Studies on the Decomposition of Nucleic Acid by Microorganisms

Part VI. On the Ribosidase of *Aspergillus oryzae* Acting on 6-Hydroxypurine Ribonucleosides and their 5'-Monophosphates*

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An enzyme preparation has been purified about forty fold from a strain of *Aspergillus oryzae*. This preparation catalyzes the hydrolytic cleavage of inosine, guanosine, 5'-IMP, and 5'-GMP to the corresponding bases (hypoxanthine and guanine) and ribose or its 5-phosphate. No change is observed in the relative rates of hydrolysis of these four substrates with purification, indicating that a single enzyme "ribosidase", which cleaves specifically the ribosidic linkages of 6-hydroxypurine ribonucleosides (inosine and guanosine) or their 5'-phosphates, does exist in *Aspergillus oryzae*. It has been found that 5'-AMP, 5'-CMP, 5'-UMP, adenosine, cytidine, uridine, and various 3'- or 2'-mononucleotides are not cleaved by the enzyme. No evidence for the requirement of ATP, P, or PP has been found. This enzyme is rather thermostable in the pH range from 5.0 to 6.5. The optimum conditions for activity are at about 45°C and pH 4.0.

According to the prevailing theory¹⁾, the phosphoric acid group in the nucleotides has to be removed before the ribosidic linkage can be resolved by enzymes. Ishikawa and Komita²⁾ discovered that dog pancreas was capable of disrupting the ribosidic linkages of guanylic and xanthylic acids without previous cleavage of the phosphate. Moreover, Komita^{3,4)} has separated this enzyme from purine nucleosidase, and named it nucleotide-N-ribosidase. However, Schlenk⁵⁾ indicated another possible interpretation of these experiments in the light of more recent observations. Thus, proof of the existence of nucleotide-N-ribosidase was lacking.

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The following abbreviations are used here: for monophosphates of adenosine, inosine, guanosine, uridine, and cytidine,—AMP, IMP, GMP, UMP, and CMP; yeast ribonucleic acid,—RNA; adenosine-5'-triphosphate,—ATP; inorganic orthophosphate,—P; inorganic pyrophosphate,—PP; ribose-5-phosphate,—R5P.

1) P. A. Levene and A. Dmochowski, *J. Biol. Chem.*, **93**, 563 (1931).

2) H. Ishikawa and Y. Komita, *J. Biochem.*, **23**, 351 (1936).

3) Y. Komita, *J. Biochem.*, **25**, 405 (1937).

4) Y. Komita, *J. Biochem.*, **27**, 23 (1938).

5) F. Schlenk, *The Nucleic Acids* (ed. E. Chargaff, J. N. Davidson), Academic Press Inc., New York, 1955, Vol. 2, p. 329.

Recently, the author^{6,7,8)} has furnished the first evidence for the conclusion that hydrolytic enzyme for the cleavage of the bond between hypoxanthine and R5P does exist in *Aspergillus oryzae*. A little later, Hurwitz et al.⁹⁾ have also independently demonstrated the enzymic cleavage of ribosidic linkage in 5'-AMP using *Azotobacter vinelandii*. In the present paper, it is indicated that the ribosidase of *Aspergillus oryzae* acts not only on 5'-IMP but also on inosine, 5'-GMP, and guanosine.

METHODS

The various nucleosides, nucleoside-3'-phosphates, 5'-AMP and ATP used in this study were commercial preparations. Preparations of the nucleoside-3'-phosphates contained the corresponding 2'-isomers together, and were used without the removal of them. 5'-IMP was prepared by the method of Marmur et al.¹⁰⁾. The

6) A. Kuninaka, *J. Agr. Chem. Soc. Japan*, **30**, 583 (1956).

7) A. Kuninaka, *Koso Kakagu Shinpojiumu*, **12**, 65 (1957).

8) A. Kuninaka, *J. Gen. Appl. Microbiol.*, **3**, 55 (1957).

9) J. Hurwitz, L. A. Heppel and B. L. Horecker, *J. Biol. Chem.*, **226**, 525 (1957).

10) J. Marmur, F. Schlenk and R. N. Overland, *Arch. Biochem. Biophys.*, **34**, 209 (1951).

other nucleoside-5'-phosphates were prepared enzymatically from RNA. The detailed procedure will be published separately.

