Hydrolytic and Alcoholic Dephosphorylation of Nucleotides by Acid Phosphatase in the Presence of Ethanol

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The effect of ethanol on the activity of acid phosphatase from wheat germ was studied, by using ribonucleoside monophosphates as the enzyme substrates. The nucleotides were effectively degraded to the corresponding nucleosides in the presence of ethanol at all concentrations tested, including a 96% (v/v) solution. However, the nucleotide dephosphorylation was accompanied by the liberation of orthophosphate only when the concentration of ethanol in the assay mixture did not exceed 15%. No inorganic phosphate was liberated when ethanol was present at higher concentrations. Instead, monoethyl phosphate was formed in quantities expected for orthophosphate. The results are explained in terms of phosphatase-catalysed alcoholysis.

We have reported previously (Buchowicz & Leśniewska, 1970*a, b*) the occurrence of an unidentified enzyme in plant tissues, which is able to catalyse an alcoholysis of UMP in the presence of high concentrations of ethanol. In the present paper we describe experiments showing that acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) may be responsible for the observed non-hydrolytic nucleotide dephosphorylation. Evidence is presented that this enzyme, depending on the solvent present, appears able to catalyse either hydrolytic or alcoholic degradation of nucleotides, according to the following general equation:

where R-OH may stand either for water or for an alcohol.

MATERIALS AND METHODS

Reagents. Monoethyl phosphate was prepared by the method of Butcher & Westheimer (1955). All other reagents, including acid phosphatase from wheat germ (Sigma Chemical Co., St Louis, Mo., U.S.A.; lot no. 36B-8580), were of commercial origins.

Standard assay conditions. The complete assay system contained: 0.1 mmol of potassium phthalate buffer, pH 4.8; 0.8 μ mol of 5'-UMP; 14 mmol of ethanol; 50 μ g of phosphatase; water to give a final volume of 1.0 ml. The mixture was incubated at 37°C for 30 min. The reaction was initiated by addition of the enzyme and stopped by 5 min heating of the sample in a boiling-water bath. To evaluate the enzyme activity the following substances were determined quantitatively in the sample: UMP, uridine, orthophosphate and ethyl phosphate. The values reported are in μ mol/assay.

Analytical methods. After inactivation, the sample was cooled in an ice-water mixture, diluted with 5 ml of 0.1 M-HCl and treated with 25 mg of activated charcoal. The suspension was filtered and ethyl phosphate and orthophosphate were determined in the filtrate. The methods used for the isolation, identification and determination of ethyl phosphate are described by Buchowicz & Leśniewska (1970*b*). Orthophosphate was measured by the method of Fiske & SubbaRow (1925).

The nucleotide and nucleoside were recovered from the charcoal with warm acetone-0.1 M-NH₃ (4:1, v/v) (Buchowicz & Reifer, 1962) and separated by paper chromatography by using methanol-1 M-ammonium acetate (7:3, v/v) as the solvent system (Cole & Ross, 1966). The separated products were eluted from the chromatogram with 0.1 M-HCl, identified and determined spectrophotometrically.

RESULTS

Incubation of UMP with acid phosphatase in 14 M-ethanol (approx. 82%, v/v) resulted in a rapid consumption of the substrate (Table 1). The UMP degradation was accompanied by the appearance of an equimolar quantity of uridine but no orthophosphate. Instead, monoethyl phosphate was formed in the quantity expected for orthophosphate. No similar product was formed in the assay when UMP was replaced by P_i. Control experiments (no enzyme, heat-inactivated enzyme, and zero-time incubation) reasonably well excluded the possibility for a non-enzymic ethyl phosphate formation. Evidently, ethyl phosphate is as normal a product of phosphatase activity in ethanol as is orthophosphate in water. By analogy with hydrolysis, the process involving the apparent participation of

Table 1. *Characteristics of the alcoholic activity of acid phosphatase*

Standard assay conditions were followed (see the Materials and Methods section), except for the indicated changes.

Assay system	UMP consumed ($\mu\text{mol/assay}$)	Products formed ($\mu\text{mol/assay}$)		
		Uridine	P _i	Ethyl phosphate
Complete	0.51	0.48	<0.05	0.43
Enzyme omitted	<0.01	<0.01	<0.05	<0.05
Ethanol omitted	0.71	0.69	0.73	<0.05
UMP omitted	—	<0.01	<0.05	<0.05
UMP omitted, P _i added*	—	<0.01	—	<0.05
Complete, boiled enzyme	<0.01	<0.01	<0.05	<0.05
Complete, zero time	0.04	0.04	<0.05	<0.05

* 0.8 μmol of KH_2PO_4 .

Table 2. *Effects of different concentrations of ethanol on hydrolytic and alcoholic dephosphorylation of UMP by acid phosphatase*

Standard assay conditions were followed (see the Materials and Methods section), except that ethanol was used in amounts sufficient to obtain the indicated final concentrations.

