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Guanine Metabolism in Fishes

I. The Occurrence of a Guanine-Deaminating Enzyme in Fish Skin

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Some properties of the guanine-deaminating enzyme of fish skin have been studied. The deaminase activity was found to be stimulated by the metal-chelating agent ethylenediaminetetraacetate (EDTA), indicating a sensitiveness of the enzyme towards metal ions. Among a number of cations tested Cu++ and Zn++ were by far the most potent inhibitors and their effect was greatly influenced by the pH. In view of this it is likely that a salt-like complex is formed between the inhibiting ions and negatively charged groups of the enzyme molecule. Experiments with iodoacetate gave no evidence for the necessity of sulfhydryl groups for the deaminase activity. Dialysis experiments failed to demonstrate any metalic-ion requirement.

As is well known guanine, chiefly recognized as one of the purine bases of the nucleic acids, also occurs in large amounts in the skin and on the scales of fishes, causing the silvery appearance characteristic of many species. Hitchings and Falco [1] and more recently Neckel [2] have convincingly demonstrated the identity of this silvery matter with pure guanine.

Fishes — like spiders — are unique in their ability to produce large amounts of non-nucleic acid guanine. In fishes this production exhibits some peculiarities, *e. g.* the rather sudden appearance of this substance, which accompanies the metamorphosis of the Salmon parr to smolt, as well as the disappearance that can be induced merely by preventing the smolt from getting into seawater as it normally does on completion of the metamorphosis. Further it was shown by Sumner [3] that the production of guanine is subject to variations caused by visual stimuli. Thus it was found that prolonged stay of the fish upon a dark background led to a decrease in the amount of guanine, while a light background produced the reverse effect. — In view of these facts fishes appear to be interesting objects for a study of the enzymes involved in the metabolism of guanine. The present investigation was intended to determine, whether or not the fishes are provided with the same or similar enzymes for the breakdown of "skin guanine", as are known to affect nucleic acid guanine.

In the present paper the existence in fish skin of a guanine deaminating enzyme is demonstrated, and some of its properties are studied.

EXPERIMENTAL

Materials. The guanine and xanthine preparations used were commercial products obtained from Schuchardt and Hoffman-La Roche Chemical Co., respectively. They were not purified prior to use.

Because of the scanty solubility of guanine at neutrality colloidal solutions according to Schmidt [4] had to be prepared for ammonia determinations. For the spectrophotometric studies, which occupied the main part of this investigation, true solutions of guanine containing, unless otherwise indicated, 10 μg per ml could be used.

Preparation of the enzyme. The fish, roach *(Leuciscus rutilus* Lin.), was skinned immediately after being killed. The skin plus scales (wet weight 8—12 g) were ground as soon as possible, and extracted at room-temperature with 3 volumes of glass-distilled water or, when indicated, with a 0.01 M solution of ethylenediaminotetraacetic acid (EDTA). The extraction was performed for 30 min in a shaking device. The mixture was centrifuged at 1 200 *g* for 15 min in order to remove insoluble material; the supernatant was then subjected to 11 000 *g* for 20 min at 0 °C. The resulting fluid containing the enzyme was not further purified, since this was not found necessary for the present study.

Dialysis of the enzyme solution. Samples of the enzyme solution were dialysed through cellophane membranes against glass-distilled water or against a 0.005 M EDTA solution in an apparatus, providing frequent and thorough stirring of both the enzyme solution and the surrounding fluid.

In some cases samples were electrodialysed against 0.005 M EDTA solutions.

Analytical. The enzymatic activity was followed either by measuring the ammonia liberated or by a spectrophotometric procedure. The ammonia estimations were carried out essentially according to Schmidt [4], involving vacuum-distillation of pre-formed ammonia according to Parnas [5] with subsequent titration, employing the Tashiro indicator. Generally the spectrophotometric procedure was chosen. It is based upon the change in optical density in U.V. caused by the deamination of guanine to xanthine. The values reported by Roush and Norris [6] for the maximum density change at 245 mμ, (for pH values below 7.5) at 250 mμ (for pH values 8—8.5) and at 255 mμ (for pH values above 9.0) obtainable with 10 μg per ml solutions of guanine were checked, employing solutions of pure guanine and pure xanthine in buffer. These values were then used in computing the per cent deamination of guanine. A Beckman model DU spectrophotometer with U.V. attachment was used throughout.

