carried out in which chicks were fed a basal fox ration supplemented with raw carp. The effect of feeding whole carp, raw or cooked, and of various fractions of carp on the incidence of polyneuritis in the chicks is summarized in Table I.

The fact that none of the chicks in group 3 developed polyneuritis gave further support to earlier observations that cooking rendered the fish safe. The factor or factors responsible for the production of the deficiency appeared to be insoluble in water and partially soluble in ether, as shown by the results obtained with groups 7 and 8, Table I. Since in all other groups there was a 100 per cent development of polyneuritis it was impossible to determine any quantitative distribution of the factor or factors in various parts of the fish. However, the chicks in group 6 developed the symptoms more rapidly than did those of groups 4 and 5, indicating a

Table I

Effect of Feeding Raw Carp on Development of Polyneuritis in Chicks

Group	Ration Fed	Per cent of Group Developing Polyneuritis
1	Basal Fox Ration (control)	0
2	Basal + 25% raw whole carp	100
3	Basal + 25% cooked whole carp	0
4	Basal + 25% raw carp heads and tails	100
5	Basal + 25% raw carp muscle meat and skin	100
6	Basal + 25% raw carp entrails	100
7	Basal + H_2O extract equivalent to 25% raw whole carp	0
8	Basal + ether extract equivalent to 25% raw whole carp	40
9	Basal + residue after water and ether extraction equivalent to 25% raw whole carp	100
10	Basal + 25% raw whole carp + 200 micrograms thiamine/100 g. of ration	100
11	Basal + 25% raw whole carp + 350 micrograms thiamine/100 g. of ration	100

higher concentration of the deficiency-producing material in the viscera.

The Wisconsin group also conducted *in vitro* studies in which portions of carp entrails (raw or cooked) were incubated at room temperature with known amounts of thiamine for varying lengths of time, after which the remaining thiamine was determined by the thiochrome method. The results, given in Table II, indicated that the fish actually destroyed or inactivated thiamine, thus rendering it unavailable; that the amount of inactivation was dependent upon the time of contact between the fish and the vitamin, and that cooked fish was incapable of destroying thiamine.

The results obtained by both the *in vitro* and *in vivo* studies indicated that the deficiency disease produced by feeding raw fish resulted from the inactivation of thiamine by some heat labile factor or factors present in raw fish, and although evidence was still too incomplete to allow making a final decision concerning the nature of this factor, the possibility of an enzymatic phenomenon was suggested.

In the same year Woolley (22) confirmed the heat lability of the thiamine destroying factor with *in vitro* studies. A suspension of carp tissue heated to the boiling point was able to destroy only one-half as much added thiamine as an unheated sample, while heating for fifteen minutes at fifteen pounds pressure destroyed all of the thiamine-destroying activity.

Table II

In vitro Studies of Effect of Raw Carp and Cooked Carp Entrails on Crystalline Vitamin B_1

Weight entrails gms.	Incubation time min.	Thiamine Added λgm.	Thiamine Destroyed per cent
2.5 raw	15	100	100.0
2.5 "	30	100	100.0
2.5 "	60	100	100.0
2.5 raw	15	200	71.0
2.5 "	30	200	90.0
2.5 "	60	200	100.0
2.5 raw	15	300	56.0
2.5 "	30	300	62.5
2.5 "	60	300	95.0
2.5 raw	15	400	45.5
2.5 "	30	400	50.0
2.5 "	60	400	70.0
2.5 raw	15	500	31.5
2.5 "	30	500	35.5
2.5 "	60	500	48.0
2.5 raw	15	600	25.0
2.5 "	30	600	25.0
2.5 "	60	600	37.0
2.5 raw	720	600	100.0
2.5 cooked	15	100	0
2.5 "	120	100	0
5.0	60	500	100.0

Wooley gave no experimental details in this note, but indicated that the active factor was extractable from freshly killed carp with ethanol and with ten per cent sodium chloride, while aqueous extracts were only one-fourth as active as suspensions of carp tissue. Aqueous extracts of dialyzed suspensions were inactive, although the active component was not dialyzable. The "fish principle" or active factor was distributed in a two to three to three ratio in the head, viscera and torso.

Suspensions or aqueous extracts of carp tissue were not only capable of destroying thiamine as determined by a chemical method, but also destroyed growth promoting activity for Endomyces vernalis. Since this organism requires either thiamine or its pyrimidine moiety for its growth, and since the growth promoting activity of thiamine was destroyed by incubation with fish extracts, Woolley concluded that the action of the "fish principle" could not be that of splitting the vitamin into its two moieties.

By using Endomyces vernalis for thiamine determination Woolley found that the thiamine destroying reaction was not instantaneous. One particular extract of carp destroyed 1.3, 4.0 and 4.3 micrograms of thiamine in 6, 16 and 24 hours respectively. Woolley made no suggestion regarding the nature of the reaction which he was studying.

In 1942 Green and his associates (3, 23) fed a basal diet (cereal 15, bread 10, carrots 9.5, cod liver oil 0.5, and horse meat and fish 65 per cent) supplemented with whole carp or carp fillets as 20 per cent of the total diet or with viscera

or trimmings of carp as 10 per cent of the total to several groups of foxes. While Chastek paralysis developed first in the group fed trimmings, fatalities were also observed in the groups fed viscera and whole carp. However, no fatalities were observed in the group receiving the muscle fraction. The "fish factor" therefore appeared to be concentrated particularly in trimmings and viscera, while it was almost absent from muscle.

The administration of 10 to 25 mg. of thiamine per day, given as part of the fish ration, gave complete protection against the production of Chastek paralysis, while 5 mg. of the vitamin per day permitted a subclinical paralysis to develop. With animals receiving less than 5 mg. of thiamine per day Chastek paralysis was observed but death was delayed beyond that of control animals receiving no thiamine.

Green and his co-workers found that one pound of whole carp fed as 20 per cent of the ration destroyed as much as 4,400 units of thiamine, but that the amount of vitamin destroyed was proportional to the amount present. This latter fact led to the suggestion that thiamine inactivation was due to "adsorption" or perhaps to the formation of a complex of the avidin-biotin type.

Hodson and Smith in the same year (24) confirmed earlier work by comparing the results obtained by feeding one group of adult silver foxes on a thiamine-deficient diet and another group on a diet containing 30 per cent frozen smelt. The progress of the disease, the effect of thiamine therapy on

several animals, and the terminal symptoms of others were the same in both groups. In order to determine the specificity of thiamine therapy, one fox in the second group was given 5 mg. of pyridoxin while another was given 5 mg. each of pyridoxin, riboflavin, and calcium pantothenate. No improvement was observed with either treatment, whereas thiamine administration caused an almost immediate improvement.

Deutsch and Ott in 1943 (25) reported that a factor present in raw smelt destroyed both thiamine and cocarboxylase of yeast, while heated smelt (100 degrees for 10 to 30 minutes) did not destroy the vitamin. Five per cent alcohol extracts of smelt were mixed with both fresh, living yeast and dry, non-viable yeast (containing 140 micrograms of vitamin B_1 per gram). The mixtures were air-dried at room temperature, allowing a 12 hour period of moist contact, and were then mixed with chick rations; these diets were analyzed for thiamine by the thiochrome method. No loss of thiamine was observed in the diets containing cooked smelt, or in those containing the raw-smelt-viable yeast mixture. Only in the raw smelt-non-viable mixture was any significant loss of thiamine observed. The failure of the raw fish to destroy the thiamine present in the viable yeast was explained on the basis that the thiamine-inactivating factor was unable to penetrate the yeast cell. In the case of the non-viable yeast, the drying process had probably caused autolysis of the cells, thus allowing

contact between the "fish principle" and the vitamin.

No increase in free thiamine was observed when the raw smelt-non-viable yeast mixtures were hydrolyzed with 0.1 N. sulfuric acid for as long as sixty minutes. This evidence made it appear very unlikely that the formation of a complex of the avidin-biotin type was the mechanism whereby the "fish factor" inactivated thiamine. Deutsch and Ott concluded with the suggestion that the inactivation of the vitamin might be due to an enzymatic reaction.

In September of 1942 Sealock, Livermore and Evans (26) made a preliminary report of <u>in vitro</u> studies in which the inactivation of thiamine upon incubation with fish tissues was measured by both fermentation and chemical procedures for thiamine determination. They defined a unit of activity as the activity causing the loss of one micromole of thiamine, when a test solution containing the fish preparation and 2.5 micromoles of thiamine at pH 7.4 was incubated for 2 hours at 37 degrees.

Stable dry powders (containing 7 to 14 units per gm.) were prepared by treatment of fish viscera (1 to 2 units per gm.) with cold acetone. The "fish principle" exhibited maximum destruction of thiamine at 60 degrees and at pH 9.0. The protein nature of the factor was suggested by the fact that it was inactivated by mild heat, by proteolytic enzymes, and

by protein precipitating agents. In addition, the action of the factor was inhibited by some of the usual enzyme inhibitors. The evidence presented by Sealock and his associates strongly suggested that the "fish principle" was an enzyme.

Sealock, Livermore and Evans in 1943 (27) published more detailed studies of their extensive work on the properties of the "fish factor". Details for the preparation of an acetone desiccated power from carp tissue were described, and it was found that 1 gm. of tissue yielded on an average 0.15 gm. of powder, which contained up to 90 per cent of the original activity. These preparations retained their activity for at least eight months when stored in the cold. The active principle could be extracted from the powder with 10 per cent sodium chloride. The solubility of the factor in dilute salt solutions and its relatively low solubility in water indicated a protein nature.

To determine thiamine destroying activity, aliquots of a fish brei or extract were adjusted to pH 7.4. To 2 or more ml. of the fish extract were added 1 ml. of a 0.2 M. phosphate buffer (pH 7.4), water to give a volume of 4 ml., and 1 ml. of a thiamine solution containing 2.5 micromoles of the vitamin. Five ml. of 20 per cent trichloroacetic acid was added at the end of two hours incubation at 37.5 degrees, and 2 ml. aliquots of the protein-free solutions thus obtained were analyzed for thiamine. Controls were made up in the same fashion, except

that the trichloroacetic acid was added immediately after the thiamine addition had been made.

The details presented in this paper regarding the effect of pH, temperature and protein precipitating agents on the "fish factor" supported the suggestion made by the authors in their initial report (26) that the factor was a protein substance. Further support for the enzymatic nature of the "fish principle" was found when it was determined that the amount of thiamine destroyed by a fish extract increased with time of incubation up to six hours. In addition velocity constants, calculated with the first order equation, exhibited good uniformity, considering the crude nature of the breis or extracts employed. The possibility of the formation of an avidin-biotin type of complex was excluded by the fact that no increase in free thiamine was observed upon heat treatment, proteolytic digestion, or acid hydrolysis of solutions containing inactivated thiamine.

In the same year Deutsch and Hasler (28) determined the thiamine-destroying activity of several species of fishes. The method employed was that described by Deutsch and Ott (25), in which yeast was used as a source of thiamine. The results indicated that the "fish principle" is found frequently in fresh-water fish, but not in salt-water fish. However, several species of marine fish have been found to be active by other workers. Green and his co-workers (1,5) reported the

production of Chastek paralysis upon feeding of salt-water fish, particularly Atlantic whiting. Deutsch and Hasler were unable to find any constant taxonomic correlation for the occurrence of the factor, nor did the food habits or the environment appear to offer a basis upon which to predict its presence in a given species.

In 1943 Owen and Ferrebee (29) published a review article in which the work dealing with the so-called "fish-disease" was summarized. No additional experimental data were presented.

In January of 1944 Krampitz and Woolley (30) published the results of their work on the mechanism of thiamine inactivation by the fish factor and reported the isolation of the products formed by the destruction of thiamine. Temperature studies showed that the activity of the fish factor increased from 0 to 37 degrees but that the temperature coefficient was small. In addition, there was increased destruction of thiamine with increase of pH from 1.0 to 8.0. Measurement of thiamine inactivation by the fish principle at different time intervals showed that the amount of reaction increased from 0 to 30 minutes, but that beyond that time no further destruction was observed. For example, a given preparation of the factor, when incubated at 25 degrees with 50 micrograms of thiamine, destroyed 24 micrograms in 5 minutes, 30 in fifteen minutes, and showed 32 micrograms destroyed at 30, 60 and 120 minutes. While the results with temperature and pH studies were in agreement with those of Sealock and his co-workers (27), those

regarding the time course of the reaction were not. However, in the latter case incubation was carried out at 37 degrees in a 0.04 M. phosphate buffer at pH 7.4 with a 5×10^{-4} M. concentration of thiamine, and the enzyme preparation was made by a sodium chloride extraction of acetone desiccated powder. Krampitz and Woolley did not indicate that their reaction mixture had been buffered at any particular pH. Furthermore their solutions were more dilute with respect to both enzyme and thiamine. Fifty micrograms of thiamine were contained in a volume of more than 20 ml., while the 5×10^{-4} M. concentration employed by Seelock, et al, represented 843 micrograms of the vitamin in an incubation mixture of 5 ml. The apparent contradiction regarding the time course of the reaction might therefore be explained by the widely different conditions employed.

The fact that the fish factor destroyed thiamine as determined by a chemical method, but did not destroy its growth promoting activity for Mucor rammannianus, a mold requiring thiamine or its thiazole moiety for optimal growth, gave evidence for the liberation of the intact thiamine thiazole moiety during the destruction of the vitamin. Assays of solutions containing the destroyed thiamine with Endomyces vernalis, a yeast requiring thiamine or its pyrimidine component for growth, revealed a difference between the action of fish tissue suspensions and that of sodium chloride extracts

of fish tissues. Solutions in which thiamine had been destroyed by the former contained approximately the same growth promoting activity for Endomyces as did an equivalent amount of unaltered thiamine. On the other hand Endomyces assay of the thiamine destroyed by the sodium chloride extracts showed only a fraction of the original growth promoting activity.

This difference between suspensions and extracts suggested that thiamine destruction took place in a step-wise manner. In the first reaction the thiazole portion was freed and a pyrimidine derivative, only slightly active for Endomyces vernalis, was liberated, while next a substance present in tissue but almost absent from the sodium chloride extracts converted this to an active pyrimidine. The sodium chloride extracts, however, exhibited increased potency for Endomyces upon standing, and in addition, treatment of the reaction mixture with strong alkali restored full activity for the yeast. Thus the overall reaction would appear to be the hydrolytic cleavage of the thiamine molecule to liberate the thiazole and pyrimidine components of the vitamin.

Krampitz and Woolley confirmed this hypothesis regarding the products of the enzymatic destruction of thiamine by the isolation and identification of 4-methyl-5- β -hydroxyethyl-thiazole and 2-methyl-6-amino-5-hydroxymethylpyrimidine. The former was isolated from a reaction mixture in 82 per cent yield and identified as the picrate, the chloroplatinate and as

the flavianate. For the isolation of the pyrimidine derivative a reaction mixture in which all of the added thiamine had been destroyed by a suspension of carp tissue was employed. The pyrimidine was first precipitated as the mercury salt and eventually isolated as the hydrochloride in a 41 per cent yield. Although the evidence to date had pointed to the enzymatic nature of the fish factor, this identification of the end products of the reaction, appeared to definitely establish this thiamine destroying reaction as an enzymatic one.

Bhagvat and Devi (31) used diluted carp blood and extracts of carp muscle and of carp viscera for studies of the thiamine destroying enzyme. The determination of enzyme activity was carried out at pH 5.6 (acetate buffer) by incubation of an enzyme extract with 25 micrograms of thiamine at 37 degrees. Muscle extracts showed an increased amount of reaction up to 24 hours, while preparations from viscera and blood produced 100 per cent destruction of the added thiamine within 1 hour. The amount of thiamine destruction increased from pH 3 to 6, but since at pH 6 and above, all of the added thiamine was destroyed no conclusions regarding an increased amount of reaction at the more alkaline pH's could be made. The results did indicate, however, that, at least with this concentration of thiamine, the reaction went to completion. A large percentage of activity was lost if the enzyme-containing solutions were allowed to stand overnight at either pH 2 or pH 10.

A comparison of water extracts, 5 per cent sodium chloride extracts and 5 per cent chloroform extracts of equal weights of carp muscle showed 14.0, 22.4 and 25.0 micrograms respectively of thiamine destroyed under similar assay conditions. On the basis of dry weight 1 mg. of muscle, viscera and blood destroyed 0.32, 8.6 and 8.3 micrograms of an added 25 micrograms of thiamine. Extracts of muscle dialyzed at 20-25 degrees for 48-72 hours against running water destroyed 31.8 per cent of the added thiamine as compared with 100 per cent for the undialyzed extracts, while similar dialysis of a visceral extract reduced the destruction from 95.2 to 64.7 per cent. Activity of dialyzed extracts was restored by the addition of a previously boiled enzyme preparation.

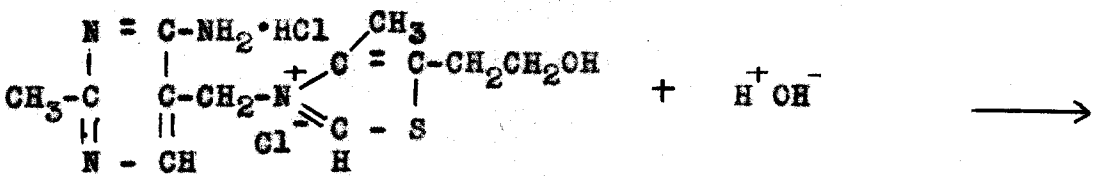
An attempt at purification of the enzyme by precipitation of a muscle extract with ammonium sulfate indicated that the active material was precipitated only at complete saturation. Treatment of a muscle extract with 3 volumes of ice-cold acetone gave a precipitate containing only one-half of the original activity, while treatment of whole muscle with 3 volumes of cold acetone gave a preparation which retained all of the original activity. No mention was made of the pH at which these precipitations were carried out.

Although no experimental details or concentrations were given, Bhagvat and Devi mentioned that the destruction of thiamine by fish extracts was not inhibited by potassium

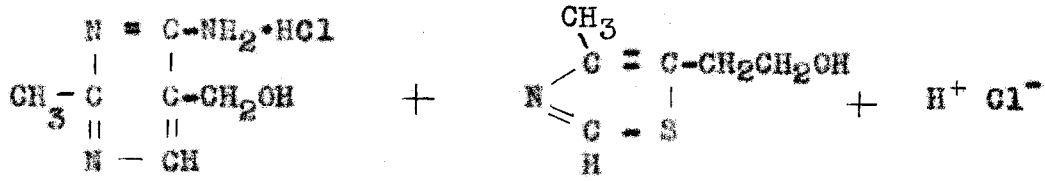
cyanide or sodium fluoride.

Smith and Proutt in 1944 (32) reported that cats receiving an exclusive diet of either raw carp or raw herring developed all of the symptoms of a thiamine deficiency within 23 to 40 days after the initiation of the fish diet. The observed symptoms were the same in all respects as those appearing when a diet of thiamine-free canned rabbit meat was fed, and subcutaneous injections of thiamine produced a prompt recovery. In contrast the feeding of catfish, butter-fish or spots produced no signs of a deficiency. Since the work with foxes and minks showed that there was a species difference with respect to susceptibility of a fish-induced thiamine deficiency, this report served to add another species to the group of susceptible animals.

If the enzymatic destruction of thiamine results from the hydrolytic cleavage of thiamine, as postulated by Krampitz and Woolley (30), the reaction could be written in the following way:



Thiamine Chloride Hydrochloride



2-methyl-6-amino-5-hydroxymethylpyrimidine hydrochloride

4-methyl-5-β-hydroxyethylthiazole

Thus the overall reaction would involve the addition of the hydroxyl ion of the water molecule to the methylene substituent in the pyrimidine, the reduction of the quaternary nitrogen of the thiazole to a tertiary nitrogen, and the release of the hydrogen ion of the water molecule. Sealock and Livermore (33), by showing that one hydrogen ion was released per molecule of thiamine-destroyed during the enzymatic destruction of the vitamin, gave support to this mechanism. This was done by carrying out the reaction in a bicarbonate buffer and measuring the carbon dioxide evolved by means of the Warburg manometric technique. In such a buffer the release of a hydrogen ion would convert a bicarbonate molecule to carbonic acid and in turn bring about the evolution of a molecule of carbon dioxide.

The relationship between carbon dioxide evolution and thiamine destruction (Table III) was good, in spite of the use of an air atmosphere which was not in equilibrium with the bicarbonate buffer, and lack of correction for the possible retention of carbon dioxide by the protein material present.

Table III

Evolution of Carbon Dioxide During Enzymatic Destruction of Thiamine

Carbon Dioxide Observed μmoles	Thiamine Destroyed μmoles	Carbon Dioxide[1] Calculated per cent
8.3	9.70	85.7
5.4	9.56	56.5
5.4	8.81	61.3

[1] Calculated assuming that the destruction of one micromole of thiamine would result in the evolution of one micromole of carbon dioxide.

In the same year Lieck and Agren (34) studied 30 species of Swedish fishes in an effort to determine the presence or absence of the thiamine inactivating factor. The fish were dissected and the viscera (excluding the testes, ovaries and swim bladder) and the gills were ground to a brei and either analyzed immediately or stored at -15 degrees. For assay 3 ml. aliquots of the brei, or dilutions thereof, were mixed with 1 ml. of a 0.04 M. phosphate buffer pH 7.4 and 1 ml. of a thiamine solution containing 700 micrograms of the vitamin. This amount of thiamine calculated as the free vitamin would be equivalent to 2.5 micromoles. The assay procedure, therefore, was similar to that of Sealock, et al, (27) except that the buffer concentration was much lower. At zero time, or at

the end of the incubation period, 5 ml. of 20 per cent trichloroacetic acid was added; after centrifugation 2 ml. aliquots of the supernatant liquid were analyzed for thiamine by the Melnick-Field method (35).

With the usual 2 hour incubation period only carp was found to contain an appreciable amount of the thiamine inactivating factor, while with a 24 hour incubation time, an increased difference between control and experimental samples indicated the presence of the factor in other species. Of the 21 species of fresh water fish, 10 were found to contain the factor and, of these, 9 belonged to the carp family. Of 9 salt water fish only one species, garfish, was found to be active. In Table IV the fishes which Leick and Agren studied are listed with respect to the presence or absence of the thiamine destroying enzyme.

During the investigations it was noted that active fish extracts were always red coloured, whereas this was not the case with extracts from fish which did not contain the enzyme. Spectroscopic studies of the extract revealed only the presence of hemoglobin. In some experiments crystallized rat hemoglobin was added to visceral extracts of inactive fish but no activation was observed. The question of whether there was any connection between this colour and the activity was left open.

Lieck and Agren measured the uptake of oxygen using the Warburg manometric technique and also measured the rate of

Table IV

Thiamine Destroying Activity of Swedish Fishes

Inactive Fishes Family and Species	Active Fishes Family and Species
Percidae	**Cyprinidae**
Perch	"Bjorkna"
Pike-perch	Bream
Cyclopteridae	Carp
Lump-fish	Crucian
Triglidae	Ide
Gurnard	Roach
Blenniidae	Rudd
Wolf-fish	Tenth
Pleuronectidae	"Vimma"
Plaice	**Scomberesocidae**
Gadidae	Gar-fish
Burbot	**Scombridae**
Cod	Mackerel
Whiting	
Salmonidae	
Char	
Melt	
Salmon-trout	
Trout	
White-fish	
Esocidae	
Pike	
Cyprinidae	
"Asp"	
Clupidae	
Baltic herring	
Herring	
Sprat	

methylene blue reduction in Thunberg tubes during incubation of enzyme extracts with thiamine. However, there was no difference between solutions containing both the extract and thiamine and those containing only the enzyme extract. It was obvious, then, that the destruction of thiamine by this enzyme was not an oxidative process.

In 1944 Goodek and Cereceda (36) reported that oxythiamine, an analogue of thiamine containing a hydroxyl group instead of an amino group on the pyrimidine ring, was inhibitory to the action of the carp enzyme on thiamine. No details with respect to relative concentrations and the amount of inhibition were given.

In the same year Sealock and Goodland (37) published results of inhibition studies with the thiamine destroying enzyme. A number of inorganic salts, including copper sulphate, zinc chloride, ferric chloride, potassium cyanide, sodium fluoride and sodium sulfite, inhibited the reaction in varying degrees. Iodoacetic acid and cysteine were both inhibitors, the latter compound being particularly effective.

In addition to these compounds a number of thiamine analogues were prepared and tested for inhibitory action. The compounds included both thiazole and pyrimidine derivatives and the inhibition produced by these compounds is shown in Table V. The importance of the amino group in producing a high degree of inhibition was shown by a comparison of the

Table V

Inhibition by Thiamine Analogues

Compound	Concentration moles/l. x 10^4	Inhibition per cent
4-methylthiazolium chloride derivatives:		
3-o-aminobenzyl-	5.0	100.0
3-o-nitrobenzyl-	5.0	0.0
3-β-aminoethyl-	5.0	56.4
3-β-phthalimidoethyl-	5.0	18.4
3-ethyl-	10.0	9.3
3-phenyl-	5.0	2.3
3-ethyl-2-methyl-	40.0	10.0
3-phenyl-2-methyl-	5.0	0.0
3-methyl-5-β-hydroxyethyl-	5.0 15.0	0.0 0.0
2-methyl-4-aminopyrimidine derivatives:		
5-bromomethyl-	5.0 20.0	34.8 48.6
5-methylenesulfonic acid-	10.0 20.0	19.7 22.7
5-ethoxymethyl-	5.0 20.0	13.2 18.8

results obtained with o-aminobenzyl- and β-aminoethyl-4-methylthiazolium chloride and o-nitrobenzyl- and β-phthalimidoethylthiazolium derivatives. The two former compounds produced a high degree of inhibition, while the latter derivatives produced no, or only slight, inhibition. However, the presence of this group alone was not in itself sufficient for good inhibition as shown by the comparatively low order of inhibition observed with similar concentrations of 4-aminopyrimidine derivatives.

More complete studies were made with the most active inhibitor, o-aminobenzyl-4-methylthiazolium chloride. Five different concentrations of thiamine ranging from 1.0 to 2.0 x 10^{-4} moles per liter were incubated with an enzyme extract both with the inhibitor (2 x 10^{-5} M.) and without. Aliquots from each solution were removed at intervals and analyzed for thiamine. The values obtained for the amount of thiamine destroyed were used for calculation of initial velocities and of dissociation constants of the enzyme-substrate and enzyme-inhibitor complexes, according to the Lineweaver-Burke modification (38) of the Michaelis-Menten equation (39).

By plotting the reciprocals of the initial velocities (both with and without inhibitor) against the reciprocals of substrate concentrations, straight lines were obtained. Since the intercepts of these lines were the same, but the slopes were different, the inhibition was shown to be of a competi-

tive nature. The reciprocals of the dissociation constants gave values of 1.2×10^4 and 50.7×10^4, representing affinity constants of the enzyme-substrate and enzyme-inhibitor complexes. It was apparent then that the affinity of the protein part of the enzyme for the inhibitor molecule was 42.2 times that of its affinity for the substrate. Thus the reason for the high degree of inhibition produced by this compound became clear; the inhibitor, by forming a less dissociable complex with the protein, effectively prevented it from combining with its substrate.

Agren in 1945 (40) reported additional studies on the action of the thiamine-destroying fish factor. His enzyme solutions were made by water extraction of fish viscera, and assays were conducted using the procedure described by Lieck and Agren (34). Employing this standard procedure, but in the absence of enzyme, he found that the presence of cysteine, glutathione and ascorbic acid resulted in low values for thiamine as determined by the Melnick-Field method (35). He suggested that these compounds were reacting with thiamine to produce a compound incapable of reacting with diazotized p-aminoacetophenone, and that perhaps the reaction was one of reduction of the double bond at the thiazole quaternary nitrogen.

Lipmann (41) and Lipmann and Perlmann (42) had reported that thiamine was reduced by hyposulfite and by activated hydrogen with the uptake of one molecule of hydrogen. One atom of this

was added to the thiamine molecule reducing the double bond at the quaternary nitrogen and the second atom was split into a proton and an electron, which neutralized the positive charge of the quaternary nitrogen. However, since thiamine, reduced with hyposulfite in a molar ratio of 1 to 5, showed no inactivation as determined by the Melnick-Field method, Agren concluded that the effect of cysteine and glutathione in decreasing the color value of thiamine could not be due to a similar type of reduction. Agren then suggested that these substances were perhaps capable of splitting the thiamine molecule in a manner similar to that of sulfite. Williams, et al, in 1935 (43) reported that sulfite split thiamine into its thiazole moiety and into the sulfonic acid derivative of the pyrimidine portion of the molecule. Agren gave no evidence to either support or disprove this hypothesis.

However, work in this laboratory has shown that diazotized p-aminoacetophenone reacts with sulfhydryl compounds to give a colored derivative, and has also suggested that the reaction of the diazotized amine with thiamine is with the sulfhydryl group of thiamine, which is formed when the thiazole ring is opened by the action of alkali on the vitamin. Therefore, two possibilities for the reduced thiamine values in the presence of cysteine or glutathione present themselves. First, the sulfhydryl compounds, by preferential combination with the diazotized amine, might reduce its concentration below that

needed for reaction with thiamine. Secondly, cysteine and glutathione might possibly be reacting with the thiamine sulfhydryl group to produce an inactive disulfide, or might be altering a sulfhydryl-disulfide equilibrium necessary for the reaction of thiamine with the diazotized amine.

Agren also found that dialysis of enzyme preparations against water and against buffers at several different pH values, reduced the enzyme activity, although it was never completely inactivated. Activity of dialyzed preparations was restored upon addition of a concentrated dialyzate and also by addition of small amounts of glutathione, Table VI. Control experiments, in which extracts from fish not containing the enzyme were employed, showed that the increased destruction of thiamine upon addition of glutathione was not due to the action of the sulfhydryl compound on the vitamin.

Upon dialysis of an enzyme extract at pH 5.5 a precipitate formed, and, if this were removed by centrifugation, the non-dialyzable extract was inactive. If, however, the precipitate were dissolved by adjusting the pH of the extract to 7.4, there was activity in this fraction. The precipitation of the enzyme at this pH could be due to its isoelectric precipitation or to its adsorption on other isoelectrically precipitated proteins.

Agren also found that glutathione was able to restore the activity of a partially purified enzyme preparation which

Table VI

Activation of Enzyme Solutions Partially Inactivated by Dialysis[1]

Fraction[2]	Thiamine Destroyed
	µgm.
Non-dialyzable	0
Non-dialyzable plus dialyzate	175
Non-dialyzable plus 2 mg. of gluthathione	175
Non-dialyzable	105
Non-dialyzable plus dialyzate	175
Non-dialyzable plus 1 mg. of gluthathione	260

[1] Dialyzed for 24 hours against 0.01 N. acetate buffer at pH 4.4.

[2] 1.5 ml. aliquots of each fraction and of the gluthathione solution were used. 1.5 ml. of water was added in the analysis of the non-dialyzable fraction.

had been inactivated by hydrogen peroxide. Since crude extracts decomposed hydrogen peroxide, partial purification and freeing of the enzyme from catalase was achieved by first precipitating at pH 5.6. The centrifugate obtained was treated with ammonium sulfate to 0.6 saturation, and then the precipitate obtained between 0.6 and 1.0 saturation with this salt was collected. After solution of this precipitate 6 ml. portions were treated with 0.4 ml. of 5 or 10 per cent hydrogen peroxide. After ten minutes, an additional 0.4 ml. of peroxide was added, and, after a second ten minute period, the excess peroxide was destroyed with catalase.

An enzyme solution purified by precipitation at pH 5.6 destroyed 520 micrograms of thiamine, while the same solution treated with 10 per cent hydrogen peroxide destroyed only 320 micrograms. Addition of 10 mg. of gluthathione to the peroxide-inactivated extract increased the amount of destruction to 470 micrograms. In a second experiment, the untreated extract (purified by both isoelectric and ammonium sulfate precipitations) destroyed 530 micrograms, while treatment with 5 per cent peroxide reduced the destruction to 175 micrograms. Addition of 5 mg. of gluthathione increased the destruction to 560 micrograms.

Agren felt that these results gave support to the hypothesis that the enzymatic reaction was the result of a reductive cleavage of thiamine. However, no other compounds similar to

gluthathione, such as cysteine, were tested to determine whether the glutathione effect was specific for that compound. Furthermore, the possibility that glutathione was affecting the sulfhydryl-disulfide equilibrium of the protein component of the enzyme should not be overlooked.

Later in the same year Agren (44) described in greater detail his methods for purifying the "fish factor". The following method was adapted and used for routine preparations:

I. The finally ground and frozen viscera--liver, spleen, intestine and gills--were shaken for 1 hour at 0 degrees with an equal volume of water, and centrifuged for 1 hour at 3,000 r.p.m. in a 4 litre cooled centrifuge. The centrifugate = 1.

II. No. 1 was acidified to pH 5.7 and again centrifuged for 1 hour. The centrifugate = 2.

III. No. 2 was brought to 0.6 saturation by the addition of solid ammonium sulfate. Together with some coarse (Hyflow) Cel the precipitate was filtered on large Buchner funnels.

IV. The clear slightly yellow coloured filtrate was brought to 1.0 saturation by addition of solid ammonium sulfate and the precipitate centrifuged off. The precipitate solved in one-tenth of the volume of No. 1 was neutralized and stored at -15 degrees when not immediately used. The solution = IV.

Using this method, a preparation from Tench which, in the first solution (No. 1 above), contained 0.14 units of activity per milligram of nitrogen gave a final solution (No. IV above) containing 0.90 units per milligram of nitrogen, thus resulting in a more than six-fold purification. The unit of activity was the same as that defined by Sealock, et al, (26,27).

A preparation from Ide containing 0.16 units of activity per milligram of nitrogen in solution 1 was purified 11.2 times, giving a final solution (No. IV) containing 1.80 units per milligram of nitrogen.

Further purification was attempted by treating 10 ml. portions of the ammonium sulfate fractionated enzyme solutions (0.8 mg. of protein nitrogen per ml.) with 0.3, 0.5 and 0.7 ml. of 0.5 N. lead acetate. Only small precipitates were obtained and the activity remained in the centrifugates.

In another experiment the centrifugate obtained after removal of material insoluble in 60 per cent ammonium sulfate solution was stored for several weeks at 0 degrees. Small precipitates formed during the first week of storage and these were removed and discarded. At the end of three weeks a rather heavy precipitate had formed and this was taken off and assayed with and without added gluthathione. The addition of this compound restored the activity of this enzyme, which appeared to have been inactivated during the period of storage. Agren believed that this work, together with the activation by glutathione of dialyzed and peroxide inactivated enzyme solutions, further supported the hypothesis that the enzymatic cleavage of thiamine was a reductive splitting. Again, however, the possibility that gluthathione was functioning by reducing disulfide linkages in the protein portion of the enzyme must not be overlooked. It would seem possible, that, if sulfhydryl

groups played an important role in the active enzyme, the spontaneous inactivation might have been due to oxidation of these groups during the storage period.

Cataphoretic studies were carried out in the Tiselius apparatus at pH 6.02, 5.44, 5.02, 4.64 and 4.32. Purified enzyme solutions showed three fractions which moved in a fairly parallel fashion at the different pH's. At pH 6.02 and 5.4 migration was anodic, at pH 4.3 and 4.6 it was cathodic, while at pH 5.0 the migration of the different fractions was very small. Agren concluded that the chances of obtaining much purification of the enzyme by cataphoresis were not great.

Also in 1945 Melnick, Hochberg and Oser (45), continuing studies of the physiological availability of the vitamins, reported that raw clams as well as certain species of fishes contained a thiamine inactivating factor. The work indicated that 50 per cent of the thiamine of a normal diet was destroyed in the gastrointestinal tract following the concomitant ingestion of raw clams, while the mixing of raw clams with the other foodstuffs caused a rapid and complete destruction of thiamine <u>in vitro</u>. Melnick and his co-workers applied the term "thiaminase" to the thiamine-inactivating factor, thus giving it a typical enzyme nomenclature.

Beloff and Stern (46) in the same year found that yeast juice, which had been incubated with extracts of carp spleen,

lost its ability to decarboxylate pyruvate. Extracts of dried bottom yeast after such incubation showed a 66 to 90 per cent decrease in carboxylase activity, irrespective of whether the incubation had been carried out at pH 6.6, 7.1 or 7.9. Addition of more pyruvate or as much as 50 micrograms of synthetic cocarboxylase failed to relieve this inhibition, which resulted from the enzymatic destruction of cocarboxylase, the pyrophosphate derivative of thiamine. The failure of added cocarboxylase to reactivate an inhibited system indicated that perhaps one of the split products from cocarboxylase was still attached to the apoenzyme of carboxylase.

The enzymatic destruction of cocarboxylase was greatest at pH 6.5 with no inactivation observed at pH 7.8, while the action of the enzyme on thiamine increased with pH up to 9.0. One milliliter of a carp spleen extract was analyzed in the Tiselius electrophoresis apparatus and three components of different electrochemical behavior were observed. One component was stationary, the one with greatest mobility was present in the highest concentration, while the enzymatic activity was associated with the component of intermediate mobility.

Hennessy and Warner (47) in 1946 reported the isolation of a pyrimidine derivative of unknown structure produced by the destructive action of raw clams on thiamine. Thiochrome, colorimetric, rat growth, and rat curative assays showed destruction of the vitamin by the factor in clams, while

solutions containing the inactivated thiamine stimulated yeast fermentation to an extent which was 65 to 85 per cent of the stimulation produced by an equivalent amount of unaltered thiamine. Sulfite treatment of such solutions destroyed their ability to stimulate yeast fermentation.

The fermentation-stimulating factor underwent base-exchange with acid-washed Decalso, and the Decalso eluate stimulated yeast in the presence of either the Decalso filtrate or of 4-methyl-5-β-hydroxyethylthiazole. Isolation of this pyrimidine derivative yielded a compound with the formula $C_8H_{16}N_4O_3S \cdot 2HCl$. The colorless needles obtained began to darken at 235 degrees and decomposed suddenly at 242 degrees. Absorption maxima at 236 and 279 millimicrons in dilute alkali and at 246 millimicrons in dilute acid were observed. Electrometric titration showed two points of inflection, indicating reaction with two equivalents of alkali. The compound had a stimulatory effect on yeast which was 75-105 per cent of that produced by the pyrimidine alcohol derivative (2-methyl-4-amino-5-hydroxymethylpyrimidine). One microgram of the pyrimidine derivative stimulated yeast fermentation in an amount corresponding to that produced by 1.01 micrograms of thiamine chloride, but after sulfite treatment stimulation was equivalent only to that produced by 0.13 micrograms of thiamine chloride.

In the same year Agren (48) reported further purification by cataphoretic means of enzyme extracts which had already

been partially purified by the isoelectric and ammonium sulfate precipitations, described in an earlier publication (43). At pH 4.7 electrophoretic analysis disclosed the presence of two components of different electrochemical behavior. The enzyme activity was associated with a small fraction exhibiting slow cathodic mobility, and a comparison of activity with nitrogen concentration indicated an additional ten-fold purification of the enzyme by this method.

The contents of the top cathodic cell from a series of electrophoretic experiments were collected with the idea of subjecting the combined solutions to further purification by this method. However, during the dialysis preliminary to electrophoresis of the solution, a rather heavy precipitate was formed. This precipitate was dissolved in 10 ml. of water by adjusting the pH to 7.4 and this solution was found to contain approximately 85 per cent of the original activity. Since this solution was clear and colourless, Agren concluded that the colour observed in less pure enzyme preparations was not associated with enzyme activity.

In 1946 Kiku Murata (49) found that a thiamine destroying factor, which he called aneurinase, was extracted from the flesh of "corbicula atrata", an oyster-like mollusk, by a 0.1 N disodium phosphate solution. Such an extract destroyed all of the added thiamine in one hour upon incubation at pH 6.1 at a temperature from 20 to 37 degrees. Murata claimed that the

enzyme deaminated the pyrimidine nucleus but did not break the thiazole ring. Unfortunately only an abstract of the report was available at the time of this review and no further details were available. However, it would be of interest to know what information led him to suggest the deamination of the pyrimidine ring, since this mechanism for the action of the fish factor has not been suggested by any other author. Of course, the available information did not justify an assumption that "aneurinase" is identical with "thiaminase", the so-called "fish principle".

In 1947 Neilands (50) reported that "thiaminase" was present in the majority of 12 fresh-water fish studied, while in the 28 marine animals tested for activity, the enzyme was found only in one teleost and infour invertebrates. Since only an abstract of this article was available, no information regarding which species were active and which inactive was obtainable.

The thiamine inactivating factor found in several species of fishes has been designated by a number of different names. It has been described as "the fish factor", "the fish principle" and the "Chastek paralysis factor". In addition it has been called "thiaminase", although as pointed out by Sealock and his co-workers (27) this name had already been suggested by Bonner and Buchman (51) for an enzyme of pea roots capable of synthesizing thiamine from its two components. However, since

the substrate for the fish enzyme is thiamine, it may be considered as a type of thiaminase, and the name would seem to be more applicable to an enzyme which is principally destructive or hydrolytic than to one which is predominantly synthetic. In the work to be described in detail in the following pages, this enzyme will be referred to by any one of the above names, and it should be noted that they are used interchangeably.

B. The Anti-thiamine Factor of Bracken Fern

In the fall of 1946 Weswig, Freed and Haag at Oregon State College (52) published a note reporting the presence of an anti-thiamine factor in bracken fern (pteris aquilina). At that time a study was being made of the so-called "fern poisoning" occurring among cattle in Oregon. Outbreaks were observed in the dry season of the year, when range cattle, unable to obtain much green food material, began to forage on this fern. Since the poisoning resulted in the death of a large number of animals, an effort was made to determine the cause of the poisoning, so that preventive or curative measures might be initiated.

Bracken fern was air-dried at room temperature, ground and included in a rat ration as 40 per cent of the total diet. The symptoms of the fern poisoning which developed in rats receiving this diet were suggestive of a thiamine deficiency. Administration of thiamine to these rats resulted in a prompt recovery. Although an analysis of the fern ration showed that

it contained 0.2 to 0.6 mg. of thiamine per 100 gm., rats receiving this diet developed deficiency symptoms, indicating that some factor present in the fern was inactivating the dietary vitamin. One group of deficient rats was maintained on this diet but a daily supplement of 0.5 mg. of thiamine was given per os. The animals in this group recovered, while those in a second group, not receiving the supplement, died.

There was no decrease in toxicity when the air-dried fern was heated at 105 degrees for 18 hours. The factor producing the thiamine deficiency was insoluble in ethyl ether and in acetone, but appeared to be slightly soluble in 92 per cent ethyl alcohol.

