Guanine Metabolism in Fishes

II. A Guanosine Phosphorylase in Fish Skin

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Aqueous extracts of fish skin, previously found to contain a guanine-deaminating enzyme, are also capable of splitting guanosine. The reaction has been studied spectrophotometrically and the enzyme has been found to be phosphorolytic.

Enzymatic splitting of guanosine may be either hydrolytic or phosphorolytic. Tarr¹ has shown recently the existence in fish muscles of two hydrolytic enzymes, one with specific action upon inosine at pH 8.6, and another, non-specific, acting upon guanosine at pH 5.6. Since Kalckar² elucidated the phosphorolytic action of the nucleosidases which had formerly been looked upon as hydrolytic enzymes, it has, on the other hand, become obvious that phosphorolytic splitting is a more wide-spread phenomenon than had been thought earlier. The purine nucleoside phosphorylase was proposed to catalyze the reaction:

and it was found to be active only with guanine or hypoxanthine as the purine.

The present investigation was intended as a study of the mechanism of the fission of guanosine which was observed to be caused by an enzyme from fish skin. The use of a spectrophotometric method for the identification of guanine was made possible by the fact that the liberated purine base was not deaminated, although the enzyme extract contained guanase in addition to the nucleoside phosphorylase, as shown in an earlier investigation³. The apparent inhibition of guanase is probably competitive, but the problem will be followed up later. Because of this inhibition, the enzymatic method of Kalckar⁴ could not be used for the quantitative determination of guanine.

Fig. 1. Enzymatic splitting of guanosine by the fish skin enzyme. Conditions as described under Experimental. $\times$ — $\times$ absorption curve of the reaction mixture at zero time, pH 7.7. O — O absorption curve of the reaction mixture after 50 minutes incubation, pH 7.7. Δ — Δ the same as O — O made alkaline to pH > 11. The extinction at maximum ($E_{\max}$) has been taken as 1.0.

Fig. 2. Absorption curves of authentic guanosine and guanine. $\times$ — $\times$ guanosine at pH 7.7, O — O guanine at pH 7.7, Δ — Δ guanine at pH > 11, $E_{\max} = 1.0$.

EXPERIMENTAL

Material. The guanosine and guanine preparations used were commercial products from Kebo Chemicals Ltd. and Schuchardt, respectively.

Preparation of enzyme. The fish (roach, *Leuciscus rutilus* Lin.) was skinned, the skins ground as soon as possible and extracted with 3 volumes of re-distilled water in a mechanical shaker at room-temperature for 30 min. The mixture was centrifuged at 1 200 g for 10 min, the supernatant was then subjected to 9 000 g for 15 min. The resulting fluid was used without further purification.

Analytical methods. The spectrophotometric method used for the identification of guanine, the reaction product, is based upon the characteristic absorption spectra of guanosine and guanine in U.V. The compounds are further differentiated and the identity of the reaction product is strengthened by raising the pH of the medium to pH > 11. A Beckman spectrophotometer model DU with U.V. attachment was used for the determinations.

3 ml of a solution of guanosine, containing 17 μg per ml in 0.067 M phosphate buffer of pH 7.7, was incubated in the Beckman cuvette with 0.3 ml of the enzyme solution. The absorption between 220 and 300 $m\mu$ was measured at zero time, and after the appropriate incubation time, usually 50 min, against a blank without guanosine. The reaction mixture was then made alkaline to pH > 11 with NaOH, and the absorption curve was re-read.

Fig. 3. The phosphate dependency of the fish skin enzyme. Conditions as described under Experimental except that phosphate buffer was exchanged for glycyl glycine buffer. $\times$ — — — $\times$ absorption curve of the reaction mixture at zero time, pH 7.8, $\bigcirc$ — — — $\bigcirc$ the same after 50 minutes incubation, pH 7.8, $\triangle$ — — — $\triangle$ the same as $\bigcirc$ — — — $\bigcirc$ but pH > 11, $\bullet$ — — — $\bullet$ authentic guanosine at pH > 11.

