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THE INFLUENCE OF THIAMINE AND RELATED COMPOUNDS ON THE IRIS OF THE FROG

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Abstract—Injection of thiamine to *Hyla arborea* and *Bufo viridis* causes closing of the iris, and the time of closing is roughly proportional to the dose used. The thiazolic and pyrimidinic components of thiamine are inactive.

Thiamine monophosphate, thiamine diphosphate, pyrithiamine and oxythiamine, act similarly to thiamine, but in higher doses. Acetylthiamine is effective almost in the same dose as thiamine.

Thiamine probably acts directly on the iris, while the other compounds must undergo hydrolysis and free thiamine must be produced in order to show activity.

Thiamine monophosphate in high doses is ineffective and its previous administration inhibits the action of thiamine diphosphate but not of thiamine.

It is supposed that there exist two different thiamine-phosphatases: Th-pyrophosphatase and Th-orthophosphatase, which contain the same or similar coenzymes but different apoenzymes.

INTRODUCTION

IN THE course of our experiments on the influence of vitamins on hormone activity an effect of thiamine (Th) on the iris of normal frogs was observed. As very few observations have been reported on the influence of Th on men and animals when not in a state of avitaminosis or hypovitaminosis, it seemed worthwhile to investigate this phenomenon.

The investigation of di Palma and Hitchcock[1] and Molitor and Emerson[2] deal principally with the toxic effect of high doses of Th in men and animals. The toxic doses given in the literature differ widely, reflecting the difference in the sensitivity of experimental animals as well as the lack of uniformity of the experimental conditions.

Toxic manifestations of Th have been attributed to curare-like activity,[3] to neuromuscular and ganglionic blockade[1] or to paralysis of the respiratory and vascular system.[4] Goldfeld[5] observed an increased visual acuity in healthy men given daily 50 mg Th from 20 to 30 days. It has also been reported that high doses of Th protect men against insect bites,[6] probably due to a particular smell of the sweat of those receiving rich treatment. Neither of these last two reports, however, as far as can be ascertained, has been confirmed.

In addition some pharmacological experiments on isolated organs have been reported. It has been found that Th antagonized acetylcholine activity in isolated frog and tortoise hearts, in rat and rabbit intestine and in virgin uterus of guinea-pig[7–14] but it increased the sensitivity of eserinized leach muscle[15] to this substance. In

avitaminotic pigeons the sensitivity to acetylcholine is decreased.[9] Furthermore, Th inhibits cholinesterase activity[3, 16] and antagonizes the effect of nicotin on isolated smooth muscles. This last effect is not specific for Th, as other substances have been reported as having the same influence.[17–20]

In the reported studies no specific action of Th in healthy animals has been noted.

The present paper presents our observations on myosis in frogs induced by Th and some chemically related compounds. The physiological aspect of this investigation will be the subject of a separate paper.

MATERIAL AND METHODS

Two species of frogs, *Bufo viridis* and *Hyla arborea*, were used after our tests with mammals (albino and wild rats, white and black mice, rabbits, dogs, cats and cows) showed no visible reaction to Th administration. Freshly caught frogs were put in individual glass vessels and kept in a cool place. No food was supplied. Their body weight varied from 5 to 25 g, but in each experimental series frogs of approximately equal weights were used. Each animal was numbered and the course of treatment was registered individually. Animals used more than once, were given a minimum of 3-days rest between experiments, and frogs showing any disturbances were eliminated.

The following substances were tested. Thiamine* (Th), thiazolic component of Th*, pyrimidinic component of Th*, oxythiamine* (Oxy-Th), thiamine pyrophosphate* (ThDP), thiamine monophosphate† (ThMP), acetyl thiamine† (Acet-Th), and pyrithiamine‡ (Pyri-Th). Doses varied from 3·75 to 60 mg/10 g body weight for each compound when injected to *Bufo*, while *Hyla* received from 1 to 16 mg/10 g body weight. In addition we experimented with acetylcholine§ (ACh) (0·001–2 mg/10 g body weight for both species), ATP‖ (2–8 mg/10 g body weight for both species), sodium sulphathiazol** (10–150 mg/10 g body weight for *Bufo*), physostigmine* (0·01–2 mg/10 g body weight for both species), KCl, KH_2PO_4 (1–20 mg/10 g body weight for *Bufo*) and HCl and NaOH diluted with saline to obtain a pH range of 1–10.

