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Full Length Research Paper

Degradation of purine ribonucleosides by extracts of *Penicillium viridicatum*

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Cell-free extracts of nitrate-grown *Penicillium viridicatum* could catalyze the hydrolytic cleavage of N-glycosidic bond of adenosine, guanosine and inosine to the corresponding base and ribose by a ribonucleoside hydrolase; however, there is no evidence for the degradation of these compounds through phosphorylation. The rate of hydrolysis of the three ribonucleosides was in the order inosine> guanosine> adenosine. It was proven that adenosine hydrolyzing enzyme is not associated with the cell membrane. Maximum enzyme activity was observed at pH 4 and 50°C. Heat inactivation kinetics and the effect of the nature of the buffer on enzyme activity revealed that, the cleavage of the three purine ribonucleosides is affected by one intracellular nucleoside hydrolase. It was proven experimentally that, all the metal ions tested had a remarkable inhibitory effect on the activity of the ribonucleoside hydrolase. Results obtained indicate that extracts of *P. viridicatum* catalyzed the conversion of guanosine into guanine and ribose by a nucleoside hydrolase and the resulting guanine was then deaminated to xanthine by an inducible guanine deaminase. In addition, xanthosine was not split into ribose and xanthine by the same extracts under the same experimental conditions and even at different pH values of the reaction mixture.

Key words: Purine ribonucleosides, adenosine, guanosine, inosine, hydrolase, *Penicillium viridicatum*.

INTRODUCTION

The purine and pyrimidine ribonucleosides and their derivatives (nucleotides) serve many diverse functions in living organisms beyond providing the monomers of the structures of nucleic acids. The catabolism of these compounds, were developed largely through studies on mammalian tissues (Mohamud et al., 1990), bacteria (Thomas, 1996) and yeast (Heppel and Hilmoe, 1952; Magni et al., 1975). Studies demonstrating the cleavage of the ribonucleosides by enzymes from the filamentous fungi are still lacking because of long standing technical difficulties of growing their mycelia; also, lack of adequate kinetic analysis of their mode of growth. According to Friedkin and Kalckar (1961), enzymes which catalyze the cleavage of N-glycosidic bonds of ribonucleosides fall into three general classes namely phosphorylases (West, 1995), transferases (Chawdhri et al., 1991) and hydrolases (Elzainy et al., 1990; Abdel-Fatah et al., 2003).

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Recently, Abdel-Fatah et al. (2009) proved that extracts of *Aspergillus terreus* could catalyze the hydrolytic cleavage of purine ribonucleosides to the corresponding base and ribose by ribonucleoside hydrolase. The aim of this prospective study is to investigate the nature and some properties of the enzymes, that catalyze the cleavage of purine ribonucleosides, adenosine, guanosine and inosine in *Penicillium viridicatum*.

MATERIALS AND METHODS

Organism

P. viridicatum, NRC 3712, was obtained from the culture collection of the National Research Centre, Cairo, Egypt.

Media

Czapek-Dox's medium was used for cultivation of the organism during the present study. The composition of this medium is as follows (g/L): glucose, 30; $NaNO_3$, 2.0; KH_2PO_4, 1.0;

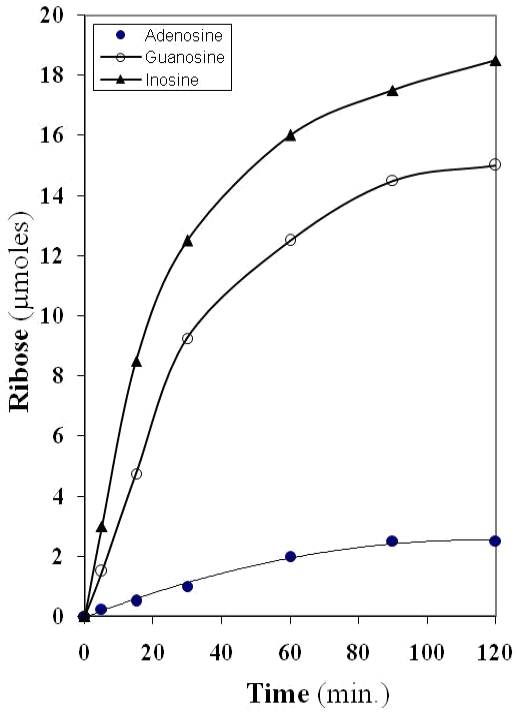

Figure 1. Comparative rate of ribose formation from purine ribonucleosides by extracts of *Penicillium viridicatum.* Reaction mixture contained: Adenosine, guanosine and inosine, 20 μmoles; citrate buffer (pH 4), 400 μmoles; extract protein, 12.32 mg; total volume, 4 ml; temperature, 50°C and reaction time, as indicated.

