composed of all the valence electrons of the specimen. In this case, the electrons of the metal are coupled via the photon gas; their actions are not completely independent. Such a situation is not the case in the usual photosynthetic system; equilibrium or quasi-equilibrium ideas may not be applied. In this situation each photosynthetic event is completely independent of the other events taking place at other sites in the material.

Although in such a complicated system as the photosynthetic unit there could conceivably be a number of events which would use up the electronic energy imparted by photon absorption (such as fluorescence, radiationless transition, etc.), the individual photoelectron interactions are beyond the region of applicability of the second law of thermodynamics and are limited only by the conservation of energy and momentum.

LITERATURE CITED

1. BOHM, D. 1951. Quantum Theory. Pp. 26–29, 165–167. Prentice Hall, New York.
2. DUYSENS, L. N. M. 1958. The path of light energy in photosynthesis. In: The Photochemical Apparatus. Brookhaven Symposia in Biology 11: 10–25.
3. DUYSENS, L. N. M. & J. AMESZ. 1959. Quantum Requirement for Phosphorpyridine nucleotide Reduction in Photosynthesis, Plant Physiol. 34: 210–213.
4. FANO, U. & L. FANO. 1959. Basic Physics of Atoms & Molecules. Pp. 36–40. J. Wiley & Sons, New York.

FORMATION OF SUCROSE FROM MALATE IN GERMINATING CASTOR BEANS [1,2]
I. CONVERSION OF MALATE TO PHOSPHOENOL-PYRUVATE

C. R. BENEDICT [3] & HARRY BEEVERS
DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, LAFAYETTE, INDIANA

It has been established that the enzymes phosphoenol-pyruvate (PEP) carboxylase (reaction i), PEP carboxykinase (reaction ii), and malic enzyme (reaction iii) are widely distributed in plants (3, 9, 16) and are present in tissues capable of an active dark CO_2 fixation (8, 17).

The finding (9) that malic acid and aspartic acid are early products of dark CO_2 fixation is in accord with the operation of these enzymes during organic acid accumulation. The question of which of these enzymes is operative during acid accumulation is unresolved (9, 17) but there is general agreement that
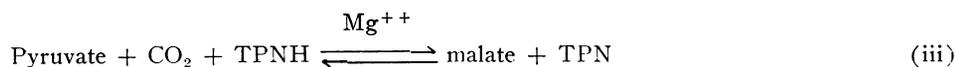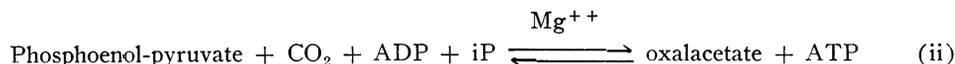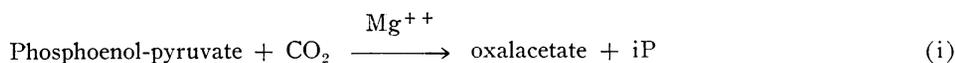
$$\text{Phosphoenol-pyruvate} + CO_2 \xrightarrow{Mg^{++}} \text{oxalacetate} + iP \qquad (i)$$

$$\text{Phosphoenol-pyruvate} + CO_2 + ADP + iP \underset{Mg^{++}}{\rightleftharpoons} \text{oxalacetate} + ATP \qquad (ii)$$

$$\text{Pyruvate} + CO_2 + TPNH \underset{Mg^{++}}{\rightleftharpoons} \text{malate} + TPN \qquad (iii)$$

during dark CO_2 fixation the direction of these enzymatic reactions favors malic acid accumulation. In the following light de-acidification period there is some evidence (8) which suggests carbohydrates are synthesized at the expense of organic acids.

In germinating castor beans which carry out a net synthesis of sucrose from acetate units (4, 6)

[3] Present address: Department of Microbiology, Dartmouth Medical School, Hanover, N. H.

an analogous problem exists since malic acid is a major early product of acetate utilization, and it has been shown that it is an intermediate in sucrose synthesis from acetate (6). Additional information is described in this paper which confirms that malate in the endosperm is converted to sugar.