Standard conditions. The incubation was carried out in 10 mm quartz cuvettes. 0.2 ml of the enzyme solution was incubated with 3.1 ml of the buffered solution of guanine containing 10 μg per ml. The pH was varied in the region 6.0—9.5, using 0.067 M phosphate or 0.05 M tris -(hydroxymethylaminomethane) buffers. The final pH was checked electrometrically at the end of the incubation. Readings were taken with one min intervals at 245, 250, or 255 mμ depending upon the actual pH against blanks without guanine. The time of incubation was generally 15 min, but was sometimes extended to 60 min. The temperature was kept at 21°C. The total volume of the samples was always kept at 3.3 ml.

RESULTS

Aqueous extracts of fish skin were found to contain a guanine deaminating enzyme. As seen from Fig. 1 there is good proportionality between the amount of enzyme added and the ammonia liberated. The spectrophotometric approach to the enzymic reaction elucidated that the ammonia formed is a result of an actual deamination and not of a breakdown of the ring structure, since xanthine, characterized by its U.V. absorption curve, appears as the other reaction product (Fig. 2).

The effect of EDTA. Preliminary experiments aiming at a determination of the enzymatic activity at different pH values showed that the time course curves for pH values above 8 were not linear. As is seen from Fig. 3 rather

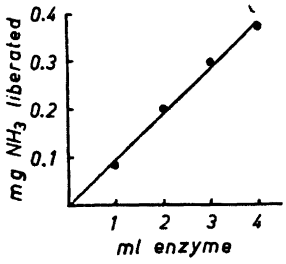

Fig. 1. The effect of varying enzyme concentration on the enzymatic activity as measured by the ammonia determination method. *Conditions:* 5.0 ml of a colloidal solution of guanine (0.88 mg per ml) and 10 ml of 0.067 M phosphate buffer pH 7.5 were incubated with the indicated amount of enzyme. The total volume of the samples was made up with distilled water to 20 ml. The incubation was carried out for 40 minutes at 40 °C, and was stopped by the addition of 4 per cent HCl. Aliquots were taken to ammonia estimations.

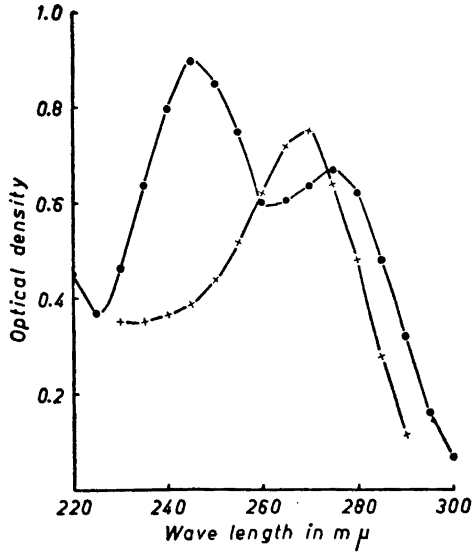

Fig. 2. The U.V. absorption changes caused by the deamination of guanine by the enzyme from fish skin. *Conditions:* 0.2 ml of the enzyme solution was incubated in a total volume of 3.3 ml with a buffered solution of guanine (15 μg per ml). Buffer: 0.067 M phosphate pH 7.5. Incubation time 30 minutes at 21 °C. ● — ●, the absorption curve at zero time, guanine. × — ×, the same after 30 minutes' incubation, xanthine.

abrupt drops in the activity occurred at these pH after about 10 min incubation. At pH 9.0 the enzyme seemed to be completely inactive. Pre-incubation of the enzyme for varying time periods with buffer at pH above 8 prior to the addition of substrate resulted in a considerably lowered deamination rate (Fig. 4). This effect of the pre-incubation increased with rising pH.