MgSO4.7H2O, 0.5; and KCl, 0.5. The medium was sterilized by autoclaving under 1.5 atmosphere for 20 min. Czapek Dox's solid medium was also used for culture maintenance.

Cultivation of the organism

Spores of 7 days old mycelia of *P. viridicatum* grown on slants, were scrapped and suspended in sterile distilled water. 2 ml portions of the obtained spore suspension were used to inoculate under aseptic conditions, 250 ml Erlenmeyer flasks each containing 50 ml sterile medium. The inoculated flasks were then incubated statically at 28°C.

Preparation of cell free extracts

The four days old mycelia were harvested by filtration, washed thoroughly with distilled water and finally blotted dry with absorbent paper. The blotted dry mycelia were ground with approximately twice its weight of washed cold sand in a cold mortar and extracted with cold distilled water. The obtained slurry was centrifuged at 5500 rpm for 10 min. The supernatant was used as the crude enzyme preparation.

Dialysis of the extracts

Dialysis of the extracts was made against 200 fold its volume of cold 0.02 M citrate buffer, pH 4 for 24 h at 4°C.

Chemical methods

Determination of ribose was made by the method described by Ashwell (1957). Ammonia was determined using Nessler's reagent as described by Schramm and Lazorik (1975). Protein was determined by the method of Lowry et al., (1951).

Enzyme assay

Purine ribonucleoside hydrolase activity was determined by measuring the reducing sugar (ribose) formed from the purine ribonucleosides as substrate, when incubated with cell -free extracts, as previously described by Elzainy et al. (1990). This was accompanied by chromatographic identification of the base. One unit of enzyme is defined as the amount that formed one micromole of ribose under the standard assay conditions. Specific activities are expressed as units/ml/mg protein.

Identification of ribose

Ribose was identified using the ascending paper chromatographic (Whatman No 1) technique according to the method of Smith and Seakins (1976). Two solvent systems were used. Solvent 1 consists of n-propanol/ethyl acetate/water (70:10:20) and solvent 2 of isopropanol/water (160:40). The developed brown spots of the identified and authentic ribose were located by using aniline oxalate reagent.

Identification of the purine ribonucleosides and their bases

Chromatographic identification of the purine ribonucleosides and their bases was made, using chromatographic Whatman No 3 MM filter paper and two solvent systems. Solvent 1 consists of n-butanol/glacial acetic acid/water (120:30:50) (Smith and Seakins, 1976) and solvent 2 of 5% K2HPO4/isoamyl alcohol (2:1) (Thomson, 1960). The spots were located with an ultraviolet lamp.

RESULTS AND DISCUSSION

Comparative rates of ribose formation from purine ribonucleosides by extracts of *P. viridicatum*. Figure 1 shows the comparative rates of ribose formation from adenosine, guanosine and inosine as a function of purine ribonucleoside hydrolase. The rates of cleavage occurred in the order inosine > guanosine > adenosine. It was also observed that, after 1 h, the rate of adenosine cleavage was about 12.5% that of inosine and 16% that of guanosine. As a result of the low rate of adenosine cleavage compared to that of inosine and the cessation of further breakdown of adenine, it might be assumed that conservation of adenine compounds is essential for the cell, because of the extremely important role they play in the metabolism (Abdel-Fattah et al., 2009).

Previous reports dealing with the mode of degradation of the same purine ribonucleosides, indicated the presence of a non particulate purine ribonucleoside hydrolase in *Aspergillus niger* (Hassan et al., 1979) and

Table 1. Ratios of ribose formation with cell-free extracts and cell homogenates of *Penicillium viridicatum.*

Source of enzyme	Ribose (µmoles) formed from			Ratio of activity (%)	
	Adenosine	Guanosine	Inosine	Aden / Guan	Aden / Inos
Cell-free extracts	0.4	3.9	4.8	10.2	8.3
Cell homogenate	0.1	2.0	3.0	5.0	3.3

Reaction mixture contained: Adenosine, guanosine and inosine 5 µmoles; citrate-phosphate buffer (pH 4), 100 µmoles; extract protein, 2.28 mg for cell-free extracts and 0.76 mg of cell homogenate; total volume, 1 ml; temperature, 50°C and reaction time, 30 min.