In deciding which of the above reactions may be of importance in converting malate to carbohydrate it is of interest that an active malic dehydrogenase is present in extracts from germinating castor beans (7), so that oxalacetate formation may be regarded as a likely first step. It should also be noticed that reaction (i) is not reversible and that free pyruvate is the substrate for reaction (ii). Since it has been shown previously that pyruvate is not an intermediate in the conversion of acetate to sucrose (11) it seems reasonable to suggest that PEP carboxykinase is the enzyme responsible for the production of a glycolytic intermediate from oxalacetate. In this paper we describe the properties of this enzyme from germinating castor beans and other results which are in harmony with this suggestion.

MATERIALS & METHODS

ENDOSPERM SLICES: Endosperm slices of germinating castor beans for $C^{14}O_2$ fixation studies were prepared as previously described (11). The slices were placed in $KHC^{14}O_3$ and 0.05 M KH_2PO_4 buffer pH 7.2. The reaction was stopped with boiling 80 % ethanol. The slices were then extracted successively with 80 %, 50 %, and 20 % ethanol and the combined extracts were concentrated under reduced pressure at 40 C. The dry residue was washed with ether, dissolved in water, and passed successively through 1 cm columns containing 6 cm Dowex 50 (X8) in the hydrogen form and 6 cm Dowex 1 (X8) in the formate form. The amino acids were eluted from the Dowex 50 resin with 50 ml 1 M NH_4OH. The acidic fraction was eluted from the Dowex 1 resin with 50 to 60 ml 8 N formic acid. The radioactivity of the fractions was determined by transferring 0.5 ml aliquots to nickel plated planchets and counting on an aluminized mylar gas flow counter.

For chromatographic resolution of the acidic fraction, the eluate was dried under reduced pressure at 40 C. The residue was then dissolved in 0.5 ml water. This was applied to paper and the components were separated with butanol-propionic acid-water [equal volumes of (1) 620 ml propionic acid & 790 ml water & (2) 1,246 ml butanol & 84 ml water] as the solvent for 20 hr. The propionic acid was removed by suspending the chromatograms over a steam outlet; the phosphate esters and acids were located by Hanes-Isherwood and brom-cresol green spray reagents, respectively. The phosphate esters, which stayed near the origin, were eluted with water, concentrated, and applied to filter paper and developed with MeOH-NH_4OH-H_2O (60/10/30) (v/v/v) at 3 C for 18 hours as described by Bandurski and Axelrod (2). The separated phosphate esters were cut out and

eluted from the chromatogram and co-chromatographed in the same solvent with known phosphate esters. The chromatograms were then exposed to x-ray film and the radioactive areas and sprayed phosphate esters were compared for coincidence.

CYTOPLASMIC PARTICLES: 4 day old germinating castor beans were used as the source of cytoplasmic particles and supernatant enzymes which were prepared as described previously (5). Soluble enzyme extracts of these particles were prepared as follows:

I. The particles from the first 18,000 × g spin were homogenized in cold 0.08 M KH_2PO_4 buffer pH 7.2. The insoluble residue was removed by centrifuging and the supernatant used as the source of enzymes.

II. The particles were homogenized in cold distilled water and lyophillized. The lyophillized powder was dissolved in 0.08 M KH_2PO_4 buffer pH 7.2, centrifuged, and the supernatant used as the source of enzymes.

BARIUM-ALCOHOL PRECIPITATION & CHROMATOGRAPHIC SEPARATION OF P^{32}-PHOSPHATE ESTERS: The phosphate esters produced in the enzyme experiments with P^{32} were separated by the method of Umbreit, Burris, and Stauffer (14) with this modification. The barium salts of the phosphate esters were dissolved and the barium removed with 1 M H_2SO_4 and the supernatant lyophillized. The lyophillized powder was dissolved in water and applied to filter paper. The chromatography and identification procedures were those described above.

ENZYMATIC ASSAYS: The PEP carboxykinase ATP-exchange reaction was assayed in castor bean extracts by the methods of Mazelis and Vennesland (10), and Utter and Kurahashi (15). The $C^{14}O_2$ released from oxalacetate-4-C^{14} by citrate treatment was collected in 0.1 M barium hydroxide. The $BaC^{14}O_3$ was plated on microporous porcelain planchets and counted on a windowless gas-flow counter. All figures are corrected for self-absorption and background. The blank which is carried throughout all the exchange reactions is subtracted from the sample values and unless the blank is low, of the order of 50 to 100 cpm, the experiment is discarded.