Inorganic and organic phosphates were determined according to the Fiske-Subbarow method¹¹⁾. The colorimetric method of Nelson¹²⁾ to observe the appearance of free ribose or ribose phosphate was employed, except that the treatments with barium hydroxide and zinc sulfate were omitted. Protein was determined by the method of Lowry et al.¹³⁾.

As the main source of ribosidase, culture filtrates of *Aspergillus oryzae* var. No. 13, a strain employed in soy-manufacture¹⁴⁾, were used. In usual cases the strain was grown at 30°C for ten days on surface of a medium having the following composition: glucose, 5%; polypeptone, 0.5%; monobasic potassium phosphate, 0.05%; dibasic potassium phosphate, 0.05%; calcium chloride, 0.04%; magnesium sulfate, 0.04%. Details concerning the procedure for each enzyme preparation will be described under the paragraph "RESULTS". Zone electrophoresis was carried out using potato starch as the supporting material. Calcium phosphate gel was prepared by the method of Keilin and Hartree¹⁵⁾.

The assay procedure of the enzyme (ribosidase) was based on the appearance of reducing sugar*. The following assay mixture was used: 0.1 ml of 25 mM substrate, 0.1 ml of 1 M sodium fluoride, 0.5 ml of 0.1 M acetate buffer, pH 4.0, 0.25 to 2.0 units of enzyme, and water to give a volume of 1.0 ml. After thirty minutes at 45°C, reducing sugar (ribose or R5P) was measured. A unit of enzyme activity was defined as equal to the amount required for the formation of 1 μ mol. of reducing sugar or purine base per hour at 45°C under the above conditions. In this assay, controls lacking enzyme or substrate were run. All enzyme reactions were performed under an atmosphere of air. The above conditions (composition of reaction mixture, pH, temperature, and time) were employed throughout all enzymic reactions described in this paper, except when otherwise indicated. Under these conditions, the

reaction rate was found to be linear with time and directly proportional to enzyme concentration. In case of using amounts of the enzyme larger than 2.0 units, proportionality between the rate and the enzyme concentration was preserved in a somewhat wider range for inosine than for 5'-IMP.

RESULTS

I. The Relation between 5'-IMP-N-Ribosidase Activity and Inosine Ribosidase Activity. In the preceding papers^{6,7,8,16)} it was shown that *Aspergillus oryzae* contained ribosidase (purine ribonucleoside hydrolase) acting on inosine and guanosine, and 5'-IMP-N-ribosidase acting on the ribosidic linkage of 5'-IMP. To begin with, the relation between 5'-IMP-N-ribosidase activity and inosine ribosidase activity was investigated by the following experiments:

(1) **Influence of culture conditions** 5'-IMP-N-ribosidase and inosine ribosidase activities in culture filtrates were measured during growth. Both activities seemed to appear simultaneously in the medium at approximately the same ratio* (Fig. 1).

(2) **Solubility in acetone** The fraction soluble in 20% and insoluble in 40% acetone at pH 6.5 contained enzymes having the majority of both original activities. (In this experiment, the fraction precipitated between 0.6 and 1.0 saturated ammonium sulfate was used as the original enzyme solution.) Treatments with methanol, ethanol, and ammonium sulfate at various pH values also, could not separate both activities.

(3) **Treatments with adsorbents** Both activities were adsorbed onto alumina, obtained from Wako Junyaku Co., 200 mesh, most effectively at about pH 5.0, and eluted together almost perfectly by 0.1 M dibasic sodium phosphate solution. For the elution of both activities, 1 M dibasic sodium phosphate solution, which was

11) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

12) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).

13) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

14) M. Kibi, *J. Agr. Chem. Soc. Japan*, **24**, 278 (1951).

15) D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London)*, **B 124**, 397 (1938). Cited by S. P. Colowick "Methods in Enzymology" Academic Press Inc., New York, 1955, Vol. 1, p. 98.

* It was preliminarily confirmed that reducing sugar and purine base are formed simultaneously. When necessary, the purine base formed was determined spectrophotometrically after separation on paper by means of either chromatography or electrophoresis^{6,7,8,16)}.

