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REAPPRAISAL OF THE INHIBITORY EFFECT OF CERTAIN SUGARS USED AS OSMOTICA ON PHENYLALANINE AMMONIA-LYASE ACTIVITY

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Key Word Index—*Melilotus alba*; Leguminosae; sweet clover; *Hordeum vulgare*; Gramineae; barley; *Nicotiana tabacum*; Solanaceae; tobacco; phenylalanine ammonia-lyase; *in vitro* inhibition; borate–carbohydrate complexes; assay pH changes.

Abstract—An earlier report that various sugars, especially D-mannitol and D-sorbitol, severely inhibited phenylalanine ammonia-lyase (PAL)§ activity from tobacco *in vitro* has been reappraised. This apparent inhibition resulted from complexing of the sugars with borate, used as buffer system in that study, leading to an undetected pH decrease. We find that D-mannitol, D-sorbitol, D-glucose and sucrose are not inhibitory to PAL preparations obtained from sweet-clover leaves or from protoplasts of tobacco and barley, when alternative buffers are used for the assay. Moreover, the inhibition of barley PAL due to borate–sugar complex formation was completely eliminated by readjusting the pH of the reaction mixture to 8.8 before assaying.

INTRODUCTION

The key role of phenylalanine ammonia-lyase (EC 4.3.1.5) in the secondary metabolism of plants is well documented [1]. Although initially regarded as a freely-soluble enzyme, PAL activity may also be associated with various organelles [2]. The nature of the osmoticum used in preparing organelles has been the subject of a recent report in which D-mannitol and D-sorbitol (and to a lesser extent D-glucose) were shown to be extremely potent inhibitors of PAL activity [3]. These data have been reevaluated here in view of complexes known to exist between boric acid, used as buffer system in many enzymic assays for PAL activity, and carbohydrate [4].

RESULTS AND DISCUSSION

The osmotica D-mannitol and D-sorbitol (at 60–200 mM concentration) potently inhibited the PAL activity of an extract from tobacco tissue [3]. The inhibition by mannitol was thought to be of a reversible non-competitive nature. D-Glucose and D-mannose were weaker inhibitors, whereas sucrose had negligible effect. Directly comparable results were obtained here under similar assay conditions with crude PAL preparations from sweet-clover leaves (Table 1) and from protoplasts and leaves of barley and tobacco. In our opinion, however, the apparent inhibition did not result from direct inhibitory action of the osmoticum upon the enzyme, but was instead caused by the known complexing of borate ions with certain carbohydrates [4].

Borate readily complexes with D-mannitol and D-sorbitol releasing protons, thereby lowering the pH of the assay medium [4]. D-Glucose shows less tendency to complex, while sucrose fails to interact with borate. The extent to which the formation of such complexes affects the pH of the assay system was tested by preparing mixtures containing borate, pH 8.8, and various concentrations of sucrose, glucose, mannitol and sorbitol (Table 1). The pH of mixtures containing sugar alcohols fell drastically with increasing concentrations, reaching a value of 4.5–5.0 at 0.5 M concentration. Sucrose had little effect, but glucose (0.5 M) reduced the pH of the mixture to 6.7.

The above results clearly show that wide deviations from the assumed assay pH of 8.8 arose in the presence of glucose and sugar alcohols. These deviations would be most critical during the assay in borate buffer of an enzyme having a pronounced pH optimum, as has been demonstrated for PAL from *Melilotus* (Fig. 1), tobacco [5], and other species [1]. The errors encountered when borate is employed can be avoided by use of alternative buffer systems. We found that Tris–HCl, glycine–NaOH and TAPS–NaOH (at 0.1 M concentration) maintained their pH value in the presence of each of the four osmotica at 0.5 M concentration (data not shown). Moreover, in contrast to results with borate buffer, the activity of these PAL preparations was not inhibited by mannitol using these buffers (e.g. Table 2). Indeed, a small stimulation (3–9%) in sweet-clover PAL activity was

§Abbreviations: PAL, phenylalanine ammonia-lyase; TAPS, tris (hydroxymethyl)-methylaminopropane sulphonic acid; MES, 2-(*N*-morpholino)ethanesulphonic acid.

Table 1. The effect of osmotica on sweet-clover PAL activity and the pH of borate buffer solutions

Sugar	Concentration (mM)	Enzyme Inhibition observed* (%)	Measured† pH
None	—	0	8.80
Sucrose	100	2	8.65
	300	2	8.35
	500	15	8.15
D-Sorbitol	100	93	6.41
	300	100	5.00
	500	100	4.54
D-Mannitol	100	78	5.72
	300	100	5.28
	500	100	4.80
D-Glucose	100	35	7.84
	300	73	7.12
	500	92	6.72

* PAL was extracted from sweet-clover leaves using 50 mM borate buffer, pH 8.8, and phenolic inhibitors were removed by Dowex treatment. Enzyme activity (60 μl aliquot) was measured in the standard assay system, which included 0.1 M borate buffer, pH 8.8, either alone or with the inclusion of osmotica at the concentrations shown above. The uninhibited reaction rate was 14 μkat/kg protein.

