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AEROBIC FERMENTATION AND PHOSPHOFRUCTOKINASE IN TISSUE SLICES OF THE CORN SCUTELLUM*

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Abstract—The study of gas exchange in tissue slices of corn scutellum incubated either in water or 0·1 M fructose showed that the presence of the hexose increased the rate of glycolysis in this tissue 4- to 7-fold depending on the experimental conditions, and that this increase was quantitatively linked with an aerobic alcoholic fermentation. The properties of crude phosphofructokinase (PFK) were studied and compared with the properties of PFK's from other higher plants, one of which does not carry out aerobic fermentation in the presence of exogenous hexose. Corn scutellum PFK appears to be similar in its properties to the PFK's of other higher plants. The tissue levels of certain metabolic intermediates, some of which influence PFK activity (citrate, ATP, inorganic orthophosphate, and fructose-1,6-diphosphate), were determined and were found not to vary in amount under regimes of low and high glycolytic activity. No evidence was found to support a regulatory role for PFK on the rate of glycolysis in corn scutellum slices. Alternative mechanisms for the control of glycolysis in scutellum slices are proposed. These controls may involve the reactions leading to the metabolic utilization of stored sucrose and the intracellular distribution of adenine nucleotides and inorganic phosphate.

INTRODUCTION

THE RATE of glycolysis is generally believed to be regulated by certain "key" enzymes among which phosphofructokinases (PFK) from various animal sources and microorganisms have been extensively studied (see reviews by Wood[1] and Atkinson[2, 3]). However, only a few reports appear in the literature describing the properties of PFK from higher plants,[4, 5] and virtually no information is available which correlates changes in endogenous levels of metabolites or "effectors" in higher plants with changes in rates of metabolic activity presumably controlled by PFK.

The investigations presented in this paper show that the addition of hexose (fructose or glucose) to corn scutellum slices results in a strong aerobic, alcoholic fermentation which represented up to a 7-fold increase in the rate of glycolysis. In addition, the properties of crude corn scutellum PFK were determined and compared with those of the enzyme isolated from the tissues of three different higher plants by other investigators. Furthermore, the endogenous levels in corn scutellum slices of certain metabolites known to affect PFK activity *in vitro* were determined under conditions of low and high glycolytic rates.

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[1] W. A. WOOD, *Ann. Rev. Biochem.* **35**, 521 (1966).
[2] D. E. ATKINSON, *Ann. Rev. Biochem.* **35**, 85 (1966).
[3] D. E. ATKINSON, *Science* **150**, 851 (1965).
[4] O. H. LOWRY and J. V. PASSONNEAU, *Arch. Exp. Pathol. Pharmakol.* **248**, 185 (1964).
[5] D. T. DENNIS and T. P. COULTATE, *Biochem. Biophys. Res. Commun.* **25**, 187 (1966).

RESULTS

Respiration and Fermentation

Respiratory patterns of corn scutellum slices were determined manometrically both in the presence and absence of exogenous hexose (fructose or glucose). In addition, the effect of a 90-min incubation period in water on the subsequent gas exchange in water or fructose was determined. These studies were made to see if the application of the hexoses which are rapidly converted to sucrose in this tissue had any effect on gas exchange. The data presented below show that the addition of fructose triggered a high degree of aerobic alcoholic fermentation in this tissue. While the data are not presented in this paper, it was found that the addition of comparable amounts of glucose to the slices produced the same effect.

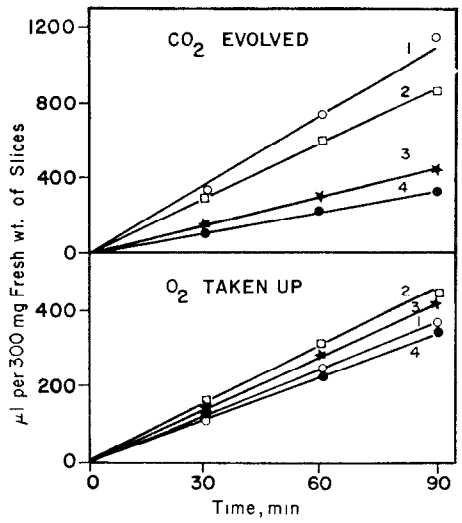

FIG. 1. EFFECT OF FRUCTOSE ON GAS EXCHANGE IN CORN SCUTELLUM SLICES.

The slices (300 mg fresh wt.) were placed in Warburg vessels immediately after their preparation or after incubation in water at 30° for 90 min. The Warburg vessels contained either water or 0·1 M fructose. The curves represent gas exchange from the following treatments: (1) 90-min preincubation, gas exchange in fructose; (2) no preincubation, gas exchange in fructose; (3) no preincubation, gas exchange in water; (4) 90-min preincubation, gas exchange in water.

The respiratory quotient (RQ) of tissue slices incubated in water was found to be near unity indicating the complete oxidation of carbohydrate substrates. As can be ascertained from Fig. 1, the incubation of the tissue slices for 90 min in water, prior to the measurement of gas exchange, slightly depressed both oxygen uptake and carbon dioxide evolution but had little effect on the RQ. The addition of 0·1 M fructose to the slices, however, resulted in a large increase in the evolution of carbon dioxide along with only the slightest increase in oxygen uptake resulting in RQ values near 2. The incubation of the slices in water for 90 min prior to placing them in 0·1 M fructose resulted in a further increase in carbon dioxide evolution along with a small decrease in oxygen consumption resulting in RQ values near 3.