When EDTA was included in the reaction mixture this phenomenon disappeared (Fig. 3), the time course curves at all pH values being linear and the activity at pH 9.0 accounting for roughly 85 % of the optimum value. Although the activating effect of EDTA was more marked in the pH region above 8, it was also evident in the region below 8. Furthermore the stability of the enzyme solution was found to be greatly enhanced by the extraction of the enzyme with EDTA solution instead of glass-distilled water.

The effect of divalent cations. As a consequence of the above-mentioned findings the effect of divalent cations on the enzymatic activity was studied. For this purpose the enzyme was prepared by extraction with EDTA followed by

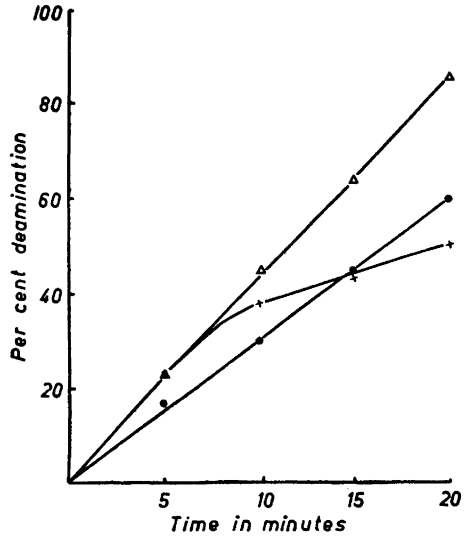

Fig. 3. The time course of the enzymatic activity. Standard conditions, 0.05 M TRIS buffer. ● — ●, obtained at pH 7.0. × — ×, obtained at pH 8.5. △ — △, obtained at pH 8.5 with the enzyme pre-treated with 0.01 M EDTA.

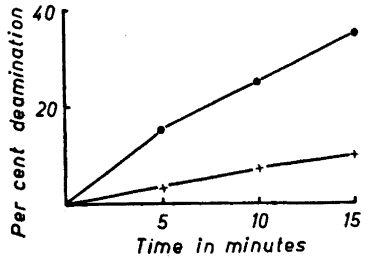

Fig. 4. The effect of pre-incubation on the enzymatic activity. Standard conditions, 0.05 M TRIS buffer pH 8.5, ● — ●, no pre-incubation. × — ×, pre-incubated for 10 minutes before the addition of substrate.

Table 1. The effect of cations on the enzymatic activity. Standard conditions: pH 8.5; 0.05 M TRIS buffer.

Inhibitor	Concentration	Relative activity
None	—	100
$CaCl_2$	1×10^{-2} M	96
$MgSO_4$	1×10^{-2} M	106
$MnCl_2$	1×10^{-2} M	104
$CoSO_4$	1×10^{-2} M	23
$ZnSO_4$	6×10^{-5} M	31
$Fe_2(SO_4)_3$	6×10^{-5} M	85
$FeSO_4$	6×10^{-5} M	85
$CoSO_4$	6×10^{-5} M	94
$CuSO_4$	6×10^{-5} M	29

Table 2. The effect of pH on the cationic inhibition. Standard conditions. The concentration of Cu^{2+} and Zn^{2+} = 6×10^{-5} M; 0.05 M TRIS buffer.

Inhibitor	Per cent inhibition at pH		
	7.3	8.5	9.0
$ZnSO_4$	0	69	100
$CuSO_4$	3	71	100

dialysis. By this treatment interferring ions together with EDTA were removed from the enzyme solution which was then suited for studies on ion promoted inhibition.

Among a number of ions tested (*cf.* Table 1) Cu^{2+} and Zn^{2+} were found to be by far the most potent inhibitors, effecting complete inhibition at concentrations of 6×10^{-5} M. The inhibition was found to be greatly influenced by the pH, being none or insignificant at pH 7, and complete at pH 9 (Table 2). When EDTA was included in the reaction mixture the mentioned ions were ineffective. However, once inhibited the enzymatic activity could not be restored by the addition of EDTA.