All substances were dissolved in saline or water and injected through the lymphatic femoral sac into the dorsal lymphatic sac. The volume of the solution injected did not exceed 0·5 ml. After injection the size of the pupil was observed for any change occurring.

The pupil of frogs, kept under normal daylight conditions, is oval in shape, and is denoted throughout this paper as 50 per cent opening. The maximal extension which is circular and which can be induced by the administration of adrenaline or noradrenaline is described as 100 per cent opening. Under the influence of contracting substances the iris becomes gradually smaller reaching finally the size of a thin line. This final state is referred to throughout this paper as a 0 per cent opening and considered to be a positive reaction (Fig. 1).

The size of the iris was evaluated approximately and without the aid of instruments. During the long course of experiments we acquired skill in evaluation to such a

* Hoffman La Roche, Basle (Switzerland).
† NBC, Cleveland (Ohio).
‡ Sigma, St. Louis (Missouri).
§ Assia, Ramath Gan (Israel).
‖ Light & Co. Ltd, Colnbrook Bucks (England).
** Boots, Nottingham (England).

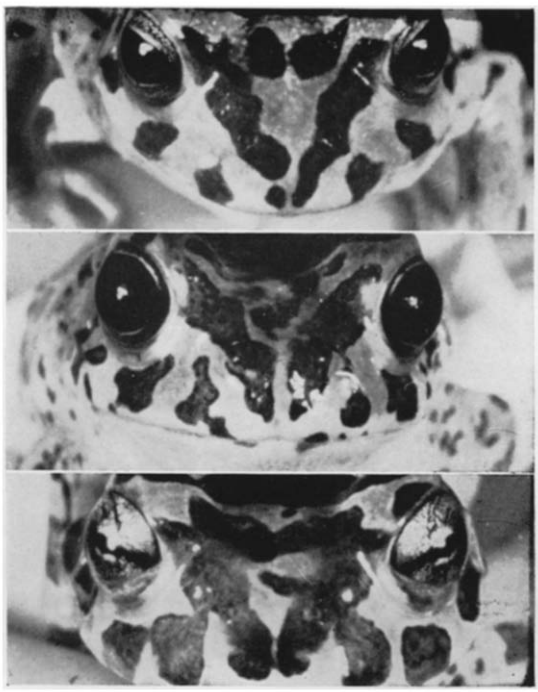

FIG. 1. A. Eyes of untreated *Bufo*. B. Eyes of *Bufo* after injection of adrenaline. C. Eyes of *Bufo* after injection of Th.

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degree, that similar estimates were obtained by different workers. Deviations, however, in the estimation of the intermediate sizes of the pupil (up to a maximum of 10 per cent) did occur.

The period which elapsed between the instant of injection of the test substance and the maximal closing of the iris is referred to as "time of closing".

The treated animals were kept under observation for 2 hr, and, in some instances, for a longer period. It often happened that the time of closing was not the same for both eyes in which case the mean value is given.

RESULTS

The results of the experiments in which Th and related substances were administered to *Bufo* and *Hyla* are shown in Table 1. Wide variations in the reaction speed were observed within the same species, even after administration of equal doses of the same preparation. There were also cases in which the same animal, when used more than once, reacted differently to the same treatment (Table 2). The difference in the time of closing of the two irises generally did not exceed 2 min, although, in rare instances, a difference of up to 20 min was noted. No prevalence of sensitivity of the left or right eye was noted.

It must be pointed out that in summer the time of reaction was much shorter than in winter. So far, we have not been able to explain this phenomenon. Although experiments were performed around the year, the results reported below relate to the period May to October (late spring and summer). The findings in other seasons show parallel but not identical trends. For a controlled study all experiments should be performed simultaneously.

All Th injections, in doses not lower than 7·5 mg/10 g body weight for *Bufo* and 2 mg/10 g body weight for *Hyla*, resulted in closing of the iris, when conditions of illumination were usual. The period during which the iris remained closed, ranged from 1 to 12 hr or more in some cases and depended on the dose. The time of closing of the iris was in reverse proportion to the dose administered (Table 1 and Fig. 2), which correlation is statistically significant. When using doses smaller than the above, not all frogs reacted and the number of the animals which did not respond increased with decreasing dose.