In order to confirm that the increase in carbon dioxide evolution in the presence of exogenous fructose resulted from an aerobic fermentation, experiments were conducted during which both ethanol production and gas exchange by the slices were measured (Table 1).

During this study, the 90-min incubation period in water prior to measurement of gas exchange and ethanol production was again used since, under these conditions, the greatest amount of carbon dioxide evolution was achieved. Furthermore, the RQ values for fructose treatment were substantially higher with this procedure than in the absence of a prior incubation period in water (see Fig. 1). This prior incubation period was conducted by placing the slices in unstoppered, 25-ml Erlenmeyer flasks containing 10 ml of water and agitating the flasks in a "Gyrotory" water bath at 30°.

When the slices were incubated in water for 90 min prior to gas exchange measurement, they produced approximately 1 μmole of ethanol/300 mg fresh wt; however, ethanol was not found in the freshly prepared tissue slices (presumably because of extensive washing of the slices). As can be seen in Table 1, during the measurement of gas exchange, a small amount of ethanol was produced in tissue slices during their incubation in Warburg vessels containing only water and that the RQ under these conditions approached unity. In the presence of 0·1 M fructose, the oxygen uptake by the tissue was little affected while the carbon dioxide

TABLE 1. THE EFFECT OF FRUCTOSE ON GAS EXCHANGE AND ETHANOL PRODUCTION IN CORN SCUTELLUM SLICES*

Bathing solution	Time period (min)	O_2 uptake (μmoles)	CO_2 production (μmoles)	Excess CO_2 (μmoles)	Ethanol production (μmoles)
Water	90	10·7	13·7	3·0	4·4
Water	120	20·5	21·8	1·3	5·3
Fructose (0·1 M)	30	5·0	11·1	6·1	6·7
Fructose (0·1 M)	90	14·3	38·1	23·8	22·7
Fructose (0·1 M)	120	21·4	58·1	36·7	36·2

* Freshly prepared slices were incubated in distilled water at 30° for 90 min. The slices were then washed with distilled water, blotted on filter paper and weighed in groups of 300 mg. Each group of slices was then placed in a Warburg vessel containing either water or fructose, and the gas exchange was measured at 30°. When ethanol production was determined, the slices and the bathing solution were washed into the distilling flask immediately after the manometer reading was obtained.

evolution was greatly increased, giving RQ values ranging from 2·2 to 2·7. It is apparent from Table 1 that the excess carbon dioxide in the presence of fructose resulted from the production of ethanol (compare CO_2 excess to ethanol production). These data clearly show that corn scutellum slices are able to carry out a high degree of aerobic ethanol production, and that exogenous hexose (fructose or glucose) increases the rate of glycolysis in this tissue.

Since prior incubation in water enhanced the effect of fructose in increasing the rate of glycolysis in this tissue, investigations were made to determine the effect of this prior incubation on sucrose synthesis from fructose. Prior incubation of the slices in water for 90 min increased sucrose production 40–50 per cent when the slices were subsequently placed in 0·5 M fructose for a period of 2 hr. When the prior incubation period was extended to 120 min, no further enhancement of sucrose production was observed.

The results given in Table 2 show that there is an equivalence between the amount of hexose passing through glycolysis and the amount of sucrose synthesized. This observation may indicate that the amount of ATP produced in glycolysis controls sucrose synthesis. This postulate would necessarily entail the synthesis of sucrose (utilization of 2 ATP's) rather than sucrose phosphate (utilization of 3 ATP's) and would preclude the availability of ATP

arising from oxidative phosphorylation. Based on the oxygen uptake values presented in Table 2, it was calculated that oxidative phosphorylation could possibly contribute 90–100 μmoles of ATP during the 2-hr incubation period in fructose. Whether this ATP is readily available for sucrose synthesis is not known. In any event, under these experimental conditions, glycolysis occurred at a rate to produce sufficient ATP for the observed sucrose synthesis, and it is significant that a 50 per cent increase in sucrose synthesis could be achieved by prior incubation of the slices in water before fructose treatment without an accompanying increase in oxygen uptake and presumably without an increase in oxidative phosphorylation.

TABLE 2. THE EFFECT OF PRIOR INCUBATION OF SCUTELLUM SLICES IN WATER ON GAS EXCHANGE AND SUCROSE SYNTHESIS*

Scutellum slices (300 mg) in 0·5 M fructose for 2 hr	Prior incubation period in water	
	0	90 min
O_2 uptake (μmoles)	16·6	15·0
CO_2 evolved (μmoles)	43·0	60·2
Hexose through glycolysis (μmoles),† calculated	16·0	25·1
ATP produced in glycolysis (μmoles), calculated	32·0	50·2
Sucrose synthesized (μmoles)‡	16 8	24·9

* The slices (300 mg) were placed in Warburg vessels containing 0·5 M fructose for 2 hr either immediately after their preparation or after a 90 min incubation period in water. The temperature for prior incubation and manometry was 30°.