16) A. Kuninaka, *J. Agr. Chem. Soc. Japan*, **29**, 801 (1955).

* In the early crude preparation, such as culture filtrates, the ratio of inosine ribosidase to 5'-IMP-N-ribosidase usually ranged from 1.4 to 2.5. However in a simple purification procedure, such as dialysis or treatment with alumina, this ratio was readily reduced to about 1.0. In the course of further purification, no change in the ratio was observed. The presence of the small molecular factor(s) affecting the enzyme reactions in culture filtrates may be suggested. This point is under investigation.

FIG. 1. Activity of Ribosidase in Culture Filtrates of *Aspergillus oryzae* at Various Periods.

Aspergillus oryzae var. No. 13 was grown in 100 ml of the glucose-peptone medium at 30°C for the periods indicated in the Figure. (The ratio of inosine ribosidase activity to 5'-IMP-N-ribosidase activity in each culture filtrate employed in this experiment seemed to be relatively high.)

Curve A: 5'-IMP-N-ribosidase activity (activity of R5P formation from 5'-IMP) in shaking culture filtrates.

Curve A': 5'-IMP-N-ribosidase activity in surface culture filtrates.

Curve B: Inosine ribosidase activity (activity of ribose formation from inosine) in shaking culture filtrates.

Curve B': Inosine ribosidase activity in surface culture filtrates.

much more effective than 0.1 M solution for elution of most of the other proteins adsorbed from culture filtrates, proved to be much less effective than 0.1 M solution. A 1 M solution of sodium bicarbonate and a 0.3 to 0.6 saturated solution of ammonium sulfate were also found effective for elution of both activities, while 0.1 M solution of sodium pyrophosphate or sodium perborate was much less effective. Both activities adsorbed onto calcium phosphate gel were eluted together with essentially the same recovery by respective eluants as described in Table I. The effect of various eluants on elution of both activities was rather different from the effect on elution of the total protein

TABLE I. ELUTION OF RIBOSIDASE FROM CALCIUM PHOSPHATE GEL

Eluants	Total protein	Recovery (%) in eluates	
		Activity against	
		5'-IMP	Inosine
1M Na_2HPO_4	2.1	14.6	12.0
0.1M Na_2HPO_4	1.6	3.9	3.4
80% glycerol	1.1	2.4	2.8
0.3 saturated $(\text{NH}_4)_2\text{SO}_4$	0.5	17.0	14.5
1M Na_2HCO_3	1.3	0	0
0.05M Borate buffer, pH 10	0.5	0	0

20 ml of partially purified enzyme solution, the fraction precipitated between 0.6 and 1.0 saturated ammonium sulfate containing 47.2 mg of total protein and 830 units of 5'-IMP-N-ribosidase or 886 units of inosine ribosidase, were treated with 12.5 g of calcium phosphate gel at pH 5.4. The both activities were adsorbed perfectly on to the gel. Elution was carried out by 4 ml of each eluant indicated in the Table from each 2 g of the gel. Recovery of total protein and ribosidase was determined.

adsorbed from culture filtrates. This was also true for adsorption onto corn starch under various conditions. For example, 40% ammonium sulfate solution seemed to prevent specific adsorption of both activities onto corn starch. Both activities were also not separated by use of a cation exchanger, Amberlite IRC-50, under various conditions.

(4) **Action of various drugs** To each 5 ml of the enzyme solution, concentrated culture filtrates containing 27.4 units of 5'-IMP-N-ribosidase per ml, 2 g of lead acetate, 2 g of urea, 0.1 g of iodine, 1 ml of water-saturated phenol and 0.1 g of benzalkonium chloride were added. After being allowed to stand at 45°C for two hours, each supernatant solution was dialyzed, and then 5'-IMP-N-ribosidase and inosine ribosidase activities were measured. Both activities were not recognized in each solution. This apparently indicates that these drugs perfectly inactivate both 5'-IMP-N-ribosidase and inosine ribosidase. On the other hand, inactivation by 0.1 g of mercuric chloride was comparatively mild under the same conditions. Thus, both enzyme activities per protein were able to be raised up to three fold by treatment with mercuric chloride, although about 70% of the total activities was lost. The splitting rates of 5'-IMP and inosine were not influenced signi-

ificantly by the addition of 0.01 M (final concentration) of potassium cyanide, monoiodoacetic acid, or oxalic acid to the reaction mixture.

