Plant Physiol. (1971) 47, 48–52

Gluconeogenesis in the Castor Bean Endosperm

I. CHANGES IN GLYCOLYTIC INTERMEDIATES

Received for publication July 13, 1970

MILOS J. KOBR[1] AND HARRY BEEVERS[2]
Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT

The control points of the Embden-Meyerhof-Parnas pathway in germinating castor bean (*Ricinus communis*) endosperms are sought in two ways: (a) by measuring the amounts of various glycolytic intermediates at intervals during the germination; (b) by determining the crossover points appearing during anoxia.

A significant departure from thermodynamic equilibrium between substrates and products is found at the level of fructose 1,6-diphosphatase and phosphofructokinase. A definite shift of this ratio is observed at the onset of active gluconeogenesis. The concentrations of phosphoenolpyruvate and 3-phosphoglyceric acid increase at the same time. Another departure from the expected equilibrium is also observed at the level of the pyruvate kinase.

The imposition of anoxia on 5-day-old endosperms reveals two crossover points, at the level of the same enzymes. It is therefore concluded that they regulate the glycolytic flow.

The maximal glycolytic flow, however, is only $\frac{1}{10}$ of the gluconeogenic one. To account for this high gluconeogenic efficiency, it is postulated that gluconeogenesis and glycolysis occur in separate intracellular regions. The consistent departure from equilibrium between adenylates observed during the early stages of anoxia supports the concepts that the pools of glycolytic and gluconeogenic intermediates are indeed compartmented and that the two processes are independently regulated.

In seedlings of castor bean (*Ricinus communis*) growing at 30 C, the conversion of fats to carbohydrates starts after the 3rd day of germination. This gluconeogenic process can be divided into three steps: (*a*) oxidation of fatty acids to yield acetyl-CoA, (*b*) conversion of acetyl-CoA to oxaloacetate, which then yields PEP[3] and CO_2, and (*c*) reversal of the Embden-Meyerhof-Parnas glycolytic sequence from PEP to G6P and subsequent formation of sucrose (5). Aspects of sequence *c* are examined in this paper.

During the most active phase of fat breakdown (day 5) sucrose accumulates at a rate of more than 3 mg/hr per seedling, implying a gluconeogenic flux equivalent to some 18 μmoles of hexose per hr in the endosperm tissue. It might therefore be anticipated that the concentrations of some glycolytic intermediates and cosubstrates *in vivo* would be distinctively different from those observed in most other tissues where the glycolytic flux is in the downward direction, *i.e.*, toward pyruvate.

In this work the levels of measurable intermediates of the sequence in the endosperm tissue have been determined at daily intervals during germination, to include periods before and after the stage of rapid sucrose accumulation. Control points were sought in two ways: (*a*) by comparing observed concentrations of substrates and products at particular enzyme steps with those predicted, and (*b*) by pinpointing crossover points following transfer to anaerobic conditions, when gluconeogenesis is prevented and a glycolytic flux toward pyruvate is induced.

It is recognized that in normal glycolysis prime regulatory points are offered at early and late irreversible reactions, namely those catalyzed by PFK and pyruvate kinase, and there is evidence that control is exerted at these levels in a variety of plant tissues (10, 14, 19). In gluconeogenesis from fats, the available evidence shows that entry into the Embden-Meyerhof-Parnas pathway occurs at the level of phosphoenol pyruvate (4) and that a specific FDPase (21, 23) circumvents the PFK reaction and allows the net production of sucrose. Particular attention was paid to changes in the content of intermediates and cosubstrates at these levels during germination and in anoxia.

MATERIALS AND METHODS

Plant Material. Seeds of *Ricinus communis* (var. Cimmaron) were soaked for about 24 hr and grown in vermiculite at 30 C for various periods of time. The seedlings were carefully selected for uniformity. After removal of the cotyledons, four endosperms were extracted and analyzed as described below. For the anaerobic experiments four endosperms were placed in small vessels kept at 30 C and flushed with moist nitrogen for various periods of time. A set of endosperms was kept in air and used as a control.

