ROTHAMSTED RESEARCH

Report for 1957

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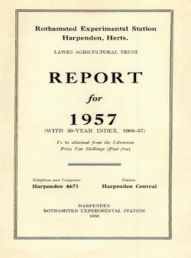

Bracken Thiaminase

R. H. Kenten

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BRACKEN THIAMINASE

By

R. H. KENTEN

Studies of enzymes *in vitro* led to the discovery, some thirty or forty years ago, that on occasion, compounds of similar structure to the substrate were capable of competitively inhibiting the enzyme activity. Later, it was demonstrated that the bacteriostatic action of sulphanilamide was antagonized by the essential metabolite *p*-aminobenzoic acid. This led to the Woods–Fildes hypothesis, which contends that because of the structural similarity of sulphanilamide and *p*-aminobenzoic acid, sulphanilamide blocks the enzyme system concerned with the metabolism of *p*-aminobenzoic acid and interferes so much with an essential metabolic function of the bacterium that growth is inhibited. Sulphanilamide and *p*-aminobenzoic acid compete for the enzyme system, and bacteriostasis results when the inhibitor is successful in the competition. It was then recognized that chemotherapeutic substances might be designed by the synthesis of substances of analogous structures to essential metabolites, thereby destroying the organism by a competitive antagonism of the essential metabolite. However, because of the present limited state of our knowledge of the multitude of co-ordinated reactions which are an essential and indispensable condition of the existence of organisms, attempts to design chemotherapeutic substances in this way have met with little success. It is certain, however, that as biochemical knowledge accumulates, living processes will become more and more susceptible to control and that an increasing number of drugs, weedkillers and insecticides will be deliberately designed and synthesized. It is for the purpose of obtaining such knowledge that a study of the enzymes of bracken (*Pteridium aquilinum* (L.) Kühn) has been undertaken; for none of the weedkillers which have been tested have proved to be practically useful for eradicating bracken specifically. The investigations so far have only occupied part of the time over the past three years and although a number of the enzymes common among other organisms have been identified in bracken, as yet our knowledge is fragmentary and unco-ordinated and only the work done on bracken thiaminase will be discussed here.

Introduction

Thiaminases were first recognized in certain species of fishes. Their discovery followed the demonstration that the disease in animal populations known as "Chastek paralysis" was in fact a thiamine (vitamin B1) deficiency and developed when certain species of raw fish were included in the diet of foxes, although thiamine was present in adequate amounts. The substance in fish responsible for the destruction of the dietary thiamine was shown to be an en-

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zyme by Sealock, Livermore and Evans (1943). The relevant literature has been reviewed by Yudkin (1949) and Harris (1951).

The thiaminases are enzymes which catalyse the fission of the methylene–quaternary-nitrogen bond of thiamine (Fig. 1). This usually occurs only in the presence of certain amines, the pyrimidine moiety being transferred to the amine according to the general equation,

$$P\text{–}CH_2\text{–}T^+ + RNH_2 \longrightarrow P\text{–}CH_2\text{–}NHR + T + H^+ \quad . \quad (1)$$

where P and T stand for the pyrimidine and thiazole components of thiamine respectively (Woolley, 1953; Fujita *et al.*, 1952; Sealock and Davis, 1949). Thiaminases catalysing the transfer reaction have been found in fishes, shellfish, bacteria and ferns (Harris, 1951; Fujita, 1954), but only with the bacterium *Bacillus aneurinolyticus* Kimura et Aoyama is there good evidence of the production of a thiaminase capable of catalysing the hydrolytic fission of thiamine (Fujita, Nose and Kuratani, 1954)

$$P\text{–}CH_2\text{–}T^+ + H_2O \longrightarrow P\text{–}CH_2OH + T + H^+ \quad . \quad . \quad (2)$$

Studies of plant thiaminase stem from the observation of Weswig, Freed and Haag (1946) that rats fed on a ration containing 40 per cent of air-dried bracken and adequate thiamine developed acute thiamine deficiency. Thomas and Walker (1949) confirmed this work and showed that bracken contained a thermolabile system capable of destroying thiamine. The active system was extracted from the dried leaf by Evans, Jones and Evans (1950), who concluded that it contained an enzyme and established that the thiazole component of thiamine was one of the products of the reaction. The situation was, however, complicated by the reports of Somogyi (1949) and Somogyi and Muralt (1949) that the factor responsible for the inactivation of thiamine in fern and bracken extracts was thermostable and passed a dialysing membrane. Subsequently, Evans and Jones (1952) obtained evidence that aqueous extracts of bracken contained an enzyme capable of catalysing the transfer reaction of equation (1), but the work of Fujita, Okamoto and Nose (1955) with the variety of bracken *var. japonicum* suggested that an enzyme was present which was capable of catalysing both the transfer and the hydrolytic fission reactions (equations 1 and 2).