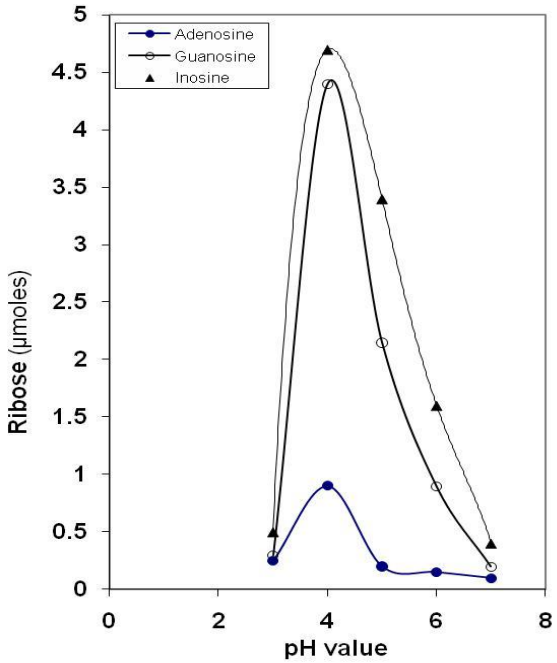

Figure 2. Purine ribonucleoside hydrolase activity as a function of pH value of extracts of *Penicillium viridicatum.* Reaction mixture contained: Adenosine, guanosine and inosine 5 µmoles; citrate-phosphate buffer (pH 4), 100 µmoles; extract protein, 3.16 mg; total volume, 1 ml; temperature, 40°C and reaction time, 30 min.

Penicillium citrinum (Elzainy et al., 1990), that catalyzes the cleavage of the purine ribonucleosides, in the order of magnitude inosine > guanosine > adenosine in agreement with our results.

Inability to detect a membrane associated adenosine hydrolyzing enzyme in *P. viridicatum*

It has been observed from the previously mentioned experiment that, the rate of hydrolysis of adenosine by the cell-free extracts of *P. viridicatum* is much less than the rates of hydrolysis of guanosine and inosine. Table 1 shows that, the ratios of activity, adenosine / guanosine, adenosine / inosine of the cell homogenate, are less than the corresponding ratio when cell-free extracts were used, which indicates that adenosine hydrolyzing enzyme is not associated with the cell membrane. In this respect *P. viridicatum* resembles *A. niger* and *Fusarium moniliforme* (Elzainy et al., 1984, 1978) and differ from *Penicillium chrysogenum* in which the adenosine hydrolyzing enzyme is suggested to be associated with the cell membrane (Elzainy et al., 1973) in contrast to our results.

Chromatographic identification of the products

Ribose was chromatographically identified in reaction mixtures containing adenosine, guanosine or inosine adjusted to pH 4.0. The developed brown spots of the identified and authentic ribose had the same Rf values of 0.89 and 0.65 in solvent 1 and 2 respectively (methods). Adenine was identified from adenosine as a product of hydrolytic activity of the extracts of *P. viridicatum*. The Rf value was 0.6 in solvent 1 and 0.26 in solvent 2 (methods). Hypoxanthine was also identified as a product of the hydrolytic activity of the same extracts on inosine with an Rf value of 0.47 in solvent 1 and 0.32 in solvent 2.

Guanine was also identified as a product from guanosine and the Rf value was found to be 0.43 and 0.21 in solvent 1 and 2 respectively. Further confirmation for such identification was obtained by determining the absorption spectrum of adenine, guanine and hypoxanthine. The spots were eluted by 1.0 N HCl (Carter, 1950) and their adsorption spectra were determined. All the previously mentioned identified products were found to be identical to their corresponding authentic samples under the same experimental conditions.

pH activity relationships of the hydrolytic cleavage of purine ribonucleosides

Figure 2 shows that the hydrolytic cleavage of the three nucleosides namely adenosine, guanosine and inosine occurs with the same profile optimally at pH4. From these results, it is suggested that the cleavage of the three nucleosides may be affected by one nucleoside

Table 2. Activities of purine ribonucleoside hydrolase in different buffer systems.