† These figures are based on the equivalence between the "extra" CO_2 and the ethanol produced and on the assumption that the O_2 uptake resulted from the complete oxidation of hexose.

‡ These data were obtained in separate experiments in which net sucrose production was determined on ethanolic extracts of the slices.

Phosphofructokinase (PFK) of Corn Scutellum

Inasmuch as hexoses (fructose and glucose) were found to greatly increase the rate of aerobic glycolysis in corn scutellum slices, it was of interest to determine whether this action was mediated through some effect on PFK activity. To achieve this end, crude PFK was prepared from corn scutella and its properties compared with those of PFK's from other higher plant sources, including one (carrot slices[6]) which does not appear to show increased aerobic glycolysis in the presence of exogenous glucose.

PFK from corn scutella was markedly inhibited by ATP (Fig. 2), but this inhibition could be partially overcome by increasing the concentration of fructose-6-phosphate (F-6-P). For example, the optimum ATP concentration was 0·67 mM when F-6-P was used at a concentration of 0·2 mM under the experimental conditions shown in Fig. 2. At this level of F-6-P, the presence of 3·33 mM ATP inhibited the enzyme activity by 37 per cent. If, however, the F-6-P concentration was increased to 2·0 mM, the inhibition by 3·33 mM ATP was reduced to 12 per cent. That increasing F-6-P diminishes ATP inhibition of PFK has also been reported for carrot,[5] parsley[4] and possibly avocado.[4] In addition, ATP inhibition could be readily overcome by increasing the Mg^{2+} concentration (Table 3). Similar results were obtained in a study of carrot PFK,[5] but the effects of Mg^{2+} on the ATP-inhibition of parsley and avocado PFK's have not been reported.

[6] H. BEEVERS, *Am. J. Botany* **40**, 91 (1953).

The effects of inorganic orthophosphate (P_i), ADP and AMP on the activity of corn scutellum PFK are shown in Table 4. At non-inhibitory levels of ATP (0·67 mM), P_i did not stimulate PFK activity, but rather caused a progressive inhibition of the rate of reaction as the concentration of P_i was increased from 0 to 33·33 mM. However, in the presence of an

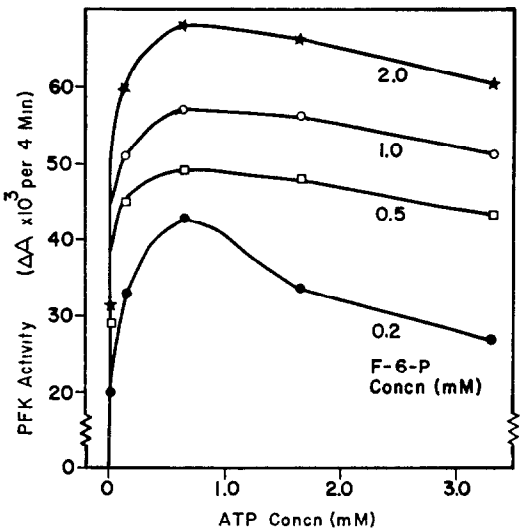

FIG. 2. THE EFFECTS OF ATP AND F-6-P CONCENTRATIONS ON THE ACTIVITY OF CORN SCUTELLUM PFK. In addition to the above, the reaction mixture contained 33·3 mM Tris (pH 7·5), 6·7 mM 2-mercaptoethanol, 6·7 mM $MgCl_2$ and 0·17 mM $NADH_2$. Appropriate amounts of aldolase, phosphohexose isomerase, triose phosphate isomerase and α-glycerophosphate dehydrogenase were mixed together and diluted with Tris buffer (pH 7·5). These enzymes were added in excess to the cuvettes. G-6-P was used as a source of F-6-P. The F-6-P concentration was calculated assuming an equilibrium between F-6-P and G-6-P in which 30 per cent of the G-6-P added to the cuvette was converted to F-6-P. The reaction was started by the addition of 0·1 ml of the diluted, crude PFK preparation to the cuvettes.

TABLE 3. THE REVERSAL OF ATP-INHIBITION OF CORN SCUTELLUM PFK BY MAGNESIUM ION*

	Rate ($\Delta A \times 10^3$ per 4 min)					
	Mg^{2+} conc., mM					
ATP conc., mM	0	3·33	6·67	13·33	20·00	26·67
0·67	1	72	72	76	76	71
6·67	2	3	25	69	71	69

* In addition to the above, the reaction mixture was the same as that given in Fig. 2 with the exception of F-6-P which was used at a 2·0 mM concentration.

inhibitory concentration of ATP (6·67 mM), the addition of P_i partially overcame the inhibition. The addition of 6·67 mM P_i nearly doubled the rate of the ATP-inhibited reaction. Increasing the P_i concentration above this level again resulted in a progressive decrease in the reaction rate. The partial reversal of ATP inhibition of PFK's from carrot[5] and parsley[4] by P_i has been reported previously. Neither ADP nor AMP were found to relieve ATP

inhibition of corn scutellum PFK. On the contrary, these compounds themselves inhibited PFK activity at a non-inhibitory ATP concentration (0·67 mM) and enhanced the inhibition of PFK at an inhibitory level of ATP (6·67 mM). AMP appeared to be a much weaker inhibitor of PFK than ADP. In this regard, the corn scutellum enzyme appears to be similar to the PFK's isolated from the other plant materials previously mentioned.

