The Partial Purification and Properties of a Thiaminase from Bracken [*Pteridium aquilinum* (L.) Kuhn]

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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 deficiency and developed when certain species of raw fishes were included in the ration of foxes. The substance in fish responsible for the destruction of the dietary thiamine was shown to be an enzyme by Sealock, Livermore & Evans (1943), and Krampitz & Woolley (1944). 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. This usually occurs only in the presence of certain amines; the pyrimidine moiety is transferred to the amine according to the general equation

$$P\cdot CH_2\cdot T^+ + R\cdot NH_2 \rightarrow P\cdot CH_2\cdot NH\cdot R + T + H^+, \quad (1)$$

where P and T stand for the pyrimidine and thiazole components of thiamine respectively (Woolley, 1953; Fujita *et al.* 1952*c*; Sealock & Davis, 1949). Thiaminases catalysing the transfer reaction have been found in fishes, shellfish, bacteria and Pteridophytes (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 & Kuratani, 1954)

$$P\cdot CH_2\cdot T^+ + H_2O \rightarrow P\cdot CH_2OH + T + H^+. \quad (2)$$

Studies of plant thiaminase stem from the observation of Weswig, Freed & Haag (1946) that rats fed on a ration containing 40 % of air-dried bracken and adequate thiamine developed acute thiamine deficiency. Thomas & Walker (1949) confirmed this work and showed that bracken contained a thermolabile system capable of destroying thiamine at pH 4·5. The active system was extracted from the dried bracken leaf by Evans, Jones & 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. However, since Somogyi (1949) and Somogyi & Muralt (1949) claimed that the factor responsible for the inactivation of thiamine in fern or bracken extracts was thermostable (1 hr. at 100°) and that it passed a dialysing membrane, Evans & Jones (1952) repeated and extended their previous work. They found that over 90 % of the thiaminase activity of aqueous extracts of bracken was destroyed by heating for 15 min. at 100° and that dialysis separated the activity into thermolabile and thermostable fractions, both of which were necessary for enzyme action. The thermostable fraction could be replaced by the amines *m*-aminobenzoic acid and 5-aminosalicylic acid, which strongly suggested that bracken thiaminase catalyses the transfer reaction according to equation (1). The work of Fujita, Okamoto & Nose (1955) with the variety of bracken *P. aquilinum* var. *japonicum* supports this suggestion in that a thermolabile fraction which was activated seven- to eight-fold by certain amines was demonstrated in the extracts; when pyridine was used in the system fluorimetric evidence was obtained which suggested that the transfer reaction with the formation of *N*-(4-amino-2-methylpyrimidin-5-yl)methylpyridine (heteropyrithiamine) had taken place. However, in partial agreement with Somogyi (1949) and Somogyi & Muralt (1949), Fujita *et al.* (1955) found that a thermostable factor which was capable of destroying thiamine and which passed a dialysing membrane was also present in the extracts. The thermolabile factor of Fujita *et al.* (1955), unlike that of Evans & Jones (1952), did not lose activity on dialysis, which suggested that it might be able to catalyse the hydrolytic fission of thiamine.

The present work describes the partial purification and some properties of a thiaminase from bracken, which catalyses the transfer reaction according to equation (1). No evidence has been obtained that the preparations catalyse the hydrolytic fission of thiamine.

MATERIALS AND METHODS

Plant material. Bracken fronds were harvested locally in late June or July and air-dried at room temperature. The stems were discarded and the leaves were passed through a Christie–Norris Junior Laboratory Hammer Mill. The powder was stored at 0–4°. Other plant material was either glasshouse-grown in John Innes Compost or found growing wild.

Plant extracts. The plant material was ground with sand in a mortar with the addition of not more than an equal

weight of water and the pulp squeezed by hand through cotton cloth. The extract was centrifuged at 10 000 *g* for 5 min. and the supernatant dialysed at 0° overnight against distilled water.

Enzyme units. One unit of enzyme activity is that amount of enzyme which catalyses the formation of 1 μmole of heteropyrithiamine in 1 hr. under the standard conditions. The specific activity is the number of enzyme units/mg. of N of the enzyme preparation.

Estimation of thiamine. Thiamine was estimated after deproteinization of the reaction mixtures with HPO_3, either by the colorimetric method of Melnick & Field (1939), the intensity of the colour produced by the Prebluda–McCollum reagent being measured at 518 mμ, or by a modification of Gaudiano's (1954) method. This modification consisted of alteration in the strength of the reagents and omission of alcohol from the H_2O_2. Oxidation of the thiamine to thiochrome was accomplished by treating the samples with 1 ml. of ferricyanide reagent [8 ml. of 20% (w/v) NaOH + 2 ml. of 1% (w/v) $K_3Fe(CN)_6$, prepared immediately before use] for 1 min. and then adding 1 ml. of 0·05% H_2O_2. After standing for 20 min. with occasional shaking the absorption at 368 mμ was measured.

Nitrogen. This was determined by a micro-Kjeldahl method and the NH_3 measured by titration with HCl.

pH *measurements.* These were made with a glass electrode.

Spectrophotometry. A Unicam SP. 500 quartz spectrophotometer was used with 1 cm. glass or quartz cells.

Thiamine chloride hydrochloride. This was of B.P. quality from Roche Products Ltd.

Calcium phosphate gel. This was prepared by the method of Singer & Kearney (1950) and was aged at 0° for at least one month before use.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 or phosphoric acid and NaOH. Acetate and borate buffers were prepared from solutions of the acids and NaOH.

4-*Amino*-5-*hydroxymethyl*-2-*methylpyrimidine.* This was prepared by treating 4-amino-2-methyl-5-methylaminopyrimidine dihydrochloride with $NaNO_2$ according to the directions of Andersag & Westphal (1937).