Preparation of the Extracts. After the prescribed time of anoxia, the endosperms were immediately frozen in liquid nitrogen and ground at −18 C in 2.0 ml of 4.8 M perchloric acid in a conical glass homogenizer. The proteins were sedimented by centrifuging at 27,000*g* for 30 min. Then 0.1 ml of the supernatant was titrated to neutrality with 2 M $KHCO_3$ or 1 M K_2CO_3, using bromocresol purple as an indicator. The bulk of the extract was neutralized accordingly with $KHCO_3$ or K_2CO_3 and the precipitate was centrifuged off. The neutralized extract was then analyzed immediately or stored at −20 C for no longer than 3 days before analysis.

This extraction procedure recovers more than 90% of the intermediates, even from young endosperms whose high fat content complicates the sampling. Recovery experiments with ATP, G6P, and FDP yielded, respectively, 100, 98, and 90% of an added amount of standardized intermediate, after deduction of the endogenous level.

[1] Present address: Central Research Department, E. I. du Pont de Nemours & Co., Experimental Station, Wilmington, Del. 19898.

[2] Present address: Division of Natural Sciences, University of California, Santa Cruz, Calif. 95060.

[3] Abbreviations: DHAP: dihydroxyacetone phosphate; G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; FDP: fructose 1,6-diphosphate; FDPase: fructose 1,6-diphosphatase; PEP: phosphoenolpyruvate; 3-PGA: 3-phosphoglyceric acid; Py: pyruvate; PFK: phosphofructokinase.

Analytical. All intermediates were analyzed by standard enzymatic methods adapted from Bergmeyer (5). The specific conditions are given below. The changes of reduced phosphopyridine nucleotides were followed at 340 nm in a Beckman DU spectrophotometer. The concentrations of the ancillary enzymes were chosen so as to produce first order reactions with a half-time between 0.5 and 2 min. Pyruvate kinase, used in the assay for ADP, also functions with uridine diphosphate as acceptor (18). Chromatographic analysis (12) showed that endosperm tissue, whether aerobic or anaerobic, did not contain significant amounts of UDP.

Enzymatic Analyses.

G6P: 0.12 M triethanolamine buffer (pH 7.8); 0.3 mM NADP; 0.2 μg/ml glucose 6-phosphate dehydrogenase.

F6P: Same plus: 2.0 μg/ml phosphoglucoisomerase.

DHAP: 0.12 M imidazole buffer (pH 6.9); 0.12 mM NADH; 10 μg/ml glycerophosphate dehydrogenase.

FDP: Same plus: 2 μg/ml triosephosphate isomerase; 2 μg/ml aldolase.

3-PGA: 0.12 M imidazole buffer (pH 7.5); 5.0 mM $MgCl_2$; 1.0 mM ATP; 5.0 mM mercaptoethanol; 0.12 mM NADH; 25 to 30 μg/ml glyceraldehyde phosphate dehydrogenase; 1.2 μg/ml phosphoglyceric kinase.

Py: 0.12 mM phosphate buffer (pH 6.9); 0.12 mM NADH; 6 μg/ml lactate dehydrogenase.

PEP: Same plus: 5.0 mM $MgCl_2$; 0.2 mM ADP; 2.0 μg/ml pyruvate kinase.

ATP: 0.12 M triethanolamine buffer (pH 7.8); 5.0 mM $MgCl_2$; 1.0 mM glucose; 0.3 mM NADP; 0.5 μg/ml glucose 6-phosphate dehydrogenase; 2.0 μg/ml hexokinase.

ADP: 0.12 M phosphate buffer (pH 6.9); 2.0 mM $MgCl_2$; 0.5 mM ATP; 0.5 mM PEP; 0.12 mM NADH; 10 μg/ml lactic dehydrogenase; 0.5 μg/ml pyruvate kinase.

