Phytochemistry, 1980, Vol. 19, pp. 1297–1301. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0701-1297 $02.00/0

IDENTIFICATION OF THE REGULATORY STEPS IN GLYCOLYSIS IN POTATO TUBERS

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(*Received* 24 *September* 1979)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato; tuber; glycolysis; control; phosphofructokinase; pyruvate kinase; cold-induced sweetening.

Abstract—The aim of this work was to identify the regulatory reactions of glycolysis in potato tubers. The amounts of glycolytic intermediates in aerobic and anoxic tubers were measured in freeze-clamped samples of tissue. Comparison of mass-action ratios with apparent equilibrium constants showed that *in vivo* the reactions catalysed by glucosephosphate isomerase, phosphoglycerate mutase and enolase were close to equilibrium. The ratios fructose-1,6-bisphosphate:fructose 6-phosphate, and pyruvate:phosphoenolpyruvate, respectively, showed that the reactions catalysed by phosphofructokinase and pyruvate kinase were considerably displaced from equilibrium. Stimulation of glycolysis by placing tubers in an atmosphere of nitrogen led to significant declines in their contents of fructose-6-phosphate and phosphoenolpyruvate. It is concluded that phosphofructokinase plays a dominant role in regulating entry into glycolysis, and that pyruvate kinase may regulate exit from glycolysis and the oxidative pentose phosphate pathway. Cold-induced sweetening of the tubers is discussed in the light of the above conclusions.

INTRODUCTION

The effects of cold on the metabolism of potato tubers suggest that cold-induced sweetening may be due, at least in part, to differential sensitivity of glycolysis to low temperature. The available data suggest that phosphofructokinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) are more sensitive to cold than are the other enzymes involved in the metabolism of hexose-6-phosphates, and that lowering the temperature diverts the latter to sucrose [1, 2]. This hypothesis presupposes that the above enzymes, phosphofructokinase in particular, play a dominant role in the control of glycolysis in potato tubers. The aim of the work reported in this paper was to see if this is so.

Comparative biochemistry and general considerations of metabolic control suggest that phosphofructokinase regulates entry into glycolysis in tubers, and that pyruvate kinase could regulate movement out of glycolysis and thus contribute to the regulation of the amounts of most of the intermediates of glycolysis and the oxidative pentose phosphate pathway in the tuber. However, adequate proof of these suppositions is lacking. Such proof requires that the steps be shown to be considerably displaced from equilibrium *in vivo*, and that the substrates be shown to change in the opposite direction to the flux when the latter is varied [3]. Although there is ample evidence of this kind that phosphofructokinase is regulatory in animals and microorganisms [4], the results of studies with higher plants are less clear cut. There are reports that hexose phosphates change in the opposite direction to the glycolytic flux when the latter is varied [5, 6] but there are also reports that increased glycolysis in plants may be accompanied by no detectable change [7] or even by an increase in these compounds [8]. The available data for potato tubers alone show considerable variation. Barker and Khan [9] reported that accelerated glycolysis in tubers was accompanied by increases in glucose-6-phosphate in three experiments, by decreases in three other experiments, and by a decrease followed by an increase in two others. Solomos and Laties [10] reported that stimulation of glycolysis by anoxia and by ethylene led to falls in glucose-6-phosphate whereas stimulation by cyanide led to marked increases. The role of pyruvate kinase in regulation of carbohydrate metabolism has yet to be elucidated in either plants [11] or animals [3]. At least some of the above apparent contradictions may be due to the technical difficulties of measuring the amounts of glycolytic intermediates in plants [12]. In none of the studies of potato was the tissue freeze-clamped and the only estimates of recovery of exogenous compounds that are presented for potatoes [9] are for compounds added to extracts after the tissue had been killed. Thus losses during killing were not investigated.

In order to assess the role of phosphofructokinase and pyruvate kinase in the regulation of glycolysis in potatoes, we first determined whether they catalysed non-equilibrium reactions *in vivo*. We did this by measuring the amounts of glycolytic intermediates in tubers and then comparing the mass-action ratios of

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the reactions *in vivo* with the apparent equilibrium constants of the enzymes. Once we had established which steps were non-equilibrium, we investigated whether they were regulatory by stimulating glycolysis and measuring whether their substrates declined in amounts. The data of Barker and el Saifi [13, 14] provide adequate evidence that anoxia stimulates glycolysis in potato tubers. They showed that the rate of release of carbon as CO_2 by aerobic tubers was 18–27% of the rate at which carbon accumulated in the products of glycolysis in anoxia. CO_2 production is not necessarily a measure of glycolysis in air because appreciable amounts of the carbon entering glycolysis as glucose-6-phosphate may be withdrawn for the synthesis of amino and organic acids. However, in a mature tuber it is most unlikely that such withdrawals would be extensive enough to account for the observed difference between CO_2 production in air and the estimate of the rate of glycolysis in anoxia.

