THE EFFECT OF MOLYBDATE ON THE ACTIVITY OF TOMATO ACID PHOSPHATASES

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Summary

The effectiveness of sodium molybdate as an inhibitor of tomato acid phosphatases was examined. Significant inhibition of cell-free dialysed extracts was obtained with as low as 10^{-7}M sodium molybdate. A range of substrates, including a number of glycolytic intermediates, was tested and all except glucose 1-phosphate were hydrolysed by tomato leaf phosphatase preparations. Hydrolysis was always inhibited by molybdate. The inhibition was competitive; $K_i = 10^{-5}$M.

A measure of the *in vivo* phosphatase activity of intact living roots was obtained by immersing roots in a solution of *p*-nitrophenyl phosphate, and measuring the rate of formation of *p*-nitrophenol in the external solution. Molybdate inhibited phosphatases in whole root systems and in cell-free root extracts to the same degree.

From the data it is concluded that molybdate, at physiological concentrations, does inhibit the phosphatases of tomato *in vivo*, at least in tomato root cells, and that the inhibition of phosphatases is a possible metabolic function of molybdenum.

I. INTRODUCTION

It has been shown that preparations of plant acid phosphatases are inhibited by molybdate (Massart and Vermeyen 1942; Bossard 1947; Courtois and Anagnostopoulos 1949). Rothstein and Meier (1949) have shown a similar effect of molybdate on the cell surface phosphatases of yeast. Phosphatases are known to be widely distributed in plants (Ignatieff and Wasteneys 1936; Yin 1945), and to occur in a variety of tissues. They have been reported as hydrolysing a wide range of substrates, including key metabolic compounds such as hexose diphosphate, glucose 6-phosphate, and adenosine triphosphate, as well as the two common phosphatase substrates β-glycerophosphate and *p*-nitrophenylphosphate (PNPP).

Experiments described in this paper were designed to examine further the effect of molybdate on plant acid phosphatases, and to test the possibility that the inhibition of acid phosphatases is a metabolic function of molybdenum in higher plants.

II. METHODS

(*a*) *Experimental Material*

The test plant was tomato (*Lycopersicon esculentum* Mill.); Pan America and Bonny Best varieties were used and no differences due to variety were ob-

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served. Plants were grown in water culture in a glass-house. The water culture medium was of the composition recommended by Cheng Tsui (1948), except that KH_2PO_4 was substituted for K_2HPO_4 and the final pH of the nutrient solution was adjusted to 6.0 with KOH at the beginning of the experiment. In order to remove traces of molybdenum, molar stock solutions of the four salts supplying the macronutrients were extracted with 1 per cent. 8-hydroxyquinoline in chloroform at pH 3-4, as suggested by Gentry and Sherrington (1950). Salts supplying the micronutrients were recrystallized at least three times before use. Plants grew well in the above nutrient solution to which 0.05 p.p.m. of molybdenum (as Na_2MoO_4) had been added. Unless otherwise stated, material used was from molybdenum-treated, normal plants and not from molybdenum-deficient plants.

(*b*) *Preparation of Enzyme Extract*

Two types of enzyme preparations were used. One was a plant powder made from leaves according to the method of Ignatieff and Wasteneys (1936). The method involves grinding the leaves, drying the grindate at 25°C for 1 hr, and re-grinding the product to a fine homogeneous powder. The second enzyme preparation used was a cell-free brei made by grinding or blending the plant material in approximately three times its weight of N/10 succinic acid-sodium succinate buffer, pH 6.0, in the cold, filtering through muslin, and centrifuging for 4 min at 3,000*g*. The supernatant was dialysed overnight at 4°C against N/20 succinate, pH 6.0, and stored at − 20°C. Before use, the brei was re-centrifuged at low speed to remove the clumped particles formed during storage. No alkaline phosphatase activity was detected in these preparations.