AMP: Same plus: 1.0 μg/ml myokinase.

Lactate: According to Holzer and Soling (5).

Ethanol: According to Bonnichsen (5).

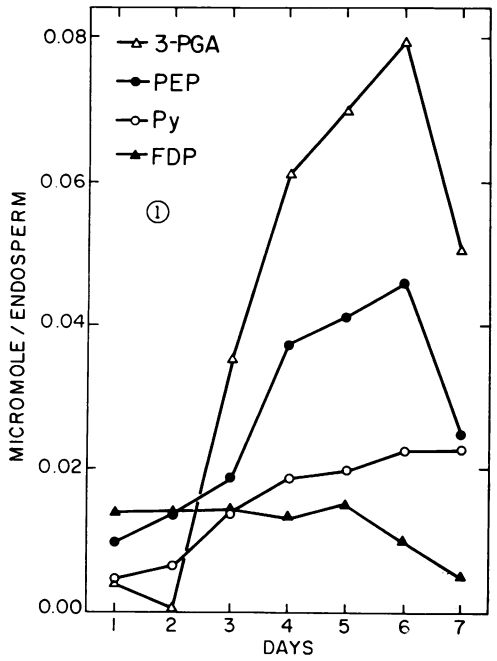
RESULTS

The levels of several intermediates and cosubstrates were measured at daily intervals (Figs. 1 to 3). As shown, particularly after 3 day, there is a marked increase in the amounts of all of the metabolites, except for pyruvate and FDP. The most striking increases are shown by PGA, ATP, and ADP, which peak at the 6th day; the hexose monophosphates peak at day 5. Throughout the 7-day period the level of ATP was three to four times that of ADP (Fig. 2). At all times the amount of PEP exceeded that of Py, and a sharp increase in PEP occurred at day 4 (Fig. 1).

The relationships between concentrations of substrates and products of three enzyme steps are shown in Table I. The G6P/F6P ratio remains remarkably constant throughout the 7-day period. The average value of 2.4 is close to that (2.5–3.1) predicted from thermodynamic considerations (13). By contrast, the ratio at the level of F6P-FDP shows a marked fall after day 3, *i.e.*, at the time of onset of rapid gluconeogenesis. At all times, however, the ratio departs by several orders of magnitude from the theoretical value of 1.2×10^3 (8). The ratio which is maintained between substrates and products of pyruvate kinase again is very different from that predicted (3.5×10^3) and remains fairly constant throughout. There is no striking change in the value after day 3, although, as shown in Figure 1, the content of PEP increases sharply at this time, coincidentally with the onset of gluconeogenesis.

Estimation of Maximal Glycolytic Flux. As emphasized earlier, gluconeogenesis prevails in the endosperm from 5-day-old endosperm, and the CO_2 output from such material originates primarily from the conversion of oxaloacetate to phosphopyruvate (4). An estimate of the maximal possible glycolytic flux was made by transferring this tissue to N_2 and measuring the amounts of ethanol and lactate produced (Fig. 4). After a short lag ethanol accumulates at a rate some four times that of lactate; the rate of hexose utilization inferred from the figure is some 1.8 μmoles per endosperm per hr. While this rate is 7-fold greater than that observed in a similar experiment with 2-day-old endosperm tissue, it is only $\frac{1}{10}$ of the rate of hexose synthesis (18 μmoles/hr) observed when the tissue is in air.

Effects of Anoxia on Glycolytic Intermediates. The imposition of anaerobic conditions results in striking changes in the adeny-



FIGS. 1 to 3. Developmental changes of some hexose and triose phosphates, adenylates, and pyruvate in germinating castor bean endosperm.