When the experiments were conducted in the dark, in which case the pupil reaches its maximal opening, the administration of Th reduced the size of the pupil up to approximately 50 per cent but no further.

Doses higher than 7·5 mg/10 g body weight in *Bufo* and than 3 mg/10 g body weight in *Hyla* were toxic and caused paralysis, increasing in severity with the amount injected and finally causing death. Such observations were already reported by other investigators.[1, 2] Similar toxic manifestations were also observed after injection of compounds chemically related to Th. Pigmentation changes were also brought about by these compounds, but this will be the subject of separate work.

A study of the components of Th separately revealed that the pyrimidinic component had no influence on the iris of either species, while the thiazolic component brought about myosis in *Bufo* only, but in a very irregular manner. When the two components were given simultaneously the action of either was not influenced by the other.

TABLE 1. EFFECT OF ADMINISTRATION OF TH AND CHEMICALLY RELATED SUBSTANCES ON FROG IRIS

Material	*Bufo*						*Hyla*					
	Number of animals	Dose mg per 10 g body weight	% of react. animal	Time of closing (min)			Number of animals	Dose mg per 10 g body weight	% of react. animal	Time of closing (min)		
				Min.	Max.	Aver.				Min.	Max.	Aver.
Vitamin B_1	13	3·75	77	8	33	19·4	20	1	50	5	17	9·2
	14	7·5	100	5	16	9·8	15	2	100	3	16	8·5
	15	15	100	3	12	6·5	15	4	100	2	11	7·1
	15	30	100	2	15	4·9	14	8	100	1	9	4·5
	9	60	100	2	10	3·6	12	16	100	1	5	2·9
4-Oxythiamine chlorid-hydrochlorid	6	3·75	0	—	—	—	8	1	0	—	—	—
	8	7·5	62·5	8	20	11·6	8	2	50	11	19	15·9
	7	15	71·4	5	15	10·6	8	4	100	5	15	8·8
	11	30	100	4	19	9·1	7	8	100	3	15	6·3
	7	60	100	3	11	6·3	8	16	100	1	7	2·9
Acetylthiamine	14	3·75	71·4	7	37	21·3	5	1	20	—	—	—
	13	7·5	92·3	12	60	21·6	14	2	64·4	5	38	16·1
	9	15	100	3	18	8·6	10	4	100	7	27	13·1
	8	30	100	1	9	3·8	10	8	100	3	23	12·6
	5	60	100	1	3	2·2	9	16	100	1	8	5
Thiamine monophosphate	4	3·75	25	—	—	—	5	1	0	—	—	—
	9	7·5	100	8	52	30·7	5	2	40	21	83	52
	11	15	100	7	65	33	10	4	80	7	42	25
	11	30	63·6	4	120	45·5	10	8	100	16	59	35·3
	6	60	0	—	—	—	5	16	100	4	31	19
Thiamine diphosphate	5	3·75	—	—	—	—	8	1	0	—	—	—
	7	7·5	42·9	33	53	44	13	2	61·5	16	30	22·6
	9	15	66·7	21	96	49·3	14	4	71·4	6	33	20·4
	10	30	70	13	110	28	11	8	100	8	32	19·6
	8	60	87·5	8	37	21·5	6	16	100	9	37	17·2

When the animals were previously injected with the pyrimidinic or the thiazolic compound, or with both, Th produced results similar to those observed in the control animals treated with Th alone.

After injection of ThDP, Acet-Th, Oxy-Th and Pyri-Th the rate of closing was proportional to the doses administered (Table 1 and Fig. 2).