4-*Amino*-5-*bromomethyl*-2-*methylpyrimidine hydrobromide.* This was prepared from the corresponding hydroxymethyl compound by treatment with HBr by the method of Cline, Williams & Finkelstein (1937).

N-(4-*Amino*-2-*methylpyrimidin*-5-*yl*)*methylpyridine bromide hydrobromide.* This was prepared by a method similar to that used by Wilson & Harris (1949) for the preparation of neopyrithiamine. 4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide (300 mg.) was dissolved in 12 ml. of *iso*propyl alcohol and 660 mg. of pyridine was added. The mixture was stood at 37° for 18 hr. The pale-yellow precipitate was washed with *iso*propyl alcohol and ether and dried *in vacuo*. Recrystallization from aqueous ethanol, and drying *in vacuo* over concentrated H_2SO_4, gave 147 mg. of white crystals, m.p. 260–261° (decomp.; uncorr.). Fujita, Nose, Uyeo & Koizumi (1952*b*) give 261° (decomp.) for material dried *in vacuo* at 100° (Found: N, 15·2; $C_{11}H_{14}N_4Br_2$ requires N, 15·45%).

Preparation of bracken thiaminase

Air-dried powdered bracken (40 g.) was stirred into 400 ml. of water containing 8 ml. of $CHCl_3$, and after stirring for 15 min. 5% (w/v) aq. NH_3 soln. was added to bring the pH to 8. Stirring was continued for a further 1·75 hr. in the cold room. The suspension was squeezed by hand through strong cotton cloth. The fibre was mixed with 200 ml. of cold water and again squeezed through the cloth. The combined extracts (about 500 ml.) contained 10 000–16 000 units of specific activity 40–50.

To the water extract at 0° solid $(NH_4)_2SO_4$ was added with stirring to a concentration of 650 g./l. After stirring for about 1 hr. the precipitate was collected by centrifuging at about 10 000 *g* for 10 min. and suspended in water (one-third of the original volume). The suspension was dialysed for 1 hr. against running tap water and then overnight against a large volume of distilled water at 0°. To the dialysed suspension sufficient 0·5 M-phosphate, pH 7·5, was added to bring the phosphate concentration to 0·01 M and it was then frozen for 24 hr. (Freezing considerably accelerated the filtration.) After thawing the suspension was filtered by suction through Hyflo Super-Cel; the pad was washed with a small volume of 0·01 M-phosphate, pH 7·5. A clear-brown filtrate, fraction A, containing 55–65% of the original activity, and with a specific activity of 100–120 was obtained.

To fraction A at 0°, solid $(NH_4)_2SO_4$ was added, with stirring, to a concentration of 500 g./l., and as soon as solution was complete the precipitate was centrifuged off at about 10 000 *g* for 5 min. The precipitate was suspended in water (about one-third of the volume of fraction A) and after brief dialysis against running tap water was dialysed against distilled water overnight at 0°. Most of the precipitate dissolved during the dialysis but a small amount of insoluble material was separated after dialysis by centrifuging at about 10 000 *g* for 5 min. The supernatant, fraction B, contained 72–84% of the activity of fraction A and had a specific activity of 450–480.

Further purification was accomplished by using calcium phosphate gel. The active material was most readily adsorbed by the gel at acid pH values and could be recovered in good yield by extraction with 0·1 M-phosphate, pH 7·5. The results were somewhat variable but the following method has given a useful purification on the three occasions it was tried.

Fraction B is mixed with the gel at 0° (about 450 mg. of gel/3000 units of enzyme) and the suspension adjusted to pH 5·5 with N-acetic acid. After standing for 20–30 min. at 0° the gel is centrifuged off and extracted 2–3 times with 0·1 M-phosphate, pH 7·5 (about 30 ml. total volume) at room temperature. The phosphate extract is then dialysed against water at 0° for 24–48 hr. In this way preparations of specific activity of 740, 960 and 1280 with 50–90% recovery of the activity have been obtained. The best preparation was obtained by successive treatment of fraction B with gel. A sample of fraction B of specific activity 460 containing 5930 units was treated with 380 mg. of gel as before and, after centrifuging, the supernatant was again treated with 170 mg. of gel. Dialysed phosphate eluates from the first gel contained 1490 units of specific activity 390, and from the second gel 3740 units of specific activity 3150. No appreciable loss of activity was observed with these purified fractions when they were stored for 3–4 weeks at −18°.

Measurement of thiaminase activity

For reasons which are discussed fully later it became desirable to develop a method of measuring thiaminase activity based on the formation of the pyrimidine–amine product. Preliminary experiments suggested that, of the

amines tested, pyridine was most active in the transfer reaction catalysed by bracken thiaminase. This reaction leads to the formation of 1 mole of heteropyrithiamine/mole of thiamine destroyed, and could be followed by measuring the rate of formation of heteropyrithiamine. A method of estimating small amounts of heteropyrithiamine in the presence of thiamine has therefore been worked out.

Estimation of heteropyrithiamine. Both thiamine and heteropyrithiamine are oxidized by alkaline ferricyanide with the formation of the fluorescent compounds thiochrome and 2-methylpyrichromine respectively (e.g. Barger, Bergel & Todd, 1935; Fujita, Nose, Ueda & Hasegawa, 1952*a*). In alkaline solution thiochrome has an absorption maximum at 368 mμ and 2-methylpyrichromine has a maximum at 386 mμ. This difference can be used for the determination of heteropyrithiamine in the presence of thiamine, but with mixtures in which the proportion of the latter is appreciable much of the absorption at 386 mμ is due to thiochrome, and attempts to develop a method based on the differing absorption spectra were finally abandoned in favour of that described below. The method is based on the fact that thiamine is unstable in alkali whereas heteropyrithiamine is unaffected. It was developed from the observation of Matsukawa & Yurugi (1954) that, when mixtures of thiamine and heteropyrithiamine were oxidized after treatment with strong alkali, the fluorescence of the solution then gave a measure of the heteropyrithiamine originally present.