In the spring of 1947 Haag and Weswig (53) reported further work in connection with this anti-thiamine factor. Ten lots of fern, from five different areas, gathered from May to October, were all toxic when fed to rats as 15 to 40 per cent of the total ration. However, groups of rats receiving such diets showed normal gains for as long as 15 weeks if a 0.5 mg. daily supplement of thiamine were given. The toxicity was destroyed when the fern was boiled in water for 30 minutes and subsequently dried.

In 1948 the Oregon group (54) reported that bracken fern rations showed decreasing toxicities in the order of fresh fern, air-dried fern in moist rations, and air- and sun-dried fern. Fresh fern which had been steamed for 30 minutes

produced no harmful effects when fed to rats as 40 per cent of the dry matter of the ration. *In vitro* studies were made using the Melnick-Field, the thiochrome and microbiological procedures for thiamine analysis. The latter two proved to be more useful, since considerable interference was observed when the former was employed for analysis of thiamine in the presence of fern extracts.

These studies on the fern anti-thiamine factor were interesting because of the similarities to the fish anti-thiamine factor. To date the nature of the factor has not been determined, but the thermostability described in the first report by the Oregon workers might indicate a non-protein nature. However, the later reports of the thermolability under moist conditions might indicate that it was a protein material, but somewhat more thermostable than most proteins.

At the time of writing there appeared to be little doubt that the fish-factor is an enzyme. However, little was known regarding the exact nature of the enzyme, and in particular nothing had been published concerning the nature of the dialyzable component. Agren's work with glutathione was suggestive of a sulfhydryl-containing coenzyme or prosthetic group, but the information available certainly did not exclude other explanations for the activating effect of this compound. Furthermore, information regarding the mechanism whereby the enzyme destroyed thiamine was still incomplete, although the

work of Krampitz and Woolley indicated that the reaction occurs in a step-wise fashion. Attention had been given to the kinetics of the reaction by Sealock and his co-workers, and the "time course" of the enzymatic destruction of thiamine had been studied by several workers. However, although under certain conditions, the reaction appeared to go to completion, no information was available regarding the equilibrium point of the reaction. Thus it is apparent that, in spite of the large amount of work already reported, much remains to be done before knowledge of "thiaminase" and its action can even approach completeness.

The problem of the anti-thiamine factor of bracken fern was of comparatively recent origin and nothing was known regarding the mechanism by which it inactivated thiamine. However, because of the possibility that it is an enzyme, any work directed towards an understanding of its properties and its mode of action would be of value, particularly to those associated with the problem of the "fish factor".

III. EXPERIMENTAL

Although attention has been given to the fern anti-thiamine factor, most of the work to be reported in the following pages deals with the thiaminase present in certain species of fishes. The end-products produced by the action of this enzyme on vitamin B_1 had been identified by Krampitz and Woolley, but the suggested pyrimidine intermediate remained to be identified. Although studies of the "time course" of the reaction have been made, to date there has been no report regarding whether or not the enzymatic destruction of thiamine reached an equilibrium point or whether the reaction was a reversible one. Agren's work with glutathione raised the question of the possible importance of sulfhydryl groups both in the protein and non-protein portions of the enzyme. Although Sealock and Livermore[1] had obtained information regarding the properties of the dialyzable component or components of this enzyme system, no conclusion regarding the exact nature of a possible coenzyme or prosthetic group could be made.

Therefore, the kinetics of the enzyme reaction were studied in an effort to determine an equilibrium point. An approach to this problem was made both by studying the enzymatic destruction of thiamine and by attempting the reversal of the reaction.

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1. Sealock and Livermore, unpublished data.

However, it became apparent that before the kinetics of the over-all reaction could be determined, more information regarding the mechanism of the reaction should be obtained. Work directed towards the identification of a possible pyrimidine intermediate was therefore initiated.

In connection with this work and in order to obtain a more com lete picture regarding the nature of thiaminase, its properties were studied in more detail. In particular, attention was given to inhibitors and activators of its action. The possible role of sulfhydryl groups in thiaminase action was studied, and efforts were made to characterize the dialyzable component of the system.

In addition preliminary experiments were carried out to determine the nature of the thiamine destroying factor of bracken fern. This work was directed towards establishing the enzymatic or non-enzymatic nature of this factor, thus establishing its similarity or non-similarity to thiaminase of fish tissues.

A. Methods

1. Preparation of acetone desiccated powder

In order to have a ready and uniform source of the thiamine-destroying enzyme, acetone desiccated powders were prepared from carp tissue by the method of Sealock, et al, (27). Viscera and gills of carp, which were received in the frozen state, were separated from swim bladders, fat and eggs; ground in a meat chopper and the minced material treated with six volumes of cold acetone. This was accomplished by blending 50 gm. portions with 300 ml. of ice-cold acetone in the Waring blendor. The combined suspensions were allowed to stand in an ice-bath for at least thirty minutes with frequent stirring. The insoluble material was removed by filtering with suction and again extracted using four volumes of ice-cold acetone. After filtration, the insoluble material was broken up and spread in a thin layer. The major portion of the acetone was removed by this air-drying, but the powder was freed from the last traces in a vacuum desiccator over phosphorous pentoxide. The dried material, in some cases freed from inactive shreds of connective tissue by sieving, was transferred to brown bottles and stored in the ice-box. Each gram of viscera yield on an average 0.16 gm. of powder, and these preparations have maintained their activity for at least 18 months. The activity per unit weight of the various powders differed and depended

upon the freshness of the original material, the degree of separation, and probably to some extent upon the source.

Sealock, et al, (27) defined the unit of activity as that amount which under the standard assay conditions, described in detail below, will cause the disappearance of one micromole (1×10^{-6} mole) of thiamine. In the work to be described in the following pages several different acetone desiccated powders were employed. Table VII lists these preparations, together with the approximate number of units of activity per gram. The calculated activity varied with the concentration used in the assay, and therefore the unitage obtained at different levels of assay are given for most of the preparations.

Preparation III-138 was a thoroughly mixed combination of approximately 27 gm. of I-142-III-A with two to three grams each of II-2 6 and II-196. The activity of all of the preparations varied from time to time and this variation appeared to depend upon the relative amounts of powder and extracting solution, upon the time of contact between the two and upon the degree of efficiency in breaking up the particles of dry material. However, all of them appeared to be quite stable. Preparation I-142-III-A was used more than any of the others. In January of 1947 an extract representing 50 mg. destroyed 1.2 micromoles of thiamine in the usual assay procedure. Similar extracts destroyed 1.62 micromoles in February of that year and 1.83 micromoles in March of 1948.

Table VII

Activity of Acetone Desiccated Powders

Preparation	Amount Assayed mg. equiv.	Thiamine Destroyed μmoles	Activity units per gm.
I-68	67	1.86	27.7
	100	1.90	19.0
	133	2.35	17.0
I-142-I-A	50	0.68	13.6
	100	1.78	17.8
I-142-II-A	50	0.59	11.8
	100	0.78	7.8
	150[1]	1.59	10.6
I-142-III-A	10	0.11	11.0
	25	0.49	19.6
	50	1.63	32.6
	75	2.50	33.4
II-87-A	50	0.59	11.8
II-87-B	50	0.37	7.4
II-196	20	0.87	43.5
	40	1.93	48.3
	50	2.21	44.2
II-236	20	0.40	20.0
	40	1.07	26.8
	50	1.59	31.8
	70	2.12	30.3
III-110	40	1.04	26.0
	50	1.48	29.6
	70	2.29	32.8

[1] Average of 5 assays

The data of Table VII and Figure I show that the amount of thiamine destroyed increased with an increasing concentration of the enzyme. With certain of the preparations the plot of thiamine destruction against the amount of enzyme gave an S-shaped curve (Preparations II-236, III-110 and I-142-III-A, Figure I). Such a relationship was probably due to the increasing dilution of the components of the enzyme system as concentration decreased.

2. Preparation of enzyme extracts and method of assay

The enzyme containing extracts were prepared from the acetone desiccated powders by the method of Sealock, et al, (27). A weighed amount of the powder was placed in a fifty ml. centrifuge tube. To this was added a 0.2 M. sodium and potassium phosphate buffer containing 10 per cent sodium chloride, adjusted to pH 7.4. After standing for at least one-half hour with frequent stirring to break up all particles the mixture was centrifuged and decanted through cotton into a graduated cylinder. The residue was extracted with three additional portions of buffer, and the combined decantates were adjusted to the desired volume with the solvent, thoroughly mixed, and the pH adjusted to 7.4. These extracts were made just prior to use.

For assay 2 ml. portions of the extract were placed in test-tubes. To this was added 2 ml. of water and then 1 ml. of a

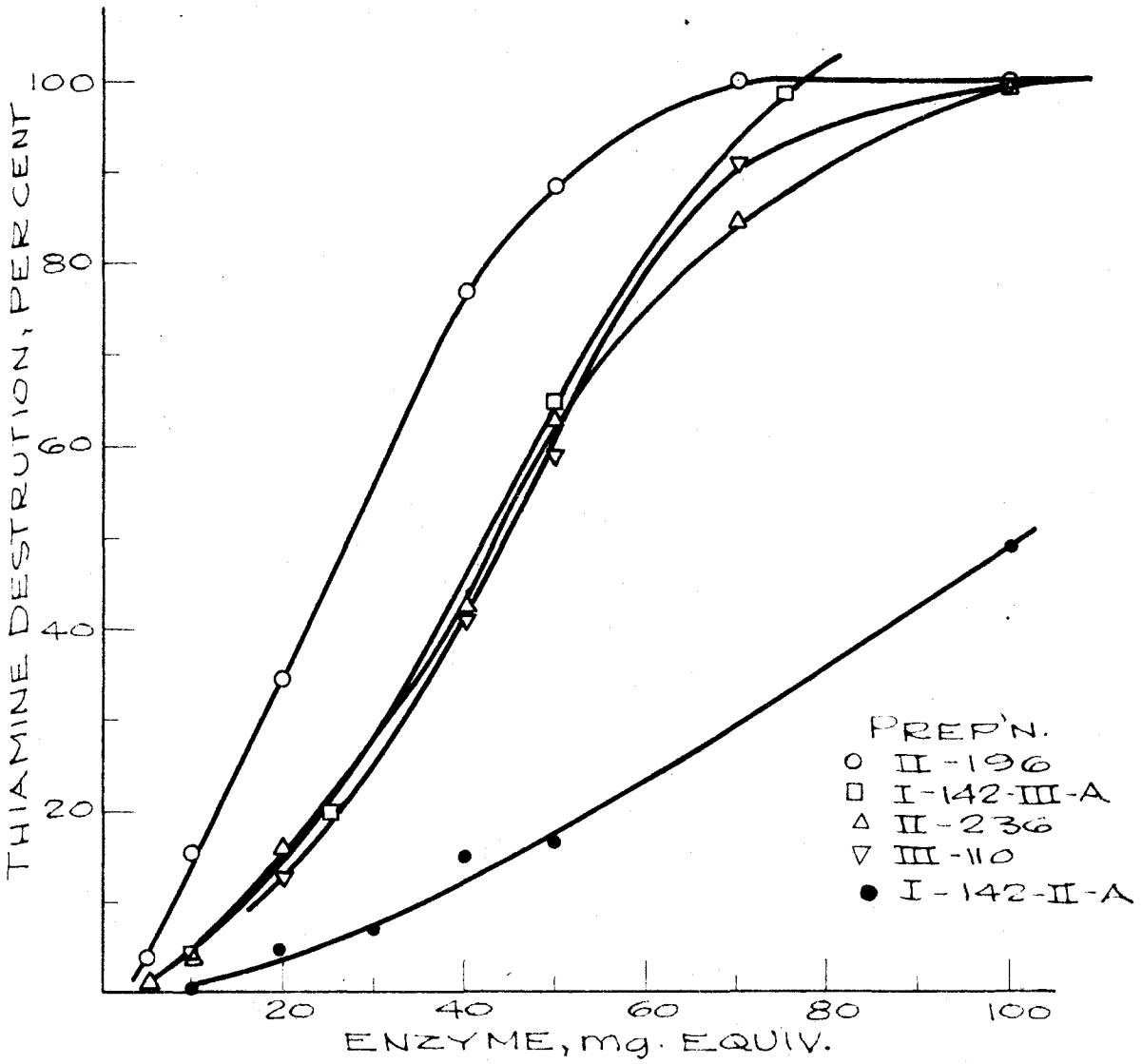


FIG. 1 ACTIVITY WITH VARYING AMOUNTS OF ACETONE DESICCATED ENZYME PREPARATIONS.

thiamine solution containing 2.5 micromoles of the vitamin. After thorough mixing the tubes were stoppered and placed in a water bath at 37.5 degrees for two hours. At the conclusion of the incubation period 5 ml. of freshly prepared 10 per cent trichloroacetic acid was added with shaking. Control tubes were made in the same fashion, except that the trichloroacetic acid was added immediately after the thiamine addition and there was no incubation. After standing for at least thirty minutes to allow the precipitated protein to flocculate the solutions were filtered.

In experiments requiring the presence of materials other than the enzyme and substrate, all or part of the water was omitted and other solutions were added. On occasion it was necessary to use fractional amounts of thiamine solutions in order to allow for the proper combination of additional substances. However, in all cases the incubation volume was 5 ml. and there was present at least 0.08 M. phosphate buffer. Except for a few experiments in which it was necessary to vary the substrate concentration, thiamine was present in the incubation solution in a concentration of 5×10^{-4} M. To determine the activity of the enzyme in the presence or absence of additional substances the thiamine remaining after incubation was determined.

3. Analytical methods for thiamine determination

a. The Melnick-Field method. The colorimetric method of Melnick and Field (35) was adopted for the majority of the analyses. The diazotized p-aminoacetophenone reagent used in this method was prepared as follows. Five ml. of 0.05 M. p-aminoacetophenone in 1 N. hydrochloric acid, and 50 ml. of 4.5 per cent freshly prepared sodium nitrite were cooled separately to 5 degrees or less in an ice-bath. Five ml. of sodium nitrite was added to the p-aminoacetophenone and the solution stirred continuously for ten minutes. An additional 20 ml. of sodium nitrite was added and stirring continued for 20 minutes. The final reagent was made by combining 20 ml. of this solution with 275 ml. of a previously cooled sodium hydroxide-sodium bicarbonate buffer (0.5 and 0.355 M.). Stirring was continued for thirty minutes. The reagent was made fresh each day and kept in the ice-bath until used.

For thiamine analysis 2 ml. portions of the protein free filtrates were transferred to 40 ml. centrifuge bottles. Ten ml. of a 50 per cent alcohol solution containing 2.5 gm. of phenol per liter was then added. To this mixture 10 ml. of the freshly prepared diazotized p-aminoacetophenone reagent was added with mixing. The solutions were allowed to stand for at least one-half hour to permit maximum reaction and color development, and if necessary the analysis could be stopped at this point and completed the following day. The colored complex

formed by the reaction of the reagent with thiamine was extracted with either 10 or 15 ml. portions of xylene. After xylene addition the solutions were shaken vigorously and centrifuged in order to obtain complete separation of the layers. The xylene was removed with a transfer pipette or by suction and read in the Klett-Summerson photoelectric colorimeter with filter 520. The instrument was first set to zero with the solvent. The amount of thiamine present was calculated by means of the following equation:

$$\text{Thiamine, micromoles} = \frac{\text{Reading, Incubated}}{\text{Reading, Zero time}} \times 0.5 \times \frac{\text{Total volume}}{\text{Aliquot volume}}$$

In general, results were expressed as the percentage of the added thiamine destroyed by the enzyme, determined as follows:

$$\text{Thiamine destroyed, per cent} = \frac{\text{Thiamine added-Thiamine present}}{\text{Thiamine added}} \times 100$$

The variability of this method was determined by analysis of ten similar solutions. These each contained 2 ml. of phosphate buffer, 2 ml. of water, 1 ml. of thiamine solution (2.5 micromoles) and 5 ml. of 0.5 N. hydrochloric acid, and 2 ml. aliquots of each were analyzed. Five samples gave a Klett-Summerson reading of 560 and five a reading of 570. The average of the ten readings was 565 $\pm$ 5 ($\pm$ 0.88 per cent).

b. The p-aminobenzoic acid method. Since 1939 when Melnick and Field (35) demonstrated that the reaction of thiamine with diazotized p-aminoacetophenone was applicable to quantitative estimation of the vitamin, several variations have been suggested, in which different solvents are used for extraction of the colored complex formed in this reaction. Since the complex is insoluble and tends to settle out upon standing, extraction is necessary.

Thiamine, however, reacts with other diazotized aromatic amines to produce a similar red-purple color, and work carried out in this laboratory[1] showed that diazotized p-aminobenzoic acid gave the expected reaction. Because of the acid radical present in this compound, the colored complex was soluble in an alkaline aqueous solution. Therefore a series of investigations were undertaken to determine whether or not this amine could be employed in a quantitative method for thiamine determination, with the color being read directly in the aqueous solutions.

The p-aminobenzoic acid was diazotized in the following way. To 10 ml. of a previously cooled solution of p-aminobenzoic acid (0.01 M. in 0.03 N. hydrochloric acid), 5 ml. of a cooled 0.02 M. sodium nitrite solution was added. After ten minutes with frequent stirring, an additional 5 ml. of sodium nitrite was added and the mixture allowed to stand for at least thirty minutes with occasional stirring. All solutions were kept in

[1] Sealock, R. R. and Williams, M. A., unpublished experiments.

an ice-bath. A few drops of an ammonium sulfamate solution (several crystals dissolved in a proximately 10 ml. of water) were added to destroy excess nitrite. This step was later omitted since it was shown that excess nitrite did not interfere with the development of color.

In the first experiment the effect of different amounts of alkali on the color development was determined. Aliquots of a thiamine solution containing 0.25 or 0.50 micromoles of the vitamin were mixed with 5 ml. of the Melnick-Field alcohol-phenol reagent. One ml. of the diazotized reagent was added followed by the addition of 1 to 5 ml. of a 20 per cent sodium carbonate solution. Water had previously been added so that the final volume was the same in each tube. Colorimetric readings, made 30 to 45 minutes after the addition of the alkali, obtained with the Klett-Summerson photoelectric colorimeter (Filter 540) did not show much difference between the thiamine solutions and control tubes to which no thiamine had been added. The colorimetric readings for the thiamine solutions containing the different amounts of alkali ranged from 238 to 248 in one case and from 256 to 266 in another. A greater difference between experimental and control solutions was obtained upon addition of an extra milliliter of the reagent after the alkali had been added, and also upon addition of 6 ml. of a 1:5 mixture of the diazotized amine and the alkali.

In several experiments colorimetric readings for the reagent

blanks or controls ranged from 234 to 325 under the above conditions, and an effort was made to reduce this high blank reading by omitting the phenol, which reacted with the reagent, probably by coupling with the diazotized amine, to produce a yellow-orange color. When a 50 per cent alcohol solution was used instead of the alcohol-phenol reagent a blank reading of 22 was obtained and a thiamine standard containing 0.25 micromoles of the vitamin gave a colorimetric reading of 110. Therefore, the substitution of the alcohol solution not only reduced the blank reading, but also resulted in a greater difference between the readings of control and experimental solutions. The presence of the alcohol served to intensify the color as indicated by readings of 265 and 47 for 1 micromole of thiamine in the presence and absence of alcohol.

The fact that mixing of the diazotized amine and the alkali prior to addition increased the intensity of color was shown by colorimetric readings of 350 and 265 for 1 micromole of thiamine obtained when the two reagents were added together and separately. Consequently for later work the diazotized amine and the alkali were mixed in a 1:5 ratio and 5 ml. of this mixture was used for color development. Because of the difficulty in keeping the sodium carbonate in solution when cold, the sodium hydroxide-sodium bicarbonate buffer employed in the Melnick-Field method was substituted.

To determine whether or not the intensity of the color produced with different amounts of thiamine followed Beer's

law, a series of tubes containing from 0.2 to 2.0 micromoles of thiamine was prepared. Water was added to give a 4 ml. volume in each, and then 5 ml. of 50 per cent alcohol and 5 ml. of the alkaline reagent were added. The solutions were allowed to stand for one-half hour to permit development of color, and were then read in the Klett-Summerson photoelectric colorimeter with Filter 540. The reading of the reagent blank containing no thiamine was substracted from the colorimetric readings of the thiamine solutions. The results of this experiment, shown in Table VIII and Figure 2 (curve a), indicated that Beer's law was observed with amounts of thiamine from 0.2 to 1.0 micromoles. Therefore, it appeared that the method could be used for the quantitative estimation of thiamine within this concentration range.

Since 1 ml. aliquots of the protein-free filtrates obtained in the work with thiaminase contained a maximum of 0.25 micromoles of thiamine, it appeared that this method would furnish a simpler means of analysis. However, in the first attempt to employ the procedure disappointing results were obtained. An aqueous thiamine standard containing 0.5 micromoles of the vitamin gave a colorimetric reading of 222, while a 1 ml. aliquot (equivalent to 0.25 micromoles of thiamine) of a standard made up with 5 ml. of 10 per cent trichloroacetic acid, 4 ml. of phosphate buffer and 1 ml. of thiamine, gave a reading of only 37. Only a pale pink color appeared instead

Table VIII

Proportionality of the
p-Aminobenzoic Acid Method for Thiamine Determination

Thiamine Present	Colorimeter Readings	Thiamine Value
μmoles		μmoles per div.
0.2	76	0.0026
0.4	154	0.0025
0.6	243	0.0024
0.8	325	0.0024
1.0	410	0.0024
1.2	475	0.0025
1.4	545	0.0025
1.6	595	0.0026
1.8	625	0.0028
2.0	645	0.0030
	Average	0.00257 ± 0.00014 (±5.4%)

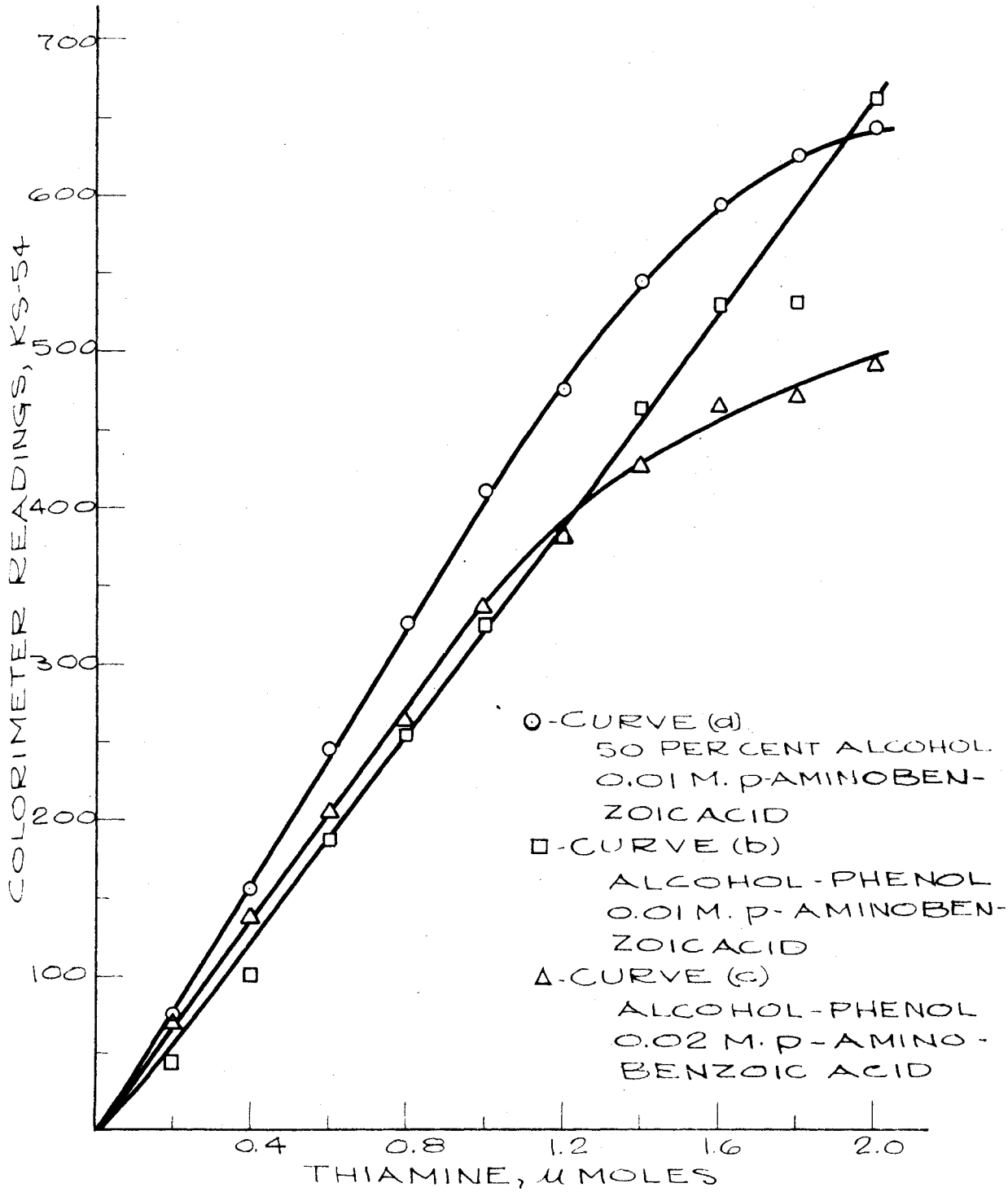


FIG. 2 PROPORTIONALITY OF p-AMINOBENZOIC ACID METHOD.

of the expected red-purple color. Apparently some substance, which would be present normally in the protein free filtrates, was interfering with the color producing reaction. Addition of extra alkali did not improve color development.

Efforts were therefore made to determine the cause of this interference. Qualitative tests showed that the use of 10 ml. of the alkaline reagent, instead of the usual 5 ml., did not better the results. Standard thiamine solutions were analyzed in the presence of the phosphate buffer, trichloroacetic acid and a phosphate-trichloroacetic acid mixture in amounts equivalent to those present in 1 ml. of a filtrate. Only in the absence of trichloroacetic acid was the expected color developed upon addition of the reagent. The acid then was apparently producing the interference.

Since trichloroacetic acid did not interfere with the Melnick-Field method, the p-aminobenzoic acid method was carried out with concentrations and proportions changed so that they were exactly equivalent to those of the former method. Again interference was observed in the presence of trichloroacetic acid, but when the alcohol-phenol reagent was used instead of 50 per cent alcohol the orange-red color characteristic of the p-aminoacetophenone method was observed. The qualitative results thus established the protein precipitant as the cause of interference in this new method of analysis and indicated that the presence of the alcohol-phenol reagent overcame this

interference. However, as shown above, the phenol produced a high blank reading.

To determine whether it might be possible to use this method in the absence of phenol if the trichloroacetic acid concentration were reduced, a series of tubes were prepared containing from 0.1 to 2.0 ml. of 5 per cent trichloroacetic acid. Whereas a thiamine standard (0.5 micromoles) gave a colorimetric reading of 226 in the absence of trichloroacetic acid, the readings obtained in its presence ranged from 41 to 101. The addition of as little as 0.1 ml. of 5 per cent trichloroacetic acid reduced the reading by more than half.

In another experiment the amount of diazotized p-aminobenzoic acid was varied. From 1 to 5 ml. portions of its solution were mixed with 5 ml. aliquots of the sodium hydroxide-sodium bicarbonate buffer, and these mixtures were added to tubes containing the thiamine standard, trichloroacetic acid, and 50 per cent alcohol. Water had been added so that the final volume would be the same for all tubes. With the higher concentrations of p-aminobenzoic acid an initial grayish-purple color developed, which disappeared upon shaking. It was evident, however, that addition of extra p-aminobenzoic acid did not overcome the trichloroacetic acid interference.

Further efforts were made to overcome this interference and thus to adapt the p-aminobenzoic acid method for analysis of thiamine in the filtrates. One ml. of 5 per cent trichloroacetic

acid and 5 ml. of alcohol-phenol reagent were added to each of two tubes, one containing a thiamine solution (0.5 micromoles) and the other containing an equal volume of water. To each was added 5 ml. of the alkaline diazotized reagent. The blank gave a colorimetric reading of 204, but when the colorimeter was set to zero with this blank the thiamine standard gave a reading of 224, which compared favorably with previous readings for this amount of thiamine. Although the blank reading was still quite high, it appeared that the phenol had overcome the interference, and by using such a blank to adjust the instrument to zero, good colorimetric readings for thiamine solutions might be obtained.

The effect of different phenol concentrations on the color development was tested with the hope that lower amounts might be used, thus reducing the blank colorimetric reading. A series of tubes containing from 4.1 to 20.5 mg. of phenol per tube was made up, and after addition of water or thiamine, colorimetric analysis was made. The results of this experiment, shown in Table IX, made it evident that while increasing concentrations of phenol did not greatly increase the blank readings there was a significant increase in the thiamine value obtained by subtracting the blank reading from that of the thiamine standard. This approached a maximum with a concentration of 12.3 to 16.4 mg. of phenol per tube, an amount comparable to that present in 5 ml. of the usual alcohol-phenol reagent, 12.5 mg.

Table IX

Effect of Varying Phenol Concentration on the p-Aminobenzoic Acid Method

Phenol Present mgm.	Colorimeter Readings Blank	Thiamine Standard	Difference
4.1	191	385	194
8.2	206	440	234
12.3	210	465	255
16.4	214	465	251
20.5	214	445	231

Therefore an experiment was carried out to determine whether the p-aminobenzoic acid method exhibited proportionality, when the alcohol-phenol reagent was used, even though a high blank reading was obtained. A series of tubes was prepared with from 0 to 4 ml. of a thiamine solution (0 to 2.0 micromoles) and water to give a 4 ml. volume in each. One ml. of 5 per cent trichloroacetic acid and 5 ml. of alcohol-phenol reagent were added to each, followed by the addition of 5 ml. of the alkaline diazotized p-aminobenzoic acid reagent. The colorimeter was adjusted to zero with the blank and readings were made one-half hour after the last addition. The results, shown in Table X and Figure 2 (curve b), indicated that the trichloroacetic acid interference had been overcome, and that this method could be used for the quantitative estimation of thiamine in amounts up to and including 1.0 micromoles of the vitamin.

Table X

Proportionality of the p-Aminobenzoic Acid Method in the Presence of Alcohol-Phenol Reagent

Thiamine Present	Colorimeter Readings	Thiamine Value
μmoles		μmoles per div.
0.2	70	0.0028
0.4	138	0.0029
0.6	204	0.0029
0.8	264	0.0030
1.0	335	0.0029
1.2	380	0.0031
1.4	425	0.0032
1.6	465	0.0034
1.8	470	0.0038
2.0	490	0.0040
	Average	0.0032 ±0.0003
		(±9.4 per cent)

A similar experiment was conducted in which the p-aminobenzoic acid and sodium nitrite solutions were doubled in concentration, giving 0.02 M. p-aminobenzoic acid and 0.04 M. sodium nitrite. The use of a reagent prepared in the usual fashion from these more concentrated solutions extended the proportionality up to 2.0 micromoles of thiamine, Table XI and curve c of Figure 2.

Table XI

Proportionality of the p-Aminobenzoic Acid Method, Using Increased Concentration of the Amine

Thiamine Present	Colorimeter Readings	Thiamine Value
μmoles		μmoles per div.
0.2	45	0.0044
0.4	100	0.0040
0.6	188	0.0032
0.8	254	0.0031
1.0	325	0.0031
1.2	380	0.0032
1.4	465	0.0030
1.6	530	0.0030
1.8	530	0.0034
2.0	660	0.0030
	Average	0.0033 ±0.0003
		(±9.1 per cent)

Although the presence of the alcohol-phenol reagent appeared to make possible the use of the p-aminobenzoic acid method in the presence of the trichloroacetic acid normally present in tissue filtrates, experiments were carried out to determine whether or not even better results might be obtained if the acid concentration were reduced. In one experiment standard thiamine solutions were made up and from 0 to 2.0 ml.

of 5 per cent trichloroacetic acid were added. With 0.0, 0.5, 1.0, 1.5 and 2.0 ml. of the trichloroacetic acid present the colorimeter readings for 0.5 micromoles of thiamine were 123, 166, 196, 189, 137. It appeared at first that increasing concentrations of the acid caused the thiamine value to increase to a maximum and then decrease. However, when blanks containing equal amounts of the acid were made up, it was found that the blank values were likewise increased. When the colorimeter was adjusted to zero with these blanks, the thiamine readings in the presence of the above amounts of trichloroacetic acid were 130, 127, 148, 147, and 75. These values were more uniform than those obtained in the first experiment, and the necessity of using a blank containing an amount of trichloroacetic acid equivalent to that of the unknown became apparent.

To determine the variability of the method as developed a series of tubes each containing 2 ml. of phosphate buffer, 2 ml. of water, 1 ml. of thiamine (2.5 micromoles) and 5 ml. of 10 per cent trichloroacetic acid was made up. In addition a solution containing five times the volume of each of these tubes was made. Two ml. aliquots from each of the ten tubes, and ten 2 ml. aliquots from the larger mixture were analyzed by the p-aminobenzoic acid method. In three experiments in which aliquots from separate tubes were analyzed, the average colorimeter readings for 10 solutions were 75.9 ± 2.8 (± 3.7 per cent), 78.1 ± 2.9 (± 3.7 per cent), and 70.4 ± 1.4 (± 2.0 per cent).

In two experiments in which ten aliquots from a larger mixture were analyzed the average colorimeter readings were 76.3 ± 2.8 (± 3.7 per cent) and 80.2 ± 1.9 (± 2.4 per cent). Although the variability was somewhat greater than that found for the Melnick-Field method, it was not so great as to prohibit the use of this method.

An additional experiment, in which actual tissue extracts were employed rather than the phosphate buffer, was carried out to determine the per cent recovery of thiamine in such solutions. Trichloroacetic acid was added to precipitate the protein immediately after the thiamine addition. The results, shown in Table XII, indicated that 96.3 per cent of the added thiamine had been recovered. Such a high degree of recovery compared well with typical results obtained with the Melnick-Field method of analysis.

Hoping to be able to omit the trichloroacetic acid and thus the alcohol-phenol reagent in this method the effect of metaphosphoric acid on the analytical procedure was determined. Varying amounts of a 10 per cent solution were added to tubes containing 2 ml. of thiamine (0.5 micromole), 5 ml. of 50 per cent alcohol, and water to give 9 ml. in each tube. Five ml. of the reagent was then added. With from 0.02 to 2.0 ml. of the metaphosphoric acid colorimeter readings of from 11 to 152 were obtained, as compared with a reading of 156 for the thiamine standard to which no metaphosphoric had been added.

Table XII

Thiamine Recovery
with Enzyme Extracts

Solution Analyzed	Colorimeter Reading	Thiamine Equivalent	Recovery
		μ moles	per cent
Reagent Blank	266 (set to zero)		
Thiamine Standard	165	0.500	
Tissue Blank	5	0.015	
Tissue plus Thiamine	165	0.500	
Tissue plus Thiamine less Tissue Blank		0.485	96.3

The results indicated that interference was produced by metaphosphoric acid as well as by trichloroacetic acid.

Finally a 0.5 N. hydrochloric acid solution was employed as a protein precipitant. This concentration was equivalent to that of the 10 per cent trichloroacetic acid previously used, and it appeared to be just as efficient as the trichloroacetic in removing the protein from solution. Recovery experiments similar to those described above were carried out, using the hydrochloric acid. In the analysis of the filtrates for thiamine by the p-aminobenzoic acid method, 50 per cent alcohol was used instead of the alcohol-phenol reagent. In two such experiments the recovery of added thiamine was 99.2 and 100.0 per cent. Therefore, it appeared that hydrochloric acid precipitation of protein not only permitted a high degree of recovery, but also allowed the omission of the alcohol-phenol

reagent with a resulting decrease in the colorimeter reading for the reagent blank.

Table XIII shows the recovery of thiamine which was obtained when several different concentrations of the vitamin were added to a constant amount of tissue extract. Five ml. of 0.5 N. hydrochloric acid was added to precipitate the protein, and 2 ml. aliquots of the filtrate thus obtained were analyzed. At the same time standard thiamine solutions, containing no extract, were prepared. The thiamine value in micromoles represented by the colorimeter readings for the tissue blank and for the solutions containing both extract and thiamine were calculated from the readings obtained for the standards. Since the thiamine equivalent of the tissue blank varies, it appears that the colorimetric readings for the standards, from which they were calculated, were not strictly proportional to concentration. The difference between the thiamine equivalents of the solution containing both tissue and thiamine and that of the tissue blank, expressed as per cent of the added thiamine, represents the recovery of the vitamin. The per cent recovery was good and tended to increase with increasing concentrations of thiamine.

Table XIII

Recovery of Thiamine Added
in Varying Amounts to a Tissue Extract

Thiamine added (2 ml. aliquots of filtrates)	Thiamine Equivalent of Tissue Blank	Tissue plus Thiamine	Thiamine Recovery
μmoles	μmoles	μmoles	per cent
0.25	0.108	0.346	95.2
0.50	0.077	0.532	91.0
0.75	0.067	0.750	91.1
1.00	0.058	0.985	91.7
1.25	0.056	1.220	93.1
1.50	0.059	1.500	96.0

In a similar experiment the colorimeter was set to zero with the tissue blank, and with 0.5, 1.0 and 1.5 micromoles of thiamine present in 2 ml. aliquots of the filtrates, the colorimetric readings were 104.0, 98.3 and 97.0 per cent of the readings obtained with equivalent thiamine standards containing no tissue extract.

Results obtained when protein-free filtrates were analyzed immediately after filtration and again twenty-four hours later, showed that such filtrates were stable for at least that time, when kept in the cold. However, in this experiment it was found that incubation increased the colorimetric reading of a tissue blank. Apparently compounds capable of producing color by reacting with the diazotized reagent were freed during

the incubation.

To determine the variability of the method in the presence of tissue extracts, six tubes all containing extracts of fish tissue and thiamine were prepared and the protein precipitated with 0.5 N. hydrochloric acid. The colorimeter readings obtained with aliquots of these filtrates were 142, 142, 139, 142, 141 and 140, with an average value of 141 ± 1.0 (±0.71 per cent). The recovery of thiamine represented by the average value was 99.2 per cent. These results further confirmed the high degree of recovery possible with this method and showed that a high degree of precision was possible.

The results of the experiments described above indicated that this modification of a diazo method for thiamine determination might be used successfully. It certainly was applicable for aqueous solutions of thiamine and appeared to give quantitative results within limits even in the presence of tissue extracts. With trichloroacetic acid employed as a protein precipitant, the necessity of using the Melnick-Field alcohol-phenol reagent was demonstrated.

With hydrochloric acid as the precipitant, colorimetric readings for the reagent blank were reduced to 10 or less. However, proper incubated and non-incubated tissue controls had to be included since tissue blanks gave significant readings which increased upon incubation. In the Melnick-Field method, the color produced by reaction of the reagent with tissue

components, is not extractable with xylene, and therefore there is no necessity for including such controls.

In actual practice, however, somewhat variable results were obtained. Standard values for 0.5 micromoles of thiamine with trichloroacetic acid present ranged from 103 to 172, when readings were made without setting the colorimeter to zero, and from 84 to 120, when the instrument was first adjusted to zero with the tissue blanks. However, the "zero time" control tubes gave values which were 97.4 to 102.7 per cent of the thiamine standard.

The results were more consistent when hydrochloric acid was employed for protein precipitation. For example, in four successive experiments standard thiamine values were 124, 119, 124 and 124. However, the colorimetric readings for the "zero time" controls ranged from 84.9 to 95.9 per cent of the standard. The values for tissue blanks were higher with extracts of acetone-desiccated powder than with extracts prepared from fresh or frozen tissue.

Difficulty was encountered, however, in experiments in which the effect of gluthathione and cysteine on the enzymatic reaction was studied. Both of these compounds interfered with the Melnick-Field method of analysis at concentrations of from 7.5 to 10.0 micromoles per assay tube. With the p-aminobenzoic acid method the presence of these compounds caused decreased colorimetric readings when the alcohol-phenol reagent was

used and increased readings when 50 per cent alcohol was employed.

Because of these difficulties the use of the p-aminobenzoic acid method was discontinued in favor of the Melnick-Field method. However, it appeared that the p-aminobenzoic acid method under certain conditions, was superior to the Melnick-Field, since the additional extraction procedure had been eliminated. Furthermore, it is possible that further investigation will show that it can be applied successfully to more complex solutions.

c. The thiochrome fluorophotometric method. In addition to the colorimetric methods just described the thiochrome fluorophotometric procedure was used (55,56). It was employed where thiamine concentrations were very low, or in solutions which because of interfering substances could not be satisfactorily analyzed by the Melnick-Field method. Its use permitted separation of thiamine from such substances and in other cases the high degree of dilution necessary for use of this method caused a "diluting out" of interfering materials.

The standard thiochrome procedure including a Decalso adsorption and elution of thiamine and an isobutanol extraction of thiochrome was carried out in the following manner. Thiamine standards, unknown thiamine solutions, and where necessary tissue blanks were all treated in the same manner. "Zero time" samples were prepared in large test-tubes graduated at 12.5 and 25 ml., and experimental samples were either prepared in these

tubes or transferred to them after incubation. The protein was precipitated and the enzyme reaction stopped by the addition of 1.25 ml. of 1 N. sulfuric acid at "zero time" or after incubation.

After dilution to 12.5 ml., giving a 0.1 N. concentration of sulfuric acid, the tubes were covered with marbles and placed in a boiling water bath for one-half to one hour. After cooling the pH was adjusted to approximately 4.5 by the addition of 2 ml. of 1.2 M. sodium acetate. Two ml. of a takadiastase solution, 1.25 gm. in 40 ml. of water, was added and the samples were incubated over-night at 40 degrees.

The following day the solutions were diluted to 25 ml., filtered, and aliquots of these filtrates or dilutions of them containing from 3 to 5 micrograms of thiamine were adsorbed on Decalso. For example, if the original thiamine addition had been 2.5 micromoles, the filtrates were diluted 1 to 100, and after the solutions had been adjusted to pH 4 to 5, 10 ml. aliquots containing a maximum of 3.36 micrograms of the vitamin were passed through a column of activated Decalso.

The exchange column consisted of a reservoir at the upper end with a capacity of at least 30 ml., which was attached to a 15 x 0.7 cm. tube ending in a 3 x 0.03 cm. capillary. A 9 cm. column of Decalso (2 gm.) was held in the narrow tube by a tiny layer of cotton.