Type of buffer (pH 4.0)	Ribose (μmoles) formed from		
	Adenosine	Guanosine	Inosine
Citrate	0.2	2.2	3.5
Citrate-phosphate	0.3	2.6	4.4
Acetate	0.2	2.3	3.9
Succinate	0.1	1.1	2.6

Reaction mixture contained: Adenosine, guanosine and inosine 5 μmoles; buffers pH 4, as indicated 100 μmoles; extract protein, 2.28 mg; total volume, 1 ml; temperature, 50°C and reaction time, 30 min.

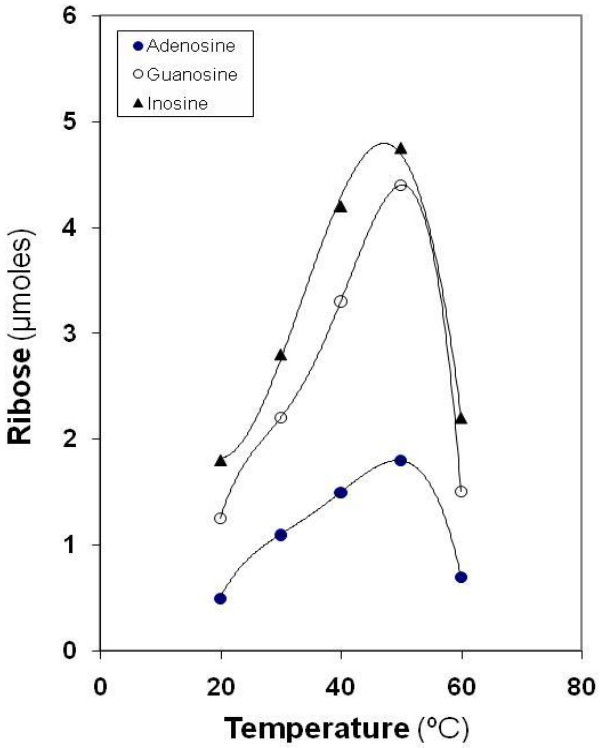

Figure 3. Temperature dependence of the hydrolytic activity with purine ribonucleosides. Reaction mixture contained: Adenosine, guanosine and inosine 5 μmoles; citrate-phosphate buffer (pH4), 100 μmoles; extract protein, 2.28 mg; total volume, 1 ml; temperature, as indicated and reaction time, 30 min.

hydrolase in accordance with the results reported by Elzainy et al. (1990) and Abdel-Fatah et al. (2003) using extracts of the two filamentous fungi namely *P. citrinum* and *Aspergillus phoenicis* respectively. Reese (1968) reported that the optimum pH for the hydrolysis of purine nucleosides was around pH 4 in some Penicillium and Aspergillus species. Abdel-Fatah et al. (2009) determined the optimum pH for the hydrolysis of these compounds by the extracts of *A. terreus* and found it to be 3.5.

Enzymatic degradation of purine ribonucleosides in different buffer systems by extracts of P. viridicatum

An experiment was made to test the ability of cell-free extracts of nitrate-grown mycelia of *P. viridicatum* to catalyze the degradation of purine ribonucleosides in different buffer systems. Four different buffer systems were used namely, citric acid-sodium citrate, citric acid-sodium mono-hydrogen phosphate, acetic acid-sodium acetate and succinic acid-sodium hydroxide. The pH value of all buffers was adjusted at pH 4.0. All the reaction mixtures were then incubated at 50°C for 30 min, after which determination of ribose was carried out. Results of these determinations which are demonstrated in Table 2 indicate that, extracts of *P. viridicatum* could catalyze the cleavage of N-glycosidic bonds of purine ribonucleosides, also the results demonstrated that almost more or less similar activities were obtained in the four buffer systems; however, a slight increase was observed with the three substrates when citrate-phosphate buffer was used as the buffering system. This pattern indicates that one enzyme is involved in the hydrolysis of the three substrates.

Temperature dependence of the hydrolytic activity with purine ribonucleosides

Figure 3 illustrates that 50°C can be considered as the degree of temperature of which optimum hydrolytic activities occur. The figure also shows the similarity of the three temperature activity profiles, obtained with the three substrates. The optimum temperature reported for the activity of this hydrolase of purine ribonucleosides seem to be similar to those obtained for analogous activities of *Streptomyces viridiviolaceus* (El-Awamry and Elzainy, 1985). However, the purine ribonucleoside hydrolase of *P. citrinum* acted optimally at 40°C (Allam et al., 1991).