In the present work it was found that solutions of thiamine incubated with NaOH and then treated with ferricyanide absorbed much less light at 386 mμ than those not previously incubated with NaOH (Fig. 1).

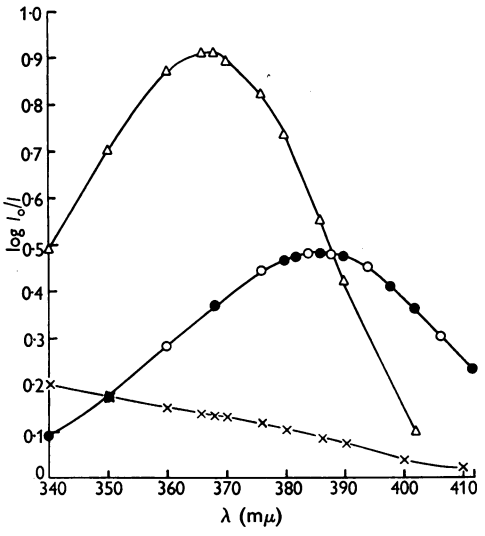

Fig. 1. Absorption spectra of the products obtained by oxidizing thiamine and heteropyrithiamine before and after treatment with NaOH as described in the text. △, Thiamine (reduced by one-half); ×, thiamine treated with NaOH before oxidation; ●, heteropyrithiamine; ○, heteropyrithiamine treated with NaOH before oxidation. Only a few of the experimental points are given.

Reaction mixtures, closely resembling those used in the enzyme assay (containing either 0·75 mg. of heteropyrithiamine bromide hydrobromide or 2 mg. of thiamine chloride hydrochloride, and 1 ml. of M-pyridine in a total volume of 10 ml., 0·025M with respect to phosphate at pH 7·5) were made up and 2 ml. samples taken into 1 ml. of 20% (w/v) metaphosphoric acid. Samples (2 ml.) from these acid mixtures were then incubated for 1 hr. at 37° with 1 ml. of 20% (w/v) NaOH. Then 0·5 ml. of the ferricyanide reagent [4 ml. of 1% (w/v) $K_3Fe(CN)_6$ + 6 ml. of 20% (w/v) NaOH] was added and after 15 min. at room temperature the excess of ferricyanide was removed by the addition of 1 ml. of 0·05% H_2O_2. After standing for 20 min., with occasional shaking, the absorption spectrum was determined with a control mixture in the blank cell from which thiamine and heteropyrithiamine had been omitted. Also, 2 ml. samples of the acid mixtures were treated with alkaline ferricyanide without prior incubation with NaOH. With these the 1 ml. of NaOH was added at the same time as the ferricyanide reagent.

The results (Fig. 1) show that whereas the absorption of the heteropyrithiamine mixtures was unaffected by previous incubation with NaOH, that of the thiamine mixtures was considerably diminished. There was little difference, on a molar basis, in the absorption of the oxidation products of thiamine and heteropyrithiamine at 386 mμ, but when the oxidation was carried out after incubation with alkali the products derived from thiamine absorbed some 14–15 times less light at 386 mμ than those of heteropyrithiamine. There was no advantage in prolonging the time of incubation of the thiamine mixtures with NaOH since the absorption at 386 mμ after oxidation was not significantly diminished by a further incubation for 1 hr.

Results obtained with solutions of heteropyrithiamine bromide hydrobromide containing amounts of phosphate, pyridine and NaOH as described above showed that the maximum absorption at 386 mμ was reached within 5 min. of addition of the ferricyanide reagent. Increasing the time of oxidation to 30 min. did not increase the absorption.

The results of Fig. 2 were obtained with mixtures of thiamine, pyridine and phosphate similar to those described above except that the amount of heteropyrithiamine was varied. Samples were acidified with HPO_3, incubated with NaOH and treated with the ferricyanide reagent and H_2O_2 exactly as previously described, and the absorption at 386 mμ was measured. It can be seen that the relationship between the amounts of heteropyrithiamine taken and the absorption is linear and that the heteropyrithiamine–thiamine mixtures when corrected for the absorption of the thiamine give essentially the same values as those found for heteropyrithiamine alone. In this and other experiments it was found that heteropyrithiamine bromide hydrobromide in amounts of 0·25–2 mg./10 ml. of mixture containing 2 mg. of thiamine chloride hydrochloride could be measured with an error of less than 10%. With amounts of heteropyrithiamine bromide hydrobromide less than 0·25 mg./10 ml., where the absorption due to the heteropyrithiamine is of the same order as, or less than, that due to the thiamine, the error increases with decrease in heteropyrithiamine concentration. The major source of uncertainty under these conditions appears to be the variability of the absorption of the products of the alkali–ferricyanide-treated thiamine. Within a single experiment the optical density (log I_0/I) varied by as much as ±0·008, although such variation was

unusual, and in different experiments the optical density has varied from 0·07 to 0·12. In a number of experiments with heteropyrithiamine bromide hydrobromide over the range 0·25–2 mg./10 ml. of mixture, the variation of the optical density among different experiments was rarely greater than 5%.

Thiaminase assay. The conditions finally adopted for enzyme assay and referred to subsequently as the standard conditions were as follows.