After adsorption the column was washed with two 20 ml.

portions of distilled water and the thiamine then eluted with approximately 25 ml. of 25 per cent potassium chloride in 0.1 N. hydrochloric acid. The eluate was collected in a 25 ml. volumetric flask and diluted to volume.

For fluorophotometric analysis 5 ml. aliquots of each eluate were placed in each of two 30 ml. glass-stoppered centrifuge tubes. To the first was added 3 ml. of a freshly mixed alkaline potassium ferricyanide solution (3 ml. of 1 per cent potassium ferricyanide diluted to 100 ml. with 15 per cent sodium hydroxide) and to the second, 3 ml. of 15 per cent sodium hydroxide. This latter served as a blank. Fifteen ml. of redistilled isobutanol was added to both, the tubes stoppered and shaken vigorously for 1.5 minutes, centrifuged at 500 r.p.m. for 0.75 minutes, and the aqueous layer discarded. The butanol layer was clarified by shaking with 1 gm. of anhydrous sodium sulfate, and transferred to cuvettes.

The Coleman fluorophotometer was adjusted so that a quinine solution gave a galvenometer reading of 85. Ten ml. of a quinine sulfate solution (0.3 mg. per liter of 0.1 N. sulfuric acid) diluted to 15 ml. with water was found to be suitable for this particular instrument. The amount of thiamine present in the unknowns was calculated by means of the equation below. This equation applied for the conditions described above and had to be modified when other dilutions were used.

$$\text{Thiamine, micrograms} = \frac{T_u - B_u}{T_s - B_s} \times 3.36 \times \frac{25}{5} \times \frac{100}{10} \times \frac{25}{1}$$

T_u = galvanometer reading of unknown

T_s = galvanometer reading of standard

B_u = galvanometer reading of unknown blank

B_s = galvanometer reading of standard blank

On occasion the complete thiochrome procedure just described was not necessary. The digestion with sulfuric acid and takadiastase was omitted except in experiments in which whole cell tissue preparations were employed. In other cases the aqueous solutions obtained after addition of the reagents to aliquots of the Decalso eluates were transferred to cuvettes and galvanometer readings made directly without isobutanol extraction.

In a number of experiments in which 2.5 micromoles of thiamine had been used, the high dilution necessary for fluorophotometric analysis made possible the direct analysis of the diluted filtrates, since interfering substances present were thus so diluted that interference became insignificant. In a typical assay the 10 ml. of filtrate obtained after trichloroacetic acid precipitation of protein contained a maximum of 0.084+ milligrams of thiamine per ml. Dilutions of 2 to 500 resulted in a concentration of 0.33 micrograms per ml. Five ml. aliquots of the diluted filtrates, representing a maximum of 1.65 micrograms of the vitamin were treated

with either 3 ml. of the alkaline ferricyanide reagent or with 3 ml. of 15 per cent sodium hydroxide. The solutions were shaken for 70 seconds, transferred to cuvettes, and galvanometer readings made 90 seconds after the addition of the reagent. This procedure gave quantitative results, and since standard thiamine solutions of this concentration gave readings of from eighty to ninety, a wide range of the galvanometer scale could be used.

B. Velocity of Thiamine Destruction

The role of the enzyme itself in enzymatic reactions is a catalytic one. Since the function of a catalyst is to alter the rate of a reaction and its presence does not change the equilibrium constant, enzymatic reactions are theoretically reversible. However, such reactions are often quite complex, and even though the final products are known, there is the possibility that the reaction occurs in step-wise fashion, with one or more of the steps being catalyzed by the enzyme, while others may be autocatalytic. Furthermore, since the products of an enzymatic reaction are sometimes inhibitory, the problem of reversal of enzyme reactions becomes even more complex.

If the enzymatic destruction of thiamine were a reversible reaction, it was expected that an equilibrium point for the reaction might be established. Although Sealock and his

co-workers (27) reported that the amount of thiamine destroyed by the enzyme increased with time up to six hours, no mention was made of a possible equilibrium point. Similar studies of the "time course" of the reaction were carried out with the enzyme preparations in use in this laboratory, since a wide variation in activity was observed with the acetone-desiccated powders, and it was possible that the powders prepared here would display different properties from those previously used. If, by determining the amount of thiamine destruction with increasing time of reaction, an equilibrium constant for the reaction could be obtained, the problem of the enzymatic synthesis of thiamine would be simplified, and would depend mainly upon knowledge of the products of the reaction.

To determine the amount of reaction at intervals of one-half hour, incubation mixtures of 100 ml., representing twenty times the usual assay volume were prepared. These contained 40 ml. of either phosphate buffer or an enzyme extract, equivalent to 50 mg. of preparation I-142-III-A per 5 ml. (3.24 mg. Nitrogen/50 mg.). To these were added sodium hydroxide (equivalent to the thiamine), water and thiamine solutions to give concentrations of 5, 10 and 20 x 10^{-4} M., respectively. From each mixture, 5 ml. aliquots were withdrawn every 30 minutes for a period of 6 hours, and the thiamine present in each determined by Melnick-Field analysis.

In order to determine the stability of the enzyme under these

conditions, a portion of the extract was incubated without substrate. Samples withdrawn at 0, 2 and 4 hours, when assayed to determine enzyme activity, were found to destroy 1.42, 1.02 and 0.80 micromoles of thiamine. The activity of the enzyme was thus reduced by 28.2 per cent and 43.7 per cent during 2 and 4 hour periods of incubation. This instability of the enzyme must be kept in mind in experiments involving times of incubation greater than the usual two hour period.

The amount of thiamine destroyed at intervals from 0 to 6 hours with the three different substrate concentrations is shown in Table XIV and Figure 3. With the lowest thiamine concentration destruction was practically complete at the end of four hours as evidenced by the 93 per cent destruction at that time. The apparent decrease observed during the last two hours was due to the destruction of the vitamin in the buffer solution, which was still increasing with time. With the intermediate thiamine concentration there appeared to be a "leveling off" of enzymatic destruction after four to five hours of incubation. This might be interpreted as an approach to an equilibrium condition, but the instability of the enzyme may also have accounted for this decrease in amount of reaction. With the highest thiamine concentration, there was also indication that the reaction was approaching an end-point, as evidenced by the fact that only 1.2 per cent of the substrate was destroyed during the last one and a half hours, as compared with the 6.4

Table XIV

Destruction of Thiamine

Time	Thiamine Destroyed [1] with Initial Concentration of 5×10^{-4} M.		10×10^{-4} M.		20×10^{-4} M.	
min.	per cent	μmoles	per cent	μmoles	per cent	μmoles
30	33.2	0.83	13.8	0.69	6.4	0.64
60	52.0	1.30	20.0	1.00	10.0	1.00
90	66.2	1.66	30.4	1.52	13.8	1.38
120	75.6	1.89	34.0	1.70	13.8	1.38
150	83.6	2.09	36.0	1.80	17.6	1.76
180	89.1	2.23	40.0	2.00	17.6	1.76
210	91.2	2.28	41.0	2.05	17.6	1.76
240	93.4	2.34	47.8	2.39	21.2	2.12
270	93.6	2.34	48.4	2.42	24.4	2.44
300	92.6	2.32	51.0	2.55	24.4	2.44
330	92.0	2.30	51.2	2.56	24.8	2.48
360	92.0	2.30	50.4	2.52	25.6	2.56

[1] Values represent enzymatic destruction of thiamine in a 5 ml. volume of the incubation mixture. The destruction in the buffer control was subtracted from that obtained in the experimental solutions.

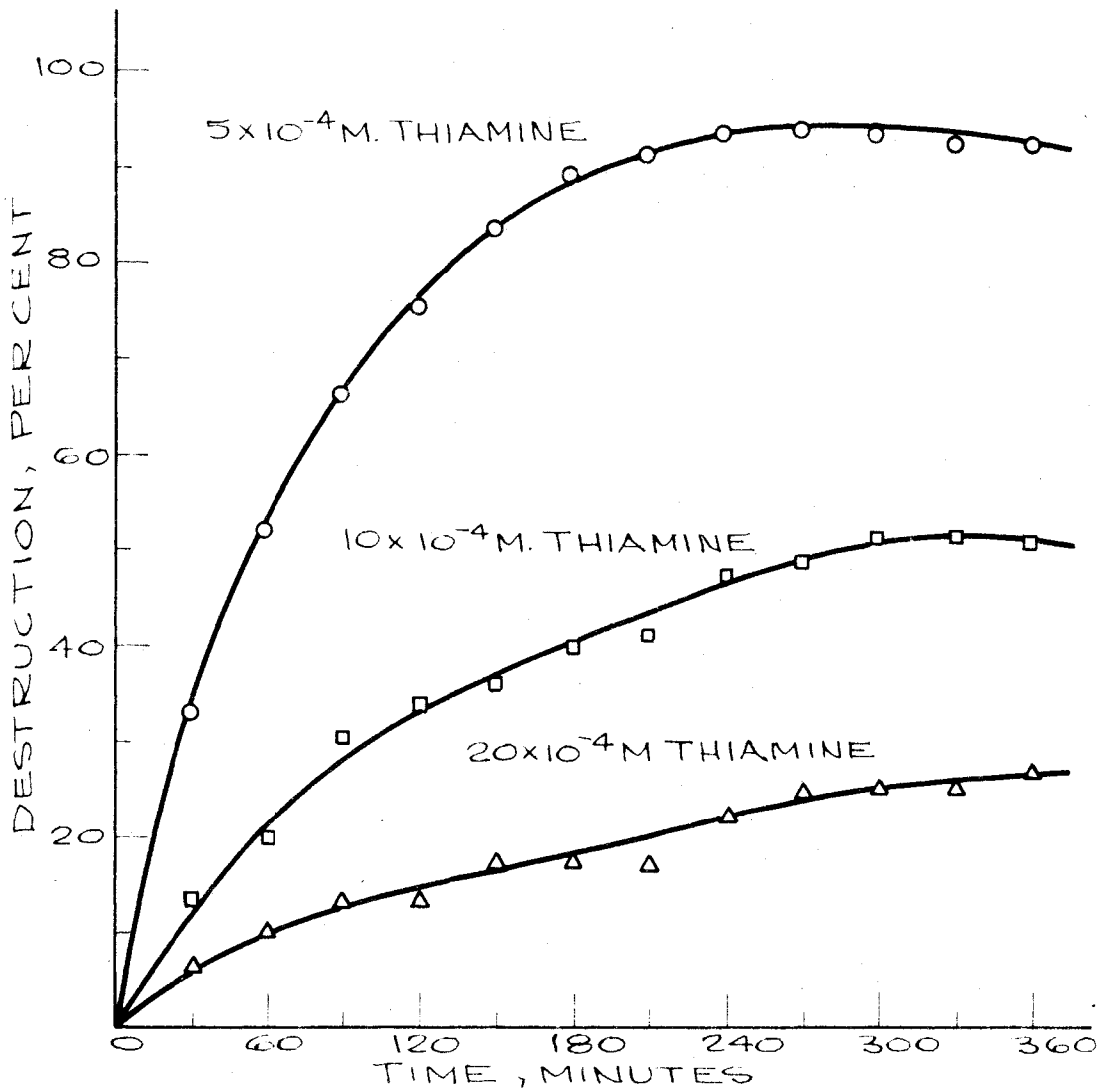


FIG. 3 TIME COURSE OF THIAMINE DESTRUCTION BY THIAMINASE WITH THREE SUBSTRATE CONCENTRATIONS.

per cent destruction observed during the initial half hour.

A comparison of the total micromoles of substrate destroyed with the three different thiamine concentrations at the time intervals up to three and one-half hours showed an "apparent substrate inhibition", since the amount of reaction decreased as the thiamine concentration increased. Because, with the lowest substrate concentration, the thiamine had been almost completely destroyed at that time, the inhibition was not so evident beyond that point.

From the amount of destruction at the half hour intervals, velocity constants were calculated using the first order equation. Considering the crude nature of the enzyme preparation these constants, shown in Table XV, exhibited a satisfactory uniformity. Since there was almost 100 per cent destruction of the substrate at the end of four hours with the lowest thiamine concentration, the velocity constants increased rapidly. As the denominator of the first order equation approaches zero, the velocity constant would obviously increase and tend to approach infinity. The decrease in velocity constants exhibited with the other two substrate concentrations could be accounted for by the decrease in enzyme activity due to its instability.

In another and similar experiment a shorter period of incubation was employed, but aliquots were withdrawn at more frequent intervals. The same three thiamine concentrations

Table XV

First Order Velocity Constants of Thiamine Destruction

Time min.	Velocity Constants ($k \times 10^3$) [1] with Initial Thiamine Concentrations of 5×10^{-4} M.	10×10^{-4} M.	20×10^{-4} M.
30	13.5	4.60	2.31
60	12.5	4.18	1.75
90	12.6	4.43	1.66
120	12.5	3.73	1.22
150	13.2	3.27	1.35
180	14.6	3.34	1.17
210	15.4	2.92	1.07
240	16.3	3.09	1.06
270	16.3	2.78	1.11
300	18.5	2.71	1.01
330		2.54	0.95
360		2.40	0.92

[1] k is expressed as moles per liter per minute.

were used, but in this experiment the enzyme concentration was equivalent to 67 mg. of preparation I-142-III-A per 5 ml. of incubation mixture. Here again the decreased amount of reaction with increased substrate concentration was observed. This can be seen from the data of Table XVI, in which the amount of reaction at intervals of 15 minutes is given.

Table XVI

Destruction of Thiamine

Time	Thiamine Destruction[1] with Initial Concentration of 5×10^{-4} M.	10×10^{-4} M.	20×10^{-4} M.
min.	μ moles	μ moles	μ moles
15	0.55	0.36	0.34
30	0.88	0.77	0.72
45	1.24	1.18	1.20
60	1.51	1.45	1.56
75	1.80	1.78	1.66
90	2.00	2.02	1.78
120	2.31	2.45	2.14

[1] The thiamine destruction represents that in 5 ml. aliquots of the incubation mixture.

The velocity constants obtained in this second experiment and recorded in Table XVII again exhibited satisfactory uniformity. The constants calculated for the 20 x 10^{-4} M. thiamine solution were in good agreement with those published by Sealock, et al (27). For two experiments, in which there was approximately 25 per cent destruction of the added thiamine, their velocity constants ranged from 1.46 to 2.78 x 10^{-3} moles per liter per minute. Here velocity constants ranging from 2.08 to 2.86 x 10^{-3} moles per liter per minute were obtained during the two hour incubation of a mixture containing 20 x 10^{-4} M. thiamine. The destruction of 2.14 micromoles of thiamine per 5 ml. in this mixture represented a 21.4 per cent destruction of added substrate.

Table XVII

First Order Velocity Constants of Thiamine Destruction

Time min.	Velocity Constants (k x 10^3) with Initial Thiamine Concentration of		
	5 x 10^{-4} M.	10 x 10^{-4} M.	20 x 10^{-4} M.
15	17.3	5.40	2.08
30	14.5	5.50	2.60
45	15.2	6.10	2.86
60	15.4	5.75	2.86
75	17.1	5.85	2.44
90	17.9	5.80	2.20
120	21.5	4.40	2.50

From these experiments it appeared that under these conditions of incubation and with these crude enzyme preparations no equilibrium point could be established. In the second experiment, in which only a two hour period of incubation was employed, the amount of reaction with all three concentrations of thiamine, was still increasing at the end of that time. With the longer incubation period, the reaction went to completion with the lowest substrate concentration, but approached an apparent "end-point" with the higher concentrations, probably because of the instability of the enzyme.

For typical enzyme reactions, the curve obtained by plotting the amount of reaction against the time of reaction, shows an initial straight-line portion, which is characteristic of zero-order reactions. For kinetic studies of the type associated with the determination of dissociation constants for enzyme-substrate and enzyme-inhibitor complexes, the amount of reaction at short intervals during the time when the reaction is of zero-order must be obtained. An examination of Figure 3 shows that in these cases with thiaminase, a zero-order type reaction existed for only a short time, probably for less than sixty minutes. A plot of the data of Table XVI indicated that with measurement at fifteen minute intervals three or four values within the period of zero-order reaction might be obtained. However, the technical difficulties involved in securing the necessary data by withdrawing samples at frequent intervals for

analysis by the Kelnick-Field method make this an undesireable procedure for kinetic studies.

Therefore work was undertaken to determine whether such studies might not be carried out using the Warburg manometric technique, a procedure which would allow more frequent and easier determinations of the amount of reaction. Sealock and Livermore (33) had reported that one hydrogen ion was released during the enzymatic destruction of one molecule of thiamine, and in a bicarbonate buffer one micromole of carbon dioxide was evolved per micromole of thiamine destroyed.

In the first experiment both a tissue suspension (400 mg. equivalent per flask) and an extract of preparation I-142-II-A (200 mg. equivalent per flask) were used. These were prepared in 0.025 M. sodium bicarbonate containing 5 per cent sodium chloride, and 2 ml. aliquots of the enzyme solutions, pH 7.4, were placed in the main compartment of 25 ml. Warburg flasks. One ml. of thiamine solution (10 micromoles), also prepared in 0.025 M. sodium bicarbonate, was placed in the side arm of the experimental flasks, while an equal amount of sodium bicarbonate was added to the control flasks. An atmosphere of 5 per cent carbon dioxide and 95 per cent nitrogen was present. After a fifteen minute equilibration period the contents of the side-arms were emptied into and mixed with the contents of the main compartments, and manometric readings made at intervals up to two and one-half hours. Although analysis of the contents made at the conclusion of the reaction, showed that

1.81 micromoles of thiamine had been destroyed by the extract and 0.49 moermoles by the tissue suspension, the carbon dioxide evolution measured for the experimental flasks was less than that of the controls.

With the idea that perhaps the bicarbonate buffer had been too dilute, a second experiment was carried out similar to the above except that the buffer used had the following composition: 0.2 M. sodium bicarbonate, 0.2 M. potassium chloride and 10 per cent sodium chloride. After a three hour incubation period using an air atmosphere, the carbon dioxide excess (experimental less control) was 18 microliters. This excess was only 23.3 per cent of the theoretical, determined from chemical analysis of the thiamine destroyed, and assuming that one micromole of carbon dioxide should be evolved per micromole of thiamine destroyed.

In the following experiment incubation was carried out at pH 8.1 in a 0.1 M. sodium bicarbonate buffer containing 0.2 M. potassium chloride and 10 per cent sodium chloride and in an atmosphere of 5 per cent carbon dioxide and 95 per cent nitrogen. At the end of three hours, the excess carbon dioxide represented only 26.7 per cent of the theoretical. However, flasks, in which the buffer had been placed in the main compartment and the thiamine solution in the side-arm, showed an uptake of 28.7 microliters of carbon. Although such a calculation might not be justified, since the reason for this was not apparent, if this uptake were added to the observed excess value, the per cent of theoretical increased to 85.

In an effort to determine the reason for the failure to duplicate the published results of Sealock and Livermore (33), a number of experiments were carried out in which carbon dioxide output was measured with standard carbonate solutions plus excess acid and with bicarbonate buffers plus a standard acid. In the first a 0.2 M. sodium bicarbonate solution containing 0.2 M. potassium chloride and 10 per cent sodium chloride at pH 7.4 was used. One ml. of 0.01 N. hydrochloric acid (10 micromoles) was placed in the side arm of one flask and one ml. of water in the side arm of a second. An air atmosphere was employed. The experimental flask at 10 minutes showed an output of 24.2 microliters of carbon dioxide, but this decreased until at 210 minutes the output was only 4.15 microliters. The control flask on the other hand showed a progressive uptake until at the end of the incubation period 53.7 microliters had been absorbed. It appeared that some phenomenum of mixing or perhaps the use of a too alkaline manometric fluid might be accounting for this retention or absorption of the gas.

With a 0.02 M. sodium bicarbonate solution at pH 7.4 the addition of 1 ml. of 0.01 N. hydrochloric acid resulted in a 54.1 per cent recovery of carbon dioxide when an air atmosphere was used and a 67.9 per cent recovery with an atmosphere of 5 per cent carbon dioxide and 95 per cent nitrogen. The carbon dioxide evolution was measured at 15 minutes and remained essentially constant for 40 minutes. The readsorption observed in the above experiment did not occur with the more dilute

bicarbonate solution.

In another experiment 2 ml. of 0.005 N. sodium carbonate, (equivalent to 10 micromoles of carbonate), yielded 75.8 per cent of the theoretical amount of carbon dioxide upon addition of 1 ml. of 1 N. sulfuric acid. Addition of sulfuric acid to 2 ml. of 0.0025 M. sodium carbonate resulted in a 92.8 per cent of the theoretical yield of carbon dioxide when the rate of shaking was 120 and 87.4 per cent, with a shaking rate of 60. Apparently the faster rate of shaking produced a greater evolution of carbon dioxide. Also since a higher per cent recovery was obtained with the more dilute carbonate solution, it appeared that an absolute amount of carbon dioxide was being held or retained in some fashion.

Further attempts were made to obtain 100 per cent recovery of carbon dioxide. A mixture of 5 per cent carbon dioxide and 95 per cent nitrogen was bubbled through a bicarbonate solution (0.03 M. at pH 7.77) and the solution was allowed to stand under this atmosphere for a day and a half before use. Upon addition of 1 ml. of 0.005 N. hydrochloric acid to 2 ml. of this bicarbonate solution, thoroughly saturated with carbon dioxide, only a 67.3 per cent of the theoretical amount of carbon dioxide was recovered.

Another attempt at measuring carbon dioxide evolution during the enzymatic destruction of thiamine was made using a more dilute enzyme preparation. Powder I-142-III-A was more active

than I-142-II-A and thus a lower concentration of the former could be used to obtain an equal amount of destruction. It was felt that with a lower concentration, there might be less tendency for protein retention of carbon dioxide through the formation of carbamino derivatives. The enzyme extract was prepared using a 0.144 M. sodium bicarbonate solution containing 10 per cent sodium chloride and 5 per cent potassium chloride. Thiamine was dissolved in 0.072 M. bicarbonate to give a concentration of 10 micromoles per ml. All solutions were adjusted to pH 7.4, additions were made so that the final bicarbonate concentration was 0.072 M., and the enzyme concentration represented the soluble fraction of 50 mg. of I-142-III-A per flask. An atmosphere of 20 per cent carbon dioxide and 80 per cent air was employed.

From the results of this experiment, shown in Table XVIII, it became clear that there was absorption of carbon dioxide. In the flask containing only the bicarbonate buffer there was an uptake of 24.7 microliters of carbon dioxide, while in the presence of the protein extract the uptake was increased to 79 microliters. Even the addition of 10 micromoles of a standard acid failed to release carbon dioxide as shown by the uptake of 23.0 microliters in the presence of the protein. The evolution of 115.7 microliters of carbon dioxide obtained upon addition of the standard acid to the buffer represented only 51.9 per cent of the theoretical.

Table XVIII

Carbon Dioxide Evolution
During Enzymatic Destruction of Thiamine

Additions Made to Flask		Carbon Dioxide Evolution (1 hr.)
Main Compartment	Side Arm	
		µl.
1 ml. 0.144 M. bicarbonate 1 ml. 0.072 M. bicarbonate	1 ml. water	-24.7
1 ml. 0.144 M. bicarbonate 1 ml. 0.072 M. bicarbonate	1 ml. 0.01 N. HCl	115.7
1 ml. enzyme extract 1 ml. 0.072 M. bicarbonate	1 ml. water	-79.0
1 ml. enzyme extract 1 ml. water	1 ml. thiamine (10 micromoles)	58.0
1 ml. enzyme extract 1 ml. 0.072 M. bicarbonate	1 ml. 0.01 N. HCl	-23.0

In the flask in which thiamine had been added to the enzyme extract there was an output of 62.0 microliters of carbon dioxide within 10 minutes, which would be equal to the amount obtained if 2.76 micromoles of thiamine had been destroyed. The value at 1 hour was 58.0 microliters, which would be equivalent to the destruction of 2.59 micromoles of thiamine. Melnick-Field analysis showed that in this case there was only a destruction of 0.8 micromoles of the substrate. The reason for the rapid evolution of carbon dioxide upon addition of the thiamine solution was not clear. The thiamine was dissolved in the bicarbonate buffer and the pH adjusted to 7.4. Excess acid from the thiamine hydrochloride should have been neutralized by this procedure.

In another experiment smaller (15 ml.) Warburg flasks were employed with the idea that in these flasks shaking and mixing should be more efficient, thus perhaps allowing a greater evolution of carbon dioxide. The enzyme extract was prepared in a buffer having the following composition: 0.025 M. sodium bicarbonate, 0.2 M. potassium chloride and 10 per cent sodium chloride. Thiamine was dissolved in the same buffer, and the pH of all solutions adjusted to 7.4. A 5 per cent carbon dioxide, 95 per cent nitrogen atmosphere was employed. Two ml. aliquots of the enzyme extract were used and represented 50 mg. of preparation I-142-III-A (3.64 mg. of nitrogen). The contents of the various flasks and the results of the experiments are given in Table XIX. The recovery of carbon dioxide upon

addition of thiamine to the enzyme extract ranged from 47.5 to 70.0 per cent of the theoretical, assuming one micromole of carbon dioxide per micromole of thiamine destroyed. Here again it appeared that there was protein retention of carbon dioxide. Although the final yield of carbon dioxide obtained, when the standard acid was added to the buffer, was 88.0 per cent of the theoretical, at one-half hour the yield was almost 100 per cent. After that time there was a progressive reabsorption of the gas, indicating retention of carbon dioxide apart from that due to protein retention. However, the results from this and earlier experiments are in agreement with the conclusion of Sealock and Livermore (33) that the enzymatic destruction of thiamine results in the release of one hydrogen ion per molecule of thiamine destroyed.

From the results obtained with the manometric studies it became apparent that this method could not be used for kinetic studies of thiaminase, unless a means for preventing carbon dioxide retention became available. Perhaps with further purification of the enzyme such a technique could be applied with success.

However, another possibility presented itself. If a hydrogen ion were released during the destruction of thiamine, perhaps the subsequent change in pH might be followed and related to the amount of reaction at different intervals of time. Therefore an extract equivalent to 100 mg. of preparation I-142-III-A was prepared in 10 per cent sodium chloride

Table XIX

Carbon Dioxide Evolution
During Enzymatic Destruction of Thiamine

Additions to Flasks: Main Compartment	Side Arm	Thiamine Destruction	Carbon Dioxide Evolution	Carbon Dioxide Yield
		μmoles	μl.	per cent of theory
extract	buffer		15.8	
extract	0.625 μmoles B_1	0.58	22.8	58.4
extract	1.25 μmoles B_1	0.79	28.2	70.0
extract	2.50 μmoles B_1	0.79	24.2	47.5
extract	5.00 μmoles B_1	0.97	29.2	61.8
extract	5.0 μmoles HCl		57.6	51.5
buffer	5.0 μmoles HCl		100.0	88.0
buffer	buffer		1.4	

and the pH adjusted to 7.4. A mixture of 40 ml. of the extract, 8 ml. of 0.005 N. sodium hydroxide, 12 ml. of water and 40 ml. of 0.02 M. thiamine was prepared and the pH adjusted to 7.4 immediately following the addition of thiamine. This solution was incubated at 37 degrees and at "zero" time and at intervals thereafter 5 ml. aliquots were withdrawn. The pH of these was measured and after precipitation of protein with 10 per cent trichloroacetic acid they were analyzed for thiamine. The pH decreased slightly with time and at the end of 2 hours

was 7.12. The initial hydrogen ion concentration was therefore 4.05×10^{-8} M. and the final concentration was 7.58×10^{-8} M. The total thiamine destruction was 103.0 micromoles, which, assuming one hydrogen ion released per molecule of thiamine destroyed, would result in the release of 1.03×10^{-3} moles per liter of hydrogen ion. Apparently the buffering capacity of the protein present was too great to permit measurement of pH change due to the release of a hydrogen ion.

Until solutions less concentrated in protein can be employed, as would be the case if the enzyme were available in a purer form, it appeared that for kinetic studies the use of Melnick-Field analysis of aliquots withdrawn at intervals from a larger incubation mixture would provide the most reliable results.

C. Attempted Reversal of Thiaminase Action

In spite of the failure to establish an equilibrium point in the thiaminase reaction by measuring the amount of destruction at different time intervals, attempts were made to reverse the reaction by incubation of enzyme preparations with various combinations of pyrimidine derivatives and the thiazole moiety of thiamine. Since the crude nature of the enzyme extracts employed may have accounted for the previous failure, it was felt that under the proper conditions an equilibrium could be approached from the opposite direction, that is by the

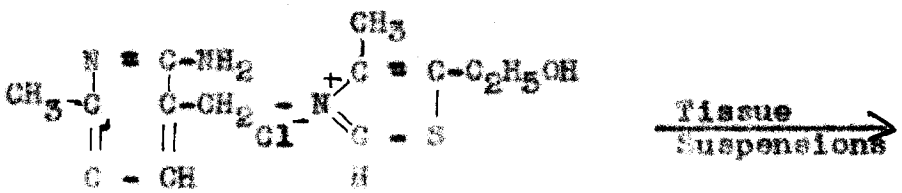
synthesis of thiamine from its component parts.

Krampitz and Woolley (30) identified the final products of the reaction as 4-methyl-5-β-hydroxyethylthiazole and 2-methyl-6-amino-5-hydroxymethylpyrimidine. However, their studies with different microorganisms indicated that the reaction occurred in at least two steps. The thiazole moiety was freed in the first, but the pyrimidine portion of the thiamine molecule was liberated as an undetermined intermediate from which pyrimidine alcohol was formed. Hennessy and Warner (47) reported the isolation of a pyrimidine derivative, which was not the alcohol, but which was formed by the action of thiaminase from clams upon thiamine. The compound was not identified except by certain physical properties and an empirical formula.

In an effort to reverse the action of thiaminase several types of enzyme preparations were used. In some cases the extract obtained from the acetone desiccated powders was employed; in others homogeneous suspensions of frozen carp tissue were used; and in certain experiments surviving tissue was utilized. For these latter experiments goldfish were killed and the viscera immediately removed and finely chopped. 250 mg. quantities were transferred to Warburg flasks, and buffer and solutions of the compounds added. After two hours of incubation with shaking, the flask contents were transferred quantitatively to large test-tubes and digested with 0.1 N. sulfuric acid prior to a thiochrome analysis for thiamine.

Several different pyrimidine derivatives were used and both the thiazole moiety of thiamine and its pyrophosphate ester were employed. Although the Melnick-Field and the p-aminobenzoic acid methods were used in a few experiments, a thiochrome analysis was more generally employed for thiamine determination. Table XX shows the concentrations of the components which were incubated with the enzyme preparations. In all cases the enzyme was incubated under the same conditions with 2.5 micromoles of thiamine to determine its activity. In no case was there evidence for the synthesis of thiamine from these components.

Considering the results obtained by Krampitz and Woolley (30) the overall reaction for the enzymatic destruction of thiamine can be written as follows:



Thiamine Chloride

I

2-methyl-6-amino-5-hydroxy-methylpyrimidine

II

4-methyl-5-β-hydroxyethyl-thiazole

III

Table XX

Attempted Reversal of Thiaminase Action

Enzyme Preparation	Thiamine Destroyed in Incubation with 2.5 micromoles	2-Methyl-6-aminopyrimidine derivatives	4-Methyl-5-β-hydroxyethylthiazole
	μmoles	μmoles	μmoles
		5-CH_2SO_3H	
Tissue suspension, 300 mg. equiv.	0.58	50.0	50.0
I-68, 100 mg. equiv.	0.71	6.3	50.0
Tissue suspension, 200 mg. equiv.	0.64	10.0	10.0
		5-CH_2Br	
I-68, 100 mg. equiv.	0.71	50.0	50.0
Tissue suspension, 200 mg. equiv.	0.64	10.0	10.0
		5-CH_2OH	
Tissue suspension 400 mg. equiv.	0.25	10.0	500.0
		10.0	10.0
		25.0	25.0
		50.0	50.0
I-142-III-A, 50 mg. equiv.	1.93	2.5	2.5[1]
		2.5	7.6[1]
I-142-II-A, 200 mg. equiv.	2.5	10.0	10.0
		25.0	25.0
		50.0	50.0
Surviving tissue, 250 mg.	0.25	2.5	2.5
		2.5	6.2
		5.0	5.0
		5.0	12.4
Surviving tissue, 250 mg.	0.19	1.0	1.0
Surviving tissue, 250 mg.	0.065	25 micrograms	25 micrograms

[1] The pyrophosphate ester of the thiazole was employed in this experiment.

For the reaction obtained with the use of a sodium chloride extract however, the pyrimidine alcohol is not produced, but instead the pyrimidine is liberated in the form of an intermediate of unknown structure:

Thiamine (I) $\xrightarrow{\text{Extract}}$ Pyrimidine Derivative + Thiazole

I IV III

The pyrimidine intermediate (IV) is converted to the pyrimidine alcohol (II) by tissue suspensions and by treatment with strong alkali.

The results obtained with the study of the velocity of the enzyme reaction indicated that under proper conditions the reaction went to completion, that is, all of the substrate was destroyed as determined by the Melnick-Field method. However, since the nature of the reaction products was not known, it was possible that the enzyme preparations were producing either the pyrimidine intermediate (IV) or the pyrimidine alcohol (II). Since perhaps only the first step of the reaction, that resulting in the formation of the pyrimidine intermediate, is enzymatic, efforts were made to reverse the reaction by adding excess quantities of the thiazole moiety at the conclusion of the two hour period of incubation and continuing incubation for another two hours.

For example, a 200 mg. equivalent of a suspension of frozen carp tissue was incubated with 2.5 micromoles of thiamine for two hours, at which time 0.5 micromoles of substrate had been

destroyed. Fifty and 100 micromoles of 4-methyl-5-β-hydroxyethylthiazole were added to additional samples and incubation continued for two hours. There was no evidence for reversal of the reaction, even in tubes to which additional enzyme had been added. Destruction continued during the second incubation period, although more slowly than in samples which were allowed to incubate for four hours without added thiazole. Similar results were obtained with a 200 mg. equivalent of acetone-desiccated powder I-68, which in two hours had destroyed 2.25 micromoles of thiamine.

In another series of experiments the pyrimidine derivative obtained by the enzymatic destruction of thiamine was used as a source of the pyrimidine component. Although previous work with the pyrimidine alcohol had given negative results, it was possible that our preparations were producing only the intermediate. The compound was not isolated but solutions of it were obtained. For example, 50 ml. of an enzyme extract was incubated with 50 ml. of a thiamine solution containing 5 micromoles per ml. Aliquots were analyzed from time to time by the p-aminobenzoic acid method until at the end of 10 hours, the colorimetric values became constant, indicating maximum destruction. The protein was then precipitated with concentrated hydrochloric acid and the solution filtered. If destruction had been complete the filtrate thus obtained would represent 2.5 micromoles per ml. of the pyrimidine derivative.

Different amounts of this filtrate were incubated with a fresh enzyme preparation in the presence of excess 4-methyl-5-β-hydroxyethylthiazole. When later analyses were made by the Melnick-Field method it was found that destruction had not been complete and therefore there was a small amount of unaltered thiamine remaining in the filtrate. However, results showed that the second incubation merely served to destroy the remaining thiamine, and that there was no indication for reversal of the reaction.

In another experiment a filtrate wasobtained from an incubation mixture in which thiamine destruction had been 99 per cent complete, and this filtrate then contained 1.24 micromoles of the pyrimidine derivative and 0.01 micromoles of unaltered thiamine per ml. One and 2 ml. portions of the filtrate were incubated with 50 micromoles of thiazole in the presence of an enzyme preparation which was capable of destroying 0.65 micromoles of thiamine in two hours. Analysis by the Melnick-Field method showed 0.008 and 0.018 micromoles of thiamine present at the end of two hours incubation with the 1 and 2 ml. additions of the filtrate. Destruction of the remaining thiamine had continued during this period, although the total amount destroyed was much less than that in the control assay.

Fairly high concentrations of the thiazole moiety, when used in efforts to reverse the reaction, were found to be inhibitory. It was possible then that the presence of both

the thiazole moiety and the pyrimidine derivative further reduced the activity of the enzyme. Such reduced activity in the presence of the two components might indicate that equilibrium had been reached but since there was still undestroyed thiamine in the filtrate used it was impossible to state definitely whether the results obtained were due to inhibition by products or due to establishment of equilibrium. The use of components marked with isotopic elements could be employed to answer this question.

Since the studies of the time course of the reaction indicated that the equilibrium point is far to the right favoring destruction rather than synthesis it may be that the concentrations employed, both of enzyme and components, were not high enough to permit measurement of synthesis by our methods. Furthermore, it must be remembered that only a few pyrimidine compounds were tested and that the filtrates used may have contained either the pyrimidine alcohol or the intermediate. If only the first step of the reaction is enzymatic, the necessity of working with the actual intermediate in reversing the reaction becomes obvious. Until further information regarding the nature of the reaction and the nature of the intermediate has been obtained, it is impossible to conclude that the reaction is not reversible. In addition, it is possible that changes in pH or temperature might be used to favor a synthetic reaction.

D. Inhibition by Tertiary Thiazoles

It has been shown that with certain enzymes, the products of the enzymatic reaction are inhibitory. In the case of thiaminase, Sealock and Goodland (37) have reported that 5-bromomethyl-, 5-methylenesulfonic acid-, and 5-ethoxymethyl-2-methyl-4-aminopyrimidine all produced inhibition. The highest degree of inhibition was observed with the 5-bromomethyl derivative, which at a concentration of 2×10^{-3} M. produced 48.6 per cent inhibition. Although there is no indication that any of these compounds could be a product of the enzymatic reaction, they are all similar to 2-methyl-4-amino-5-hydroxymethylpyrimidine, which Krampitz and Woolley (30) reported to be one of the products.

In the efforts to reverse thiaminase action by the addition of pyrimidine derivatives and the thiazole moiety of thiamine, it was noted that excess thiazole appeared to inhibit the destructive action of the enzyme. Additional analysis of this inhibition with several different concentrations of 4-methyl-5-β-hydroxyethylthiazole was therefore undertaken. The results, shown in Table XXI, indicate that this compound is inhibitory although the concentrations necessary to produce a significant degree of inhibition are from ten to five hundred times the concentrations of the thiaminase inhibitors previously studied by Sealock and Goodland. For purpose of further illustrating thiazole inhibition the results from the second experiment of

Table XXI are shown in graphic form in Figure 4. The S-shaped curve is similar to that published by Sealock and Goodland (37) for inhibition by 3-o-aminobenzyl-4-methylthiazolium chloride. The inhibition of the enzyme reaction is expressed as inhibition in per cent and is calculated by means of the following equation:

$$\text{Inhibition, per cent} = \frac{\% \text{ Dest. (Uninhibited)} - \% \text{ Dest. (Inhibited)}}{\% \text{ Dest. (Uninhibited)}} \times 100$$

In addition to 4-methyl-5-β-hydroxyethylthiazole, 4-methylthiazole was tested for inhibitory action. In the first experiment solutions of this compound were added in varying amounts to an enzyme extract representing 150 mg. of preparation I-142-II-A, and the results, shown in Table XXII, demonstrate that the compound, at least in these concentrations, has only an insignificant inhibitory effect on enzyme activity.

Table XXI

Inhibition by 4-Methyl-5-β-hydroxyethylthiazole

Enzyme Preparation	Thiazole Concentration moles/l. x 10^2	Thiamine Destruction per cent	Inhibition per cent
Tissue suspension, 220 mg. equiv.	0	35.2	
	1.00	23.8	32.4
	2.00	14.3	59.5
	4.00	5.8	83.5
	6.00	3.7	89.5
	8.00	3.7	89.5
	10.00	3.7	89.5
I-142-II-A, 133 mg. equiv.	0	88.3	
	0.20	85.8	2.8
	0.40	79.3	10.2
	1.00	71.8	18.7
	2.00	33.4	62.3
	4.00	14.3	84.0
	6.00	8.4	90.5
	10.00	6.0	93.3
Tissue suspension, 140 mg. equiv.	0	45.4	
	0.02	45.5	0
	0.10	42.7	5.9
	0.50	34.9	25.3
I-68, 200 mg. equiv.	0	96.8	
	0.02	97.0	- 0.2
	0.04	97.5	- 0.8
	0.08	97.7	- 1.1
	0.16	97.7	- 1.1
	0.20	96.5	0.4
	0.40	93.7	3.7
	1.00	89.0	8.1
	2.00	74.1	23.5
Tissue suspension, 200 mg. equiv.	0	17.6	
	0.50	13.9	21.1
	1.00	16.4	6.8
	1.50	10.4	41.0
	2.00	10.4	41.0
	3.00	7.2	59.1
	4.00	5.6	68.2
	6.00	8.4	52.4

(continued next page)

Table XXI (con'd).