(5) **Influence of temperature and pH** Both 5'-IMP-N-ribosidase and inosine ribosidase were rather thermostable from pH 5.5 to pH 6.0 (Fig. 2). After heating the enzyme solution

FIG. 2. Effect of pH on Stability of Ribosidase at 60°C.

The enzyme solution corresponding to "ethanol fraction" (described in "RESULTS II") was employed.

Curve A: 5'-IMP-N-ribosidase activity.

Curve B: Inosine ribosidase activity.

("first zone fraction" described in "RESULTS II") for 10 min., 20 min., 30 min., 40 min., and 60 min. at 55°C, pH 5.5, 80%, 70%, 58%, 50%, and 32% of both activities were retained respectively. Furthermore, no difference was observed between stability of 5'-IMP-N-ribosidase against various temperatures and that of inosine ribosidase (Fig. 3). The optimum conditions for the activities of 5'-IMP-N-ribosidase and inosine ribosidase were found to be at about 45°C and pH 4.0. The results are given in Figs. 4 & 5.

(6) Action of ribosidase on mixed substrates

The results described above suggest that two

FIG. 3. Effect of Temperature on Stability of Ribosidase at pH 5.5.

"Acetone fraction" diluted 10-fold (described in "RESULTS II") was used as the enzyme solution.

Curve A: 5'-IMP-N-ribosidase activity.

Curve B: Inosine ribosidase activity.

enzyme activities are associated with the same protein. The same results were also obtained when the enzyme acted on mixtures of the two substrates. The data given in Table II may be assumed to indicate that both 5'-IMP and inosine are attacked by the same enzyme.

(7) **Zone electrophoresis** Additional evidence for the association of the two activities results from their behaviour on zone electrophoresis. When electrophoresis was carried out using starch as a supporting material in a trench of $46 \times 2.5 \times 1.0$ cm, both activities migrated equally towards the cathode or the anode at various pH values between 4.5 and 9.0. Fig. 6 shows typical results obtained at pH 4.54 and pH 8.62. No separation of the two enzyme activities was observed, nor was either activity resolved into the two fractions.

This series of experiments may be considered to indicate that a single enzyme ribosidase catalyzes the hydrolytic cleavage of the ribosidic linkages in both 5'-IMP and inosine. From

FIG. 4. Activity of Ribosidase at Various Temperatures.

Culture filtrates were employed after mild dialysis as the enzyme solution.

Curve A: 5'-IMP-N-ribosidase activity.

Curve B: Inosine ribosidase activity.

FIG. 5. Activity of Ribosidase at Various pH Values.

The enzyme solution corresponding to "ethanol fraction" was used.

Curve A: 5'-IMP-N-ribosidase activity.

Curve B: Inosine ribosidase activity.

TABLE II. ACTION OF *ASPERGILLUS* RIBOSIDASE ON MIXED SUBSTRATES

Time (min.)	Reducing sugar liberated (μ mol./ml)				
	From 5'-IMP		From inosine		From 5'-IMP (2.5 μ mol./ml) and inosine (2.5 μ mol./ml)
	2.5 μ mol. /ml	5.0 μ mol. /ml	2.5 μ mol. /ml	5.0 μ mol. /ml	
15	0.380	0.470	0.320	0.330	0.330
30	0.655	0.830	0.630	0.705	0.710
45	0.800	1.060	0.840	1.020	1.000
60	0.950	1.350	1.100	1.335	1.250

The enzyme solution corresponding to "acetone fraction" diluted 10-fold was used.

this point, the action of several molds on the ribosidic linkage of 5'-IMP or inosine was studied. Culture filtrates of *Aspergillus oryzae* A*, *Aspergillus oryzae* S-4-15*, *Aspergillus niger* a*, and *Penicillium multicolor* G.M. and P*, as well as *Aspergillus oryzae* var. No. 13 were observed to be capable of splitting the ribosidic linkages of both 5'-IMP and inosine at pH 5.0

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and 45°C. As a contrast, those of *Rhizopus nigricans* NRRL No. 45*, *Rhizopus delmer* var. minus R-1-4*, *Mucor spinescens* Mu-3*, *Monascus purpureus* 3-1*, *Penicillium citrinum* Thom 1131*, *Penicillium spinulosum* Thom*, *Penicillium expansum* 1327*, and *Aspergillus fumigatus* 26-12* were observed to be incapable of appreciably splitting the ribosidic linkages of both 5'-IMP and inosine at least under the present conditions. It thus seems probable that

FIG. 6a. Zone Electrophoresis in 0.05 M Acetate Buffer, pH 4.54, with 400 V and 10 to 11.8 mamp for 14.5 hr.