The effect of other inhibitors. The observation of Schmidt [4] that sodium fluoride inhibits the deamination of guanine could not be verified under the conditions of the present experiments. Neither when tested directly nor after pre-incubation with the enzyme before the addition of substrate did sodium fluoride exert any significant effect in concentrations as high as 0.01 M.

Iodoacetate was used for testing the possibility of an interaction between the inhibiting ions and sulfhydryl groups of the enzyme molecule. The enzyme was pre-incubated with iodoacetate at a concentration of 0.01 M for periods up to 60 min, yet without any effect (Table 4).

Table 3. The effect of sodium fluoride on the enzymatic activity. Standard conditions. 0.05 M Tris buffer pH 8.0.

Inhibitor	Per cent deamination	Per cent inhibition
None	37	0
NaF, 0.01 M not pre-incubated	36	2
NaF, 0.01 M pre-incubated for 20 min	35	5

Table 4. The effect of iodoacetate on the enzymatic activity. Standard conditions. 0.05 M Tris buffer pH 8.0.

Inhibitor	Per cent deamination	Per cent inhibition
None	35	0
Iodoacetate, 0.01 M not pre-incubated	35	0
Iodoacetate, 0.01 M pre-incubated for 20 min	33	5

Ammonia in a concentration of 0.01 M did not affect the enzymatic activity.

The effect of dialysis. When dialysis against 0.005 M EDTA was extended over 16 h no loss in activity occurred. On the contrary, when the enzyme had been prepared by extraction with glass-distilled water dialysis resulted in an activation (Fig. 5). No change in activity occurred when an enzyme solution prepared by extraction with EDTA was dialysed against glass-distilled water.

Electrodialysis during 5 h produced no inactivation.

The pH-activity curve and the effect of phosphate. The pH-activity curve is based on time course curves obtained for different pH values, while using enzyme solution freed from contaminating ions (Fig. 6). When studied in TRIS buffer the optimum pH was found to lie between pH 8.0 and 8.25. This is in agreement with Roush and Norris [6] using mammalian guanase. However, the

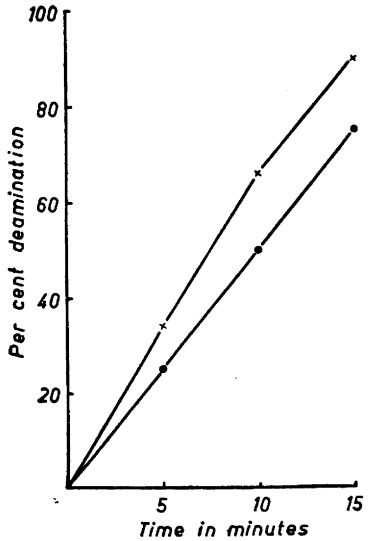

Fig. 5. The effect of dialysis on the enzymatic activity. The enzyme solution prepared by extraction with glass-distilled water was dialysed for 16 hours against 0.005 M EDTA. The activity was measured under standard conditions. ● — ●, non-dialysed enzyme. × — ×, dialysed enzyme.

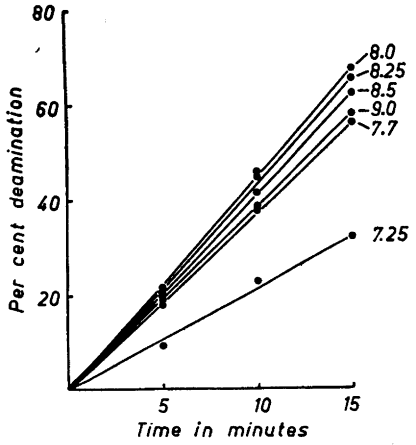

Fig. 6. Time course curve for the enzymatic activity at different pH values. Standard conditions, EDTA prepared enzyme, 0.05 M TRIS buffer, pH as indicated in the figure.