Enzyme Preparation	Thiazole Concentration moles/l. x 10^2	Thiamine Destruction per cent	Inhibition per cent
Tissue suspension, 200 mg. equiv.	0	15.6	
	0.20	10.0	36.0
	0.40	10.0	36.0
	1.00	7.7	50.7
	2.00	3.4	78.2
	4.00	1.2	92.5
	6.00	2.2	86.0
	8.00	0	100.0
	10.00	4.5	71.2

Table XXII

Inhibition by 4-Methylthiazole

Thiazole Concentration moles/l. x 10^2	Thiamine Destruction per cent	Inhibition per cent
0	40.0	
0.05	39.3	1.8
0.10	38.3	4.2
0.30	38.9	2.8
0.60	38.1	4.7

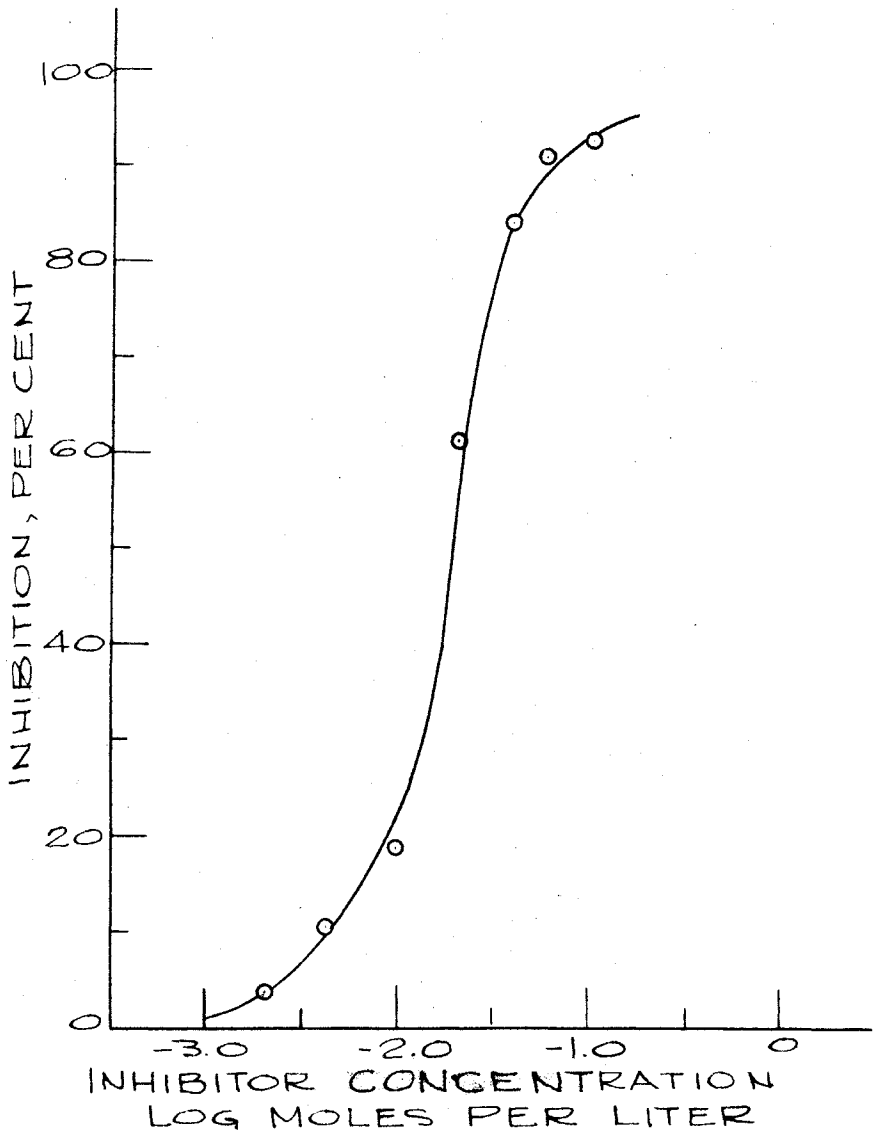


FIG. 4 INHIBITION BY 4-METHYL-5-β-HYDROXY ETHYLTHIAZOLE

In another experiment the inhibition produced by the two tertiary thiazoles was compared at equal concentrations of both. Again the results, shown in Table XXIII, indicate that 4-methylthiazole is a much less active inhibitor than the thiamine thiazole. This would appear to emphasize the importance of the hydroxyethylgroup in inhibition. However, thiamine analogues,

Table XXIII

Inhibition by 4-Methylthiazole and by 4-Methyl-5-β-hydroxyethylthiazole

Thiazole Concentration	Thiamine Destruction	Inhibition
moles/l. x 10^2	per cent	per cent
0	75.2	
4-Methyl-5-β-hydroxyethylthiazole:		
2.0	29.4	60.9
4.0	20.2	73.2
4-Methylthiazole:		
2.0	66.9	11.0
4.0	55.5	26.2

such as o-aminobenzyl-4-methylthiazole, although lacking an hydroxyethyl group are even more active inhibitors than the vitamin thiazole. Perhaps the absence of the quaternary nitrogen in these two thiazole compounds is an important factor accounting for the relatively low degree of inhibition. Furthermore, it must be remembered that the apparent inhibition by the thiamine thiazole moiety may have been due to the effect of this compound in establishing equilibrium.

E. Synthesis of Analogue Molecules

In addition to the analysis of inhibition produced by the two tertiary thiazole derivatives, a number of thiamine analogues were tested for inhibitory action. The previously reviewed work of Sealock and Goodland (37) on the inhibition produced by o-aminobenzyl- and β-aminoethyl-4-methylthiazolium chlorides has emphasized the importance of the free amino group in the inhibitor molecules. In the former compound the amino group is three carbons removed from the thiazole ring and is attached to the benzene ring; in the latter, the amino group is two carbons removed. The m-aminobenzyl derivative on the other hand, in which the amino group is four carbons removed from the thiazole ring has proved to be a potent activator for thiaminase[1]. In an effort to further clarify the relationship of structure and inhibition, and in particular to elucidate the importance of the spatial relationship between the thiazole

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[1] Sealock, R. R. and Livermore, A. H., unpublished.

ring and the free amino group, 3-γ-aminopropyl- and 3-δ-aminobutyl-4-methylthiazolium derivatives were prepared and tested for inhibition. With respect to the number of carbons separating the amino group from the thiazole ring, the former is structurally similar to the o-aminobenzylthiazolium compound and the latter compares with the m-aminobenzyl derivative.

In addition, o-aminobenzylpyridinium chloride and benzylpyridinium chloride were studied to determine whether or not the presence of the thiazole ring was necessary in an active inhibitor. The spatial relationship of the amino group and the quaternary nitrogen of o-aminobenzyl pyridinium chloride is like that of the o-aminobenzylthiazolium compound. It was felt that perhaps the presence of the amino group and the quaternary nitrogen three carbons removed from each other might be sufficient to produce an inhibitor molecule.

The two thiazolium derivatives were prepared by the method of Clarke (57) in the following manner.

γ-Bromopropylphthalimide: 75 gm.of potassium phthalimide (0.41 moles) and 246 gm. of trimethylene bromide (1.22 moles) were placed in a one liter two-necked flask fitted with a motor stirrer and a reflux condenser. The mixture was heated on an oil bath at 180-190° for 12 hours. The condenser was placed for distillation and the excess trimethylene bromide distilled under reduced pressure. When the material in the flask became too concentrated to distill without bumping, it was filtered with suction.

The crude product was separated from potassium bromide by refluxing with 150 ml. of absolute alcohol for one-half hour. The hot solution was filtered with suction, the salt residue washed with hot absolute alcohol, and the alcohol removed by distillation in vacuo. The residue was refluxed with 255 ml. of carbon disulfide for 15 minutes and the hot solution then filtered. The filter paper was colored but there was no apparent residue of a di-phthalimide derivative. The carbon disulfide was removed from the product by distillation under reduced pressure. The γ-bromopropylphthalimide was recrystallized from 150 ml. of 95 per cent alcohol, using Norite to decolorize. The white crystalline material melted at 71-72.5°. (Beilstein gives 72-73°). The yield was 42.4 gm. or 39.4 per cent of the theoretical.

An attempt to determine the per cent nitrogen in these compounds by the Kjeldahl method was unsuccessful. However, the method of synthesis employed for the preparation of the analogue molecules was such that there could be little doubt as to their structure.

3-γ-Phthalimidopropyl-4-methylthiazolium bromide: 1.989 gm. of 4-methylthiazole was mixed with 5.363 gm. of γ-bromopropylphthalimide (0.02 moles of each) and sealed in a Pyrex tube. The tube was allowed to stand for one day in an oven at 60° and for 3 days at 100°. In a second preparation the same amounts were used but the mixture was allowed to stand unsealed at room temperature for 3 days, and then for 4 hours in an oven

at 130°.

In both preparations the resulting solid material was washed five times with 10 ml. portions of ether. In the first, the residue was then dissolved in 12 ml. of hot water, giving a very dark solution. The water was removed in a desiccator and the residue dissolved in 30 ml. of hot absolute alcohol, from which the compound crystallized upon cooling. In the second preparation the residue after ether washing was dissolved immediately in hot absolute alcohol, filtered and allowed to cool. Both products were recrystallized from hot absolute alcohol. The product from the first preparation decomposed at 184-185°, and from the second, at 179-182°. The yield in the first case was 3.8 gm. or 51.7 per cent of the theoretical, and in the second, 3.5 gm. or 47.6 per cent of the theoretical.

3-γ-aminopropyl-4-methylthiazolium bromide hydrobromide: 2.5 gm. of 3-γ-phthalimidopropyl-4-methylthiazolium bromide was mixed with 11 ml. of 48 per cent hydrobromic acid and refluxed for 10 hours. At the end of one hour crystals of phthalic acid began to appear on the surface of the solution. After refluxing, the crystals were filtered and washed well with water. The filtrate and washings were concentrated to a dark syrup, from which crystals appeared upon cooling. These were washed several times with absolute alcohol and allowed to dry. The material was dissolved in a small volume of water, and after partial concentration of the solution, absolute alcohol was added. Crystallization was thus induced and repetition of

the concentration and alcohol addition yielded additional crystals. The recrystallized compound melted at 214-215° with darkening and apparent decomposition. The yield was 1.7 gm. or 79 per cent of the theoretical.

δ-Bromobutylphthalimide: 14.3 gm. of potassium phthalimide (0.08 moles) and 50 gm. of 1,4-dibromobutane (0.23 moles) were mixed in a 500 ml. two-necked flask fitted with a stirrer and reflux condenser. The mixture was heated on an oil bath at approximately 170° for 14 hours. The condenser was placed for distillation and the excess 1,4-dibromobutane distilled in vacuo. The residue was refluxed with 50 ml. of absolute alcohol for one-half hour, the hot solution filtered with suction, and the residue of potassium bromide washed with hot alcohol. The filtrate and washings were then concentrated in vacuo.

The residue was refluxed with 60 ml. of carbon disulfide for 20 minutes and filtered while hot. After removing the carbon disulfide by distillation under reduced pressure, the residue was dissolved in 100 ml. of hot 95 per cent alcohol and treated with Norite. The filtered solution was allowed to stand overnight in the cold, whereupon crystals were obtained. The yield was 16.3 gm. or 46.6 per cent of the theoretical. The recrystallized compound melted at 79-80°. (Beilstein gives 80.5°.)

3- δ -Phthalimidobutyl-4-methylthiazolium bromide: 1.28 gm. of δ -bromobutylphthalimide was mixed with 0.472 gm. of 4-methylthiazole (0.0048 moles of each), stoppered and placed

in an oven at 110° for 2 days. The solid product was washed with five 10 ml. portions of anhydrous ether and dissolved in 30 ml. of hot absolute alcohol. Upon cooling the solution, crystals appeared. The yield was 1.1 gm. or 62.9 per cent of the theoretical. The compound melted at 116-117°.

3- δ -Aminobutyl-4-methylthiazolium bromide hydrobromide: 1.1 gm. of 3- δ -phthalimidobutyl-4-methylthiazolium bromide was hydrolyzed with 10 ml. of 48 per cent hydrobromic acid for 15 hours. The material was washed into a distilling flask and concentrated. The residue was washed with absolute alcohol and a few drops of water into a test-tube. After cooling crystals appeared, and these were filtered and washed with absolute alcohol and ether. The yield was 0.794 gm. or 82.6 per cent of the theoretical. The compound melted with decomposition at 180-185°.

o-Aminobenzylpyridinium chloride was prepared by the reduction of o-nitrobenzylpyridinium chloride. 3.5 gm. of the nitrobenzylpyridinium compound (0.014 moles) was dissolved in a mixture of 140 ml. of water and 24 ml. of concentrated hydrochloric acid. To this mixture 6.7 gm. of granular tin and 9.5 gm. of stannous chloride ($SnCl_2 . 4\ H_2O$) were added. The mixture was heated just to boiling and then placed in a water bath at 50-60° for 19 hours. The solution was filtered from unreacted tin, and the filtrate, cooled to 5°, was treated with hydrogen sulfide, until free of tin. This required

sulfide treatment for approximately 35 minutes. The mixture was filtered with suction and the residue washed with water. The filtrate and washings were concentrated in vacuo to approximately 3 ml., when crystals appeared on the sides of the flask. Absolute alcohol did not dissolve these, but the addition of a few drops of water caused the residue to go into solution readily. Absolute alcohol was added to a volume of 25 ml. Although no crystals appeared upon standing overnight in the ice-box, further concentration and addition of more absolute alcohol induced crystallization. The total yield was 2.39 gm. or 77.6 per cent of the theoretical. The compound began to darken at 160°, and complete melting with some decomposition was observed at 174-175°.

F. Inhibition by Quaternary Salts

The initial experiment with the 3-γ-aminopropyl-4-methylthiazolium analogue indicated that it was an active inhibitor. Table XXIV shows the results of a second experiment in which a wide range of inhibitor concentrations was employed. Figure 5 shows that the S-shaped curve typical of competitive inhibition was obtained when the per cent inhibition was plotted against the logarithm of the inhibitor concentration.

A comparison was made of the inhibition produced by several aminothiazolium derivatives, in which the chief point of difference was the spatial relationship between the amino group and the quaternary nitrogen of the thiazole ring. The results, shown in Table XXV, indicate that at a concentration of 2×10^{-4} M. the o-aminobenzyl-4-methylthiazolium derivative was the most active inhibitor, while less inhibition was produced by 3-γ-aminopropyl-4-methylthiazolium bromide, and even less with the 3-β-aminoethyl-4-methylthiazolium compound. Such a relationship might be expected on the basis of a comparison of the structure of these compounds with that of thiamine, in which the amino group is three carbons removed from the thiazole nitrogen. In both the aminobenzyl and the aminopropyl compounds these two functional groups are also separated by three carbons, while the benzyl compound, because of the presence of a second ring, possesses an additional point of similarity to thiamine.

The aminoethyl compound on the other hand has only two carbons separating the amino group from the thiazole ring. Since inhibitors of this type act by competing with the substrate for the enzyme, it would be expected that the highest degree of inhibition would be produced by the compound structurally most similar to the substrate. However, this order of inhibition, exhibited by the three aminothiazolium compounds, was not observed at all concentrations of the inhibitor molecules.

Table XXIV

Inhibition by
3-γ-Aminopropyl-4-methylthiazolium bromide

(Preparation I-142-II-A, 150 mg. equiv.)

Inhibitor Concentration	Thiamine Destruction	Inhibition
moles/l. x 10^4	per cent	per cent
0	74.2	
0.05	70.2	5.4
0.20	58.1	21.8
0.50	44.2	40.5
2.00	23.0	69.0
4.0	17.8	76.1
5.00	15.5	79.4
10.00	12.7	83.0
20.00	9.6	87.2
40.00	6.7	91.9

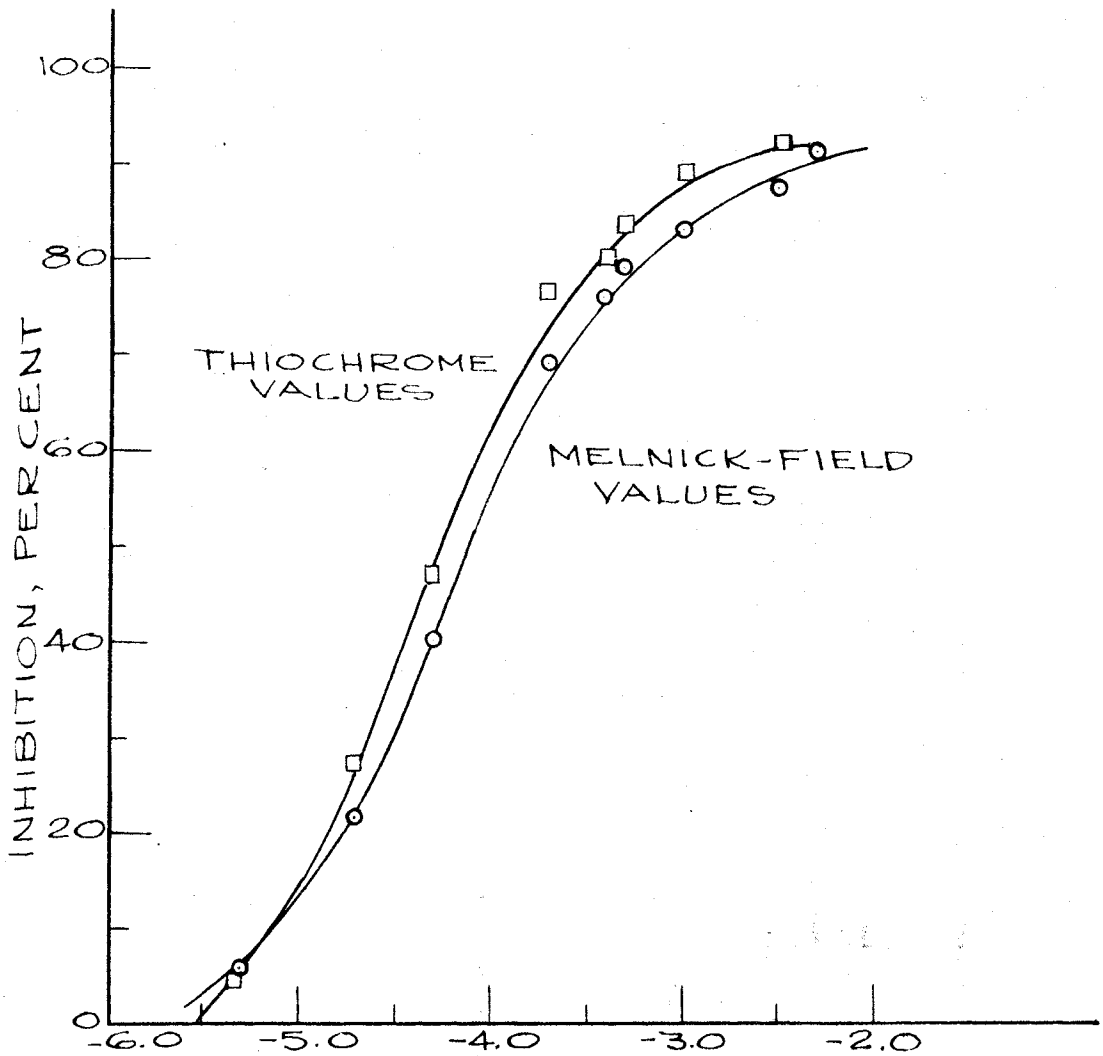

FIG. 5 INHIBITION BY 3-r-AMINOPROPYL-4-METHYLTHIAZOLIUM BROMIDE

Reasoning from the activating effect observed with the m-aminobenzyl-4-methylthiazolium compound, it might have been predicted that 3-β-aminobutyl-4-methylthiazolium bromide would likewise be an activator, since in both of these compounds the amino group is four carbons removed from the thiazole ring. On the contrary, when the enzyme preparation used for the analysis of the inhibitor analogues was employed, the aminobutyl compound had no significant effect upon thiaminase activity in concentrations of from 0.5 to 2.0 x 10^{-4} M. However, as is also shown in the third experiment of Table XXV, an inhibition of 11.8 per cent was produced by this compound in a concentration of 10 x 10^{-4} M. With a different enzyme preparation and with fairly high concentrations of the analogue a trend towards activation was observed, as evidenced by the results shown in the last two experiments of Table XXV.

In Table XXVI the results of several experiments, in which the effect of o-aminobenzylpyridinium chloride on the enzyme was analyzed, are given. This compound can be compared with the aminobenzylthiazolium derivative, with the chief point of difference being the substitution of a pyridine ring for the thiazole ring. With an enzyme extract prepared from I-142-II-A the effect of the pyridinium derivative was insignificant, while with this preparation the benzyl derivative had been strong inhibitor. However, o-aminobenzylpyridinium chloride proved to be an activator for both preparations

Table XXV

Inhibition by 4-Methylthiazolium Derivatives

Enzyme Preparation	Inhibitor Concentration	Thiamine Destruction	Inhibition
	moles/l. x 10^4	per cent	per cent
I-142-II-A	0	74.8	
150 mg. equiv.	γ-aminopropyl-		
	1.0	30.4	59.4
	2.0	20.0	73.3
	β-aminoethyl-		
	1.0	29.0	61.3
	2.0	23.8	68.2
	o-aminobenzyl-		
	1.0	18.0	76.0
	2.0	13.0	82.7
I-142-II-A	0	62.8	
150 mg. equiv.	γ-aminopropyl-		
(8.4 mg N/150 mg.	0.5	38.2	39.2
equiv.)	1.0	27.8	55.7
	2.0	17.4	72.3
	β-aminoethyl-		
	0.5	29.0	53.8
	1.0	23.2	63.0
	2.0	19.8	68.5
	o-aminobenzyl-		
	0.5	21.0	66.6
	1.0	12.8	79.4
	2.0	10.6	83.0
I-142-II-A	0	64.1	
150 mg. equiv.	δ-aminobutyl-		
(8.3 mg. N/150 mg.	0.5	65.6	- 2.3
equiv.)	1.0	63.6	0.8
	2.0	63.5	0.9
	10.0	56.5	11.8
I-142-III-A	0	37.8	
33 mg. equiv.	δ-aminobutyl-		
	9.0	39.7	- 5.0
	18.0	41.3	- 9.3
I-142-III-A	0	35.6	
33 mg. equiv.	δ-aminobutyl-		
	2.3	35.8	- 0.6
	4.6	37.1	- 4.2
	9.0	36.8	- 3.4

Table XXVI

Activation by
o-Aminobenzylpyridinium Chloride

Enzyme Preparation	Activator Concentration moles/l. x 10^4	Thiamine Destruction per cent	Activation per cent
I-142-II-A	0	64.1	
150 mg. equiv.	0.5	63.7	- 0.6
	1.0	63.4	- 1.1
	2.0	64.1	0
	10.0	64.1	0
I-142-III-A	0	40.0	
33 mg. equiv.	2.0	63.2	58.0
	4.0	71.6	79.0
	5.0	73.1	82.9
I-142-III-A	0	75.6	
50 mg. equiv.	2.0	84.4	11.6
	4.0	86.6	14.6
	5.0	87.2	15.3
II-196			
20 mg. equiv.	0	23.5	
	2.0	37.8	60.8
	4.0	46.5	97.9
	5.0	49.9	112.0
	10.0	54.9	134.0
II-196,			
43 mg. equiv.	0	83.3	
	2.0	89.1	7.0
	4.0	91.5	9.9
	5.0	91.5	9.9
	10.0	89.7	7.7

I-142-III-A and II-196, with the activating effect more pronounced at lower enzyme concentrations. Activation was calculated by means of the following equation:

$$\text{Activation, per cent} = \frac{\text{\% Dest.(with activator)} - \text{\% Dest. (without activator)}}{\text{\% Dest. (without activator)}} \times 100$$

Later a comparison of the effects produced by o-aminobenzyl- and benzylpyridinium chlorides was made. The results, shown in Table XXVII indicated that, although some activation was produced with benzylpyridinium chloride, the presence of the amino group, as in o-aminobenzylpyridinium chloride, produced a much more potent activator. The importance of the amino group in activating molecules as well as in inhibitor compounds was thus emphasized.

3-γ-Aminopropyl-4-methylthiazolium bromide has been used to the largest extent in the inhibition studies reported here, and the previous tables have shown that with certain of the enzyme preparations it is a good inhibitor. However, as is shown in Table XXVIII, this compound produced activation with two concentrations of preparation II-196, while only with the highest enzyme concentration was inhibition observed. The reason for the variation in the action of this compound with different enzyme preparations and concentrations was not apparent.

Table XXVII

Activation by Benzylpyridinium
and o-Aminobenzylpyridinium Chlorides

(Preparation II-236, 30 mg. equiv.)

Pyridinium Concentration	Thiamine Destruction	Activation
moles/l. x 10^4	per cent	per cent
0	40.0	
o-aminobenzyl-		
2.0	60.0	50.0
4.0	79.7	99.3
5.0	84.6	112.0
10.0	88.8	122.0
benzyl-		
2.0	44.6	11.5
4.0	42.7	6.8
5.0	42.1	5.3
10.0	40.9	2.3

Table XXVIII

Inhibition of Preparation II-196
by 3-γ-Aminopropyl-4-methylthiazolium Bromide

Amount of Enzyme	Inhibitor Concentration	Thiamine Destruction	Inhibition
mg. equiv.	moles/l. x 10^4	per cent	per cent
20	0	31.3	
	1.0	35.4	- 13.1
	2.0	33.1	- 5.8
	3.0	34.8	- 11.2
	4.0	32.5	- 3.8
	5.0	31.3	0
40[1]	0	31.0	
	4.0	48.0	- 54.8
48	0	60.6	
	1.0	40.9	32.6
	2.0	35.0	42.3
	3.0	33.2	45.3
	5.0	28.2	53.5

[1] In this experiment an incubation time of one-half hour was employed instead of the usual two hour period.

G. Inhibition in the Presence of Boiled Extract

Since it had been previously shown that the presence of the manganous ion, which activates thiaminase, produced increased inhibition, when added together with one of the inhibitory analogues[1], similar studies of the effect of boiled extract on inhibition were undertaken. Extracts of acetone desiccated powders were made in the usual manner. After adjusting to pH 4.0 to 5.5, the extract was placed in a boiling water bath for ten to fifteen minutes, during which time it was mechanically stirred. The precipitated protein was removed by centrifugation, and the clear supernatant decanted and adjusted to the desired volume. The boiled extract thus obtained contained a factor or factors which activated enzyme extracts, and which was particularly effective in activating dialyzed extracts. The effect of a boiled extract representing 37.5 mg. of preparation I-142-I-A on different amounts of an enzyme extract of I-142-III-A is shown in Table XXIX. The results show that the boiled extract is a potent activator particularly for dilute enzyme preparations.

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[1] Seelock, R. R. and Livermore, A. H., unpublished.

Table XXIX

Activation by Boiled Extract

Amount of Enzyme	Thiamine Destruction without boiled extract	Thiamine Destruction with boiled extract	Activation
mg. equiv.	per cent	per cent	per cent
10	1.6	8.7	444.0
25	17.4	31.2	79.3
40	51.2	64.1	25.2
50	67.5	77.8	15.3

Table XXX shows again that the activating effect of a boiled extract is more pronounced with dilute enzyme preparations. This could be accounted for, partially at least, by the fact that the presence of the boiled extract helps to overcome the decrease in activity caused by dilution of the system as the enzyme concentration is reduced. However, increasing amounts of boiled extract do not produce a proportional increase in activity. A boiled extract representing 16.8 mg. of preparation I-142-I-A produced 3.1 and 27.3 per cent activation of two different amounts of enzyme, while four times that amount of boiled extract produced only 8.7 and 68.2 per cent activation.

After it had been established that boiled extracts prepared as described above were capable of activating enzyme preparations, a number of experiments were conducted in which the effect of boiled extract on the inhibition produced by

Table XXX

Activation by Varying
Amounts of Boiled Extract

Amount of Boiled Extract	Enzyme Preparation I-142-III-A, 25 mg. equiv.		Enzyme Preparation I-142-III-A, 50 mg. equiv.	
	Thiamine Destruction	Activation	Thiamine Destruction	Activation
mg. equiv.	per cent	per cent	per cent	per cent
0	26.4		78.2	
18.8	33.5	26.9	80.9	3.5
37.5	38.3	45.2	83.8	7.2
56.3	41.6	57.8	83.8	7.2
75.0	44.5	68.7	85.1	8.8

3-γ-aminopropyl-4-methylthiazolium bromide was studied. Table XXXI gives the results of several such experiments in which different amounts of enzyme were employed and in which a boiled extract representing a 75 mg. equivalent of preparation I-142-I-A was used. The per cent inhibition obtained in the absence of boiled extract was calculated in the usual way, and is shown in column 3, headed Inhib. (1). The inhibition obtained in the presence of the boiled extract was calculated in two ways. For column 5, headed Inhib. (2), the per cent inhibition was calculated on the basis of the amount of destruction observed with the uninhibited enzyme in the absence of the boiled extract, while for column 6, headed Inhib. (3), the inhibition was calculated on the basis of the thiamine destruction observed with the uninhibited enzyme in the presence of the boiled extract.

With the dilute enzyme preparation used for experiment 1 of Table XXXI, there was no significant inhibition in the absence of the boiled extract. In the presence of both the boiled extract and the inhibitor, the amount of destruction was greater than that observed with the uninhibited enzyme in the absence of boiled extract. However, the per cent inhibition shown in column 5 is significantly greater than that of column 3.

For the other experiments in which higher enzyme concentrations were employed inhibition was produced both in the

Table XXXI

Effect of Boiled Extract on Inhibition
by 3-γ-Aminopropyl-4-methylthiazolium Bromide

Thiazole Concentration	No Boiled Extract Dest.	Inhib.(1)	Boiled Extract Present Dest.	Inhib.(2)	Inhib.(3)
moles/l.x10^4	per cent	per cent	per cent	per cent	per cent
Preparation I-142-III-A, 25 mg. equiv.					
0	22.5		39.3		
1.0	23.1	- 2.7	32.6	- 44.8	17.2
2.5	21.2	5.8	28.2	- 27.2	28.3
5.0	22.5	0	26.3	- 16.9	33.1
Preparation I-142-III-A, 33 mg. equiv.					
0	39.3		58.8		
1.0	31.5	19.9	38.5	2.0	34.5
2.5	25.0	36.4	24.5	37.7	58.3
5.0	21.9	44.3	17.5	55.5	70.2
Preparation I-142-III-A, 33 mg. equiv.					
0	42.4		60.6		
1.0	31.6	25.5	43.2	- 1.9	28.7
2.0	22.5	47.1	32.7	22.9	46.0
3.0	26.7	37.2	30.2	28.8	50.2
4.0	21.8	48.7	24.8	41.6	59.1
5.0	25.5	39.9	24.2	43.0	60.1
Preparation I-142-III-A, 50 mg. equiv.					
0	70.3		78.8		
1.0	52.4	25.5	62.8	10.7	20.3
2.5	61.3	12.8	51.7	26.5	34.6
5.0	39.3	44.2	36.5	48.2	53.8
Preparation I-142-III-A, 50 mg. equiv.					
0	71.0		76.8		
1.0	45.1	36.5	57.1	19.6	25.7
2.5	46.1	35.1	42.6	40.1	44.6
5.0	35.2	50.5	28.4	60.0	63.0

presence and absence of the boiled extract. In general increased inhibition was observed in the presence of the boiled extract, as evidenced by a comparison of the inhibition values of column 5 with those of column 3. Furthermore a comparison of the values of column 6 with those of column 3 shows an even greater number of examples of increased inhibition with the addition of the boiled extract. Thus it appears that manganese and boiled extract, both of which activate the enzyme, cause an increased inhibition when added together with an inhibitor molecule.

As is shown in Table XXVIII the addition of 3-γ-amino-propyl-4-methylthiazolium bromide to certain concentrations of preparation II-196 activated rather than inhibited the enzyme. The effect of boiled extract on the action of this compound with this particular enzyme preparation was also studied and the results are shown in Table XXXII. With the lower enzyme concentration, the thiamine analogue produced slight activation in the absence of the boiled extract, but when the compound was added together with the boiled extract a good degree of inhibition was obtained as evidenced by inhibitions of from 11.3 to 31.5 per cent. With the higher enzyme concentration inhibition was observed both with and without added boiled extract. However, in contrast to the results obtained with preparation I-142-III-A (Table XXXI) inhibition was not increased in the presence of the boiled extract. With II-196 then an increased inhibition resulting

from the addition of boiled extract appears to depend upon the amount of enzyme present.

Table XXXII

Effect of Boiled Extract on Inhibition of Preparation II-196 by 3-τ-Aminopropyl-4-methylthiazolium Bromide

Thiazole Concentration	No Boiled Extract Dest.	Inhib.(1)	Boiled Extract Present Dest.	Inhib.(2)	Inhib.(3)
moles/l.x10^4	per cent	per cent	per cent	per cent	per cent
Preparation II-196, 20 mg. equiv. Boiled Extract, I-142-I-A, 75 mg. equiv.					
0	31.3		44.1		
1.0	35.4	- 13.1	39.1	- 24.9	11.3
2.0	33.1	- 5.8			
3.0	34.8	- 11.2	33.1	- 5.8	24.9
4.0	32.5	- 3.8	31.3	- 0	29.0
5.0	31.3	0	30.2	3.5	31.5
Preparation II-196, 48 mg. equiv. Boiled Extract, II-196, 60 mg. equiv.					
0	60.6		69.6		
1.0	40.9	32.5	50.4	16.8	27.6
2.0	35.0	42.3	41.5	31.5	40.4
3.0	33.2	45.3	38.5	36.4	44.7
5.0	28.2	53.5	33.2	45.2	52.4

The effect of boiled extract on the action of 3-δ-aminobutyl-4-methylthiazolium bromide and of o-aminobenzylpyridinium chloride was also studied. Table XXV shows that the butylthiazole derivative has very little effect on the enzyme, exhibiting only a slight degree of activation. On the other hand the benzylpyridinium compound proved to be a potent activator for the enzyme (Table XXVI). The data of Table XXXIII show

that the addition of boiled extract does not significantly alter the effect of 3-δ-aminobutyl-4-methylthiazolium bromide, if at all. In the first experiment shown in this table, with fairly high concentrations of the analogue slight activation was observed in the absence of boiled extract and slight inhibition in its presence. In both experiments the enzyme extract represented 33 mg. of preparation I-142-III-A, and the boiled extract represented 75 mg. of I-142-I-A.

Table XXXIII

Effect of Boiled Extract on Action of 3-δ-Aminobutyl-4-methylthiazolium Bromide

Thiazole Concentration	No Boiled Extract		Boiled Extract Present	
	Dest.	Inhib.	Dest.	Inhib.
moles/l.x10^4	per cent	per cent	per cent	per cent
0	37.8		56.8	
9.0	39.7	- 5.0	53.5	5.8
18.0	41.3	- 9.3	53.8	5.3
0	35.6		55.0	
2.3	35.8	- 0.6	57.2	- 4.0
4.6	37.1	- 4.2	57.4	- 4.3
9.0	36.8	- 3.4	55.4	- 0.7

The effect of boiled extract on the activation of thiaminase by o-aminobenzylpyridinium chloride is shown by the data of Table XXXIV. The use of a high enzyme concentration producing a high degree of thiamine destruction prevents critical analysis of activation, as can be seen from experiment 1 of Table XXXIV. In experiment 2, in which a lower enzyme concentration was employed, the addition of boiled extract (in the absence of the pyridinium compound) increased thiamine destruction from 23.5 to 34.6 per cent, an activation of 47.3 per cent. Addition of the analogue alone to the enzyme extract increased the amount of thiamine destruction, and at the highest concentration of the pyridinium compound an activation of 134 per cent was observed.

However, only in the case of the lowest concentration of the analogue, did the addition of boiled extract produce increased destruction, over and above that obtained in the presence of the compound alone. At the higher concentrations of the pyridinium derivative, the increased destruction could be accounted for by the presence of the analogue itself. In other words the two activators in combination did not produce a cumulative effect.

Table XXXIV

Effect of Boiled Extract
on Activation by o-Aminobenzylpyridinium Chloride

Pyridinium Concentration	No Boiled Extract Dest.	Act.	Boiled Extract Present Dest.	Act.
	per cent	per cent	per cent	per cent
Enzyme extract, II-196, 43 mg. equiv. Boiled extract, II-196, 60 mg. equiv.				
0	83.3		83.0	
2.0	89.1	7.0	88.2	6.4
4.0	91.5	9.9	90.3	8.8
5.0	89.7	9.9	90.6	9.2
10.0	89.7	7.7	91.8	10.6
Enzyme extract, II-196, 20 mg. equiv. Boiled extract, II-196, 50 mg. equiv.				
0	23.5		34.6	
2.0	37.8	60.8	42.8	23.8
4.0	46.5	97.8	46.6	34.8
5.0	49.9	112.0	49.2	42.3
10.0	54.9	134.0	54.3	57.1

H. Varied Substrate Concentration

Theoretically the amount of an enzymatic reaction should be independent of the substrate concentration, if the substrate is present in sufficient excess. As long as there is adequate substrate to keep the enzyme saturated, or in other words to maintain it all in the form of the enzyme-substrate complex, additional unbound substrate should not influence the total amount of reaction. This of course depends upon the dissociation constant for the enzyme-substrate complex, and if this is large, excess substrate by virtue of the mass action effect could maintain more of the enzyme in its active complex form. Furthermore, such a zero order type of reaction will ordinarily be observed only during a fairly short initial period of reaction.

For velocity measurements, which are necessary in the determination of dissociation constants for enzyme-substrate and enzyme-inhibitor complexes, at least five different substrate concentrations should be employed. Therefore, before kinetic studies of this type could be undertaken, it was necessary first to study the effect of varied substrate concentration on the enzyme. Table XXXV shows the results obtained when extracts representing 50 mg. equivalents of five acetone-desiccated powders were incubated with two different thiamine concentrations, 5 and 20 x 10^{-4} M. For purpose of comparison the results are given in terms of the total micromoles of the vitamin destroyed during the two hour period of incubation.

With the exception of powder I-142-III-A the expected relationship was observed. The total amount of reaction was greater at the higher substrate concentration with all of the other preparations. Since the last two powders were difficult to extract and since their activities were of a fairly low order, little use was made of them.

Table XXXV

Thiamine Destruction with Five Acetone-desiccated Powders

Enzyme Preparation	Thiamine Destroyed with Initial Concentration of 5×10^{-4} M.	20×10^{-4} M
	μ moles	μ moles
I-142-I-A	1.08	1.23
I-142-II-A	0.59	0.75
I-142-III-A	2.11	1.75
II-87-A	0.59	0.65
II-87-B	0.37	0.45

Powder I-142-III-A, which was the most active, was studied in more detail with a greater number of thiamine concentrations in an effort to find a range of concentrations in which the "apparent substrate inhibition" did not occur. In general a two hour incubation period was not employed, since velocity measurements within incubation times of one hour or less would be necessary to obtain values representative of the zero-order

type of reaction. Table XXXVI shows that the "apparent substrate inhibition" was exhibited at thiamine concentrations greater than 5×10^{-4} M. in a number of experiments in which several different amounts of enzyme were employed.

Table XXXVI

Varied Substrate Concentrations
Preparation I-142-III-A

Thiamine Conc.	Thiamine Destroyed with Enzyme Equivalent of					
	16.6 mg.	20 mg.	20 mg.	25 mg.	50 mg.	50 mg.[1]
moles/l.x10^4	µmoles	µmoles	µmoles	µmoles	µmoles	µmoles
1.0		0.34	0.38			0.99
2.0	0.27	0.33	0.37	0.49	0.98	1.85
4.0	0.26	0.29	0.31	0.43	1.34	1.96
5.0	0.24	0.30		0.43	1.22	1.66
10.0	0.53	0.18	0.24	0	1.09	1.45
20.0	0.15	0		0.15	1.05	

[1] Two hour period of incubation; all others one hour incubation.

Similar experiments in which a wide range of thiamine concentrations were used indicated that both preparations II-196 and I-142-I-A exhibited a decrease in total amount of thiamine destruction as substrate concentrations increased above 5×10^{-4} M. Preparation I-142-II-A was the only one to show increasing destruction with increasing thiamine concentration. The results obtained with these acetone-desiccated powders

are shown in Table XXXVII. It appeared then that for kinetic studies a series of thiamine concentrations of less than 5×10^{-4} moles per liter would have to be used.

Table XXXVII

Varied Substrate Concentration

Thiamine Concentration	Thiamine Destroyed in One Hour with		
	I-142-I-A 50 mg. equiv.	I-142-II-A 50 mg. equiv.	II-196 17 mg. equiv.
moles/l. x 10^4	μ moles	μ moles	μ moles
1.0	0.48	0.48	
2.0	0.63	0.69	0.73
4.0			0.72
5.0	0.61	0.78	0.75
10.0	0.53	0.81	0.60
15.0			0.62
20.0	0.45	1.05	0.64

In another experiment, in which the thiamine concentrations ranged from 2 to 20 x 10^{-4} moles per liter, boiled extract was added to determine whether or not a better uniformity of reaction could be obtained. The amount of thiamine destroyed by an enzyme preparation representing 25 mg. of I-142-III-A was determined with and without the addition of a boiled extract representing 38 mg. of powder I-142-I-A. The results, shown in Table XXXVIII, indicated that for both one and two hour incubation periods a better uniformity of reaction was obtained

in the presence of boiled extract than in its absence. The addition of boiled extract therefore appeared to give results which were in better agreement with those expected; that is, the amount of thiamine destroyed was approximately the same for all substrate concentrations. The presence of the boiled extract at least partially overcame the "apparent substrate inhibition".

Table XXXVIII

Effect of Boiled Extract
with Varied Substrate Concentration

Thiamine Concentration	Thiamine Destroyed with No Boiled Extract		Thiamine Destroyed with Boiled Extract Present	
	1 hr.	2 hr.	1 hr.	2 hr.
moles/l. x 10^4	μmoles	μmoles	μmoles	μmoles
2.0	0.63	0.80	0.67	0.87
4.0	0.50	0.65	0.65	0.84
5.0	0.53	0.69	0.69	0.88
10.0	1.6 (?)	0.84	0.65	0.82
20.0	0.73	0.93	0.67	0.79

For preliminary work, directed towards a kinetic study of inhibition similar to that described by Sealock and Goodland (37) for o-aminobenzyl-4-methylthiazole, the inhibition produced by a 2 x 10^{-4} M. concentration of 3-γ-aminopropyl-4-methylthiazolium bromide was determined with several substrate concentrations. Table XXXIX shows the results obtained in two such experiments, in which a 50 mg. equivalent of preparation

I-142-III-A was employed. A Kjeldahl analysis of the extract used in the first experiment showed 3.20 mg. of nitrogen per 50 mg. equivalent of extract. Boiled extract was present in all tubes, and as can be seen from the table, the "apparent substrate inhibition" was not observed. Efforts to calculate the dissociation constants for enzyme-substrate and enzyme-inhibitor complexes from the data of Table XXXIX were disappointing, but since only one value for each thiamine concentration was available for the determination of velocity constants, such a calculation would not be too significant.

Similar experiments were carried out with preparation II-196. In the first experiment shown in Table XL, the "apparent substrate inhibition" was observed, while in the second, the previously observed activation of this preparation by 3-γ-aminopropyl-4-methylthiazolium bromide was again obtained, making impossible the calculation of inhibition. However, in the second experiment, the amount of thiamine destruction was approximately the same with all except the highest substrate concentration.

