5. Irving, G. C. J. and Cosgrove, D. J. (1971) *Aust. J. Biol. Sci.* **24**, 559.
6. Irving, G. C. J. and Cosgrove, D. J. (1974) *Aust. J. Biol. Sci.* **27**, 361.
7. Maiti, I. B., Majumder, A. L. and Biswas, B. B. (1974) *Phytochemistry* **13**, 1047.
8. Bitar, K. and Reinhold, J. G. (1972) *Biochim. Biophys. Acta* **268**, 442.
9. Pizer, F. L. and Ballou, C. E. (1959) *J. Am. Chem. Soc.* **81**, 915.
10. Axelrod, B. and Bandurski, R. S. (1951) *J. Biol. Chem.* **193**, 405.
11. Anet, E. F. L. J. and Reynolds, T. M. (1954) *Nature* **174**, 930.
12. Mandal, N. C. and Biswas, B. B. (1972) *Indian J. Biochem.* **7**, 63.
13. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.

Phytochemistry, 1979, Vol. 18, pp. 318–320. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0201-0318 $02.00/0

PHOSPHOFRUCTOKINASE OF CARROT ROOTS

ROBERT A. CALDWELL and JOHN F. TURNER
Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia

(*Received* 12 *June* 1978)

Key Word Index—*Daucus carota*; Umbelliferae; carrots; phosphofructokinase; monovalent cations; P-enolpyruvate; regulation of glycolysis; salt respiration.

Abstract—Phosphofructokinase was partially purified from carrot root extracts. Monovalent cations stimulated carrot phosphofructokinase activity. The enzyme was strongly inhibited by P-enolpyruvate and this inhibition was relieved by NaCl or KCl. Pi inhibited the enzyme at pH 7.9 but was stimulatory at pH 6.6.

INTRODUCTION

The properties of phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) have been studied with preparations from a variety of tissues. Phosphofructokinases from both mammalian tissues [1, 2] and higher plants [3] are inhibited by ATP and citrate and, in general, stimulated by Pi. A distinctive property of the plant enzyme is the strong cooperative inhibition given by low concentrations of P-enolpyruvate [4, 5] and by relatively low concentrations of 2-P-glycerate and 3-P-glycerate [6]. It is believed that the effects of metabolites on the activity of phosphofructokinase are of major significance in the control of glycolysis in plants [3, 7].

In view of the regulatory significance of phosphofructokinase, the study of the plant enzyme has been extended to include carrot roots which have been used extensively for physiological work. The present communication describes the effects of monovalent cations, P-enolpyruvate and Pi on carrot phosphofructokinase.

RESULTS

There was considerable activation of carrot phosphofructokinase by monovalent cations. The concentration of Na^+ in the assay mixture without added monovalent cation was 1.5 mM and the further addition of 12 mM (final concentration) LiCl, NaCl, KCl and RbCl increased phosphofructokinase activity by 40, 53, 59 and 47%, respectively. As most of the modifiers of phosphofructokinase activity used in this investigation were Na salts, the standard assays were performed at constant (24 mM) Na^+ ion concentration.

Carrot phosphofructokinase was subject to strong cooperative inhibition by low concentrations of P-enolpyruvate (Fig. 1). Increasing the concentration of Na^+ in the reaction mixtures relieved the inhibition and increased the degree of cooperativity shown in the P-enolpyruvate inhibition. Hill plots for P-enolpyruvate had slopes of 2.2, 2.5 and 3.1 with Na^+ ion concentrations 1.5, 12 and 24 mM, respectively. The addition of KCl also relieved the inhibition by P-enolpyruvate.

The effect of increasing concentrations of Pi on the activity of carrot phosphofructokinase at different concentrations of $MgCl_2$ is shown in Fig. 2. At pH 7.9 Pi inhibited the enzyme except at low concentrations of both $MgCl_2$ and Pi when a slight stimulation was observed. When the pH of the reaction mixtures was 6.6 there was significant stimulation by Pi.