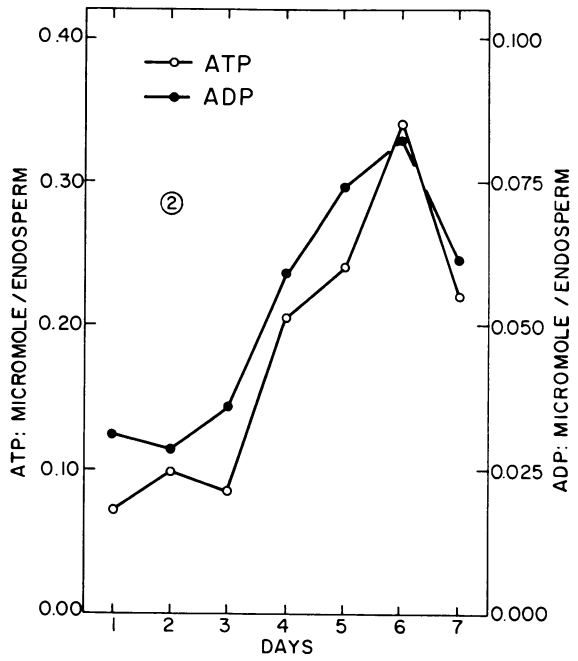


FIG. 2

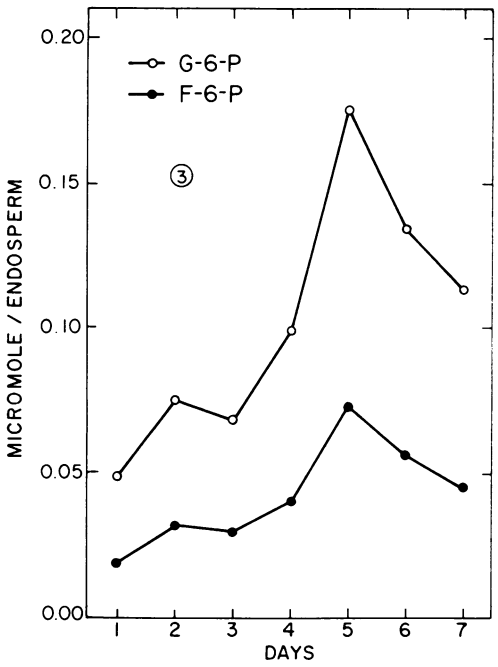


FIG. 3

Table I. *Relationships among G6P-F6P, F6P-FDP, and PEP-Py in the Castor Bean Endosperm during Germination*

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
$\frac{G6P}{F6P}$	2.7	2.3	2.3	2.5	2.4	2.4	2.5
$\frac{(FDP)\ (ADP)}{(F6P)\ (ATP)}$	0.35	0.17	0.29	0.09	0.02	0.04	0.03
$\frac{(Py)\ (ATP)}{(PEP)\ (ADP)}$	1.1	2.0	1.8	1.7	1.6	2.0	3.0

late levels in the 5-day-old endosperm (Fig. 5). Within 15 min the ATP level drops to one-third of the value in air and remains stationary thereafter. Only during the first 5 min is there a corresponding increase in ADP and AMP, and this does not fully account for the fall in ATP. When equilibrium is reached, after 30 min, AMP has returned to its aerobic level and there has been a net loss of 0.15 μmole of ATP and of 0.03 μmole of ADP. The fall in ATP level is a characteristic feature of aerobic tissues made anoxic. However, the changes in ADP and AMP observed here are of relatively small amplitude and quite transitory. This contrasts with the pattern observed in animal tissues (20), other higher plant tissues (6), and lower fungi (17).

Chromatographic analysis on Dowex with formate elution showed that no significant accumulation of inosine monophosphate occurred in N_2; we have not investigated the possibility that deamination of AMP was followed by hydrolysis of inosine monophosphate.