(*c*) *Measurement of Phosphatase Activity*

The activity of phosphatase preparations was determined either by measurement of the amount of inorganic phosphate liberated from the substrate, or, when PNPP was the substrate, by measuring the *p*-nitrophenol (PNP) liberated as described by Axelrod (1947). Inorganic phosphate was determined by the method of Waygood (1948), which uses ascorbic acid at pH 4.0 to reduce the phosphomolybdate. Incubated substrate controls were run with each experiment to account for non-enzymic substrate hydrolysis under experimental conditions. With β-glycerophosphate as the substrate, maximum phosphatase activity was obtained at pH 5.9-6.2, and all incubations were carried out in this pH range. The activity of the enzyme preparation was not affected by 10^{-2}M KCN, 10^{-2}M ICH_2COOK, 2.5×10^{-4}M 2,4-dinitrophenol, or 10^{-4}M *p*-chloromercuribenzoate. Sodium fluoride inhibited the enzyme preparation by 59 per cent. at 10^{-2}M.

III. Results

(*a*) *Substrate Specificity and Inhibition by Molybdate*

A range of organic phosphate compounds of physiological interest was tested as potential substrates for tomato-leaf acid phosphatases in the presence and absence of added molybdate (Table 1). The range of substrates hydrolysed

was essentially in agreement with the results of Axelrod (1947) for citrus fruit phosphatase, Nakamura and Becker (1951) for ragweed pollen phosphatase, and Hoagland (1952) for tomato acid phosphatase. Glucose 1-phosphate was comparatively slowly hydrolysed. Triose phosphate, which has not been tested previously, was readily hydrolysed. With all substrates tested phosphatase activity was markedly inhibited by 5×10^{-4}M Na_2MoO_4.

TABLE 1

ACTIVITY OF TOMATO ACID PHOSPHATASES TOWARDS A RANGE OF SUBSTRATES, AND THE EFFECT OF MOLYBDATE ON ENZYME ACTIVITY

Phosphatase preparation = dialysed leaf brei (see Methods). Substrate concentration 2×10^{-3}M. Sodium molybdate 5×10^{-4}M. Phosphatase activity = μg inorganic P liberated in 20 min at 30°C

Substrate	Phosphatase Activity	
	Without Molybdate	With Molybdate
Glucose 1-phosphate	8	Nil
β-Glycerophosphate	28	Nil
Fructose 6-phosphate	22	Nil
Fructose 1, 6-diphosphate	26	Nil
3-Phosphoglycerate	35	Nil
p-Nitrophenyl phosphate	49	7
Adenosine triphosphate	78	25
Adenosine 3-phosphate	34	3
Inosine 3-phosphate	30	5
Triose phosphate*	236	44

*Figures for triose phosphate are not strictly comparable since they were obtained from another experiment using plant powder as enzyme source, and incubated 60 min at 31°C. The rate of hydrolysis of triose phosphate was approximately equal to that of fructose 6-phosphate. Triose phosphate was prepared according to Myerhof (1938).

(*b*) *Phosphatase Activity in Relation to Molybdate Concentration*

Using β-glycerophosphate (0.02M), the activity of a phosphatase preparation was measured over a range of molybdate concentrations (Fig. 1). At this substrate concentration 6.25×10^{-6}M Na_2MoO_4 caused 50 per cent. inhibition.

(*c*) *Type of Inhibition Caused by Molybdate*

The construction of curves relating initial velocity of a phosphatase-catalysed reaction to substrate concentration (sodium β-glycerophosphate) in the presence and absence of inhibitor (5×10^{-6}M Na_2MoO_4) showed that molybdate inhibits the enzyme competitively, i.e. when reciprocals of both initial velocity and substrate concentration are plotted, according to the treatment of Lineweaver and Burk (1934), two straight lines are obtained having a common intercept on the y axis, but significantly differing slopes (Fig. 2). This result is in agreement with those of Rothstein and Meier (1949) for the surface phos-

phatases of intact yeast cells. From the data of Figure 2 the Michaelis constant for the enzyme-substrate complex was calculated, as described by Wilson (1950), to be 1.58×10^{-2}M, and the dissociation constant for the enzyme-inhibitor complex to be 10^{-5}M.