DISCUSSION

This investigation has shown that carrot root phosphofructokinase is stimulated by monovalent cations and strongly inhibited by low concentrations of P-enolpyruvate. K^+ and NH_4^+ ions are known to stimulate phosphofructokinase from a number of animal tissues [2] and yeast [8]. Na^+ and K^+ ions also had a pro-

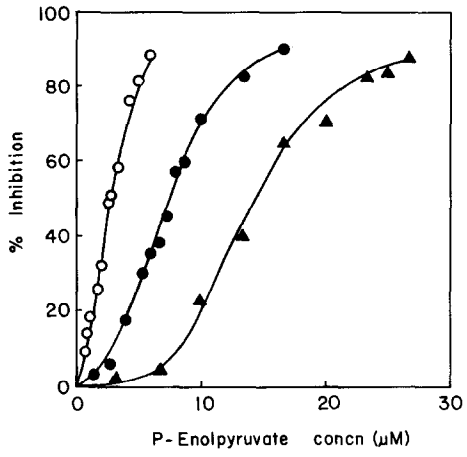

Fig. 1. Effect of P-enolpyruvate on carrot root phosphofructokinase activity at different levels of NaCl. The reaction mixtures were of the composition described for the standard assay with *P*-enolpyruvate added as shown. ○, no addition; ●, 12 mM NaCl; ▲, 24 mM NaCl.

nounced effect in decreasing the inhibition of the carrot enzyme by P-enolpyruvate. With 1.5 mM Na^+, 5 μM P-enolpyruvate inhibited carrot phosphofructokinase by 82% and this inhibition was reduced to 25 and 5% when the Na^+ concentration in the reaction mixtures was increased to 12 and 24 mM, respectively. This suggests an important role for the monovalent cations in determining phosphofructokinase activity and hence in regulating glycolysis in plant tissues [3]. Monovalent cations may affect phosphofructokinase activity directly. In addition, the effect of the monovalent cations will be markedly enhanced if phosphofructokinase in aerobic tissues is, as seems probable, subject to a degree of P-enolpyruvate inhibition [3]. It is of interest that

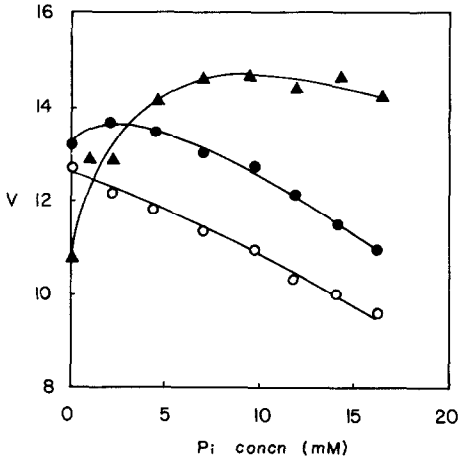

Fig. 2. Effect of Pi on carrot root phosphofructokinase activity at different concentrations of $MgCl_2$. The reaction mixtures were of the composition described for the standard assay with the addition of Pi as shown. The concentration of Na^+ was 48 mM. The velocity (V) is expressed as nmol fructose-1,6-P_2 produced per min/reaction mixture. At pH 7.9: ●, 1.6 mM $MgCl_2$; ○, 16 mM $MgCl_2$. At pH 6.6: ▲, 1.6 mM $MgCl_2$.

NaCl and KCl increase glycolysis in carrot slices [9, 10]. The present findings may have relevance to salt respiration: it is well known that the respiration of washed discs of storage tissue is stimulated by a number of inorganic salts [11].

Carrot phosphofructokinase was inhibited by Pi at pH 7.9 except when the Pi and Mg^{2+} concentrations were low. On the other hand the enzyme was stimulated by Pi at pH 6.6 These experiments were carried out at constant monovalent cation concentration. Pi is generally accepted as an activator of mammalian phosphofructokinase [1, 2] and of the plant enzyme [2, 4, 10] but inhibition by Pi of corn scutellum and spinach chloroplast phosphofructokinases has been reported [12, 13]. It is clear from the present work that interpretation of the effects of Pi and other anions may require consideration of the pH and of the cations present.

