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Studies on the Ribose-5-Phosphatase of Sugarcane

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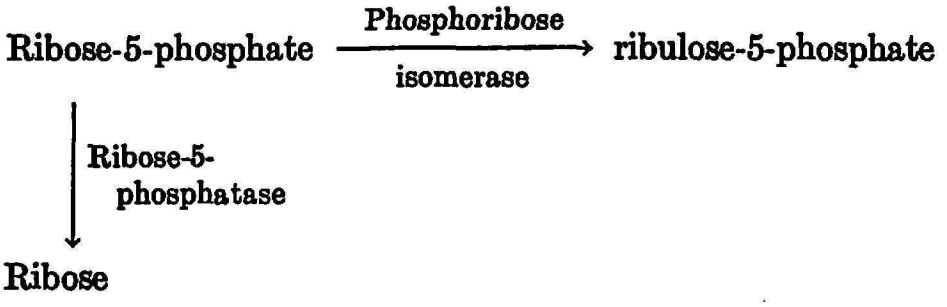
INTRODUCTION

It has been shown recently that ribose accumulates in the leaves of sugarcane treated with the bipyridylium compounds, Paraquat and Diquat (*4*).[2] After ribose-5-phosphate is formed during the photosynthetic carbon reduction cycle, an enzyme, phosphoribose isomerase, converts it into ribulose-5-phosphate. To account for ribose accumulations in Paraquat- and Diquat-treated plants, it was proposed that the above reaction did not proceed beyond the formation of ribose-5-phosphate, and that the latter was hydrolyzed by ribose-5-phosphatase to yield free ribose. The general reaction can be written:



The present paper describes the isolation and properties of ribose-5-phosphatase from sugarcane leaves.

EXPERIMENTAL PROCEDURE

PREPARATION OF ENZYME

Fresh leaf-tissue of 7-month old sugarcane of the variety P.R. 980 was used. Twenty gm. of leaves +1 and +2 were homogenized with chilled, distilled water at high speed with a Waring blendor. The slurry was passed through six layers of cheesecloth and clarified by centrifuging at 2000 r.p.m. for 10 minutes. Ribose-5-phosphatase was removed from the clarified

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[2] Italic numbers in parentheses refer to Literature cited, p. 11.

water extracts, pH 5.6, by precipitation with ammonium sulfate. The salt was added slowly in increments of 5 percent up to a maximum saturation of 90 percent (*1*). The protein from each increment was removed by the centrifuge and taken up in distilled water. Most of the enzyme was precipitated between 35- and 60-percent saturation. This fraction was employed as the enzyme source throughout the remainder of the study.

PHOSPHATASE ASSAY

Ribose-5-phosphatase was measured by determining the amount of inorganic phosphorus released from the substrate by enzyme action under standardized conditions. The reaction mixture for the standard assay was composed of 0.5 ml. of 0.1 M acetate buffer (pH 5.5), 0.5 ml. of ribose-5-phosphate (Ba salt, 0.1 M), and 0.5 ml. of a suitably diluted enzyme preparation. The reaction proceeded for 30 minutes at 37° C. One ml. of 10 percent trichloroacetic acid was used to stop the reaction. Color was developed by the phospho-molybdic acid technique (*1,2,10*), allowing 10 minutes for color stabilization. The precipitated Ba was removed by centrifugation and the percent transmittance was recorded at 640 mμ. Phosphorus was determined by reference to a standard curve representing 0.001 to 0.04 mg. per ml. Enzyme action was computed as specific activity (activity units/mg. of protein). The phosphatase activity unit was arbitrarily defined as the amount of enzyme catalyzing the production of 0.01 mg. of phosphorus under the prescribed conditions of assay. Protein content of the enzyme preparations was determined colorimetrically by the micromethod of Sutherland *et al.* (*12*).

Ribose-5-phosphatase was further purified by gel filtration and electrophoresis. Protein obtained by saturation with ammonium sulfate (35 to 60 percent) was taken up in 10 ml. of distilled water and dialyzed overnight at 6° C before passage through a 3 × 37 cm. column of Sephadex G-200.[3] Four-ml. samples were collected under gravity flow at rates of 1 to 1.5 ml./min. (*3*).