RESULTS AND DISCUSSION

The need to freeze-clamp bulky tissue to ensure immediate cessation of metabolism has already been emphasized [12]. It is not practicable to freeze-clamp whole tubers so we sampled them by removing cylinders of tissue with a cork borer. For measurements on anoxic tubers the cores were removed under nitrogen so as to prevent resumption of aerobic metabolism. For both aerobic and anoxic tubers the time between the removal of the core and freeze-clamping was kept to 4 sec so as to reduce wound effects. We investigated whether the cores were representative samples of the tubers by measuring the amounts of hexose-6-phosphates in triplicate cores from the same tuber. We could not take more than three cores from one tuber; nonetheless, even with this small number of samples, the standard errors were small in relation to the mean values. For glucose-6-phosphate these were 98.6 ± 1.3 and 62.6 ± 3.7 nmol/g fr. wt for aerobic and anoxic tubers, respectively. The corresponding values for fructose-6-phosphate were 26.5 ± 0.35 and 15.5 ± 1.7. We conclude that the cores were adequate samples.

We tested the validity of our analytical techniques in the following type of recovery experiment. For each test we took duplicate cores from the same tuber. One core was freeze-clamped, killed and extracted in the usual way; the other was treated similarly except that the $HClO_4$ used to kill the tissue contained measured amounts of the intermediates to be assayed. The difference between the amounts of an intermediate found in the extracts of the two samples was then expressed as a percentage of the amount added to give an estimate of the recovery of that compound (Table 1). For each compound we consistently obtained values close enough to 100% to indicate that our techniques were reliable. The least satisfactory recoveries are those for fructose-1,6-bisphosphate; in order to obtain even these values we had to vary the procedure considerably and use a much shorter incubation in $HClO_4$ to kill the tissues than for the other intermediates. If the period during which the freeze-clamped tissue stood in $HClO_4$ was extended beyond 2 hr the recovery of fructose-1,6-bisphosphate fell dramatically so that none could be detected after 4 hr. Freeze-clamping disrupted the cores of tissue. Thus the added compounds were accessible to the enzymes and contents of the tissue during the killing in $HClO_4$. We think that this type of recovery experiment is a much more stringent test than those in which exogenous compounds are added either after killing the tissue or to tissue that is not disintegrated until after it has been killed. In both the last instances the endogenous compounds are likely to be at substantially greater risk of modification during killing and extraction than the exogenous compounds. Thus high recoveries for the latter may not be significant.

We estimated the amounts of glycolytic intermediates in aerobic tubers and in tubers that had been in nitrogen for 90 min (Table 2). We also measured the amounts of glucose-6-phosphate and fructose-6-phosphate in tubers that had been in nitrogen for 20, 40, 55 and 100 min. Decreases could be detected after 40 min but were not fully developed until 90 min. Therefore our major analyses were carried out after 90 min in nitrogen. We attribute the relatively slow changes to the time taken for the intact tuber to become anaerobic. The data of Burton [15] indicate that at 20° the oxygen content of a tuber of 100 g fr. wt is about equal to the amount of oxygen consumed by the tuber in 40–80 min.

Considering that for any one intermediate each measurement was made on a different tuber in a separate experiment, the values in Table 2 show remarkably little variation between tubers. There are previously published estimates of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate and phosphoenolpyruvate [9, 10] with which our

Table 1. Recovery of glycolytic intermediates during killing and extraction of potato tubers

Intermediate	Amount added (nmol/g fr. wt)	Estimate of recovery of added intermediate (%)*
Glucose-6-phosphate	140	96±4
Fructose-6-phosphate	30	89±7
Fructose-1,6-bisphosphate	10	122±2
3-Phosphoglycerate	50	93±4
2-Phosphoglycerate	15	95±1
Phosphoenolpyruvate	25	93±3
Pyruvate	15	93±2

*Values are means ± s.e. of estimates made on 6 different tubers.