EXPERIMENTAL

Partial purification of carrot root phosphofructokinase. Fresh carrots (*Daucus carota* L.) (*ca* 1 kg) were peeled, the xylem removed and the remaining tissue placed in a juice extractor. To the juice was added 0.33 vol. of an extraction medium composed of 0.1 M Tris, 50 mM NaF, 10 mM $MgCl_2$ and 8 mM EDTA (adjusted to pH 8 with HOAc) and the mixture centrifuged at 20000 *g* for 10 min. The supernatant was decanted through glass wool and the filtrate adjusted to pH 8 with NH_4OH. Saturated $(NH_4)_2SO_4$ (pH 8) was added to 35% satn and the ppt. removed by centrifuging at 20000 *g* for 20 min. The supernatant, after decantation through glass wool, was raised to 55% satn. The pellet obtained by centrifuging at 20000 g for 20 min was resuspended in a 1 in 10 dilution of the extraction medium containing 0.1 mM ATP (*ca* 7 ml per 100 ml initial carrot juice) and heated at 55° for 3 min. After cooling and centrifugation at 27000 *g* for 15 min, the supernatant was decanted through glass wool and a further $(NH_4)_2SO_4$ fractionation carried out. The pellet obtained between 25 and 35% satn was resuspended in 6 ml of a 1 in 10 dilution of the extraction medium containing 0.1 mM ATP and dialysed against 1 in 10 diluted extraction medium to remove $(NH_4)_2SO_4$. The dialysed material was frozen and thawed twice and the resultant ppt. removed by centrifuging. The final prepn had a sp. act. of 0.7 μmol fructose-1,6-P_2 produced per min/mg protein, which represented a 20-fold purification from the crude extract. There was no loss in activity during storage for 3 months at −15°. Phosphatases acting on fructose-6-P, fructose-1,6-P_2 and ATP were not detected.

Assay of phosphofructokinase activity. Enzyme activity was assayed by coupling the production of fructose-1,6-P_2 with the oxidation of NADH through aldolase, triosephosphate isomerase and α-glycerophosphate dehydrogenase. To remove $(NH_4)_2SO_4$, suspensions of the coupling enzymes were dialysed before use against a soln containing 10 mM Tris, 5 mM NaF, 1 mM $MgCl_2$ and 0.8 mM EDTA (adjusted to pH 8 with HOAc). Reaction mixtures for the standard assay contained in a total vol. of 3 ml, 60 μmol imidazole, 8 μmol HOAc, 0.42 μmol β-NADH, 0.75 μmol ATP, 1.5 μmol fructose-6-P, 4.8 μmol $MgCl_2$, 72 μmol Na^+ (total), 0.3 units aldolase, 3 units α-glycerophosphate dehydrogenase and 15 units triosephosphate isomerase. The pH of the assay mixture was 7.9. Assays at pH 6.6 contained an additional 36 μmol HOAc. The Na^+ concn was adjusted by the addition of NaCl soln. The reaction was started by the addition of 5 μl of enzyme prepn containing *ca* 7–12 μg of protein. Reaction mixtures were maintained at 30° and the decrease in *A* at 340 nm was followed.

Acknowledgements—This investigation was supported by the University of Sydney Research Grant. The authors wish to thank Dr. L. Copeland for helpful discussion.

REFERENCES

1. Passonneau, J. V. and Lowry, O. H. (1964) *Advances in Enzyme Regulation* (Weber, G., ed.) Vol. 2, p. 256. Macmillan, New York.
2. Bloxham, D. P. and Lardy, H. A. (1973) *The Enzymes* (Boyer, P. D., ed.) Vol. 8, p. 239. Academic Press, New York.
3. Turner, J. F. and Turner, D. H. (1975) *Annu. Rev. Plant Physiol.* **26**, 159.
4. Kelly, G. J. and Turner, J. F. (1969) *Biochem. J.* **115**, 481.
5. Kelly, G. J. and Turner, J. F. (1968) *Biochem. Biophys. Res. Commun.* **30**, 195.
6. Kelly, G. J. and Turner, J. F. (1970) *Biochim. Biophys. Acta* **208**, 360.
7. ap Rees, T. (1974) *MTP International Review of Science. Biochemistry Series One. Plant Biochemistry* (Northcote, D. H., ed.) Vol. 11, p. 89. Butterworths, London.
8. Mavis, R. D. and Stellwagen, E. (1970) *J. Biol. Chem.* **245**, 674.
9. Adams, P. B. and Rowan, K. S. (1972) *Plant Physiol.* **50**, 682.
10. Faiz-Ur-Rahman, A. T. M., Davies, D. D. and Trewavas, A. J. (1974) *Planta* **118**, 211.
11. Briggs, G. E., Hope, A. B. and Robertson, R. N. 1969) *Electrolytes and Plant Cells.* Blackwell, Oxford.
12. Garrard, L. A. and Humphreys, T. E. (1968) *Phytochemistry* **7**, 1949.
13. Kelly, G. J. and Latzko, E. (1977) *Plant Physiol.* **60**, 295.