Following assay for ribose-5-phosphatase and protein, peak activity fractions were combined, concentrated by lyophilization and analyzed electrophoretically with a Beckman Model R paper electrophoresis system.[4] Standard separations were run for 2 to 9 hours with Beckman buffer B-2, pH 8.6, and a constant current supply of 2.5 MA. Paper strips were air-dried at the end of the run and then cut into 1-cm. sections. These were eluted with distilled water and aliquots were drawn for ribose-5-phosphatase and protein assays. Enzyme action for ribose-5-phosphatase was

[3] Supplied by the Pharmacia of Fine Chemicals, Rochester, Minn.

[4] Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

expressed as specific activity for both the gel filtration and electrophoresis studies.

RESULTS AND DISCUSSION

EXTRACTION, FRACTIONATION, AND DISTRIBUTION WITHIN THE PLANT

Preliminary extraction of cane-leaf in distilled water and distilled water containing 0.01 M cysteine revealed that cysteine afforded no protection to the enzyme. All extractions were thereafter done in chilled, distilled water alone.

Fractionation of the cane-leaf extracts revealed that most of the protein containing ribose-5-phosphatase was precipitated between 35- to 60-percent

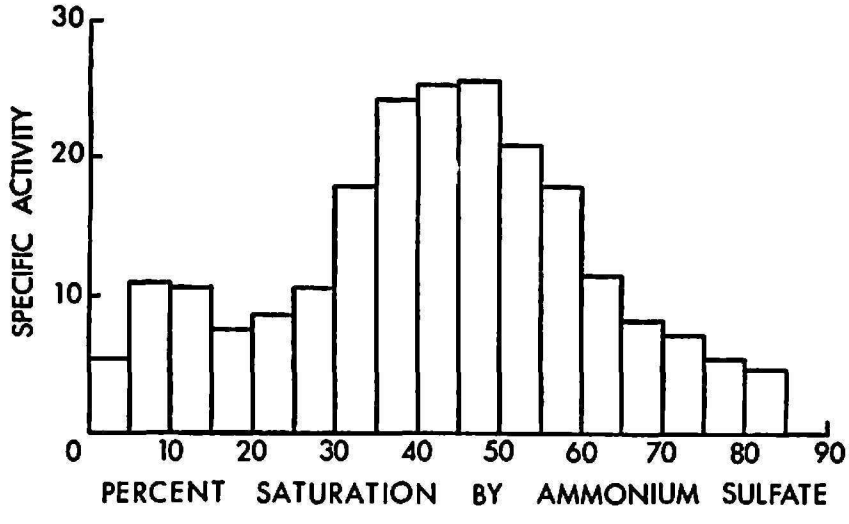

FIG. 1.—Ribose-5-phosphatase activity among protein increments precipitated from sugarcane leaf extracts.

saturation with ammonium sulfate (fig. 1). Other sugarcane phosphatases have shown predominant activity between 40- and 60-percent saturation (*2*,*3*).

Distribution of ribose-5-phosphatase within the cane plant was studied. Distilled-water extracts were prepared for leaves +1 and +2, leaves +3 and +4, spindle leaves, and immature storage tissue. Maximum ribose-5-phosphatase activity was found in spindle leaves. This contrasts with ATP-ase and β-glycerophosphatase, which had greatest activity in meristem and 8- and 10-internode preparations and lowest activity in leaves +5 and +6.

OPTIMUM TEMPERATURE AND PH

Activity of ribose-5-phosphatase was studied at varying temperatures ranging from 16° C. to 60° C. (fig. 2). As pointed out by Alexander (*2*), the

lower temperature is well below the optimum for known sugarcane enzymes, and the higher temperature will retard most heat-sensitive enzymes. Our results show that an increase in temperature from 16° C. to 52° C. caused progressively greater activity. At higher temperatures, 56° C. and beyond, there was a subsequent decline in enzyme action. This enzyme seems to be