Table 2. Amounts of glycolytic intermediates in aerobic and anoxic tubers of potato

Compound	Amount (nmol/g fr. wt)* Aerobic	Anoxic	Fisher's *P* values† aerobic vs anoxic
Glucose-6-phosphate	96.6±3.4	46.8±1.4	<0.001
Fructose-6-phosphate	24.7±0.8	13.1±0.7	<0.001
Fructose-1,6-bisphosphate	4.5±0.2	6.1±0.2	<0.001
3-Phosphoglycerate	68.5±1.8	57.6±2.1	<0.01
2-Phosphoglycerate	8.4±0.5	8.2±0.5	n.s.
Phosphoenolpyruvate	23.0±0.6	14.5±0.6	<0.001
Pyruvate	12.6±0.5	19.6±1.0	<0.001

*Each value is the mean ± s.e. of estimates made on 6 different tubers in 6 separate experiments.

†Values of 0.05 or less are considered significant. Values greater than 0.05 are given as n.s. (not significant).

estimates may be compared. We found less variation between tubers, somewhat lower values for glucose-6-phosphate and phosphoenolpyruvate, and very much higher values for fructose-1,6-bisphosphate. For the first two compounds the differences between our results and the published values may reasonably be ascribed to variation in the type and physiological state of the tubers. However such variations will not explain the fact that we found 4.5–6.1 nmol fructose-1,6-bisphosphate per g fr. wt, whereas Barker and Khan [9] reported 0.2 and Solomos and Laties [10] values that ranged from <0.05 to 0.24. Our higher values were not due to selection of tubers with abnormally high contents of glycolytic intermediates. This is shown by comparison of our estimates of hexose 6-phosphates and phosphoenolpyruvate (Table 2) with the previously published values [9, 10]. We stress that the ratio of fructose-6-phosphate to fructose-1,6-bisphosphate that we found, 5.5, is comparable to those reported for a range of animal tissues (16–18) but differs radically from that of 90 reported by Barker and Khan [9]. Solomos and Laties [10] measured glucose-6-phosphate, not fructose-6-phosphate, but on the assumption that the two are in equilibrium, their ratios of fructose-6-phosphate to fructose-1,6-bisphosphate may be calculated, and be shown to have ranged from 210 to 1500. We suggest that it is particularly difficult to measure fructose-1,6-bisphosphate in some, if not all, higher plants. We think that previously published estimates for potatoes, and quite possibly other plants, are serious underestimates. It is conceivable that during killing and extraction fructose-1,6-bisphosphate is, in part, converted to fructose-6-phosphate, thus inflating estimates of the latter. Consequently, attempts to assess the regulatory role of phosphofructokinase in plants from unsubstantiated measurements of these two compounds should be treated cautiously.

The data in Table 2 have been used to calculate the mass-action ratios of some of the glycolytic reactions (Table 3). Lack of a suitable technique for measuring cytoplasmic, as opposed to mitochondrial or total, ATP and ADP in potato tubers precluded calculation of the mass-action ratios for phosphofructokinase and pyruvate kinase. However, we have used the lower values of the apparent equilibrium constants in Table 3, and our estimates of the ratios of fructose-1,6-bisphosphate:fructose-6-phosphate; and pyruvate: phosphoenolpyruvate to calculate the ATP:ADP ratio that would have to exist in the cytoplasm if the phosphofructokinase and pyruvate kinase steps were at equilibrium *in vivo*. For phosphofructokinase in aerobic and anoxic tubers these ATP:ADP ratios would have to be 2×10^{-4} and 5.1×10^{-4}, respectively. The corresponding values for pyruvate kinase are 3636 and 1481. All these values are so far from the ratios likely to exist in the cytoplasm [19] that we conclude that phosphofructokinase and pyruvate kinase catalyse non-equilibrium reactions in both aerobic and anoxic tubers. Thus both reactions are capable of acting as regulatory steps. The mass-action ratios for glucosephosphate isomerase (EC 5.3.1.9),

Table 3. Comparison of apparent equilibrium constants of glycolytic reactions with ratios of substrates in aerobic and anoxic tubers of potatoes

Reaction	Apparent equilibrium constant*	Substrates	Ratios† Aerobic	Anoxic
Glucosephosphate isomerase	0.36–0.47	Fru-6-*P*:Glc-6-*P*	0.26	0.28
Phosphofructokinase	900–1200	Fru-1,6-P_2:Fru-6-*P*	0.18	0.47
Phosphoglycerate mutase	0.1–0.2	2-*P*-Glyceric acid:3-*P*-Glyceric acid	0.12	0.14
Enolase	2.8–4.6	*P*-Enolpyruvate:2-*P*-Glyceric acid	2.74	1.77
Pyruvate kinase	2000–20000	Pyruvate:*P*-enolpyruvate	0.55	1.35

*The sources of these values are in ap Rees *et al.* [7]

†Ratios of mean values shown in Table 2.

phosphoglycerate mutase (EC 2.7.5.3), and enolase (EC 4.2.1.11) are sufficiently close to their apparent equilibrium constants to establish that these reactions are close to equilibrium in both aerobic and anoxic tubers and are thus unlikely to exert appreciable direct control over glycolytic flux in the tubers.