REAGENTS: Oxalacetic acid was obtained from the Sigma Chemical Co. and adjusted to pH 6.5 with KOH prior to use. L-Malic acid was obtained from Sigma Chemical Co. Adenosine-t r i p h o s p h a t e (ATP), and adenosine-diphosphate were obtained from Pabst Laboratories and adjusted to pH 6.5 with KOH before use. Phosphopyruvic acid-tricyclhexylamine salt was obtained from California Corp. Diphosphopyridine nucleotide (DPN) was obtained from Pabst Laboratories.

RADIOACTIVE COMPOUNDS: Phosphorus 32 was obtained in the form of $H_3P^{32}O_4$ from Oak Ridge

National Laboratory. $BaC^{14}O_3$ was obtained from Tracerlab in isotope concentrations of 0.044 and 0.24 mc/mg. $BaC^{14}O_3$ was converted to $KHC^{14}O_3$ by acidifying with lactic acid and trapping the $C^{14}O_2$ in KOH.

RESULTS

FORMATION OF SUCROSE FROM MALATE IN VIVO. It is already known that castor bean endosperm tissue, like that of other seedlings rich in fat is able to fix CO_2 into the carboxyl groups of organic acids, preponderantly malic, in the dark, and that sucrose, with C^{14} largely confined to carbons 3 and 4, emerges as the principal product of the fixation (12, 13). In order to demonstrate that the tissue is indeed capable of forming sucrose from malate, endosperm slices were exposed to $HC^{14}O'_3$ in two types of experiment. In the first, samples were withdrawn at intervals and the C^{14} incorporation into organic acid, amino acid, and neutral fractions was determined. The results are shown in figure 1. From this it is clear that incorporation into organic acids (& amino acids) is a primary event and that the form of the curve for C^{14} in sugars is that expected if they had arisen in a subsequent reaction with the organic acids acting as precursors. In view of the suggestion that PEP is an intermediate in these reactions, it is significant that a minor component of the $C^{14}O_2$ incorporated

into the acid fraction was identified by chromatography and co-chromatography as PEP-C^{14}. In a further experiment, it was shown that when slices which had been exposed to $HC^{14}O'_3$ for 10 minutes were transferred to water, the C^{14} in the acid fraction decreased during the subsequent 6 minutes and there was a corresponding increase in the C^{14} content of the sugar fraction. The above relationships would hold true if sucrose was being synthesized from malate (with C^{14} in the carboxyl groups) by reactions which included a single decarboxylative step and a reversal of glycolysis.