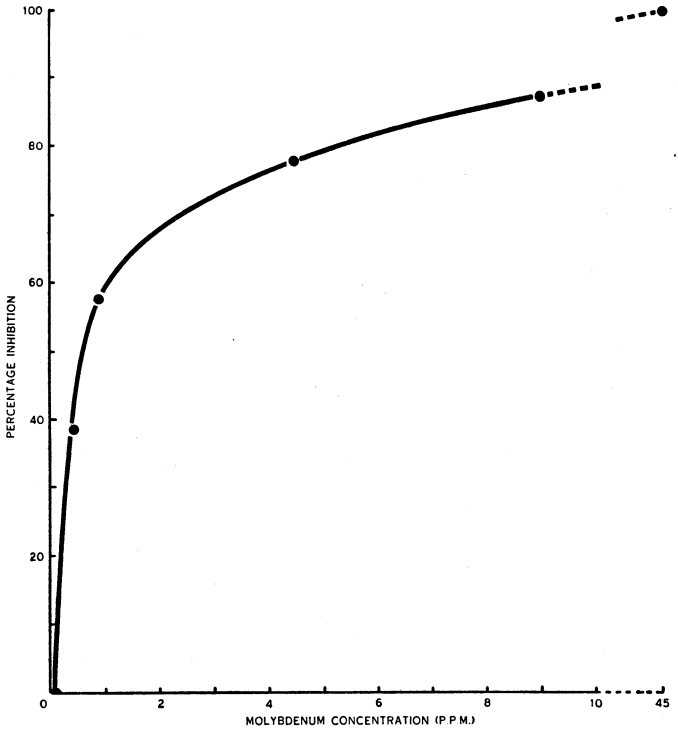

Fig. 1.—Effect of sodium molybdate on the activity of tomato acid phosphatase *in vitro*. Each tube contained 1.0 ml 0.2M glycerophosphate; 0.05 g plant powder (see Methods); the appropriate amount of Na_2MoO_4; and distilled water to make 10.0 ml. Incubated at 30°C for 30 min.

(*d*) *Measurement of the Phosphatase Activity of Intact Plant Cells*

In order to test whether the molybdate status of the plant could affect phosphatase activity *in vivo* it was first necessary to devise a technique by which the phosphatase activity of whole, intact plant cells could be measured. This was achieved by use of PNPP as substrate. If roots of intact plants are immersed in a solution of PNPP the latter is taken up and hydrolysed, and the product of hydrolysis (PNP) diffuses out into the surrounding solution. By taking aliquots of this external solution, making alkaline, and reading the optical transmittance at 400 mμ, the rate of breakdown of the substrate can be followed, and a measure is thus obtained of the activity of the phosphatases of the intact root cells.

The following experiment illustrates this fact: molybdenum-deficient plants 5 wk old were removed from water cultures, roots were washed first in running tap water, and then in distilled water, and each plant transferred to a beaker containing 20 ml of 5×10^{-3}M PNPP, the roots being immersed in the solution. The solution of substrate was adjusted to pH 6.0, and, although unbuffered, the pH did not change more than 0.2 units during the experiment. After 2 hr the roots were washed, and the external solution and the roots were analysed for PNPP and PNP. Roots were ground in 2.5 per cent. trichloracetic acid and residual PNPP estimated as the increase in PNP after hydrolysis in 3N HCl for