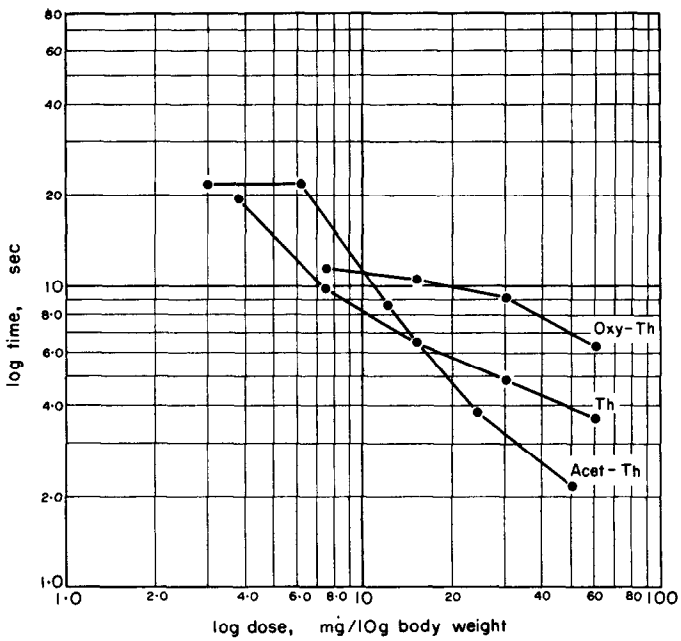


FIG. 2. Log time–log dose relationship of iris closing time in *Bufo* after injection of three different compounds. The amounts given in the curves correspond to the molecular weight of Th.

TABLE 2. VARIATION IN IRIS CLOSING TIME AFTER REPEATED INJECTIONS OF TH (7·5 MG/10 G BODY WEIGHT) TO THE SAME FROGS

Date	Time of iris closing (min)		
	Bufo 1	*Bufo* 2	*Bufo* 3
10.9.59	12	11	9
13.9.59	2	—	33
17.9.59	10	13	17
21.9.59	6	8	20

A dose of 1 mg/10 g body weight of pyrithiamine given to *Hyla* still brought about the closing of the iris, while 0·5 mg/10 g body weight, had no effect. Unfortunately, our supply of Pyri-Th was very limited and we could not perform a complete series of experiments, but we were able to show a correlation between the injected dose and the time of closing of the iris. In the case of Oxy-Th this correlation was clearly evident

as seen in Table 1 and Fig. 2. When this compound was injected simultaneously with Th the time of the reaction was shorter than anticipated from the sum of the two compounds.

ThMP was effective but no correlation was found between the dose and the speed of action. A dose of 60 mg/10 g body weight of this compound had no effect on *Bufo*, while in *Hyla* the reaction was irregular. In *Bufo* injections of ThMP in high doses which had no effect on the size of the pupil did not alter the action of Th injected simultaneously or after 10–15 min. On the other hand ThDP when injected after previous administration of high doses of ThMP proved to be ineffective.

Administration of ACh, physostigmine or both simultaneously, KH_2PO_4 or KCl dissolved in saline or water had no influence on the iris. High doses of potassium salts caused paralysis in frogs notably in the rear limbs and subsequent death. ATP administered alone or with either pyrimidinic or thiazolic compound, or with both, had no effect on the iris of frogs. Sodium sulphathiazole and diluted solutions of HCl and NaOH at pH ranging from 1 to 10, also had no influence.

DISCUSSION

The main purpose of the present study was to determine whether the specific action of Th on the frog iris was direct or through activation of other substances capable of influencing the size of the pupil of frogs. We found that ACh and physostigmine, which are known to exert an influence on mammalian eyes, were without effect on frog eyes when administered simultaneously. Potassium salts, found by us to be potent in inducing myosis of isolated frog eyes, had no effect *in vivo* even when doses causing paralysis of the limbs were used. ATP known as energy source for physiological Th activity did not show any effect on the frog iris.

Most of the solutions examined were acidic, but injections of HCl and NaOH with pH's ranging from 1 to 10 were ineffective, showing that the pH did not play a significant role. We were led to assume therefore that the myosis was effected directly by Th, or possibly by one or more substances derived from it.

Neither of the components of Th, the pyrimidinic or the thiazolic could be acknowledged as the active substance since the former did not show any activity, while the latter acted only on *Bufo* and in quantities much larger than Th. In addition the effect of these compounds was not correlated with the dose.

Th is known to inhibit the action of nicotine on isolated smooth muscle of the intestine of rabbits, frogs and guinea-pigs. A similar effect of the thiazolic component of Th, as well as of sulphathiazole has been reported.[17–20] In our experiments, however, no action of the sodium salt of sulphathiazole on the iris was noted.