PHOSPHOENOL-PYRUVATE PRODUCTION IN CYTOPLASMIC PARTICLES. Mitochondria from castor bean endosperm are known to be able to carry out the reactions of the TCA cycle (5) and to produce ATP efficiently during the oxidation of α-ketoglutarate (1). The ability of such particles to produce PEP under these conditions was demonstrated by incubating with α-ketoglutarate, ADP, and $KH_2PO^{32}_4$. The radioactive compounds were isolated from the incubation mixture by the barium-alcohol precipitation method and chromatographed. The P^{32}-phosphate ester which showed up as a clear band running ahead of the other P^{32} labeled products was eluted and co-chromatographed with known PEP. The two compounds coincided nicely. This suggests that ATP^{32} generated during the oxidation of α-ketoglutarate had reacted with oxalacetate to produce PEP.
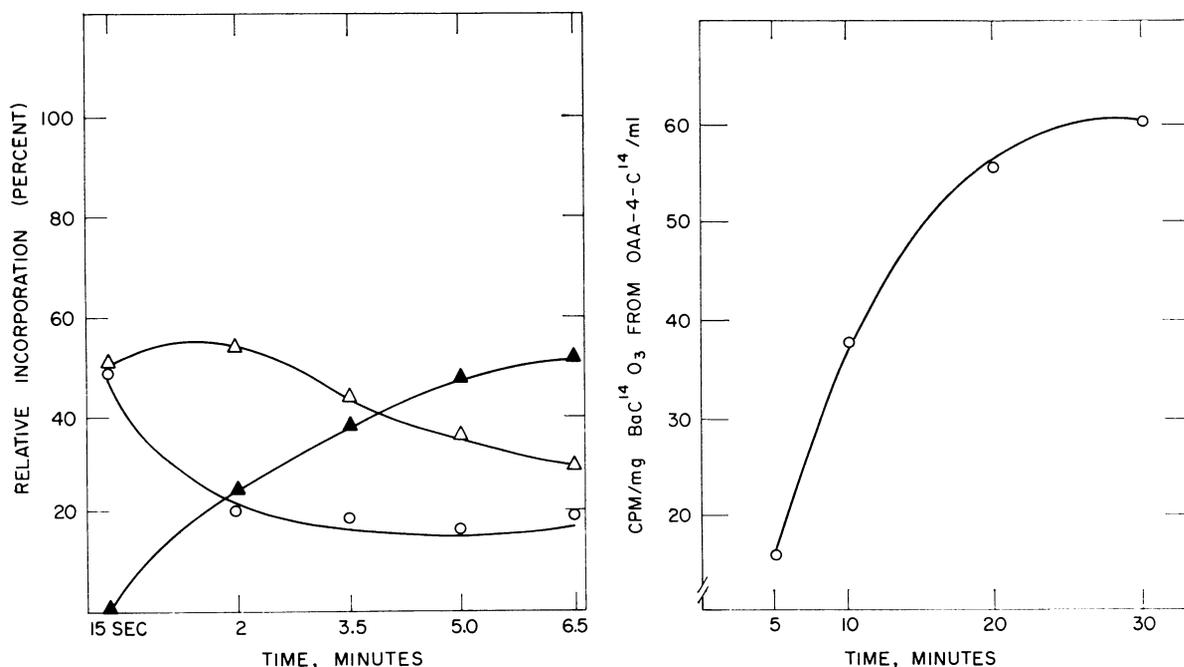
FIG. 1. Dark fixation of $HC^{14}O'_3$ in castor bean endosperm slices. ▲. neutral fraction (sugars) ; O, amino acids; △, organic acids.

FIG. 2. PEP carboxykinase exchange reaction. The reaction mixture contains in micromoles: Oxalacetate (OAA), 100; sodium fluoride, 15.0; ATP, 15.0; $MgCl_2$, 7.5; $KHC^{14}O_3$, 10(1.10 × 10^6 cpm) ; 0.5 ml particulate enzyme (solubilized in 0.08 M KH_2PO_4 buffer pH 7.2) and 0.5 ml supernatant enzyme in 200 micromoles potassium phosphate pH 7.2. Total volume 3.2 ml. Incubation at 25 C.

PROPERTIES OF PEP CARBOXYLASE & PEP CAR-
BOXYKINASE: If oxalacetate (OAA) is an inter-
mediate in the formation of PEP by the cytoplasmic
particles, the enzymatic reaction converting OAA to
PEP should be directly demonstrable. To study this
reaction soluble particulate extracts were used (see
Materials & Methods). In the exchange reaction,
the oxalacetate is phosphorylated and decarboxylated
by PEP carboxykinase to give PEP which then re-
acts with $HC^{14}O'_3$ to generate OAA-4-C^{14} by the
action of PEP carboxylase. A determination of the
activity of overall reaction of the combined enzymes
is made by measuring the amount of $C^{14}O_2$ liberated
from the OAA-4-C^{14}. Neither enzyme can be assay-
ed individually in a crude extract using this method.
A large pool of OAA is added to the reaction mixture
to assure saturation of the PEP carboxykinase, to
offset possible losses of OAA through decarboxylation
by Mg^{++} or OAA decarboxylase and to provide a
trap for the incoming OAA-4-C^{14}.

The results in table I show that incorporation of
$HC^{14}O'_3$ into the β-carboxyl group of OAA does
indeed occur when ATP is provided. At higher
ATP levels than those shown in table I, there was a
progressive inhibition of the overall reaction. The
results in table I establish that Mg^{++} stimulates the
overall reaction in accordance with its known effects
on PEP carboxykinase and PEP carboxylase.