Dennis and Coultate[5] reported that the PFK from carrot was strongly inhibited by citrate, and that this inhibition could be diminished by increasing the concentrations of F-6-P, Mg^{2+} or P_i, while the inhibition could not be reversed by AMP, ADP and 3′,5′-AMP. Also, citrate was found to act synergistically with ATP in inhibiting carrot PFK. In addition, these

TABLE 4. THE EFFECT OF P_i, AMP AND ADP ON THE ACTIVITY OF CORN SCUTELLUM PFK*

Addition to cuvette	Conc. mM	Rate ($\Delta A \times 10^3$ per 4 min) ATP conc., mM 0·67	6·67
P_i	0	72	24
	6·67	67	44
	13·33	61	41
	20·00	59	35
	26·67	54	33
	33·33	49	29
ADP	0	59	17
	0·17	59	12
	0·67	56	7
	1·67	53	6
	3·33	44	5
	6 67	24	5
AMP	0	69	22
	0·17	67	20
	0·67	67	19
	1·67	66	16
	3·33	65	15
	6·67	58	13

* In addition to the above, the reaction mixtures contained 33·3 mM Tris (pH 7·5), 6·7 mM 2-mercaptoethanol, 6·7 mM $MgCl_2$, 0·17 mM $NADH_2$ and 2·0 mM F-6-P.

investigators found DL-isocitrate to be much less inhibitory than citrate on the enzyme from carrot. The effects of citrate and DL-isocitrate on the activity of PFK from the corn scutellum are shown in Table 5. It is apparent that citrate strongly inhibited corn scutellum PFK and that increasing Mg^{2+} diminished this inhibition. DL-Isocitrate also inhibited the PFK from corn scutellum and appeared to be an even stronger inhibitor than citrate at the lowest concentration tested (3·33 mM). However, at higher concentrations (6·67 and 13·33 mM), citrate inhibited the scutellum PFK more strongly than DL-isocitrate, and the inhibition caused by citrate was more readily reversed by increasing the Mg^{2+} concentration than in the case of inhibition by DL-isocitrate.

The synergism of ATP and citrate in inhibiting corn scutellum PFK is shown in Table 6. At an ATP concentration of 0·17 mM, the presence of 3·33 mM citrate had little if any effect

on the activity of the enzyme while the presence of 6·67 mM citrate inhibited the activity of PFK 36 per cent. If, however, the ATP concentration was increased to 3·33 mM, the PFK activity at citrate concentrations of 3·33 mM and 6·67 mM was inhibited 45 per cent and 90 per cent, respectively. As can be seen, this inhibition was readily reversed by increasing Mg^{2+}. In contrast to the properties of PFK from carrot tissue, P_i had little effect in relieving citrate inhibition of corn scutellum PFK. Under the experimental conditions given in

TABLE 5. THE INHIBITION OF CORN SCUTELLUM PFK BY CITRATE AND DL-ISOCITRATE AND ITS REVERSAL BY MAGNESIUM ION*

Organic acid added to cuvette	Mg^{2+} conc. (mM)	Percent inhibition of PFK activity		
		Conc. of acid, mM		
		3·33	6·67	13·33
Citrate	6·67	3	36	95
	13·33	—	—	66
	26·67	—	—	16
DL-Isocitrate	6·67	19	28	52
	13·33	—	—	39
	26·67	—	—	35

* In addition to the above, the reaction mixture contained 33·3 mM Tris (pH 7·5), 6·7 mM 2-mercaptoethanol, 0·17 mM ATP, 0·17 mM $NADH_2$ and 1·0 mM F-6-P. Citrate was obtained in the free acid form and was neutralized with 10 per cent KOH before it was added to the reaction mixtures. DL-Isocitrate was obtained and used as the trisodium salt. The per cents of inhibition given above were based on reaction rates of controls run simultaneously without addition of the acids.

TABLE 6. SYNERGISM OF ATP AND CITRATE IN INHIBITING CORN SCUTELLUM PFK AND THE REVERSAL OF INHIBITION BY MAGNESIUM ION*

ATP conc. (mM)	Mg^{2+} conc. (mM)	PFK activity ($\Delta A \times 10^3$ per 4 min)		
		Citrate conc., mM		
		0	3·33	6·67
0·17	6·67	59	57	38
3·33	6·67	62	34	6
3·33	13·33	66	—	55
3·33	20·00	65	—	63

* In addition to the above, the reaction mixtures contained 33·3 mM Tris (pH 7·5), 6·7 mM 2-mercaptoethanol, 0·17 mM $NADH_2$ and 1·0 mM F-6-P.

Table 5, a concentration of 6·67 mM citrate inhibited PFK activity between 33 and 36 per cent. This inhibition was slightly reduced (to 29 per cent) in the presence of 6·67 mM P_i, and no reduction of citrate inhibition was observed at a concentration of 20·00 mM P_i.