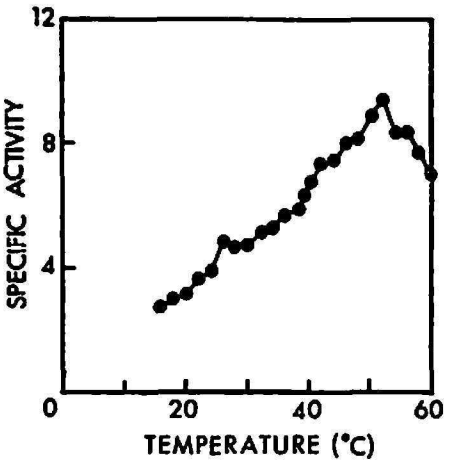

FIG. 2.—Effects of varying temperature upon ribose-5-phosphatase activity.

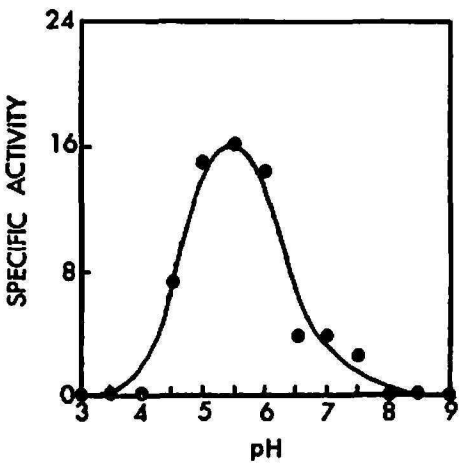

FIG. 3.—Effects of variable pH on ribose-5-phosphatase activity in sugarcane leaf preparations. The following buffers were employed: Citrate, pH 3.0–6.0; acetate, pH 4.5–6.0; succinate, pH 6.5; and tris (trishydroxymethylamino methane), pH 7.0–9.0.

more heat stable than ATP-ase (*2*) but is somewhat similar to β-glycerophosphatase in its temperature response.

Ribose-5-phosphatase was also studied within a pH range of 3.0 to 9.0 (fig. 3, table 1). Maximum activity was recorded at pH 5.5. This is in general agreement with other phosphatases from sugarcane (*2*). Workers in Australia reported a pH optimum between 6.4 and 6.7 for a specific sucrose-phosphate phosphatase (*9*).

SUBSTRATE CONCENTRATION K_m,

The ribose-5-phosphatase reaction was tested against substrate levels ranging from 0.05 to 20 μmoles/ml. The reaction achieved maximum velocity at 10 μmoles, while substrate concentrations greater than 12 μmoles inhibited the reaction (fig. 4). ATP-ase and ADP-ase are also sensitive to high substrate levels (*2*). K_m for ribose-5-phosphatase was calculated to be 2.5×10^{-3} moles of ribose-5-phosphate per liter.

TABLE 1.—*Effect of variable pH upon ribose-5-phosphatase from sugarcane leaves*[1]

	Specific activity at pH—												
	3.0	*3.5*	*4.0*	*4.5*	*5.0*	*5.5*	*6.0*	*6.5*	*7.0*	*7.5*	*8.0*	*8.5*	*9.0*
Ribose-5-phosphatase	0	0	0	7.2	13.9	16.3	14.5	3.9	4.1	2.6	0	0	0

[1] Each reaction mixture was composed of 0.5 ml. of buffer, 0.5 ml. of substrate, and 0.5 ml. of diluted enzyme preparation. Each reaction proceeded for 30 minutes at 37° C.

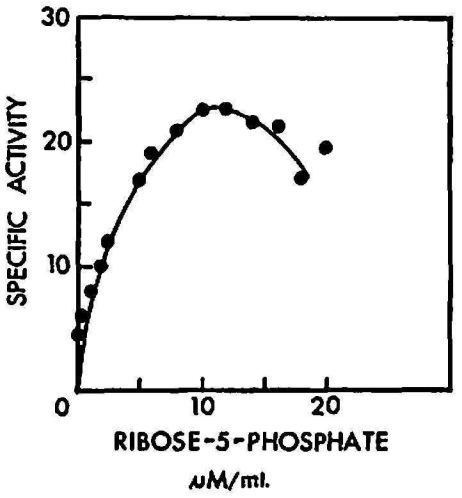

FIG. 4.—Effect of variable substrate concentration on the activity of ribose-5-phosphatase from sugarcane leaves.