Administration of both thiaminic components simultaneously, whether in the presence or absence of ATP, had also no effect on the iris and all above-mentioned substances were unable to inhibit the activity of Th injected simultaneously or shortly after.

It may be concluded therefore that the complete molecule of Th is required to obtain thorough closing of the iris. Consequently it may be assumed that *Hyla* is unable to synthesize Th from its components. On the other hand such a synthesis could take place at a low rate in the case of *Bufo* as seen from the weak and irregular action of the thiazolic component in the latter. However, this is questionable as in

Bufo the pyrimidinic component did not promote the activity of the thiazolic component.

It may be supposed that in order to obtain activity, both components have to be attached to a reacting centre (enzyme protein? muscle protein?) and that the methylenic bridge between the two components is essential, since simultaneous administration of both components did not cause a positive reaction. One possibility would be, that the reacting centre tends to be linked to the molecule of Th, at least at three points, i.e. one at the thiazolic component, one at the connecting methylenic bridge and one at the pyrimidinic component.

Another possibility is, that the reacting centre tends to be linked solely to the two components of Th, the methylenic bridge serving only as a factor conditioning their spatial arrangement. None of these links would be very strong, since previous administration of both components did not inhibit the action of Th. The latter probably expels the linked compounds from their complexes with the reacting centre, and takes over, forming much stronger links. Such a mechanism would be analogous to the action of ACh upon active centre of intracellular protein, as described by Nachmanson and Wilson[21]. The comparison seems permissible since both Th and ACh belong to the group of biologically very active "onium" compounds, which are derivatives of quaternary ammonium salts.

The possibility also exists that Th does not act directly upon the reacting centre but that it produces enzymatically another active factor whose components x, y are first linked at the appropriate place to the Th molecule (x–Th–y), then linked with each other and finally separated from their complex with Th in the form x–y, which would be the active compound. The role of the methylenic bridge in this case would be to provide the necessary spatial arrangement for the binding of x and y with each other after linking to the components of Th. This pathway would resemble the model of hydrolysis of glucose by glucosidase as suggested by Pigman[22].

The generally accepted view is that the main and perhaps the only role of Th is its inducement of the active cocarboxylase, and only few investigators[23–30] attribute some activity to free Th itself. In our observations the reactivity to ThDP was much lower than to Th when administered in corresponding amounts (Table 1 and Fig. 2). The difference in dose required to produce the same time of closing excludes the possibility that Th acted through its pyrophosphate derivative. The opposite is more probable, i.e. that the degradation of ThDP to Th is necessary in order to obtain positive results. Possibly the pyrophosphate chain prevents joining of the molecule to the reacting centre.

The possibility, however, cannot be excluded that the Th molecule is able to penetrate the cell more easily than ThDP and therein undergo conversion to ThDP, which last derivative may be the active factor. To solve this problem we examined compounds analogous to Th, namely Pyri-Th and Oxy-Th which are known to antagonize Th in animals (e.g. mice) and yeasts. Their mode of action is not definitely known, but the accepted view is that Pyri-Th antagonizes Th itself, while Oxy-Th is rather an antagonist of ThDP. Woolley[26] postulated that Pyri-Th together with pyrophosphate and a specific enzyme form a stable complex. In this way the enzyme becomes blocked and is not available for the synthesis of ThDP from Th. Oxy-Th acts in a similar way, but its pyrophosphate is easily separated from the enzyme

complex. Oxy-Th-pyrophosphate would bind with apocarboxylase making the synthesis of holo-carboxylase from cocarboxylase impossible (Fig. 3).

Some investigators (Muralt, for example), on the basis of these assumptions, tried to establish whether some specific pharmacological reactions were due to Th or to its pyrophosphate. In our experiments neither Pyri-Th nor Oxy-Th inhibited the action of Th or ThDP on the closing of the iris. Moreover, they attributed an activity of their own as seen in Table 1 and Fig. 2.

