Phytochemistry, Vol. 25, No. 2, pp. 345–349, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Press Ltd.

PHOSPHOFRUCTOKINASE FROM *LYCOPERSICON ESCULENTUM* FRUITS—II. CHANGES IN THE REGULATORY PROPERTIES WITH DISSOCIATION

J. E. ISAAC and M. J. C. RHODES*

Department of Life Sciences, Bethlehem University, West Bank, Bethlehem, Via Israel; *Food Research Institute, Colney Lane, Norwich, U.K.

(*Revised received* 31 *July* 1985)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato fruit; phosphofructokinase; kinetic properties; allosteric interactions.

Abstract—The regulatory properties of PFK from the tomato are discussed in relation to the dissociation of the oligomeric form of the enzyme. Both the oligomeric and monomeric forms of PFK were inhibited by citrate, malate, PEP, 2-phosphoglycerate, phosphoglycolate and ammonium sulphate. PEP was the most potent inhibitor of PFK activity with 9 and 10 μm PEP causing 50% inhibition of the oligomeric and monomeric forms of PFK respectively. The inhibition by all these metabolites of the oligomeric form of PFK was sigmoidal while their inhibition of the monomeric form was hyperbolic. The magnitude of inhibition by these metabolites is affected by the levels of Mg^{2+}. The oligomeric form of the enzyme is more resistant to citrate inhibition than the monomeric form. In the presence of citrate or ammonium sulphate, the kinetics of the oligomeric form of PFK with F6P yielded positive cooperativity while in their absence, the kinetics revealed negative cooperative interactions. Phosphoenolpyruvate had no effect on the nature of the kinetics with F6P. ADP is stimulatory to the oligomeric form while it is slightly inhibitory to the monomeric form. The significance of these properties and their relation with the regulation of PFK activity *in vivo* are discussed.

INTRODUCTION

The major characteristic of regulatory enzymes is that their activity is modulated by metabolites as well as their catalytically active substrates [1, 2]. Phosphofructokinase (ATP-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11; hereafter PFK) is sensitive to a large number of modulators. Studying the effects of modulators on PFK in the tomato is complicated by the finding that this enzyme exists in more than one molecular form. Gel permeating chromatography revealed that at pH 7.5, the enzyme extracted from tomatoes at the breaker stage exists solely in an oligomeric form which has an M_r of 180 000. Raising the pH promotes the dissociation of the oligomeric form of the enzyme to give a catalytically active monomer which has a M_r of 35 000 [3]. The dissociation of PFK oligomer was irreversible *in vitro* [3] and it seems likely that the oligomeric form is the dominant form of PFK *in vivo* under normal conditions.

In the previous paper [4], the kinetics of this enzyme with regard to its substrates have been discussed and in this report the effect of different modulators on the two forms of PFK is discussed.

RESULTS

Effect of organic acids

Figures 1 and 2 show the effect of citrate and malate on the oligomeric and monomeric forms of PFK and the kinetic parameters resulting from the analysis of this data are summarized in Table 1. The results show that citrate is

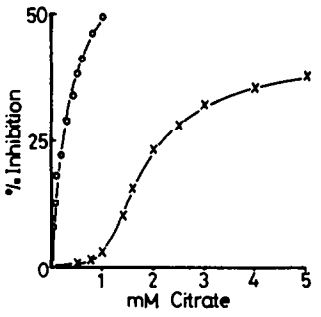

Fig. 1. Effect of citrate on the activity of the oligomeric (×) and monomeric (○) forms of tomato PFK.

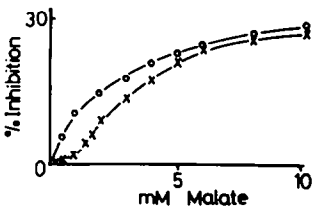

Fig. 2. Effect of malate on the activity of the oligomeric (×) and monomeric (○) forms of tomato PFK.

