THIAMINE PYROPHOSPHATASE, ACID AND ALKALINE PHOSPHATASE ACTIVITY IN THE CHICKEN BRAIN IN VITAMIN B_1 DEFICIENCY

by

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SHIMIZU, HANDA, HANDA AND KUMOMOTO[1] examined nervous tissue of thiamine-deficient pigeons in respect to both alkaline and acid phosphatases. Acetone fixed tissue showed that thiamine-deficient nerve cells "acquired increased amounts of alkaline phosphatase" whereas "acid phosphatase of the axis cylinders and cytoplasm of the nerve cells is diminished in amount". There has been much criticism of the acid phosphatase technique when chemically fixed tissue is used particularly since loss in activity may be considerable[2,3,4,5], and since results with nervous tissue may be variable[5,6]. It is now generally agreed that freeze-drying methods are to be preferred for histological enzyme study. Further, NAIDOO AND PRATT[7] demonstrated a specific enzyme in rat brain splitting inorganic phosphate from thiamine pyrophosphate. This was active in both the acid and alkaline ranges. At pH 9.1 thiamine pyrophosphatase was present in different histological sites from the non-specific alkaline phosphatase. In the acid range, however, although the sites of activity of the thiamine pyrophosphatase were histologically similar to those of the non-specific acid phosphatase, these enzymes were functionally distinguishable by their differences in activation and inhibition.

It was considered that a study of the distribution and activity of thiamine pyrophosphatase and of the non-specific acid and alkaline phosphatases in frozen-dried nervous tissue from thiamine-deficient chickens was necessary to confirm and extend the findings of SHIMIZU, HANDA, HANDA AND KUMOMOTO[1]. This paper illustrates the histochemical findings and compares the activity of these enzymes in normal and in thiamine-deficient brain.

EXPERIMENTAL

Management of Birds. Day-old Rhode Island Red cockerels obtained from an accredited commercial hatchery were housed in an electrically heated Hover-Brooder. The brooder temperature was set at first at 35° C and progressively reduced during the first three weeks to room temperature which was maintained at 22° by thermostatically controlled steam heaters. The room was well lit by daylight. The birds were kept on a wire mesh floor, the droppings falling onto metal trays which were cleaned out twice weekly. Clean drinking water was provided and during the first three weeks a commercial baby chick mash (Spillers Ltd., London) was allowed without restriction.

At the end of the third week all weaklings or abnormal birds were rejected and the remainder was divided into two similar groups in respect of weight. The two groups were placed in smaller adjacent cages and the points of their upper beaks blunted by clipping in order to prevent feather

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pecking. The commercial mash was withheld and the birds fed on a synthetic diet which was mixed with water to a thick creamy consistency and introduced directly into the crop. The feeding was carried out twice daily, at 9 and 17 hours approximately, with a metal syringe of a type normally used to apply ornamental icing to cakes. The diet was prepared by grinding the mixture through a fine metal seive and mixing 100 g with 100 ml of water. A 12 cm length of rubber tubing (external diameter 6 mm, internal diameter 3 mm) was attached to the nozzle of the syringe, moistened and passed down the oesophagus into the crop. The bird was held in the left hand with the first two fingers placed upon the crop and the food mixture injected until the crop was palpably full. The birds were allowed to feed freely on a mixture of gelatin and cellulose powder.

Each bird in the control group received every second day an intramuscular injection containing 100 μ of thiamine in saline. All birds were weighed daily immediately before the morning feed. Four sets of 12 birds each were used in this work. The synthetic diet was a modification of that of COATES, KON AND SHEPHEARD[8], omitting the thiamine. The constituents were as shown in Table I.

TABLE I

COMPOSITION OF SYNTHETIC DIET

Dextrin	1200 g
Casein (B.D.H. vitamin free)	360 g
Mixed salts IV (HEGSTED, MILLS, ELVEHJEM AND HART[9])	100 g
$CaHPO_4$	20 g
Cystine	6 g
Inositol	2 g
Sulphite treated liver extract[10]	15 ml
Lard	100 g
Cod liver oil	20 g
Heptane extract from 60 g dried grass	
Choline chloride	3 g
Riboflavin	12 mg
Ca pantothenate	30 mg
Pyridoxin	8 mg
Nicotinamide	100 mg
2-Methyl-1:4-naphthoquinone	100 mg

The solid constituents were first ground. The last six items were dissolved in 25 ml 90% aqueous ethanol. The lard, cod liver oil and grass extract were mixed together and added to a little dextrin before incorporation with the bulk of the solid constituents. The liver extract and the alcoholic solution of vitamins were added to the mixture in a similar manner.