In preliminary studies of corn scutellum PFK it was determined that GTP could also serve as a phosphate donor in the PFK-catalyzed reaction, but that this compound would only support about two-thirds the reaction rate observed when an equivalent amount of ATP was used. GTP was also found to be inhibitory at high concentrations. This is in

contrast with results obtained for yeast PFK, inasmuch as GTP was a substrate for yeast PFK but did not inhibit the enzyme at higher concentrations.[7]

Levels of Metabolic Intermediates During Periods of Low and High Rates of Glycolysis

In view of the fact that a strong aerobic fermentation could be triggered in corn scutellum slices by the addition of either fructose or glucose, it was of interest to investigate the levels of certain metabolic intermediates (some of which have been shown to affect PFK activity) under conditions of both low and high rates of glycolysis. These results are shown in Table

TABLE 7. THE LEVELS OF CERTAIN METABOLIC INTERMEDIATES IN INTACT SCUTELLA, FRESHLY PREPARED SCUTELLUM SLICES, AND SCUTELLUM SLICES FOLLOWING THEIR INCUBATION IN EITHER WATER OR FRUCTOSE*

	Content of intermediate (μmoles/g fresh wt.)			
	Intact scutella	Freshly prepared slices	Following 90 min incubation in:	
Intermediate			Water	Fructose (0·1 M)
Citrate	—	2·40	1·60	1·64
P_i	—	15·84	15·73	15·11
		15·84	15·95	16·57
G-6-P	1·20	1·23	0·95	1·64
	1·09	1·12	0·89	1·45
F-6-P	0·19	0·23	0·16	0·32
	0·17	0·21	0·16	0 30
FDP	0·33	0·15	<0·05	<0·05
	0·20	0·14	<0·05	<0·05
ATP	0·34	0·30	0·35	0·30
	—	0·30	0·34	0·30

* Freshly prepared slices (1 g fresh wt.) were incubated at 30° for 90 min, after which they were removed from the bathing mediums and washed thoroughly with distilled water. The citrate contents of the slices were determined by assay of ethanolic extracts while the other intermediates were assayed in TCA extracts. With the exception of citrate, the data given above represents the results of duplicate experiments utilizing 1 g quantities of slices In the case of citrate, duplicate experiments were run but the slices were pooled to give the 2 g sample necessary for assay. In the case of whole scutella, neither citrate nor P_i were determined and only one ATP determination was made.

7. As can be seen, the levels of citrate, P_i, ATP and fructose-1,6-diphosphate (FDP) found in the slices which were incubated in water were essentially the same as those found in the slices which were incubated in 0·1 M fructose although in the latter case glycolysis was taking place at a much faster rate (four times greater). The most pronounced effect of exogenous fructose on the levels of metabolic intermediates was in increasing the tissue levels of glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P).

Data for the intact corn scutellum are also presented in Table 7 to show that the metabolic activity of the intact scutellum differes from that of the freshly prepared tissue slices, presumably as a result of the restricted oxygen availability in the intact scutellum. This thesis is

[7] E. VIÑUELA, M. L. SALAS and A. SOLS, *Biochem. Biophys Res Commun* **12**, 140 (1963).

reinforced by the fact that the oxygen uptake of intact corn scutella was found to be only 40 per cent that of an equivalent weight of tissue slices when they were incubated in water, and the RQ for intact scutella was near 3, while the RQ for the tissue slices approached unity. The values for the metabolic intermediates in freshly prepared tissue slices (Table 7) can perhaps best be regarded as transient levels as the tissue passes from a state of restricted oxygen availability to a state of greater oxygen availability. It will be observed in Table 7, that the G-6-P/F-6-P ratio for the tissue slices incubated under both regimes was different from that expected to be found as a result of the phosphohexose isomerase-catalyzed reaction equilibrium. This variance was observed repeatedly throughout these experiments as well as many others not reported in this paper. This may indicate the existence of a G-6-P pool within a region of the cell isolated from phosphohexose isomerase.

In order to determine the effect of endogenous ATP levels on the rate of glycolysis, and to test the postulate of ATP-inhibition of PFK *in vivo*, additional experiments were conducted.

TABLE 8. ENDOGENOUS ATP LEVELS AND GAS EXCHANGE IN CORN SCUTELLUM SLICES*

Bathing solution	Tissue ATP† (μmoles/g)	O_2 uptake (μmoles)	CO_2 production (μmoles)	Excess CO_2 (μmoles)
(A) Water	0·5–0·6	11·8	13·0	1·2
Fructose (0·5 M)	0·5–0·6	7·3	27·2	19·9
(B) Water	0·9–1·0	9·9	10·9	1·0
Fructose (0·5 M)	0·9–1·0	8·2	28·6	20·4

* Freshly prepared slices were incubated at 30° in distilled water (A) or 0·01 M adenosine (B) for 90 min. At the end of this period, the slices were washed in water, blotted on filter paper and weighed in groups of 300 mg. The slices were placed in Warburg vessels containing either water or fructose and gas exchange was measured at 30° for 60 min.

† In separate experiments, the ATP levels of the slices were determined after 90 min incubations in 0·01 M adenosine (high ATP level) or water (low ATP level).