The total incorporation of C^{14} into OAA in the
presence of ATP and Mg^{++} increases with time up
to 30 minutes (fig 2).

An attempt to localize the enzymes, PEP car-

TABLE I

EFFECTS OF ATP & MG^{++} ON PEP CARBOXYKINASE
EXCHANGE REACTION

(a) ATP added (micromoles)	CPM/mg $BaC^{14}O_3$ from OAA-4-C^{14}/ml
0	0.77
24.3	239.0
48.6	324.0
(b) $MgCl_2$ added (micromoles)	
0	48.2
18.7	62.8
37.5	81.8
75.0	125.4

All of the reaction mixtures contained, in micromoles:
oxalacetate (OAA), 100; sodium fluoride, 37.5;
$KHC^{14}O_3$, 50 (5.5 x 10^6 cpm) ; 5 ml of lyophillized par-
ticulate powder in 200 micromoles potassium phosphate
pH 7.2. Total volume 7.6 ml. Time of incubation at
25 C, 30 minutes.
In (a) the $MgCl_2$ was held at 18.7 micromoles/ml and
the ATP varied as shown.
In (b) the ATP was held constant at 48.6 micro-
moles/ml and the $MgCl_2$ varied as shown. The lyophil-
lized enzyme preparation was held in storage for several
days between experiments (a) and (b).

TABLE II

EFFECTS OF MALATE ON PEP CARBOXYKINASE
EXCHANGE REACTION

CPM IN OAA-4-C^{14}/ml	CPM IN MALATE-4-C^{14}/ml
51.3	269. 5

The reaction mixture contained, in micromoles:
malate, 50; sodium fluoride, 15; $KHC^{14}O_3$, 50 (5.5 x
10^6 cpm) ; DPN, 11.2; 1.0 ml particulate enzymes in 160
micromoles potassium phosphate pH 7.2. Total volume
4.1 ml. Time of incubation at 25 C, 30 minutes.

boxykinase and PEP carboxylase, using the exchange
reaction showed that the supernatant solution remain-
ing after sedimenting the mitochondria was more
than three times as active (on a protein basis) as the
particles. However, since in the exchange reaction
two enzymes are assayed and there is no reason to
expect an identical distribution, the only conclusion
from these results is that neither the PEP car-
boxylase nor the PEP carboxykinase is confined to
the particles.

The results in table II show the effect of adding
malate and DPN, rather than oxalacetate to the ex-
change reaction mixture. C^{14} is incorporated into
the large malate pool under these conditions, and C^{14}
was also present in oxalacetate after 30 minutes. The
enzymes in the particulate preparation are therefore
capable of establishing an equilibrium between malate,
oxalacetate, and phosphoenol pyruvate.

DISCUSSION

In germinating castor beans the reactions of the
glyoxylate cycle when coupled to the production of
acetyl CoA from the fatty acid spiral convert fatty
acids to malate and then to sucrose (6,7). The
results presented in this paper show that PEP car-
boxykinase is present in extracts from this material,
so that it is now reasonable to invoke the action of
this enzyme in the sequence by which malate is con-
verted into a glycolytic intermediate and thence to
sucrose. The demonstration that radioactive PEP is
one of the early products of dark $C^{14}O_2$ fixation and
the production of PEP^{32} from α-ketoglutarate and
ATP^{32} in the cytoplasmic particles is in accord with
the operation of these enzymes in the castor bean
endosperm. By studying the combined reactions of
PEP carboxykinase and PEP carboxylase it has been
shown that given the necessary conditions such as
sufficient substrate and ATP, the PEP carboxykinase
reaction is easily reversed in the direction of PEP
formation from oxalacetate.

It will be noted that in addition to possessing the
mechanism bringing about a net synthesis of oxala-
cetate from acetyl units, tissues such as the endo-
sperm of the germinating castor bean generate re-
duced flavins and pyridine nucleotide in the act of

producing these acetyl units from long chain fatty acids. ATP formed during the subsequent oxidation of these coenzymes would provide the other condition essential for an efficient entry into the glycolytic sequence at the level of PEP. In the somewhat analogous situation of light deacidification in succulent leaves (8) both elements of the assimilatory power required for the production of sucrose from preformed acids arise no doubt from the light reactions in photosynthesis.