Each bird received 35–50 g daily of the diet mixture when forced feeding was started. The birds given the thiamine supplement continued to receive this quantity of diet daily throughout the experiment but the birds not given thiamine received less of the diet mixture towards the end of the experiment, owing to delayed emptying of the crop. Manipulation of the crop did not appear to hasten its emptying. The birds were killed when the weight had fallen sharply and they were unable to stand or hold their heads up. When placed on their backs they were unable to right themselves.

The activity of acid and alkaline phosphatases was estimated by inorganic phosphate liberation from glycerophosphate by suspensions of tectum ground in saline, as previously described[11]. The histological distribution of these enzymes was studied in sections prepared from frozen dried blocks of tissue as described elsewhere[7,12]. A modified medium was employed for the study of thiamine pyrophosphatase as follows:

	Assay medium M	*Histological medium* M
Medinal buffer (pH 8.7)	0.025	0.025
$MgCl_2$	0.020	0.020
$CaCl_2$	0.025	0.100
Thiamine pyrophosphate	0.008	0.008

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The substrate was added immediately before use and the final pH was 8.4 (as measured by a glass electrode). For quantitative estimation of activity, the medium included sufficient tissue suspension to give 1 mg of fresh tissue/ml.

RESULTS

The first difference seen between the two groups was a greater increase in weight of the thiamine supplemented group. Weakness of leg and neck muscles appeared first in two birds of the deficient group on the eleventh day but two did not show these symptoms until the twenty-fourth day. Enzyme activity found in suspensions prepared from the tectum of birds with and without the thiamine supplement are given in Table II. The acid or alkaline phosphatase activity did not appear to be related to the duration of the experimental feeding period, but the thiamine pyrophosphatase activity tended to fall with increasing survival on the experimental diet in both the deficient and the thiamine supplemented groups. Table III compares the mean enzyme activity of each group and shows that there was a significant increase in the alkaline phosphatase and thiamine pyrophosphatase but no significant change in acid phosphatase activity.

TABLE II

ACID AND ALKALINE PHOSPHATASE AND THIAMINE PYROPHOSPHATASE ACTIVITY IN NORMAL AND THIAMINE-DEFICIENT CHICKEN TECTUM

At 21 days of age all animals were fed the diet of Table I. One series received in addition regular thiamine injections. Deficient birds were killed when nervous symptoms were severe and controls were sacrificed at similar intervals.

Chicken No.	*Duration of experiment days*	*Change in weight g*	*Acid phosphatase units*	*Alkaline phosphatase units*	*Thiamine pyrophosphatase units*
		Basal diet + Thiamine			
99	15	+ 210	—	81	—
100	18	+ 212	26	80	59
111	21	+ 247	50	70	45
101	22	+ 270	46	65	58
102	23	+ 262	39	—	34
103	23	+ 243	37	74	—
104	24	+ 261	40	—	39
112	25	+ 287	—	—	37
113	25	+ 305	44	78	34
114	26	+ 301	46	71	38
		Basal diet only			
93	13	+ 23	40	131	65
105	15	— 13	33	119	76
94	15	— 21	38	115	63
95	18	+ 20	40	120	108
96	21	+ 14	43	107	68
97	22	— 21	42	89	34
107	22	— 10	49	—	47
98	23	— 12	40	—	44
108	25	— 28	53	—	49
109	26	— 3	—	112	52

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TABLE III

COMPARISON OF MEAN ENZYME ACTIVITY IN TECTUM WITH AND WITHOUT THIAMINE

Enzyme	Thiamine		No thiamine		P-value of difference of means
	No. expts	Mean units	No. expts	Mean units	
Acid phosphatase	8	41.9	9	42.0	> 0.3
Alkaline phosphatase	7	74.1	7	113.3	< 0.001
Thiamine pyrophosphatase	8	43.9	10	66.6	< 0.05