In preliminary studies it was noted that incubation of corn scutellum slices in solutions containing adenosine caused an increase in the endogenous ATP. Thus tissue slices were incubated for a 90-min period in either adenosine or water before they were placed in manometer vessels containing either water or 0·5 fructose and the gas exchange determined. The results of these studies are given in Table 8. It is clear from these data that elevation of the level of endogenous ATP had no effect on the gas exchange of the tissue. It will be observed that the ATP levels of the tissue slices which were prior incubated in water were somewhat higher than those previously shown for water-incubated tissue slices (Table 7). This variability is thought to be the result of some differences in the physiological age of the seedlings used in these experiments, and while quantitative results have varied to some degree, qualitative relationships have been constant throughout these studies. Measurements of ADP and AMP following treatment with adenosine were not made since neither compound is effective in diminishing ATP inhibition. It should also be mentioned that incubation of tissue slices in adenosine plus fructose did not elevate the endogenous levels of G-6-P and F-6-P above the levels found in the tissue slices when they were incubated in fructose alone.

DISCUSSION

The possible role of PFK as a regulator or "pacemaker" for glycolysis in tissue slices of the corn scutellum was studied. Such a role has been assigned, based on *in vitro* studies, for the PFK of carrot,[5] a tissue which shows no aerobic fermentation in the presence of exogenous glucose.[6] *In vitro* studies have shown corn scutellum PFK to be similar in its properties to the enzyme isolated from carrot with the exception that citrate-inhibition of corn scutellum PFK is not as strongly reversed by P_i as citrate-inhibition of PFK from carrot. The levels of certain metabolic intermediates were assayed in scutellum slices which were subjected to regimes giving essentially no aerobic fermentation (lower glycolytic rate) and regimes which resulted in a very strong aerobic alcoholic fermentation (greatly increased glycolytic rates). The tissue levels of certain intermediates known to influence the activity of PFK *in vitro* (citrate, P_i, FDP and ATP) were essentially the same under both regimes (Table 7). Although citrate was found to be a potent inhibitor of scutellum PFK, it appears that citrate-inhibition of the enzyme is not a factor in regulating the rate of glycolysis in this instance, provided there is an equivalent intracellular distribution of the acid in the tissues from both treatments. It has also been shown that corn scutellum PFK is strongly inhibited by high levels of ATP and that this inhibition could be partially relieved by P_i. The fact that the P_i contents of the slices did not differ under the two regimes would make it unlikely that PFK activity was enhanced by a P_i-mediated reversal of ATP inhibition, again provided there was an equivalent distribution of P_i within the cells of the tissues from the two treatments. In addition, incubation of the tissue slices for 90 min in 0·05 M KH_2PO_4 had no effect on gas exchange when the slices were subsequently incubated in water alone or in 0·5 M fructose.

The most pronounced effect of exogenous fructose on the levels of metabolic intermediates was in increasing the tissue levels of both G-6-P and F-6-P. This observation may provide some basis for postulating a control mechanism for the rate of glycolysis at the PFK-catalyzed reaction. At the present time there appears to be no way of determining the ATP concentration at the point of PFK activity *in vivo*, and to suggest that ATP inhibition occurs at all under either treatment would be at best speculative. If, however, ATP inhibition of PFK does have a role in regulating the rate of glycolysis in these tissue slices, an increase in F-6-P might account for an increase in the rate of the PFK-catalyzed reaction thus increasing the overall rate of glycolysis. Following this line of thought, the rates of ATP-inhibited, PFK-catalyzed reactions would increase with increases in the F-6-P/ATP ratio since it has been established previously that this hexose phosphate diminishes ATP-inhibition of scutellum PFK. It may be ascertained from Table 7 that the F-6-P/ATP ratio doubled in the presence of exogenous 0·1 M fructose (0·5 for water incubation and 1 for fructose treatment). However, the data presented in Table 8 do not support ATP-inhibition of PFK activity in the control of glycolytic rate. Approximately doubling the ATP content of tissue slices by incubation in adenosine prior to gas exchange measurements did not increase the rate of glycolysis above the rate observed when the slices were incubated in water prior to gas exchange measurements. Also, incubation of tissue slices in adenosine plus fructose did not elevate the endogenous levels of G-6-P and F-6-P above the levels found when the slices were incubated in fructose alone. Thus, provided some of the ATP produced as a result of adenosine treatment is available within the glycolytic volume of the cell, the F-6-P/ATP ratio would decrease and any ATP-inhibition of PFK would be enhanced (the rate of glycolysis curtailed). That this occurs under the experimental conditions given above is not supported by the observed results.

Inasmuch as incubation of tissue slices in fructose does increase the tissue contents of G-6-P and F-6-P as well as the rate of glycolysis, it may be argued that the rate of glycolysis is increased merely by an increase in readily available substrate for PFK. We fail to see, however, how doubling the F-6-P level of the tissue would result, in this instance, in a 4- to 7-fold increase in glycolytic rate. While the control of glycolysis by PFK in higher plants has been inferred by *in vitro* studies of the enzyme and the subsequent comparison of the properties of plant PFK to the PFK's from various mammalian sources,[4, 5] we do not believe that our data support a regulatory role for this enzyme in controlling the rate of glycolysis in corn scutellum slices.