Heat inactivation kinetics of enzyme activity

Results presented in Table 3, as percentage of remaining activities, indicate that incubation of the extracts which was previously incubated at 50°C for 10 min (in absence of substrate) with guanosine and inosine, resulted in a complete loss of activity. The same effect was observed after 8 min when adenosine was used as a substrate under the same experimental conditions. The results also indicate that incubation of the pretreated extract incubated previously at 60°C for 4 min, in the absence of substrate with each of the three ribonucleosides, resulted in a complete loss of activity. These results can be interpreted by the fact that adenosine, guanosine or inosine plays a major role in the protection of catalytic site(s) of the enzyme at high temperature.

Table 3. Heat inactivation kinetics of the hydrolytic activity on purine ribonucleosides.

Time of incubation (min)	Remaining activity (%)					
	Adenosine		Guanosine		Inosine	
	50°C	60°C	50°C	60°C	50°C	60°C
0	100	100	100	100	100	100
4	38.09	0.0	58.92	0.0	68.18	0.0
6	4.76	0.0	35.71	0.0	45.45	0.0
8	0.0	0.0	21.42	0.0	28.78	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0

Reaction mixture contained: Adenosine, guanosine and inosine 5 μmoles; citrate buffer (pH 4), 100 μmoles; extract protein, (2.4 mg for reaction mixture incubated at 60°C and 3.12 mg for reaction mixture incubated at 50°C); total volume, 1 ml; temperature, 50°C and reaction time, 30 min.

Table 4. Effect of frequent freezing and thawing on purine ribonucleoside hydrolase activity.

Incubation at -5°C (h)	Remaining activity (%)		
	Adenosine	Guanosine	Inosine
0	100	100	100
24	100	100	100
48	33.3	88.6	95.7
72	22.2	86.3	93.6
120	11.1	75.0	82.9

Reaction mixture contained: Adenosine, guanosine and inosine 5 μmoles; citrate-phosphate buffer (pH 4), 100 μmoles; extract protein, 3.16 mg; total volume, 1 ml; temperature, 40°C and reaction time, 30 min.

Effect of frequent freezing and thawing on purine ribonucleoside hydrolase activity

The enzyme preparation was assayed for activity in the usual manner, stored in the refrigerator at -5°C for 24 h after which it was thawed and an aliquot was withdrawn for assay of activity under the same experimental conditions. This procedure was made after 24, 48, 72 and 120 h of incubation at -5°C. Results of analysis of the products are presented in Table 4 as mole ribose, from which it appears that by the fifth day about 88.9, 25 and 17% of hydrolytic activity was lost when adenosine, guanosine and inosine were used separately in the reaction mixture respectively. These results together with those obtained from studies on the effect of the nature of the buffer and subjecting the extracts to 50 and 60°C, indicate that hydrolytic cleavage of the three purine ribonucleosides might be affected by one hydrolase.

Effect of dialysis and some metal salts on purine ribonucleosides degradation

Results obtained in the preceding experiments indicate that, the enzymatic activity(s) that catalyzes the formation of ribose and the corresponding base from each of adenosine, guanosine or inosine is due to a hydrolase, not to a phosphorylase activity. This is based on the fact that, ribose is the pentose formed from the three purine ribonucleosides without the addition of arsenate to the reaction mixture.

However, one may attribute ribose formation to be a result of the combined activities of nucleoside hydrolase and ribose-phosphate phosphatase, in cases where inorganic phosphate is present, as an endogenous substance, in the cell free extracts. In order to clarify this point, dialyzed cell extracts that proved to be phosphate free were tested for their ability to catalyze ribose formation from the three purine ribonucleosides. The obtained activity was compared with the analogous activity of the nondialyzed extracts. The results obtained show that, the specific activities with the three substrates did not decrease due to dialyzing the extracts indicating that, all the amount of ribose formed from each nucleoside resulted by the action of hydrolase activity only (data not shown), in agreement with the results obtained in the case of *P. citrinum* (Elzainy et al., 1990) and A. terreus (Abdel-Fatah et al., 2009). In addition, the results indicate that the purine ribonucleoside hydrolase does not seem to require a dialyzable factor for its activity. Further confirmations of these results were obtained by studying the effect of addition of inorganic metal ions to the reaction mixture. Data presented in Table 5 shows that, all metal salts used caused inhibition of the purine ribonucleoside hydrolase activity. $MnCl_2$ caused a complete inhibition of enzyme activity. The previously mentioned results indicate that, all metal ions under study had a remarkable inhibitory effect on purine ribonucleoside hydrolase activity.