It was known that horses suffer from thiamine deficiency when the proportion of bracken in their diet is high (Roberts, Evans and Evans, 1949) and it seemed likely that the deficiency was brought about by the destruction of the dietary thiamine by factors in the bracken. It follows therefore that such factors are unlikely to be present in the common plants of pastures. Also, Fujita (1954) examined large numbers of higher plants for the presence of thiaminase and found activity only in the herb *Celosia crista*.

Apart from the need to clarify the conflicting reports of the nature of the thiamine-destroying factors which are present in bracken, it was of particular importance to establish whether an enzyme system which attacked thiamine was present in bracken but absent from most of the higher plants. Such a difference might be exploited for the development of a specific bracken-killing agent.

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Preliminary experiments

Examination of aqueous extracts of dried and fresh bracken showed that the destruction of added thiamine was small and that it could be greatly increased by the addition of certain amines. Further, over 90 per cent of the activity was abolished by heating the extracts for 15 minutes at 100°. These results, in agreement with those of Evans and co-workers, suggested that the bracken extracts contained a thiaminase capable of catalysing the transfer reaction according to equation (1). Previously, thiaminase activity had been followed by measuring the disappearance of thiamine, although evidence for the transfer reaction had been got by the isolation of the pyrimidine–amine product, or by fluorimetric or chromatographic techniques. As a result of the preliminary experiments, it became clear that it would be desirable to develop a method by which the formation of the pyrimidine–amine product could be measured. The availability of such a method would make it possible to determine the precise nature of the reaction by which thiamine was destroyed. For the activating effect of amines might have been due either to their stimulating the hydrolytic fission of thiamine or to their entering into the transfer reaction.

Spectrophotometric method of estimating thiaminase transfer activity

Tests with a variety of amines suggested that bracken thiaminase destroyed thiamine most readily in the presence of pyridine. If the transfer reaction was involved it would lead to the production of 1 mol. *N* - (4 - amino - 2 - methylpyrimidin - 5 - yl) - methylpyridine heteropyrithiamine, called HPT hereafter, Fig. 1) per mol. of thiamine destroyed and could be followed by measuring the rate of formation of HPT. A method of estimating small amounts of HPT in the presence of thiamine was therefore worked out (Kenten 1957). The method depends on the destruction of thiamine by incubation with strong alkali and subsequent oxidation of the HPT to 2-methyl-pyrichromine by alkaline ferricyanide. The 2-methyl-pyrichromine is estimated spectrophotometrically at 386 mμ. Using this method, studies were made of the effect of pH, thiamine concentration and pyridine concentration on the rate of formation of HPT by extracts and partially purified thiaminase preparations from bracken.

These studies helped to establish standard conditions for the estimation of thiaminase activity under which the rate of formation of HPT was directly proportional to the amount of extract or thiaminase preparation present. This new method was of value in following the thiaminase activity in work on the purification of bracken thiaminase. Kenten (1957) defined one unit of thiaminase activity as that amount of enzyme which catalyses the formation of 1 μmole of HPT in 1 hour under the standard conditions, and the specific activity as the number of enzyme units/mg. of N of the enzyme preparation.

The course of the reaction

Using either water extracts of dried bracken leaf or partially purified thiaminase preparations, it was shown that in the presence

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of pyridine 90–95 per cent of the thiamine added was converted to HPT. The activity was virtually completely destroyed by heating the extracts or thiaminase preparations for 15 minutes at 100° before testing. With the small amounts of extract used no destruction of thiamine in the absence of pyridine could be detected. These results suggested that destruction of thiamine with both the extracts and the partially purified thiaminase preparations took place through the transfer reaction and that side reactions were small.

By fractionation with ammonium sulphate and calcium phosphate gel, concentrated, partially purified thiaminase preparations were made from water extracts of dried bracken leaves. The best preparation represented a purification of about sixty-fold. It catalysed the formation of 3,150 μmole of HPT/hour/mg. of N of the preparation under the standard conditions. Using such preparations, no evidence was obtained that they were capable of catalysing the destruction of thiamine in the absence of amines.