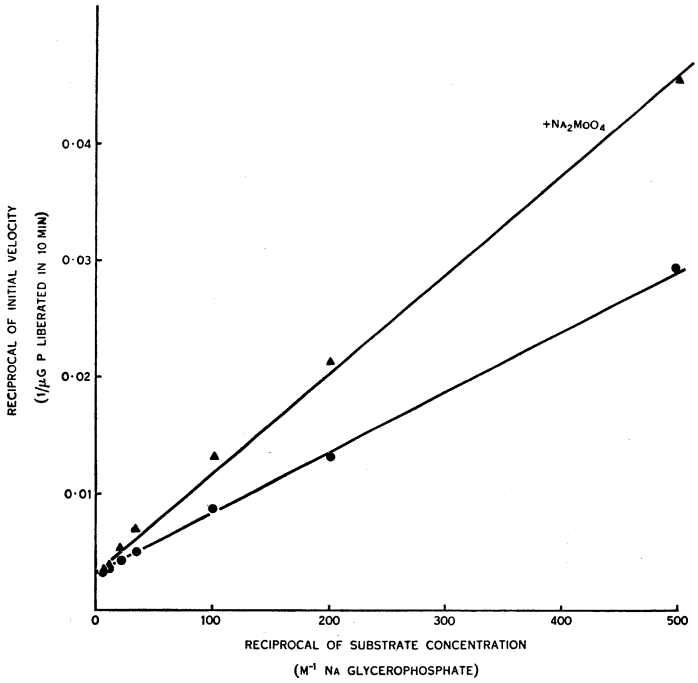

Fig. 2.—Competitive inhibition of tomato acid phosphatases by sodium molybdate. The common origin of the two lines on the y axis is significant at $P = 0.07$. Each tube contained 0.05 g plant powder (see Methods); appropriate concentrations of β-glycerophosphate; 5×10^{-6}M Na_2MoO_4 in the curve indicated; and distilled water to make a total volume of 5.0 ml. Incubated 10 min at 30°C.

3 hr on a boiling water-bath (Bessey and Love 1952). Roots of a plant similarly treated and stood in water for the same period were used to correct for transmittance due to endogenous absorbing substances. Analyses showed (Table 2) that 96 per cent. of the PNP could be recovered in the external solution, so that sampling the external solution for PNP provides a reliable means of determining the rate of the reaction.

Microscopic examination and plating of root segments on potato dextrose agar failed to disclose any contamination by microorganisms. The experiment

was carried out in the glass-house and a solution of PNPP was held under the same conditions and analysed as above in order to account for non-enzymic hydrolysis. When solutions were shielded from light, non-enzymic hydrolysis was slight.

TABLE 2

UPTAKE AND HYDROLYSIS OF PNPP BY WHOLE TOMATO ROOTS IN THE PRESENCE AND ABSENCE OF MOLYBDATE

External Medium (Total vol. 20 ml)	PNPP in Roots (μM/g fresh wt.)	PNP in Roots (μM/g fresh wt.)	PNP in External Solution (μM/g fresh wt.)	$\frac{\text{PNP in External Solution}}{\text{Total PNP Formed}} \times 100$
PNPP (5×10^{-3}M)	0·86	0·68	16·3	96
PNPP (5×10^{-3}M) + Na_2MoO_4 ($7{\cdot}5\times10^{-4}$M)	2·02	0·19	4·61	96

(*e*) *Effect of Molybdate on the Activity of Phosphatases* in vivo

Using the technique described in the preceding section, the effect of molybdate on the phosphatases of intact roots of 5-wk-old molybdenum-deficient plants was tested. Figure 3 shows the inhibitory effect of molybdate added to the external solution on the liberation of PNP into this solution. The volume of each aliquot (0.2 ml) taken from the external solution was small in comparison to the total volume (20 ml), and hence the concentrating effect of withdrawing aliquots can be ignored. Analysis of the roots for PNP and unhydrolysed PNPP showed that in the presence of molybdate, roots contained more PNPP than in its absence; i.e. the hydrolysis of PNPP to PNP had been markedly inhibited (Table 2). Thus *in vivo* as well as *in vitro*, molybdate inhibits tomato phosphatases.

(*f*) *Phosphatase Activity of Roots in Relation to the Molybdate Concentration of the External Solution*

This was measured by the rate of appearance of PNP in the external solution when roots of whole plants were immersed in a PNPP solution (10^{-4}M) containing varying concentrations of molybdate. At the end of an experiment roots were removed from the plant, washed, and dried at 100°C, and the phosphatase activity was expressed as the PNP concentration of the external solution per unit dry weight of roots. Plants used were 4-5 wk old, and molybdenum-deficient. Results (Fig. 4) showed that the phosphatases of intact tomato roots were inhibited by 50 per cent. when the concentration of molybdate in the external solution was approximately 6×10^{-6}M. Significant inhibition was obtained with 10^{-7}M Na_2MoO_4 ($\equiv$ 0.0096 p.p.m. Mo).