Acceleration of glycolysis by putting tubers in nitrogen was accompanied by striking changes in the amounts of glycolytic intermediates (Table 2). Both fructose-6-phosphate and glucose-6-phosphate declined. This establishes that phosphofructokinase plays a dominant role in the regulation of glycolysis in potato tubers. The attendant rise in fructose-1,6-bisphosphate supports this conclusion, but, for the reasons given by Rolleston [20], and Newsholme and Start [3], does not, on its own, prove that phosphofructokinase is regulatory. The behaviour of fructose-6-phosphate and fructose-1,6-bisphosphate when potatoes are made anoxic is very similar to that which has often been reported for animal tissues [4]. Similar studies with plant tissues as a whole often show the rise in fructose-1,6-bisphosphate but less marked changes in fructose-6-phosphate [11]. Whether the pattern in potato, or those referred to above, is the more usual behaviour for plants cannot be decided until further authenticated data are available.

The other major changes in glycolytic intermediates that occur when potatoes are made anoxic are a decline in phosphoenolpyruvate and a rise in pyruvate. These changes indicate a regulatory role for pyruvate kinase. As this step is preceded by phosphofructokinase which catalyses an irreversible reaction, pyruvate kinase cannot regulate glycolytic flux directly as it cannot directly control the entry of glucose-6-phosphate into glycolysis. However pyruvate kinase could play a role in the regulation of the movement of carbon out of glycolysis and the oxidative pentose phosphate pathway and thus contribute to the control of the amounts of the intermediates of these pathways in the tissue. As a number of these intermediates are effectors of phosphofructokinase [21], control of pyruvate kinase could influence glycolytic flux indirectly.

We conclude that in mature tubers of potato entry into glycolysis is regulated largely by phosphofructokinase. We suggest that pyruvate kinase plays an important role in determining the levels of intermediates of glycolysis and the oxidative pentose phosphate pathway and that this may influence indirectly the activity of phosphofructokinase. As carbohydrate oxidation in potato tubers, as in plant tissues as a whole, is almost certainly predominantly via glycolysis [22], then cold-lability of phosphofructokinase and pyruvate kinase could lead to a rapid reduction in hexose phosphate consumption that could cause their diversion to sucrose.

EXPERIMENTAL

Materials. Substrates, cofactors and enzymes were from Boehringer, Mannheim. Tubers of *Solanum tuberosum* L. cv Record were provided by the National Institute of Agricultural Botany, Cambridge, and were kept at 10° in the dark until used. For each experiment we selected regularly shaped tubers of about 100 g fr. wt. All experiments were done with mature tubers 2–8 weeks after harvest.

Methods. Tubers were removed from the store and kept at 20° for 18 hr before being used in the experiments. For measurements on aerobic tubers, a core of tissue (3 cm × 0.7 cm; fr. wt 3–4 g) was removed with a No. 4 cork borer. One core was taken per tuber through the centre parallel with the long acis of the tuber, suspended by a loop of cotton and freeze-clamped as described in ref. [7]. Anoxia was achieved by placing a tuber, after it had equilibrated to 20°, in a Perspex box (30 cm × 25 cm × 30 cm) previously been flushed with O_2-free N_2 for 30 min. N_2 was flushed through the closed box throughout the incubation at 20°. After incubation, cores were removed from the tubers as described above in the box under N_2. The cores were freeze-clamped at once (within 4 sec). The weight of the cores was determined by weighing the tubers before and after core removal. The freeze-clamped material was ground in liquid N_2 in a pestle and mortar. Before the liquid N_2 had evapd the suspension was quantitatively transferred to a plastic centrifuge tube and 5 ml 1.41 M $HClO_4$ was added. The frozen mass that resulted was transferred to −4° and kept at this temp. for 18 hr, except when fructose-1,6-bisphosphate was to be measured, when the time was reduced to 2 hr. Glycolytic intermediates were extracted from the freeze-clamped samples as described in ref. [23] and assayed spectrophotometrically at once as described in ref. [24]: the methods marked I were used for fructose-1,6-bisphosphate and 3-phosphoglycerate. Fisher's *P* values were calculated by Student's *t*-test.

Acknowledgements—We thank Mr. M. Bailey of the National Institute of Agricultural Botany for the potatoes. W.L.D. thanks the Potato Marketing Board for a postgraduate studentship.

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