The relative amounts of the three adenylates in aerobic conditions (Table II) are close to those predicted if the myokinase equilibrium was established (1). However, the ratio (ATP) (AMP)/(ADP)^2 declined on transfer to N_2 and reached its

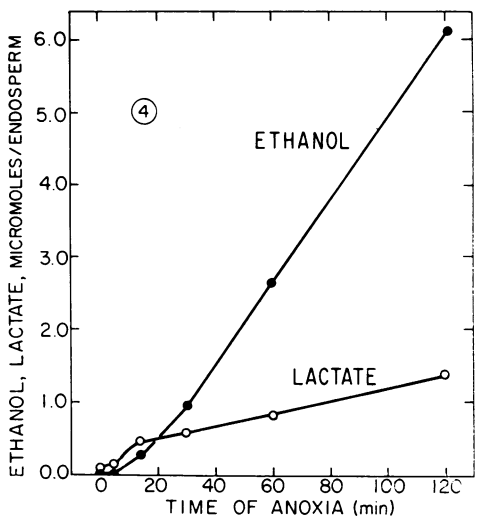


FIG. 4. Time course of ethanol and lactate accumulation in anoxic 5-day-old castor bean endosperm.

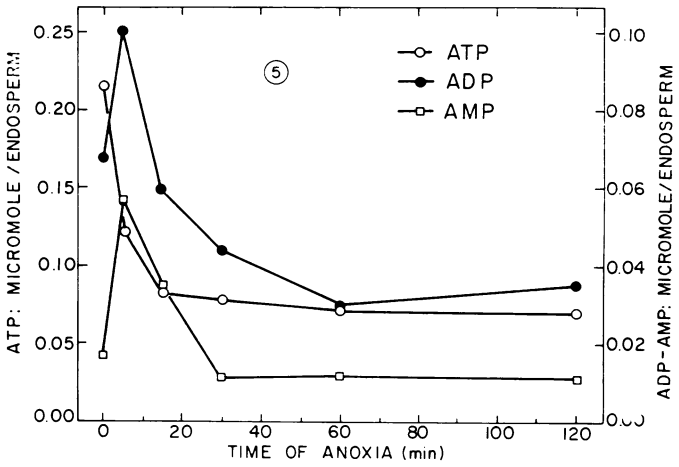


FIG. 5. Time course of adenylate fluctuations in anoxic 5-day-old castor bean endosperm.

Table II. *Relationships between Adenylates in 5-day-old Endosperm in N_2*

Time of Anoxia	$\frac{(AMP)\ (ATP)}{(ADP)^2}$	
	Experiment I	Experiment II
min		
0	0.59	0.62
5	0.40	0.25
15	0.33	0.12
30	0.50	0.39
60	0.41	0.41
120	0.37	0.40

lowest point after 15 min. After 2 hr of anoxia, it was still significantly lower than the aerobic level.

The levels of hexose and triose phosphates are also markedly affected by anoxia (Fig. 6). G6P and F6P fall immediately, but the equilibrium between them is retained. DHAP and FDP rise considerably above the air values and return to new equilibrium

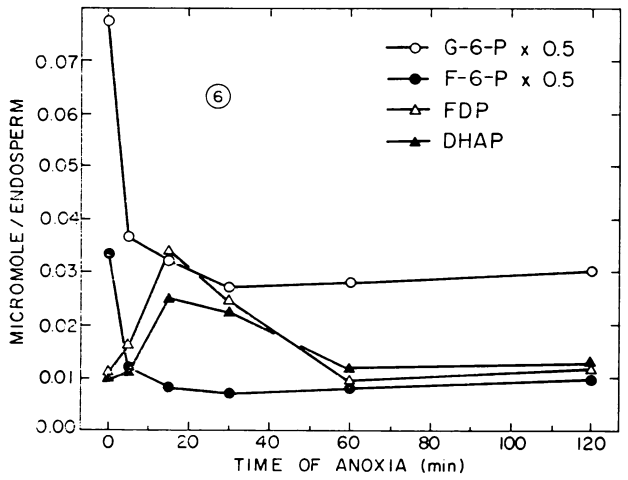


FIG. 6. Crossover point between G6P-F6P and FDP-DHAP in anoxic 5-day-old castor bean endosperm.