At zero time enzyme was added to reaction mixtures containing 2 mg. (5·9 μmoles) of thiamine chloride hydrochloride, 1 ml. of 0·1 M-pyridine, 0·5 ml. of 0·5 M-phosphate and water to a total volume of 10 ml., at pH 7·5 and 37°. A 2 ml. sample was taken immediately into 1 ml. of 20% (w/v) HPO_3; after incubation for 0·5 hr. at 37°, a second sample was taken. After centrifuging, 2 ml. of the supernatant was incubated with 1 ml. of 20% (w/v) NaOH for 1 hr. at 37°. Oxidation with the ferricyanide reagent and H_2O_2 was carried out as described previously and 20 min. later the absorption at 386 mμ was measured, samples of a mixture from which thiamine and enzyme had been omitted, but treated with the other reagents, being used in the blank cell. The absorption does not alter appreciably in 1 hr. but a small decrease takes place on standing at room temperature overnight. Blank determinations with mixtures from which enzyme or thiamine had been omitted and with enzyme which had been heated for 15 min. before testing were run simultaneously with the experimental reaction mixtures. The increase in optical density in the blank determinations was always less than one-tenth of that found with the experimental mixtures. After subtracting the blank values the amount of heteropyrithiamine formed (μmoles) is calculated, a value for the molar extinction coefficient (ε) of 8000 for the oxidation product of heteropyrithiamine being used. Since the formation of heteropyrithiamine decreases the concentration of thiamine and hence its contribution to the absorption at 386 mμ, a correction allowing for this is calculated based on the absorption of the thiamine control mixture from which enzyme has been omitted. It was found that the absorption of such mixtures was proportional to the amount of thiamine present over the range 0·5–2 mg. of thiamine/10 ml. of mixture. It was also determined that small amounts (1 mg./10 ml. of mixture) of the thiazole moiety of thiamine, the other product of the enzyme action, did not contribute any significant absorption at 386 mμ after incubation with the NaOH and ferricyanide reagents.

Under the conditions for the enzyme assay described above, the rate of formation of heteropyrithiamine is directly proportional to the amount of enzyme used provided that the amount of heteropyrithiamine formed does not exceed 1·2 μmoles (Fig. 3). In practice it is preferable to work in the top part of the linear range with amounts of enzyme that will form about 0·7–1 μmole of heteropyrithiamine, since the estimation of heteropyrithiamine becomes increasingly subject to error with decrease in its concentration.

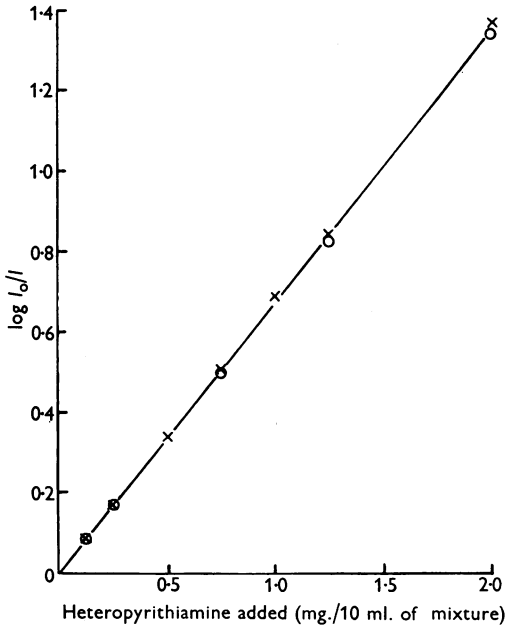

Fig. 2. Absorption of light at 386 mμ of heteropyrithiamine and heteropyrithiamine–thiamine mixtures after treatment with NaOH and ferricyanide. ○, Heteropyrithiamine alone; ×, heteropyrithiamine–thiamine mixtures corrected for the absorption of thiamine. Thiamine blank absorption was 0·102.

RESULTS

Effect of varying different factors on the activity of bracken thiaminase

Effect of pH. This was studied under the standard conditions except that different buffers were used. At pH values above 7 the final buffer concentration

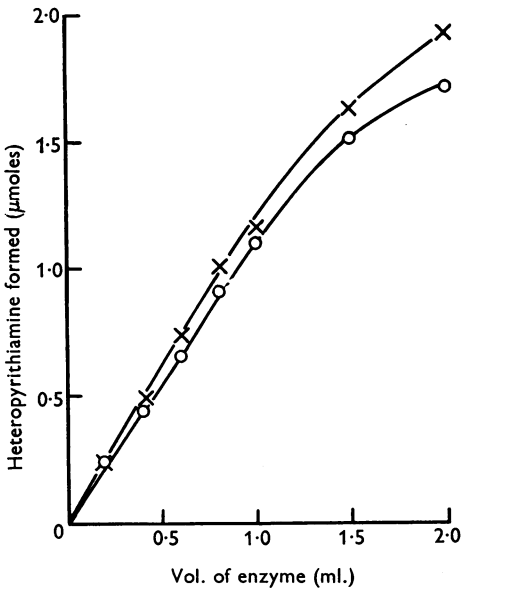

Fig. 3. Effect of enzyme concentration on the rate of formation of heteropyrithiamine. ○, Water extract of dried bracken leaf, specific activity 42; ×, bracken-thiaminase preparation (fraction B), specific activity 460.

was 0·01 M (phosphate and borate) and below pH 7 it was 0·01 M (phosphate and acetate). Where necessary the pyridine was adjusted to the appropriate pH value with N-HCl. The incubation period was 0·5 hr. and the enzyme used had specific activity 740. The difference between the initial and final pH of the reaction mixtures was within 0·2 pH unit. The results (Fig. 4) show that the optimum pH is in the neighbourhood of pH 7·5.

Effect of pyridine concentration. This was tested under the standard conditions, except that the amounts of pyridine were varied, an enzyme preparation of specific activity 740 being used. The results show (Fig. 5) that the activity increases with increase in pyridine concentration up to about 0·08 M and remains approximately constant over the range 0·08–0·3 M but decreases with further increase in pyridine concentration.