REACTION VELOCITY VS. ENZYME CONCENTRATION AND TIME

Suitable protein aliquots having concentrations in the range 5.2×10^{-2} to 43.7×10^{-2} mgs./ml. were drawn to obtain a linear relationship between enzyme concentration and reaction velocity. The linear relationship illustrated in figure 5 was achieved. Ordinarily about 1.5 units of enzyme were employed per ml. of digest.

A linear time-velocity curve was also obtained (fig. 6). For this purpose 1-hour experiments were conducted with the otherwise standard reaction.

GEL FILTRATION AND ELECTROPHORESIS

Gel-filtration studies were undertaken in an effort to further purify ribose-5-phosphatase. The enzyme was separated from the contaminant protein by passage through Sephadex as shown in figure 7. Initial traces of ribose-5-phosphatase appeared in tube no. 15, and maximum activity appeared in tubes 21 and 22. No appreciable protein was detected in these

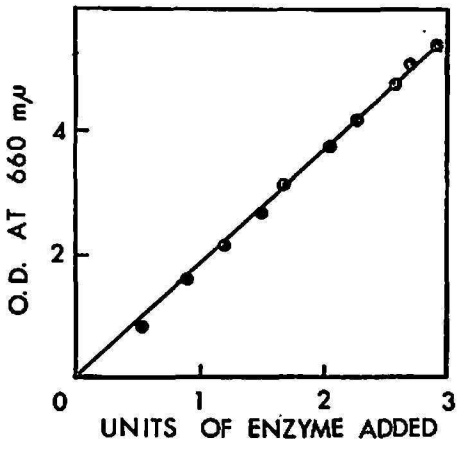

FIG. 5.—Effects of varying enzyme concentrations on the reaction velocity of ribose-5-phosphatase.

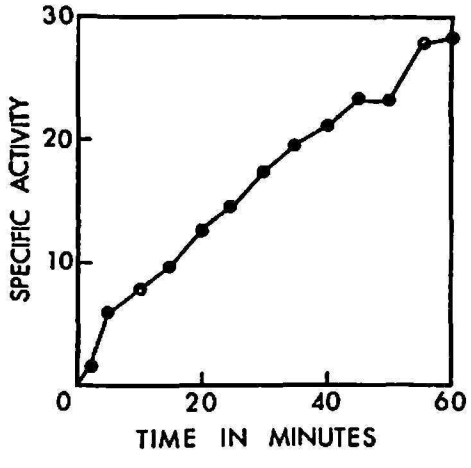

FIG. 6.—Time-velocity curve for ribose-5-phosphatase.

fractions. The mass of protein was obtained in tubes 28 to 45, well after the enzyme had been collected. Similar filtration patterns were obtained with other cane phosphatases (*2*). A two-fold increase in the specific activity of this enzyme indicated that gel filtration did yield a purer enzyme as compared to that obtained during salt fractionation.

Alexander (*3*) reported that amylase peaks could be separated electrophoretically into distinct positively and negatively charged components. Working with sugarcane phosphatases he observed that almost all phos-

phatase activity remained near the point of application during a 2-hour run, and only slight migration towards the negative pole was achieved after a 7-hour run. Only a single phosphatase peak was evident after each experiment. An inspection of figure 8 shows that during a 2-hour run most of the ribose-5-phosphatase was localized near the point of application; but after 4 hours much of the enzyme had migrated toward the positive pole and two distinct peaks were obtained. This suggests that the Sephadex peak fractions were composed of a mixture of catalytic proteins having similar properties with respect to solubility and molecular weight, but having distinct net charges. After 7-hours migration only one peak remained on the strip.

