# STUDIES OF BRACKEN FERN THIAMINASE: CONDITIONS INFLUENCING THE PROCEDURE, STABILITY OF THE ENZYME, AND ITS DISTRIBUTION IN THE BRACKEN FERN AT DIFFERENT STAGES OF MATURITY

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EVELYN G. CATLI
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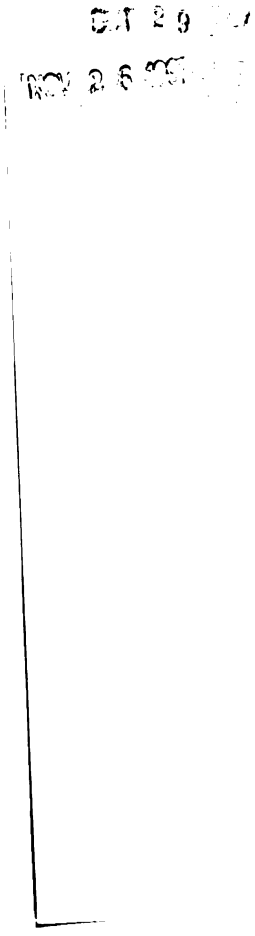

ABSTRACT

STUDIES OF BRACKEN FERN THIAMINASE: CONDITIONS INFLUENCING ITS PRODUCTION, STABILITY OF THE ENZYME, AND ITS DISTRIBUTION IN THE BRACKEN FERN AT DIFFERENT STAGES OF MATURITY

By

Evelyn G. Catli

Bracken fern is a toxic plant growing in open fields and along the edge of wooded areas. It is a potent source of a thiamin destroying factor (thiaminase) affecting monogastric animals, it produces irreversible hematological changes in ruminants and contains a carcinogenic factor for both monogastric animals and ruminants. Considerable work has been done with this fern because it grows in many fields, is difficult to eradicate and has been reported to be eaten by man in some areas of the world.

There appears to be disagreement concerning the chemical nature and mode of action of the thiamin destroying factor in bracken fern. Reports indicate that this factor is thermolabile or thermostable or that two factors are present.

This study was designed to extend investigations on bracken thiaminase particularly on the methods of enzyme extraction and preservation of enzyme activity in the fern from the time of harvest to initiation of analysis. Since suggestions have been made that bracken fern may be toxic only at certain stages of its development, plants collected

at different stages of maturity were analyzed for thiaminase activity.

Measurement of thiaminase activity was carried out using the procedure devised by Kenten[1] which was based on the formation of a pyrimidine-amine product. Pyridine is active in the transfer reaction catalyzed by bracken thiaminase leading to the formation of one mole of heteropyrithiamin (HPT) per mole of thiamin destroyed. Heteropyrithiamin is converted to a fluorescing substance which can be measured in a photofluorometer. Thiaminase activity was expressed as the quantity of thiamin in micrograms inactivated by 10 milligrams of fern (dry weight basis).

The results show that there are several factors influencing the various steps involved in the assay of bracken thiaminase. The preparation of bracken homogenate was found to be influenced by the pH and the kind of homogenizing medium used; the optimum pH was about 8.0 and among the different solutions tested for homogenizing the fern, Tris buffer plus cysteine and VERONAL buffer were the best. Complete extraction of the enzyme was not achieved when bracken fern was homogenized with Tris buffer plus cysteine; 75 per cent of the total thiaminase activity appeared in the aqueous phase and 25 per cent in the residue. For this reason, bracken homogenates were used as a source of thiaminase. This homogenate was stable for 24 hours at 5°C and its activity was destroyed by heating.

Time of incubation during the assay procedure influenced

the amount of thiamin destroyed. The rate of thiamin destruction continued to increase after the first hour; however, the ratio of the amount of thiamin inactivated was proportional to homogenate concentration. The thiamin remaining after enzymatic reaction was terminated by incubation with strong alkali and the remaining HMP was oxidized to the fluorescing compound, 2-methylpyrichromine. The strong alkali was found to have no effect on the galvanometer readings for the standard HMP solutions even after incubation for 2 hours.

To provide information on the change in activity of bracken thiaminase from the time the plant was harvested until analysis was started, fresh bracken fern was brought to the laboratory and subjected to either air drying, vacuum oven drying, freezing, freeze-drying, refrigeration and preparation of an acetone powder. All treatments resulted in considerable destruction of enzyme activity. Bracken fern brought to the laboratory in a ball of earth surrounding the stems and the roots was found to be the best way to secure samples for determining the concentration of thiaminase in the fresh form.

Thiaminase was found to be present in bracken fern at all stages of development. Its concentration increased as the dry weight per leaf increased. Activity was found to be high in the blade and stem but not in the roots and stipe.

[1] R. H. Kenten, The partial purification and properties of a thiaminase from bracken [Pteridium aquilinum (L.) Kuhn], Biochem. J. 67, 25 (1957).

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By

Evelyn G. Ostli

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# INTRODUCTION

Bracken fern has been described by Braid (1949/1950) as a botanist's plaything, on account of its characteristic methods of growth and development, and as an agricultural pest because it grows in many pastures and is difficult to eradicate. It attracted the interest of entomologists when ecdysones, which regulate molting and metamorphosis in insects, were isolated from the dry pinnae (Kaplanis, et al., 1967). Added to these are the veterinarian's problem and the nutritionist's concern about the toxic properties of this plant.

The poisonous nature of bracken fern for both monogastric animals and ruminants has been reviewed by Kingsbury (1964) and by Nicholson and Kang (1966). It is now recognized that bracken fern destroys thiamin in the ration, and this is responsible for the symptoms seen in monogastric animals. This is not the case with ruminants fed bracken fern. They develop irreversible hematological changes and as yet no substance is known to alleviate the condition.

In recent years, attention has also been focused on a certain toxin(s) in young, actively growing bracken fern which reproduces many of the effects of radiation; these include intestinal adenocarcinoma in rats and Japanese quail,

pulmonary adenomas in mice, and hematuria and bladder tumors in guinea pigs and cows. The nature of this toxin(s) is reviewed by Evans (1960).

The thiamin destroying factor in bracken fern is of interest not only because the fern is eaten by animals when found in their beddings or when they graze in the pasture but also because it is used for human consumption. Fujimura and Matsui (1963) reported that a variety of this fern is eaten by the Japanese. Marsh and Worrell (1962) stated that the young shoots or "crosiers" of bracken are used in fresh and cooked salads in some areas of the United States. This may also be true in other parts of the world.

There is still a controversy concerning the chemical nature and mode of action of the thiamin destroying factor. Reports have been made that this factor is either thermolabile or thermostable or that two factors are present in the fern.

The present work was designed to extend studies on bracken thiaminase particularly on the methods of enzyme extraction and preservation of enzyme activity in the fern from the time of collection to initiation of analysis. Since bracken fern is used as food, experiments were also conducted to find out if there are stages during its growth when the concentration of thiaminase is low or absent. These included determination of variations in activity in the different parts of the fern, in individual ferns

classified under the same stage of maturity and at different stages of development.

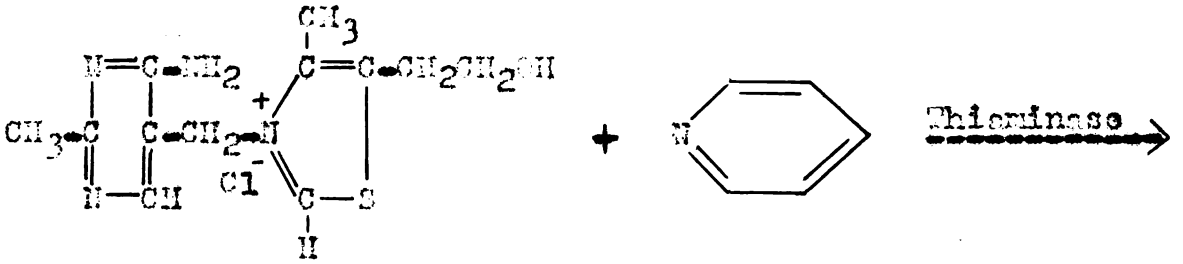
## REVIEW OF THE LITERATURE

### Historical Background

The presence of a thiamin destroying factor in fish was first reported by Green, Carlson and Evans (1941) from their observation in 1932 of a paralysing disease in foxes on a ranch owned by Mr. J. S. Chastek near Glencoe, Minnesota. They demonstrated that the disease which has been referred to as "Chastek paralysis" was a thiamin deficiency due to feeding certain species of raw or frozen fish. The deficiency occurred not because the diet was low in thiamin, but rather because the fish destroyed the thiamin. This observation was confirmed in chicks by Spitzer and others (1941) and in a later experiment with foxes by Green, et al. (1942).