† Observed pH at 37° of mixture containing 0.1 M borate buffer, pH 8.8, and each of the stated osmotica added singly at the concentration indicated.

observed when mannitol was present. Any direct inhibitory effect of mannitol upon PAL activity was further discounted by the finding that the borate–sugar inhibition of PAL in whole shoot homogenates of barley could be completely reversed by readjusting the pH of the reaction mixture to 8.8 before assaying. For example, the PAL activity (26.5 pkat/mg protein) of a homogenate containing 100 mM mannitol in 25 mM borate at a pH of 6.6 was increased to 43.0 pkat/mg protein (96% of control) when the pH was adjusted to 8.6 with 1.0 M NaOH.

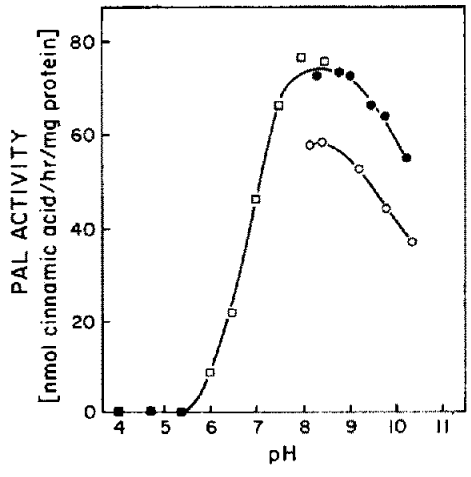

Fig. 1. Effect of pH on PAL activity. The enzyme preparation (0.26 mg protein) was incubated for 30 min with 0.32 μmol of L-phenylalanine-[U-^{14}C] as described in the Experimental, but using 100 mM concentrations of the following buffers: potassium phosphate (□–□), sodium borate (●–●), glycine–NaOH (○–○), and sodium acetate (■–■).

Table 2. Effects of sugars and buffers on PAL activity in enzyme preparations from tobacco and barley protoplasts

Sugar*	Buffer†	Per cent enzyme inhibition‡ and pH of reaction mixture: Tobacco % inhibition	Tobacco pH	Barley % inhibition	Barley pH
D-Sorbitol	Borate	68	7.0	64	6.8
	Tris–HCl	0	8.8	0	8.8
D-Mannitol	Borate	68	7.2	61	6.9
	Tris–HCl	0	8.8	0	8.8
D-Glucose	Borate	42	8.0	16	8.0
	Tris–HCl	0	8.8	0	8.8
Sucrose	Borate	0	8.7	14	8.6
	Tris–HCl	0	8.8	0	8.8

* Final sugar concentration: 0.1 M.

‡ Final buffer concentration: 25 mM.

‡ The uninhibited reaction rates (expressed as pkat/mg Chl) were as follows: tobacco PAL, 5.0 (borate) and 6.3 (Tris); barley PAL, 11.2 (borate) and 20.0 (Tris).

It may be concluded that the apparent inhibition of mannitol and certain other osmotica [3] was in fact due to an undetected change in assay pH. It is advisable that special care be taken when borate buffer is used to assay enzyme activity when such osmotica are present. Our data show that alternative buffer systems could be used advantageously in cases where sugar alcohols are necessary in the preparation of intact organelles.

EXPERIMENTAL

Chemicals. L-Phenylalanine-[U-^{14}C] (460 mCi/mmol) was obtained from Schwarz-Mann, Orangeburg, NY, and diluted with unlabelled L-phenylalanine from Aldrich Chemical Co.

Enzyme preparation. Apical buds and axillary leaves from the first 2–3 macroscopic nodes were harvested from sweet-clover plants (*Melilotus alba* var. White Blossom), which were grown in greenhouses. Plant material was homogenized in either 50 mM Na borate or 30 mM Tris–HCl (both at pH 8.8), followed by centrifugation, as described previously [6]. Before assaying, the supernatant liquid was treated with Dowex 1×2 resin to remove phenolic inhibitors [6]. Protoplasts were isolated from primary leaves of 5–6-day-old light-grown barley (*Hordeum vulgare* L. cv Atlas 68) seedlings as described elsewhere [7]. Tobacco protoplasts were prepared by a described method [8], except that 25 mM MES buffer (pH 5.7) was used. Protoplasts were pelleted by centrifugation at 100g for 60 sec, washed free of isolating medium with *ca* 10 ml 0.6 M glucose in 25 mM Tris–HCl (pH 8.8) and repelleted. Washed protoplasts were taken up in 2 ml 25 mM Tris–HCl (pH 8.8) or 25 mM borate buffer (pH 8.8) containing 5 mM 2-mercaptoethanol and sonicated for 20 sec (50 W, 20 kc, Branson Sonifier Cell Disruptor). Whole-tissue homogenates of barley and tobacco leaves were prepared essentially as described previously [9].

Enzyme assay. Sweet-clover PAL activity was assayed by measuring the rate of formation of cinnamate-[^{14}U] from L-phenylalanine-[U-^{14}C] which were separated by PC and estimated by scintillation spectrometry [6]. A similar method, but involving Si gel TLC of the reaction products, was used for tobacco and barley PAL activities. *Trans*-cinnamic acid

was identified as the product of the reaction with *Melilotus* preparations by cochromatography with an authentic sample on Whatman 3 MM chromatography paper using four different solvent systems. Using a modification of the 'double-decker two-dimensional' technique of Stafford [10], it was demonstrated here that *Melilotus* extracts catalysed the formation of phenylacetate at less then 4% of the rate of *trans*-cinnamic acid synthesis.

Other methods. Protein content was determined by the sulphosalicylic acid method [11] and chlorophyll content by the method of Arnon [12].

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