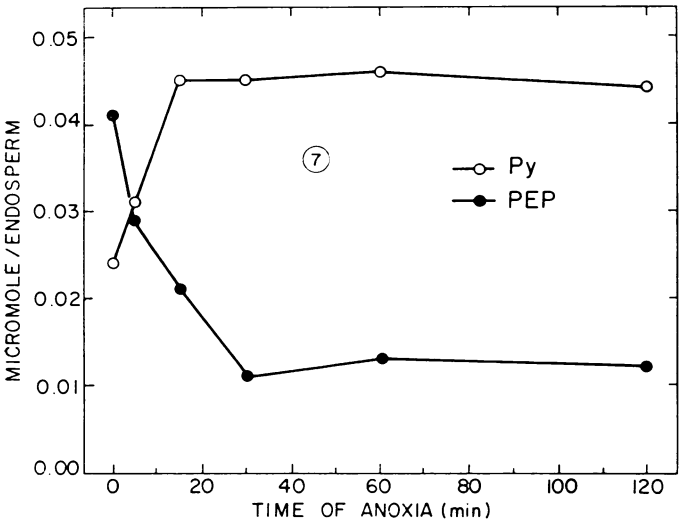


FIG. 7. Crossover point between PEP and Py in anoxic 5-day-old castor bean endosperm.

conditions at roughly twice the control levels. The amount of GAP is very low at all times and close to the limit of detection, as would be expected if equilibrium was maintained at the level of triose phosphate isomerase.

Striking and immediate changes are seen in the levels of PEP and pyruvate (Fig. 7). The PEP content declines sharply for 30 min, after which it is maintained at about one quarter of the aerobic level. The increase in pyruvate level, roughly a doubling, is completed in 15 min. This coincides with the time necessary to establish linear rates of ethanol and lactate accumulation (Fig. 4).

DISCUSSION

In considering what the observed results imply for the control of gluconeogenesis in the castor bean endosperm, it may be profitable to compare them to those obtained from other plant tissues in which gluconeogenesis does not occur. For various technical reasons there is no plant tissue for which a complete and simultaneous analysis of all of the intermediates and cosubstrates of the glycolytic sequence is available, and our analysis of the castor bean endosperm is also incomplete. However, fragmentary data are available for some tissues; and for a few, *e.g.*, rhododendron leaves (7), buckwheat seedlings (11), and particularly peas and other tissues studied by Barker's group (2), fairly complete analyses are available.

In several respects the relationships between the glycolytic intermediates in the castor bean endosperm are remarkably similar to those observed previously in other tissues. Thus the relative levels of F6P, G6P, the triose phosphates, 3-PGA, and PEP show that equilibrium is maintained at several of the enzymatic stages of the sequence and these equilibria are not drastically altered in N_2. Only at two points in the sequence, F6P-FDP and PEP-Py (the irreversible steps in glycolysis) are the relative concentrations of substrates very different from those predicted, and these are the stages at which the imposition of anoxia has its most marked effect. In this respect, too, the castor bean endosperm does not differ strikingly from nongluconeogenic tissues.

In the endosperm, G6P, F6P, and PEP decline simultaneously in the first 15 min of anoxia, whereas FDP, DHAP, and Py increase. The situation is only partly duplicated in peas where G6P and F6P remain constant during 40 min after oxygen withdrawal (2). In buckwheat seedlings, however, FDP and DHAP decrease upon imposition of anoxia, whereas the hexose monophosphates drop drastically only after an initial rise (11). With respect to hexose phosphates, the castor bean endosperm behaves in anoxia exactly like animal tissues (20), roots (Kobr, M. J. and O.M. Lowry, unpublished), and lower fungi (17).

Because the changes in PEP in peas precede those in FDP, and in potatoes and apples are not always accompanied by an accumulation of FDP and G6P, Barker (3) has stressed that the pyruvate kinase is the first glycolytic enzyme to respond to anaerobic conditions. However, such a sequential response of the pyruvate kinase and phosphofructokinase steps has not been observed in our material within the time span explored.