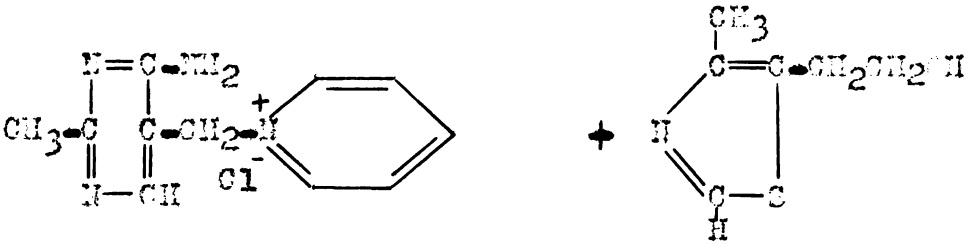
Experiments conducted by Woolley (1941) showed that the active principle in carp extract was not dialysable, unstable to heat and that the thiamin destroying action was not instantaneous. Evidence of the catalytic nature of the reaction suggesting that the thiamin destroying factor is an enzyme was achieved by Sealock and co-workers (1943) and by Krampitz and Woolley (1944). Literature on the Chastek paralysis factor has been reviewed by Rudkin (1949) and others (Nutr. Rev., 1943).

Thiaminase is an enzyme that catalyses the fission of the methylene-quarternary-nitrogen bond of thiamin in the presence of certain amines (Kodicek and Davis, 1949; Fujita, et al., 1952a; Woolley, 1953). The reaction involves the transfer of the pyrimidine moiety of thiamin to the amine. Using a specific amine as an example, the reaction catalyzed by thiaminase is shown in the following equation




Pyrimidine Moiety | Thiazole Moiety | Pyridine

Thiamin
3-(4-amino-2-methyl-pyrimidyl-5-methyl)-4-methyl-5,β-hydroxy-ethylthiazolium chloride



Heteropyrithiamin
1-(4-amino-2-methyl-pyrimidyl-5-methyl)-pyridinium chloride

Thiazole

Although the species of animals and plants containing thiaminase are not numerous, the transfer reaction has been shown in fishes, shellfishes, bacteria and Pteridophytes (Fujita, 1954). In contrast to this reaction, Fujita, Hose and Kuratani (1954) showed that the bacterium Bacillus

aneurinolyticus Kimura et Aoyama produces thiaminase capable of catalysing the hydrolytic cleavage of thiamin according to the equation

$$P - CH_2 - T^+ + H_2O \longrightarrow P - CH_2OH + T + H^+$$

where P and T represent the pyrimidine and thiazole components of thiamin respectively.

The presence of an antithiamin factor in ferns was first demonstrated experimentally by Woswig, Freed and Haag (1946) when rats were fed a ration containing 40 per cent air-dried bracken and adequate thiamin. This work was duplicated and confirmed by Evans and Evans (1949b) who suspected the tannin fraction of bracken as antagonistic to thiamin. In a similar study, Thomas and Walker (1949) showed that bracken contained a thermolabile factor capable of destroying thiamin and that this substance was not absorbed in the gut of the rat as indicated by the relatively high capacity for inactivation retained by the feces. Studies by Roberts and others (1949) reproduced the bracken staggers in horses similar to those reported by Madsen and Bruce (1920, 1933) which they successfully treated by thiamin therapy. Further experiments were conducted by Carpenter and others (1950) using rats, ponies and heifers. It was observed that the last group of animals did not respond to the administration of vitamin $B_1$.

Evans, et al. (1950) and Haag and others (1950) were able to extract the thiamin destroying factor from air-dried and fresh bracken, respectively, and reported it to be an

enzyme. The former group supported their finding by showing chromatographic evidence that thiamole was one of the products of the enzymatic reaction.

At this time independent studies conducted by Somogyi (1949) in Switzerland showed that the active principle in the extract of bracken fern was thermolabile and passed a dialysing membrane. Further studies were made on the chemical nature (Somogyi and Koller, 1959) and the isolation (Koller and Somogyi, 1961) of the thermostable factor which was finally achieved by Berüter and Somogyi (1967) and identified as 3,4-dihydroxycinnamic acid.

Pursuing their earlier findings on the presence of a thermolabile thiamin destroying factor in bracken fern, Evans and Jones (1952) observed that dialysis of bracken extract separated the enzyme into a thermostable "coenzyme" and a thermolabile "apoenzyme" fraction, both of which are required for complete enzyme action on thiamin. The identity of the "co-factor" in bracken thiaminase was studied by Watkin and others (1953). They reported that several meta-substituted aromatic amines could act as artificial "co-factors" to the apoenzyme of bracken and that this effect was similar to the enzyme system present in carp (Seelock and Davis, 1949), shellfish and bacterial thiaminase (Fujita and Toshiro, 1952). The work of Fujiwara and Matsui (1953) is in agreement with the findings of Watkin and others (1953) as shown by their observation that bracken fern has two different kinds of anti-thiamin factors;

thiaminase and a thermostable factor. These two factors were characterized by Fujita and co-workers (1955).

In plants, the thermolabile and thermostable thiamin destroying factor are present in ferns (Fujiwara and Matsui, 1953) and in cockscomb (Fujita, 1954). On the other hand, the thermostable factor has been shown by Bhagvat and Devi (1944a, 1944b), Fujita (1954), Kundig and Somogyi (1964) and Sakamoto and Fujita (1956) to be present in certain cereals, fruits and vegetables.

## Assay of Bracken Thiaminase Activity

The procedure for the quantitative assay of thiaminase activity appearing in the literature varies considerably. Because of differences in activity between fish, shellfish, bacteria and plant thiaminases (Fujita, 1954), this section will deal mainly with bracken thiaminase. Thiaminase activity from other sources will be discussed only to clarify certain points.

### Preparation of thiaminase extract

The importance of pH and buffer used to prepare bracken homogenate has been reported by various workers. Evans and others (1950) and Kenten (1957b) showed that the active factor could be extracted from bracken leaf with chloroform-water at pH 8.0. Fujita, et al. (1955) also found this buffer to be effective as well as other solutions like phosphate buffer at pH 7.0 and 10 per cent acetone. On the other hand, Fujiwara and Matsui (1953)

used water to prepare bracken extracts.

Published studies on bracken thiaminase have dealt primarily with characterization of its enzymatic activity. Only two studies have included purification procedures for this enzyme. Matlin and co-workers (1953) reported a method for purifying the enzyme which can be preserved in the freeze-dried state while Kenton (1957b) described a partially purified thiaminase preparation from water extracts of dried fern leaves which gave a 30 per cent yield representing a purification of about 60-fold.

Reaction of thiaminase extract with thiamin

Cleavage of thiamin by bracken thiaminase is influenced by different factors. These include pH of the reaction mixture, the incubation temperature and the presence of activators or inhibitors.

(1) pH

Results of experiments conducted by Haag, et al. (1950) showed that enzymatic activity increases from pH 2.0 to 5.5 or 6.5, the concentration and nature of the buffers used affecting the enzymatic process. Similarly, Fujimura and Matsui (1953) found thiamin inactivation to be greatest between 7 and 8, Fujita, et al. (1955) at pH 6.0 and Kenton (1957b) in the neighborhood of 7.5.

(2) Temperature

Incubation temperature has a marked effect on the reaction rate. Haag and others (1950) found the enzyme activity to be low at 25° and increased to 60° but at

70° the activity decreased. Fujita, et al. (1955) showed that with a rise in temperature the decomposition of thiamin became higher, but at 50 and 60°C, decomposition remained the same.

(3) Inhibitors

Several substances have been implicated as inhibitory to the enzymatic reaction. Among these are $AgIO_3$ (Haag, et al., 1950), $Cu^{++}$ and $Mn^{++}$ (Fujita and others, 1955) and $Fe^{++}$ and $Fe^{+++}$ (Fujita, 1954).

(4) Activators

With the finding of Sealock and Livermore (1949) that m-aminobenzyl-4-methylthiazolium chloride increased the destruction of thiamin by carp thiaminase in contrast to the o-amino analogue, they hypothesized that the activation was due to the former compound's combination through its amino group with the 5-methylene group of the pyimidine moiety as it is split from thiamin in the course of the enzyme reaction. In a subsequent study, Sealock and Davis (1949) incubated desiccated carp viscera, thiamin and m-nitroaniline and isolated N-(2-methyl-6-amino-pyrimidyl-5-methyl)-m-nitro-aniline. They concluded that amines enter into the reaction at a stage which probably follows the formation of the enzyme-substrate complex and are converted to a similar pyrimidylmethyl derivative. They further stated that because of the relatively small amount of enzyme intentionally used in the experiment, the artificially added reactor amine overwhelmed the naturally occurring one.

With bracken thiaminase, Evans and Jones (1952) showed that m-aminobenzoic acid and 5-amino-salicylic acid were able to replace the natural coenzyme or activate the apoenzyme produced after dialysis of bracken extract. Fujiwara and Matsui (1953) and Fujita, et al. (1955) showed that the aromatic amine aniline and the heterocyclic amines pyridine and quinoline produced marked activation of enzymatic destruction of thiamin. The presence of any of these compounds is not sufficient to cause maximal thiamin destruction but as indicated by Kenten (1957b) the concentration of the activator is also important. He observed that thiaminase activity increased in pyridine concentration up to about 0.03 M and remained constant over the range of 0.08- 0.3 M but with further increase in concentration, the enzymatic activity decreased.