In order to obtain a measure of the molybdate concentration at the site of phosphatase activity in the root cells, a cell-free brei was made from the roots

of plants from the same batch as those used in the above experiment. The phosphatase activity of this preparation was measured under the same conditions of substrate and molybdate concentrations as used in the above "whole root" experiment, and the degree of inhibition by molybdate was compared. It will be seen (Fig. 4) that the order of inhibition of the cell-free preparation was the same as that for the intact root system.

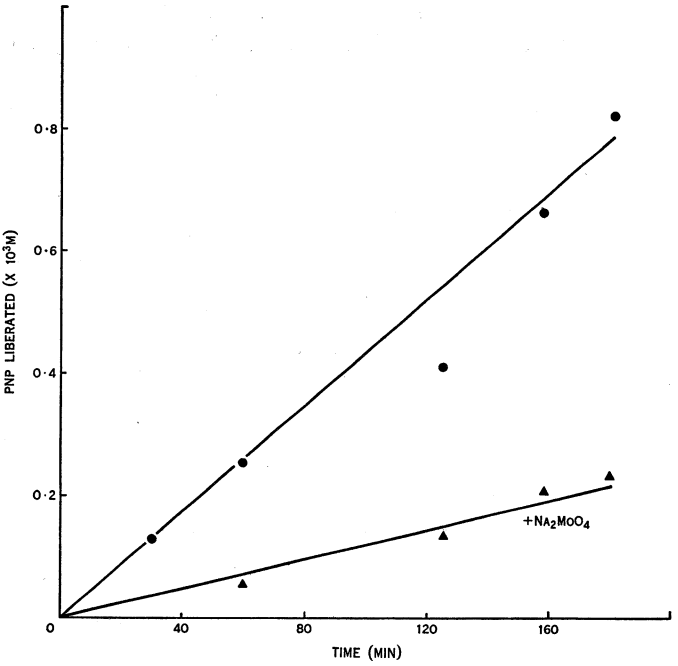

Fig. 3.—Effect of molybdate on the hydrolysis of PNPP by the phosphatases of intact tomato roots. Substrate 5×10^{-3}M PNPP; Na_2MoO_4 7.5×10^{-4}M. Total volume 20 ml.

IV. Discussion

Experiments reported here were designed to examine the possibility that molybdate normally functions as a metabolic inhibitor of acid phosphatases in plants. Results showed that acid phosphatase preparations from tomato were significantly inhibited by concentrations of molybdate as low as 10^{-7}M (Figs. 1 and 4). This is within the physiological range of molybdenum concentrations normally found in higher plants (Williams and Moore 1952), and together with the possibility of local concentrations of molybdenum within the cell (Whatley, Ordin, and Arnon 1951), indicates that molybdate could significantly inhibit phosphatases in intact cells providing it were present at the sites of phosphatase action.

The inhibition is shown to be competitive (Fig. 2), and hence its degree will vary with the substrate concentration. Since neither the natural substrates of plant phosphatases nor their concentrations under physiological conditions

are known, it is not possible from a knowledge of the molybdenum status of the plant to predict the extent of inhibition *in vivo*. It was therefore necessary to test the hypothesis with intact living cells. This was achieved by measuring the hydrolysis of PNPP by whole roots of tomato in the presence of graded amounts of molybdate. These experiments (Table 2; Fig. 3) showed that inhibition of phosphatase by physiological concentrations of molybdate does occur, at least in the special case of the root cells. Whether this result can be extrapolated to other plant tissues is not known. Evidence that this is a possibility is given by the data of Figure 4, which shows that inhibition by molybdate was

