Phosphodiesterases active against glucose cyclic phosphates

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Virtually no attention has hithero been devoted to enzymes potentially active against phosphodiester bonds in compounds other than nucleic derivatives (Schmidt & Laskowski, 1961). Evidence for the existence of such enzymes was presented recently in connection with studies on the properties of cyclic esters of phosphoric acid. It was shown, e.g., that glucose-3:6- and -4:6- cyclic phosphates (Żmudzka & Shugar, 1964), and 1-(β-D-glucopyranosyl)uracil-2':4'-, -3':6'- and -4':6'-cyclic phosphates (Żmudzka, Szer & Shugar, 1962) all undergo scission of the cyclic phosphate rings in the presence of a crude rabbit brain extract. These findings appeared to warrant further examination in view of the absence of any published data on enzymes attacking phosphodiester linkages in free sugar derivatives. Of possible interest in this connection are the reports of Zamenhof et al. (1953) and Rosenberg & Zamenhof (1960) indicating that poly-ribosephosphate, extracted from Hemophilus influenzae, is slowly hydrolyzed by a preparation of pancreatic ribonuclease.

Additional evidence for the existence of such enzymes is presented here, with particular reference to one with specific activity against D-glucose-1:2-cyclic phosphate.

The foregoing compound was prepared as elsewhere described by treatment of D-glucose-1-phosphate with dicyclohexylcarbodiimide (Żmudzka

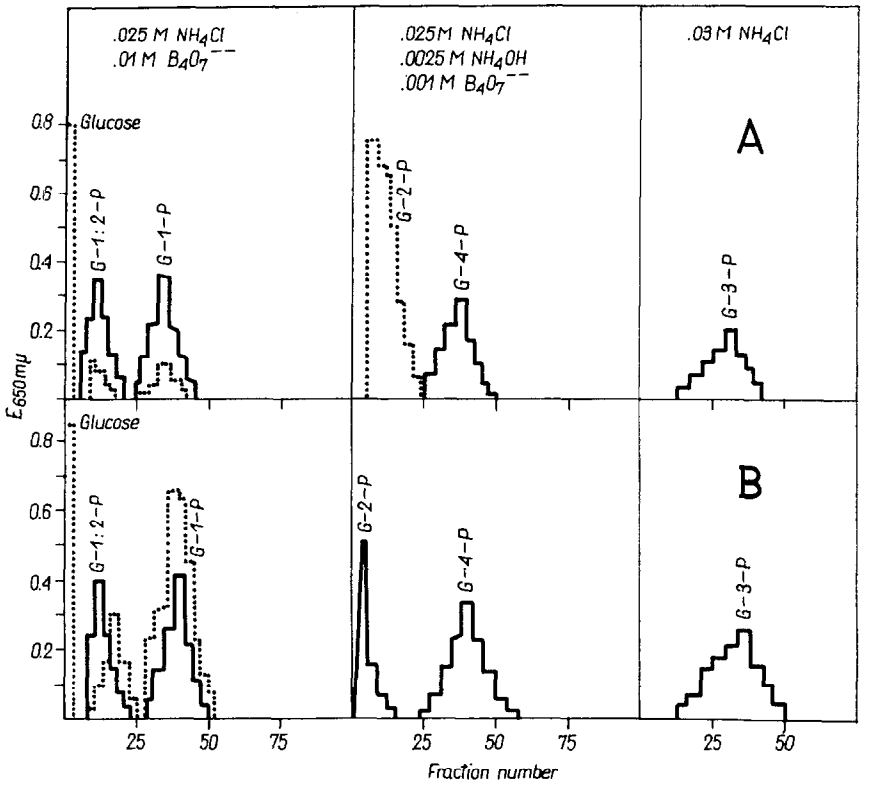

Fig. 1: Column chromatography of the products of acid (A) and enzymatic (B) hydrolysis of D-glucose-1:2-cyclic phosphate on Dowex 1 x 8 (Cl^-):

(————) calibration of column with the use of about 5 mg each of D-glucose-1-, -1:2-, -2-, -3- and -4- phosphates.

(- - - - -) products of acid hydrolysis (about 55 mg placed on column) or enzymatic hydrolysis (about 25 mg product).

Acid hydrolysis conditions: 0.1N HCl, 100°, 4 mins.

Enzymatic hydrolysis conditions: 25 mg substrate incubated in 1.5 ml. of yeast autolysate at pH 7.5 and 37° for 60 mins. Reaction was interrupted by suddenly heating sample to 100° for several minutes. The protein precipitate was centrifuged off, the supernatant diluted to 65 ml., brought to pH 8.5 and deposited on the column.

Conditions of chromatography: The column was 10.1 x 2 cms. and elution with buffers as described by Khym & Cohn (1953), 10 ml. fractions being collected at intervals at 4 mins.; sugar content was estimated by anthrone method, colorimetrically, at 650 mu.