The sulfhydryl compound cysteine has also been shown to influence thiaminase activity by Haag, et al. (1950) and Fujita and others (1955). Sodium thiosulfate and γ-acetoγ-mercaptopropyl acetate were also cited by Fujita (1954) as activators.

Measurement of thiaminase activity

The activity of thiaminase has been studied by estimating its destructive action on thiamin. Of the various chemical methods used for the estimation of thiamin, the colorimetric procedure of Melnick and Field (1939) has been used by Peelock and others (1943) and modified by

Krampitz and Woolley (1944) in their investigations of the characteristics of carp thiaminase. This method was later replaced by other procedures after Seelock and Goodland (1944) reported that the addition of cysteine resulted in interference of color development.

Another method for estimating thiamin remaining after enzymatic reaction is by the thiochrome procedure. Thiamin is converted to a blue fluorescing substance which can be measured in a photofluorometer. To measure thiaminase activity in fish, Spitzer and others (1941) used the method described by Hennessy (1941) while Gnaedinger (1965) worked out a thiochrome procedure eliminating enzyme hydrolysis and base exchange techniques.

Fujita and others (1952a) also modified Hennessy's procedure (1941) to study bracken thiaminase activity. Their new method was used by the Japanese workers in their subsequent studies of thiaminase from various sources.

The disadvantage of using the thiochrome method when working with plant materials was reported by Grob (1947). He showed that thiochrome can be reduced by many substances present in plants and converted to a non fluorescing substance. Reduction was particularly promoted by polyphenols, gallic acid, tannins, ascorbic acid and cysteine. This observation was confirmed in a similar experiment conducted by Evans and Evans (1949a).

Another method for estimating thiaminase activity is by the use of the base exchange reaction. Its use started

from the observation of Fujita, et al. (1952a) that thiamin breakdown by thiaminase is activated by certain bases. In the presence of thiamin and pyridine, thiaminase catalyses a base exchange reaction forming heteropyrithiamin and thiazole and that the former compound can be converted by alkaline oxidation to pyrichromine which has a greenish-yellow fluorescence. From this information, Kenten (1957b) developed a method for estimating small amounts of heteropyrithiamin in the presence of thiamin. He showed with this method using both crude extracts and partially purified preparations that in the presence of excess enzyme, 90-95 per cent of the thiamin added was converted to heteropyrithiamin.

The manometric method has also been suggested for the measurement of thiaminase activity. Sealock and Livermore (1944) and Kenten (1950) have shown that since the cleavage of one molecule of thiamin is accompanied by the release of a proton, the liberation of extra carbon dioxide in a bicarbonate buffer could be measured. Working with carp thiaminase, Sealock and Livermore (1944) found that the method they developed did not give results similar to those obtained by other chemical methods. With bracken thiaminase, Kenten (1950) reported that with his procedure about 5-10 per cent thiamin may be lost in side reactions but a reasonable agreement between the calculated and experimental carbon dioxide was found.

## The Physiological Significance of Thiaminase

The presence of the thiamin destroying enzyme in fishes, shellfishes, bacteria and ferns would suggest that it has a special role in the species containing it. Since there was no constant correlation between taxonomic classification or the feeding habits or habitat and the presence of the anti-thiamin factor in certain families of fish as reported by Hilker and Peter (1966) and by Deutsch and Hasler (1943), Kenten (1957a) and Fujita (1954) suggested that perhaps thiaminase has some special role in these organisms in which it is found. In vivo, it may be involved in a reaction common to most organisms while in vitro it may only show activity with thiamin.

Fujita and others (1952b) have shown that the transfer reaction is reversible. Incubation of various pyrimidyl bases and thiazole with shellfish, fish and bacterial thiaminase produced thiamin. Although Kenten (1957b) found no evidence of such a reaction taking place with bracken thiaminase, he suggested that such a role of thiaminase would presuppose different pathways of thiamin synthesis in, for example, different but closely related fishes (1957a).

The review written by Fujita (1954) and the work by Kenten (1957a) show that thiaminase will also act on several thiamin analogues. Woolley (1952) has also shown that pteroic acid or pteroylglutamic acid could be synthesized by a transfer reaction between the pteridine analogue of thiamin and p-aminobenzoic acid or p-aminobenzoylglutamic acid using carp

thiaminase. Although he does not consider this the mode of biosynthesis of these compounds since the yields are extremely small, this suggests the possibility of thiaminase as a synthesizing enzyme.

Importance and Possible Uses of Thiaminase

The significance of thiaminase in human and animal nutrition is still difficult to evaluate at this time. It has been pointed out in two reviews (Sudkin, 1949; Nutr. Rev., 1943) that this thiamin destroying factor may be used in the preparation of thiamin free diets as a substitute for prolonged autoclaving. Evidence of success in the production of $B_1$ avitaminosis experimentally has been shown by Spitzer and others (1941) in chicks, Smith and Proutt (1944) in cats and by Green and others (1941) in foxes, by mixing raw carp or herring in the diet. Vitamin $B_1$ deficiency symptoms due to consumption of bracken ferns were demonstrated by Carpenter and co-workers (1950) in rats and ponies and by Roberts and others (1949) in horses.

Recently, great interest has been given to a certain antitumor agent in the common quahog, mercenaria, mercenaria (Hegyeli, 1964; Schmeer, 1964). Studies by Judge (1966, 1968) demonstrated that DBA/2 mice infected with Friend leukemia virus and maintained on a thiamin free diet showed no clinical manifestation of the disease as compared with the controls maintained on a normal diet. It was suggested that a thiaminase may be the modality of action of various seafood

extracts as oncostatic or oncocidal agents.

The thiamin destroying enzyme has been regarded as having no direct bearing on human nutrition since it is unlikely that many people consume uncooked fish (Nutr. Rev., 1943). Melnick and others (1945) considered it otherwise and stated that consideration should be given to thiaminase in fish products as a possible conditioning factor in malnutrition. They demonstrated that a diet containing raw clams reduced the availability of dietary thiamin as measured by the thiamin excretion of human subjects. The same opinion was shared by Miller and Peter (1946) when they showed that two of the most popular species of fish, the "aku" and "ahi", which are frequently eaten raw in Hawaii as "sashimi" contain thiaminase. There is some concern about the possible presence of anti-thiamin activity in fish flours used for high protein food supplements for human use and also the use of fish products in animal feeds.

Although bracken fern is not as popular a food as fish, it has been reported by Fujimura and Matsui (1953) and by Proebloch and Worrell (1962) to be a common food in certain parts of the world. A possibility, therefore, exists that destruction of dietary thiamin could result from eating raw or partially cooked fern if consumed in sufficient quantity.

## MATERIALS AND METHODS

### Plant Material

Bracken fern [*Pteridium aquilinum* (L.) Kuhn var. *latiusculum* (Desv.) Underw.] was collected at different stages of maturity from April to August 1968. It grew in a wooded area at the Rose Lake Wildlife Experiment Station near East Lansing.

A number of procedures were tried in an effort to preserve the enzymatic activity from the time the fern was harvested until the analytical work could be started in the laboratory. These included (1) freshly cut fern transported in a dry state or kept moist with crushed ice, (2) within ten minutes after harvesting, the fern was made into an acetone powder (Mason, 1955) or (3) transporting the intact fern and the adhering earth to the laboratory.

At the laboratory, the ferns were sorted according to their stage of development. Description of the various stages of maturity and definition of the different parts of bracken fern are shown in Tables 1 and 2. Each fern was divided into the blade (B) and stipe (S) which were treated individually. Separation of these two parts was not possible for ferns at the first few stages of maturity because the blade appeared as a bud or as a fiddlehead in a tight 'hook'

Table 1

Description of Bracken Fern at Different Stages of Maturity

| Designated Stage of Maturity | Description |
|---|---|
| 0 | Secondary stem bud--found underground adjacent to a frond bud. It remains dormant and grows out as a secondary shoot if the frond bud is damaged. |
| 1 | Frond bud--club-like bud which will eventually expand above soil level. |
| 2 | Fiddlehead or 'crozier' in a tight 'hook' found 1-2 inches above ground level. |
| 3 | Fiddlehead or 'crozier' about 4-6 inches above the ground. The 3 lobes of the tripartite blade could be distinguished but the blade still forms a hook. |
| 4 | Frond is about 15 inches tall and the blade is about 3 inches long. The latter has started to turn erect but the tips of the blade have not completely unfolded. |
| 5 | Frond is about 20 inches tall and the blade is about 6 inches long. Tips of the blade lobes are well unfolded. |
| 6 | Frond is about 22-23 inches tall. The blade is about 8-9 inches long with sparse development of blade tissue. |
| 7 | The tripartite blade is fully expanded and dark green in color. |
| 8 | The tripartite blade has turned olive green in color. |

and therefore each form was analyzed as one. Samples of the stem and roots were also obtained for analysis.

Table 2

Definition of Terms

Blade -- Expanded portion of the leaf.

Stipe -- Leafstalk

Whole Leaf -- Blade and stipe; frond.

Rhizome -- Dark-brown horizontal stem found about 5 inches below the surface of the ground.