Table XXXIX

Inhibition by 3-γ-Aminopropyl-4-methyl-thiazolium Bromide with Varied Substrate Concentration

Thiamine Concentration	Thiamine Destroyed in One-half Hour with		
	No Inhibitor	Inhibitor	Inhibition
moles/l. x 10^4	μ moles	μ moles	per cent
5.0	0.62	0.44	29.0
6.0	0.66	0.43	34.9
10.0	0.59	0.34	42.3
15.0	0.77	0.59	23.4
20.0	0.83	0.48	42.2
2.0	0.71	0.42	40.8
4.0	0.82	0.45	45.2
5.0	0.76	0.44	42.1
8.0	0.83	0.37	55.4
12.0	0.99	0.59	40.4

Table XL

Inhibition by 3-γ-Aminopropyl-4-methyl-thiazolium Bromide with Varied Substrate Concentration

Thiamine Concentration	Thiamine Destroyed in One-half Hour with No Inhibitor	Inhibitor	Inhibition
moles/l.x10^4	μmoles	μmoles	per cent
2.0	0.43	0.30	30.2
3.0	0.53	0.37	30.2
4.0	0.45	0.54	- 19.9
5.0	0.36	0.28	22.2
7.5	0.38	0.29	23.7
2.0	0.75	0.82	- 9.3
3.0	0.74	1.05	- 41.8
4.0	0.74	1.11	- 49.9
5.0	0.77	1.20	- 55.8
7.5	0.70	1.27	- 81.4

In the first experiment a boiled extract representing 60 mg. of II-196 was present in all tubes. The enzyme extract was equivalent to 20 mg. of II-196, and the inhibitor was added to give a concentration of 2×10^{-4} M.

In the second experiment the enzyme extract represented 40 mg. of II-196 (1.54 mg. N/40 mg. equiv.), and the inhibitor was added to give a concentration of 4×10^{-4} M. No boiled extract was present.

Although inhibition was not observed, the dissociation constant for the enzyme-substrate complex was determined from the values obtained in the absence of the aminopropyl compound for the second experiment shown in Table XL. From the micromoles of thiamine destroyed for each substrate concentration the velocity as moles per liter per minute was determined. Using the method of least squares the intercept and slope of the line, which would be obtained by plotting the reciprocal of the velocity against the reciprocal of the substrate concentration, were calculated. The slope as determined was 8.8 and the intercept had a value of 1.78×10^{5}. The reciprocal of the intercept, which represents the maximal velocity, was 5.6×10^{-6} moles per liter per minute, a value which compares favorably with that obtained by Sealock and Goodland (37), 2.04×10^{-6} moles per liter per minute. The dissociation constant for the enzyme-substrate complex as determined by the Lineweaver-Burke equation was 4.93×10^{-5} as compared with the previously published value of 8.31×10^{-5}. The agreement between these values was excellent, considering the fact that here only one value for each thiamine concentration was available, and that this value was obtained after one-half hour of incubation, a time which is on the border-line of the maximum limit of the zero-order reaction.

I. The Importance of Sulfhydryl Groups

In view of Agren's finding (40) that glutathione activated dialyzed preparations of the fish factor, work was initiated here to determine, if possible, the importance of the sulfhydryldisulfide relationship in both the protein part of the enzyme and the non-protein dialyzable component. Sealock and Goodland (37) have reported that cysteine, another sulfhydryl-containing compound, in a concentration of 1×10^{-4} M. produced 56.7 per cent inhibition of the thiamine-destroying enzyme.

When this work was started the p-aminobenzoic acid method was being used for thiamine determinations, but it soon became apparent that both cysteine and glutathione in concentrations of from 1 to 2×10^{-4} M. interfered with the determination. When the alcohol-phenol reagent was employed the presence of the sulfhydryl compounds caused decreased colorimetric readings for standard thiamine solutions, whereas, if a 50 per cent alcohol solution were used in its place, the readings were increased. However, these compounds could be present in concentrations as high as 10×10^{-4} M. without producing significant interference in the Melnick-Field method for thiamine determination.

When an analysis was made of the effect of glutathione on thiaminase, the results, shown in Table XLI, indicated that in general the compound activated the enzyme, although never in any significant degree. The activation was not as great as

that obtained by Agren, and it certainly was not the type of activation which would be expected, if gluthathione were a coenzyme for thiaminase.

Table XLI

Effect of Glutathione

Enzyme Preparation	Glutathione Concentration moles/l.x10^4	Thiamine Destruction per cent	Remarks
Tissue suspension, 200 mg. equiv.	0	82.9	
	2.0	83.0	No effect
Preparation I-68 100 mg. equiv.	0	76.0	
	1.6	86.5	13.8 % Activation
Tissue suspension, 75 mg. equiv.	0	14.0	
	4.0	22.0	57.1 % Activation
Tissue suspension, 132 mg. equiv.	0	10.0	
	4.0	7.0	30 % Inhibition
Preparation I-68, 100 mg. equiv.	0	70.0	
	4.0	74.0	5.7 % Activation
	8.0	76.0	8.6 % "
	20.0	78.0	11.4 % "
Preparation I-142-III-A, 40 mg. equiv. (1.35 mg. N/50 mg. equiv.)	0	52.8	
	5.0	50.4	4.5% Inhibition
	10.0	52.0	1.5 % Inhibition

Although the activation of the enzyme by glutathione was not great, it was important to determine whether the effect was specific for this compound or whether another sulfhydryl compound would produce similar effects. Therefore, an analysis was made of the effect of cysteine on similar enzyme preparations. Control tubes, in which cysteine was incubated with thiamine in the absence of the enzyme, were included and it was found that cysteine in concentrations of from 1 to 20 x 10^{-4} M. caused the disappearance of from 4 to 10 per cent of the added thiamine. However, these values can not be considered too critically, since the presence of protein appears to stabilize thiamine, and since probably the sulfhydryl compound would not in itself cause the destruction or disappearance of that amount of the vitamin in the presence of the enzyme extract. Table XLII shows that the effect of cysteine on thiaminase was even less significant than that of glutathione. Inhibition was observed in some cases, and activation in others.

It was felt that the effect of these compounds might be more pronounced with dialyzed enzyme preparations. However, glutathione in concentrations of 5 and 10 x 10^{-4} M. produced only 4.5 and 6.3 per cent activation respectively of a dialyzed extract, which alone destroyed 76.0 per cent of the added thiamine, while similar concentrations of cysteine produced 8.0 and 12.1 per cent inhibition. For another experiment an extract of preparation II-196 was dialyzed so that a 25 mg.

Table XLII

Effect of Cysteine

Enzyme Preparation	Cysteine Concentration moles/l.x10^4	Thiamine Destruction per cent	Remarks
Tissue suspension, 80 mg. equiv.	0	8.0	
	4.0	4.0	50 % Inhibition
Preparation I-68, 100 mg. equiv.	0	62.0	
	4.0	60.0	3.2 % Inhibition
	8.0	60.0	3.2 % Inhibition
	20.0	70.0	12.9 % Activation
Preparation I-142-III-A, 40 mg. equiv.	0	52.8	
	5.0	61.2	17.0 % Activation
	10.0	52.8	No effect
Preparation I-142-III-A, 40 mg. equiv.	0	50.8	
	0.1	50.8	No effect
	1.0	49.6	2.4 % Inhibition
	2.0	48.6	4.3 % "
	5.0	51.2	0.8 % Activation
Preparation II-196, 25 mg. equiv.	0	49.5	
	0.2	49.5	No effect
	0.4	50.0	No effect

equivalent destroyed only 13.5 per cent of the added thiamine as compared with a 50.1 per cent destruction for an equal amount of an undialyzed portion of the extract. Kjeldahl analysis showed that a 25 mg. equivalent of the undialyzed extract contained 1.03 mg. of nitrogen, while after 18 hours of dialysis a 25 mg. equivalent contained only 0.34 mg. of nitrogen. Activation of this dialyzed enzyme preparation was observed with 0.001 M. concentrations of cysteine or glutatione, and in addition activation was observed with a 0.001 M. concentration

of manganese. However, as is shown in Table XLIII, the range of thiamine destruction from 13.6 to 17.1 per cent for the various additions was not wide enough to be particularly significant.

Table XLIII

Effect of Sulfhydryl Compounds on a Dialyzed Enzyme Extract

Additions to Dialyzed Extract	Thiamine Destruction	Activation
	per cent	per cent
None	13.5	
Cysteine, 0.001 M.	14.8	9.6
Glutathione, 0.001 M.	15.2	12.6
Cysteine + Mn, 0.001 M.	16.8	24.4
Glutathione + Mn, 0.001 M.	13.6	0
Manganese, 0.001 M.	17.1	26.7

When the effect of cysteine was analyzed with a dialyzed extract which had been allowed to stand over-night in the ice-box, activations of 4.6, 11.0 and 25.4 per cent were obtained with 2.5, 5.0 and 10.0 x 10^{-4} M. concentrations of the compound. The extract had not lost activity during the storage period as evidenced by the fact that it destroyed 39.1 per cent of the added thiamine, while on the previous day an equal amount produced 35.4 per cent destruction. Therefore, the higher degree of activation observed in this experiment could probably

not be accounted for by the reduction by cysteine of disulfide linkages formed by oxidation of sulfhydryl groups during the period of storage.

Activation by cysteine and glutathione could be interpreted as an indication that sulfhydryl groups of the enzyme, essential for its action, were being maintained in the reduced state by these compounds. Further evidence for the importance of sulfhydryl groups in thiaminase was obtained, when it was found that phenylmercuric chloride, considered to be a highly specific inhibitor for enzymes dependent upon sulfhydryl groups, in concentrations of from 0.1 to 2 x 10^{-4} M. produced from 76.7 to 89.3 per cent inhibition of the enzyme. In another experiment a 0.1 x 10^{-4} M. concentration of phenylmercuric chloride reduced thiamine destruction from 100 per cent to 21.5 per cent. When cysteine in concentrations of 0.1, 1.0, 5.0 and 10.0 x 10^{-4} M. was added to the phenylmercuric chloride inhibited enzyme, thiamine destruction of 18.7, 12.8, 10.7, and 20.3 per cent respectively was observed. Although this inhibition was not reversed by cysteine, the very fact that such a high degree of inhibition was exhibited with phenylmercuric chloride strongly suggested that sulfhydryl groups were playing an important role in thiaminase action.

Following these first experiments, which indicated that thiaminase action was dependent upon sulfhydryl groups, efforts were made to determine whether these groups were essential components of the protein or of the non-protein

portion of the enzyme. Since iodine, which oxidizes sulfhydryl groups to the disulfide form, has been extensively used for the quantitative estimation of sulfhydryl-containing compounds, boiled extract and both dialyzed and undialyzed enzyme extracts were titrated with a standard iodine solution. Three ml. of 1 per cent potassium iodide was added to the aliquots of the extracts, at pH 7.4; five drops of a 1 per cent starch solution were added and the solutions kept in an ice-bath during the titration with 0.001 N. iodine. The appearance of a blue starch-iodine color, which persisted for 30 seconds, was considered to be the end-point.

A three ml. aliquot of a boiled extract, representing 50 mg. per ml. of preparation II-236, reduced 0.90 ml. of 0.001 N. iodine, indicating the possible presence of sulfhydryl compounds. Furthermore, since 1 ml. of 0.001 N. iodine was required for the titration of a 2 ml. aliquot of a dialyzed enzyme extract, while an equal amount of an undialyzed portion reduced 1.7 ml. of iodine, it appeared that there was a decrease in iodine reducing compounds, perhaps sulfhydryl-containing molecules, during dialysis. However, since iodine does not specifically oxidize sulfhydryl groups, these results do not give absolute evidence for the presence of sulfhydryl groups in the non-protein fractions of enzyme preparations.

In an effort to obtain more definite evidence for the presence of sulfhydryl molecules in the dialyzable component of the enzyme, portions of the boiled extract described above

were treated with varying amounts of phenylmercuric chloride and of cysteine, and the activating effect of these extracts on a dialyzed enzyme preparation compared with that of an equivalent amount of untreated boiled extract. Five ml. aliquots of the boiled extract were transferred to 10 ml. volumetric flasks, and phenylmercuric chloride and cysteine were added as shown in Table XLIV. After dilution to 10 ml., and adjustment to pH 7.4, 2 ml. aliquots were tested for their ability to activate a dialyzed enzyme extract.

The results, also given in Table XLIV, show that the addition of boiled extracts, which had been treated with phenylmercuric chloride, produced inhibition of the enzyme, while those treated with cysteine produced a slightly greater activating effect than the untreated portion of the boiled extract. However, the boiled extracts, to which both phenylmercuric chloride and cysteine had been added, did not produce an effect significantly different from that of the phenylmercuric chloride treated extracts, indicating that the addition of cysteine in amounts equivalent to the phenylmercuric chloride, did not reverse inhibition by this latter compound.

An experiment parallel to the above was conducted in which additions of phenylmercuric chloride and cysteine were made directly to the enzyme extract in amounts to give concentrations in the 5 ml. incubation volume equal to those which were present when the boiled extracts were added.

Table XLIV

Activation by Boiled Extracts
Treated with Phenylmercuric Chloride and Cysteine

Additions to Boiled Extract		Effect of Boiled Extract on Dialyzed Extract	
Phenylmercuric Chloride (0.00025 M.)	Cysteine (0.00025 M.)	Thiamine Destruction	Activation
ml.	ml.	per cent	per cent
0	0	40.7	24.9[1]
0.2	0	28.6	- 12.3
0.6	0	19.4	- 40.4
1.0	0	13.8	- 57.6
0	0.2	41.8	28.2
0	0.6	41.3	26.7
0	1.0	42.1	29.2
0.2	0.2	29.9	- 8.3
0.6	0.6	18.8	- 42.3
1.0	1.0	16.3	- 50.0

[1] Dialyzed enzyme extract alone destroyed 32.6 per cent of the added thiamine.

The phenylmercuric chloride additions resulted in inhibition of from 33.7 to 64.8 per cent, while cysteine addition also produced a slight degree of inhibition, ranging from 0.0 to 14.7 per cent with the three decreasing concentrations of the compound. Again no significant reversal of phenylmercuric chloride inhibition was observed when cysteine was added in combination with this compound, as evidenced by the inhibition of from 33.7 to 53.4 per cent exhibited in the presence of both compounds.

A second experiment, similar to that described on page 160 in Table XLIV, was conducted and the results were comparable. The untreated boiled extract increased the thiamine destruction of a dialyzed enzyme extract from 12.2 to 26.4 per cent, an activation of 116.0 per cent. The phenylmercuric chloride treated boiled extracts produced inhibition, ranging from 9.8 to 73.8 per cent with increasing concentrations of the compound. Again cysteine failed to reverse the inhibition, and in fact, the boiled extracts treated only with cysteine produced activations of the dialyzed extract of only 97.5, 104.0, and 107.0 per cent as compared with 116.0 per cent activation observed upon addition of the untreated extract.

Although these experiments would not allow any conclusions to be made regarding whether phenylmercuric chloride was reacting with sulfhydryl groups of the protein or of the non-protein portion of the enzyme, they served to further emphasize the importance of sulfhydryl groups in thiaminase.

Next, the activating effect of boiled extracts treated with iodine and with cysteine was determined. It would be expected that if sulfhydryl groups were essential for the activation by boiled extract, iodine by virtue of its oxidizing action on these groups, would reduce or destroy the activating ability of such extracts. Iodine was added to aliquots of a boiled extract in amounts equal to 0.2, 0.4, 0.6, 0.8, and 1.0 times the amount required for titration of an equivalent amount of the extract. Unfortunately the iodine-treated extracts produced activation of the dialyzed enzyme, which was not significantly less than that of the untreated extract. The results of this experiment, given in Table XLV, show that the extracts treated with both iodine and cysteine produced an effect not much different from that of the extracts treated only with iodine. Therefore, since no real inhibition was produced by addition of iodine in these concentrations, no conclusions regarding cysteine reversal of iodine inhibition could be made.

A second similar experiment, the results of which are also shown in Table XLV, served to confirm the above results. The addition of an untreated boiled extract to a dialyzed enzyme preparation increased the destruction of thiamine from 12.8 to 20.0 per cent, an activation of 56.3 per cent. The destruction observed upon addition of the iodine treated extracts and those treated with both iodine and cysteine ranged from 16.9 to 20.0 per cent. The activating ability of the boiled extracts was therefore not destroyed by the addition

of iodine.

Table XLV

Activation of Dialyzed Extract
by Boiled Extract Treated with Iodine and Cysteine

Additions to Boiled Extract	Thiamine Destruction in Presence of Boiled Extract Treated with		Activation by Boiled Extracts Treated with	
Iodine or Cysteine equivalents[1]	Iodine per cent	Iodine and Cysteine per cent	Iodine per cent	Iodine and Cysteine per cent
0	34.3		53.1	
0.2	34.3	33.7	53.1	50.4
0.4	34.3	34.3	53.1	53.1
0.6	33.3	34.3	48.7	53.1
0.8	31.5	32.8	40.6	46.4
1.0	32.8	33.3	46.4	48.7
0	20.0		56.3	
0.2	20.0	20.0	56.3	56.3
0.6	17.6	18.7	36.7	46.1
1.0	16.9	18.7	32.1	46.1

[1] The iodine equivalent was determined by titration of an aliquot of the boiled extract. Cysteine was added in amounts equivalent to the iodine.

However, when boiled extracts were treated with larger amounts of iodine, the activating ability of the extract was significantly reduced. Table XLVI shows the results of an experiment in which the activating effect of a boiled extract treated with 1 to 5 times the amount of iodine, which was required for titration of an equivalent aliquot of the extract, was determined. At the same time other portions of the extract were treated with varying amounts of phenylmercuric chloride.

The addition of iodine served to reduce the activation produced by the boiled extracts below that exhibited by an untreated portion, but except for the extract containing the highest concentration of iodine, the thiamine destruction was greater than that obtained with the dialyzed extract alone. In contrast, the phenylmercuric chloride-treated extracts, except for that containing the lowest concentration of the compound, actually inhibited the dialyzed enzyme.

In general the results obtained in this series of experiments indicate that thiaminase is a sulfhydryl-dependent enzyme. However, it appears that the activating ability of boiled extract is not dependent entirely at least, upon sulfhydryl compounds. If under the conditions employed for iodine titration, the sulfhydryl compounds are oxidized; and if the activating effect of boiled extract depends upon the presence of such groups; then the addition of one equivalent of iodine to a boiled extract would be expected to destroy or significantly reduce its activating ability. In contrast, the addition of as much as four equivalents of iodine did not completely destroy the activating ability of such an extract. The reduction of activity produced by amounts of iodine greater than one equivalent may be due to the action of iodine upon other oxidizable compounds of the boiled extract, or may be due to the iodine oxidation of sulfhydryl groups of the protein portion of the enzyme upon addition of the iodine-treated extract to dialyzed enzyme preparations.

Table XLVI

Activation by Boiled Extracts
Treated with Phenylmercuric Chloride and with Iodine

Undialyzed enzyme extract - 50.8 per cent destruction.
Dialyzed enzyme extract - 34.8 per cent destruction.

Additions to Boiled Extract	Effect of Boiled Extract on Dialyzed Extract: Thiamine Destruction	Activation
ml.	per cent	per cent
0	46.5	33.6
Phenylmercuric Chloride, 0.00025 M.		
0.2	36.3	4.3
0.4	29.2	- 16.1
0.6	23.0	- 33.9
0.8	18.9	- 45.7
1.0	17.6	- 49.4
Iodine, 0.01 N.		
0.33[1]	40.8	17.3
0.66	43.7	25.6
1.32	36.7	5.5
1.98	34.8	0
2.64	29.8	- 14.4

[1] This amount of iodine was equivalent to the amount of 0.001 N. iodine reduced by the boiled extract. The other iodine additions represent two to five times the iodine equivalent.

J. Activation by Manganese

As mentioned earlier, the manganous ion has been found to be an activator for thiaminase. A series of experiments were therefore conducted in an effort to determine whether this ion was the activating factor of the boiled extract or whether its action was perhaps a non-specific one. Manganese was present in such extracts, for a determination on a boiled extract, representing 2.5 gm. of preparation II-196, indicated the presence of 0.0281 mg. of manganese.

The manganese determination was carried out using the method of Richards (58). Thirty ml. of the boiled extract was evaporated to dryness in a platinum crucible, carbon was burned off and the residue ashed in a muffle furnace. The ash was transferred to a beaker with 20 ml. of 33 per cent sulfuric acid and the solution evaporated almost to dryness. An additional 15 ml. of sulfuric acid was added and the evaporation repeated. The residue was then dissolved in approximately 20 ml. of a mixture of 5 ml. of 85 per cent phosphoric acid and 45 ml. of water. This solution was heated on a steam bath for one-half hour, filtered and washed into a 50 ml. beaker. Approximately 0.3 gm. of potassium periodate was added and the solution boiled until maximum color developed. This solution was transferred to a 25 ml. volumetric flask and diluted to volume with the dilute phosphoric acid.

Against a blank of the diluted phosphoric acid, which was used to adjust the Klett-Summerson colorimeter to zero with Filter 52, the colorimetric reading for the unknown solution was 27. This value represented 2.81×10^{-2} mg. of manganese as determined from a previously constructed calibration curve.

To determine the activating effect of this metal, a solution of manganous chloride was added to different amounts of an enzyme extract, prepared from I-142-III-A, to give a 5×10^{-4} M. concentration of manganese in the incubation mixtures. As shown by the data of Table XLVII, manganese had no significant effect even at the lower concentrations of the enzyme. However, the manganese solution had not been freshly prepared, and it was possible that the manganous ion had been oxidized.

Table XLVII

Activation by Manganese

Amount of Enzyme	Thiamine Destruction	
	without manganese	with manganese
mg. equiv.	per cent	per cent
6.25	1.5	1.5
12.50	5.8	3.8
25.00	15.5	18.7
37.50	41.3	41.7
50.00	71.8	71.4

Slight activation of enzyme extracts prepared from acetone-desiccated powders II-236 and II-196 was observed upon addition of manganese. With a concentration of 6.25×10^{-4} M. the thiamine destruction of a 25 mg. equivalent of the former was increased from 34.0 to 36.4 per cent, an activation of 7.1 per cent. With an equal amount of an extract of preparation II-196, and with manganese in concentrations of 2×10^{-5} to 2×10^{-4} M., thiamine destruction of from 51.2 to 52.3 per cent was observed. Since the extract itself destroyed 49.5 per cent of the added thiamine, this increased destruction in the presence of manganese represented an activation of only 3.4 to 5.6 per cent.

The activating effect of manganese was however more obvious when dialyzed enzyme extracts were employed, as evidenced by the data shown in Table XLVIII. In all of the experiments represented here manganese was present in a concentration of 6.25×10^{-4} M., and the results show that manganese in this concentration is an activator of the enzyme. However, the degree of activation was never great and in only a few instances was activity restored to that of the undialyzed enzyme by the addition of manganese. These results may indicate that a factor other than manganese is removed by dialysis, and they may also be interpreted as meaning that manganese activation is non-specific. On the other hand the failure to restore initial activity by manganese addition may be due to denaturation or perhaps oxidation of sulfhydryl groups of the non-dialyzable fraction during the period of dialysis.

Table XLVIII

Manganese Activation of Dialyzed Extracts

Enzyme Preparation	Thiamine Destruction			
	Undialyzed	Dialyzed	Dialyzed + Mn	Activation
	per cent	per cent	per cent	per cent
I-142-III-A, 25 mg. equiv.	21.6	6.1	7.1	16.4
I-142-III-A, 50 mg. equiv.	69.9	18.4	17.8	- 3.3
I-142-III-A, 50 mg. equiv.	73.2	23.2	29.5	27.2
II-196, 25 mg. equiv.	50.0	24.2	26.4	9.1
II-196, 25 mg. equiv.	47.6	26.4	28.5	8.0
II-196, 25 mg. equiv.	41.8	28.6	30.4	6.3
II-236, 25 mg. equiv.	25.8	20.3	25.8	27.1
II-236, 25 mg. equiv.	23.0	23.3	24.8	6.4
II-236, 40 mg. equiv.	54.2	45.7	51.2	12.0
III-110, 50 mg. equiv.	45.5	35.4	41.3	16.7
III-110, 50 mg. equiv.	43.7	34.7	36.4	4.9
III-138, 50 mg. equiv.	71.7	29.6	37.7	27.4

Several inhibitors, which might indicate the importance of a metallic ion, although not specific for manganese were studied. Sealock and Goodland (37) have reported that cyanide in a concentration of 1 x 10^{-3} moles per liter produced 23.8 per cent inhibition of thiaminase, but, since no values for the amounts of thiamine destroyed were given, the significance of this inhibition can not be considered too critically. In this laboratory an extract, representing a 50 mg. equivalent of preparation I-142-III-A, destroyed 71.9 and 72.6 per cent of the substrate in the presence of 1 and 2 x 10^{-3} M. cyanide, respectively, while with no added cyanide the extract destroyed only 69.2 per cent of the added thiamine. It appeared then that at least with this enzyme preparation and at these concentrations, cyanide was not inhibitory. Addition of 2 x 10^{-4} M. manganese in combination with the cyanide did not cause any significant change as evidenced by the thiamine destruction of 68.2 and 71.5 per cent for the two cyanide concentrations plus manganese.

During the course of these experiments MacLeod and Snell (59) reported that citrate inhibited the growth of certain microorganisms which required manganese for growth. Citrate forms a complex with manganese and other divalent metals. Therefore, it would be expected that citrate would inhibit an enzyme system, which was dependent upon manganese. In the initial experiment to determine the effect of citrate upon thiaminase action an extract representing 50 mg. of I-142-III-A, which destroyed 75.2 per cent of the thiamine, was employed. The

addition of citrate to give six different concentrations ranging from 2 to 100 x 10^{-4} M. resulted in thiamine destruction from 74.4 to 76.6 per cent, indicating that at least in these concentrations, the effect of citrate was insignificant.

However, with a similar enzyme preparation, producing 74.4 per cent thiamine destruction, citrate in concentrations of 0.027, 0.053 and 0.106 M. produced 2.0, 2.5 and 5.2 per cent inhibition. The results of a further experiment in which even higher concentrations of citrate were employed are shown in Table XLIX. Both 25 mg. and 50 mg. equivalents of preparation I-142-III-A were used, and inhibition, increasing with increasing citrate concentrations, was observed with both concentrations of the enzyme. The citrate solution was prepared in the following manner: 52.5 gm. of citric acid was moistened with a small amount of water, saturated sodium hydroxide was added slowly, and the pH of the solution checked from time to time. When the acid had been neutralized the volume was adjusted to 200 ml. giving a 1.25 M. solution of citrate.

Table XLIX

Inhibition by Citrate

Citrate Concentration	I-142-III-A, 25 mg. equiv.		I-142-III-A, 50 mg. equiv.	
	Destruction	Inhibition	Destruction	Inhibition
moles per liter	per cent	per cent	per cent	per cent
0	24.8		74.2	
0.100	18.8	24.2	62.4	15.9
0.250	17.6	29.0	59.7	19.6
0.375	15.8	36.4	55.4	25.4
0.500	14.6	41.2	52.4	29.4

If the inhibition observed in the presence of citrate were due to the inactivation of manganese through the formation of a complex, the addition of manganese would be expected to reverse that inhibition. Table L shows the results of an experiment in which both manganese and citrate were added to an enzyme extract, representing 50 mg. of I-142-III-A. Although at the higher citrate concentrations, slightly less inhibition was observed upon addition of manganese, it appeared that citrate still produced inhibition, even in the presence of this metal. This could perhaps be explained on the basis of the relative concentrations of manganese and citrate, but, because of interference in the Melnick-Field determination, concentrations of manganese higher than 0.001 M. could not be employed.

Table L

Citrate Inhibition in the Presence of Manganese

Citrate conc.	Destruction with Manganese Concentration of 0 M.	0.0005 M.	0.001 M.	Inhibition with Manganese Concentration of 0 M.	0.0005 M.	0.001 M.
moles/l.	per cent	per cent	per cent	per cent	per cent	per cent
0	78.1					
0.050	71.8	72.4	70.3	8.1	7.3	10.0
0.100	70.6	70.3	70.0	9.6	10.0	10.4
0.250	66.9	68.7	68.3	14.3	12.1	12.6
0.375	61.6	62.3	65.1	21.2	20.2	16.7

Although the results obtained with cyanide and citrate did not furnish any conclusive evidence for the presence or absence of a metallic coenzyme or activator for thiaminase, work carried out in this laboratory[1] showed that the presence of sulfide ion in concentrations from 2×10^{-8} to 2×10^{-5} M. inhibited the enzyme from 0.0 to 91.4 per cent. A number of metallic ions including manganese form insoluble or slightly dissociable salts with sulfide, and this inhibition may therefore have been due to the inactivation of an essential metallic ion. In an effort to determine whether the inhibition was produced by the action of sulfide upon the non-protein component of the enzyme, a boiled extract was treated with hydrogen sulfide and its

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1 Sealock, R. R. and Davis, N. C., unpublished.

activating effect on a dialyzed enzyme extract compared with that observed upon addition of an untreated portion of the extract.

The extract which had been treated at pH 7.6 with hydrogen sulfide was freed of the precipitated sulfides by centrifugation and filtration, and then the excess hydrogen sulfide was removed in a vacuum desiccator. Addition of this extract to a dialyzed enzyme preparation reduced the destruction of thiamine from 34.6 to 17.7 per cent, an inhibition of 48.8 per cent. On the other hand addition of an equivalent amount of the extract, which had not been treated with hydrogen sulfide, increased thiamine destruction to 46.0 per cent, an activation of 32.9 per cent. The hydrogen sulfide treatment therefore not only destroyed the activating ability of the boiled extract, but caused it to be inhibitory.

Addition of manganese in combination with the sulfide-treated boiled extract did not restore its activating ability, as evidenced by the fact that thiamine destruction ranging from 18.1 to 20.7 per cent was observed in the presence of manganese in concentrations from0.01 to 6.25×10^{-4} M. Even with the highest manganese concentration, the treated boiled extract produced an inhibition of 40.2 per cent. Manganese in these concentrations added to the dialyzed extract, in the absence of any boiled extract, produced activation of from 2.6 to 16.2 per cent.

Although the treated extract had given a negative lead acetate test for the sulfide ion, it was adjusted to an acid pH and aerated for approximately four hours to insure complete removal of excess hydrogen sulfide. Further analysis of this extract showed that it was still inhibitory. Therefore the inhibition could not be accounted for by the presence of excess sulfide.

In another series of experiments enzyme preparations were dialyzed against solutions containing manganese. If the dialyzable component were manganese, the inactivation of the enzyme produced by such dialysis would be expected to be less than that obtained by dialysis against solutions, which were identical except that no manganese was present.

In the first experiment, one portion of an extract, representing 50 mg. per ml. of I-142-III-4, was dialyzed for five and one-half hours with continuous stirring against a solution of 2 per cent sodium chloride containing 0.001 M. manganese, while a second portion was dialyzed under identical conditions except that the outside solution contained no manganese. After dialysis, these extracts and a portion which had not been dialyzed were diluted to equivalent volumes and analyzed directly and with added manganese and citrate.

The results obtained when both 25 and 50 mg. equivalents of the extracts were analyzed, are shown in Table LI. Both of the dialyzed extracts destroyed less thiamine than did the undialyzed portion, although the extract dialyzed against

Table LI

Dialysis of Enzyme Extract Against Manganese

Solution analyzed	Thiamine Destruction	Amount of Original Activity[1]
	per cent	per cent
25 mg. equiv.		
Undialyzed	21.6	100.0
Dialyzed (A)	6.9	41.2
plus manganese[2]	15.5	71.7
plus citrate[2]	5.8	26.9
plus citrate + Mn	6.2	28.7
Dialyzed (B)	6.1	28.3
plus manganese	7.1	32.9
plus citrate	10.4	48.2
plus citrate + Mn	0.5 (?)	
50 mg. equiv.		
Undialyzed	69.9	100.0
Dialyzed (A)	24.1	34.5
plus manganese	21.2	30.4
Dialyzed (B)	18.4	26.4
plus manganese	17.8	25.5

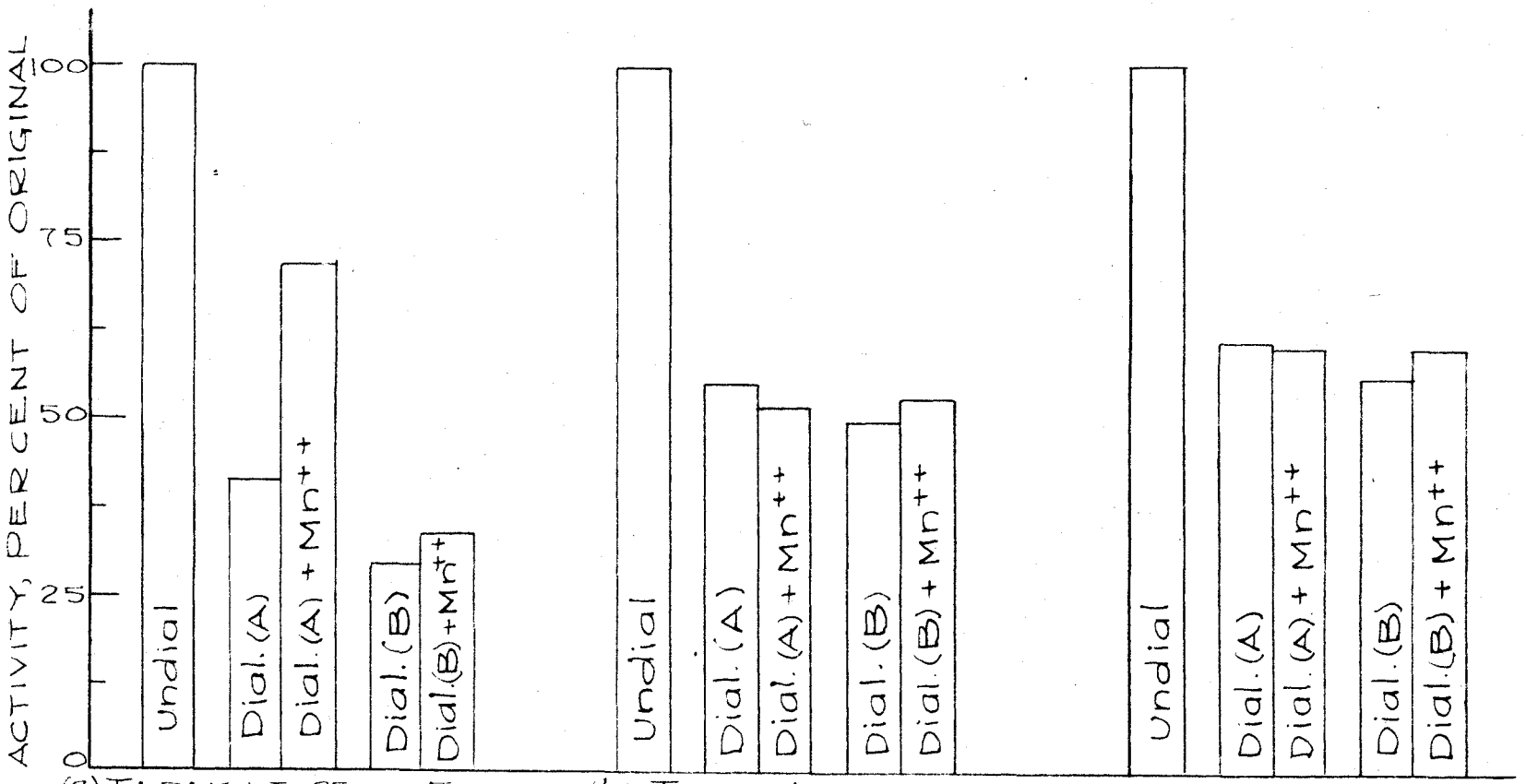
Dialyzed (A) - dialysis against 2 % NaCl, plus 0.001 M. Mn.
Dialyzed (B) - dialysis against 2 % NaCl.

1 Expressed as the per cent of the destruction observed with the undialyzed extract.

2 Manganese added to give 6.25×10^{-4} M.; citrate added to give 0.25 M.

manganese retained a slightly greater amount of activity. This may, of course, have been due to a non-specific activating effect of manganese, which would enter the extract from the dialyzate. Addition of manganese to the dialyzed extracts resulted in inhibition, when they were analyzed at a 50 mg. concentration, and activation, when analyzed at a 25 mg. concentration. Figure 6 (a) shows in graphic form the effect of dialysis against manganese as indicated by the analysis at the 25 mg. concentration of the enzyme.

Two similar experiments were carried out in which an extract representing 50 mg. per ml. of II-196 was employed. In the first, the dialyzate for one portion of the extract was 0.2 M. phosphate buffer at pH 7.4 containing 10 per cent sodium chloride, while for a second portion the dialyzate was the same except that 0.001 M. manganese was present. At the conclusion of dialysis the extracts were diluted to equivalent volumes and assayed at a concentration equivalent to 25 mg. A portion of the extract, which had not been dialyzed, contained 2.28 mg. of nitrogen per 25 mg. equivalent, while the portion dialyzed against manganese contained 1.01 mg. of nitrogen per 25 mg. equivalent, and the portion which was dialyzed against the manganese-free solution contained 1.07 mg. of nitrogen per 25 mg. equivalent. The results obtained, when these solutions were analyzed, are shown in Table LII and in Figure 6 (b), and are comparable to those obtained above. The extract dialyzed against manganese retained a greater amount of activity than did



(a) TABLE LI, 25mg. EQUIV. DIALYZED AGAINST
(A) 2% NaCL + 0.001 M. Mn^{++}
(B) 2% NaCL

(b) TABLE LII, EXP. 1 DIALYZED AGAINST
(A) 0.2 M. PHOSPHATE +10% NaCL + 0.001 M. MN^{++}
(B) 0.2 M. PHOSPHATE + 10% NaCL

(c) TABLE LII, EXP. 2 DIALYZED AGAINST
(A) 0.04 M. PHOSPHATE +2% NaCL + 5×10^{-5} M. Mn^{++}
(B) 0.04 M. PHOSPHATE +2% NaCL

FIG. 6 DIALYSIS OF ENZYME EXTRACTS AGAINST MANGANESE

Table LII

Dialysis of Enzyme Extracts Against Manganese

Solution Analyzed	Thiamine Destruction	Amount of Original Activity
	per cent	per cent
Undialyzed	50.0	100.0
Dialyzed (A)	27.4	54.9
plus citrate	17.4	34.9
plus manganese	25.4	50.8
Dialyzed (B)	24.2	48.5
plus citrate	18.4	36.9
plus manganese	26.4	52.8
Undialyzed	47.6	100.0
Dialyzed (A)	28.8	60.5
plus citrate	20.5	43.1
plus manganese	28.2	59.3
Dialyzed (B)	26.4	55.5
plus citrate	19.8	41.6
plus manganese	28.5	59.8

Dialyzed (A) - Manganese present in dialyzate.
Dialyzed (B) - No manganese present in dialyzate.

that dialyzed against the manganese-free solution. The addition of manganese to give a 6.25×10^{-4} M. concentration reduced the activity of the former, but increased the activity of the latter; while citrate in a concentration of 0.25 M. inhibited both.

For the second experiment the dialyzing solution was a 0.04 M. phosphate buffer containing 2 per cent sodium chloride for one portion of the extract, and the same plus 5×10^{-5} M. manganese for the second. Here again the amount of nitrogen present in the dialyzed extracts was about one-half that of the undialyzed. The latter contained 1.89 mg. of nitrogen per 25 mg. equivalent. The extract dialyzed against manganese contained 1.15 mg. of nitrogen per 25 mg. equivalent, and that dialyzed against the manganese-free solution, 1.08 mg. of nitrogen per 25 mg. equivalent. The results of this experiment shown also in Table LII and in Figure 6 (c) were similar to those obtained in the first experiment described in this table.

Although in these experiments, the extracts dialyzed against manganese retained a slightly greater amount of activity than did those dialyzed against solutions containing no manganese, the difference in activity was not great. Since inactivation was observed in all cases, it would appear that loss of activity upon dialysis was due to loss of some component other than or in addition to manganese. A comparison of the activating effect of boiled extract on dialyzed extracts similar to these may lend further support to this hypothesis.

K. The Activating Factor of Boiled Extract

Since it appeared that manganese, although it was capable of activating the enzyme, was not alone responsible for the activation produced by boiled extract, efforts were made to further characterize the activating factor or factors. First, experiments were carried out to gain information regarding the solubility properties of the factor. Twelve ml. of an enzyme extract at pH 7.2 was extracted with three 5 ml. portions of redistilled isobutanol. The butanol fractions were evaporated to dryness, and the residue dissolved in 10 ml. of water. The enzyme extract was stirred and aerated briefly, but a faint butanol odor was still evident at the time it was analyzed.

This extract destroyed 16.8 per cent of the added thiamine, while an equal amount of the extract, which had not been extracted with butanol, destroyed 46.7 per cent of the substrate. The butanol soluble fraction produced a 4.0 per cent destruction of the thiamine, while its addition to the untreated and extracted enzyme preparations produced 55.3 and 18.2 per cent destruction, respectively. The reduction of activity following the butanol extraction may have been due either to the removal of a butanol soluble component of the system or to the alcohol denaturation of the protein. Since extraction and separation of the layers was difficult, the activity of the butanol soluble fraction may not have been due to the actual solubility of the non-protein factor in the alcohol. However, this factor

appeared to be quite stable, since this fraction, which had been evaporated to dryness in an air-stream, still had an activating effect on the enzyme.

Later 10 ml. portions of a boiled extract at pH 5.5 and 9.5 were extracted with three 4 ml. portions of isobutanol. Here again separation of the layers was difficult and there may have been a "carry-over" into the organic layer of materials not actually soluble. The residues obtained after evaporation of the solvent were each dissolved in 10 ml. of 0.2 M. phosphate buffer containing 10 per cent sodium chloride. In addition equal amounts of the boiled extract at the same pH's were extracted in a similar manner with ether, and the residues obtained after evaporation of the ether fractions also dissolved in the phosphate-sodium chloride solution. The activating effects of these solutions on a dialyzed enzyme extract are shown in Table LIII. The results appeared to indicate that some factor, capable of activating the dialyzed enzyme was extracted from the boiled extract at pH 9.5 by ether.

A portion of the enzyme extract prepared from II-196, which was not dialyzed, and which contained 1.09 mg. of nitrogen per 12.5 mg. equivalent, destroyed 53.2 per cent of the added thiamine. An equal amount of the extract, which had been dialyzed for 14 hours against a 2 per cent solution of sodium chloride in 0.04 M. phosphate buffer, contained 0.215 mg. of nitrogen per 12.5 mg. equivalent, and destroyed only 15.6 per cent of the added thiamine.