Barker *et al.*[8, 9] have suggested that the sugar phosphorylating and glycolytic enzymes of pea are located in an organized structure or organelle which is more permeable to ADP than to ATP, and that the increased rate of glycolysis in anoxia could be explained by a faster production of "glycolytic" ATP and a hastening of the phosphorylation of both glucose and F-6-P. These authors also review additional evidence for the glycolytic structure.[9] It is quite possible that a highly organized glycolytic system, perhaps even an organelle, is present in the corn scutellum. Assuming this to be true, we propose the following mechanisms for the control of glycolysis in corn scutellum slices. During the incubation of the slices in water, "glycolytic" ATP must move from the region of glycolysis to other regions of the cell where it is utilized with the formation of ADP and P_i. These compounds in turn must diffuse back to the glycolytic structure. The rate at which ADP becomes available for phosphoglyceric kinase and pyruvic kinase could control the rate of glycolysis at the level of these enzymes. In addition, the rate of movement of P_i back into the glycolytic area could control the rate of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase catalyzed reaction. However, since these control points all occur after the PFK-catalyzed reaction one would not expect to find the extremely low level of FDP observed during these studies (Table 7). In addition, a decrease in the levels of hexose monophosphates would not be expected to occur. However, in a previous study,[10] hexose monophosphate levels steadily decreased with time (up to 4 hr) in tissue slices incubated in water. These observations suggest a control step prior to hexose phosphorylation. During incubation of tissue slices in water, hexose entering glycolysis comes from the breakdown of stored sucrose. This utilization of stored sucrose has been shown to occur at a rate of 5–6 μmoles/hr/g fresh wt.[11] It is possible that a control for glycolysis mediated through substrate availability exists at the point of sucrose breakdown, and that the hydrolysis of sucrose itself may be coupled with its removal from storage. We believe that this breakdown of sucrose is dependent on either inorganic ortho- or pyrophosphate, and have demonstrated that incubation of tissue slices in 0·05 M KH_2PO_4 increased the levels of hexose monophosphates (a 3-fold increase in 180 min) without affecting the rate of glycolysis. This observation reinforces the suggestion that dual control mechanisms exist for the regulation of glycolysis in tissue slices incubated in water.

We further postulate that sucrose synthesis takes place either within the glycolytic structure or within a region of the cell proximate to the glycolytic system, and that these regions of the cell are within the "hexose space" which makes up about 12 per cent of the water volume of the cell.[12] During incubation of the tissue slices in fructose, utilization of ATP for sucrose synthesis would increase the concentrations of ADP and P_i within the glycolytic

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[9] J. Barker, M. A. A. Khan and T. Solomos, *Nature* **211**, 547 (1966).
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structure thus increasing the rate of glycolysis. Therefore, the increase in the rate of glycolysis during sucrose synthesis (incubation in fructose) would be due to a change in the cellular distribution of adenine nucleotides and P_i and not necessarily due to changes in tissue levels of these compounds.

EXPERIMENTAL

Plant Material

Corn grains (*Zea mays* L., var. Funks G-76) were soaked in running tap-water for 24 hr and then placed on moist filter paper in the dark at 24–25° for 72 hr. The scutella were excised from the germinating grains and cut transversely into slices 0·5 mm or less in thickness. The slices were washed in distilled water until the wash water remained clear, and then were blotted on filter paper and weighed in groups of 1 g. These scutellum slices were used immediately for experiments involving incubation of the tissue under various regimes. In instances where larger amounts of material were required for the preparation of enzyme extracts, the whole scutella were excised and immediately frozen without slicing. In order to obtain sufficient material for these preparations, it was necessary to collect whole scutella over a period of several months during which time storage temperature was maintained at −20°.

Preparation of Phosphofructokinase

Frozen, whole scutella (100 g) were ground in a Waring blender with 125 ml of an ice-cold solution containing 0·25 M sucrose, 0·05 M Tris (pH 7·5) and 0·01 M EDTA. After grinding the homogenate was washed into a beaker with an additional 25 ml of the above solution and was allowed to stand in the cold for 10–15 min. The homogenate was then centrifuged in the cold at 21000 × *g* for 30 min after which the supernatant fraction was decanted through glass wool. The supernatant fraction was dialyzed overnight against 2 l. of 0·05 M Tris buffer (pH 7·5) with one change of dialyzing medium. Following dialysis the extract was again centrifuged at 21,000 × *g* for 30 min, and the supernatant fraction was immediately subjected to ammonium sulfate fractionation. Centrifugation (10,000 × *g* for 20 min) was carried out after each addition of salt, and the precipitates taken up in a small volume of 0·05 M Tris buffer (pH 7·5). The various fractions were dialyzed against 2 l. of 0 05 M Tris (pH 7·5) for 24 hr with one change of dialyzing medium. The fractions were subsequently centrifuged at 11,000 × *g* for 25 min and the supernatant fractions decanted into plastic tubes and frozen until they could be assayed for PFK activity. Preliminary studies showed that the protein that precipitated upon increasing the ammonium sulfate content from 20 per cent to 40 per cent saturation contained the major portion of the PFK activity. This fraction was again treated with ammonium sulfate, and the protein precipitating as the salt content was increased from zero to 30 per cent saturation was collected by centrifugation. This precipitate was taken up in a small volume of 0·01 M Tris (pH 7·5), and the fraction was dialyzed overnight against 2 l. of 0·002 M Tris (pH 7·5) with one change of bathing medium. This preparation was found to contain virtually all the PFK activity of the original 20–40 fraction. This preparation was divided into small aliquots (0·2 ml) which were immediately frozen in plastic tubes and stored at −20°. The preparation was found to be quite stable under these conditions and no loss of activity could be detected for a period of several months. Before use the enzyme preparation was diluted 21-fold with 0·12 per cent bovine serum albumin. Upon dilution the enzyme slowly lost activity; however, fresh dilutions were made frequently during these experiments.