Roots -- Black, brittle, hair-like structures which arise from the rhizome, most frequently close to the buds.

Bracken fern was subjected to various procedures. These included (1) air drying at room temperature (27-29°C) for ten days, (2) drying in a vacuum oven at room temperature for two days, (3) freezing at -5°C, (4) refrigeration at 5°C, (5) preparation of an acetone powder and (6) freeze-drying (freezing at -29°C, platen temperature of 24-27°C, vacuum pressure of 5μ and a condenser temperature of -51°C) for one day. Frozen samples were wrapped in aluminum foil while refrigerated samples were wrapped in moist paper towels and kept in a plastic bag to avoid drying. When the fern was dried, water loss was estimated from the original and final weights of the sample. After drying, the fern was ground in a Wiley Mill and kept in a desiccator or refrigerated.

## Preparation of Bracken Homogenate

Dried or fresh bracken fern was homogenized for 15 minutes in a Virtis "45" Hi-Speed Homogeniser with crushed ice around the container. Preliminary determinations have shown that preparation of bracken homogenate for 0.25, 0.5, 0.75, 1.0 and 2.00 hours does not seem to improve the extraction of the enzyme.

The suspension was kept in ice until used. When bracken fern was homogenized in Tris buffer and cysteine, the suspension was stable under refrigeration for 24 hours while freezing for the same time appeared to result in a 10 per cent loss of activity. Storing the homogenate was not necessary because the analyses could be carried out within six hours.

For reasons that will be explained under Results and Discussion, bracken homogenate was used in the assay procedure instead of the suspension.

## Measurement of Thiaminase Activity

Measurement of thiaminase activity was carried out using the procedure devised by Kenton (1957b) which was based on the formation of a pyrimidine-amine product. Pyridine is active in the transfer reaction catalysed by bracken thiaminase leading to the formation of one mole of heteropyrithiamin (called HPT hereafter) per mole of thiamin destroyed; the amount destroyed was followed by measuring the rate of formation of HPT. The latter was

oxidised to 2-methylpyrichromine by alkaline ferricyanide. The pyrichromine was estimated by measuring fluorescence at 306mμ with a Turner Fluorometer equipped with an activation (primary) filter number 110-812 (excitation wavelength of 405 mμ) and a secondary filter number 110-823 (emission wavelength of 465 mμ).

The methods used in the assay of bracken thiaminase and HPT standard are found in the Appendix. The thiaminase activity of bracken fern was expressed as the quantity of thiamin in micrograms inactivated by 10 milligrams dry weight of fern or its equivalent. All calculations were made on a dry weight basis so that thiaminase activity of ferns at different stages of maturity could be compared. Moisture determinations were carried out by drying aliquot samples of bracken homogenate in an oven at 90°C for 48 hours in small aluminum pans.

## RESULTS AND DISCUSSION

### Factors Influencing the Assay of Bracken Thiaminase

An evaluation of the factors affecting the assay of bracken thiaminase will be discussed under the various steps involved in the procedure.

#### Preparation of bracken homogenate

An attempt was made to extract the thiamin destroying factor in bracken fern by Kenten's method (1955b). To test whether all the thiaminase could be extracted from the fern, a freeze-dried sample was homogenized with Tris buffer and cysteine. The volume of the homogenate was 25 ml. From this amount, 0.1 ml. was taken for analysis and 10 ml. was centrifuged at high speed for 10 minutes. The supernatant was decanted and the volume made up to 10 ml. with Tris buffer and cysteine. This was also done to the residue. A 0.1 ml. sample from each of these two fractions was taken and analysed for thiaminase activity. A combination of 0.1 ml. sample from the two fractions was also prepared and analyzed. The results of the experiment are shown in Table 3. About 75 per cent of the total thiaminase activity of the fern homogenate appeared in the aqueous phase and 25 per cent in the residue. This extraction

Table 3

Thiaminase Activity of Bracken Fern Homogenate Versus the Aqueous Phase and Residue of the Homogenate and a Recombination of the Latter Two

| Form of Bracken Fern Added to the Assay Solution | Thiamin (γ) Destroyed by 10 mg. Freeze-dried 7B[1] Sample |
|---|---|
| Homogenate | 1275 |
| Extract | 750 |
| Residue | 225 |
| Extract and Residue | 1088 |

[1] The number refers to the stage of development and the letter indicates that the blade of the frond was used for analysis.

procedure apparently resulted in the destruction of some activity since combining the residue and extract produced only 85 per cent of the activity of the untreated homogenate. Although most studies appearing in the literature have used tissue extracts to carry out the assay procedure, the report of Woolley (1941) showed that the aqueous extract of carp viscera was only 25 per cent as active as the carp suspension.

In Kenten's procedure (1957b) the time suggested for homogenizing bracken fern is 2.00 hours. In the present study the enzyme was not completely extracted into the supernatant after homogenizing the sample for the suggested length of time. For this reason an experiment was conducted to find out if there was a difference in the activity of bracken fern homogenized for 0.25, 0.50, 0.75, 1.00 and 2.00 hours. The results indicated that about the same amount of thiamin was destroyed by the homogenates prepared at different lengths of time. From this observation, the homogenates used in subsequent determinations were all prepared for 15 minutes.

Since complete extraction of the thiamin destroying factor in bracken fern was not achieved, the activity of this enzyme was studied when different solutions were used for the preparation of the fern homogenates. A comparison of the various media for homogenizing the fern is shown in Table 4. For these experiments, the same batches of freeze-dried fern at stage 6 and another at stage 7 were used for

Table 4

Influence of Different Buffers on Thiaminase Activity of Bracken Fern[1]

| Homogenizing Media[2] Water Plus: | Thiamin Destroyed by 10 mg. Freeze-dried Bracken Fern | | | |
|---|---|---|---|---|
| | γ Thiamin Destroyed | | Relative Thiaminase Activity | |
| | 6B[3] | 7B | 6B | 7B |
| Control[4] | 938 ( 900, 975) | 900 ( 900, 900) | 100.0 | 100.0 |
| 10% NaCl[5] | 450 ( 450, 450) | 750 ( 600, 900) | 48.0 | 83.3 |
| SHLP[6] | 825 ( 750, 900) | 863 ( 750, 975) | 88.0 | 95.9 |
| $NH_4OH$ | 1125 (1125,1125) | 900 ( 900, 900) | 120.0 | 100.0 |
| MSPP[6] | 1275 (1200,1350) | 975 ( 900,1050) | 136.0 | 108.3 |
| $CHCl_3$ + $NH_4OH$[7] | 750 ( 750, 750) | 1125 (1050,1200) | 80.0 | 125.0 |
| MOPS[6] | 863 ( 825, 900) | 1163 ( 975,1350) | 92.0 | 129.2 |
| Tris | 1425 (1350,1500) | 1163 (1050,1275) | 152.0 | 129.2 |
| Tris + Cysteine | 1950 (1950,1950) | 1763 (1500,2025) | 208.0 | 195.9 |
| TRICINE[6] | 2100 (2100,2100) | 2100 (2100,2100) | 224.0 | 233.0 |
| TRICINE + Cysteine | 2175 (2100,2250) | 2101 (1913,2288) | 232.0 | 233.4 |

[1] Duplicates were run for each homogenizing medium; the individual values and averages are given.

[2] pH adjusted to 8.0 except for the control which was only water.

[3] The number refers to the stage of development and the letter indicates that the blade of the frond was used for analysis.

[4] As described by Fujiwara and Katsui (1953).

[5] As described by Woolley (1941).

[6] For composition of buffer see section entitled Special Reagents in Appendix.

[7] As described by Kenten (1957b).

[8] 0.1 M L-Cysteine HCl added to Tris buffer at a 4.5 level gave the highest activity as compared to 0.025, 0.05 which was equal to that of 0.12 M concentrations.

all assays. The enzyme activity was apparently reduced when the fern was suspended in 10 per cent saline solution. This was the method used by Woolley (1941) for extracting thiaminase from fish. Water has been used by a number of Japanese workers for extraction of the enzyme from both bracken fern (Fujiwara and Katsui, 1953; Fujita, et al., 1955) and clam viscera (Fujita and others, 1952 a). Fujita, et al. (1955) extracted dried fern with water and phosphate buffer at pH 7.0. This extract from 5 mg. of fern was reported to destroy 0.67γ of thiamin when the solution contained 1γ of this vitamin. If it is assumed that this was the maximum activity, then the 10 mg. of dried fern extract would destroy 1.34γ of thiamin. The present results show that 10 mg. freeze-dried fern homogenized with water (pH 6.6) destroyed 900γ of thiamin and in the case of sample 63, the amount of thiamin destroyed increased to 1125γ with the addition of $NH_4OH$ to increase the pH of water to 8.0. This observation agrees with the reports of Fujita, et al. (1955) and Kenten (1957b) that extraction of bracken thiaminase is maximum at pH 7 to 8.

Kenten (1957b) extracted air-dried ferns with chloroform-water at pH 8.0. After incubation with 2 mg. thiamin for 1 hour, about 1000γ thiamin was inactivated by 10 mg. dried leaf. This is similar to the results in Table 4 using the same medium.