Table LIII

Activation by Butanol and
Ether Soluble Fractions of Boiled Extract

Addition to Dialyzed Enzyme	Thiamine Destruction per cent	Activation per cent
None	15.6	
Untreated boiled extract	39.4	152.0
Butanol soluble, pH 9.5	16.2	3.9
Butanol soluble, pH 5.5	15.2	0
Ether soluble, pH 9.5	25.8	65.3
Ether soluble, pH 5.5	15.6	0

The ether extraction of an alkaline boiled extract was repeated to determine whether the activation observed in the above experiment was due to a "carry-over" of the activating factor or to its actual solubility in ether. Fifteen ml. of a boiled extract at pH 9.3 was extracted with eight 5 ml. portions of anhydrous ether, and the layers separated by centrifugation. The combined ether fractions were evaporated to dryness at room temperature, and the residue dissolved in 15 ml. of the phosphate-sodium chloride buffer. The aqueous layer was freed from ether by aeration and the volume returned to 15 ml. The results obtained, when these extracts were tested for activity with a dialyzed enzyme extract, indicated that the boiled extract did not contain an ether soluble

activator. An untreated portion of the boiled extract increased the thiamine destruction of the dialyzed enzyme from 13.6 to 26.5 per cent, while an equal amount of the ether extracted solution increased the destruction to 25.0 per cent. The ether soluble fraction itself reduced the thiamine destruction to 8.9 per cent.

Therefore, the activation observed in the previous experiment, when a similar ether soluble fraction was added to a dialyzed enzyme preparation, was probably due to incomplete separation of the aqueous and organic layers. However, the fact that the extracted portion of the boiled extract, which had been kept at an alkaline pH during the extraction, and which had been freed from ether by aeration, still possessed a large proportion of the original activating ability, was further indication for the presence of a fairly stable activator.

In another attempt to show the solubility of an activating factor in an organic solvent, 15 ml. of a boiled extract at pH 8.6 was extracted with two 10 ml., two 8 ml., and four 7 ml. portions of redistilled n-butanol, and the layers separated by centrifugation. The combined butanol fractions were made acid and then concentrated to dryness in vacuo in an atmosphere of carbon dioxide. The residue was washed from the flask with water and diluted to 15 ml. The aqueous layer was washed into a distilling flask, concentrated to dryness in the same fashion, and the residue also dissolved in 15 ml.

of water. Whereas a portion of untreated boiled extract produced a 62.0 per cent activation of a dialyzed enzyme preparation, the butanol soluble fraction produced 58.7 per cent inhibition and the butanol insoluble fraction, 26.1 per cent inhibition. The combination of the two butanol fractions produced an inhibition of 46.9 per cent. The reason for this inhibition was not apparent, but obviously no information regarding the butanol solubility of the activating factor could be obtained.

In certain of the boiled extracts, particularly those prepared from III-110, a flocculent precipitate appeared upon adjustment to pH 7.4. Although at first this was kept in suspension for the analyses, later an effort was made to determine whether the insoluble material possessed activity. Therefore one portion of such an extract was centrifuged, and the activation produced by both the clear decantate and the residue, suspended in an equal volume of water, compared with that of the non-centrifuged extract. The decantate and the non-centrifuged portion both activated a dialyzed enzyme extract to an extent of 38.4 per cent, while the residue-containing solution produced 17.2 per cent activation. It appeared then that the boiled extract could be freed from this insoluble material to facilitate its use. The activation produced by the residue was probably due to the fact that it was not washed, but merely drained free from the supernatant liquid.

In another experiment a 18.7 per cent activation was produced by a similar decantate, while the non-filtered portion of the same extract produced only 11.3 per cent activation. At the same time two portions of the unfiltered extract at pH 4.2 and 8.6 were evaporated to dryness in an air-stream and the residues redissolved in equal volumes of water. The solutions obtained from these residues both activated a dialyzed enzyme to an extent of 14.7 per cent as compared with the 11.3 and 15.7 per cent activations observed with the portions which were not evaporated. This gave further indication that the activating component of boiled extract possessed a high degree of stability.

Later air was bubbled through a 10 ml. portion of a boiled extract at pH 8.6 for 40 minutes. The tube containing the extract was kept in a bath of warm water at a temperature from 50 to 60 degrees. The aerated sample produced 25.6 per cent activation of a dialyzed enzyme preparation, while an untreated portion gave 34.0 per cent activation. Thinking that the inactivation of a boiled extract produced by boiling at an alkaline pH, as described by Sealock and Livermore[1], might have been due to the loss of a volatile base, the tube containing the boiled extract was following by one containing an equal volume of 0.1 N. hydrochloric acid. However, this solution, after

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1 Sealock, R. R. and Livermore, A. H., unpublished.

adjustment to pH 7.4 produced no activation of the dialyzed extract. It must be remembered, however, that Sealock and Livermore found that a boiled extract made by a phosphate extraction of a desiccated powder lost activity upon boiling at an alkaline pH, whereas one made by a water extraction was much more stable under these conditions.

Here a 63.7 per cent reduction of activity was observed with a boiled extract, made by water extraction of an acetone-desiccated powder, which had been placed at pH 8.2 in a boiling water bath for 10 minutes. This heated sample produced only 4.1 per cent activation of a dialyzed enzyme, whereas an equal amount of an unheated sample produced 11.3 per cent activation.

In general these experiments indicated that the activating factor was not completely thermostable at an alkaline pH. On the other hand, it appeared to be stable at both acid and alkaline pH's at room temperature and under oxidizing conditions, as evidenced by the fact that little activity was lost upon evaporation or aeration.

Several adsorption procedures were also employed in an effort to determine the nature of this factor, or factors. In the first experiment a boiled extract, representing 25 mg. per ml. of preparation II-196, was adsorbed on activated Decalso, one portion of the extract at an initial pH of 4.2 and a second at a pH of 7.3. The columns were washed with water and then eluted with a solution of 2 per cent potassium chloride in 0.1 N. hydrochloric acid. Here and in all following experiments

the eluate was diluted to a volume equivalent to that of the adsorbed extract. After adjustment to pH 7.4, the activating ability of filtrates and eluates was determined by their addition to a dialyzed enzyme extract, and compared with the activation produced by an untreated portion of the boiled extract. This extract had been prepared in the usual fashion using the phosphate buffer containing sodium chloride, but in all of the following experiments the extraction of the acetone-desiccated powder was made with distilled water to reduce the cation concentration of the solution.

Table LIV shows the results obtained when the activating effect was determined with a dialyzed enzyme extract, representing 25 mg. per ml. of II-196. An equal amount of an undialyzed portion destroyed 41.9 per cent of the added thiamine. Since the analysis of the eluates was not carried out at the same time, values for the dialyzed extract are included twice. It appeared that even in the presence of a high concentration of cations, some factor necessary for activation was being adsorbed by Decalso, and that the initial pH of the solution did not materially affect the amount of adsorption.

Both eluates contained an activating factor. The combination of the filtrate and eluate in the one case produced an activation greater than that of either alone, perhaps indicating the presence of both an anionic and a cationic activator. The addition of manganese did not however restore the activating ability of the Decalso filtrates.

Table LIV

Decalso Treatment of Boiled Extract

Additions to Dialyzed Extract	Thiamine Destruction		With 6.25×10^{-4} M. Mn. Thiamine Destruction	Activation
	per cent	per cent	per cent	per cent
None	28.6		30.4	6.3
Untreated boiled extract	50.2	75.7	50.7	77.2
Decalso filtrate, pH 4.2	44.3	54.9	46.5	61.5
Decalso filtrate, pH 7.3	41.8	46.2	45.9	53.3
None	25.7			
Decalso filtrate, pH 4.2	43.8	70.4		
Decalso filtrate, pH 7.3	43.2	68.1		
Eluate, pH 4.2	28.2	9.7		
Eluate, pH 7.3	33.7	31.2		
Filtrate + Eluate, pH 7.3	47.2	83.8		

Table LV shows the results of two similar experiments in which elution was carried out with a 25 per cent solution of potassium chloride in 0.1 N. hydrochloric acid. The activating ability of the boiled extract was reduced in both of these experiments, but not completely destroyed. The eluates in both cases possessed some activity and the combination of filtrate and eluate caused a higher per cent destruction of the thiamine, than did either alone, but the activity was not restored to that of the untreated extract. An examination of the bar graphs shown in Figure 7, indicates that the increase in activity observed, when manganese was added to the dialyzed extract in combination with the Decalso filtrate, was not significantly greater than that obtained, when it alone was added to the dialyzed extract, or when it was added in combination with the untreated boiled extract. This would seem to indicate that the factor adsorbed by Decalso was not manganese. In the first experiment the addition of cysteine to give a 5×10^{-4} M. concentration did not increase the activity of the Decalso filtrate.

Table LV

Decalso Treatment of Boiled Extract
Elution with 25 per cent KCl in 0.1 N. HCl

Addition to Dialyzed Extract	Thiamine Destruction		With 6.25×10^{-4} M. Mn. Thiamine Destruction	Activation
	per cent	per cent	per cent	per cent
None[1]	35.4		41.3	16.7
Untreated boiled extract	52.5	48.3	54.7	54.5
Decalso filtrate	39.4	11.3	45.4	28.2
Eluate	45.5	28.5		
Filtrate + eluate	50.5	42.6	52.5	48.3
Filtrate + cysteine	38.4	8.5	41.7	17.8
Cysteine	56.8 (?)	60.4 (?)		
None[2]	23.2		29.5	27.2
Untreated boiled extract	31.8	37.1	36.6	57.8
Decalso filtrate	24.5	5.6	29.5	27.2
Decalso eluate	24.7	5.4		
Filtrate + eluate	27.4	18.1	38.0	63.8

[1] The boiled extract in this experiment represented 40 mg. of preparation III-110. The pH was adjusted to 6.5 before adsorption. The undialyzed portion of the enzyme extract destroyed 45.5 per cent of the thiamine.

[2] The boiled extract in this experiment represented 50 mg. of preparation III-110. The pH was adjusted to 4.5 before adsorption. The undialyzed portion of the enzyme extract destroyed 73.2 per cent of the thiamine.

FIG. 7 ACTIVATION OF DIALYZED ENZYME PREPARATIONS BY DECALSO TREATED BOILED EXTRACTS

Table LVI shows the results obtained in further experiments in which boiled extracts were adsorbed by Decalso. Here several different solutions were employed in an effort to obtain a more efficient elution of the Decalso. In all of these experiments the activity of the boiled extract was again reduced by means of this treatment. However, elution was still unsatisfactory. Eluates obtained with 5 per cent potassium chloride in 0.1 N. hydrochloric acid and with 5 per cent acetic acid possessed some activity, and the addition of the eluate in combination with the filtrate produced a greater activation than either solution alone. On the other hand the eluate obtained with a 20 per cent potassium chloride solution inhibited the dialyzed enzyme, indicating that an acid solution was necessary for elution. An examination of the bar graphs, Figure 8, representing the first two experiments of Table LVI, indicated again that, although manganese increased the activating ability of the Decalso filtrates, the increase due to manganese was not significantly greater than that produced when manganese alone was added to the dialyzed enzyme.

Table LVI

Decalso Treatment of Boiled Extracts

Addition to Dialyzed Extract	Thiamine Destruction	With 6.25×10^{-4} M. Mn. Thiamine		
		Activation	Destruction	Activation
	per cent	per cent	per cent	per cent
None[1]	29.6		37.7	27.4
Untreated boiled extract	47.9	62.0	49.2	66.3
Decalso filtrate	43.8	48.0	50.2	69.7
Eluate	30.2	2.0	38.4	29.8
Filtrate + eluate	46.6	57.5		
None[2]	23.3		24.8	6.4
Untreated boiled extract	30.7	31.8	33.7	44.6
Decalso filtrate	24.2	3.9	28.3	21.4
Eluate	22.4	- 3.9		
Filtrate + eluate	23.5	0.0	26.0	11.6
None[3]	27.5			
Untreated boiled extract	36.9	34.2		
Decalso filtrate	31.7	15.3		
Eluate	30.4	10.5		
Filtrate + eluate	32.2	17.1		

1 Undialyzed portion of enzyme extract produced 71.7 per cent destruction. Elution of Decalso was made with 5 per cent KCl in 0.1 N. HCl.

2 Undialyzed enzyme extract, representing 25 mg. equiv. of II-236 contained 1.29 mg. of nitrogen and destroyed 23.0 per cent of the thiamine. The dialyzed extract contained only 0.952 mg. of nitrogen per 25 mg. equivalent. The Decalso was eluted with 20 per cent KCl.

3 The undialyzed portion of the enzyme extract destroyed 41.6 per cent of the added thiamine. Elution was made with 5 per cent acetic acid.

FIG. 8 ACTIVATION OF DIALYZED ENZYME PREPARATIONS BY DECALSO TREATED BOILED EXTRACTS

The Amerlite exchange resins[1] were also employed in the study of the activating factor or factors of boiled extract. Amberlite IR-100-H, analytical grade, the cation exchange resin in its hydrogen form, was used most extensively. The procedure was similar to that used in the Decalso experiments, and the boiled extracts, prepared by extracting acetone-dessicated powders with distilled water, were passed through columns containing approximately 7 gm. of the resin. An examination of Table LVII, which includes a summary of four such experiments, and the bar graphs of Figures 9 and 10, indicates that there is a loss of some factor of cationic nature by treatment of the boiled extract with Amberlite IR-100-H. Also, a 31 mg. equivalent of the boiled extract used in the third experiment contained 1.23 mg. of nitrogen, while an equal amount of the extract, after treatment with Amberlite, contained only 0.87 mg. of nitrogen. An equivalent amount of the eluate contained 0.048 mg. of nitrogen. This would appear to indicate that nitrogenous bases were being adsorbed on the Amberlite and eluted with 0.1 N. hydrochloric acid.

The results obtained when manganese was added to give a concentration of 6.25×10^{-4} M. were similar to those observed in the Decalso experiments. The addition of manganese in combination with the Amberlite treated extract caused increased activation of the dialyzed enzyme extract. However, this increase was not significantly greater than the increased activation observed when manganese was added in combination

[1] Resinous Products and Chemical Co.

Table LVII

Amberlite IR-100-H Treatment of Boiled Extracts

Additions to Dialyzed Extract	Thiamine Destruction	Activation	With Manganese Thiamine Destruction	Activation
	per cent	per cent	per cent	per cent
None[1]	45.7		51.2	12.0
Untreated boiled extract	54.2	18.6	59.4	30.0
Amberlite filtrate, pH 4.2	48.8	6.8	53.3	16.7
Amberlite filtrate, pH 7.5	48.2	5.5	53.3	16.7
None[2]	34.0		36.4	7.1
Untreated boiled extract	42.4	24.8	42.4	24.8
Amberlite filtrate	36.7	7.9	39.4	15.9
Amberlite eluate	32.5	- 4.4	34.6	11.8
Filtrate + eluate	35.8	5.3	38.6	13.5
None[3]	20.3		25.8	26.2
Untreated boiled extract	33.2	63.5	35.5	74.8
Amberlite filtrate	21.8	7.4	28.2	39.0
Amberlite eluate	21.7	6.9		
Filtrate + eluate	24.2	19.2		
None[4]	23.3		24.8	6.4
Untreated boiled extract	30.7	31.8	33.7	44.6
Amberlite filtrate	24.8	6.4	28.3	21.4
Amberlite eluate	16.2	-30.5		
Filtrate + eluate	14.6	-37.4	19.2	- 17.6

1 Boiled extract, 30 mg./ml., II-236. Portions at pH 4.2 and pH 7.5 adsorbed on Amberlite. No elution. Undialyzed enzyme extract destroyed 54.2 per cent of the added thiamine.

2 Boiled extract, 30 mg./ml., II-236. Adsorbed at pH 7.0. Elution with 0.1 N. HCl. Dilute enzyme extract, 25 mg. equiv. of II-236 used for analysis. No dialysis.

3 Boiled extract, 25 mg./ml., II-236. Adsorbed at pH 7.1. Elution with 0.1 N. HCl. Undialyzed enzyme extract produced 25.8 per cent destruction, and contained 1.50 mg. of nitrogen per 25 mg. equiv. The dialyzed extract contained 1.13 mg. of nitrogen per 25 mg. equiv.

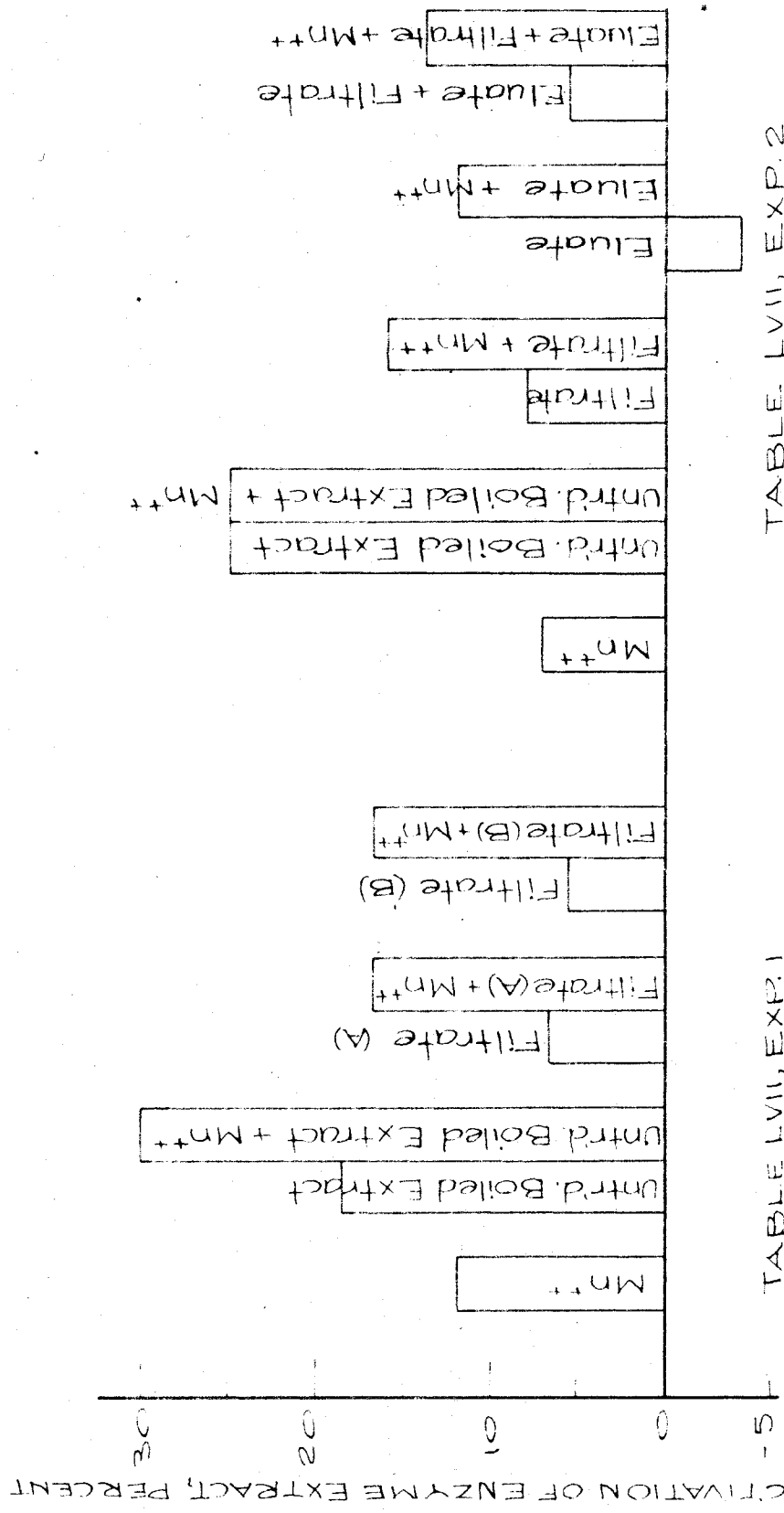

TABLE LVII, EXP. 1
FILTRATE (A) TREATED AT pH 4.2
FILTRATE (B) TREATED AT pH 7.5

TABLE LVII, EXP. 2
ELUTION WITH 0.1 N HCL

FIG. 9 ACTIVATION OF ENZYME PREPARATIONS BY BOILED EXTRACTS TREATED WITH AMBERLITE IR-100-H.

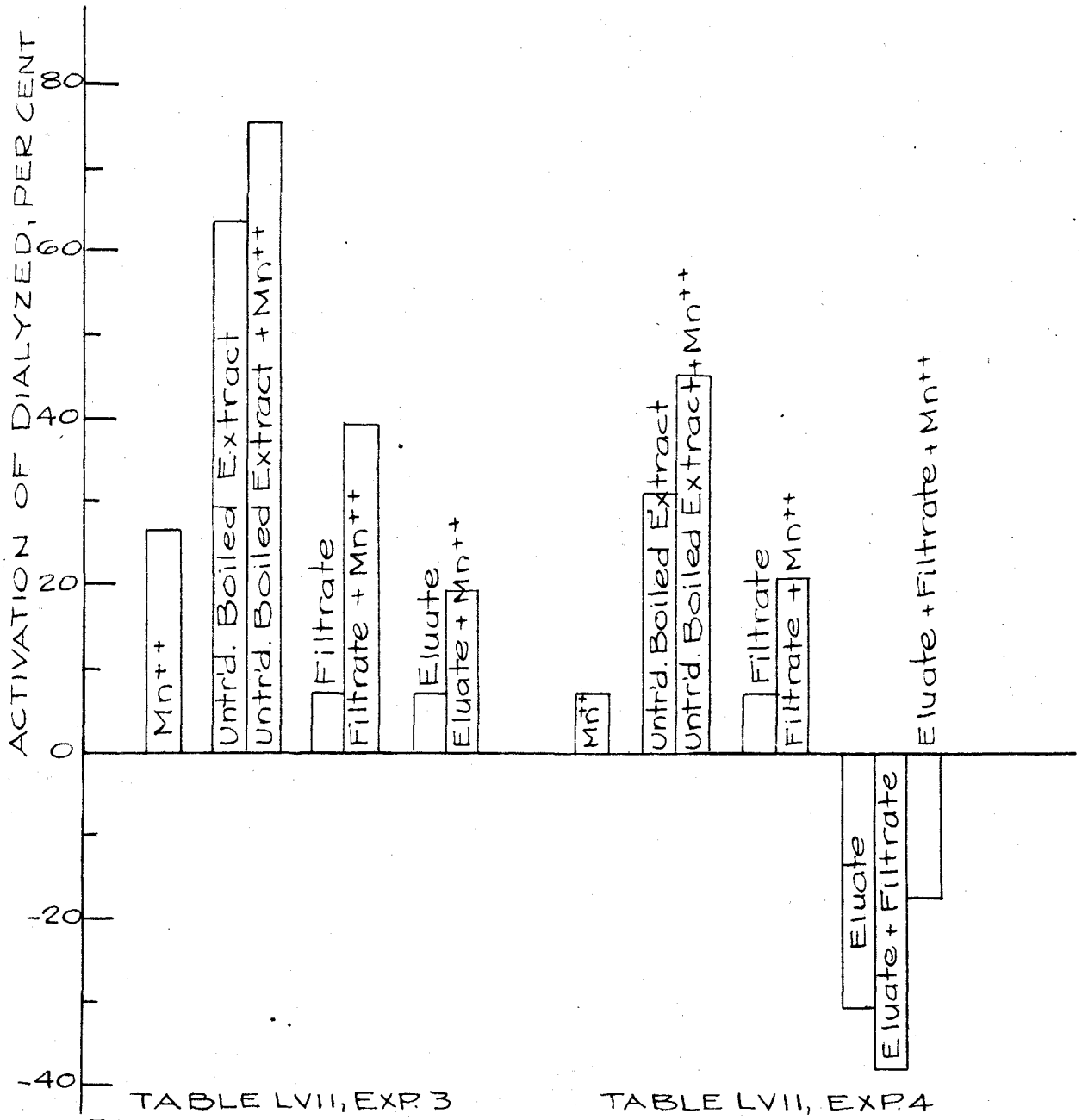

FIG. 10 ACTIVATION OF DIALYZED ENZYME EXTRACTS BY BOILED EXTRACTS TREATED WITH AMBERLITE IR-100-H

with the untreated extract, nor greater than the activation produced by the addition of manganese alone to the dialyzed extract. These results, therefore, support the hypothesis made in connection with the Decalso adsorption experiments, that a cationic material, other than manganese, is responsible, partially at least, for the activating ability of a boiled extract.

Again, however, elution was difficult. The eluate obtained in the third experiment of Table LVII possessed only a slight activating ability, while that obtained in the second and fourth experiments proved to be inhibitory.

A portion of the boiled extract at pH 7.1, described in the third experiment of Table LVI, was passed through a column containing Amberlite 4-B, the anionic exchange resin, and the column eluted with 2 per cent sodium carbonate. When the Amberlite filtrate and eluate were added to a dialyzed enzyme extract, which destroyed 20.3 per cent of the added thiamine, inhibition was observed. The presence of the filtrate decreased the thiamine destruction to 18.8 per cent, an inhibition of 7.4 per cent, while the eluate produced an inhibition of 14.0 per cent and the two together, an inhibition of 18.7 per cent. Since inhibition was observed in all cases, it was impossible

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Footnote continued from page 198

[4] Boiled extract, 25 mg./ml., II-236. Adsorbed at pH 7.0. Elution with 20 per cent KCl. Heavy, orange-red precipitate appeared in eluate upon adjustment to pH 7.4. Undialyzed enzyme extract destroyed 23.0 per cent of the added thiamine.

to determine whether or not an anionic compound, necessary for activation, was being removed by the resin.

In another experiment the cation exchange resin was washed twice with a 5 per cent solution of ammonium chloride followed by a 5 per cent solution of hydrochloric acid. The Amberlite was then washed with water until the washings gave no test for chloride ion. This was done to insure the complete activation of the resin and to insure its being in the hydrogen form. Fifty ml. of a boiled extract, representing 25 mg. per ml. of preparation III-110, was passed through a column containing the resin; and the column washed with water. The filtrate and washings were concentrated in vacuo, and the residue obtained dissolved in water to give a solution representing 50 mg. per ml. The column was then eluted with 5 per cent hydrochloric acid and the eluate likewise concentrated. The residue was dissolved in water to give a concentration equivalent to that of the filtrate.

The results obtained when the untreated boiled extract and the Amberlite solutions were added to a dialyzed enzyme are given in Table LVIII. The Amberlite treatment reduced the activity of the boiled extract, and activity was not restored by addition of either the eluate or manganese. When a 100 mg. equivalent of the eluate was added, inhibition was observed, while the addition of a 50 mg. equivalent, produced slight activation. Furthermore, the results indicated that the addition of two equivalents of the boiled extract did not activate

the enzyme much more than did one equivalent.

Table LVIII

Amberlite IR-100-H Treatment of Boiled Extract

Additions to Dialyzed Extract	Thiamine Destruction	Activation	With Manganese Thiamine Destruction	Activation
	per cent	per cent	per cent	per cent
None	34.7		36.4	4.9
Untreated boiled extract, 100 mg. equiv.	48.1	38.6		
50 mg. equiv.	46.4	33.7	49.3	42.1
Amberlite filtrate, 100 mg. equiv.	40.3	16.2		
50 mg. equiv.	39.7	14.4	45.4	30.9
Amberlite eluate, 100 mg. equiv.	33.4	- 3.7		
50 mg. equiv.	36.5	5.2	39.7	14.4
Filtrate + eluate, 50 mg. equiv. of each	39.7	14.4		

Another boiled extract was treated with Amberlite IR-100-H by a static method instead of by the column exchange technique. Five gm. of the resin was washed with 30 ml. of 5 per cent ammonium chloride, followed by a wash with 5 per cent hydrochloric acid. The material was then washed with water until the washings gave no test for chloride ion and until the pH of the wash solution became constant. Thirty ml. of a boiled extract at pH 4.52 was added to 3 gm. of the washed Amberlite. The mixture was continuously stirred and the pH

checked at intervals until after 11 minutes, the pH was constant at 2.4. The extract was decanted onto a second 2 gm. portion of the Amberlite and again stirred until after 8 minutes the pH was constant at 2.29. After removing the extract, the Amberlite was washed with water and then with 10, 8 and 6 ml. portions of 5 per cent hydrochloric acid. The filtrate and eluate thus obtained were diluted to equal volumes, and their activating effect determined with a dialyzed enzyme preparation. The results, shown in Table LXIX, indicated that the reduction in activity by the static method was not as great as with the column exchange procedure. Furthermore, the efficiency of elution was not improved by this method.

Table LIX

Amberlite IR-100-H Treatment
of Boiled Extract, Static Method

Additions to Dialyzed Extract	Thiamine Destruction	Activation
	per cent	per cent
None	27.0	
Untreated boiled extract	35.4	31.1
Amberlite filtrate	32.3	19.6
Amberlite eluate	25.8	- 4.4
Filtrate + eluate	29.8	10.4

In addition to the Amberlite and Decalso treatments of boiled extracts, solutions containing the residues obtained by ashing portions of the extract were treated in a similar fashion. It was felt that, if the reduction of activity resulting from adsorption were due to the removal of a metallic cation, a solution containing the ash should behave in the same way. In the first experiment a boiled extract, representing 1.0 gm. of preparation II-236, was evaporated to dryness in a platinum crucible, the carbon burned off, and the ashing completed in a muffle furnace at 750-900 degrees. The residue was dissolved in water with a few drops of dilute hydrochloric acid added to effect solution, and diluted to 40 ml., giving a solution containing a 25 mg. equivalent of the original preparation per ml. A portion of this at pH 7.1 was passed through a column containing Amberlite IR-100-H, the column washed, and eluted with 20 per cent potassium chloride. Upon adjustment to pH 7.4, the eluate became yellow-orange in color and finally a precipitate similar to that of ferric hydroxide formed. For analysis of the eluate, this precipitate was kept in suspension.

The results obtained, when these solutions were analyzed for their activating effect on a dialyzed enzyme, are shown in Table LX. Inhibition was observed upon addition of the untreated ash solution, the Amberlite filtrate, and the Amberlite eluate. The addition of manganese overcame this inhibition, but the activation observed in its presence was not very great.

Table LX

Amberlite Treatment of Ash Solution

Additions to Dialyzed Extract	Thiamine Destruction	Activation	With 6.25×10^{-4} M. Mn. Thiamine Destruction	Activation
	per cent	per cent	per cent	per cent
None	23.3		24.8	6.4
Untreated ash solution	21.8	- 6.4	25.2	8.1
Amberlite filtrate	15.9	- 31.8	25.4	9.1
Amberlite eluate	13.7	- 41.2		
Filtrate + eluate	17.5	- 24.9	23.7	0.0

The inactivity of the ash was in contrast to results previously obtained[1]. However, it was possible that oxidation of metallic ions had occurred during the "burning off" of the carbon. Therefore, another boiled extract, representing 1.5 gm. of preparation III-110, was evaporated in the same way, but the crucible was placed in the muffle furance (500-600 degrees) before all of the carbon had been burned off. The white ash was dissolved in water by adding a few drops of dilute hydrochloric acid and diluted to 30 ml., giving a solution containing a 50 mg. equivalent of the original preparation per ml. Twenty ml. of this solution at pH 2.4 was passed through a column containing 7 gm. of activated Decalso, the column washed, and then eluted with a solution of 25 per cent potassium chloride in 0.1 N. hydrochloric acid.

[1] Sealock, R. R., Livermore, A. H. and Davis, N. C., unpublished.

When these solutions were analyzed with a dialyzed enzyme preparation, no activating effect was observed upon addition of the untreated ash solution, although in this case it was not inhibitory. The Decalso filtrate and eluate both inhibited the dialyzed enzyme, while the two in combination produced activation, although this was not as great as that observed with a portion of unashed boiled extract. The thiamine destruction and the activation observed upon addition of these solutions to the dialyzed enzyme are given in Table LXI.

Table LXI

Decalso Treatment of Ash Solution

Additions to Dialyzed Extract	Thiamine Destruction	Activation	With 6.25×10^{-4} M. Mn. Thiamine Destruction	Activation
	per cent	per cent	per cent	per cent
None	23.2		29.5	27.2
Untreated boiled extract	31.8	37.1	36.6	57.8
Untreated ash solution	23.5	0.0	30.8	32.8
Decalso filtrate	22.6	- 2.6	27.0	16.4
Decalso eluate	22.7	- 2.2		
Filtrate + eluate	25.9	11.6		

In general the results obtained with Decalso and Amberlite IR-100-H adsorption of boiled extracts indicate the presence in these extracts of an activating factor of cationic nature. The evidence, however, does not permit the conclusion that

the failure to completely inactivate the extracts is due to the presence of another factor of anionic nature, since adsorption of the cationic factor may have been incomplete. Furthermore, these experiments lend support to the hypothesis that manganese, although it does activate the enzyme, is not the factor or even one of the factors responsible for the activation by a boiled extract.

Several efforts were made to fractionate the boiled extract, in order to obtain the activating factor in a more purified form, so that it could be studied in greater detail. In the first experiment 10 ml. of a boiled extract representing 50 mg. per ml. of preparation III-110 was fractionated with alcohol in the following way:

1. Ten ml. of the boiled extract at pH 5.4 was mixed with 1 volume of absolute alcohol, and allowed to stand in an ice-bath with frequent stirring for 40 minutes. The solution was then centrifuged and decanted. The residue was dried in vacuo and dissolved in 5 ml. of water, thus giving a concentration equivalent to 100 mg. per ml. This fraction, insoluble in 50 per cent alcohol was labeled Fraction I.

2. The mother liquor from 1 was treated with 20 ml. of absolute alcohol and kept in an ice-bath with frequent stirring for 30 minutes, after which time it was centrifuged and decanted. The residue was dried in vacuo, and dissolved in 5 ml. of water, thus giving a concentration equivalent to 100 mg. per ml. This fraction, insoluble in 75 per cent alcohol was labeled Fraction II.

3. The mother liquor from 2 was treated with 70 ml. of absolute alcohol and kept in an ice-bath with frequent stirring for 1 hour, after which time it was centrifuged and decanted. The residue was dried in vacuo, and dissolved in water, thus giving a concentration equivalent to 100 mg. per ml. This fraction, insoluble in 91 per cent alcohol was labeled Fraction III.

4. The mother liquor from 3 was treated with 300 ml. of absolute alcohol and kept in an ice-bath for one and one-half hours. Since no visible precipitate was observed at this time, the solution was concentrated in vacuo in an atmosphere of carbon dioxide. The dry residue was washed from the flask and diluted to 5 ml. with water. This fraction, soluble in alcohol was labeled Fraction IV.

The activating effect of each of these fractions, adjusted to pH 7.4, was determined with a dialyzed enzyme extract, using 50 mg. equivalents of each. The results, shown in Table LXII, indicate that the activating factor is precipitated with both Fractions II and III. However, complete activity was not recovered, as is evidenced by a comparison of the activation observed with a portion of untreated boiled extract and the sum of the activations produced by Fractions I, II and III. The reason for the inhibition by Fraction IV was not apparent, but a similar effect was observed when butanol fractions, which had been concentrated in vacuo, were analyzed. It may be that the process of concentration or the effect of the carbon dioxide present during the concentration altered a constituent of the extract in such a way as to make it inhibitory.

Table LXII

Alcohol Fractionation of Boiled Extract

Addition to Dialyzed Extract	Thiamine Destruction	Activation
	per cent	per cent
None	17.0	
Untreated boiled extract	23.7	39.4
Fraction I, insoluble in 50 per cent alcohol	17.6	2.3
Fraction II, insoluble in 75 per cent alcohol	19.4	14.1
Fraction III, insoluble in 91 per cent alcohol	18.8	10.6
Fraction IV, alcohol soluble	8.9	- 47.6
Fractions I, II, III, and IV	12.3	- 27.7

A similar experiment was carried out with fractions obtained at different alcohol concentrations and with the final alcohol soluble fraction concentrated in an air atmosphere. The boiled extract represented 75 mg. per ml. of I-142-I-A, and it was prepared by extracting the powder with 0.04 M. phosphate containing 2 per cent sodium chloride. An outline of this fractionation follows:

1. Fifteen ml. of boiled extract, at pH 4.5, was mixed with an equal volume of absolute alcohol, and allowed to stand in an ice-bath with frequent stirring for 1 hour. It was then centrifuged and decanted, and the residue dried in vacuo. The colorless dry residue was taken up in 7.5 ml. of water, but since it was not completely soluble, it was kept in suspension for analysis. This fraction, insoluble in 50 per cent alcohol was labeled Fraction I.

2. The mother liquor from 1 was treated with 150 ml. of absolute alcohol and allowed to stand in an ice-bath for 2 hours, after which time it was centrifuged and decanted. The vacuum dried residue was not homogeneous but contained a yellow glassy material as well as some white powdery material. It dissolved easily in 7.5 ml. of water. This fraction, insoluble in 91 per cent alcohol was labeled Fraction II.

3. The mother liquor from 2 was treated with 150 ml. of absolute alcohol and kept in an ice-bath for 2 hours, after which time it was centrifuged and decanted. The white residue obtained was dried in vacuo and dissolved in 7.5 ml. of water. This fraction, insoluble in 95.5 per cent alcohol was labeled Fraction III.

4. The mother liquor from 3 was concentrated to dryness in vacuo with an air atmosphere. The residue was washed from the flask and diluted to 7.5 ml. with water, giving a clear yellow solution. This fraction, soluble in 95.5 per cent alcohol was labeled Fraction IV.

The activity of these fractions was determined at a concentration equivalent to 50 mg. of the original preparation, and the results of this determination are shown in Table LXIII. Here again activity was divided between two fractions, II and III, although there was not a complete recovery of activity. Again the alcohol soluble fraction, which this time had been concentrated in an air atmosphere, was inhibitory.

Table LXIII

Alcohol Fractionation of Boiled Extract

Additions to Dialyzed Extract	Thiamine Destruction	Activation
	per cent	per cent
None	36.5	
Untreated boiled extract	47.8	31.0
Fraction I, insoluble in 50 per cent alcohol	34.2	- 6.3
Fraction II, insoluble in 91 per cent alcohol	40.0	9.6
Fraction III, insoluble in 95.5 per cent alchol	40.7	11.5
Fraction IV, alcohol soluble	17.7	- 51.5
Fractions I, II, III and IV	10.7	- 70.7

In a third experiment, similar fractionations were made, but in an effort to overcome the inhibitory effect observed previously with the alcohol soluble fraction, this was concentrated in vacuo using a nitrogen atmosphere. The tank nitrogen used was passed through a solution of pyrogallol before admission to the distilling flask. In this fractionation the boiled extract was the same as that used in the preceding experiment, and an outline of its fractionation follows:

1. Ten ml. of the boiled extract at pH 4.5 was mixed with 15 ml. of absolute alcohol and allowed to stand overnight in the ice-box to permit complete precipitation, and then the solution was centrifuged and decanted. The residue, after drying in vacuo, was dissolved in 10 ml. of water. The solution was colorless, but the residue was not completely soluble. However, the insoluble material was kept in suspension for analysis. This fraction, insoluble in 50 per cent alchol was labeled Fraction I.

2. The mother liquor from 1 was treated with 300 ml. of absolute alcohol and allowed to stand in an ice-bath for 3 hours, after which time it was centrifuged and decanted. The residue, after drying in vacuo, was readily soluble in 10 ml. of water, giving a clear yellow solution. This fraction, insoluble in 95 per cent alcohol was labeled Fraction II.

3. The mother liquor from 2 was concentrated to dryness in vacuo using a nitrogen atmosphere, as described above. The residue was washed from the flask and diluted to 10 ml. with water. It was readily soluble, giving a clear yellow solution. This fraction, soluble in alchol, was labeled Fraction III.

These fractions were analyzed with a dialyzed enzyme preparation and the results, shown in Teble LXIV, again indicate the alcohol insolubility of the activating factor. However, the alcohol soluble fraction was again inhibitory. This inhibition was probably not due to oxidation of some component of the boiled extract, since concentration in both nitrogen and carbon dioxide atmospheres produced fractions which were inhibitory. Then, too, the previous work dealing with the stability of boiled extracts, indicated that aeration, under oxidizing conditions, did not cause the fractions to be inhibitory. These results might be interpreted to mean that a naturally occurring inhibitor had been isolated by the alcohol fractionation, but at present no definite conclusions regarding this possibility can be made.

Table LXIV

Alcohol Fractionation of Boiled Extract

Additions to Dialyzed Extract	Thiamine Destruction	Activation
	per cent	per cent
None	27.9	
Untreated boiled extract	44.8	60.6
Fraction I, insoluble in 60 per cent alcohol	26.9	- 3.6
Fraction II, insoluble in 95 per cent alcohol	29.1	4.3
Fraction III, alcohol soluble	13.5	- 51.6
Fraction I plus Fraction II	32.6	16.8
Fraction II plus Fraction III	13.2	- 52.6

Although there was never complete recovery of activity in any of the fractions obtained by alcohol precipitation of the boiled extract, the results indicated that at least one of the factors responsible for the activating effect of boiled extract is insoluble in high concentrations of alcohol. These fractionation experiments raised the question of why the alcohol soluble fractions, concentrated in air, carbon dioxide or in nitrogen, were such potent inhibitors. However, no answer to this question is available.

L. Characterization of the Pyrimidine Derivative

Because of the failure to reverse thiaminase action, the importance of characterizing the products of the enzymatic reaction became obvious. It has been generally accepted that 4-methyl-5-β-hydroxyethylthiazole is liberated by the action of the enzyme on thiamine, but the fate of the pyrimidine portion of the vitamin has not been clearly established. Krampitz and Woolley (30) suggested that 2-methyl-6-amino-5-hydroxymethylpyrimidine is the final product, but that the pyrimidine is first liberated in the form of an unknown derivative. Although Hennessey and Warner (47) reported the isolation of a pyrimidine derivative, the compound was not identified, nor has any further information regarding its nature been published since the initial report in 1946.

The identification of the pyrimidine derivative or intermediate would furnish information regarding the mechanism of thiamine destruction and would aid in determining whether it is a one or two step reaction. If it proved to be a complex reaction, knowledge of the intermediate would help in establishing whether one or both steps are enzymatic. Only after the solution of these problems, can the question of whether or not thiaminase action is reversible be answered.