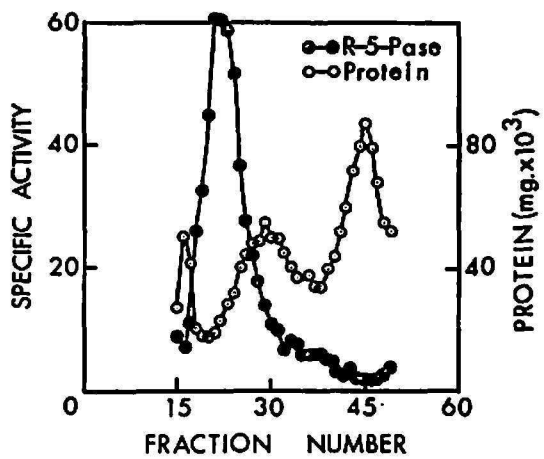

FIG. 7.—Separation of ribose-5-phosphatase from contaminant protein by passage through Sephadex G-200.

ACTIVATION AND INHIBITION

A number of additives were tested for their effects on ribose-5-phosphatase. The effects and significance of molybdenum (Mo), tungsten (W), boron (B), fluoride (F), and β-glycerophosphate (β-GP) on acid phosphatases have already been reported (*2*). These were tested against ribose-5-phosphatase at an initial concentration of 10 μmoles/ml. of digest. Molybdenum and F brought about an inhibition of the enzyme's activity. Tungsten, contrary to the results with acid phosphatases, increased activity, while B and β-glycerophosphate gave slight stimulation.

To analyze the effects of the additives in more detail, Mo, W, and F were tested at varying concentrations ranging from 0 to 10 μmoles per ml. of digest. The results are summarized in table 2. Tungsten, like Mo, exerted an inhibitory effect when given in lower concentrations. Again, this inhibitory effect was reversed at higher concentrations.

Another experiment was performed to determine whether the inhibition

is competitive or specific. Molybdenum, because of its powerful inhibitory action, was selected and used at varying concentrations. Substrate concentration was also varied. Increasing substrate concentrations had no effect on the Mo inhibition of ribose-5-phosphatase, which suggests that a specific or noncompetitive inhibition was in effect. Molybdenum has been