Mode of guanosine degradation by extracts of *P. viridicatum*

Two possibilities were suggested for the hydrolytic and

Table 5. Effect of addition of some metal salts to dialyzed extracts on purine ribonucleosides degradation.

Metal salt 10^{-2}M	Relative activity (%)		
	Adenosine	Guanosine	Inosine
--	100	100	100
$CoSO_4$	33.3	25.8	24.2
$CuSO_4$	0.0	9.67	10.6
$ZnCl_2$	41.6	45.2	57.5
NaCN	41.6	38.7	60.6
$MgSO_4$	75.0	74.2	62.1
FeO_4	66.6	54.8	60.6
$MnCl_2$	0.0	0.0	0.0

Reaction mixture contained: Adenosine, guanosine and inosine 5 μmoles; citrate buffer (pH 4), 100 μmoles; metal salts (10^{-2} M), as indicated; extract protein, 1.92 mg; temperature, 50°C and reaction time, 30 min.

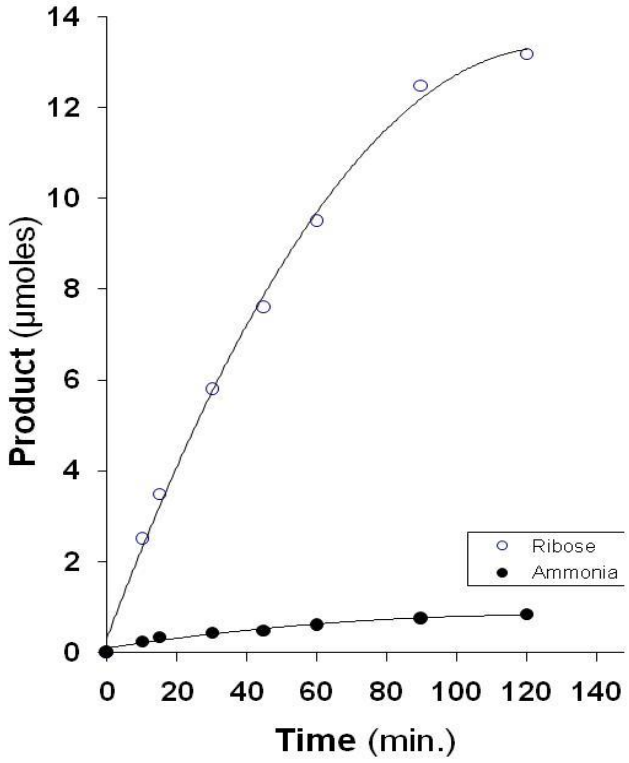

Figure 4. Mode of guanosine degradation by extracts of *Penicillium viridicatum.* Reaction mixture contained: Guanosine 15 μmoles; citrate-phosphate buffer (pH5), 300 μmoles; extract protein, 0.96 mg; total volume, 3 ml; temperature, 40 C and reaction time, as indicated.

deaminating activities that play a role in this degradation; as shown in Figure 5 (pathways 1 and 2).

Figure 4 demonstrates that ribose and ammonia were formed in the reaction mixture that contained fresh enzyme and guanosine up to 2 h, and the amounts of ribose were higher than the corresponding amounts of ammonia under the same experimental conditions. The possibility of xanthosine formation as an intermediate was excluded, by finding out that xanthosine was not split into ribose and xanthine by the same extracts, under the same conditions of the guanosine reaction and even at different pH values of the reaction; thus, it seems that