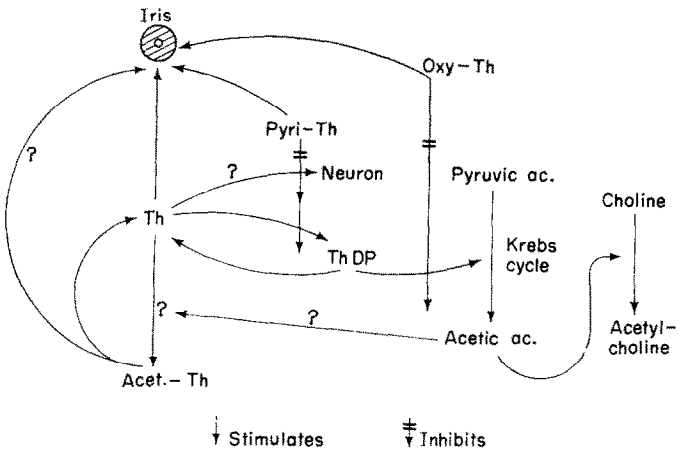
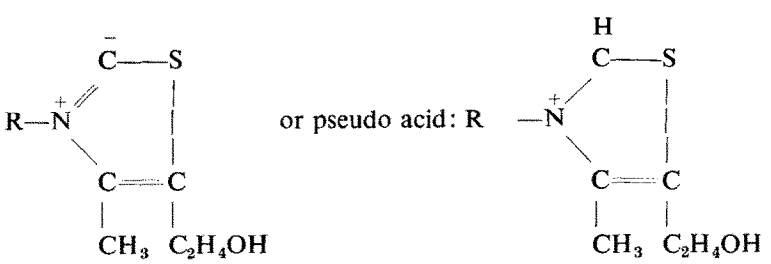


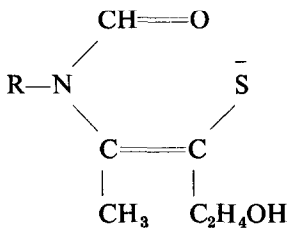
FIG. 3. Some pathways of the supposed action of Th and related compounds. For explanation see the text.

The analogy found in the activity of Th, Pyri-Th and Oxy-Th is not surprising, since it is known, that substances with related chemical structures and steric configurations (isosteric compounds) reveal similar physiological activity. This is encountered in the case of benzenic and thiophenic, thiazolic and pyridinic compounds. As a matter of fact Pyri-Th was originally synthesized for the purpose of obtaining a compound with an activity analogous to Th and not antagonistic to it.[31]

The active group in the process of decarboxylation of α-keto acids is probably a bipolaric ion:[32]



We suppose that in our experiments also these groups are the active ones, or at least partially so. We base this on the fact that substitution of the thiazolic ring by a pyridinic one, as in the case of Pyri-Th, does not affect the specific physiological activity. This obviously, could happen, if the thiazolic component acted in the form of the thiolic pseudobase:



The fact that Pyri-Th and Oxy-Th are active in inducing myosis in frogs similarly to Th, speaks for a different mechanism than the one involved in the known process of Th activities. The antagonistic action of these antimetabolites apparently involve receptors other than those related to the influence on the iris (Fig. 3).

It should be noted that these compounds inhibit also the action of ACh on isolated frog heart, similarly to Th and that Oxy-Th potentiate the action of Th,[14] as also found in the present study.

The results obtained on administration of ThMP are of special interest. This compound acts much slower (in winter) than Th and ThDP and augmentation of its dose not only does not increase the speed of the reaction, but from a certain level upwards the reaction does not occur at all (especially in *Bufo*). Doses higher than 60 mg/10 g body weight were ineffective in *Bufo*, while in *Hyla* the increase of the dose caused an irregular response. Probably ThMP, in high doses, inhibits the activity of the phosphatase which converts ThMP to Th. Many cases of non-competitive inhibition of enzymes caused by excess of substrate concentration are known in enzymology, e.g. inhibition of invertase by excess of fructose, β-glucosidase by excess of glucose, urease by excess of urea, or cholinesterase by excess of ACh.[33, 34]

The difference in behaviour between ThDP and ThMP points to the probable existence in frogs and perhaps in other animals of two different enzymes, namely Th-orthophosphatase and Th-pyrophosphatase, the last being much more active.

