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# Contribution of Phosphoenolpyruvate Carboxylase to the Carbon Economy of cv. Fuerte Avocado Fruit - Categorization of Photosynthesis and Effects of Simulated Salinity, $CO_2$ Shock and CA-Storage

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***Abstract.*** **Phosphoenolpyruvate carboxylase (PEPC, orthophosphate: oxaloacetate carboxylase [phosphorylating], EC 4.1.1.31) was extracted and partially purified from the mesocarp of pre-climacteric avocado cv. Fuerte *(Persea americana* Mill.) fruit. From determinations of enzyme activity, it was concluded that PEPC refixed $CO_2$ in quantities which were of the same order of magnitude as the amount of $CO_2$ lost from the fruit through the peel. Kinetic properties of PEPC were determined by initial rate reactions of 30 sec. The effects of simulated chloride-induced salinity, $CO_2$ shock of 20 to 30% and CA-storage in 10% $CO_2$ on PEPC from 'Fuerte' fruit were examined. PEPC was assayed under 0 to 180 mM Cl at pH 7.8. Chloride (180 mM) caused an increase in apparent Km (PEP) from 80 to 212 //M, accompanied by a 14% decrease in $V_{max}$. PEPC was also assayed up to 125 mM $HCO_3$ at pH 7.0, equivalent to 87% $CO_2$, without loss in activity. Simulation of salinity, $CO_2$ shock and CA-storage showed that the activity of PEPC was reduced by chloride ions, but not by the $CO_2$ concentrations used in both CA storage and $CO_2$ shock. These findings and comparison of kinetics of avocado fruit PEPC suggests a new type of (avocado) fruit photosynthesis different from established $C_3$, $C_4$ or CAM photosynthesis in leaves or entirely heterotrophic metabolism in nongreen storage organs rather than its categorization within one of these existing types.**

Abbreviations: dithiothreitol (DTT); malate dehydrogenase (MDH); 3-[N-morpholino] propanesulfonic acid (MOPS); nicotinamide adenosine dinucleotide [reduced form] (NADH); oxaloacetic acid (OAA); Phosphoenolpyruvate (PEP); N-tris[hydroxymethyl]-methyl glycine (Tricine).

Avocado (*Persea americana* Mill.) is specified as a $C_3$ species; its fruit however, appear to exhibit some properties different from $C_3$ photosynthesis (Blanke *et al*., 1986). In order to categorize photosynthesis of avocado fruit and examine effects of salinity, $CO_2$ shock and controlled atmosphere (CA) storage, Phosphoenolpyruvate carboxylase

(PEPC) was extracted from the mesocarp of pre-climacteric avocado fruit and its properties examined.

## Materials and Methods

Fruit. Pre-climacteric avocado (*Persea americana* Mill.) cv. Fuerte fruit, free of any skin blemish, were purchased locally in the United Kingdom and exposed to bright daylight at an ambient temperature of 20C prior to respiration measurement and to tissue extraction.

Enzyme extraction. Extraction buffer was used appropriate to the particular experiments, either 200 mM Tricine/NaOH (pH 7.8) or 200 mM MOPS/NaOH, (pH 7.0), containing 3 mM DTT, 2.5 mM $MgSO_4$ and, except for the bicarbonate kinetics experiments, 5 mM $NaHCO_3^-$ All extracts were prepared at 4C. The mesocarp was diced and the tissue ground with a cold pestle and mortar in appropriate buffer in a 3:1 ratio together with clean sand and 2% (w/v) PVP to respectively aid extraction and to prevent browning of the solution. The homogenate was filtered through muslin and the filtrate centrifuged at 20,000 g for 30 min. The aqueous phase was removed from debris and floating oils using a hypodermic needle and syringe. The clear solution was made 65% saturated with respect to $(NH_4)_2SO_4$ by addition of the appropriate quantity of a cold, saturated solution of $(NH_4)_2SO_4$ previously adjusted to pH 7.8 with $NH_4OH$. After 45 min stirring, the mixture was centrifuged at 20,000 g for 30 min and the resultant pellet dissolved in 10% of the initial volume of the extracting buffer, using 50 mM Tricine (pH 7.8) or 50 mM MOPS buffer (pH 7.0) containing 1 mM DTT, 2.5 mM $MgSO_4$, 5% glycerol and, as appropriate, 5 mM $NaHCO_3$.

Enzyme assays. Activity of PEPC was determined spectrophotometrically as previously described (Blanke *et al.,* 1986) at 340 nm by coupling the reaction to the oxidation of NADH in the presence of MDH. One unit of PEPC activity is expressed as OD change at 340 nm of 1.0 per minute per gram tissue. The standard assay medium contained enzyme, 10 units of MDH, and 0.1 mM NADH, 2.5 mM $MgSO_4$ and 5 mM $NaHCO_3$ in a total volume of 2.95 ml 50 mM Tricine buffer (pH 7.8), at 27C. The reaction was started by the addition of 50 $\mu$L of PEP at 2.2 mM final concentration. The assay mixture, in the spectrophotometer cuvette, was rapidly mixed *in situ* using a mini magnetic bar and an electronic stirrer type 200 (Rank Brothers, Cambridge CB59DA, United Kingdom) during and after the addition of the substrates. The rate of oxidation of NADH was measured 10 sec after the addition of the PEP over two 30 sec periods. The reaction was observed using the visual display of the spectrophotometer type PU 8720 (Pye Unicam, Cambridge, United Kingdom) to confirm both that adequate mixing of the cuvette contents had taken place and that NADH oxidation caused by the reaction was linear. Assays were done in duplicate on multiple extracts.