One ml of the partially purified enzyme solution corresponding to "ethanol fraction" containing about 70 to 100 units was put on the band of 1 cm wide of the starch column. After running each band of 1 cm wide was eluted with 5 ml of distilled water. The supernatant fluid was used for measurement of activity.

Curve A: 5'-IMP-N-ribosidase activity.

Curve B: Inosine ribosidase activity.

FIG. 6b. Zone Electrophoresis in 0.1 M Veronal-Hydrochloric Acid Buffer, pH 8.62, with 200 V and 10 to 11 mamp for 15.0 hr.

the enzyme "ribosidase" acting on both 5'-IMP and inosine is distributed among various fungi, especially, among the strains which belong to the *Aspergillus* genus.

The K_m values for 5'-IMP and inosine calculated were found to be approximately 5.46×10^{-3} M and 2.63×10^{-3} M, respectively (Fig. 7). Thus, the affinity of ribosidase with inosine may be closer than that with 5'-IMP.

In contrast with AMP-ribosidase of *Azotobacter vinelandii*⁹⁾, it is apparent from the following experiment that ribosidase of *Aspergillus oryzae* is not activated by ATP. The partially purified enzyme preparation, corresponding to the "ethanol fraction", was incubated with 2.5 μ mol. of 5'-IMP or inosine in the presence of 2 μ mol. of ATP at 45°C and pH 4.0 for 30 minutes (total volume, 1.0 ml). In the absence of ATP, 0.54 μ mol. of reducing sugar was formed from 5'-IMP and inosine, respectively. In the presence of ATP, 0.49 and

FIG. 7. Determination of the K_m Values.

The K_m values were determined for 5'-IMP and inosine at 45°C and pH 4.0, using "acetone fraction" diluted 10-fold as the enzyme solution.

K_m (5'-IMP) = $M/183 = 5.46 \times 10^{-3}$ M (Curve A)

K_m (inosine) = $M/380 = 2.63 \times 10^{-3}$ M (Curve B)

0.51 μ mol. of reducing sugar were formed from 5'-IMP and inosine, respectively.

II. Purification and Substrate Specificity of *Aspergillus oryzae* Ribosidase. As described above, *Aspergillus oryzae* seems to contain an enzyme "ribosidase" having both 5'-IMP-N-ribosidase and inosine ribosidase activities. The enzyme was then purified as follows: 4940 ml of the culture filtrate of *Aspergillus oryzae* var. No. 13 was concentrated in vacuo, and dialyzed overnight against running water. To the undialyzable fraction (180 ml) four volumes of ethanol were added. The resulting precipitate was separated from the supernatant by centrifugation, and dried up in a desiccator (ethanol fraction, 1.5 g). The "ethanol fraction" was dissolved in 21 ml of distilled water and subjected to zone electrophoresis in a trench of 45 $\times$ 2 $\times$ 5 cm using 0.1 M veronal-hydrochloric acid buffer at pH 9.02 with 150 V and about 23 mamp., for 24 hours. After running, the band containing ribosidase activity (15 cm-wide) was eluted with 407 ml of distilled water (first zone fraction). This fraction was further subjected to second zone electrophoresis in 0.1 M veronal-hydrochloric acid buffer at pH 8.85 with 200 V and 40 mamp. for 20 hours. After running, the band with high ribosidase activity (3 cm-wide) was eluted with 272 ml of distilled water (second zone fraction). This "second zone fraction" was concentrated to 46 ml in