It seems clear that prime regulatory points in the glycolytic sequence occur at the F6P-FDP and PEP-Py steps. In this respect, the castor bean endosperm is not unusual. It is of interest that in this tissue the AMP-insensitive FDPase activity increases strikingly between the 2nd and 4th day of germination (25) and that the equilibium (FDP) (ADP)/(F6P) (ATP) decreases sharply at day 4 (Table I). The increased FDP/F6P ratio at this time when gluconeogenesis is underway is related to this enzymatic change. PFK is also present in the endosperm with an activity high enough (5.6 μmoles per hr per endosperm) to accommodate the highest glycolytic flux observed. Since the castor bean FDPases are apparently not sensitive to ATP (23), the changing amounts of FDP and F6P in anoxia may be ascribed to the stimulatory influence of the declining level of ATP on the PFK reaction (10, 19). At the PEP-Py level, the increases of PEP (and PGA) observed at day 4 are consistent with the onset of gluconeogenesis. The altered levels of ATP and ADP in anoxia are expected to influence the PEP carboxykinase equilibrium and so to contribute to the decreasing level of PEP. This drop in turn may trigger a further activation of the PFK by an allosteric effect (15).

Another feature of interest in seeking for an explanation of the gluconeogenic flux is the relationship between the adenylates. The ratio (ATP) (AMP)/(ADP)^2 in air remains at all times close to the value of 0.6 expected if the myokinase equilibrium is maintained. As shown in Table II (which records the smallest and largest changes observed in 4 separate experiments) this ratio falls appreciably in N_2. We have observed (Kobr and Beevers, unpublished) that there is both a soluble and mitochrondrial myokinase in this tissue. Although a direct effect of anoxia on the myokinase cannot be ruled out, it is tempting to suggest that a fraction of the total adenylate content is segregated in a region not accessible to myokinase and that the existence of this pool is revealed only when anoxia is imposed.

Barker and his colleagues (3) have emphasized that compart-

mentation of enzymes and metabolites must be considered in interpreting data on the total amounts of glycolytic intermediates present in tissue. They have argued that a glycolytic granule containing enzymes of the Embden-Meyerhof-Parnas sequence and with special permeability properties toward ADP and ATP would offer the best explanation of their extensive results.

We have no data bearing on this possibility, but it is of interest that the castor bean and other fatty seedling tissues contain proplastids which have a second set of enzymes of the Embden-Meyerhof-Parnas sequence (16). It is, therefore, conceivable that gluconeogenesis from PEP occurs on this organelle as it does from PGA in the chloroplast in photosynthesis, and that glycolysis occurs elsewhere in the cell.

Such a division of labor would account for the finding that the maximal glycolytic flux is but $\frac{1}{10}$ that of gluconeogenesis, even in conditions which would favor highly activated glycolysis. The two processes could conceivably occur together in the same cell and be independently regulated. Endosperm tissue from 5-day-old seedlings, while producing sucrose from endogenous fat, will nevertheless convert added sucrose-^{14}C to $^{14}CO_2$ at a slow rate. Any pyruvate produced glycolytically would be expected from previous experiments (22) to be converted to acetyl-CoA and, after metabolism in the glyoxylate cycle, join the gluconeogenic flux from PEP.

Acknowledgments—This work was supported by Atomic Energy Commission Contract At (11-1) 330. Seeds were kindly furnished by Dr. W. E. Domingo, Baker Castor Oil Company.