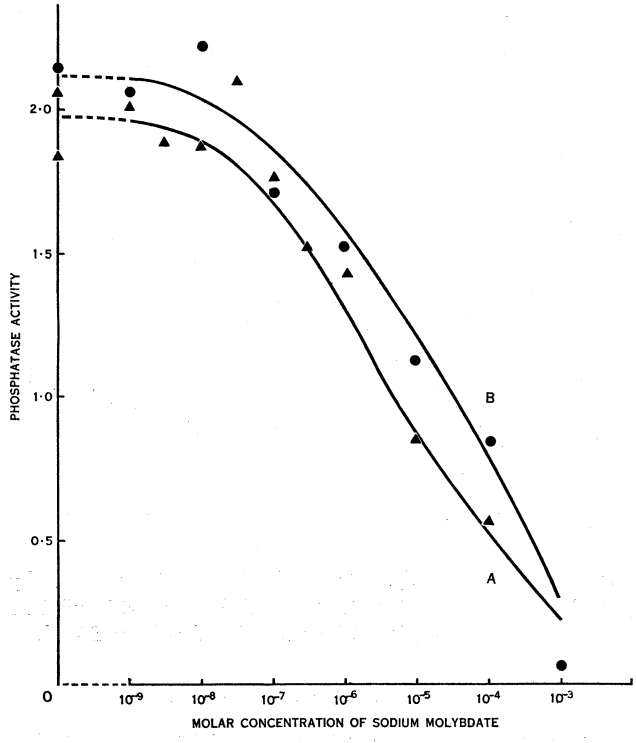

Fig. 4.—Effect of molybdate concentration on the *in vivo* and *in vitro* phosphatase activity of tomato roots. *A* (▲) = intact roots; activity expressed as PNP liberated per 0.1 g dry wt. of root in 60 min. *B* (●) = cell-free root brei; activity expressed as concentration PNP liberated in 20 min at 30°C.

of the same order in both "whole root" and "root brei" experiments. Since significant inhibition was obtained with concentrations of 10^{-7}M in both cases, and since this is of the order of physiological concentrations of molybdenum, it does suggest that a similar inhibition of phosphatases could occur in other plant tissues.

Attempts to introduce PNPP into the leaves by immersing cut stems in a solution of the substrate, a technique used by Rautenen (1948) to follow assimi-

lation of amino acids, were unsuccessful. The very high blank due to endogenous substances which absorb strongly at 400 mμ made it impossible to measure the relatively small change due to the formation of PNP under these conditions.

Bossard (1947) has shown that the effect of molybdate on phosphatases is specific, and that molybdate does not affect the activity of β-glucosidases, proteases, lipase, oxidases, peroxidase, catalase, dehydrogenases, and carboxylases found in extracts of plant tissues. In addition, potato apyrase is unaffected by sodium molybdate concentrations as high as 5×10^{-2}M (Rosenberg, personal communication).

If the hypothesis is correct it should follow that the phosphatase activity of molybdenum-deficient plants is higher than that of plants with adequate molybdenum. However, a comparison of the phosphatase activity of intact roots of normal and deficient plants on a protein basis would not provide an unequivocal answer to the question of the possible *in vivo* effect of molybdate on phosphatase activity, since it is known that the relative content of any one enzyme in a plant tissue may vary with its physiological stage and nutritional state. Axelrod and Jagendorf (1951) demonstrated that phosphatase, catalase, and invertase activities of detached leaves (per unit dry weight) remained at their original level for 14 days, in which time the protein content of the leaves decreased by 45 per cent. As has been emphasized by Wood (1953), in such a case as the present one it is only valid to compare the enzyme activities of physiologically uniform material.

Indirect evidence of increased phosphatase activity, relative to other enzymes, in molybdenum-deficient plants is given by the fact that in the author's experience the inorganic phosphate content of the leaves per unit dry weight is from four to ten times greater than that of leaves from normal plants, both series being grown in water culture with identical phosphorus nutrition. On a protein basis this ratio would be further widened since the molybdenum-deficient plants were found to contain low concentrations of protein. Furthermore, it has been found (Possingham, personal communication) that upon addition of molybdate to deficient plants there is a rapid redistribution of phosphate from the inorganic to the organic fractions. Also Wilson and Cutter (1952), in a histochemical study of developing coconut, demonstrated the coincidence of high inorganic phosphate concentrations with sites of increased phosphatase activity. Although indirect, this evidence does support the hypothesis of increased phosphatase activity in molybdenum-deficient plants.

V. Acknowledgment

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