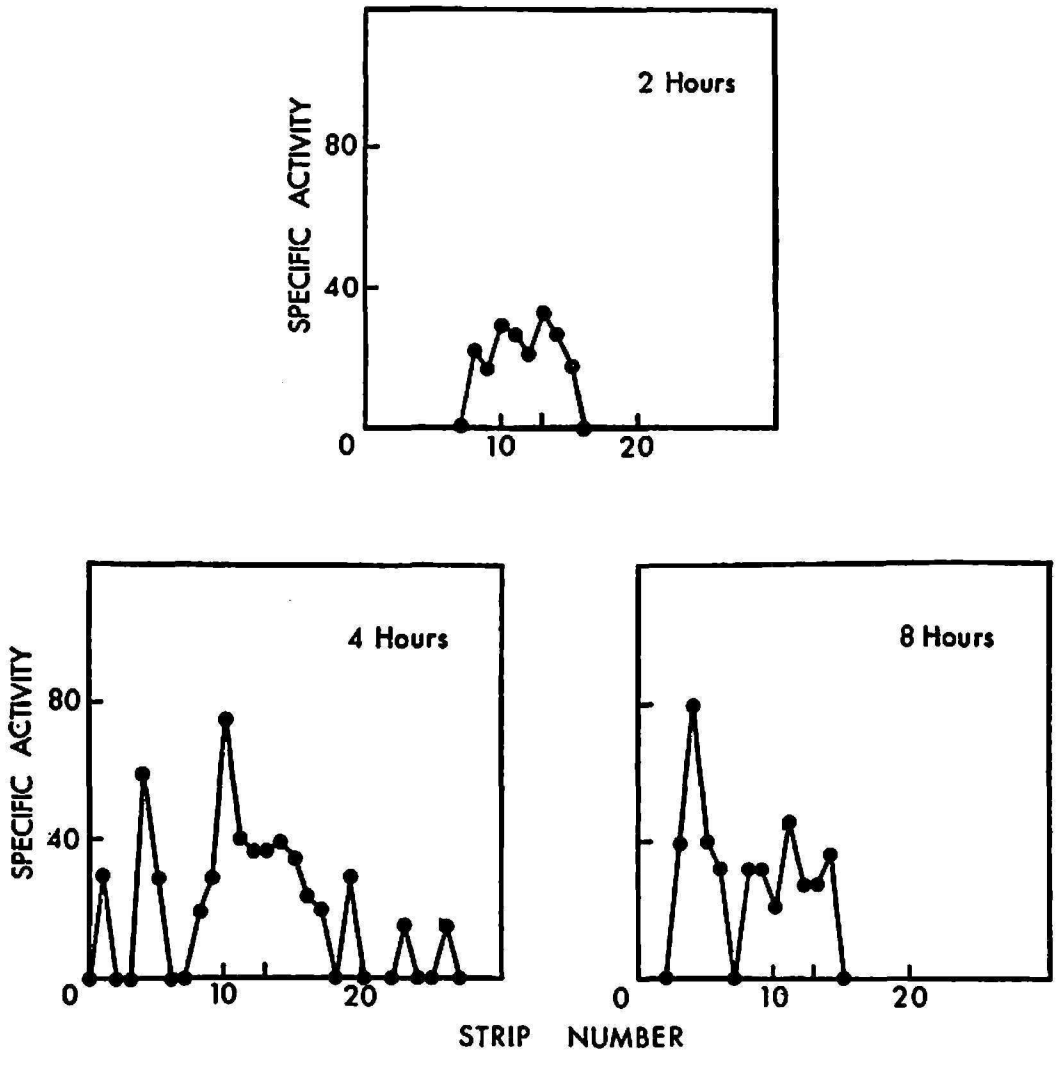

FIG. 8.—Ribose-5-phosphatase activity of Sephadex peak areas subjected to paper electrophoresis for varying periods of time. One-centimeter strips were cut and eluted with water. Strip number 13 includes the area of sample application.

shown to be a competitive inhibitor of acid phosphatase of cane (*2*) and tomato (*11*).

Dialyzing the inhibited enzyme system has often resulted in its recovery to almost normal activity. Experiments were conducted in which the Mo-inhibited system was dialyzed for 24 hours against two changes of distilled water. This resulted in almost complete recovery of the ribose-5-phosphatase activity.

EFFECTS OF DIALYSIS

Enzyme preparations were dialyzed against several changes of distilled water for periods ranging up to 72 hours (table 3). Activity declined about 20 percent within 4 hours, although activity thereafter increased to its former level within 8 hours. At 24 hours there was a 60-percent increase over the 0-hour level. It appears that a natural inhibitor of the enzyme present in the crude preparation was removed by dialysis. Other cane phosphatases have not responded to dialysis (*2*).

TABLE 2.—*Effects of variable molybdenum, tungsten, and fluoride on the specific activity of ribose-5-phosphatase from sugarcane leaves*[1]

Additive	*Additive concentration (μmoles of digest)*					
	0	*0.001*	*0.01*	*0.1*	*1.0*	*10.0*
Mo	4.71	3.98	3.29	1.94	2.35	3.89
W	4.71	3.76	2.35	2.82	2.76	11.28
F	4.71	4.35	4.17	4.11	3.86	4.24

[1] Assay procedures were described under Materials and Methods.

TABLE 3.—*Specific activity of ribose-5-phosphatase during prolonged dialysis against distilled water*[1]

	Data for indicated dialysis time (hours)									
	0	*½*	*1*	*2*	*4*	*8*	*12*	*24*	*48*	*72*
Specific activity	10.4	10.5	11.1	9.4	8.5	10.6	11.6	16.5	13.1	15.9

[1] Enzyme samples are dialyzed at 2° C. for the indicated period of time. After running the assay under standard conditions, the reaction was stopped by the addition of 1 ml. of 10-percent TCA and samples were stored at 2° C. Color was developed at the end of the 72-hour experiment.

SIGNIFICANCE OF RIBOSE-5-PHOSPHATASE IN SUGARCANE

It has been reported by Alexander and Montalvo-Zapata (*4,5*) that ribose appears in sugarcane leaves within 48 hours after treatment with 0.5 percent Paraquat, and by as little as 0.004 percent Paraquat after 3 days. They postulate that there is a block at the site of action of phosphoribose isomerase in the photosynthetic carbon reduction cycle. This in turn brings ribose-5-phosphatase into play, resulting in the accumulation of free ribose. The appearance of ribose thus constitutes an indicator of the time and biochemical position of photosynthetic disruption.