Therefore, before making further attempts to reverse the reaction of thiaminase, an effort was made to characterize

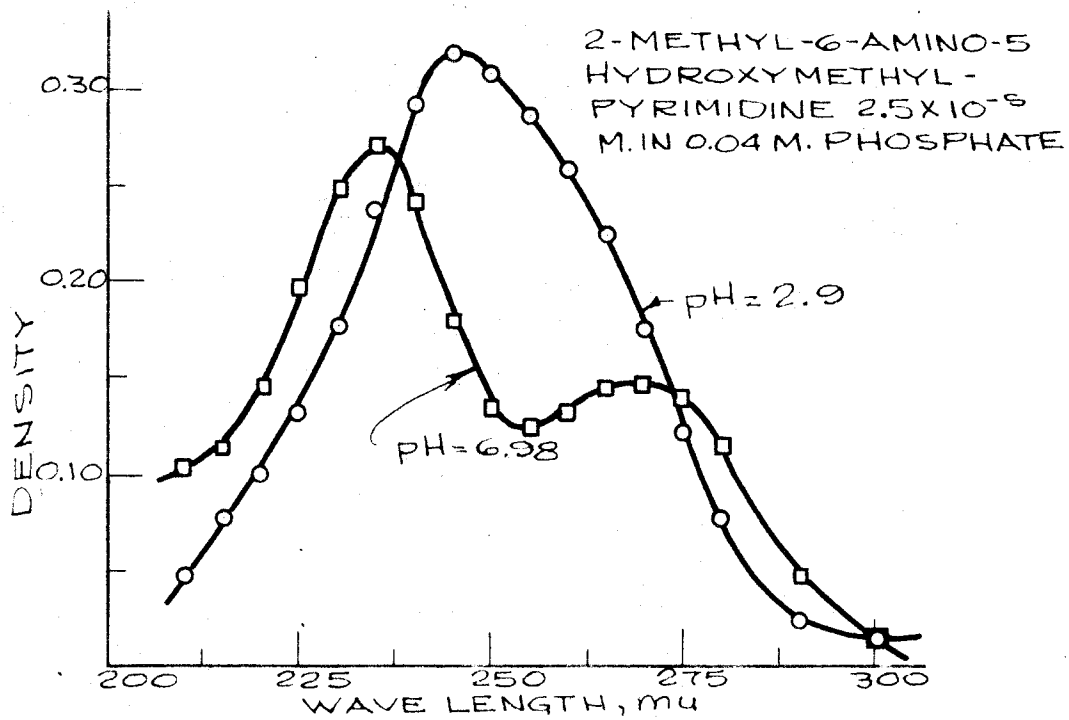
the pyrimidine intermediate. At the time no chemical methods for direct analysis of amino pyrimidines similar to the pyrimidine moiety of thiamine were available. Microbiological assays of the type which led Krampitz and Woolley (30) to postulate the existence of an intermediate might be employed in this work. Recently Tinker and Brown (60) have reported an adaptation of the Craig counter-current procedure for the separation and identification of pyrimidine derivatives. However, since pyrimidines exhibit characteristic absorption spectra, it was felt that the pyrimidine derivative formed by the enzymatic destruction of thiamine could be studied and perhaps characterized by its ultraviolet absorption spectrum. Spectrophotometric analyses of reaction mixtures were therefore carried out using the Beckman spectrophotometer and determining absorption between 200 and 300 millimicrons.

In the 1930's, when efforts were being made to establish the structure of thiamine, the ultraviolet absorption spectra of a number of different pyrimidines were determined (61-63). It was found that mono- and di-methyl derivatives of 6-amino and 6-hydroxy pyrimidines exhibited two absorption maxima in neutral and alkaline solution, while only one maximum was exhibited in acid solution. The spectrum for thiamine also exhibited two absorption maxima in alkaline solution and one in acid solution. Loofbourow (64) reviewed the earlier work concerning the absorption spectra of thiamine and pyrimidines,

and stated that the "changes of absorption maxima of 6-amino pyrimidines as a function of pH are qualitatively analogous to those observed in thiamine". From these results it appeared that the characteristic thiamine spectrum was a function of its pyrimidine nucleus, and that its thiazole ring contributed but little to absorption in the ultraviolet range.

In order to make comparisons of the absorption curve obtained upon analysis of the pyrimidine derivative formed by the enzymatic destruction of vitamin B_1 with those of thiamine and of typical pyrimidines, the absorption spectra of the vitamin and of 5-bromomethyl-, 5-methylenesulfonic acid, and 5-hydroxymethyl-2-methyl-6-aminopyrimidine were determined. In Figures 11 and 12 the absorption curves, plotted as density vs. wave length are given. Each compound was dissolved in 0.04 M. phosphate buffer to give a concentration of 2.5×10^{-5} M., and as indicated, absorption in the ultraviolet region was determined in both acid and neutral solution. In neutral solution all four compounds exhibited two absorption bands, as evidenced by an absorption maximum at 230 to 240 millimicrons and one at 265 to 275 millimicrons. In acid solution, only one absorption band with a maximum at 245 to 250 millimicrons was observed.

In Table LXV the molecular extinction coefficients for these compounds at three different wave lengths are listed. These were calculated by dividing the density, which is equal



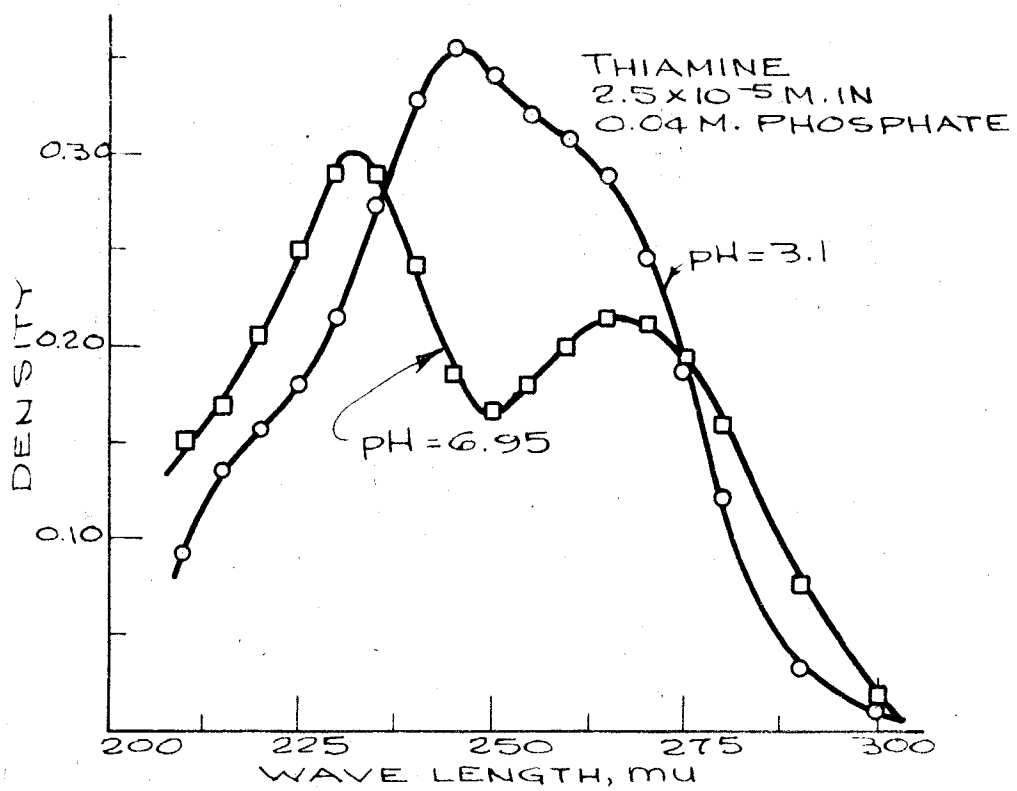


FIG. 11 ABSORPTION SPECTRA OF THIAMINE AND 2-METHL-6-AMINO-5-HYDROXY-METHYL PYRIMIDINE.

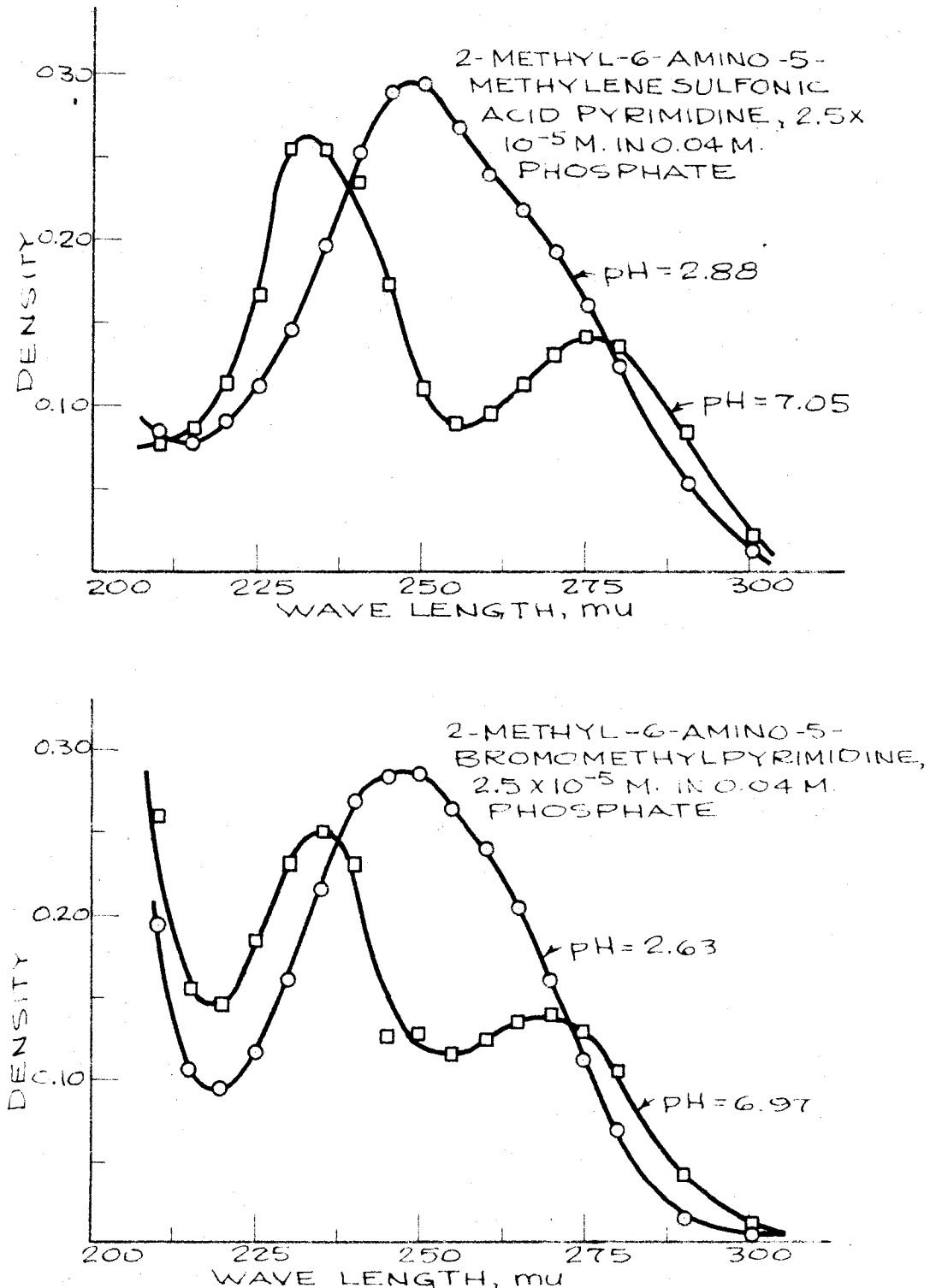


FIG. 12 ABSORPTION SPECTRA OF 2-METHYL-6-AMINO-5-BROMOMETHYLPYRIMIDINE AND 2-METHYL-6-AMINO-5-METHYLENESULFONIC ACID PYRIMIDINE.

to log I_o / I, observed at these wave lengths by the molar concentration (M.). As can be seen from the equation below, the depth (d) of the solution did not have to be considered, since 1 cm. cells were used.

$$\epsilon \text{(Molecular extinction coefficient)} = \frac{1}{M. \times d} \times \log \frac{I_o}{I}$$

Table LXV

Molecular Extinction Coefficients for Thiamine and for Three Pyrimidines

Compound	Wave Length					
	265 mμ		250 mμ		235 mμ	
	acid	neutral	acid	neutral	acid	neutral
Thiamine	11,600	8,550	13,700	6,720	10,900	11,600
2-Methyl-3-aminopyrimidines:						
5-Bromomethyl-	8,230	5,470	11,400	5,150	8,660	10,000
5-Hydroxymethyl-	8,890	5,760	12,300	5,330	9,400	10,800
5-Methylene-sulfonic acid	8,770	4,560	11,800	4,440	7,920	10,200

In order to carry out spectrophotometric analyses of the pyrimidine formed by enzymatic destruction of thiamine, it was necessary first to determine what relative concentrations of protein (enzyme) and thiamine were adaptable to such studies. Efforts were made to insure complete destruction of thiamine so that there would be no interference due

to remaining unaltered substrate; and yet in order to keep absorption due to materials other than the thiamine breakdown products at a minimum, the enzyme concentration had to be kept as low as possible.

In the first series of experiments the protein was not removed prior to spectrophotometric analysis, but proper controls were included so that absorption due to protein and other materials present in the extract could be determined. For example, an extract representing 75 mg. of preparation II-196, incubated with 2.5 micromoles of thiamine, completely destroyed the vitamin in two hours. The experimental solutions (2 ml. enzyme extract, 2 ml. water and 1 ml. of thiamine), both incubated and unincubated, were diluted with 5 ml. of water and filtered. In addition control tubes containing no thiamine, both incubated and unincubated, were prepared and treated in a similar fashion.

Further dilutions of 1 to 100 and 1 to 10 were made with 0.04 M. phosphate buffer (pH 7.4) and spectrophotometric readings made with the Beckman spectrophotometer using the buffer as a blank. With both of these dilutions the absorption curves for experimental and control solutions were parallel. However, in the sample diluted 1 to 100, the calculated concentration of thiamine and of its products was only 2.5×10^{-6} M.

and perhaps a good separation of the curves could not be expected. A portion of the control solution (the unincubated solution containing no thiamine) was diluted 1 to 1000 and to a part of this thiamine was added to give a concentration of 2.5×10^{-5} M. With this dilution the absorption due to the protein had been reduced to such an extent that a good absorption curve for thiamine was obtained.

Later a 10 mg. equivalent of preparation II-196 was found to completely destroy 0.25 micromoles of thiamine in 4 hours of incubation. The incubated solutions (both with and without thiamine) and the "zero time" samples (both with and without thiamine) were diluted to 10 ml. and spectrophotometrically analyzed. The concentration of thiamine and of the thiamine breakdown products in the "zero time" and incubated samples respectively was 2.5×10^{-5} M. The plot of density vs. wave length for each of these solutions is shown in Figure 13. In addition the plot of the density values obtained by subtracting the densities of the control solutions (containing no thiamine) from those of the experimental solutions is shown in Figure 14. By this procedure the absorption due to materials present in the enzyme extract was eliminated and the resulting curve should be that of the added substrate or its products. The curve thus obtained for the "zero time" solutions is typical of thiamine, showing two absorption bands with maxima at 230 and 265 millimicrons. However, a similar

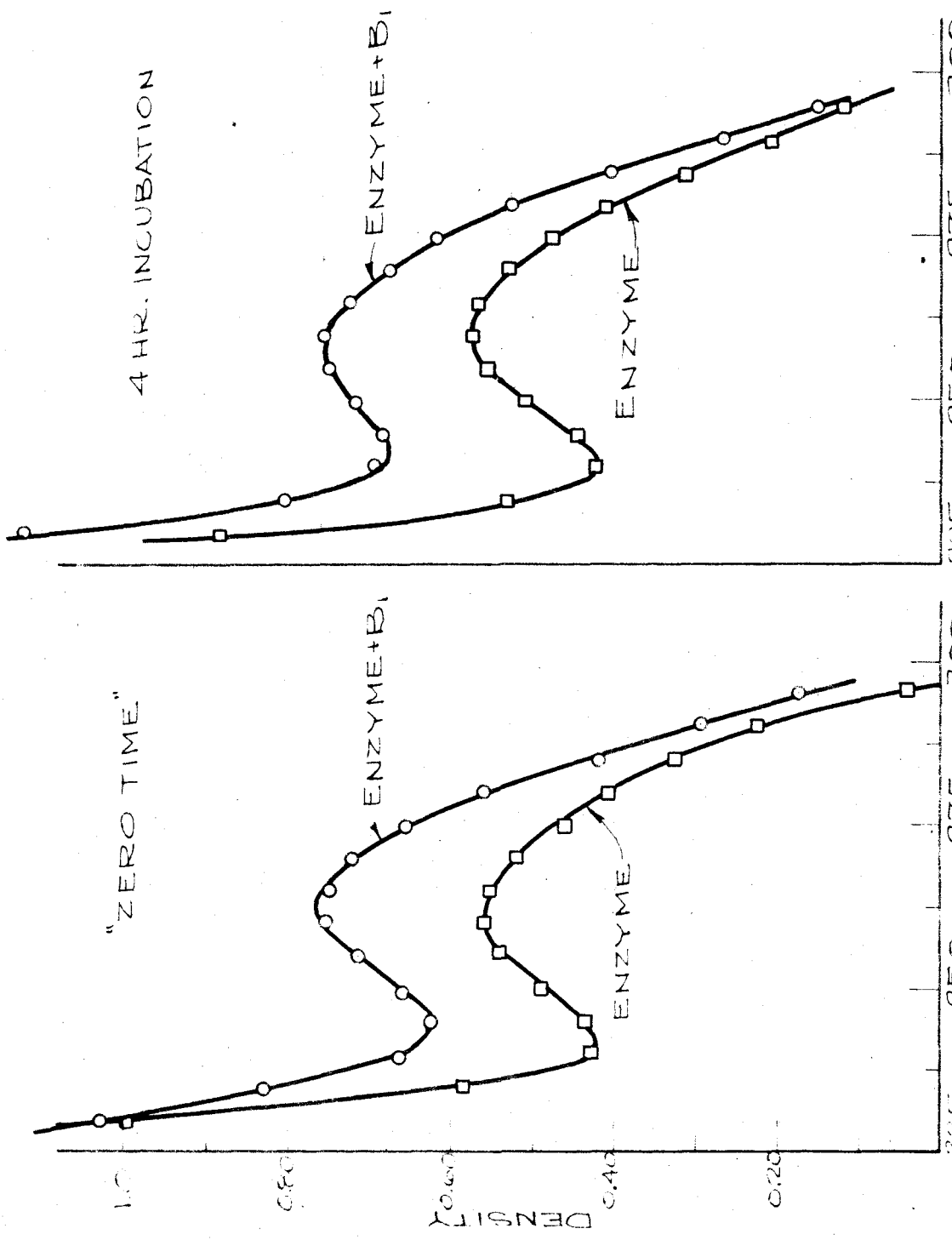


FIG. 13 ABSORPTION SPECTRA OF REACTION MIXTURES WITH AND WITHOUT THIAMINE AT "ZERO TIME" AND AFTER 4 HR. INCUBATION.

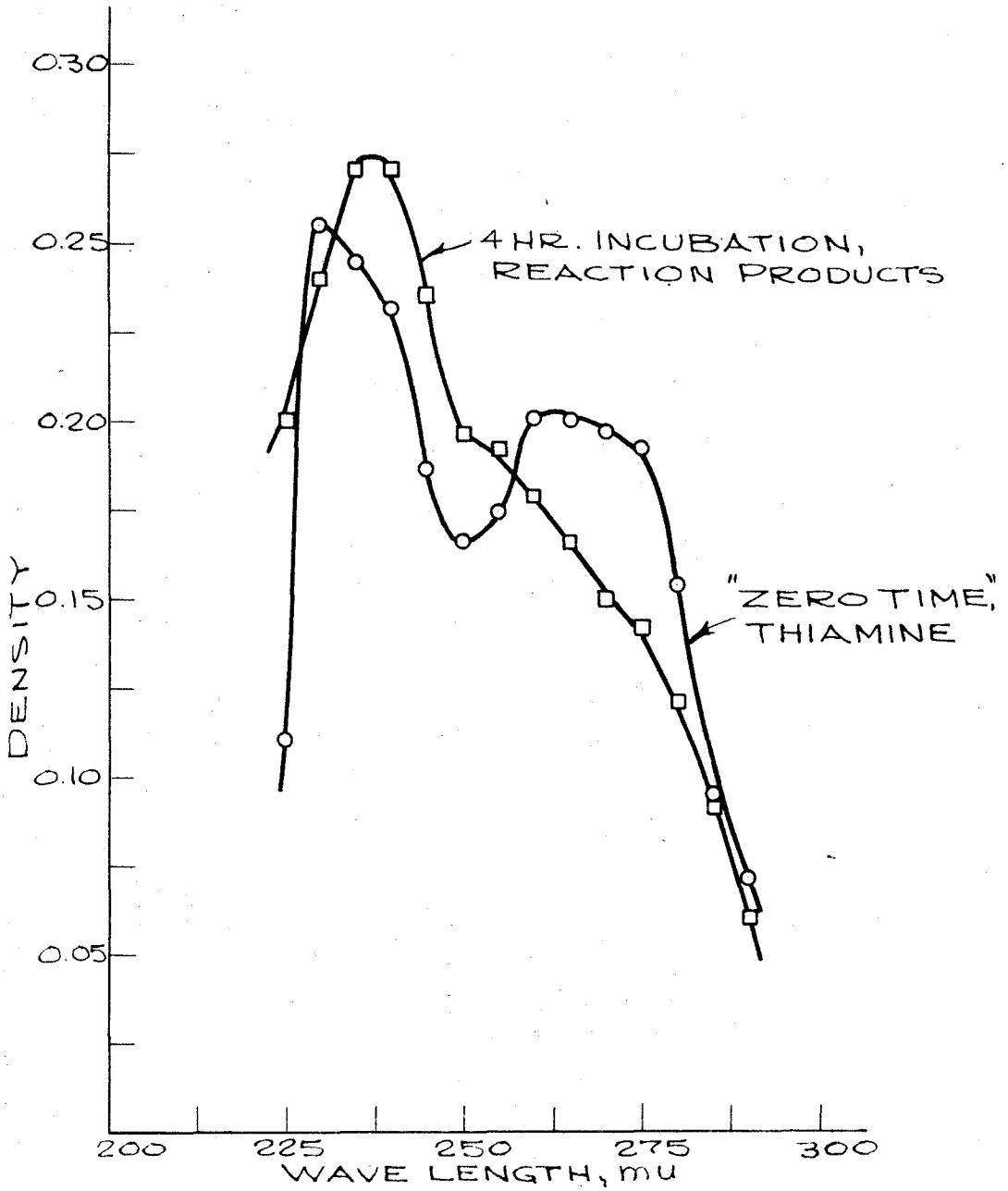


FIG. 14 ABSORPTION SPECTRA OF THIAMINE AND REACTION PRODUCTS REPRESENTED BY SUBTRACTED DENSITY VALUES.

curve for the incubated samples shows only one absorption maximum at 235 millimicrons. The absorption maximum at 270 to 275 millimicrons, characteristic of thiamine and amino pyrimidine compounds is not present.

Later the protein was removed by both acid and alcohol precipitation before spectrophotometric analyses were made. A 50 mg. equivalent of preparation II-196 was incubated with 2.5 micromoles of thiamine until destruction of the vitamin was complete, and then the protein was precipitated by the addition of an equal volume of 0.5 N. hydrochloric acid. The filtrates obtained were diluted 1 to 10 with a solution of 4 per cent sodium chloride in 0.08 M. phosphate buffer giving a theoretical concentration of thiamine or its products in "zero time" and incubated samples respectively of 2.5×10^{-5} M. A plot of the subtracted density values (density of experimental solution less that of the control) gave curves which were similar to those shown in Figure 14. These results indicated then that it was possible to free the solutions from protein without altering the compounds. Even though protein had been removed, the control samples still showed a high degree of absorption between 200 and 300 millimicrons, but nevertheless, the precipitation of protein permitted the use of higher enzyme and substrate concentrations

in the initial "zero time" and incubation mixtures.

The procedure used in this experiment for obtaining the absorption curves for thiamine and its products by subtracting the density values was used in all of the following work. Unless otherwise stated, it should be understood that in all cases both incubated and unincubated control solutions containing no thiamine were prepared and treated in the same way as the experimental solutions.

Alcohol precipitation was also employed and the results were similar to those obtained by hydrochloric acid precipitation of the protein. The absorption curve for the products of thiamine destruction again showed the flattening out of the typical thiamine and pyrimidine peak at 260 to 270 millimicrons.

Since Krampitz and Woolley (30) had shown that the pyrimidine intermediate was changed to the pyrimidine alcohol upon alkali treatment, a filtrate, containing a 2.5×10^{-5} M. concentration of the pyrimidine derivative, was allowed to stand over-night in the ice-box at pH 10.5. However, this treatment did not alter the shape of the absorption curve. If the failure to obtain a good pyrimidine curve were due to the nature of the derivative, conversion to the pyrimidine alcohol, which gives the characteristic pyrimidine absorption curve (Figure 11), might have been expected to give such a curve. However, at least under these conditions, no change was apparent.

Since the failure to obtain the pyrimidine absorption curve might have been due to the presence of interfering substances or perhaps to the formation of a complex with other materials present in the enzyme extracts, attempts were made to further purify the pyrimidine derivative. Five ml. aliquots of solutions containing 2.5×10^{-4} M. thiamine or the derivative, which had not been deproteinized, were extracted at pH 8.5 with three 5 ml. portions of n-butanol. The butanol was evaporated to dryness in an air-stream and the residue dissolved in 10 ml. of 0.04 M. phosphate containing 2 per cent sodium chloride. If extraction had been complete, these solutions would then contain 1.25×10^{-4} moles per liter of thiamine or of the pyrimidine. The spectrophotometric curve obtained for the "zero time" samples was typical of thiamine, while the curve for the pyrimidine was similar to those previously observed. The characteristic absorption peak in the region of 265 millimicrons was not evident. These results, however, indicated that the thiamine breakdown products were extracted from the alkaline reaction mixture by butanol. A portion of these solutions was then made alkaline and incubated for 48 hours at 37 degrees. Again this alkali treatment produced no change in the shape of the pyrimidine absorption curve.

Since Hennessy and Warner (47) reported that the pyrimidine intermediate, which they obtained from enzymatic destruction of thiamine by raw clams, was adsorbed on Decalso, attempts were made to partially purify the pyrimidine derivative by such a procedure. Thiamine was enzymatically destroyed with an extract of preparation II-196, and 4 ml. portions of "zero time" and incubated samples were diluted to 100 ml. and each adjusted to pH 4 to 4.5. Fifty ml. of each of these solutions was adsorbed on 3 gm. of activated Decalso, the columns washed with water, and then eluted with 25 per cent potassium chloride in 0.1 N. hydrochloric acid. When the eluates, diluted to 25 ml., were adjusted to pH 7.4, a white gelatinous precipitate formed, and this was removed by filtration.

If adsorption and elution had been complete the eluates of the experimental samples should have contained 4×10^{-5} moles per liter of thiamine and its products for the "zero time" and incubated solutions respectively. Spectrophotometric analysis of these solutions was made using the potassium chloride-hydrochloric acid solution as a blank. The absorption curves obtained by plotting the subtracted density values are shown in Figure 15 (A). The typical thiamine curve was observed with the zero time samples, while the incubated solutions containing the pyrimidine derivative gave

an absorption curve, in which there was an indication of the typical pyrimidine absorption maximum at approximately 265 millimicrons. However, in further experiments it was observed that the removal of the gelatinous precipitate from the eluate left the solution at a slightly acid pH. Pyrimidine absorption curves have already been shown to be pH dependent and this may have been the case here. Later, when a Decalso eluate was adjusted to pH 9.0 after filtration, a good pyrimidine curve with two absorption maxima at 235 and 275 millimicrons was obtained for an incubated sample containing the pyrimidine derivative, Figure 15 (B). These absorption maxima were almost identical with those obtained by Hennessy and Warner (47) for their derivative. They reported absorption maxima at 236 and 279 millimicrons in dilute alkali and at 246 millimicrons in dilute acid.

From these results it appeared that the pyrimidine intermediate had been purified to such an extent that its absorption curve became similar to those of the pyrimidine compounds first studied. It also became apparent that the effect of pH on the absorption of the derivative was similar to the pH effect on the absorption of the other pyrimidine compounds.

In a further attempt to purify the pyrimidine intermediate, 5 ml. aliquots of filtrates obtained by hydrochloric acid precipitation of protein were adjusted to pH 8.8 and extracted with three 3 ml. portions of anhydrous ether. After

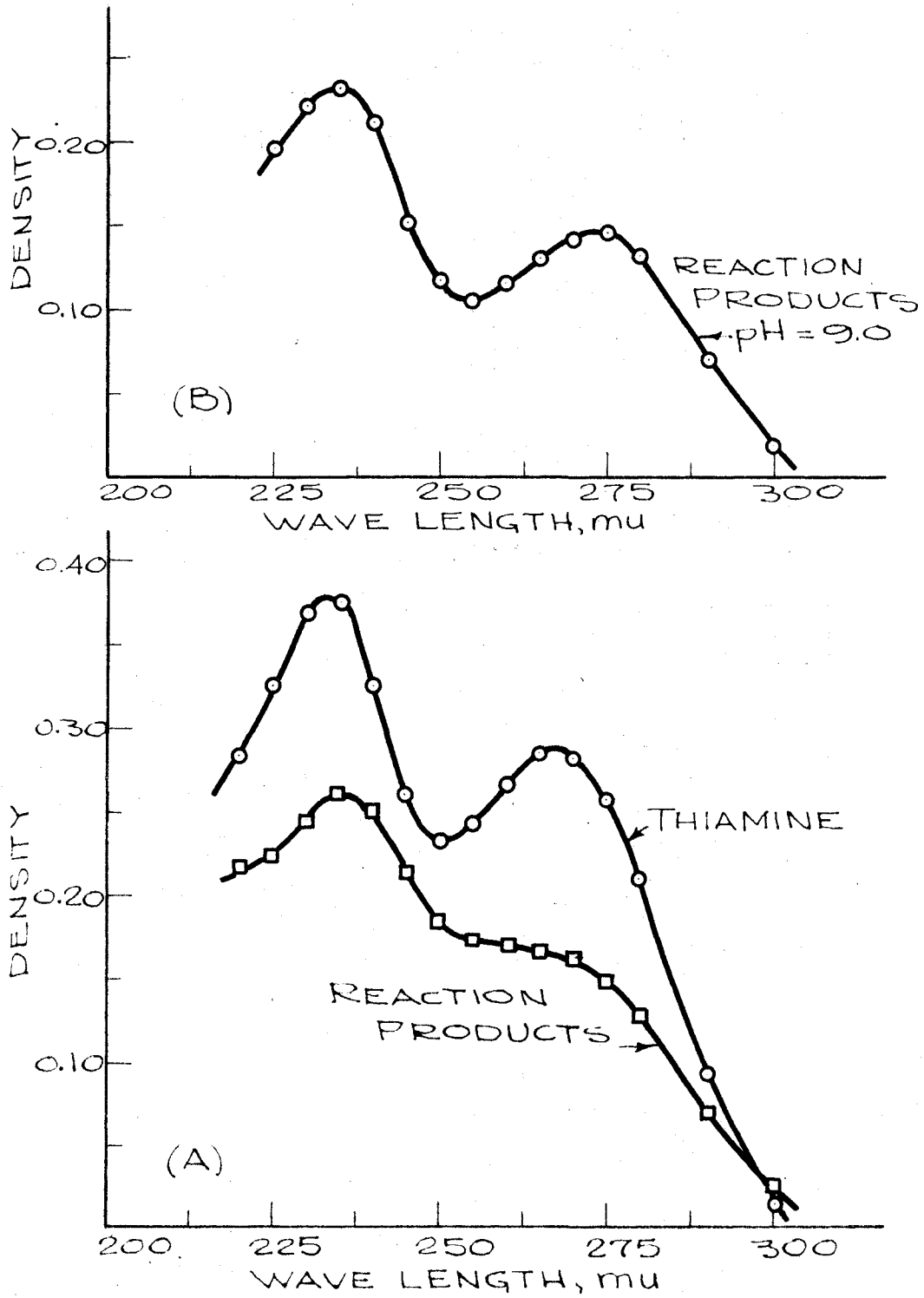


FIG. 15 ABSORPTION SPECTRA OF THIAMINE AND REACTION PRODUCTS OBTAINED BY ANALYSIS OF DECALSO ELUATES.

removing the ether by aeration the aqueous layers were diluted to 10 ml. with a dilute phosphate buffer (pH 7.4) to give a 1.25×10^{-4} M. concentration of the derivative. In addition 5 ml. aliquots of Decalso eluates containing a theoretical 4×10^{-5} M. concentration of the derivative, after adjustment to pH 9.0, were extracted with five 3 ml. and five 2 ml. portions of ether. After freeing them from ether these solutions were also analyzed spectrophotometrically.

The absorption curve for the derivative obtained with the ether extracted hydrochloric acid filtrate gave more indication of a pyrimidine curve than did the filtrate itself. However, here again this was evidenced more by a flattening and levelling off of the curve than by the actual appearance of an absorption maximum. The ether extracted Decalso eluate, on the other hand, gave a curve which was quite similar to the typical pyrimidine curve, but which was also similar to the absorption curve obtained when a Decalso eluate was spectrophotometrically analyzed at pH 9.0 (Figure 15 (B)). Although ether extraction did not significantly alter the character of the absorption curve obtained for Decalso eluates, it appeared to be removing some substance from the filtrates, which had been interfering with the characteristic pyrimidine absorption. However, extraction was probably incomplete.

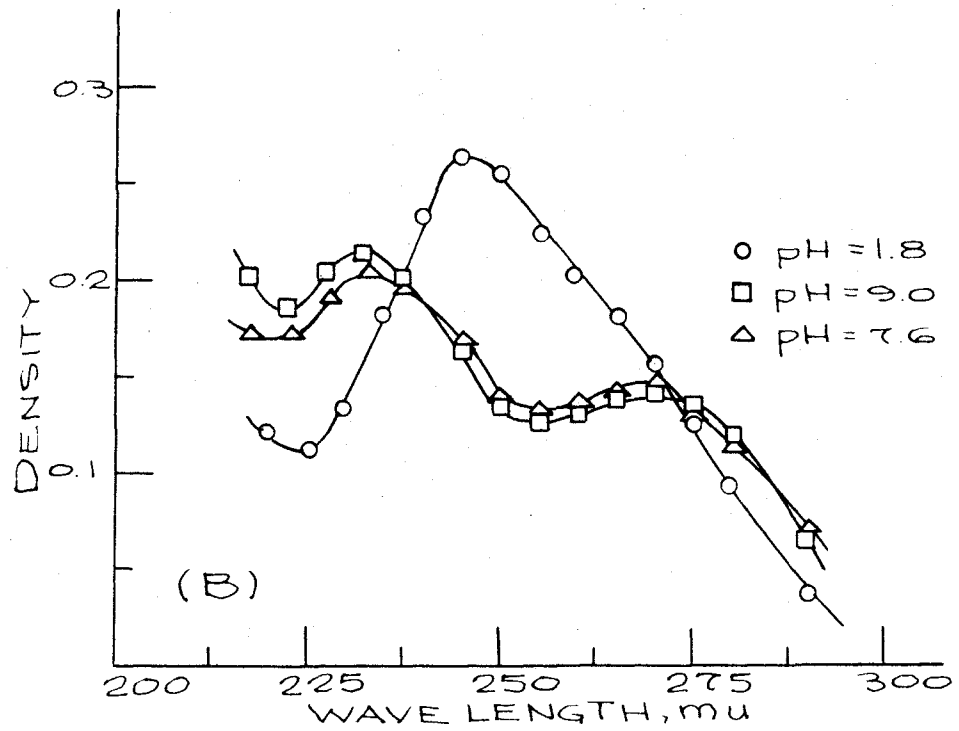
The effect of pH on the shape of the absorption curve of the pyrimidine derivative was emphasized by an experiment

in which hydrochloric acid filtrates were treated with equal volumes of 6 N. sodium hydroxide and heated in a boiling water bath for one-half hour. After dilution, the solutions containing the derivative in a concentration of 2×10^{-5} M. were analyzed spectrophotometrically at both pH 7.2 and pH 9.5. At the higher pH a fair pyrimidine curve was obtained whereas at the lower pH the flat curve previously observed was obtained. Therefore the appearance of the pyrimidine curve at pH 9.5 may have been due to the change of pH of reading rather than to the alkali treatment. However, for the pyrimidines first studied, typical pyrimidine absorption curves were obtained when their solutions were analyzed both at a neutral and at an alkaline pH. It was possible that in contrast to these pyrimidines, the derivative itself was of such a nature that a slight change in pH altered its absorption, or it may be that it was in combination with some other substance from which it was freed at the higher pH.

The pH effect on the nature of the absorption curve for the pyrimidine derivative was further studied. Fifteen ml. portions of hydrochloric acid filtrates at pH 9.3 were extracted with nine 5 ml. portions of butanol. The residue after evaporation was dissolved in dilute phosphate and after dilution to give a theoretical concentration (assuming complete extraction) of 2.5×10^{-5} M., was spectrophotometrically analyzed at pH 7.5 and pH 9.3.

Portions of these solutions were submitted to additional purification by Decalso adsorption, and the eluates were analyzed at pH 1.9, 7.6 and 9.0. The absorption curves for the pyrimidine derivative are shown in Figure 16. Those for the butanol soluble fraction at both pH 7.5 and 9.3 were similar to those previously obtained with no appearance of a maximum in the region of 265 millimicrons. The failure to obtain a pyrimidine curve with such fractions might indicate that an interfering substance was extracted with the pyrimidine derivative. The absorption curves obtained with the Decalso eluates were characteristic of pyrimidines in acid, neutral and alkaline solutions. Two absorption maxima at 235 and 270 millimicrons were observed in alkaline and neutral solutions, while only one maximum at 245 millimicrons was evident with the acid solution. These results, however, indicated that the Decalso treatment was either freeing the pyrimidine from an interfering substance or altering its nature in such a way that it exhibited the typical pyrimidine absorption spectrum.

Although the similarity with respect to wave lengths of maximum and minimum absorption of the pyrimidine absorption curves to those of thiamine made it appear that the thiazole portion of the molecule was contributing but little to the characteristic absorption of the vitamin, an effort was made to determine whether 4-methyl-5-β-hydroxyethylthiazole might not be the substance, which was apparently interfering with



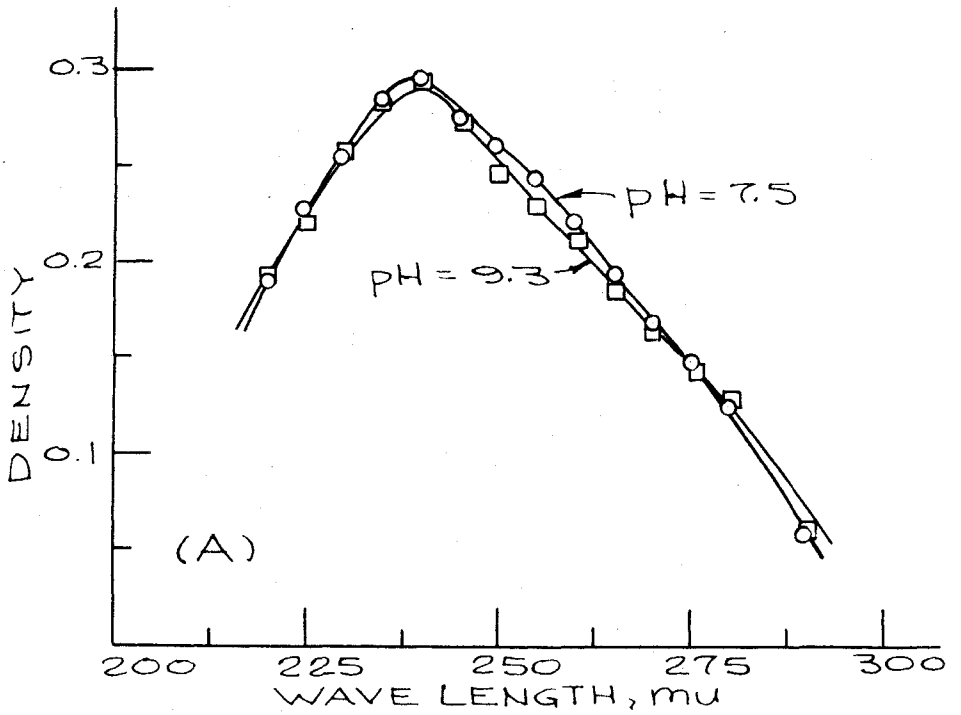


FIG. 16 ABSORPTION SPECTRA OF REACTION PRODUCTS, (A) BUTANOL SOLUBLE FRACTION OF REACTION MIXTURE (B) DECALSO ELUATE PREPARED FROM BUTANOL SOLUBLE FRACTION.

the pyrimidine absorption. A solution containing both 2-methyl-6-amino-5-hydroxymethylpyrimidine and the thiazole in concentrations of 2.5×10^{-5} M. in 0.04 M. phosphate was spectrophotometrically analyzed at pH 2.9, 6.84 and 9.3. The absorption curves exhibited in the neutral and alkaline solutions (Figure 17) were similar to those obtained when filtrates and butanol soluble fractions of the enzyme-thiamine reaction mixtures were studied.

Thus it appeared that 4-methyl-5-β-hydroxyethyl-thiazole, also a product of thiamine destruction, had been responsible for the failure to obtain a typical absorption curve for the pyrimidine derivative. Since thiazole is not adsorbed on Decalso, the reason for the appearance of typical absorption curves, when Decalso eluates were analyzed, becomes evident. It also appeared that ether extraction of a reaction mixture removed the thiazole and left the pyrimidine derivative in the aqueous solution. The effect of the thiazole molecule on the pyrimidine absorption curve was not as marked in the acid solution. Here one absorption maximum at 250 millimicrons was observed. Figures 11 and 12 show that thiamine and aminopyrimidines exhibit absorption maxima from 245 to 250 millimicrons in acid solution.