vacuo. The precipitate was removed. To the supernatant adjusted to pH 5.5 with acetic acid, a two-third-volume of acetone was added. The precipitate formed was dissolved in distilled water and dialyzed against running water. Fifteen ml of a colorless and clear solution was obtained (acetone-fraction). The above procedure resulted in a purification of approximately forty fold. A summary of the purification procedure is given in Table III. The final preparation was free of phosphomonoesterase activity. As described in Table III, the final preparation was recognized to split 5'-GMP and guanosine, as well as 5'-IMP and inosine. Furthermore, the ratio of the activities of "ethanol fraction" against 5'-IMP, inosine, 5'-GMP, and guanosine was not appreciably altered by further purification. The reducing sugars formed from 5'-GMP and guanosine were identified by the method described previously^{6,7,8)} as R5P and ribose, respectively. Both P and PP were recognized to be involved in the enzymic splitting of 5'-GMP and guanosine, as well as in the splitting of 5'-IMP and inosine⁸⁾. It may be concluded that these reactions are not (pyro)phosphorolysis but hydrolysis. Evidence for requirement of ATP was also not recognized in the splitting of 5'-GMP and guanosine. The ribosidic linkages in adenosine, uridine, cytidine, 3'- and 2'-isomers of AMP, GMP, IMP, XMP, CMP,

TABLE III. SUMMARY OF PURIFICATION PROCEDURE

Fraction	Total Protein mg	Specific activity† units per mg protein	Relative activity for 4 substrates			
			5'-IMP	Inosine	5'-GMP	Guanosine
Culture filtrate	1580.8	11.9	100	142		
Ethanol fraction	565.0	16.3	100	107	12	32
First zone fraction	151.4	50.8	100	106	13	30
Second zone fraction	90.3	70.5	100	98	15	27
Acetone fraction	5.7	447.4	100	96	12	27

† Specific activity was determined using 5'-IMP as substrate.

and UMP, as well as 5'-isomers of AMP, CMP, and UMP, were not recognized to be splitted by *Aspergillus oryzae* ribosidase. It is as yet undecided whether ribosidase splits 5'-XMP or xanthosine, although preliminary experiments suggest that both compounds are split by the enzyme at rather small rates.

DISCUSSION

On the basis of the results described in this paper, *Aspergillus oryzae* var. No. 13 has been shown to contain a ribosidase acting on the ribosidic linkages of the compounds with the structure shown in Fig. 8. Conditions necessary

FIG. 8. The General Structure of the Substrates of *Aspergillus oryzae* Ribosidase.

for the structure of the substrate may be summarized as follows:

(i) The base moiety is to be purine nucleus containing a hydroxy group in the 6 position. (An amino group in the 6 position seems to completely interfere with the enzymic cleavage of ribosidic linkage. Pyrimidine nucleosides or their phosphates are not attacked by the enzyme.)

(ii) Both the 2' and 3' positions of ribose moiety are not to be esterified with P.

Although the presence of an amino group in the position 2 in purine moiety seems to some-

what interfere with the cleavage of ribosidic linkage, the influence of the kind of the group in this position on ribosidase action is not recognized to be vital. It is interesting to note that the 5'-phosphate group does not appear to have a remarkable effect on reaction. Further experiments on substrate specificity are now being carried out.

Both ribosidase from *Aspergillus oryzae* and 5'-AMP-ribosidase from *Azotobacter vinelandii*⁹⁾ are nonphosphorolytic hydrolases, and act on nucleoside-5'-phosphates but do not act on 2'- or 3'-isomers*. However, these enzymes are distinguishable from each other in regard to either detailed substrate specificity or relation to ATP. The special feature of *Aspergillus* ribosidase may be to hydrolyze both nucleosides and 5'-nucleotides.

There are various hydrolytic or (pyro)phosphorolytic enzymes, acting on the ribosidic linkages in either nucleosides or nucleotides. Therefore, the confirmation of the existence of a particular enzyme, nonphosphorolytic ribosidase acting on both nucleosides and 5'-nucleotides, in *Aspergillus oryzae* may present interesting evidence from the view point of comparative biochemistry. Furthermore, this enzyme may be employed to establish the location of the phosphate group in guanylic or inosinic acid.

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* It is very interesting to find that both hydrolases and (pyro)phosphorylases, studied so far, act on nucleoside-5'-phosphates but do not act on 2'- or 3'-isomers.