The use of other buffers like HEPPS, MOPS, Tris and TRICINE for homogenizing bracken fern resulted in higher

amounts of thiamin destroyed compared to the control solution. Bracken fern homogenized with TRICINE inactivated the greatest amount of thiamin. Unfortunately, this buffer became available only toward the end of the study. For this reason, all of the assays were carried out with the fern homogenized in Tris plus cysteine.

Cysteine has been reported to activate the enzymatic reaction (Haag, et al., 1950, Fujita and others, 1952a). When it was added to the Tris buffer, it did increase the enzyme activity by about a third or more. However, the addition of cysteine to the TRICINE buffer had no effect on the results.

To determine whether Tris, Tris plus cysteine and TRICINE buffers influenced the extraction of the enzyme or the assay procedure, freeze-dried bracken fern was homogenized in water-ammonia solution at pH 8.0. The bracken homogenate and one of the three buffers were added to the reaction mixture and the assay procedure was carried out. The results indicated that the addition of either Tris plus cysteine or TRICINE buffer increased the amount of thiamin destroyed as compared to a solution without any of the three mentioned buffers. Tris had no effect on the assay procedure. From this observation, it appears that TRICINE may also be acting as an activator of the enzymatic reaction in a manner similar to cysteine.

As a check on the linearity of the response produced by graded amounts of bracken fern, from 2 to 10 mg. of an

air-dried sample homogenized in the Tris plus cysteine solution were incubated with 2000 γ thiamin. There was a linear relation between the amount of bracken fern in the assay tube and the thiamin destroyed as shown in Figure 1. This agrees with the observation of Kenten (1957b).

Several reports have indicated that thiaminase in bracken fern extract loses its activity slowly even when preparations of the enzyme are stored at low temperatures. Seelock and co-workers (1943) found that aqueous extracts of fresh fish tissue lost up to 36 per cent of their thiaminase activity in 10 days at 5°C. The fish thiaminase extract prepared with 5 per cent potassium chloride by Gnaedinger (1965) lost no enzymatic activity during 4 hours of storage at 1°C but after 24 hours at this temperature, a loss in activity of about 10 per cent occurred. In this study, experiments were conducted to determine the stability of thiaminase in bracken fern homogenized in Tris buffer plus cysteine. The results were presented in Materials and Methods but it is well to point out that the homogenate was found to be stable under refrigeration for 24 hours at 5°C while freezing at -5°C for the same number of hours resulted in a 10 per cent loss in activity. Although no comparison could be made with bracken fern homogenized with other solutions, the stability of the bracken homogenate prepared with Tris buffer plus cysteine under refrigeration for one day may have been due to the fact that the latter solution is a reducing agent.

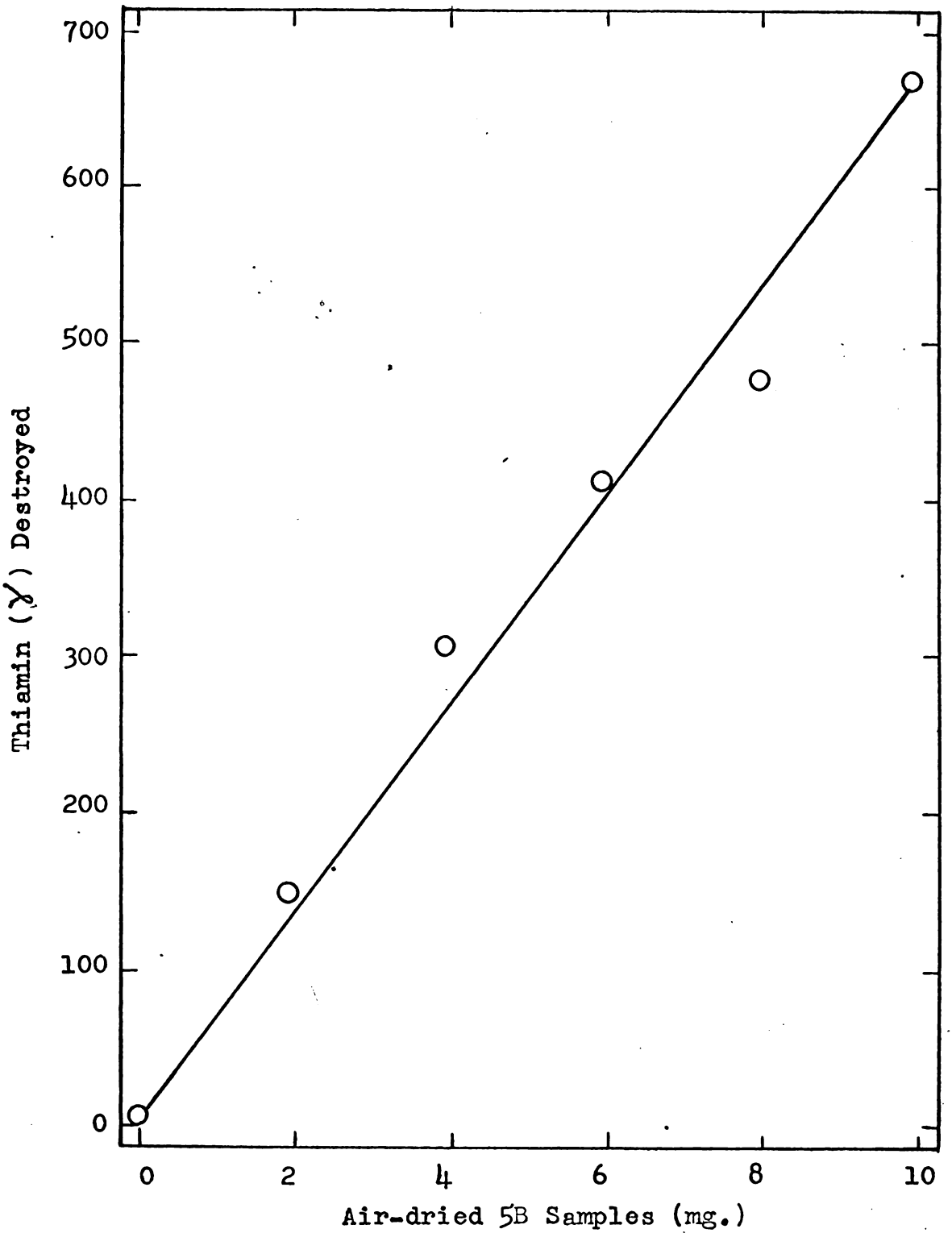



Figure 1. Effect of varying bracken fern concentration on amount of thiamin destroyed; 2000 γ vitamin $B_1$ in each tube.

The effect of heat on thiaminase activity was also determined. When the homogenate was boiled, it no longer destroyed thiamin. This agrees with the observations of Fujita, et al. (1935) who reported that there was a thermolabile factor in bracken fern and when it was heated, HMT was not formed.

Thiaminase assay procedure

A bracken homogenate was incubated according to the procedure described in the Appendix (with thiamin, pyridine and phosphate buffer) for 1, 3, 6 and 24 hours. Two volumes of this homogenate were used, one twice the other. After incubation, the HMT formed was converted to pyrichromine. The results are presented in Figure 2. Although the rate of thiamin destruction continued to increase after the first hour, the ratio of the amount of thiamin inactivated at each incubation period was proportional to the volume of homogenate used.

Heteropyrithiamin formed in the preceding reaction was not destroyed during the longest incubation period. This was shown when crystalline HMT was incubated for comparable periods. The HMT standard solutions carried through the alkaline ferricyanide oxidation after the incubation gave galvanometer readings which were the same as the freshly prepared standards. This suggests that HMT formed by bracken thiaminase during the incubation period was stable.