Difference between bracken and higher plants and its bearing on bracken eradication

It was therefore clearly established that bracken contained a thiaminase capable of catalysing the transfer reaction (equation 1), but without significant hydrolytic activity (equation 2). Tests with a number of higher plants failed to provide any evidence for the presence of an enzyme system capable of destroying thiamine. This difference suggested two possible lines of approach towards the development of a specific bracken-killing agent. In the first place, it might be possible to synthesize a thiamine analogue which would not be attacked by thiaminase but which would combine with it and destroy its catalytic activity. If the thiaminase system is essential, then introduction of such a compound into the bracken plant might inactivate this system and so disturb the metabolism of the plant that its death would follow. Secondly, it might be possible to introduce a substance into the bracken plant on which the thiaminase would act to release a toxic compound. That this latter type of approach can be successful has been demonstrated by Wain and co-workers (Wain, 1956). With certain homologues of the substituted phenoxyacetic acids, selectivity was achieved by exploiting differences in the β-oxidation systems of plants; only certain plants having the capacity to convert the homologues into the phytotoxic substituted phenoxyacetic acids.

To facilitate studies of the action of bracken thiaminase on thiamine analogues it was necessary to develop a general method for following thiaminase action. The spectrophotometric method described previously can be used only when pyridine is the acceptor amine and heteropyrithiamine is one of the products of the reaction.

Manometric studies of bracken thiaminase

Since both the reactants and the products of the thiaminase transfer reaction are bases and the extent of ionization of the products, at about neutral pH, may differ from that of the reactants, acid may be released. If the reaction takes place in bicarbonate–CO_2 buffer, the amount of acid released, and hence the extent of

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thiaminase action, can be followed manometrically by measuring the evolution of CO_2.

The following examples of systems in bicarbonate–CO_2 at pH 7·5 make this clear. If the acceptor amine is a strong, or moderately strong base (pKa 10 or greater), then the two extreme possibilities may be written

$$P\text{–}CH_2\text{–}T^+ + RNH_3^+ \longrightarrow P\text{–}CH_2\text{–}NH_2^+R + T + H^+ \quad (3)$$

$$P\text{–}CH_2\text{–}T^+ + RNH_3^+ \longrightarrow P\text{–}CH_2\text{–}NHR + T + 2H^+ \quad (4)$$

and between 1 and 2 mol. of acid would be released according to the extent of ionization of the pyrimidylmethyl–amine product. If the acceptor amine is a very weak base (pKa 5 or less) and the pyrimidylmethyl–amine product is a strong base, then the reaction can be formulated

$$P\text{–}CH_2\text{–}T^+ + RNH_2 \longrightarrow P\text{–}CH_2\text{–}NH_2^+R + T \quad . \quad (5)$$

and no release of acid, and hence no evolution of CO_2, would take place. For studying such systems (equation 5) therefore, the manometric method is not suitable, but they can be avoided by the choice of an amine acceptor of suitable pKa.

Quantitative studies using aniline, pyridine, piperidine and trimethylamine showed that, although a small part (5–10 per cent) of the thiamine was lost by side reactions under certain conditions, there was a reasonable agreement (within 10 per cent) between the calculated and experimental outputs of CO_2 (Kenten, 1958). This suggested that the method was suitable for studying the action of thiaminase, and it was accordingly used to test the activity of thiaminase towards structural analogues of thiamine.

Behaviour of thiaminase with structural analogues of thiamine

Thiamine analogues were synthesized which differed in structure from that of thiamine in that only the thiazole portion was replaced by different amines.

The results of manometric tests with these compounds showed that some of them were attacked by the bracken thiaminase and that none of them, when present in relatively small amounts ($5 \times 10^{-3}M$), was capable of appreciably inhibiting the action of bracken thiaminase on thiamine. In particular, heteropyrithiamine and quinilinothiamine (Fig. 1), in which pyridine and quinoline replace the thiazole heterocycle, were readily attacked by thiaminase at about half the rate of that with thiamine. When the primary amino group of thiamine, which is carried on the pyrimidine portion of the molecule, was replaced by hydroxyl the resultant compound, oxythiamine, was attacked very slowly. Oxythiamine at concentrations of $5 \times 10^{-3}M$ was, however, without appreciable effect on the rate of the thiaminase reaction with thiamine. These results suggested that in searching for a structural inhibitor of thiaminase a more profitable approach would be to vary the nature of the pyrimidine component of thiamine. Accordingly, several analogues were synthesized in which the pyrimidine heterocycle was replaced by a substituted benzene ring and in which the thiazole component was the same, or very nearly the same, as that of thiamine. Tests