A comparison of the absorption curves for the pyrimidine alcohol (Figure 11) with those for the pyrimidine produced by the destruction of thiamine, which were obtained by

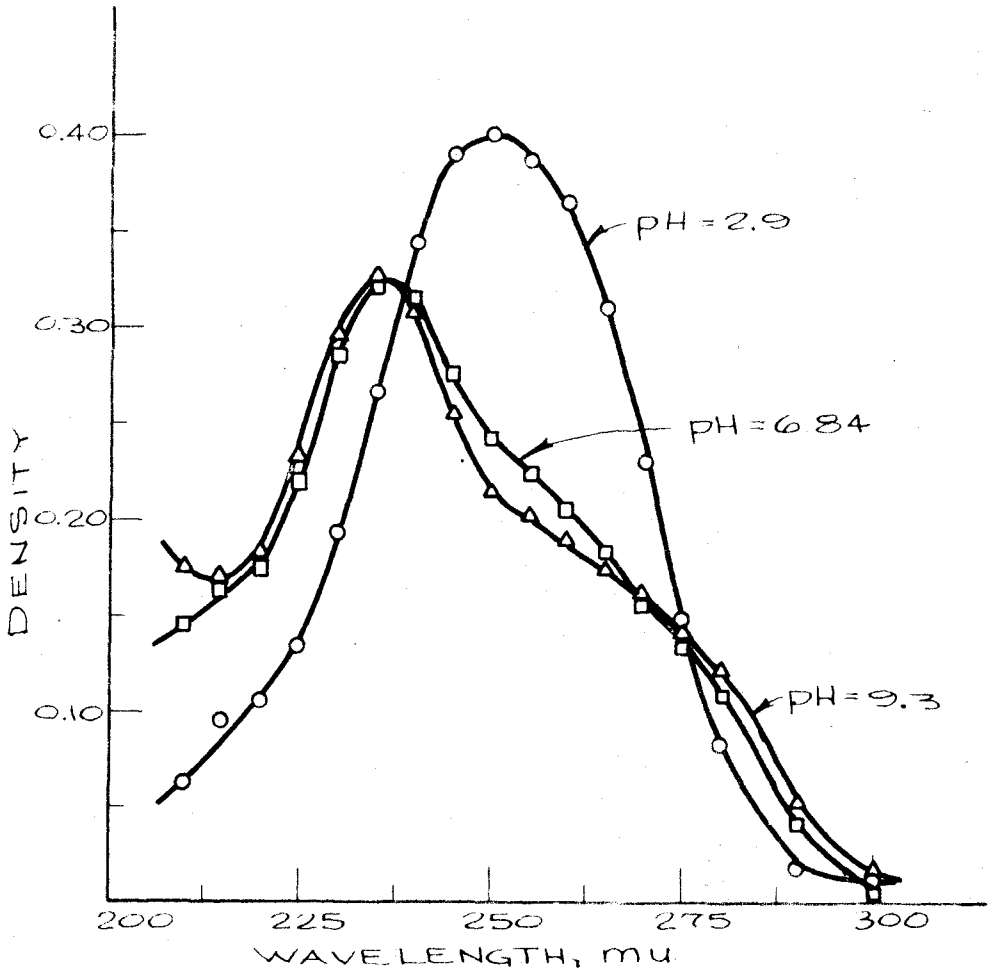


FIG. 17 ABSORPTION SPECTRA OF MIXTURE OF 2-METHYL-6-AMINO-5-HYDROXYMETHYL PYRIMIDINE AND 4-METHYL-5-β-HYDROXY ETHYLTHIAZOLE. EACH 2.5×10^{-5} M. IN 0.04 M. PHOSPHATE.

analysis of a Decalso eluate (Figure 15 (B)), shows identical absorption maxima at 235 and 270 millimicrons in neutral solution and at 245 millimicrons in acid solution. The absorption minimum in neutral solution at 255 millimicrons is the same for both. Because of the similarity in the wave lengths of maximum and minimum absorption shown by pyrimidines of different structures, this indicates that the derivative being formed could be the pyrimidine alcohol or a closely similar compound.

Because of the report by Krampitz and Woolley (30) that different pyrimidine derivatives were formed when sodium chloride extracts and tissue suspensions were employed as sources of the enzyme, thiamine was completely destroyed by a suspension of frozen carp tissue and the reaction mixture spectrophotometrically analyzed. The protein was precipitated with hydrochloric acid and the mixture was purified by a Decalso treatment as described above. The typical pyrimidine absorption curve was exhibited with absorption maxima at 235 and 275 millimicrons in alkaline solution and 245 millimicrons in acid solution (Figure 18). The absorption minimum at 255 millimicrons was observed in the alkaline solution. The only difference between the maximum and minimum absorption of the pyrimidine alcohol and the pyrimidine obtained by use of tissue suspensions was that the former exhibited an absorption maximum in alkaline solution at 270 millimicrons.

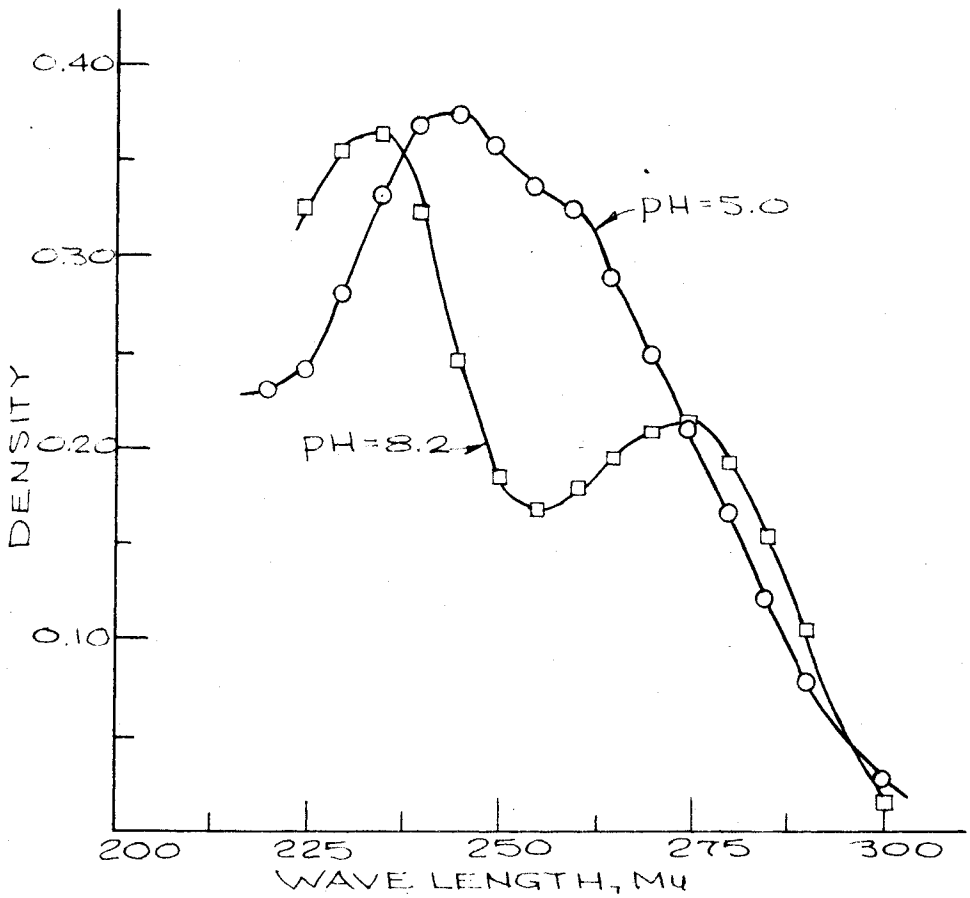


FIG. 18 ABSORPTION SPECTRA OF REACTION PRODUCTS FROM THIAMINE DESTRUCTION BY TISSUE SUSPENSION - ANALYSIS OF DECALSO ELUATES.

The theoretical concentration of the pyrimidine derivative in the Decalso eluates was calculated, assuming that adsorption and elution had been complete. With these values the molecular extinction coefficients of the pyrimidine were calculated at several different wave lengths and compared with those of the pyrimidine alcohol. These values, shown in Table LXVI, indicate a greater similarity between the alcohol and the derivative formed by tissue suspensions than that produced with a sodium chloride extract. The values for the pyrimidine produced by the action of a sodium chloride extract were obtained with a solution, which had been purified by butanol extraction prior to Decalso adsorption. The coefficients shown in parenthesis for this derivative were obtained in another experiment, in which a filtrate was adsorbed without butanol extraction, but the Decalso eluate was analyzed at only one pH. If the two-step reaction postulated by Krampitz and Woolley (30) is actually the case, a closer similarity would be expected between the pyrimidine alcohol and the pyrimidine derivative produced by a tissue suspension than between the alcohol and the derivative obtained by employing a sodium chloride extract.

It would appear that a comparison of the wave lengths at which maximum and minimum absorption were observed would not allow any decision to be made regarding the identity of the pyrimidine derivatives obtained by sodium chloride

Table LXVI

Molecular Extinction Coefficients of
Pyrimidine Alcohol and Pyrimidine Derivatives

Wave Length	Molecular Extinction Coefficient (ε) of Pyrimidine Alcohol	Pyrimidine Derivative Tissue Suspension	Pyrimidine Derivative NaCl Extract
mμ			
235, acid	9,400	8,250	4,580
neutral	10,800		5,150
basic	11,600	9,050	5,380
255, acid	11,400	8,380	5,680
neutral	4,920		3,330
basic	4,320	4,200	3,200 (2,120)
270, acid	6,880	6,230	3,960
neutral	5,880		3,010
basic	6,120	5,230	3,060 (2,850)
275, acid	4,800	5,230	3,180
neutral	5,520		3,280
basic	5,950	5,250	3,400 (2,910)

extracts and by tissue suspensions with pyrimidine alcohol or with each other. On the other hand the comparison of the molecular extinction coefficients suggests that the derivative obtained by tissue suspensions is more similar to the alcohol, than is the derivative obtained by use of sodium chloride extracts. However, the results obtained by spectrophotometric analysis of reaction mixtures do not at present permit any definite conclusion to be made regarding the nature of the

pyrimidine derivative. Nevertheless, it is apparent that the pyrimidine produced by the enzymatic destruction of thiamine can be studied by this procedure, and that spectrophotometric methods can be adapted in work directed towards the isolation of this derivative.

M. The Anti-thiamine Factor of Bracken Fern

At the time the work with the thiamine-destroying fish factor was in progress in this laboratory, Weswig, Freed and Haag (52) reported that "fern-poisoning", which was observed in cattle foraging on bracken fern, was actually a thiamine deficiency. Therefore, it was concluded than an anti-thiamine, or a thiamine-destroying factor, was present in bracken fern. Thus, from the initial report, it appeared that here was another thiamine-destroying factor, which might or might not be similar to the Chastek-paralysis factor. Although Weswig and his co-workers reported that air-drying of bracken fern at 110 degrees for 18 hours did not reduce its "toxicity", the fact that the material was not in contact with water during the heating period still left the possibility that the factor was protein in nature.

Therefore <u>in vitro</u> studies of this factor were undertaken in an effort to determine the nature of this new thiamine destroying substance, and to determine if it were related to the thiamine-destroying enzyme of fish tissues. For the initial

experiment 20 gm. of fresh fern (species unknown) was blended with 80 ml. of phosphate buffer and squeezed through cloth. The resulting solution contained particles which settled out upon standing, but which were kept in suspension for determination of thiamine-destroying activity.

When the Melnick-Field method was used to determine whether or not this fern suspension had caused the disappearance of thiamine during a two hour incubation period, interference was observed. The "zero time" control samples gave colorimetric readings, which were 44 to 53 per cent of the reading for the thiamine standard; but readings for the incubated samples were still lower, indicating a decrease in thiamine during incubation. A direct thiochrome analysis gave "zero time" values which were 95 to 100 per cent of the standard, and as is shown in Table LXVII, a loss of thiamine occurred during incubation. The fact that a previously heated extract destroyed only 35.5 per cent as much thiamine as the unheated portion, indicated that the anti-thiamine factor of fern might be a protein.

Following the initial experiment, which indicated that the anti-thiamine factor of fern could be studied by <u>in vitro</u> methods, several attempts were made to determine whether or not it was protein in nature. Although there was always some loss of activity upon heating, complete inactivation was never obtained. The results of several such experiments with heated samples, shown in Table LXVIII, make it evident that, if the

Table LXVII

Thiamine Destruction by Fern Extract

Extract Assayed		Thiamine Destroyed	
		μmoles	per cent
Fern extract, 2 hr. incubation		0.48	19.0
		0.61	24.3
	Ave.	0.55	21.7
Fern extract, 1 hr. incubation		0.34	13.4
Fern extract, heated for 20 min. in boiling water bath, 2 hr. incubation		0.06	2.6
		0.32	12.7
	Ave.	0.19	7.7

factor is protein, it must be much more thermostable than most proteins. For example, a sample of acetone desiccated fish powder, when heated at 108 degrees for approximately 20 hours, lost 95 per cent of its activity, as compared with the 31.5 per cent inactivation of a fern sample heated under identical conditions (sample 6, Table LXVIII).

If the factor were protein, it would be expected that its activity would be reduced or destroyed by the action of proteolytic enzymes. Portions of a fern extract were therefore incubated overnight at 37 degrees with pepsin at pH 3.5 and with trypsin at pH 7.5. Another portion at pH 5.5 was incubated with no further additions, while a similar fraction at pH 5.5 was stored overnight in the ice-box. The following day all were adjusted to

Table LXVIII

Inactivation of Fern by Heat

Amount of Fern	Method of Heating	Thiamine Destruction Unheated	Heated	Inactivation
mg. equiv.		per cent	per cent	per cent
400[1]	Boiling water bath, pH 6.6, 25 min.	29.0	11.8	59.3
250[2]	Open flame, 10-15 min.	8.5	6.0	29.4
	Boiling water bath, pH 6.7, 1 hr.	8.5	6.0	29.4
250[3]	Open flame, 10 min.	27.8	17.1	42.1
333[4]	Boiling water bath, pH 4.7, ½ hr., briefly over open flame	30.4	11.0	63.9
100[5]	Boiling water bath, pH 4.1, 35 min.	14.4	7.2	50.0
125[6]	Dry fern heated at 108° for 19½ hr.	28.6	17.4	31.5

1. Species, similar to bracken, gathered in North Woods, used when fresh.
2. Bracken fern from Franklin avenue, used when fresh.
3. Unknown species from North Woods, used when fresh.
4. Same as (1), but dried for several days.
5. Air-dried bracken fern from Pennsylvania.
6. Same as (2), air-dried for three days, desiccator dried for two days.

pH 7.4 and assayed to determine their thiamine-destroying activity. The fraction, which had not been incubated, destroyed 12.8 per cent of the added thiamine, while incubation alone decreased

the destruction to 9.2 per cent. The samples incubated with pepsin and trypsin, however, destroyed 16.5 and 13.8 per cent of the added thiamine. These results did not indicate that the factor was a protein material.

On the other hand, the activity of a fern extract was inhibited by mercuric chloride, a typical protein precipitant and enzyme inhibitor. An uninhibited extract destroyed 22.6 per cent of the added thiamine, while the same extract in the presence of 5.0 and 50.0 x 10^{-6} M. mercuric chloride destroyed only 15.7 and 16.6 per cent of the substrate.

The loss of thiamine produced by the fern factor increased with increasing time of incubation in a manner similar to the enzymatic destruction of thiamine by the fish principle. Figure 19 shows graphically the results of two experiments, in which thiamine destruction was measured at intervals from 0 to 2 hours, and shows the increase in destruction with time.

Although the use of fern suspensions, prepared as described above, prevented the use of the Melnick-Field method for thiamine determination, it was found that in certain cases the filtration of these suspensions gave clear dark filtrates which did not produce interference in the method. While such a filtrate prepared from a sample of air-dried bracken fern did not produce interference, filtrates prepared from samples of fern powders did cause interference. Although filtrates prepared from fresh fern collected from the latter part of May

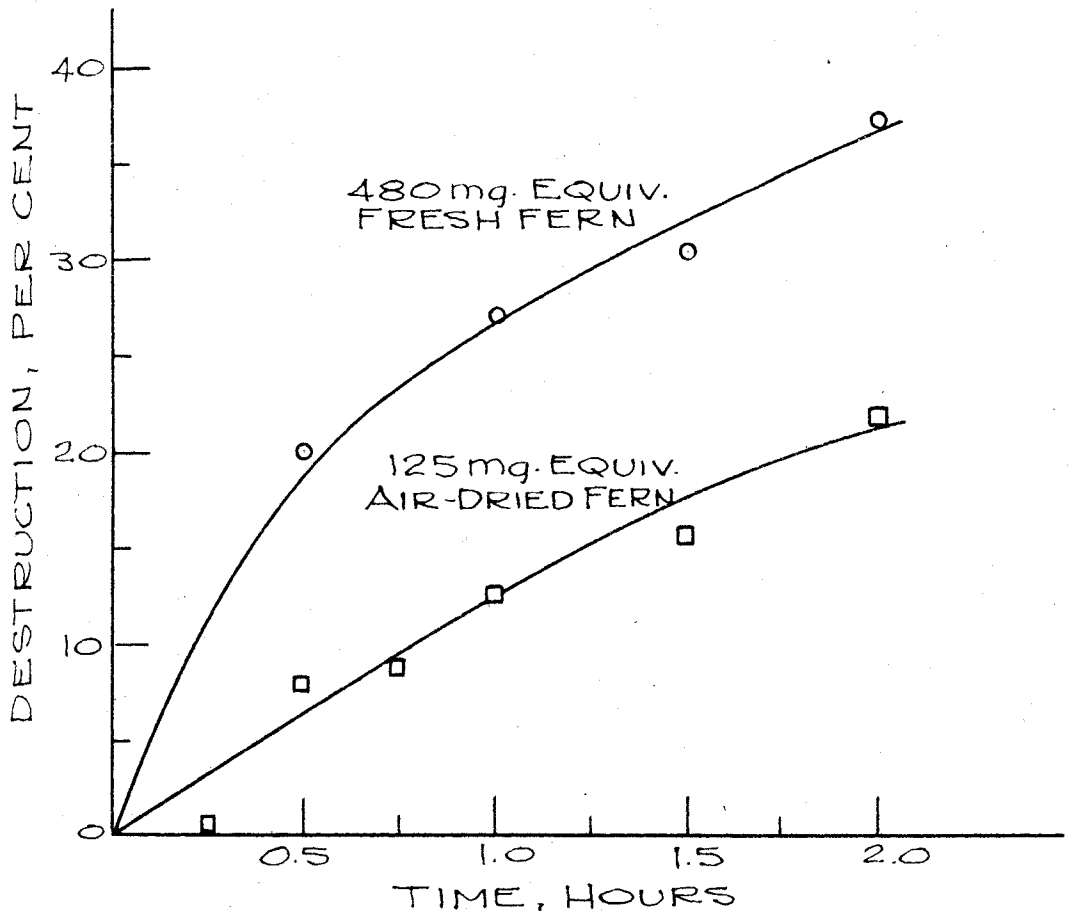


FIG. 19 TIME COURSE OF THIAMINE DESTRUCTION BY FERN EXTRACTS

through the middle of June produced no such interference, a sample collected and analyzed on July 10th did cause interference. Furthermore, a sample collected on June 3rd and air-dried for two days was analyzed successfully with the Melnick-Field method, the "zero time" control giving a colorimetric reading, which was 95.4 per cent of that of the standard, but when this sample was further dried interference increased. A "zero time" control prepared from the sample after it had been dried in a desiccator gave a colorimetric reading which was 86.1 per cent of the standard reading, while heating for 20 hours at 108 degrees caused the "zero time" value to decrease to 50.5 per cent of the standard.

It must of course be remembered that there was a decrease in weight during the drying and, even though lower concentrations of the desiccator-dried and heated samples were used, there may have been a higher concentration of the interfering substance. However, there was only a slight decrease in weight, when the desiccator-dried sample was heated for 20 hours at 108 degrees, and yet at the same concentration, interference was greater with the heated sample.

It appeared then that the interference increased with the age of the fern, being less with samples collected in the spring of the year, and that it also increased upon heating and drying. Direct thiochrome analysis was used successfully with only slight interference usually observed. A comparison

of results obtained by several methods of analysis is given in Table LXIX. The extract used was prepared from air-dried bracken fern and represented 125 mg. of the fern.

Table LXIX

Interference by Fern Extract

Method of Analysis	Reading of Thiamine Standard[1]	Reading of "Zero Time"[1]	Interference per cent
Melnick-Field	320	160	50.0
Direct thiochrome on diluted filtrates	89.5	81	9.6
Direct thiochrome on Decalso eluates	27.5	31	- 13.0
Thiochrome on Decalso eluates with isobutanol extraction	69.5	70.5	- 1.0

1. All values, except for those of the Melnick-Field method, represent an average of two.

The results shown in the above table indicate that interference is eliminated by the use of a complete thiochrome analysis, including adsorption on and elution from Decalso, and isobutanol extraction of thiochrome. However, most of the results given in the following work were obtained by direct thiochrome analysis of the filtrates, in which "zero time" values were 85 per cent or more of the thiamine standard.

The effect of 3-γ-aminopropyl-4-methylthiazolium bromide and o-aminobenzyl-4-methylthiazolium chloride on the activity of the fern anti-thiamine factor was determined. Since both of these compounds are inhibitors for the carp enzyme, it is interesting to note that they also inhibit the thiamine-destroying activity of fern extracts. An uninhibited fern extract destroyed 30.4 per cent of the added thiamine. The addition of the aminopropyl analogue to give concentrations of 2.0 and 5.0 x 10^{-4} M. reduced the amount of destruction to 16.9 and 14.7 per cent respectively. This represented inhibition of 44.4 and 51.7 per cent for the two concentrations of the analogue. Equivalent concentrations of the aminobenzyl analogue reduced the destruction to 18.2 and 4.8 per cent, resulting in inhibition of 40.0 and 84.1 per cent. The inhibition thus increased with increasing concentration of the thiazolium compounds, and the benzyl derivative, which is a more active inhibitor for the fish factor than is the propyl compound, also proved to be a better inhibitor of fern activity.

In order to determine whether the loss of thiamine produced by fern extracts could have been due to the formation of a complex of an avidin-biotin type, the incubated mixtures were hydrolyzed with 0.1 N. sulfuric acid, by placing the solutions in a boiling water bath for one hour. At the conclusion of the hydrolysis, the solutions were cooled, diluted to 25 ml.

and the thiamine present determined by thiochrome analysis. At the same time aliquots of the incubated mixtures were analyzed without sulfuric acid hydrolysis. As shown in Table LXX, evidence for destruction of thiamine was obtained, regardless of the method of analysis. It would appear then that the loss or destruction of the vitamin by fern extracts is not due to the formation of a sulfuric acid-labile complex.

Table LXX

Thiamine Destruction by Fern
Analysis with and without Sulfuric Acid Hydrolysis

Amount of Fern	Thiamine Destruction	
	without H_2SO_4 hydrolysis	with H_2SO_4 hydrolysis
mg. equiv.	per cent	per cent
31.2	8.7	4.8
62.4	9.2	12.2
124.8	26.0	12.8

In order to obtain more information regarding the nature of the fern factor, the activities of dialyzed extracts were compared with those of equivalent amounts of undialyzed extracts. In the first experiment a filtrate representing 122 mg. per ml. of air-dried fern was dialyzed for 9 hours against redistilled water. The extract was continuously stirred during the dialysis. A second undialyzed portion was

diluted so that its concentration was equal to that of the dialyzed extract. The dialyzate was divided into two portions and evaporated to dryness in an air-stream, one-half at an initial pH of 4.9 and the other half at an initial pH of 8.5. The residues were dissolved in water so that 1 ml. was equivalent to 2 ml. of the dialyzed extract. The thiamine-destroying activity of both the dialyzed and undialyzed extracts was determined with and without the addition of equivalent amounts of the dialyzates. The results, shown in Table LXXI, show that there was a complete loss of activity upon dialysis and that neither portion of the dialyzate was capable of destroying thiamine. However, the activation of both of the extracts produced upon addition of the basic dialyzate indicates that the thiamine-inactivating factor of fern may have two components, one dialyzable and the other non-dialyzable.

In the second experiment a fern extract was dialyzed for 8 hours against redistilled water, and the dialyzate at a neutral pH evaporated in an air-stream to 10 ml., and then diluted with water so that 1 ml. of the solution was equivalent to 2 ml. of the extract. Here again, although the extract was not completely inactivated by dialysis, there is evidence for a two component system, as indicated by the results shown in Table LXXII. Manganese, which activates the fish enzyme, also produced a striking activation of the dialyzed fern extract. The effect of dialysis upon fern extracts and the activation by

Table LXXI

Dialysis of Fern Extract

Fraction Analyzed	Thiamine Destruction	Remarks
	Per cent	
Undialyzed	10.3	
Dialyzed	0	Complete inactivation by dialysis
Dialyzed plus acid dialyzate	0	No activation of dialyzed
Dialyzed plus basic dialyzate	6.2	Activation of dialyzed
Acid dialyzate	0	No active material dialyzable
Basic dialyzate	0	No active material dialyzable
Undialyzed plus acid dialyzate	2.4	76.7 per cent inhibition
Undialyzed plus basic dialyzate	12.6	22.3 per cent activation

manganese of a dialyzed extract show additional points of similarity between the fish factor and the fern factor.

Several samples of air-dried and powdered bracken fern were sent to us by Dr. Haag of Oregon State College. Hoping that these might be used as a stable source of the "fern factor", they were assayed in the same way as were the acetone desiccated fish powders. However, since extracts of these powders produced interference, the Melnick-Field method could not be employed for thiamine determination.

Table LXXII

Dialysis of Fern Extract

Fraction Analyzed	Thiamine Destruction per cent	Remarks
Undialyzed	19.8	
Dialyzed	9.2	53.3 per cent inactivation by dialysis
Dialyzed plus dialyzate	15.7	70.8 per cent activation
Undialyzed plus dialyzate	11.2	43.4 per cent inhibition
Dialyzate	0 to 2.0	No significant activity
Dialyzed plus 1 x 10^{-4} M. Mn.	27.6	200 per cent activation
Undialyzed plus 1 x 10^{-4} M. Mn.	11.6	41.4 per cent inhibition

In order to obtain a preparation even more similar to the desiccated fish powders, one sample, No. H-8440, was treated with 12 volumes of cold acetone, which dissolved a large amount of the colored pigments. After filtration and a second treatment with 8 volumes of cold acetone, the material was dried. An extract of the acetone-treated powder, representing 125 mg. of the material, destroyed 31.8 per cent of the added thiamine, while an equivalent extract of a portion of untreated powder destroyed 18.8 per cent of the thiamine. Nitrogen analysis of the extracts showed 0.244 mg. of nitrogen present in an extract representing 125 mg. of the

untreated sample and 0.548 mg. of nitrogen for an equivalent amount of the extract prepared from the acetone-treated sample.

Additional assays were made with extracts prepared from the fern powders and the results are recorded in Table LXXIII. All of the samples inactivated or destroyed thiamine to some extent. The last three samples show an increased destruction with increased concentrations, but the first two samples do not show this relationship. However, they represent assays made at different times with different extracts, and variability in the efficiency of extraction may account for the lack of proportionality.

In an effort to reduce interference an extract, representing a 25 mg. equivalent of H-8966 per ml., was assayed directly and also after two extractions with equal volumes of ether. However, no reduction of interference in the Melnick-Field method was observed with the ether-extracted solution. Therefore a thiochrome analysis was made on the eluates obtained after Decalso adsorption of the reaction mixtures. The "zero time" values for the ether-treated extract were higher than those of the untreated sample, indicating a decrease in interference. A portion of the untreated extract, representing a 50 mg. equivalent, destroyed 2.0 per cent of the thiamine, while an equivalent amount of the ether-extracted sample caused 11.0 per cent destruction. At a 100 mg. concentration

Table LXXIII

Thiamine Destruction by Fern Powders

Fern Powder	Amount of Powder	Thiamine Destruction	Nitrogen Present in Equivalent Used
	mg. equiv.	per cent	mg.
H-8440	40	18.6	0.078
	50	9.2	0.156
	80	7.8	0.110
	100	9.7	0.220
H-8966	40	26.3	
	50	8.3	0.310
	80	14.0	
	100	11.5	0.620
	125	18.3	0.174
	100[1]		
	10 ml. adsorbed	4.0	
	20 ml. adsorbed	17.0	
	30 ml. adsorbed	5.0	
H-7950	50	3.7	0.182
	100	6.7	0.364
H-8360	50	6.7	0.202
	100	13.0	0.404
H-8984	50	4.1	0.758
	100	7.6	1.516

1. Analysis made by a direct thiochrome method on Decalso eluates. Different amounts of a diluted filtrate were adsorbed as indicated. All were eluted to the same volume. All other analyses were made by the direct thiochrome procedure on the diluted filtrates.

the destruction observed was 23.3 and 29.4 per cent for the untreated and treated extracts, respectively.

The work on the fern factor was not carried beyond this point. The difficulties encountered in the analytical methods made progress slow. However, from the results obtained here and from those reported by Haag and his co-workers, it seems obvious that there is an anti-thiamine factor in fern. The possibility that this factor is protein in nature still exists and is supported by its thermolability, although the factor appears to be more heat stable than most proteins. However, the present information does not definitely establish the thiamine-destroying factor as protein or non-protein. Furthermore, the possibility that the observed decrease in thiamine upon incubation with fern extracts is associated with the factor responsible for interference in the analytical methods must not be overlooked.

IV. DISCUSSION

The occurrence of a thiamine-destroying enzyme in fish tissues presents several problems. The nutritional problem associated with the production of Chastek paralysis, actually a thiamine deficiency, upon inclusion of raw fish in fox rations, was the first to arise. Withdrawal of fish from the diet prevents outbreaks of this disease. However, since fish is an inexpensive source of protein for the rations, efforts were made to find ways of utilizing fish as a diet constituent without causing a thiamine deficiency. Previously cooked fish produces no harmful effects, while an alternation of the fish ration with a non-fish ration also prevents the development of Chastek paralysis.

The fact that an enzyme capable of destroying a vitamin is present in the tissues of animals requiring that same vitamin, raises the question of the role played by the enzyme in the living animal. Perhaps the most attractive hypothesis is that under physiological conditions the enzyme functions in a reverse manner and that it actually synthesizes thiamine. However, the fact that whole-cell preparations of gold-fish viscera proved to be capable of destroying added thiamine, and did not give any evidence of synthesis upon addition of certain precursors, argues

against this hypothesis. Consequently, at present one can only speculate regarding the physiological role of this enzyme.

A third problem associated with this enzyme has received attention. Here, efforts have been made to determine the fundamental nature of "thiaminase" and to clarify the mechanism of its thiamine-destroying action. The "time course" of thiamine destruction by the "fish principle" was studied with several substrate and enzyme concentrations. However, under the conditions employed, no equilibrium point for the reaction could be determined. Within the time, in which the enzyme maintains most of its activity, the reaction goes to completion with low substrate concentrations. In the presence of larger amounts of thiamine, there is no evidence for a "levelling-off" of the amount of reaction, as would be expected, if equilibrium were being approached. Attempts to approach equilibrium from the opposite direction by enzymatic synthesis of thiamine were also unsuccessful.

However, these results emphasized the importance of clarifying the mechanism of the over-all reaction for the enzymatic destruction of vitamin B_1. Therefore, preliminary investigations directed towards the identification of the pyrimidine derivative were undertaken. When this compound has been identified, then the question of whether thiamine destruction by the enzyme is a two-step reaction may be more readily answered.

The observation that 4-methyl-5-β-hydroxyethylthiazole, which has been identified as a product of thiaminase action, inhibits thiamine destruction by the enzyme, has given rise to the question of whether its action is strictly one of competitive inhibition or whether it acts by altering the time required to attain equilibrium. If the reaction does reach equilibrium, it would be logical to suppose that the presence of an excess amount of one of the products, by virtue of its mass action effect, would cause the equilibrium point to be reached more quickly, thus giving the appearance of inhibition. The possible application of isotope techniques to obtain a final answer to this question is obvious.

However, the complexity of reactions catalyzed either in whole or in part by enzymes, makes the problem of determining equilibrium points and of effecting reversal of the reactions a difficult and yet challenging one. In the case of thiaminase no conclusions regarding these points can be made until the mechanism of the reaction has been clarified and until the enzyme is available in a more purified form.

The importance of inhibition studies as an aid in establishing the enzymatic nature of a given reaction has received much attention in recent years. In the case of thiaminase, Sealock and Goodland (37) have reported that inhibition is produced by o-aminobenzyl- and β-aminoethyl-4-methylthiazolium chloride. This inhibition has been

confirmed and in addition 3-γ-aminopropyl-4-methylthiazolium bromide has been added to the list of active inhibitors. Thus support is given to the hypothesis made by the above authors that, since the combination between enzyme and the aminothiazolium inhibitors appears to be dependent upon the presence of the free amino group, then the combination between the enzyme and its substrate must be a function of the amino group of the pyrimidine ring of the thiamine molecule. Since these molecules act by competing with the substrate for the enzyme, it would be logical to suppose that the enzyme combines with both inhibitors and substrate through the amino group, which is common to both.

In contrast to the high degree of inhibition produced by the aminoethyl and the aminopropyl thiazolium derivatives, the next higher homolog, 3-δ-aminobutyl-4-methylthiazolium bromide produced no significant effect upon thiamine destruction by the enzyme. The fact that o-aminobenzylpyridinium chloride produced an increase in the enzymatic destruction of thiamine, while benzylpyridinium chloride produced a much lower degree of activation, suggests that an amino group is also important in activating molecules. This is supported by the high degree of activation observed in the presence of m-aminobenzyl-4-methylthiazolium chloride[1]. The observation that certain analogues of thiamine increase the destruction

[1] Sealock, R.R. and Livermore, A.H., unpublished.

of the vitamin by the enzyme may well be an aid in the eventual identification of the dialyzable component of this enzyme system.

Sincer certain structural analogues of thiamine are capable of combining with the thiamine-destroying enzyme, it is also possible that such molecules could combine with the apoenzyme of enzymes, for which the vitamin is the prosthetic group. This suggests the application of the thiazolium analogues, studied in connection with the thiamine-destroying enzyme, to inhibition of reactions dependent upon the catalysis of thiamine-contianing enzymes. For example, the inhibition of bacterial growth by the use of such compounds, which are possibly capable of competing with a prosthetic group for a protein portion of an enzyme, would be worthy of much attention. Preliminary investigations toward this end have been recently undertaken in this laboratory (65). Similar studies have been reported by Woolley and White (66), who found that pyrithiamine inhibits the growth of certain microorganisms, which require thiamine. Pyrithiamine is an analogue of the vitamin, in which a substituted pyridine ring replaces the thiazole ring.

During the course of the inhibition studies it was found that with most of the enzyme preparations, there was an "apparent substrate inhibition", that is, the amount of thiamine destroyed decreased with increasing substrate

concentration, instead of increasing in the expected manner. One may suggest that this inhibition results from the reaction between the enzyme and two molecules of the substrate to give an inactive complex of the type ES_2 instead of the active complex form, ES. Although such a situation is not commonly encountered, it is not without parallel among known enzymes. The addition of excess substrate would thus reduce the amount of the active enzyme complex.

Some support for the hypothesis that the "apparent substrate inhibition" is due to the formation of inactive complex, ES_2, was found in the observation that the addition of a boiled extract partially overcame the inhibition. The S-shaped curves obtained when the amount of thiamine destruction was plotted against the enzyme concentration are typical of dilution curves. Such dilution would be expected to materially effect the dissociation of the apoenzyme-coenzyme complex. It is logical to suppose then that the addition of the boiled extract, which contains the thermostable, dialyzable component of the enzyme, by virtue of a mass action effect, would maintain more of the enzyme in its active form. This in turn would make available more enzyme for combination with the substrate, thus increasing the amount of the ES complex and reducing the amount of the ES_2 form.

A number of studies were undertaken in an effort to determine the nature of the activating factor present in the

boiled extracts. Since manganese had previously been shown to activate thiaminase, attempts were made to determine if manganese were the activating factor of boiled extract, or whether its activation was merely a non-specific effect. The dialyzable component appeared to be quite thermostable, particularly at an acid pH. It was not readily susceptible to inactivation by oxidation under conditions which would be expected to oxidize the manganous ion to the manganic form. It was not extractable from acid or alkaline solution by either n-butanol or ether, but it was precipitated from boiled extracts by alcohol.

Treatment of boiled extracts with activated Decalso and with the cationic Amberlite exchange resin reduced the activating ability of these extracts, but never completely destroyed the ability to activate dialyzed enzyme preparations. This would appear to indicate that the boiled extracts contained two components necessary for complete activation, one of cationic nature and one of anionic nature. The cation may be either an organic substance or a metallic ion, but does not appear to be manganese. Since the activating ability of the boiled extracts, which had been treated with Decalso or Amberlite IR-100-H. was never completely restored upon addition of manganese, it would seem logical to suppose that the activator removed by these substances was something other than or in addition to manganese. Furthermore, since the

increased activation observed upon addition of manganese in combination with the treated boiled extract was not significantly greater than the increased activation exhibited, when it was added in combination with an untreated extract, it appears that manganese cannot be one of the activating factors present in boiled extracts.

Since Agren had reported that glutathione restored, partially at least, the activity of a dialyzed enzyme preparation, it was possible that glutathione or perhaps another sulfhydryl compound was one of the dialyzable components of thiaminase. However, the stability of the boiled extracts to oxidation argues against this hypothesis. The fact, too, that the activating ability of boiled extracts was not destroyed by the addition of four times the amount of iodine, which was required to titrate the extract, does not support the idea that a sulfhydryl containing compound can be an essential component of the extract.

However, since thiaminase activity was strongly inhibited by phenylmercuric chloride, considered to be a highly specific inhibitor for sulfhydryl-containing enzymes, it appears likely that this enzyme is also sulfhydryl-dependent. Considering this inhibition and the results obtained with boiled extract, it seems apparent that sulfhydryl groups in the protein portion of the enzyme are essential for its activity. Assuming this to be true, the slight degree of activation observed

upon addition of glutathione and cysteine to enzyme extracts can be explained on the basis that these compounds help to maintain the protein sulfhydryl groups in a reduced form.

In addition to the studies with the thiamine-destroying enzyme of fish tissues, efforts were made to determine the nature of the anti-thiamine factor present in bracken fern. If this factor were also to prove to be an enzyme, then another possibility of being able to synthesize thiamine enzymatically by *in vitro* methods would be available. However, chiefly due to interferences produced in the analytical methods for thiamine determination, the work with the fern factor was not entirely successful. Nevertheless, several points of similarity to the fish thiaminase were observed. The anti-thiamine factor of fern is thermolabile, although not to the same extent as is the fish factor. The factor appears to contain both a non-dialyzable and a dialyzable component. The inactivation of thiamine by the factor is inhibited by mercuric ion, and by two of the aminothiazolium analogues, which are strong inhibitors for the fish enzyme. The time course of thiamine inactivation by this factor is similar to that of thiaminase of fish tissues.

The thermolability, the inhibition by mercury, and the presence of a non-dialyzable component all point to a protein nature. Furthermore the presence also of a dialyzable

component and the fact that the amount of inactivation increases with time of incubation suggest the possibility that the factor is an enzyme. However, at present no definite conclusions may be made regarding the enzymatic or non-enzymatic nature of the thiamine-destroying factor present in fern.

V. SUMMARY

1. A new method for the determination of thiamine has been developed. The method is similar to the Melnick-Field determination in that it depends upon the formation of a colored derivative of the vitamin, when it is allowed to react in alkaline solution with a diazotized aromatic amine. However, the substitution of p-aminobenzoic acid for p-aminoacetophenone, which is used in the Melnick-Field method, permits colorimeter readings to be made directly on the aqueous solution and eliminates extraction of the colored complex with an organic solvent.

2. Efforts were made to establish an equilibrium point for the reaction of the enzyme "thiaminase". However, the instability of the enzyme preparations prevents accurate measurement of activity over a long period of time. With low substrate concentrations the reaction goes to completion, as evidenced by the complete destruction of thiamine. The equilibrium point, if one exists, is far to the right favoring thiamine destruction. Efforts to approach equilibrium from the opposite direction by synthesizing thiamine enzymatically from its component parts were unsuccessful. No evidence for the synthesis of thiamine was obtained, when pyrimidine derivatives and the thiamine thiazole moiety were incubated with enzyme preparations.

3. The measurement of the carbon dioxide evolved during the enzymatic destruction of thiamine, carried out in a bicarbonate buffer, supports the hypothesis that the destruction of thiamine by "thiaminase" results in the release of one hydrogen ion per molecule of vitamin destroyed.

4. The thiamine thiazole moiety, 4-methyl-5β-hydroxyethylthiazole is an inhibitor of thiaminase activity, while 4-methylthiazole is inhibitory to a much less degree. The inhibition by the thiamine thiazole suggests the possibility that it acts by virture of a mass action effect and that the decreased thiamine destruction in its presence is due to an approach to an equilibrium condition.

5. Earlier work reporting that o-aminobenzyl-4-methylthiazolium chloride and the corresponding β-aminoethyl thiazolium derivative inhibit thiaminase action has been confirmed. In addition, γ-aminopropyl-4methylthiazolium bromide has been added to the list of strong inhibitors. The higher homolog, δ-aminobutyl-4-methylthiazolium bromide, has only a slight activating effect on the enzymatic destruction of thiamine. o-Aminobenzylpyridinium chloride produces a high degree of activation of the enzyme, while benzylpyridinium chloride activates thiaminase to a much less degree. The addition of boiled extract in combination with γ-aminopropyl-4-methylthiazolium bromide, produces increased inhibition.

6. With most of the acetone-desiccated powders used in this laboratory, the amount of thiamine destroyed decreases with an increasing concentration of substrate. The addition of boiled extracts partially overcomes this "apparent substrate inhibition".

7. The importance of sulfhydryl groups in thiaminase action is emphasized by the fact that inhibition is produced by iodine and phenylmercuric chloride. Experiments designed to locate the essential sulfhydryl groups in the protein or non-protein portion of the enzyme, indicate that the sulfydryl groups necessary for its effective action are contained in the apoenzyme, or protein portion.

8. The manganous ion activates thiaminase. However, the activation appears to be a non-specific effect of this ion. The evidence indicates that the activating effect of boiled extract is due partially at least to the presence of a factor or factors of cationic nature. The activating factor cannot be extracted from boiled extract at an alkaline or acid pH with either ether or butanol, and is precipitated from the extract by alcohol. The activator appears to be fairly stable to heat and to oxidation.

9. Spectrophotometric analyses of reaction mixtures containing enzymatically destroyed thiamine show that the pyrimidine product of the reaction is extractable from alkaline solution with n-butanol and that it is adsorbed by

Decalso. It exhibits absorption maxima and minima similar to those of 2-methyl-6-aminopyrimidine derivatives.

10. Studies of the anti-thiamine factor of bracken fern emphasize certain points of similarity with the thiaminase of fish tissues. The factor is thermolabile, although it is not as readily inactivated by heat as is thiaminase. Its ability to inactivate thiamine is inhibited by mercury and by two of the thiazolium derivatives, which inhibit the fish thiaminase. The inactivation of thiamine by fern extracts increases with increasing time of incubation. The evidence indicates that the fern factor contains both dialyzable and non-dialyzable components. There is present in extracts of fern a substance which produces interference in both the Melnick-Field and thiochrome methods for thiamine determination, particularly in the former.

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