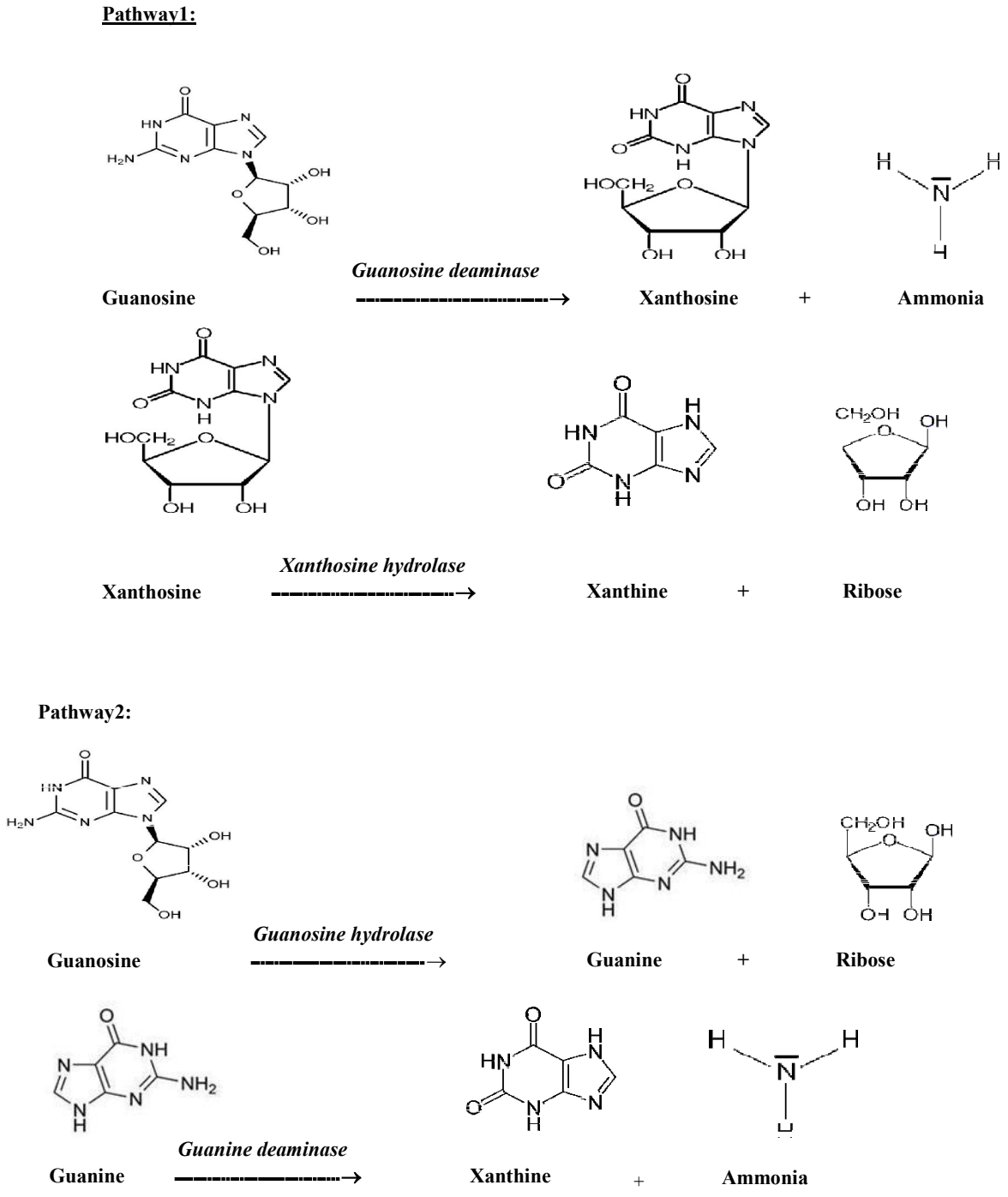

Figure 5. Pathways showing hydrolytic and deaminating activities.

guanosine is catabolized to give guanine and ribose. The resulting guanine was then deaminated to give xanthine by guanine deaminase.

Also, application of chromatographic procedure on the reaction mixture proved that, guanine and small quantity of xanthine were the products of the enzyme action. According to the mentioned results, it can be concluded that guanosine cleavage in *P. viridicatum* was operated through pathway 2 (Figure 5). Similarly, Elzainy and Allam (1973) proved that extracts of *P. chrysogenum* catalyzed the conversion of guanosine into xanthine and ribose by the combined activities of nucleoside hydrolase and guanine deaminase. Alternatively, studies with *A. niger* (Elzainy et al., 1978) and *P. citrinum* (Elzainy et al., 1990) revealed absence of a deaminating activity with guanine.

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