Phytochemistry, 1979, Vol. 18, pp. 320–321. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0201-0320 $02.00/0

A REAPPRAISAL OF THE FREE AMINO ACIDS IN SEEDS OF *CROTALARIA JUNCEA* (LEGUMINOSAE)

DAVID J. PILBEAM and E. ARTHUR BELL

Department of Plant Sciences, King's College, 68 Half Moon Lane, London SE24 9JF, U.K.

(*Received* 7 *September* 1978)

Key Word Index—*Crotalaria juncea*; Leguminosae; non-protein amino acids; δ-hydroxynorleucine.

Abstract—The seeds of *Crotalaria juncea* have been reported at different times to contain β-hydroxy-*N*-methyl-DL-norvaline and δ-hydroxynorleucine, which are isomers. We detected only one non-protein amino acid in seeds obtained from various sources; it has been isolated and its identity as δ-hydroxynorleucine confirmed.

INTRODUCTION

Crotalaria juncea (Leguminosae) is cultivated in the Indian subcontinent as a source of fibre [1]. The seeds are used medicinally [2] and the green parts are toxic to horses and sheep [3, 4]. Seeds of *C. juncea* have been reported to contain β-hydroxy-*N*-methyl-DL-norvaline [5] and δ-hydroxynorleucine (5-hydroxy-2-amino-hexanoic acid) [6], the last-named also occurring in *C. tetragona* Roxb. [7].

Chromatographic studies in this laboratory revealed the presence of only one major non-protein amino acid in extracts of *C. juncea* seeds obtained from four different sources. This amino acid designated NJ has been isolated and its identity as δ-hydroxynorleucine confirmed.

RESULTS AND DISCUSSION

When seeds of *C. juncea* were extracted with either 70% aqueous ethanol or with methanol (the solvent reported to extract β-hydroxy-*N*-methyl-DL-norvaline [5]) the extracts were found to contain major concentrations of only one non-protein amino acid. This amino acid was isolated by ion-exchange chromatography. The compound formed a chelate with cupric ions showing it to be an α-amino-α-carboxylic acid [8], and it failed to react as an *N*-methyl compound with *p*-nitrobenzoyl chloride [9].

The MS spectrum showed a parent peak at *m/e* 147 (Found: 147.0900; calc. for $C_6H_{13}NO_3$: 147.0895). The base peak at *m/e* 84 indicated the loss of COOH + H_2O from the parent molecule (Found: 84.0811; calc. for $C_5H_{10}N$: 84.0813). A peak at *m/e* 74 (intensity = 63.5%) corresponded to a $CH(NH_2)COOH$ fragment (Found: 74.039; calc. for $C_2H_4NO_2$: 74.0242). There was no peak at *m/e* 89, which would be expected if a $CH(NH—CH_3)COOH$ fragment had been produced.

The PMR spectrum in D_2O showed a doublet δ 1.18 (3H, *d*, *J* = 6 Hz, C-6), attributable to a CH_3 group split by a lone proton, multiplets δ 1.52 (2H, *m*, C-3) and 1.84 (2H, *m*, C-4)) attributable to two CH_2 groups, each split by a CH_2 group and a lone proton (one occurred further down field than the other attributable to its proximity to an OH group), and a multiplet δ 3.81 (2H, *m*, C-2 and C-5) attributable to a CH group split by a CH_3 and a CH_2 group plus a CH group split by a COOH and a CH_2 group. These peaks have the relative intensities 3:2:2:2. Irradiation of the low field multiplet reduced the high field doublet to a singlet, a further indication that the CH_3 signal is split by a lone proton.