Table 1. The kinetics of citrate and malate inhibition of PFK (initial activity 5.3 pkat/μg protein)

Acid	Form of enzyme	V at maximal inhibition (pkat/μg protein)	h	$I_{0.5}$ (mM)
Citrate	oligomer	3.36	3.76	1.78
	monomer	1.43	1	0.47
Malate	oligomer	3.92	2.4	3
	monomer	3.85	1	2

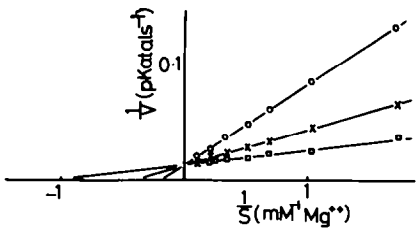

Fig. 4. A double reciprocal plot of the kinetics of PFK oligomer with Mg^{2+} in the absence of (□) and presence of 2 mM citrate (×) and 10 μM PEP (○) (ATP concentration was held constant at 2 mM).

more inhibitory to PFK than malate and that for both acids, the inhibition is greater with the monomeric form. The inhibition of the oligomeric form by these acids is of a sigmoidal nature which manifests itself in h values (Hill coefficient) of more than unity. On the other hand, the inhibition of the monomeric form by both acids is hyperbolic in nature and h is equal to one.

The effect of citrate on the kinetics of the PFK oligomer with regard to its substrates was investigated. The saturation plot of Fig. 3 shows the effect of 2 mM citrate on the kinetics with regard to F6P. The kinetic parameters for the data were 12.8 pkat/μg protein, 0.69 and 6.53 mM for V_{max}, h and $S_{0.5}$ (substrate concentration giving half maximal rate) respectively in the absence of citrate and 7.4 pkat/μg protein, 1.65 and 2.3 mM in the presence of 2 mM citrate. Citrate caused a shift in PFK cooperativity towards F6P from apparent negative to positive as revealed by the values of h. The presence of 2 mM citrate decreased both V_{max} and $S_{0.5}$ for PFK with respect to F6P. This suggests that citrate increased both the sensitivity of PFK to small changes in substrate concentration and the affinity of the enzyme towards F6P. The low activity and instability of the monomeric form of PFK rendered it difficult to carry out as detailed kinetic studies of this form of the enzyme as of the oligomer [3].

In view of the effect of Mg^{2+} in overcoming ATP inhibition [4], the effect of Mg^{2+} on the inhibition of the oligomeric form by citrate was investigated in the presence of 1 mM ATP (Fig. 4). The kinetics revealed simple Michaelis–Menten pattern in the presence and absence of 2 mM citrate. Citrate had no effect on V_{max} but it increased the K_m for Mg^{2+} from 0.84 to 2.4 mM. This shows that increasing Mg^{2+} concentration decreased the inhibition of PFK by citrate, and that citrate decreased the affinity of the enzyme to Mg^{2+}. Since the Mg-ATP complex is the real substrate of the enzyme and free ATP is a potent inhibitor for PFK [4], it is not clear from these data whether the inhibitory effect of citrate is directly upon the enzyme or due to an indirect effect of complexing Mg^{2+} and thus raising the concentration of free ATP to inhibit the enzyme.

The previous data provide further evidence for the important role of Mg^{2+} in controlling the activity of PFK. The inhibition by citrate could be accentuated or diminished by changing the concentration of Mg^{2+} relative to ATP.

Effect of phosphoenolpyruvate (PEP)

Figure 5 shows the effect of PEP on the oligomeric and monomeric forms of PFK. PEP is a very effective inhibitor of both forms of the enzyme and the kinetic parameters of the inhibition were 1.5 and 9 μM for h and $I_{0.5}$ respectively for the oligomeric, and 1 and 10 μM for the monomeric form of PFK.

The effect of 10 μM PEP on the kinetics of the oligomeric form of PFK with respect to ATP and Mg^{2+} (Fig. 4) showed that in the presence of 1 mM ATP, PEP had no effect on V_{max} but increased the K_m for Mg^{2+} from 0.84 in its absence to 5.3 mM in its presence. This indicates that the inhibition by PEP could be decreased by increasing Mg^{2+} concentration and it could be accentuated by decreasing it. PEP decreased the affinity of the enzyme to Mg-ATP. The very low concentrations of PEP required to induce inhibition of the enzyme makes it

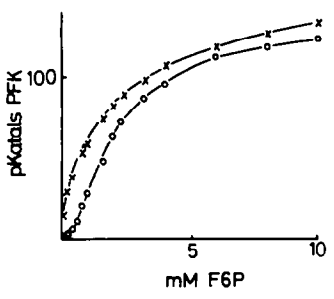

Fig. 3. A saturation plot of the oligomeric PFK activity against F6P concentration in the absence (×) and presence (○) of 2 mM citrate.