& Shugar, 1964); and was identified by periodate oxidation, which demonstrated simultaneously the absence of D-glucose-1:3-cyclic phosphate, the alternative cyclic phosphate ester which could theoretically be formed from glucose-1-phosphate. This has been further confirmed by a column chromatographic analysis of the acid hydrolysis products of the presumed glucose-1:2-cyclic phosphate (Fig. 1-A). It will be seen from this figure that the acid hydrolysate contains no glucose-3-phosphate, which is the principal acid hydrolysis product of glucose-1:3-cyclic phosphate (Żmudzka, Lepoutre & Shugar, 1964).

Initial experiments on the enzymatic hydrolysis of D-glucose--1:2-cyclic phosphate were based on the use of a variety of homogenates and extracts of various microorganisms and plant and animal materials. The qualitative findings, listed in Table I, show that enzyme(s) active against the foregoing substrate are by no means uncommon, particularly in material of plant origin.

Subsequent experiments were directed towards the characterization of some of the properties of this enzyme, the most active and convenient source of which appeared to be Saccharomyces fragilis autolysates. The yeast organisms were cultivated on a galactose-supplemented medium for 48 hours at 30° and then subjected to autolysis in the presence of 2.2% $(NH_4)_2HPO_4$ for 48 hours at 4°. The suspension was cleared by centrifugation, the supernatant dialyzed for 4 hours at 4° against 0.01M tris buffer pH 7, and finally diluted with buffer so that the protein concentration was 25 mg/ml. When stored at -60°, no loss in activity was noted over a period of several months.

The activity of this autolysate against glucose-1:2-cyclic phosphate exhibited a pH optimum at about 7.5, although some activity was still observable at pH 5 and 10. Sodium versenate, 0.025M, markedly inhibited activity; but the divalent cations Mg^{++}, Co^{++} and Zn^{++} at a concentration of 10^{-3}M were without effect, while Cu^{++} was a strong

Table I: Hydrolysis of D-glucose-1:2-cyclic phosphate to D-glucose--1-phosphate by extracts or homogenates of various biological materials.[x]

Source of enzyme	Activity[xx]	Source of enzyme	Activity
Rat heart	+	Hen's egg yolk	-
Rat epididymis	-	Saccharomyces fragilis	++
Rat duodenum	-	Bacillus subtilis	-
Rat pancreas	+	Micrococcus lysodeicticus	-
Rat lung	+	Beet root	++
Rat spleen	-	Potato tuber	-
Rat kidney	-	Onion (thickened leaf)	++
Rat liver	+	Apple mesocarp	-
Cow's milk	+	Tomato mesocarp	++

[x] Enzymatic hydrolysis conditions: To 0.025 ml. of a 5-10% homogenate in 0.05M tris buffer pH 8, was added 0.010 ml. of a 0.15M solution of D-glucose-1:2-cyclic phosphate, and incubation conducted for 2 hours at 37°. The extent of hydrolysis of the substrate was then controlled by paper chromatography as described in text.

[xx] Enzyme activity is expressed arbitrarily (-) no detectable hydrolysis of substrate; (+) partial hydrolysis of substrate; (++) substrate completely hydrolyzed.

inhibitor. The autolysate lost all its activity either by heating at 100° for 5 mins., or following lengthy dialysis.

Column chromatography on Dowex 1 x 8 (Cl^-), as shown in Fig. 1-B, demonstrated that the only initial product of enzymatic hydrolysis of D-glucose-1:2-cyclic phosphate was D-glucose-1-phosphate. The final hydrolysis products also contained free glucose, due to the presence in the autolysates of non-specific monophosphatase. This is to be contrasted with the results of acid hydrolysis of this substance (Fig. 1-A), leading to the formation of a mixture of glucose-1-phosphate (which, under the

conditions of acid hydrolysis, is dephosphorylated to glucose) and glucose-2-phosphate. The latter, which is the more stable, was the major product of hydrolysis, about 70%.

The yeast autolysate, under analogous conditions, was only slightly active against D-glucose-3:6- and -4:6- cyclic phosphates, and entirely devoid of activity with respect to uridine-2':3'-cyclic phosphate and the methyl esters of glucose-2-phosphate and glucose-1:2-cyclic phosphate. The methyl ester of glucose-1:2-cyclic phosphate was obtained by treatment of the latter with diazomethane in anhydrous ethanol; acid hydrolysis of the methyl ester of the cyclic phosphate led to the formation of glucose-2-methyl phosphate.

It is clear from the foregoing that the described hydrolysis of D-glucose-1:2-cyclic phosphate by the yeast autolysate and extracts of other biological materials is enzymatic in character, and that the enzyme(s) in question exhibits some specificity towards glucose analogues. The question naturally arises as to the nature of the substrate(s) of this enzyme under natural conditions, bearing in mind that no free sugar cyclic phosphate esters have as yet been reported from natural sources.

The course of enzymatic or acid hydrolysis was followed by ascending chromatography on Whatman No. 1 paper, using the solvent system n-propanol-2-ol - ammonia (d = 0.88) - water (7:1:2, v/v/v). The compounds were located on the paper by spraying (Bandurski, 1951; Partridge, 1949) or with a dark UV lamp. The chromatograms were frequently photographed to increase contrast. The course of the reaction was estimated from the visual intensity of the spots. The R_f values of the cyclic phosphate, the monophosphates and free glucose were, respectively, 0.26, 0.05, 0.45.

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