By using a one hour incubation, it was possible to carry

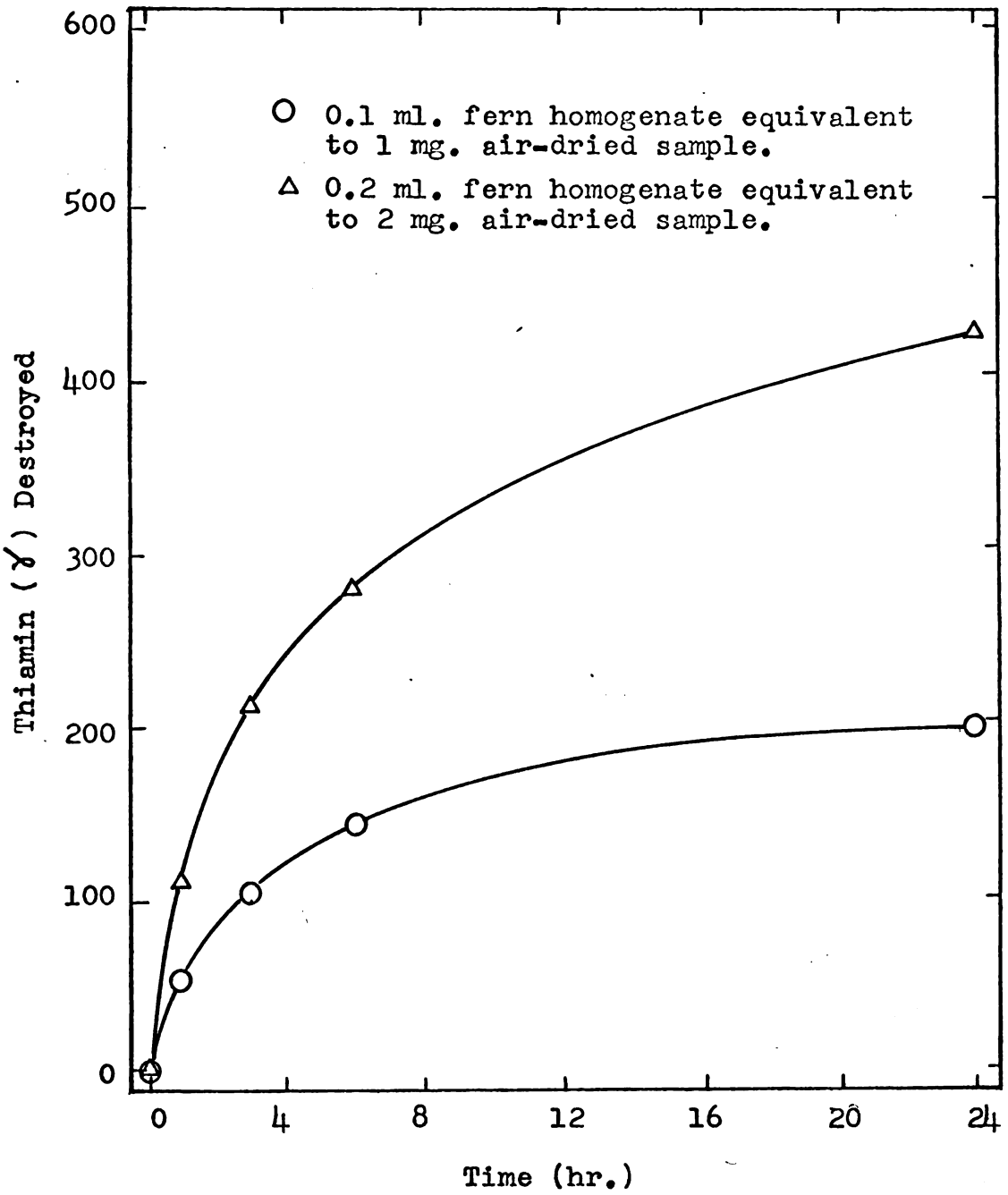



Figure 2. Effect of varying the time of incubation on the amount of thiamin destroyed using two levels of air-dried 3S bracken fern homogenate.

out the assay in one day. The agreement secured for duplicates put through the procedure further confirmed the reliability of this technique.

After the incubation period the enzymatic reaction was stopped with 20 per cent meta-phosphoric acid. This was followed by the addition of 20 per cent sodium hydroxide to the acid solution and incubation at 37°C for one hour. The thiamin remaining in the solution after the enzymatic reaction was completed must be destroyed since Kenton (1957b) showed that thiochrome (formed from thiamin) absorbs a considerable amount of energy at 366 mμ which is the wavelength for maximum absorption of 2-methyl pyrichromine (formed from HPT). The thiamin remaining in the solution can be destroyed by incubation with concentrated sodium hydroxide. To check on the completeness of thiamin destruction and stability of HPT under these conditions, the following procedures were carried out:

(1) Quadruplicate solutions similar to the ones used in the assay were prepared with 2000γ thiamin in each tube but no bracken fern. Two ml. of each solution were incubated at 37°C for 1 hour after which 1 ml. meta-phosphoric acid was added. To 2 ml. of each solution, 1 ml. of 20 per cent sodium hydroxide was added. The latter solutions were incubated for periods up to 2 hours. At the end of the incubation period, 0.5 ml. of alkaline ferricyanide was added; the excess ferricyanide was destroyed by adding 1 ml. of 0.05 per cent hydrogen peroxide. The resulting solution was

diluted with water to 100 ml. The fluorescence of the diluted solutions was measured at 386 mμ in a Turner Fluorometer.

The results (Table 5) show that when a reaction mixture with thiamin was incubated with 20 per cent sodium hydroxide at 37°C, thiamin destruction was completed within 30 minutes. Thiamin destruction occurred at a slower rate when the incubation temperature was set at 27°C.

(2) The preceding experiment was repeated except that HPT was used at two levels (200γ and 400γ per tube) in place of thiamin and the incubation carried out only at 37°C.

The results presented in Table 6 show that HPT was stable to concentrated sodium hydroxide during varying periods of incubation at 37°C. Thus with the assay procedure used in this study, the HPT formed by the enzymatic reaction was unaffected by a strong base during the one hour incubation period to destroy the residual thiamin. This observation agrees with the findings of Kenten (1957b).

A comparison of the galvanometer readings at zero time for thiochrome (Table 5) and 2-methylpyrichromine (Table 6) shows that 2000γ thiamin had a galvanometer reading of 56 after the assay procedure had been carried through while 200γ HPT, which is one tenth the thiamin concentration, had a reading of 28. This indicates that at 386 mμ the fluorescence of 2-methylpyrichromine is about 5 times greater than that shown by thiochrome. This means that should any thiamin remain after incubation with the

Table 5

Influence of Varying Time and Temperature of Sodium Hydroxide Incubation on Destruction of Thiamin

| Reaction Mixture with 2000γ Thiamin Incubated at: | Time (hour) of Incubation with 20% NaOH | Galvanometer Reading |
|---|---|---|
| 27°C | 0 | 57.5 |
| | 0.5 | 5.0 |
| | 1.0 | 2.0 |
| | 2.0 | 1.5 |
| 37°C | 0 | 56.0 |
| | 0.5 | 1.0 |
| | 1.0 | 1.0 |
| | 2.0 | 1.0 |

Table 6

Stability of Heteropyrithiamin to Concentrated Sodium Hydroxide During Incubation for Varying Lengths of Time

| Reaction Mixture Plus: | Time (hour) of Incubation with 20% NaOH at 37°C | Galvanometer Reading |
|---|---|---|
| 200γ HPT | 0 | 27.5 |
| | 0.5 | 27.0 |
| | 1.0 | 27.5 |
| | 2.0 | 27.5 |
| 400γ HPT | 0 | 55.0 |
| | 0.5 | 54.5 |
| | 1.0 | 54.5 |
| | 2.0 | 55.0 |

concentrated sodium hydroxide solution, it will have only a minor effect on the over-all results.

## Stability of Bracken Thiaminase

There is little information on the stability of thiaminase in the intact leaf from the time it is collected to the time it is analyzed. Working with carp thiaminase, Seelock, et al. (1943) reported that preparation of acetone powder yielded after such treatment 90 per cent of the original activity. Evans and co-workers (1950) showed that the antithiamin factor was stable in the intact leaf after drying at room temperature. From their report, it is not known to what extent there was a change in activity from the time the plant was harvested until the initiation of such treatment.

To provide information on the preceding, fresh bracken fern was brought to the laboratory where 1 batch of fern was analyzed immediately while another batch from the same stage of maturity was subjected to one of the procedures listed in Table 7. Such procedures as air drying, vacuum oven drying or freezing (at -5°C for 14 days) resulted in considerable destruction of enzyme activity. Even freeze-drying produced as much loss of activity as refrigeration for 24 hours. The preparation of an acetone powder even after purifying the acetone and using it in the chilled state did not obviate the loss of about 1/4 of the enzyme activity. Actually, a greater loss occurred in the acetone

Table 7

Stability of Thiaminase in Bracken Fern in the Fresh State and After Various Treatments

| Stage of Maturity | Treatment | Thiamin (γ) Destroyed by 10 mg. Sample (Dry Wt. Basis) | | Percentage Destruction of Enzyme Activity |
|---|---|---|---|---|
| | | Fresh | Treated | |
| 1 | Air Dried | 5607 | 1753 | 69 |
| 1 | Vacuum Oven Dried | 5607 | 750 | 87 |
| 63 | Frozen (14 days) | 3208 | 1903 | 41 |
| 83 | Acetone Dried | 6692 | 4856 | 27 |
| 63 | Freeze-dried | 3025 | 2322 | 23 |
| 33 | Refrigerated(1 day) | 4163 | 3007 | 28 |

powder prepared when the fern was brought into the laboratory in its ball of earth (Table 8). When that was done, the acetone powder prepared immediately showed less than 50 per cent of the activity of the homogenate prepared from the freshly cut frond (4006 versus 8077 γ thiamin destroyed by the respective samples). Although freezing has long been recognized as a method used to retard enzyme activity, Joslyn (1949) has reviewed some of the deleterious effects of low temperature on vegetable tissues. The data in the present study is accompanied by the destruction of about 1/4 of the activity present in the fresh fern.

The results in Table 8 show that the best method of transporting bracken fern from its source to the laboratory is by removing it in the ball of earth surrounding the stems and roots since a considerable amount of activity is lost from the time the fern is cut to the time it is analyzed. Keeping the fern moist after cutting does not facilitate retaining its activity but once the leaf is dry, the enzyme seems to be stable.