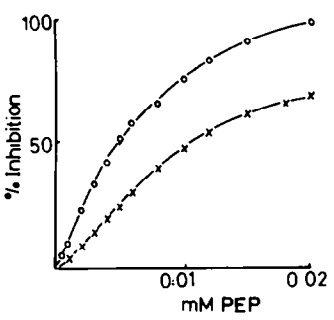

Fig. 5. Effect of PEP on the activity of the oligomeric (×) and monomeric (○) forms of PFK.

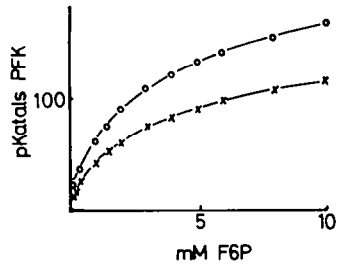

Fig. 6. A saturation plot of PFK activity against F6P concentration in the absence (○) and presence (×) of 10 μM PEP.

unlikely that PEP acts by complexing Mg^{2+} leading to an increase in the concentration of free ATP which would inhibit the enzyme.

Figure 6 shows the effect of 10 μM PEP on the nature of the kinetics of PFK oligomer in relation to F6P. Analysis of the data gave values of 9.9 pkat/μg protein, 0.65 and 6.9 mM for V_{max}, h and $S_{0.5}$ respectively in the absence of PEP and 8 pkat/μg protein, 0.72 and 8.6 mM in the presence of 10 μM PEP. The presence of PEP did not alter the apparent negative cooperativity of the enzyme with F6P, but caused a decrease in V_{max} and slight increases in both h and $S_{0.5}$. This indicates that PEP decreased the affinity of the oligomeric form of the enzyme to F6P. In addition, it shows that increasing the F6P concentration did not relieve PEP inhibition completely.

Effect of other metabolites

The effect of other metabolites such as 2-phosphoglycerate and phosphoglycolate on PFK activity were investigated and Table 2 shows the kinetic parameters for these inhibitors in comparison with PEP. The results show that all these metabolites are inhibitory for PFK but less so than PEP. However, the inhibition by these metabolites is not greatly affected by the molecular form of the enzyme although they tend to inhibit the oligomer in a sigmoidal fashion. The tomato enzyme was not affected by fructose-2,6-bisphosphate.

Effect of ADP

Figure 7 (a and b) shows the effect of ADP, at various Mg^{2+} concentrations on both the oligomeric and monomeric forms of PFK. ADP stimulated the activity of the oligomer and this stimulation was enhanced by increasing the concentration of Mg^{2+}. However, ADP inhibited the

Table 2. Kinetic parameters of PFK inhibition by some metabolic intermediates

Metabolite	Form of enzyme	h	$I_{0.5}$(mM)
Phosphoenolpyruvate	oligomer	1.5	0.009
	monomer	1.00	0.01
2-Phosphoglycerate	oligomer	1.56	0.49
	monomer	1.00	0.66
Phosphoglycolate	oligomer	1.54	0.58
	monomer	1.00	0.78

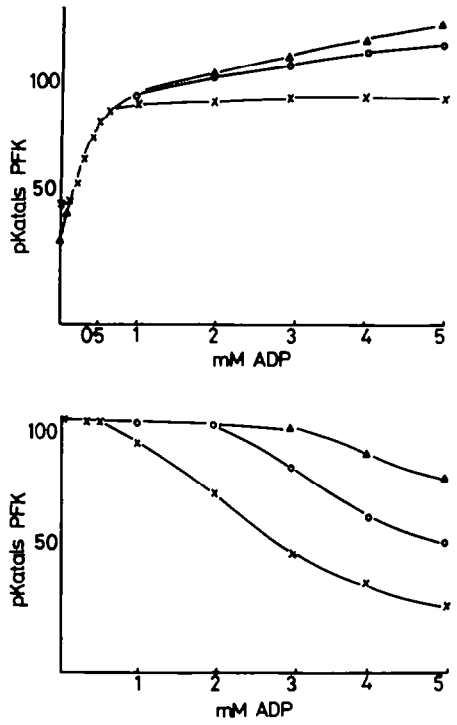

Fig. 7. (a) Effect of ADP on the oligomeric form of PFK in the presence of 2 (Δ), 5 (×), 10 (○) and 20 (Δ) mM Mg^{2+}. (b) Effect of ADP on the monomeric form of PFK in the presence of 5 (×), 10 (○) and 20 (Δ) mM Mg^{2+}.

monomer but increasing Mg^{2+} concentration decreased this inhibition. These results indicate that the effect of both ATP and ADP are influenced by Mg^{2+} concentration.