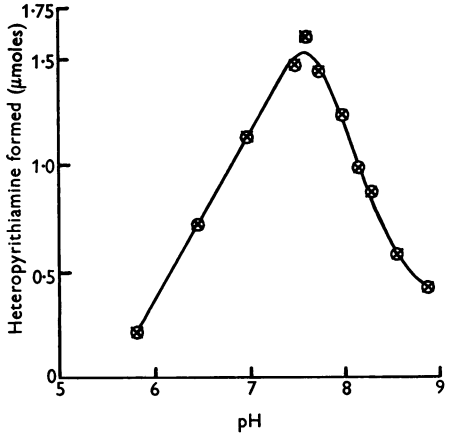

Fig. 4. Effect of pH on the rate of formation of heteropyrithiamine by a bracken-thiaminase preparation of specific activity 740.

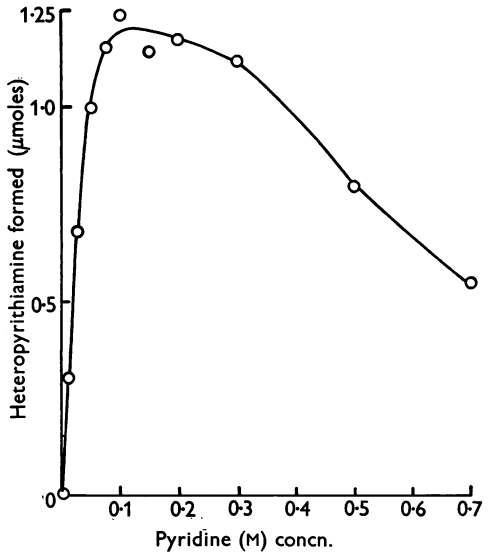

Fig. 5. Effect of varying the concentration of pyridine on the rate of formation of heteropyrithiamine by a bracken-thiaminase preparation of specific activity 740.

Effect of thiamine concentration. The method used for the determination of heteropyrithiamine does not lend itself to a study of the variation of enzyme activity with thiamine concentration. It is desirable to have at least 0·5 μmole of heteropyrithiamine formed in the reaction mixture, and this limits the lowest amount of thiamine which can be used. Further, if an amount of enzyme giving a reasonable activity at low thiamine concentration is employed with high thiamine concentration then the amount of thiamine remaining unchanged at the end of the experiment is high relative to the amount of heteropyrithiamine formed, and the thiamine blank absorption in the determination of heteropyrithiamine becomes unreasonably high. These difficulties could be partly overcome by using absorption cells with 4 cm. light path with thiamine concentrations below $1{\cdot}5 \times 10^{-4}$ M to increase the range of estimation of heteropyrithiamine, and, by using more enzyme, higher ranges of thiamine concentration could be investigated. In the present work the thiamine concentration was varied from 1·5 to $7{\cdot}4 \times 10^{-4}$ M (0·5–2·5 mg. of thiamine chloride hydrochloride/10 ml.) and the other conditions were standard. The results (Table 1) suggest that the maximum activity is reached at concentrations greater than $7{\cdot}4 \times 10^{-4}$ M.

Course of the reaction

Formation of heteropyrithiamine. Fig. 6 shows the rate of formation of heteropyrithiamine when either a crude enzyme (centrifuged water extract of dried bracken, specific activity 55) or a highly purified preparation (specific activity 3150) was

Table 1. *Effect of varying thiamine concentration on the rate of the thiaminase reaction*

Standard conditions were used (see Materials and Methods) and the thiamine concentration was varied. A partially purified bracken thiaminase of specific activity 3150 was used.

$10^4 \times$ Thiamine concn. (M)	Heteropyrithiamine formation (μmoles)
1·5	0·51
3·0	0·62
4·4	0·64
5·9	0·72
7·4	0·81

used. It can be seen that, provided sufficient enzyme is used, 90–95% of the thiamine added is finally present as heteropyrithiamine. With the complete reaction mixtures, only in those containing the lowest amount of water extract could the presence of thiamine be detected at the end of the experiment. In this mixture 5 μmoles of heteropyrithiamine had been formed and 0·3 μmole of thiamine remained out of the 5·9 μmoles of thiamine originally present. The blank values with enzyme, thiamine or pyridine omitted or with heated enzyme (15 min. at 100°) were very small, the largest being with heated enzyme, which is shown on Fig. 6. The results suggest that losses of thiamine by side reactions must be small.

Tests for hydrolytic fission of thiamine. With relatively large amounts of the purified preparations, tests were made to determine whether they contained thermolabile factors capable of catalysing the destruction of thiamine in the absence of added amine. Reaction mixtures consisted of 1 mg. of thiamine chloride hydrochloride, 1 ml. of phosphate–acetate buffer (0·25 M) and enzyme in a total volume of 10 ml. at pH 3, 4·5, 6 or 7·5. Two enzyme preparations were used: (1) 70 units of specific activity 740; (2) 154 units of specific activity 3150.

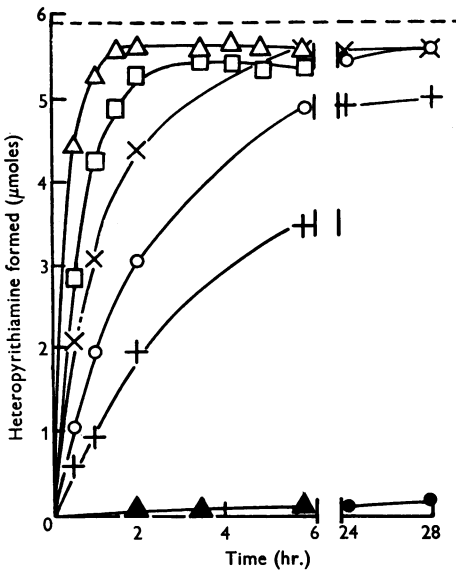

Fig. 6. Course of the reaction. Rate of formation of heteropyrithiamine with 0·05 ml. (+), 0·1 ml. (○) and 0·2 ml. (×) of centrifuged water extract of dried bracken leaf, and with bracken-thiaminase preparation of specific activity of 3150: 0·12 ml. (□), 0·24 ml. (△), and blank (●) with 0·2 ml. of water extract previously heated at 100°; ▲, blank with 0·24 ml. of thiaminase preparation previously heated at 100°. Other blanks with enzyme, thiamine or pyridine omitted were negligible.