Because of the apparent disruption of phosphoribose isomerase activity, ribose-5-phosphate will not be converted into ribulose-5-phosphate. An alternate pathway for cane has been suggested by Alexander and Montalvo-

Zapata, based on the appearance of fructose and glucose in leaves *after* treatment with Paraquat (*6*). The production of xylulose-5-phosphate from thiamine pyrophosphate-glycolaldehyde and glyceraldehyde phosphate has been described in *Chlorella pyrenoidosa* by Bassham (*7*, pp. 888–89). Ribulose phosphate-xylulose phosphate isomerase, if also operative in cane, might then convert xylulose-5-phosphate into ribulose-5-phosphate. On the other hand, the very appearance of free ribose following Paraquat treatment seems to confirm that the CO_2 fixation pathway originally proposed by Calvin (*8*) is operative at least to the point of CO_2 capture.

SUMMARY

A study has been made of ribose-5-phosphatase in sugarcane. The enzyme catalyzes the hydrolysis of ribose-5-phosphate to yield free ribose and phosphoric acid. The enzyme was extracted from fresh leaf tissues of 7-month old sugarcane grown in sand culture.

Fractionation of cane extracts with ammonium sulfate showed that most of the enzyme was precipitated between 35 to 60 percent saturation. The richest source was spindle tissue. Leaves +1 and +2 were employed for studying the enzyme's properties. Dialysis up to 72 hours against several changes of distilled water had no appreciable effect on the phosphatase activity.

Optimum pH was 5.5 and the K_m was 2.5×10^{-3} moles of ribose-5-phosphate per liter. Good resolution of the enzyme and other protein constituents was obtained by gel filtration on Sephadex G-200. Molybdenum and tungsten inhibited at low concentrations. Molybdenum at 0.1 μmole and tungsten at 1 μmole per ml. severely inhibited the enzyme's action. Inhibition was reversed at higher concentrations. Molybdenum action was specific. Inhibition was lost during prolonged dialysis.

It is proposed that ribose-5-phosphatase is a distinctly different enzyme than the other acid phosphatases previously reported for sugarcane. It appears to be functional in the CO_2 fixation pathway elucidated by Calvin.

RESUMEN

Se hizo un estudio de la ribosa-5-fosfatasa en la caña de azúcar. La enzima cataliza la hidrólisis de la ribosa-5-fosfato y produce ribosa libre y ácido fosfórico. La enzima se extrajo de los tejidos foliares verdes de plantas de 7 meses cultivadas en arena.

El fraccionamiento de los extractos de la caña con sulfato amónico indicó que la mayor parte de la enzima se precipitó entre un 35 y un 60 por ciento de saturación. La fuente más rica de enzima fue el tejido de la zona apical del cogollo. Se utilizaron las hojas +1 y +2 para estudiar las propiedades

de la enzima. La diálisis de las preparaciones enzimáticas durante períodos de hasta 72 horas, con varios cambios de agua destilada, no tuvo efecto apreciable sobre la actividad de la ribosa-5-fosfatasa.

El nivel óptimo del pH fue 5.5 y el K_m 2.5 $\times$ 10^{-3} moles de ribosa-5-fosfato por litro. Se obtuvo una buena resolución de la enzima y de los componentes proteicos al emplear el gel de Sephadex G-200 para la filtración. Se encontró que el molibdeno y el tungsteno inhibían la enzima a concentraciones bajas. El molibdeno a una concentración de 0.1 μmol y el tungsteno a 1 μmol por ml. inhibieron su acción severamente, pero a concentraciones más altas ambos invirtieron el proceso. La acción del molibdeno fue específica. La diálisis prolongada hizo cesar la inhibición.

Se propone que la ribosa-5-fosfatasa es una fosfatasa manifiestamente distinta a las otras fosfatasas ácidas de la caña de azúcar previamente informadas. La enzima parece tener una función en el proceso de fijación del CO_2 según lo explica Calvin.

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