## Distribution of Thiaminase in Bracken Fern

In the spring, bracken fern exists in various states of development; some frond buds are just coming through the surface of the ground while other ferns in the immediate vicinity are about 15 inches tall. The individual variation in thiaminase activity did not appear related

Table 8

Enzyme Activity of Bracken Fern Fronds When Subjected to Various Kinds of Treatment After Harvesting

| Treatment | Thiamin (γ) Destroyed by 10 mg. Sample (Dry Weight Basis) | |
|---|---|---|
| | Stage 7B[1] | Stage 8[2] |
| (1) Freshly cut and left at room temperature for 1 hour. | 7838 ± 640 | 6753 ± 251 |
| (2) Freshly cut, kept moist at room temperature for 1 hour. | 6502 ± 803 | |
| (3) Freshly cut, acetone powder prepared immediately. | 3173 ± 108 | 4006 ± 749 |
| (4) Fern and stem removed to laboratory in ball of earth. | | 8877 ± 838 |

[1] Five tripartite fern blades were used. Each blade or frond consisted of three parts; one part from each of the five blades was treated as described after (1), another was treated as described after (2) and the third as described after (3). The values listed are the averages for the five parts ± the standard deviation.

[2] Same as footnote 1 except the procedures are described after (1), (3) and (4).

to the moisture in the ground or the degree of sunlight filtering through the tree. No attempt was made to get frond buds from the same rhizome. This would be difficult since ferns at the same stage of maturity were usually separated by a number of feet.

To find out if there are variations of thiaminase activity in bracken fern collected on the same day or on different days, bracken buds (stage 1 of development) were collected about 2 weeks apart and air-dried at room temperature for 10 days. The moisture content of air-dried samples was found to be 2 per cent more than the oven dried ferns. As shown in Table 9, samples A and B collected on different days, destroyed practically the same amount of thiamin. This was not the case for sample C which was collected on the same day as sample B. However, when several ferns were ground together and analyzed, the thiaminase activity was similar to that of samples A and B.

Since the results presented in Table 9 indicated that there are individual variations in the thiaminase activity of bracken fern, two ferns were brought to the laboratory in a ball of earth and each portion of the tripartite blade, stipe, stem and roots were analyzed. The results presented in Table 10 show that there were no variation in activity among the three divisions of the fern blade but as indicated in Table 9, there were differences in activity among individual ferns classified under the same stage of maturity. Although ferns 1 and 2 in Table 10 had about the same fresh

Table 9

Variations of Thiaminase Activity of Bracken Buds (Stage 1 of Development) When Collected on the Same Day or on Different Days. This Form Exists in a Number of Developmental Stages During the Spring

| Sample | Number of Ferns Used | Date Collected | Thiamin (γ) Destroyed by 10 mg. Air Dried Sample |
|---|---|---|---|
| A | One | 5- 3-68 | 1509 |
| B | One | 5-20-68 | 1753 |
| C | One | 5-20-68 | 975 |
| D | Several | 5-20-68 | 1809 |

All frond buds were air-dried. Samples A,B and C were individual frond buds--in each case the entire frond was used. Sample D represented about 7 of those frond buds which were ground and a portion was assayed.

Table 10

Thiaminase Activity of Different Parts of Bracken Fern Leaf[1] (Stage 3 of Development)

| | Fern 1 | | | | | Fern 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Portion of Blade | | | | | Portion of Blade | | | | |
| | Right | Middle | Left | Stipe | Whole Leaf | Right | Middle | Left | Stipe | Whole Leaf |
| Fresh Wt. (gm.) | 9.12 | 14.45 | 10.96 | 13.14 | 47.67 | 8.06 | 12.56 | 10.21 | 13.88 | 44.71 |
| Thiamin (mg.) Destroyed by 10 mg. Dry Sample | 7.05 | 6.89 | 6.79 | 0.78 | --- | 4.84 | 4.68 | 4.11 | 0.25 | --- |
| Thiamin (gm.) Destroyed by Entire Part (Dry Wt. Basis) | 1.62 | 2.50 | 1.88 | 0.27 | 6.27 | 0.99 | 1.48 | 1.06 | 0.08 | 3.61 |

[1] For definition of different parts of the bracken leaf see Table 2.

weight, the activity of the blade of fern 1 was about twice that of fern 2. The thiamin destroying activity of the stipe appears to be proportional to the activity of its blade. Thiaminase activity of the stipes of ferns 1 and 2 in relation to their blades are 4.5 and 2.2 per cent respectively. The stem or rhizome inactivated 12.23 mg. while the roots destroyed 0.28 mg. thiamin per 10 mg. sample on a dry weight basis.

The results in Tables 9 and 10 indicating that differences in thiaminase activity exist among ferns classified under the same stage of maturity may imply that either variations really exist or perhaps the gross method of classifying bracken fern in different developmental stages was not adequate to detect more detailed changes that occur in the plant as it matures which could be a better index for classification.

It appeared in Table 10 that there were differences in thiaminase activity in the different parts of the bracken leaf. One fern frond was taken and separated into different parts as indicated in Table 11. The greater portion of the activity was found in the blade. Since the stipe has two separate colors, a green portion close to the blade and a dark brown portion close to the stem, it was suspected that this difference might be due to a difference in thiaminase content. Results show that the activity for both portions were the same.

Table 11

Distribution of Thiaminase Activity in Bracken Fern at Stage 3 of Development

| Part of Fresh Fern | Thiamin (γ) Destroyed by 10 mg. Sample |
|---|---|
| Frond | 493 |
| Stipe, green portion close to the blade | 169 |
| Stipe, dark brown portion close to the stem | 172 |

It is interesting to note that the actively growing parts of the fern, the stem and the frond blades, were observed to possess the highest thiaminase activity. This is in agreement with the observation of Evans and Evans (1949a) in their *in vitro* and animal feeding experiments.

There are very few reports on the thiaminase activity of bracken fern at different stages of maturity. Smith and Fenton (1944), Shearer (1945) and Evans and Evans (1949a) found a decreasing crude protein and increasing crude fiber with aging of this plant. Wenig, *et al.* (1946) and Haag and others (1947) fed rats air-dried fern collected from different lots and at different stages of maturity. All produced thiamin deficiency symptoms but no quantitative data on thiaminase activity were presented.

Studies were conducted by Evans and Evans (1949a) using air-dried bracken leaves harvested in July when the plant was at full growth and still green, and those harvested in October when the plant was completely withered.

They showed that the former batch of ferns destroyed 90 per cent of the 100γ thiamin added to an assay solution containing 0.5 gm. of fern. This batch of fern also proved toxic when added to the ration of rats at a 40 per cent level. On the other hand, the latter batch of ferns inactivated only 5 per cent of the thiamin added to the assay solution and was not toxic to rats. Similar observations were also made on horses (Evans and Evans, 1949a; Evans, et al., 1951). It was postulated by these authors that bracken fern is more toxic at certain stages of growth.

Since the preceding reports indicate the possibility of a difference in concentration of the thiamin destroying factor as bracken fern matures, ferns at different stages of development as described in Table 1 were collected and analyzed for their thiaminase activity. When the dry weight and thiaminase activity were plotted on a graph as shown in Figure 3, there appeared to be a relationship between the two. As the dry weight of the leaf increased, the amount of thiamin destroyed per leaf also increased. The values plotted in Figure 3 are shown in Table 12 in the Appendix.

A considerable amount of enzyme activity was lost when the mature fern dried up. Dried fern fronds were gathered in late November of 1967 in the same area where the present samples were collected. The blade of the withered fern destroyed 0.33 mg. thiamin while fresh fern blade at full maturity (stage 8) inactivated 6.69 mg. thiamin. Both values are expressed per 10 mg. sample on a dry weight basis.