The mixtures were incubated at 37° for 6 hr.; samples were taken into metaphosphoric acid at 0, 2 and 6 hr. After centrifuging, the thiamine in the supernatant was estimated by the modification of Gaudiano's method. Control mixtures with heated enzyme (15 min. at 100°), or from which thiamine or enzyme had been omitted, were also incubated along with the experimental mixtures. No evidence was obtained that either of the enzyme preparations contained a thermolabile factor capable of catalysing the destruction of thiamine. Preparation (1), 0·8 ml. (70 units), was mixed with 0·4 ml. of 0·001 M-$MnSO_4$ and set aside at room temperature for 15 min. The mixture was then tested as above but again with negative results. Also the enzyme–$MnSO_4$ mixture was diluted and tested for transfer activity with added pyridine, but the rate of formation of heteropyrithiamine was not affected by the treatment with $MnSO_4$.

Tests for synthesis of thiamine. Reaction mixtures containing 3 μmoles of 4-amino-5-hydroxymethyl-2-methylpyrimidine, 3 μmoles of 5-β-hydroxyethyl-4-methylthiazole, 1 ml. of phosphate–acetate buffer (0·25 M) and enzyme (70 units of specific activity 740) in a total volume of 10 ml. at pH 3, 4·5, 6 or 7·5 were incubated at 37° for 6 hr. After deproteinization with metaphosphoric acid, samples were tested for thiamine by the procedure of Melnick & Field (1939). No evidence of the formation of thiamine was obtained.

Other plants

Four species of higher plants and two species of ferns have been tested for transfer activity under the standard conditions. Extracts were made as described previously and tested immediately after dialysis. With the leaves of the ferns *Phyllitis scolopendrium* (L.) Newm. and *Dryopteris filix-mas* (L.) Schott, the specific activities of the dialysed extracts were 105 and 100 respectively as compared with 25 for a sample of fresh bracken leaf harvested and tested at the same time. By using larger amounts of enzyme and a longer incubation period the formation of heteropyrithiamine as a major product can be confirmed by determining the absorption spectra (350–415 mμ) of samples after treatment with NaOH and ferricyanide–H_2O_2. With both ferns the absorption spectra obtained in this way were almost identical with that found for heteropyrithiamine. Extracts of germinating soya bean (*Glycine soja* Sieb. & Zucc), parsley leaves [*Petroselinum crispum* (Mill.) Airy-Shaw], cabbage leaves (*Brassica oleracea* L.) and parsnip leaves (*Pastinaca sativa* L.) gave negative results even when large amounts (3 ml. of extract, 4–9 mg. of N) were used and incubation was prolonged to 3–5 hr.

DISCUSSION

The work referred to in the introduction of this paper shows that although it is generally agreed that extracts of bracken fronds are capable of destroying thiamine the nature of the factors involved have not yet been clearly identified. It seemed possible that there might be a thiaminase in bracken capable of catalysing both the hydrolytic fission of thiamine and the transfer reaction. Previously thiaminase activity has been followed by measuring the disappearance of thiamine, although evidence for the transfer reaction has been obtained by the isolation of the pyrimidine-amine product or by fluorimetric or chromatographic techniques. It seemed desirable therefore 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 whether the activating effect of amines was wholly due to the transfer reaction or partly due to a stimulation of the hydrolytic fission of thiamine. Preliminary tests suggested that pyridine was highly active in the transfer reaction catalysed by bracken thiaminase, and a method of estimating the product (heteropyrithiamine) in the presence of thiamine was accordingly worked out. It has been shown by this method with both crude extracts of bracken and partially purified preparations that in the presence of excess of pyridine 90–95 % of the added thiamine is converted into heteropyrithiamine. This suggests that the destruction of thiamine by side reactions must be small. In the absence of pyridine, with amounts of enzyme capable of catalysing the destruction of 70–150 μmoles of thiamine/hr. in the transfer reaction, no evidence of a thermolabile factor capable of catalysing the destruction of thiamine was found. It has been reported that small amounts of Mn^{2+} ions increase the rate of destruction of thiamine by certain animal thiaminase preparations (Deolalkar & Sohonie, 1954; Reddy, Giri & Das, 1948). However, one of the partially purified preparations, after previous incubation with a small amount of Mn^{2+} ion, failed to destroy thiamine in the absence of pyridine. The transfer activity of the preparation with pyridine was not affected by the treatment with Mn^{2+} ion. No evidence of thiamine synthesis was obtained when one of the partially purified thiaminase preparations was incubated with 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-β-hydroxyethyl-4-methylthiazole. It must be concluded therefore that if the enzyme responsible for the transfer reaction is capable of catalysing the hydrolytic fission of thiamine its hydrolytic activity must be negligible in comparison with its transfer activity.

By analogy with the mechanism which is thought to hold for the other transferring enzymes the first step in the reaction would involve the formation of a pyrimidine–enzyme complex and 5-β-hydroxyethyl-4-methylthiazole (equation 3):

$$P\cdot CH_2\cdot T^+ + \text{enzyme} \rightarrow P\cdot CH_2\cdot \text{enzyme}^+ + T, \quad (3)$$

$$P\cdot CH_2\cdot \text{enzyme}^+ + C_5H_5N \rightarrow P\cdot CH_2\cdot N^+C_5H_5 + \text{enzyme}. \quad (4)$$

The pyrimidine–enzyme complex would then react with certain amines, e.g. pyridine (equation 4), but not to any appreciable extent with water. In this way it would differ from those enzymes which possess both hydrolytic and transferring activity.