general appearance of the pH-activity curve for the enzyme in fish skin (Fig. 7) differs somewhat from that given by these authors, the discrepancies being essentially confined to the pH region below 8. This was found to be due to the kind of buffer substance used, and its concentration. When the reaction was studied in phosphate buffer of low concentration the curve more closely resembled that of mammalian guanase, the activity values at 7.3 accounting for 85 % of the optimum value as compared with 60 % in TRIS buffer (Fig. 8). With increasing concentration of the phosphate buffer the optimum pH was shifted towards the acid side. At a buffer concentration of 0.1 M the optimum was found to lie in the vicinity of 7.5.

DISCUSSION

In its pH dependency the guanine-deaminating enzyme of fish skin agrees with guanase of other origin studied previously [6]. The present authors could, however, not demonstrate any inhibition by sodium fluoride as reported by Schmidt [4]. Using bacterial guanase Rakosky, Zimmerman and Beck [7] were also unable to confirm this observation.

On account of the ineffectiveness of iodoacetate it is concluded that sulfhydryl groups are not essential for the enzymatic activity.

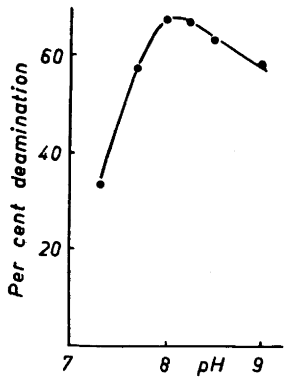

Fig. 7. The pH-activity curve of the fish skin enzyme. The curve is based on the time course curves in Fig. 6. The values corresponding to 10 minutes' incubation are plotted against the respective pH.

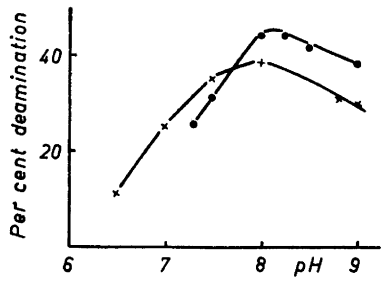

Fig. 8. The influence of the buffer system used on the pH-activity curve. Standard conditions, EDTA prepared enzyme solution. ● — ● Measured in 0.05 TRIS buffer. × — × Measured in 0.067 M phosphate buffer; the pH values 8.8 and 9.0 were obtained by the addition of phosphate up to 0.067 M concentration to TRIS buffer which latter determined the pH. The values correspond to 5 minutes' incubation time.

There is no evidence for a metal-ion requirement of the enzyme, as might be suspected from Schmidt's [4] observation. The dialysis experiments failed to demonstrate any need for a dialysable activator. On the contrary, some divalent cations were found to be potent inhibitors, and it was observed that their effect was counteracted by EDTA. On the basis of this fact the activating effect of EDTA was interpreted as being due to its metal-chelating property. It is likely that the enzyme solutions prepared by extraction with glass-distilled water are contaminated by ions which are thus removed by the action of EDTA.

As to the effect of dialysis illustrated in Fig. 5, the activation observed may most likely be ascribed to EDTA, since dialysis of an EDTA treated enzyme solution against glass-distilled water was unable to bring about any change in enzymatic activity.

In view of the dependency of the effect of the inhibiting ions on the pH it is likely that a salt-like complex is formed between the inhibiting cations and negatively charged groups of the enzyme molecule. This complex seems to be stable, since EDTA could not restore the activity when once inhibited. No macroscopic precipitate was, however, observed.

Ammonia, one of the reaction products, was found not to inhibit the activity at any concentration up to 0.1 M. Xanthine was not tested in this respect.

With respect to the pH-activity curve the enzyme from fish skin shows definite similarities with the mammalian guanase as studied by Roush and Norris [6], especially when measured in phosphate buffer of low concentration.

The mechanism behind the displacement of the optimum by increasing concentrations of the phosphate buffer as well as the somewhat different shape of the curve when measured in TRIS buffer remains, however, obscure.

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