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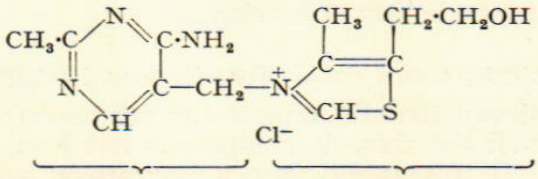
showed that none of these compounds was attacked by thiaminase. One of them, 3-(*o*-aminobenzyl)-4-methylthiazole (ABMT, Fig. 1), was found to be a very powerful inhibitor of thiaminase action. Concentrations of 2X $10^{-6}M$ ABMT inhibited bracken thiaminase by 15–20 per cent and at $5 \times 10^{-5}M$ inhibition was virtually complete (Kenten, 1958). The structural requirements for inhibition appear to be very exacting, for if the amino-group of ABMT is replaced by a nitro-group the capacity to inhibit thiaminase is lost; $5 \times 10^{-3}M$ concentrations of the nitro-compound being without

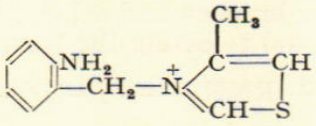
FIG. 1.



Pyrimidine moiety (P) Thiazole moiety (T)

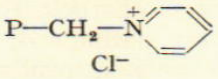
Thiamine

N-(4-amino-2-methylpyrimidin-5-yl)methyl-5-(β-hydroxyethyl)-4-methyl thiazolium chloride



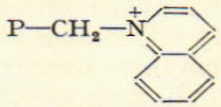
ABMT

3-(*o*-aminobenzyl)-4-methylthiazolium chloride



Heteropyrithiamine

N-(4-amino-2-methylpyrimidin-5-yl) methylpyridinium chloride



Quinilinothiamine

N-(4-amino-2-methylpyrimidin-5-yl) methylquinolium chloride

effect. Inhibition of thiaminase by ABMT would be expected to be competitive because of its structural resemblance to thiamine, and in fact Sealock and Goodland (1944) have obtained evidence that fish thiaminase is inhibited competitively by ABMT. With bracken thiaminase, however, the inhibition is not competitive because increasing the concentration of thiamine fails to decrease the amount of inhibition. Also, the amount of inhibition increases with increase in time of exposure of the thiaminase to the ABMT. It is possible that the ABMT initially combines reversibly with the enzyme at the same site as thiamine but that a second irreversible reaction follows which gives an inactive ABMT–enzyme complex.

Injection of ABMT into bracken

Single injections of up to 5 mg. of ABMT into the stems of bracken fronds caused no obvious damage to develop in a period

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of 3–4 weeks. These injections were made into pot-grown bracken in early August, when growth was almost complete. The size of the fronds was such that, assuming even distribution, the concentration of ABMT in the frond would be at least $3 \times 10^{-4}M$; *in vitro* $5 \times 10^{-5}M$ ABMT is sufficient to bring about nearly complete inhibition of the thiaminase system.

There are several reasons why the ABMT should have failed to affect the bracken. In the first place, it may not have been translocated to the site of thiaminase action. Secondly, it may have been attacked by another enzyme system in the bracken and its capacity to inhibit thiaminase destroyed. Thirdly, it may be that the thiaminase system is not important, or only important at certain stages of growth, to the metabolism of bracken.

Thiaminase substrates which could give rise to a phytotoxic substance

It has been shown that thiaminase can act on certain thiamine analogues in which the thiazole component has been replaced by another amine. If this amine were a phytotoxic substance (X), then it is possible that combination with the pyrimidylmethyl component of thiamine might mask its toxic properties. Thus, if the pyrimidylmethyl-X compound were administered to a mixed population of plants it might be expected that only those containing the thiaminase system (i.e., bracken and other Pteridophytes) would be capable of splitting it and releasing the toxic substance X.

$$\text{P–CH}_2\text{–X} + \text{RNH}_2 \longrightarrow \text{P–CH}_2\text{–NHR} + \text{X}$$

Few amines are known which are toxic to plants when administered in very small quantities. The amino-compounds, 1-*iso*propyl-2-nonyl-4 : 4-dimethyl-2-imidazoline (Allen and Skoog, 1951) and (5-chloro-2-benzimidazolylthio)-acetic acid (Rebstock, Ball, Hamner and Sell, 1957) are known to cause severe damage or kill certain plants, but when these were injected into bracken fronds in amounts of up to 5 mg. the only obvious effect was a slight chlorosis at the tip of the frond with the higher amounts of the latter compound.