The identification of the reaction product was further strengthened by subjecting to paper chromatography aliquots of a reaction mixture. This contained 5 μ moles of guanosine and 3 μ moles of phosphate in 0.02 M glycyl glycine buffer pH 7.8 which had been allowed to incubate with the enzyme at 37 °C for 60 min. A mixture of *isobutyric acid-ammonia-ethylenediaminetetraacetic acid*, according to Krebs and Hems⁵, was used as solvent. The spots were detected with the aid of a U.V. lamp.

Guanine was determined quantitatively with Folin's phenol reagent as described by Hitchings⁶ with minor modifications. Of reaction mixtures containing guanosine, phosphate, and enzyme solution in glycyl glycine buffer of pH 7.8, 1.0 ml was taken for guanine determination. The proteins were precipitated with 0.3 ml of 3 % perchloric acid, as recommended by Lowry and Lopez⁷ for phosphate determination (see below). 1.0 ml of the protein-free centrifugate was mixed with 2.0 ml of the phenol reagent and 5.0 ml of a saturated solution of sodium carbonate. The mixture was brought up to 15 ml with distilled water, and readings were taken at 650 m μ in a Beckman spectrophotometer.

Compared with guanine, guanosine produces a very weak colour with the phenol reagent. This makes possible a quantitative determination of guanine in the presence of guanosine, as is also pointed out by Kalekar⁴. Before the addition of enzyme a 1.0 ml sample of the reaction mixture was taken and treated in the same way as the tests, and was used as a blank in these determinations.

Phosphate was determined according to Lowry and Lopez⁷. The colour reaction was made with 0.2 ml of the protein-free centrifugate mentioned in connection with the guanine determination.

RESULTS AND DISCUSSION

A comparison between Figs. 1 and 2 shows the similarity between the absorption curves of authentic guanine and the reaction product after 50 min incubation. The two figures in addition show the agreement between the respective absorption spectra at alkaline pH. The ratio values for authentic guanine at neutral pH: $E_{290} / E_{280} = 0.50$, $E_{250} / E_{260} = 1.37$, and $E_{280} / E_{250} =$

Table 1. Phosphorolysis of guanosine. 4.85 μ moles of guanosine and 3.35 μ moles of phosphate incubated with enzyme solution in glycyl glycine buffer at pH 7.8. Temp. 37 °C.

Incub. time, min	Inorg. phosph. μ moles	Inorg. + org. phosph. μ moles	Org. phosph. μ moles
0	3.33	3.35	0.02
200	2.55	3.31	0.76

Table 2. Phosphorolysis of guanosine and the formation of equivalent amounts of organic phosphate. 3.0 μ moles guanosine and 10.70 μ moles phosphate incubated with enzyme solution in glycyl glycine buffer. pH 7.8. Temp. 37 °C.

Incub. time, min	Inorg. phosph. μ moles	Org. phosph. μ moles	Guanine μ moles
0	10.68	0.02	—
10	9.99	0.71	0.76
20	9.24	1.46	1.53
30	8.91	1.79	1.81
40	8.76	1.94	2.04

0.71, given in the literature ⁸ should be compared with the corresponding values for the reaction product: 0.45, 1.35, and 0.70, respectively.

The paper chromatography confirmed the liberation of guanine during incubation of guanosine with the fish skin enzyme. The R_F values for authentic guanine and the fission product were identical and equal to 0.66, whereas guanosine had an R_F of 0.53.

Early experiments showed that the splitting of guanosine is effected only in the presence of inorganic phosphate. As is apparent from Fig. 3 no or only slight activity was noted when glycyl glycine was substituted for phosphate buffer.

The phosphate analysis indicated that inorganic phosphate disappears from the reaction mixture during the fission of guanosine. The phosphate obviously entered into a labile organic linkage, the substance formed being probably ribose-1-phosphate. The mild hydrolytic action of perchloric acid at 30 °C liberated all the organically bound phosphate (Table 1).

As is seen from the results of a typical experiment summarized in Table 2 there is good agreement between the liberated guanine and the phosphate bound in organic linkage. That the phosphate values throughout are somewhat too low might be explained by a slight, but unavoidable splitting of ribose-1-phosphate during the procedure of the determination as is pointed out by Lowry and Lopez ⁷, or by the existence in the enzyme solution of phosphatases as proposed by Kalckar ².

The above-mentioned data show that the observed enzymatic splitting of guanosine by an enzyme extracted from fish skin is a phosphorolytic one.

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