LITERATURE CITED

1. Atkinson, M. R., R. Burton, and R. K. Morton. 1961. Equilibrium constant of phosphoryl transfer from adenosine triphosphate to galactose in the presence of galactokinase. Biochem. J. 78: 813–820.
2. Barker, J., M. A. A. Khan, and T. Solomos. 1964. Mechanism of the Pasteur effect. Nature 201: 1126–1127.
3. Barker, J., M. A. A. Khan, and T. Solomos. 1966. Mechanism of the Pasteur effect. Nature 211: 547–548.
4. Benedict, C. R. and H. Beevers. 1961. Formation of sucrose from malate in germinating castor beans. I. Conversion of malate to phosphoenolpyruvate. Plant Physiol. 36:540–544.
5. Bergmeyer, M. V. 1962. Methoden der Enzymatischen Analyse. Verlag Chemie, Weinheim.
6. Bomsel, J. L. and A. Pradet. 1968. Study of adenosine 5′-mono-, di- and triphosphates in plant tissues. IV. Regulation of the level of nucleotides *in vivo* by adenylate kinase: Theoretical and experimental study. Biochim. Biophys. Acta 162: 230–242.
7. Bourne, D. T. and S. L. Ranson. 1965. Respiratory metabolism in detached rhododendron leaves. Plant Physiol. 40: 1178–1190.
8. Burton, K. 1957. Free energy data of biological interest. Ergeb. Physiol. Biol. Chem. Exp. Pharmakol. 49: 275–298.
9. Canvin, D. T. and H. Beevers. 1961. Sucrose synthesis from acetate in germinating castor bean: Kinetics and pathway. J. Biol. Chem. 236: 988–995.
10. Dennis, D. T. and T. P. Coultate. 1966. Phosphofructokinase, a regulatory enzyme in plants. Biochem. Biophys. Res. Commun. 25: 187–191.
11. Effer, W. R. and S. L. Ranson. 1967. Some effects of oxygen concentration on levels of respiratory intermediates in buckwheat seedlings. Plant Physiol. 42: 1053–1058.
12. Hurlbert, R. B., H. Schmitz, A. F. Brumm, and V. R. Potter. 1954. Nucleotide metabolism. II. Chromatographic separation of acid-soluble nucleotides. J. Biol. Chem. 209: 23–39.
13. Kahana, S. E., O. H. Lowry, D. W. Schulz, J. Passonneau, and E. J. Crawford. 1960. The kinetics of phosphoglucoisomerase. J. Biol. Chem. 235: 2178–2184.
14. Kelly, G. J. and J. F. Turner. 1968. Inhibition of pea-seed phosphofructokinase by phosphoenolpyruvate. Biochem. Biophys. Res. Commun. 30: 195–199.
15. Kelly, G. J. and J. F. Turner. 1969. The regulation of pea-seed phosphofructokinase by phosphoenolpyruvate. Biochem. J. 115: 481–487.
16. Kobr, M. J. and H. Beevers. 1968. Distribution of the gluconeogenic enzymes in the castor bean endosperm. Plant Physiol. 43: S-17.
17. Kobr, M. J. and G. Turian. 1967. Metabolic changes during sexual differentiation in *Allomyces*. Arch. Mikrobiol. 57: 271–279.
18. Leloir, L. F. and S. H. Goldemberg. 1960. Synthesis of glycogen from uridine diphosphate glucose in liver. J. Biol. Chem. 235: 919–923.
19. Lowry, O. H. and J. Passonneau. 1964. A comparison of the kinetic properties of phosphofructokinase from bacterial, plant and animal sources. Arch. Exp. Pathol. Pharmakol. 248: 185–194.
20. Lowry, O. H., J. V. Passonneau, F. X. Hasselberger, and D. W. Schulz. 1964. Effect of ischemia on known substrates and cofactors of the glycolytic pathway of the brain. J. Biol. Chem. 239: 18–30.
21. Marx, J. 1967. Fructose-1,6-diphosphatase in germinating seedlings. Ph.D. thesis, Purdue University, Lafayette, Ind.
22. Neal, G. E. and H. Beevers. 1960. Pyruvate utilization in castor bean endosperm and other tissues. Biochem. J. 74: 409–416.
23. Scala, J., C. Patrick, and G. Macbeth. 1968. FDPases of the castor bean endosperm and leaf: Properties and partial purification. Arch. Biochem. Biophys. 127: 576–584.