The results of the present work are therefore in close agreement with those of Evans & Jones (1952), who found that both a thermolabile and a thermostable factor were necessary for enzyme action and that the thermostable fraction could be replaced by an aromatic amine.

The effect of pH and variation in pyridine and thiamine concentration on the transfer reaction catalysed by bracken thiaminase have been studied in order to find suitable conditions for the measurement of thiaminase activity. With these results a relatively simple method was worked out for the assay of bracken thiaminase. The method has proved useful in the preparation of partially purified thiaminase preparations, the best of which represented a 60-fold purification and a yield of about 30 %. With the method, thiaminase transfer activity was demonstrated in two other species of fern but no evidence of such activity was found in the few species of higher plants, including germinating soya beans, which were examined. Although Fujita (1954) found no thiaminase in germinating soya beans, Murata & Shiga (1952) have reported the presence of a thermolabile thiamine-destroying factor in extracts of such material. The results of the present work do not therefore conflict with those given by Fujita (1954), who examined a large number of Angiosperms and Pteridophytes for the presence of thiaminase. He found activity in all of the Pteridophytes tested but among the Angiosperms only in *Celosia crista*. Horses suffer from thiamine deficiency when the proportion of bracken in their diet is high (Roberts, Evans & Evans, 1949). It seems likely that the deficiency is brought about by destruction of the dietary thiamine by bracken thiaminase. It follows therefore that thiaminase is not likely to be present in the common plants of pastures.

Woolley (1953) has pointed out that many results on the activity and distribution of thiaminases are of doubtful value because the activity may have been limited by the amount of amines in the preparations tested. Kawamori (1955) failed

to detect thiaminase in the faeces of some human subjects unless pyridine was added to the reaction mixtures. Under the conditions used in the present work the activity of thiaminase increased with increase in pyridine concentration up to about 0·08 M. If the reaction mechanism outlined above is correct then the rate of formation of heteropyrithiamine would be expected to increase with increase in pyridine concentration. The fact that a maximum was obtained experimentally suggests that high pyridine concentrations may have undesirable effects on the enzyme or interfere with the reaction in some other way.

It has been shown (Fujita, 1954; Fujita & Tashiro, 1952; Fujita *et al.* 1952*a*, *c*) that pyridine increases the rate of destruction of thiamine by extracts and preparations from fishes, shellfish and bacteria, and fluorimetric or chromatographic evidence has been obtained (with the clam *Meretrix meretrix* L. proof was obtained by isolation of a derivative) of the formation of heteropyrithiamine. Although with some preparations, under the conditions used by these authors, amines other than pyridine were more active it appears probable that the present method of following transfer activity of bracken thiaminase may prove useful in studies of other thiaminases.

It is doubtful whether the enzyme approaches the maximum activity of which it is capable under the conditions used here for the assay. It has been shown that the enzyme is not saturated at the thiamine concentration used ($5{\cdot}9 \times 10^{-4}$ M), and further work may show that there are amines with a much higher activity than pyridine in the transfer reaction. From the results obtained, by assuming that the rate of thiaminase action at 17° is one-quarter of that found at 37° and that the dry weight of bracken frond is 20% of the fresh weight, the rate of metabolism of thiamine by thiaminase in the fresh leaf could be of the order of 4000 μg./g. of leaf/hr. at 17°. Watanabe (1952) finds that bracken contains 0·66 μg. of thiamine/g. fresh wt. It is by no means certain that thiamine is the substrate of thiaminase *in vivo*. If it is, then thiaminase would catalyse the synthesis of thiamine analogues having an amine other than 5-β-hydroxyethyl-4-methylthiazole attached to the methylene bridge. Such compounds are as yet unknown in biological material. Thiaminase will act on substrates having structural features resembling thiamine (Fujita, 1954), and it appears probable that *in vivo* it may play some role in the synthesis of compounds other than thiamine analogues. Woolley (1951) has provided some experimental support for this by demonstrating that carp thiaminase catalyses the transfer reaction between the pteridine analogue of thiamine [3-(2-amino-4-hydroxypteridin-6-ylmethyl)-4-methyl-5-β-hydroxyethylthiazolium bromide] and *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid, with the formation of pteroic acid or pteroylglutamic acid, although the yields were extremely small. 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. Thiaminase 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.

With certain amines the transfer reaction (equations 3 and 4) is reversible (Fujita *et al.* 1952*b*). Therefore the possibility exists that thiaminase takes part in the synthesis of thiamine by catalysing the exchange of 5-β-hydroxyethyl-4-methylthiazole with a pyrimidine–amine precursor. Such a role for thiaminase would appear to presuppose different pathways of thiamine synthesis in, for example, different but closely related fishes.

SUMMARY

1. A method for the estimation of small amounts of heteropyrithiamine [*N*-(4-amino-2-methylpyrimidin-5-yl)methylpyridine] in the presence of thiamine is described. It depends on the destruction of thiamine by incubation with strong alkali and subsequent oxidation of heteropyrithiamine to 2-methylpyrichromine by ferricyanide. The 2-methylpyrichromine is estimated spectrophotometrically at 386 mμ.

2. With the method it is shown that bracken thiaminase catalyses a transfer reaction between thiamine and pyridine with the formation of heteropyrithiamine. In the presence of excess of pyridine the amount of heteropyrithiamine formed accounts for 90–95% of the thiamine added.

3. A relatively simple method for the measurement of bracken-thiaminase activity is described.

4. By fractionation with ammonium sulphate and calcium phosphate gel, concentrated partially purified thiaminase preparations have been made from water extracts of dried bracken leaves. The best preparation was obtained in 30% yield and represented a purification of about 60-fold. It catalysed the formation of 3150 μmoles of heteropyrithiamine/hr./mg. of N at 37°.