Histological. Nissl and myelin preparations of the deficient brain did not show any histological abnormality. Sections of optic tectum incubated with glycerophosphate at pH 5.3 showed no differences of staining between the normal and thiamine-deficient at incubation periods of up to 2 hours (Fig. 1 and 2). In the alkaline range at pH 9.1 definite differences of activity and distribution were evident in sections incubated for different intervals up to 2 hours. The deficient tectum showed an increase in the activity of the stratum opticum and the subjacent outer layers (Fig. 4). In the inner layers nuclei of the neurones showed moderate staining. In the layers overlying the ependyma staining was deep. In the normal control (Fig. 3) no nuclei could be made out on microscopy and the outer layer immediately below the stratum opticum stained comparatively lightly. None of the zones of the tecta showed capillary endothelial staining. The neuropile between inner layers was lighter in the deficient brain. In the sections incubated with thiamine pyrophosphate, the deepest stained layer was the stratum opticum in both control (Fig. 5) and experimental (Fig. 6) tissues. In the other layers staining of the nerve cell bodies was moderately deep in the normal, but considerably deeper in the deficient bird. The neuropile in the deficient tectum was darker than that in the control.

DISCUSSION

The synthetic thiamine-deficient diet makes possible controlled feeding of measured amounts of a diet of known constitution which is adequate for normal growth when supplemented with thiamine.

In the later stages in the experimental group delayed emptying of the crop, a phenomenon possibly inseparable from thiamine deficiency, probably accounted for some degree of inevitable interference with general nutrition. But, since crop stasis is not severe until general paralysis is so pronounced that the birds are sacrificed, it appears unlikely that the effects of this under-nutrition will complicate the picture of thiamine deficiency.

Alkaline phosphatase in the normal chicken brain appears to be much more active than in rat brain[13]. Chicken brain thiamine pyrophosphatase differs in some respects from the enzyme previously studied in rat brain[7,14]. The rat enzyme shows high activity over a wide range of H^+ concentration, including both pH 6.9 and 9.1, but the chicken enzyme only acts over a narrower range close to pH 8.4. The latter enzyme is activated only by Mg^{+2}, whereas the rat thiamine pyrophosphatase can be activated by either Ca^{+2} or Mg^{+2}. The activity of this enzyme tends to fall with survival of the birds, even in the group fed on a thiamine-supplemented diet. This finding suggests that the activity of this enzyme may alter with maturation of the nervous system.

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Fig. 1. × 80. Glycerophosphate substrate pH 5.3 (supplemented diet). Showing deep staining of the nerve cell and glial element throughout all layers.

Fig. 2. × 80. Glycerophosphate substrate pH 5.3 (deficient diet). Showing no differences between cytological findings in deficient and supplemented diets.

Fig. 3. × 80. Glycerophosphate substrate pH 9.1 (supplemented diet). Lightly stained outer layer and no nuclei present.

Measurement of enzyme activity clearly shows differences between the deficient and the control groups. There is a significant increase in the activity both of alkaline phosphatase and of thiamine pyrophosphatase in the thiamine-deficient brains, but no significant changes in acid phosphatase activity. The optic tectum is chosen for histological study as it is clearly laminated and changes can be described in terms of the intensity of staining of the distinct layers. The changes in the staining are consistent with the measured increase in alkaline phosphatase and in thiamine pyrophosphatase activity. The quantitative results therefore confirm the histological findings.

The change in alkaline phosphatase activity is in agreement with the findings of SHIMIZU, HANDA, HANDA AND KUMOMOTO[1], but the fall in acid phosphatase activity described by these authors is not seen in the present work. It should be noted that the frozen-dried chicken tectum shows little acid phosphatase activity in axons or dendrites, unlike the chemically fixed pigeon brain studied by SHIMIZU, HANDA, HANDA AND KUMOMOTO[1]. An explanation of this disagreement may lie in the different methods of tissue preparation, or in the probability that brain acid phosphatase activity represents more than one enzyme, as reported by LOWRY, ROBERTS, WU, HIXON AND CRAWFORD[15].

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Thiamine pyrophosphate undergoes a series of reversible and irreversible changes in alkaline solution[16]. If sufficient breakdown products accumulate during the incubation of the chicken brain sections at pH 8.4 suitable substrates may be provided for alkaline phosphatase action. However, not more than a small part of the increase in thiamine pyrophosphatase activity in the deficient chicken brain is likely to be accounted for in this manner.