Because of the readiness with which heteropyrithiamine and quinilinothiamine were attacked by bracken thiaminase, it seemed likely that a thiamine analogue containing a substituted pyridine ring in place of the thiazole would also be attacked. It was decided therefore to synthesize some pyridine analogues of the phytotoxic phenoxyacetic acids. Since quite small modification of a molecule can abolish phytotoxicity, the chances that the pyridine compounds would have toxic properties were small. However, the structural requirements for phytotoxicity are not clearly understood, and there were no other grounds on which to base the choice of structures. Two compounds were synthesized (Fig. 2), 6-chloro-2-methylpyridin-3-oxyacetic acid and 5-bromopyridin-3-oxyacetic acid. These compounds had no obvious effect on bracken when injected into the fronds as described above; they were also without significant activity as compared with 2 : 4-D or MCPA in inhibiting the germination of cress seeds.

Because of the lack of toxicity of these imidazole and pyridine derivatives, no attempt was made to synthesize and test the pyri-

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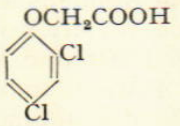
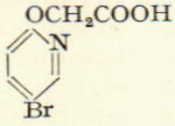
midylmethyl-substituted compounds. It may be that other amines, for example, pyridine compounds with halogen or halogen and methyl substituents in other positions, will prove to be toxic to bracken, and there is scope here for much further work.

FIG. 2.

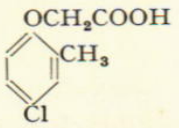


2 : 4D

2 : 4-dichlorophenoxyacetic acid

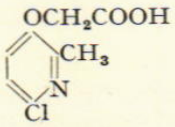


5-bromopyridin-2-oxyacetic acid



MCPA

4-chloro-2-methylphenoxyacetic acid



6-chloro-2-methylpyridin-3-oxyacetic acid

The role of thiaminase

The physiological role of thiaminase is not clear. Thiamine apparently has a limited and erratic distribution (Harris, 1951; Fujita, 1954). Among the fishes, for example, it occurs only in some fresh-water and salt-water species. It could be that thiaminase has some special role in those organisms in which it occurs. Alternatively, *in vivo* it may catalyse a reaction common to most organisms and only show activity with thiamine *in vitro* in certain cases. If thiamine is the substrate of thiaminase *in vivo*, then thiaminase would catalyse the synthesis of thiamine analogues having an amine other than the thiazole moiety of thiamine attached to the methylene bridge. Such compounds are as yet unknown in biological material. From the activity of thiaminase in bracken extracts it can be calculated that the rate of metabolism of thiamine by thiaminase in the fresh leaf could be of the order of 4,000 μg./g. of leaf/hour at 17° C. Watanabe (1952) finds that bracken contains 0·66 μg. of thiamine/g. fresh weight.

With certain amines the transfer reaction is reversible. Therefore the possibility exists that thiaminase takes part in the synthesis of thiamine by catalysing the exchange of 5-β-hydroxyethyl-4-methyl thiazole with a pyrimidinmethylamine precursor. Such a role for thiaminase would appear to presuppose different pathways of thiamine synthesis in, for example, different but closely related fishes.

The present work and that of Fujita (1954) show that thiaminase will act on certain thiamine analogues. Also Woolley (1953) has found that carp thiaminase catalyses the transfer reaction between the pteridine analogue of thiamine and *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid, with the formation of pteroic acid or pteroylglutamic acid, although the yields were extremely small.

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Woolley does not consider that this is the mode of biosynthesis of these compounds. The results do, however, indicate the potentialities of thiaminase as a synthesizing enzyme.

The results of the present work with bracken thiaminase and that with other thiaminases suggests that for the transfer reaction to take place readily the substrate must have a pyrimidine ring attached by a methylene bridge to an amine. The nature of the amine may be varied widely, but little variation in the pyrimidine ring is permissible. It is therefore of particular interest that preliminary results suggest that certain purines and pyrimidines are active as acceptor amines in the bracken thiaminase transfer reaction. It may be that the physiological function of thiaminase lies in the metabolism of these compounds or in helping in such actions as the nucleic acids may be concerned in, and that is not restricted to reactions involving thiamine.

Conclusion

Although the investigations have as yet produced no results of immediate practical value for the eradication of bracken, a start has been made on the synthesis of compounds which may be of value, and a good deal of information about the thiaminase system has been obtained. New techniques for studying thiaminase action have been developed, and the information obtained by these methods will help to elucidate the physiological role of thiaminase. In particular, the investigations have shown that ABMT is a powerful inhibitor of thiaminase *in vitro*, although it is apparently without effect on the bracken plant. Further studies of compounds having a close structural resemblance to ABMT would appear to be worthwhile, as the availability of compounds which would inhibit thiaminase *in vivo* might help to throw light on the role of this enzyme and might also be useful as bracken-killing agents.

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