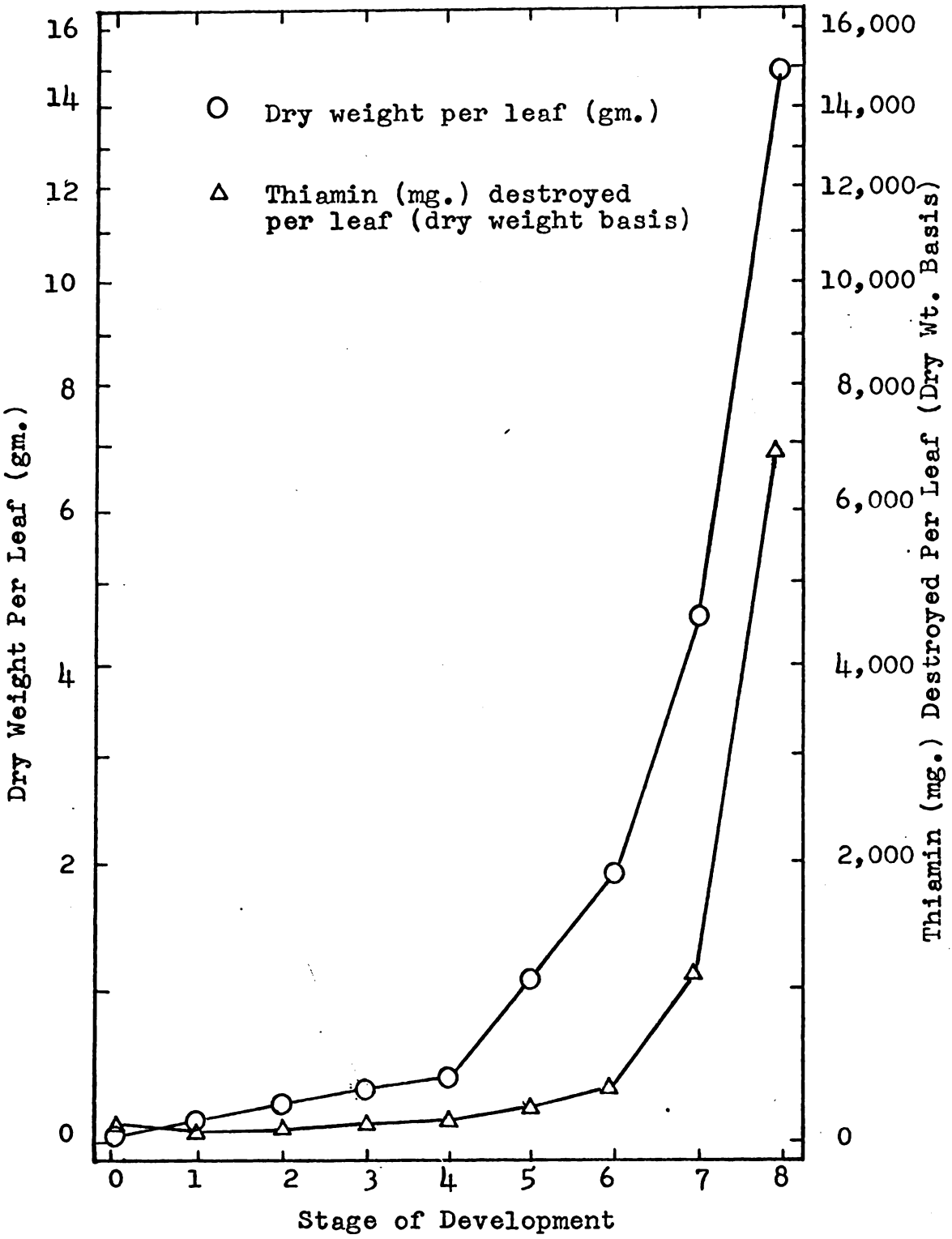



Figure 3. Relationship of dry weight of bracken fern to its thiamin destroying activity.

The results of the present study show that thiaminase is found in bracken fern at all stages of development, its activity increases as the dry weight per leaf increases. This enzyme was observed to be unstable as soon as the bracken leaf was separated from its stem and its activity was found to be influenced by various conditions involved in the assay procedure.

## SUMMARY

Experiments were conducted to extend studies on thiaminase activity in bracken fern particularly on the methods of enzyme extraction and preservation of enzyme activity in the fern from the time of collection to initiation of analysis. Ferns were also harvested and analyzed at different developmental stages to establish whether a change in activity occurred as the plant matured. Enzyme activity was measured by the procedure of Kenten (1957b).

In the presence of excess thiamin, thiaminase activity was found to vary with the kind and pH of the homogenising media. Complete enzyme extraction was not achieved. Centrifuged extracts possessed high activity but some remained in the residue.

Thiamin inactivation was found to be proportional to the amount of bracken homogenate used. This fact together with the evidence that the active factor is thermolabile support the findings of other workers that the principle effecting thiamin loss is an enzyme.

Enzymatic activity was found to increase as the dry weight of the fresh leaf increased. The activity varied in the different parts of the plant and among ferns at the same stage of maturity. As soon as the leaf was separated

from its stem, loss of activity was observed but once the form was dry the activity appeared to be stable.

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# APPENDIX

## Special Reagents

(1) 1-(4-amino-2-methyl-pyrimidyl-5-methyl)-pyridinium bromide hydrobromide was prepared by the method of Kenten (1957b) from 4-amino-5-bromomethyl-2-methyl-pyrimidine hydrobromide.

(2) Thiamine chloride hydrochloride [1]

(3) Buffers

(a) 0.2 M Tris [(hydroxymethyl) aminomethane] pH 8.0.

(b) 0.05 M MOPS [2] (Morpholine sultone) pH 8.0.

(c) 0.05 M TRICINE [2] [Tris (hydroxymethyl) methyl glycine] pH 8.0.

(d) 0.05 M HEPPS [2] (Hydroxyethylpiperizine propane sulfonic acid) pH 8.0.

(e) 0.05 M SHEP [2] (Succinyl hydroxyethylpiperizine) pH 8.0.

[1] Purchased from General Biochemicals, Chagrin Falls, Ohio. This was equal to the U. S. P. Thiamine Hydrochloride Reference Standard.

[2] Buffers furnished by Dr. Norman Good of the Department of Botany and Plant Pathology, Michigan State University. MOPS and TRICINE are available from Calbiochem.

## Thiaminase Assay

Preliminary experiments were conducted to determine the amount of bracken homogenate that could be added to a known amount of thiamin chloride hydrochloride so that the rate of enzymatic reaction would be independent of the substrate concentration.

Each assay involved the addition of 0.1 ml. of fern homogenate (equivalent to 1 mg. of dried fern) to a mixture containing 2000γ thiamin, 1 ml. of 0.1 N pyridine, 1 ml. of 0.2 M phosphate buffer (Gomori, 1955) at pH 7.5 and water to a total volume of 10 ml. The assay solution was prepared by taking a 2 ml. sample of the reaction mixture and incubating it for 1 hour at 37°C. Enzyme activity was stopped with 1 ml. of 20 per cent metaphosphoric acid. The blank solution was prepared by taking 2 ml. of the reaction mixture and adding 1 ml. of the metaphosphoric acid at zero time. Both acid mixtures were centrifuged and 2 ml. of the supernatant from each sample was incubated for 1 hour at 37°C with 1 ml. of 20 per cent sodium hydroxide to destroy the residual thiamin. The 0.5 ml. of ferricyanide reagent [4 ml. of 1% potassium ferricyanide plus 6 ml. of 20% sodium hydroxide] was added and after 15 minutes at room temperature the excess

ferricyanide was removed by the addition of 0.05% hydrogen peroxide. After thorough mixing, the fluorescence of the mixture was measured.

## Standard Heteropyrithiamin

Using 1-(4-amino-2-methyl-pyrimidyl-5-methyl)-pyridinium bromide hydrobromide which is commonly referred to as heteropyrithiamin, standards were treated in a similar manner as the thiaminase assay except bracken homogenate and thiamin were omitted from the reaction mixture. Two levels of heteropyrithiamin were used in each determination. The readings from the two levels of heteropyrithiamin were plotted on a graph. Using this graph, the amount of heteropyrithiamin formed from the thiaminase assay using a known amount of bracken fern was computed according to dilutions made.

Table 12

Thiaminase Activity of Bracken Fern at Different Stages of Maturity

| Sample Code[2] | Fresh Weight[3] (gm.) | Percentage Dry Matter | Thiamin (mg.) Destroyed (Dry Weight Basis) | | |
|---|---|---|---|---|---|
| | | | 10 mg. Sample[4] | Part of Leaf[5] | Entire Leaf |
| 0 | 0.076 | 16.00 | 14.64 | 17.90 | 17.90 |
| 1 | 0.564 | 8.36 | 5.61 | 26.45 | 26.45 |
| 2 | 2.135 | 8.65 | 1.41 | 26.15 | 26.15 |
| 3B | 0.089 | 16.21 | 4.16 | 6.00 | 35.31 |
| 3S | 2.904 | 8.75 | 1.18 | 29.31 | |
| 4B | 0.042 | 17.49 | 5.25 | 3.88 | 51.74 |
| 4S | 3.927 | 8.23 | 1.48 | 47.86 | |
| 5B | 1.325 | 11.02 | 3.49 | 50.96 | 137.03 |
| 5S | 9.177 | 10.17 | 0.92 | 86.07 | |
| 6B | 0.789 | 14.04 | 3.21 | 214.13 | 251.36 |
| 6S | 3.732 | 11.55 | 0.32 | 37.23 | |
| 7B | 15.957 | 15.29 | 2.39 | 583.57 | 1072.18 |
| 7S | 13.033 | 15.70 | 0.24 | 488.60 | |
| 8B | 32.680 | 25.25 | 6.69 | 6456.28 | 6761.69 |
| 8S | 13.510 | 26.00 | 0.58 | 305.41 | |

[1] For description of bracken fern at different stages of maturity see Table 1.

[2] The number refers to the stage of development and the letter indicates that the blade (B) or stipe (S) of the frond was used for analysis.

[3] Average of two samples.

[4] Average of two determinations.

[5] The blade and stipe were not analyzed separately for the three earliest stages of growth. Thereafter each of these parts of the leaf were analyzed and the sum of these values are given in the last column.