Analysis of the kinetic data for ADP effect on the oligomer under conditions of excess Mg^{2+} revealed that h and $A_{0.5}$ (activator concentration giving 50% activation) were 2.2 and 0.4 mM respectively. For the monomer, ADP had no inhibitory effect up to a concentration of 3 mM. At higher concentrations of ADP, the inhibition of the monomer could be attributed to a salt effect.

The effect of 1 mM ADP on the kinetics of the oligomeric form of PFK with respect to F6P are shown in Fig. 8. V_{max}, h and $S_{0.5}$ were 10.4 pkat/μg protein, 0.66 and 7.5 mM, respectively in the absence of ADP while in the presence of 1 mM ADP they were 12.25 pkat/μg protein, 0.78 and 4.1 mM, respectively. ADP caused an increase in both V_{max} and h and a decrease in $S_{0.5}$. This indicates that ADP caused an increase in the affinity of PFK for F6P.

Effect of AMP and cAMP

AMP had a slight inhibitory effect on PFK oligomer and monomer and this inhibition was relieved by increasing Mg^{2+} concentration, e.g. 5 mM AMP caused a 35.7, 20 and 11.4% inhibition of PFK oligomer in the presence of 5, 10 and 20 mM Mg^{2+}, respectively. On the other hand, cAMP had a slight stimulatory effect on both forms of PFK causing a maximum of 10% activation at a concentration of 60 μM. At higher concentration, 1 and 2 mM cAMP caused a 9 and 17.4% inhibition, respectively and

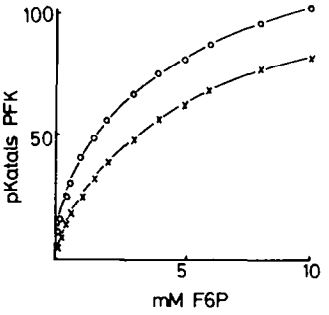

Fig. 8. A saturation plot of the activity of PFK oligomer against F6P concentration in the absence (×) and presence (○) of 1 mM ADP.

again this inhibition was reduced by increasing Mg^{2+} concentration.

Ammonium sulphate inhibited both the oligomeric and monomeric forms of the enzyme but the inhibition of the monomer is greater ($I_{0.5}$ of 27.5 and 46.3 mM for the monomer and oligomer, respectively).

DISCUSSION

Phosphofructokinase catalyses the first unique step in the glycolytic pathway and its regulatory properties derive from its complex kinetic interactions with its substrates and with a large number of allosteric modifiers [1, 2]. The literature on plant PFKs is confusing on the details of the interaction between PFK and these substrates and modifiers, enzymes from different sources appearing to be vastly different in their regulatory properties [5]. The most striking regulatory effector of tomato PFK is PEP, which acts at concentrations well within the physiological range. The concentration of PEP in tomatoes ranges from 13 to 30 μM [5]. PEP inhibition is a feature of PFKs from other plant sources [5–8] but PFK from wheat grain was reported to be unaffected by PEP [9]. PEP inhibition of plant PFK is more potent than that for PFK from animal and microbial sources [10–12], e.g. a K_i value of 148 μM for PEP inhibition of mammalian PFK has been quoted [10]. On the other hand, the plant PFK [12–16], in contrast to animal PFK, is relatively insensitive to inhibition by citrate and malate. Reports show a K_i value of 8 μM for citrate inhibition of mammalian PFK [10], as compared to 470 and 1780 μM for half-maximal inhibition by citrate for the monomeric and oligomeric forms of the tomato enzyme respectively. It could be that citrate inhibition is of less regulatory significance in plants and it is possible that its effect is indirect via ATP due to a decrease in the ratio of Mg^{2+} : ATP required for complexing. Differences in the regulatory properties of enzymes in general, and PFK in particular, from the various sources are possible and may reflect adaptations to particular physiological situations.