Although in acute vitamin B_1 deficiency no structural changes are seen in the brain by the usual histological staining methods, small haemorrhages and a glio-mesodermal reaction may be found in the tecta, in the periventricular grey matter, and particularly in the mamillary bodies of chronic deficient animals. The changes in enzyme activity may possibly be a sequel to this tissue damage but the tecta examined in this study showed no evidence of histological abnormality by ordinary methods. Moreover, it is difficult to explain on this basis why activity should increase rather than decrease, and it is surprising that there is no change in acid phosphatase activity, especially as activation of this enzyme has been reported after axon section[17, 18] or after electrical stimulation[19]. It has been found that alkaline phosphatase and thiamine pyrophosphatase activity does not undergo rapid changes post-mortem[13]. It seems unlikely that the changes in alkaline phosphatase and thiamine pyrophosphatase activity can be due to destruction of the tissue but rather to a more specific metabolic injury.

Fig. 4. × 80. Glycerophosphate substrate pH 9.1 (deficient diet). Deeply staining outer layers and nuclei present.

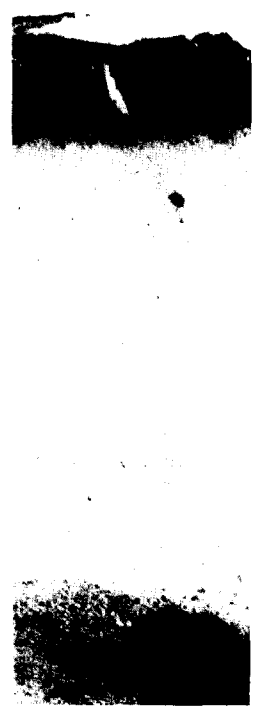

Fig. 5. × 80. Thiamine pyrophosphate substrate pH 8.4 (supplemented diet). Deeply staining stratum opticum and light neuropile with faintly staining nuclei.

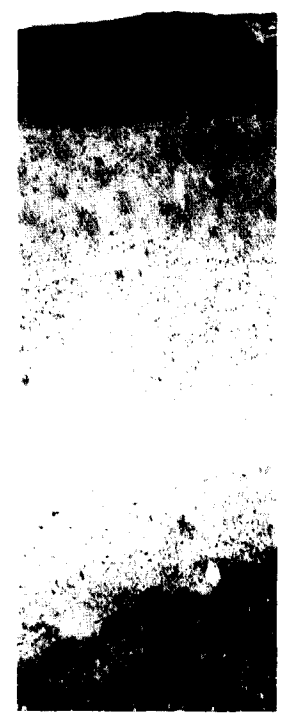

Fig. 6. × 80. Thiamine pyrophosphate substrate pH 8.4 (deficient diet). Deeply staining stratum opticum with moderately staining neuropile and more darkly staining cell bodies.

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SUMMARY

1. The overall activity and the histological distribution of thiamine pyrophosphatase, of acid and alkaline phosphatases have been studied in nervous tissue of the normal and of the thiamine-deficient chicken.

2. A Mg^{-2}-activated thiamine pyrophosphatase with optimal activity about pH 8.4 is present in chicken brain in addition to the acid and alkaline phosphomonoesterases.

3. In the optic tectum of thiamine-deficient chickens there is a significant increase in the activity of the thiamine pyrophosphatase and of alkaline phosphatase, but no significant change in the activity of the acid phosphatase.

RÉSUMÉ

1. L'activité totale et la répartition histologique de la thiamine pyrophosphatase et des phosphatases acide et alcaline ont été étudiées dans le tissu nerveux du poulet normal et du poulet carencé en thiamine.

2. Outre les phosphomonoestérases acide et alcaline, le cerveau du poulet renferme une thiamine pyrophosphatase, activée par le Mg^{+2} et dont le pH optimum est d'environ 8.4.

3. Dans le tectum optique de poulets carencés en thiamine, on observe une augmentation significative de l'activité de la thiamine pyrophosphatase et de la phosphatase alcaline, mais aucune modification de l'activité de la phosphatase acide.

ZUSAMMENFASSUNG

1. Die Gesamtaktivität und die histologische Verteilung der Aneurinpyrophosphatase, der sauren und der alkalischen Phosphatasen wurden im Nervensystem der normalen und der an Aneurin-Mangel leidenden Küken untersucht.

2. Eine durch Mg^{++}-aktivierte Aneurinpyrophosphatase mit optimaler Aktivität bei pH 8.4 ist im Gehirn der Küken noch ausser der sauren und alkalischen Phosphomonoesterasen vorhanden.

3. Im Tectum des Mittelhirns der an Aneurinmangel leidenden Küken ist eine bemerkenswerte Zunahme der Aktivität der Aneurinpyrophosphatase und der alkalischen Phosphatasen aber keine besondere Veränderung in der Aktivität der sauren Phosphatasen zu beobachten.

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