5. With the concentrated partially purified preparations no evidence was obtained of the presence of a thermolabile factor capable of catalysing the hydrolytic fission of thiamine.

REFERENCES

Andersag, E. & Westphal, K. (1937). *Ber. dtsch. chem. Ges.* **70**, 2035.
Barger, G., Bergel, F. & Todd, A. R. (1935). *Ber. dtsch. chem. Ges.* **68**, 2257.
Cline, J. K., Williams, R. R. & Finkelstein, J. (1937). *J. Amer. chem. Soc.* **59**, 1052.
Deolalkar, S. T. & Sohonie, K. (1954). *Nature, Lond.*, **173**, 489.
Evans, W. C. & Jones, N. R. (1952). *Biochem. J.* **50**, xxviii.
Evans, W. C., Jones, N. R. & Evans, R. A. (1950). *Biochem. J.* **46**, xxxviii.
Fujita, A. (1954). *Advanc. Enzymol.* **15**, 389.
Fujita, A., Nose, Y. & Kuratani, K. (1954). *J. Vitaminol., Japan*, **1**, 1.
Fujita, A., Nose, Y., Ueda, K. & Hasegawa, E. (1952*a*). *J. biol. Chem.* **196**, 296.
Fujita, A., Nose, Y., Uyeo, S. & Koizumi, J. (1952*b*). *J. biol. Chem.* **196**, 305.
Fujita, A., Nose, Y., Kozuka, S., Tashiro, T., Ueda, K. & Sakamoto, S. (1952*c*). *J. biol. Chem.* **196**, 289.
Fujita, A., Okamoto, T. & Nose, Y. (1955). *J. Vitaminol., Japan*, **1**, 24.
Fujita, A. & Tashiro, T. (1952). *J. biol. Chem.* **196**, 305.
Gaudiano, A. (1954). *R.C. Ist. sup. Sanit.* **17**, 591.
Harris, R. S. (1951). *The Enzymes*, vol. 1, part 2, p. 1186. New York: Academic Press.
Kawamori, T. (1955). *Vitamins, Japan*, **8**, 213. [Cited in *Chem. Abstr.* (1955), **49**, 14077i.]
Krampitz, L. O. & Woolley, D. W. (1944). *J. biol. Chem.* **152**, 9.
Matsukawa, T. & Yurugi, S. (1954). *J. Vitaminol., Japan*, **1**, 27.
Melnick, D. & Field, H. (1939). *J. biol. Chem.* **127**, 505.
Murata, K. & Shiga, S. (1952). *Vitamins, Japan*, **5**, 574. [Cited in *Chem. Abstr.* (1955), **49**, 14110b.]
Reddy, K. K., Giri, K. V. & Das, R. (1948). *Enzymologia*, **12**, 238.
Roberts, H. E., Evans, E. T. R. & Evans, W. C. (1949). *Vet. Rec.* **61**, 549.
Sealock, R. R. & Davis, N. C. (1949). *J. biol. Chem.* **177**, 987.
Sealock, R. R., Livermore, A. H. & Evans, C. A. (1943). *J. Amer. chem. Soc.* **65**, 935.
Singer, T. P. & Kearney, E. B. (1950). *Arch. Biochem. Biophys.* **29**, 190.
Somogyi, J. C. (1949). *Int. Z. Vitaminforsch.* **21**, 341.
Somogyi, J. C. & Muralt, A. (1949). *Helv. physiol. acta*, **7**, C 56.
Thomas, B. & Walker, H. F. (1949). *J. Soc. chem. Ind., Lond.*, **68**, 6.
Watanabe, H. (1952). *Japan. J. Nation's Health*, **21**, 134. [Cited in *Chem. Abstr.* (1953), **47**, 11384c.]
Weswig, P. H., Freed, A. M. & Haag, J. R. (1946). *J. biol. Chem.* **165**, 737.
Wilson, A. N. & Harris, S. A. (1949). *J. Amer. chem. Soc.* **71**, 2231.
Woolley, D. W. (1951). *J. Amer. chem. Soc.* **73**, 1898.
Woolley, D. W. (1953). *Nature, Lond.*, **171**, 323.
Yudkin, W. H. (1949). *Physiol. Rev.* **29**, 389.

Tissue Components of the Domestic Fowl

2. BLOOD UREA*

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The domestic fowl, besides being of great economic importance, is often used as an experimental animal. Pathological abnormalities of various organs which arise from time to time, even under the best husbandry, are rarely detectable except at post-mortem examination. The investigation of avian urine for pathological constituents is at present impracticable; blood examination and tissue biopsy afford the only approaches to diagnosis by chemical means. The flock of brown leghorns at the Poultry Research Centre is carefully isolated and is not subject to contagion from outside; nevertheless, organ dysfunctions, e.g. of the kidney, are sometimes encountered. The possibility that some aspect of blood analysis might lead to early detection of kidney dysfunction is under investigation.

The literature from 1923 to 1952 contains conflicting reports on blood urea of presumably healthy fowls; the figures quoted cover a remarkable range despite the view generally accepted by biologists that urea is found only in low concentrations in normal avian tissues (cf. Clementi, 1914, 1915, 1918, 1922, 1932; Hunter & Dauphinée, 1925; Pitts & Korr, 1938; Howell, 1939*a*).

In the literature results have been expressed in two ways: as 'mg. of urea' and as 'mg. of urea nitrogen'. As the former is quantitatively more than twice the latter, this is a matter of some moment, since papers exist where both modes of expression are used indiscriminately to cover the same or similar sets of values (e.g. Hogan, Shrewsbury & Kempster, 1929; Herrmann, 1946*a*, *b*). The collated summaries of Shimer (1937), Howell (1939*a*) and Sturkie (1954) contain errors arising from confusion of the two values.

This paper describes an examination of the blood-

* Part 1: Bell (1957).