Another major difference between PFK from plants and other sources is in its response to fructose-2,6-bisphosphate. In agreement with the findings for other plant enzymes [7, 16–21], it has no effect on tomato PFK. This compound is now considered to be the most potent activator for PFK from mammalian and other sources [21]. PFP, a PPi dependent phosphofructokinase (pyrophosphate: fructose-6-phosphate 1 phosphotransferase, EC 2.7.1.90) has been found in plants [22] and it has been reported that there is a metabolite mediated interconversion between PFK and PFP in spinach leaves [23]. However, there were three recent reports which cast doubt on this interconversion [24–26]. Needless to say, the role of fructose-2,6-bisphosphate and PFP in the control of glycolysis is still not known [22].

Another difference between PFK from plant and other sources is its response to ADP, AMP and cAMP. In general, PFK from most animal and microbial sources is stimulated by ADP, AMP and cAMP [1, 2, 10]. However, ADP inhibited PFK from wheat grain [9], avocado [12] and banana [15] and cAMP had no effect on PFK from avocado, parsley, pea and banana [12, 15, 27]. AMP was found to be inhibitory to PFK from many plant sources [6, 12, 14–16, 27]. On the other hand, PFK from *Atriplex* and *Kalanchoe* was stimulated by AMP and cAMP [28], but chloroplast PFK from spinach was inhibited by AMP and cAMP [29]. In this work, it has been shown that the effect of adenine nucleotides depends on the levels of Mg^{2+} relative to the nucleotide and changes in Mg^{2+} can accentuate or diminish the effect of these compounds on PFK. The PFK oligomer was stimulated by ADP while the monomeric form was not. ADP increased the negative cooperativity of the oligomer with F6P which suggests that ADP increased the affinity of the enzyme for F6P and it relieved the 'strain' imposed by negative cooperativity. AMP and cAMP had no significant effect on tomato PFK.

The present work shows that tomato PFK exhibits complex kinetic properties with its substrates and other modulators which is characteristic of regulatory enzyme. However, the large number of modulators that could affect PFK and the interactions between them in addition to the liability of the PFK oligomer to dissociation, make it difficult to compare the kinetic and regulatory properties of PFK from different sources. It may well be that the inconsistencies in the literature on the properties of plant PFK are due to these effects.

Nevertheless, the present work points to major differences in regulatory properties between PFK from plant and animal sources with the plant enzyme inhibited more by PEP and less by citrate while it is not affected by AMP, cAMP or fructose-1,6-bisphosphate. These differences suggest that the regulation of PFK from plant and animal sources may be different.

The most significant finding presented in this work is the change in the regulatory properties of tomato PFK oligomer with dissociation. On one hand, the PFK oligomer exhibits negative cooperativity with F6P and is stimulated by ADP while it is relatively more resistant to inhibitors than the monomer. On the other hand, the PFK monomer exhibits non-cooperative interactions with F6P and is not stimulated by ADP while it is more sensitive to inhibitors than the oligomer. The physiological significance of this finding is that the tomato PFK oligomer *in vivo* may be operating under constrained conditions imposed by the negative cooperativity with F6P, which is not affected by PEP inhibition. The negative cooperativity with F6P would ensure that total inhibition of tomato PFK oligomer is unlikely and it would also maintain a constant rate of PFK reaction despite minor fluctuations in the concentrations of its modulators. Consequently, it is argued here that a true understanding of PFK regulation *in vivo* can only be achieved through the understanding of the factors that may control the

dissociation of the PFK oligomer and bring about the contrasting regulatory properties between PFK oligomer and monomer. The effect of Pi on the activity of tomato PFK and on the dissociation of the oligomeric form will be discussed in a forthcoming report.

EXPERIMENTAL

Tomatoes (*Lycopersicon esculentum* var. Eurocross BB) were grown in the greenhouse of the Food Research Institute and taken for analysis at the 'breaker' stage of ripening. Biochemicals were purchased from Boehringer and other chemicals were obtained from BDH.

PFK was purified 260 fold using a procedure involving affinity chromatography on Blue Sepharose and ATP-Sepharose and was assayed using the methods previously described [3, 4]. All work on the oligomeric form of PFK was carried out on a preparation made in Tris (pH 7.5) under conditions in which the enzyme exists exclusively in this form and is stable for several days at 0° [3]. Work on the monomeric form relates to preparations made at high pH (8.0–8.5) and stored at 0° under conditions in which dissociation is promoted and the monomer becomes the major species [3].