Preliminary experiments with products of incubation of ThDP and ThMP with blood of rats (known to be rich in phosphatases), injected into frogs, were made. It was found that when ThDP was used, the time of closing of the iris was much shorter than when no incubated material in the same dose was injected. It almost corresponded to the time calculated from the time–dose relationships when the same amount of the free Th, as contained in ThDP, was injected. In contrast, incubation of ThMP did not alter its activity. This problem needs more additional work and will be investigated separately, but it may be pointed out that ThMP in acidic medium containing myosin, is much more stable than ThDP or ThTP, as described in the work of Greiling and

Kiesow[35]. There is probably a correlation between this and the weak activity of ThMP hydrolyzing enzymes.

It was found that previous administration of high inactive doses of ThMP to *Bufo* did not change the action of Th, while ThDP injected under the same conditions was completely inactive. It may be concluded from these results, that the unhydrolyzed ThMP does not bind the active centre, or if it does, such a link is very weak so that Th may easily replace it.

The lack of reaction to ThDP after previous administration of ThMP, is indicative, that excess of circulating ThMP inhibits not only the enzyme which hydrolyses ThMP to Th, but also the enzyme which hydrolyses ThDP to Th. One possible explanation is that both enzymes possess a common coenzyme but different apoenzymes. It cannot be excluded, however, that the same enzyme acts on both compounds and that hydrolysis of the enzyme–ThMP complex is much slower than that of the enzyme–ThDP. It may be concluded, whether there are one or two thiamine-phosphatases, that there is a chemical competition between ThDP and ThMP for the hydrolysing enzyme (or enzymes); therefore a anticocarboxylase action of ThMP possibly exists under certain conditions. Of course the blackade of the enzyme would not affect the action of Th, a fact proved by us experimentally.

In contrast to ThMP, Acet-Th was found to be as active as free Th. It may be supposed that it undergoes a very rapid hydrolysis, releasing active free Th or that, unlike Th-phosphates, it binds the active centre in the unchanged form. Probably the acetyl group does not affect the ability of the molecule to become linked to the reacting centre, as it is known that the ability of molecules to bind the proper receptors, greatly depends on their steric configuration and on their rigidity or elasticity.

One of the main roles of carboxylase is to enable the splitting of pyruvic acid, liberating acetate which participates in the synthesis of ACh. It cannot be, however, excluded, that Th undergoes acetylation simultaneously and that Acet-Th participates in some metabolic processes. This supposition is supported by the work of Kuhn *et al.*[36], who attributed to Acet-Th a role similar to ACh in nerve metabolism.

The present observations indicate that Th is able to participate in active metabolism in frogs, independently from cocarboxylase. Support for this assumption may be found in some investigations dealing with experiments *in vitro*. Ochoa[37,38] observed that free Th stimulated the decarboxylation of pyruvic acid by alkaline-washed yeasts in the presence of an excess of cocarboxylase, and methods for separate determination of Th and ThDP were based on this observation. Westerbrink[39] showed that Acet-Th acts similar to Th although to weaker degree (50 per cent). Recent experiments[40, 41] have shown that free Th is able to catalyse the decarboxylation of α-keto acids in the absence of other enzymes, though to a much less extent than ThDP.

Experiments performed on vertebrates *in vivo* are less convincing. Woolley and Merrifield,[29,30] as well as Muralt,[25] basing their conclusions on results obtained by using Oxy-Th and Pyri-Th in rats and mice, believe that Th has a direct specific action on the nervous system not mediated through ThDP (Fig. 3). On the other hand, experiments of Caro *et al.*[42, 43] demonstrate that Oxy-Th and Pyri-Th have a similar action, the first decreasing the amount of Th mainly in muscles and liver, the second decreasing the amount mainly in the brain. Correspondingly, clinical manifestations, when using both compounds, are quite different. Di Palma and Hitchcock[1] attributed the difference

in action between Oxy-Th and Pyri-Th to the fact that Pyri-Th is much more active in causing neuromuscular and ganglionic blockade than Oxy-Th and not to their influence on Th activity.

It may be concluded that the presently observed *in vivo* action of Th and possibly also of Acet-Th, is not related to its classic metabolic role. The mode of action seems to be direct and not mediated through cocarboxylase. The later acts probably after undergoing hydrolysis to produce free Th (Fig. 3).

The question whether Th is involved in the physiological regulation of the size of the pupil in the frog, as yet cannot be answered, and will be a subject of further investigation.

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