Concn. of ethanol (%, v/v)	UMP consumed ($\mu\text{mol/assay}$)	Products formed ($\mu\text{mol/assay}$)		
		Uridine	P _i	Ethyl phosphate
None	0.71	0.71	0.70	<0.05
5	0.69	0.69	0.68	<0.05
10	0.68	0.68	0.65	<0.05
15	0.68	0.67	0.66	<0.05
20	0.66	0.65	<0.05	0.60
25	0.64	0.64	<0.05	0.60
33	0.63	0.63	<0.05	0.61
50	0.55	0.54	<0.05	0.52
67	0.50	0.49	<0.05	0.45
82	0.47	0.48	<0.05	0.45

ethanol is referred to as ethanolysis or, more generally, alcoholysis.

The results in Table 1 showed that in water phosphatase displays only hydrolytic activity and in 82% (v/v) ethanol only the alcoholic one. Further experiments were designed to establish the concentration of ethanol at which the transition from the hydrolytic to alcoholic mechanism of UMP dephosphorylation occurs. Table 2 shows that no ethyl phosphate is formed when dephosphorylation of UMP catalysed by phosphatase is carried out in the presence of an ethanol concentration not exceeding 15% (v/v). When the alcohol was present in the incubation mixture at concentrations of 20% and above, only ethyl phosphate was formed in addition to the dephosphorylated product. The overall rate of uridine liberation was influenced by ethanol, but even in the 96% solution it still remained relatively high (Fig. 1). The reaction was also influenced by changes in pH and

temperature of incubation. In 82% ethanol, as in water, uridine was released with the maximum rate at pH 4.8. The optimum temperature was 40°C. The reaction ceased at 65°C but remained rather high at low temperature values. At 0°C and -15°C it proceeded with rates equal to 60% and 25% respectively of that observed at the standard incubation temperature, 37°C.

In attempts to characterize further the solvent-dependent alteration of the mechanism of phosphatase-catalysed dephosphorylation, the enzyme was preincubated with 82% ethanol at 37°C for 15 min and, after freeze-drying, was tested for activity toward UMP in the presence and absence of alcohol. The results obtained were essentially the same as those given in Table 1 for the non-pretreated enzyme.

As with 5'-UMP, all other nucleotides tested were degraded to the corresponding nucleosides by phosphatase in concentrated ethanol solution

(Table 3). In addition to the dephosphorylated product ethyl phosphate was formed and no P_i was liberated. Methanol could replace ethanol and methyl phosphate formed was distinguished from ethyl phosphate by its chromatographic behaviour.

DISCUSSION

The present work lends additional support to the earlier reports (Benson, 1955; Ullrich & Calvin,

1962; Ullrich, 1963; Bielecki, 1963, 1964; Bielecki & Young, 1963; Cole & Ross, 1966; Bledose, Cole & Ross, 1969) that phosphatases can display considerable activity in the presence of high concentrations of alcohol. However, in contradistinction to those reports, our experiments indicate that the hydrolytic activity of acid phosphatase is abolished when ethanol is present in the system at concentrations higher than 15% (v/v). Dephosphorylation which may then be observed is probably due to an alcoholic activity of the phosphatase, as ethyl phosphate is formed in the place of orthophosphate. Evidently, phosphatase is able to use both the solvents to split its substrates, and the transition from the hydrolytic to the alcoholic mechanism of dephosphorylation takes place when the concentration of ethanol exceeds a limit value. Although alcoholysis is most commonly known as a typical non-enzymic process, the possibility of substitution of water by alcohol in reactions catalysed by some hydrolytic enzymes has been envisaged (Koshland & Herr, 1957; Bender & Glasson, 1960; Findlay, Mathias & Rabin, 1962).

The phosphatase-catalysed alcoholysis of nucleotides may be of interest for both analytical and physiological reasons. This process may lead to considerable losses of nucleotides and to the accumulation of toxic alkyl phosphates when plant material is stored in, or extracted with, concentrated alcohol solutions. Similar enzymic reactions may also represent an additional way in which ethanol enters the cellular metabolism.

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Fig. 1. Comparison of rates of phosphatase-catalysed UMP dephosphorylation in the absence (○) and presence of ethanol at 20% (▲), 82% (■) and 96% (●) concentrations (v/v). Experimental details are given in the Materials and Methods section.

Table 3. *Dephosphorylation of various nucleotides by acid phosphatase in 82% (v/v) ethanol*

The listed nucleotides were used as substrates for acid phosphatase assayed under standard conditions (see the Materials and Methods section).

Nucleotide	Nucleotide consumed (μmol/assay)	Products formed (μmol/assay)		
		Nucleoside	P_i	Ethyl phosphate
2'-(or 3')-AMP	0.42	0.42	<0.05	0.38
5'-AMP	0.46	0.45	<0.05	0.41
2'-(or 3')-CMP	0.35	0.35	<0.05	0.34
5'-CMP	0.38	0.36	<0.05	0.35
2'-(or 3')-GMP	0.53	0.54	<0.05	0.50
2'-(or 3')-UMP	0.40	0.42	<0.05	0.39

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