For kinetic studies, the Hill equation was used in the form $V = \frac{V_0 I^h}{K_i + I^h}$ for inhibition studies and in the form $V = \frac{V_a A^h}{K_a + A^h}$ for activation studies [30] where V is the reaction rate in the presence of modulators; V_0 is the initial velocity in the absence of modulators; I is the inhibitor concentration; K_i and K_a are constants; V_a is the maximum velocity in the presence of an activator; A is the activator concentration and h is the Hill coefficient. This analysis provides the concentration required for half maximal activation ($A_{0.5}$) or inhibition ($I_{0.5}$).

Acknowledgement—The authors would like to thank the British Council for the scholarship awarded to J.E.I. during this work.

REFERENCES

1. Uyeda, K. (1979) *Adv. Enzymol.* **48**, 193.
2. Sols, A., Castano, J. G., Aragon, J. J., Domenech, C., Lazo, P. A. and Nieto, A. (1981) in *Metabolic Interconversion of Enzymes* (Holzer, H., ed.) pp. 111–123. Springer, Berlin.
3. Isaac, J. E. and Rhodes, M. J. C. (1982) *Phytochemistry* **21**, 1553.
4. Isaac, J. E. and Rhodes, M. J. C. (1986) *Phytochemistry* **25**, (Ms 996).
5. Chalmers, D. J. and Rowan, K. S. (1971) *Plant Physiol.* **48**, 235.
6. Turner, J. F. and Turner, D. H. (1980) in *The Biochemistry of Plants* (Davies, D. D., ed.) Vol. 2, pp. 279–316. Academic Press, New York.
7. Sabularse, D. C. and Anderson, R. L. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1423.
8. Garland, W. J. and Dennis, D. T. (1980) *Arch. Biochem. Biophys.* **204**, 310.
9. Goyal, R. K., Kumar, R., Malhotra, S. and Singh, R. (1984) *Phytochemistry* **23**, 2159.
10. Mansour, T. E. (1972) *Curr. Top. Cell. Regul.* **5**, 1.
11. Blangy, D., Buc, H. and Monod, J. (1969) *J. Mol. Biol.* **31**, 13.
12. Kelly, G. J. and Turner, J. E. (1969) *Biochem. J.* **115**, 481.
13. Ashihara, H., Komamine, A. and Shimokoriyama, M. (1972) *Phytochemistry* **11**, 2717.
14. Dennis, D. T. and Coultate, T. P. (1967) *Biochim. Biophys. Acta* **146**, 129.
15. Salminen, S. O. and Young, R. E. (1975) *Plant Physiol.* **55**, 45.
16. Sasaki, T., Tadokora, K. and Suzuki, S. (1973) *Phytochemistry* **12**, 2843.
17. Carnal, N. W. and Black, C. C. (1983) *Plant Physiol.* **71**, 150.
18. Cseke, C., Weeden, N. F., Buchanan, B. B. and Uyeda, K. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4322.
19. Stitt, M., Meiskes, G., Soling, H. D. and Heldt, H. W. (1982) *FEBS Letters* **145**, 217.
20. Van Shaftigen, E. B., Lederer, B. and Hers, H. G. (1981) *Biochem. Biophys. Res. Commun.* **101**, 1078.
21. Kruger, N. H., Kombrink, E. and Beevers, H. (1983) *FEBS Letters* **153**, 409.
22. Hers, H. G. (1984) *Biochem. Soc. Trans.* **12**, 729.
23. Balogh, A., Wong, J. H., Wötzel, C., Soll, J., Cseke, C. and Buchanan, B. (1984) *FEBS Letters* **169**, 287.
24. Gancedo, J. (1984) *FEBS Letters* **175**, 369.
25. Kruger, N. J. and Dennis, D. T. (1985) *Biochem. Biophys. Res. Commun.* **126**, 320.
26. Ap Rees, T., Green, J. H. and Wilson, P. M. (1985) *Biochem. J.* **227**, 299.
27. Lowry, O. H. and Passonneau, J. V. (1964) *Arch. Exp. Pathol. Pharmacol.* **248**, 85.
28. Sutton, B. G. (1975) *Aust. J. Plant Physiol.* **2**, 403.
29. Kelly, G. J. and Latzko, E. (1977) *Plant Physiol.* **60**, 295.
30. Taketa, K. and Pogell, B. M. (1965) *J. Biol. Chem.* **240**, 651.