SUMMARY

Both phosphoenol-pyruvic carboxylase and phosphoenol-pyruvic carboxykinase are present in castor bean extracts. When ATP was provided the conversion of oxalacetate to PEP was strongly stimulated. Magnesium ion is required as a cofactor in this reaction. P^{32}-labeled phosphoenol-pyruvate was produced when cytoplasmic particles were provided with P^{32}-labeled phosphate and α-ketoglutarate, and C^{14} labeled phosphoenol-pyruvate was detected when slices of castor bean endosperm were converting malate-C^{14} (produced in vivo from $C^{14}O_2$) to sugars. The results presented support the suggestion that phosphoenol pyruvic carboxykinase is one of the enzymes involved in the conversion of malate to carbohydrate which occurs in germinating castor beans.

LITERATURE CITED

1. AKAZAWA, T. & H. BEEVERS. 1957. Mitochondria in the endosperm of the germinating castor bean: A developmental study. Biochem. J. 67: 115–118.
2. BANDURSKI, R. S. & B. AXELROD. 1951. The chromatographic identification of some biologically important phosphate esters. J. Biol. Chem. 193: 405–410.
3. BANDURSKI, R. S. & C. M. GREINER. 1953. The enzymatic synthesis of oxalacetate from phosphorylenol-pyruvate & carbon dioxide. J. Biol. Chem. 204: 781–786.
4. BEEVERS, H. 1957. Incorporation of acetate carbon into sucrose in castor bean tissue. Biochem. J. 66: 23p.
5. BEEVERS, H. & D. A. WALKER. 1956. The oxidative activity of particulate fractions from germinating castor beans. Biochem. J. 62: 114–120.
6. CANVIN, D. T. & H. BEEVERS. 1961. Sucrose synthesis from acetate in the germinating castor bean. In: Kinetics & Pathway. J. Biol. Chem. 236: 988–995.
7. KORNBERG, H. L. & H. BEEVERS. 1957. The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor bean. Biochim. Biophys. Acta. 26: 531–537.
8. KUNITAKE, G. M. & P. SALTMAN. 1958. Dark Fixation of CO_2 by succulent leaves: Conservation of the dark fixed CO_2 under diurnal conditions. Plant Physiol. 33: 400–403.
9. KUNITAKE, G. M., C. STITT, & P. SALTMAN. 1959. Dark fixation of CO_2 by tobacco leaves. Plant Physiol. 34: 123–127.
10. MAZELIS, M. & B. VENNESLAND. 1957. Carbon dioxide fixation into oxalacetate in higher plants. Plant Physiol. 32: 591–600.
11. NEAL, G. E. & H. BEEVERS. 1960. Pyruvate utilization in castor bean endosperm & other tissues. Biochem. J. 74: 409–416.
12. STILLER, M. L. 1959. The mechanism of malate synthesis in crassulacean leaves. Ph.D. Thesis, Purdue University, Lafayette.
13. STILLER, M. L., G. E. NEAL, & H. BEEVERS. 1958. CO_2 fixation during the conversion of carbohydrate to fat in the castor bean. Plant Physiol. 33 suppl.: xxxiv.
14. UMBREIT, W. W., R. H. BURRISS, & J. F. STAUFFER. 1949. Manometric techniques & tissue metabolism. Burgess Publish. Co., Minneapolis.
15. UTTER, M. F. & K. KURAHASHI. 1954. Mechanism of action of oxalacetic carboxylase. J. Biol. Chem. 207: 821–841.
16. VENNESLAND, B. & E. E. CONN. 1952. Carboxylating enzymes in plants. Ann. Rev. Plant Physiol. 3: 307–332.
17. WALKER, D. A. 1957. Physiological studies on acid metabolism. 4. Phosphoenol pyruvic carboxylase activity in extracts of crassulacean plants. Biochem. J. 67: 73–79.