The activity of PFK was assayed by determining the rate of formation of FDP. The rate of formation of this intermediate was determined by following the oxidation of $NADH_2$ in the presence of excess quantities of aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase $NADH_2$ oxidation was followed at 340 nm using a Gilford Model 220 spectrophotometer. The volume of each reaction mixture was 3·1 ml. Specific conditions of assay are given in the tables and figures of the Results section of this paper.

Measurement of Metabolic Intermediates

Measurements of certain metabolic intermediates were made following incubation of tissue slices with either water or 0·1 M fructose. Each group of slices (1 g fresh wt.) was placed in a 25 ml Erlenmeyer flask containing 10·0 ml of the appropriate bathing medium. Incubation of the slices was conducted at 30° in a "Gyrotory" water bath (New Brunswick Scientific Company, New Brunswick, N.J.) set to provide approximately 180 rev/min. Incubation was usually carried out for a period of 90 min after which the slices were removed from the bathing solutions, washed thoroughly with distilled water, blotted on filter paper and extracted with either ethanol or cold TCA. Any deviations from the above procedure are given in the figures and tables of the Results section of this paper.

The tissue levels of P_i, G-6-P, F-6-P, FDP and ATP were determined following the extraction of the tissue slices in cold TCA. Each group of slices (1 g fresh wt.) were ground thoroughly in an all glass, motor-driven homogenizer with 5·0 ml of cold 10% TCA An additional 5 0 ml of cold TCA was used to wash the homo-

genate into a plastic centrifuge tube. The extracts were allowed to stand in the cold for 10–15 min before they were centrifuged (10,000 × *g* for 20 min at 0°). The supernatant fluids were collected and freed of TCA by ether extraction. Ether was removed from the extracts with a stream of nitrogen. The extracts were then brought to approximately pH 7·5 by the addition of 10% KOH and 0·1 ml of 0·5 M $MgCl_2$ was added for the precipitation of phytate. The extracts were then centrifuged for approximately 5 min in a clinical centrifuge and the supernatant fluids decanted into plastic tubes and frozen until they could be assayed. All operations during the preparation of TCA extracts were performed at 2–3°.

G-6-P and F-6-P were assayed by measuring the amount of $NADPH_2$ formed in the presence of the scutellum extract and the proper combination of G-6-P dehydrogenase and phosphoglucose isomerase. This system has been reported in a previous paper.[10] The assay of ATP was accomplished using the same procedure with the exception that glucose (22 μmoles) was initially added to the cuvette and excess hexokinase added to the reaction mixture following the complete oxidation of the hexose monophosphates.

P_i was measured by the procedure of Gomori.[13] In these determinations the TCA was not removed from the extracts. Known samples of P_i used to establish a standard curve also contained TCA in an amount equivalent to that found in the tissue extracts.

FDP was measured by determining the oxidation of $NADH_2$ in the presence of the scutellum extract and appropriate amounts of aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase. In addition to the extract and enzymes, the reaction mixture (3·1 ml) contained 33 mM Tris buffer (pH 7·5) and 0·17 mM $NADH_2$. The decrease in absorbance (A) at 340 nm resulting from $NADH_2$ oxidation was measured using a Gilford Model 220 spectrophotometer.

The citrate content of the tissue slices was determined by assay of ethanolic extracts. The slices (2 g fresh wt.) were placed in a 50 ml beaker and killed by the addition of 25 ml of boiling 80% ethanol, the boiling was continued for 30 sec, and after 1 hr at room temperature the ethanol extract was decanted. This was repeated with 50% ethanol and the slices were then washed with three 5-ml portions of 80% ethanol. The combined extracts were taken to dryness under reduced pressure and the residue removed from the drying flasks by washing with three 5-ml portions of distilled water (the second portion of water contained 1 drop of *N* HCL). The washings were combined and centrifuged at 10,000 × *g* for 20 min. The supernatant fluid was assayed for citrate by the method of Luke and Freeman[14] modified by Shiralipour.[15]

Gas Exchange

The manometric experiments were conducted in the Warburg apparatus at 30°. The "direct method" for the measurement of carbon dioxide was used.[16]

Ethanol Determination

When ethanol production was to be determined following the gas exchange experiments, the tissue slices and bathing medium were washed into an alcohol distilling flask and made alkaline with KOH. The distillate was collected in a cold receiving tube until essentially all the alcohol had been cleared from the distilling flask contents. The volume of the distillate was recorded and an aliquot taken for ethanol determination. Ethanol was determined enzymatically using a commercially available kit based on alcohol dehydrogenase (Sigma Chemical Company, Technical Bulletin No. 330).

Sucrose Determination

The sucrose contents of the tissue slices were determined by analyzing ethanolic extracts for glucose before and after treatment with invertase (The British Drug Houses Ltd., Laboratory Chemicals Division, Poole, England). Glucose was determined by the glucose oxidase method (Glucostat, Worthington Biochemical Corp., Freehold, N.J.).

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