Enzyme kinetics. Values for Kpp, and $V_{max}$ were calculated as previously described (Blanke *et al.,* 1987) using a kinetics computer programme (Transcribe, 4 Royal York Crescent, Clifton, Bristol, United Kingdom) based on Hanes' transformations of s/v against s. Values are derived from duplicate samples of three extracts.

Simulated $CO_2$ shock. CA storage and salinity. $CO_2$shock, CA storage and salinity were simulated by exposing avocado fruit extracts to the equivalent $CO_2/HCO_3$ (Table 1) or NaCI concentration during the assay for PEPC at 27C.

Measurement of ambient $CO_2$ . The ambient $CO_2$ concentration during the $CO_2/HCO_3$ kinetics experiments was monitored using a digital portable infrared gas analyzer type LCA 2 (ADC, The Analytical Development Co., Hoddesdon, Herts., United Kingdom).

Fruit porometry. Respiration of avocado fruit was measured by a portable porometer type LCA 3 from ADC. The fruit cuvette consisted of two transparent hemispheres (Blanke and ADC, 1992). Flow rates into the fruit cuvette and into the LCA 3 were controlled by the two mass flowmeters of the LCA 3. Fruit temperature was recorded by a thermistor on a flying lead pushed into the mesocarp.

**Results and Discussion**

Role of PEPC in fruit. The enzyme PEPC combines $HCO_3$ from intracellular $CO_2$ with PEP to produce oxaloacetate (OAA), which in turn is converted to malate (Fig. 1). The substrate PEP originates from metabolic conversion of starch or sugars. The properties of the enzyme PEPC have been widely used to examine the photosynthesis of a plant organ such as fruit, leaf or root (Blanke and Lenz, 1989).

Carbon conservation by PEPC in pre-climacteric avocado fruit. The maximal activity of $CO_2$ refixation potential of PEPC in the mesocarp of pre-climacteric 'Fuerte' avocado fruit was 106 /$\mu$mol $CO_2$/fruit/h, thereby exceeding the respiratory $CO_2$ efflux from the fruit of 50 $\mu$mol $CO_2$/fruit/h by ca. two-fold (Fig. 2). However, the $CO_2$ refixation potential included any enzyme loss during the extraction and was determined under optimum conditions for maximal enzyme activity, i.e. saturating concentrations of cofactors and substrates at pH 7.8. It may be assumed that the enzyme activity or $CO_2$ refixation potential is significantly lower in the fruit than in the test tube. Metabolic regulation of PEPC by supply of substrate and cofactors, makes it more likely that the enzyme refixes $CO_2$ in quantities which are of the same order of magnitude as the $CO_2$ lost from the fruit through the peel. Thus a substantial amount of carbon (ca. 50%) was conserved in pre-climacteric avocado fruit by the activity of PEPC and this contributed to the carbon economy of the fruit.

Effects of salinity on avocado fruit PEPC. The vast water requirement of the fully grown avocado tree can often only be met by irrigation, e.g. in Southern California and Israel. Concentrations of up to 350 ppm salt (NaCI plus $CaCl_2$) in Southern California's irrigation water produces chloride-induced salinity disorders which become visual as leaf tip burn and also increase the chloride concentrations in the avocado fruit. In order to study direct effects of chloride-salinity on PEPC without interference from tree effects, changes in PEPC activity and kinetics were examined.

Chloride inhibited activity of PEPC from pre-climacteric 'Fuerte' avocado fruit in a concentration-dependent manner at pH 7.8 (Fig. 3); this effect on PEPC activity had been observed with PEPCs from $C_3$ and $C_4$. photosynthetic leaves (Ting and Osmond, 1973). Chloride at a concentration of 180 mM NaCI in the PEPC assay decreased PEPC activity by 30% at saturating (2.2 mM) PEP concentration. We have shown (Blanke and Notton, 1991) that the degree of inhibition is dependent on the concentration of the substrate PEP, e.g. PEPC activity was inhibited 50% by 180 mM NaCI when the PEP was at a subsaturating concentration of 0.5 mM. The concentration of PEP occurring in fruit is likely to be subsaturating with respect to PEPC, thereby enhancing chloride inhibition effects. Chloride at 180 mM caused an almost three-fold increase in apparent $K_m$ (PEP) from 80 $\mu$M to 212 $\mu$M PEP, accompanied by a 14% decrease in $V_{max}$. This effect on apparent $K_m$ and $V_{max}$ resembles that reported for the chloride inhibition of PEPC from $C_3$ photosynthetic leaves (Ting and Osmond, 1973) which was thought to be due to competition of chloride with PEP binding to PEPC.

Effects of $CO_2$ shock and CA storage on intercellular $CO_2$; and cellular $HCO_3$ levels. Mature, unripe, refrigerated avocado fruits are shipped from California, Israel, Chile or SA to Europe, a journey which lasts up to 20 to 30 days and often enhances physiological disorders such as mesocarp discoloration and vascular browning. Research with apple had shown the benefits for fruit quality of a postharvest $CO_2$ shock and subsequent CA storage. At harvest, avocado fruit may contain 1 to 3% intercellular $CO_2$ (Table 1) when the refrigerated fruit is exposed to a 48 to 72 h $CO_2$ shock of 20 to 30% $CO_2$. This $CO_2$ will eventually increase the intercellular $CO_2$ and equilibrate as bicarbonate ($HCO_3^-$ in the cell sap or cytosol (Fig. 2). Assuming pH 7 in the cytosol, 20, 25 or 30% $CO_2$ equilibrate as 28, 35 or 42 mM $HCO_3$ (Table 1). CA storage in 10% $CO_2$ similarly equilibrates as 14 mM $HCO_3$ at pH 7. Carbonic anhydrase is an enzyme which enhances the equilibrium between $CO_2$ and $HCO_3^-$. In certain leaves, the presence of carbonic anhydrase has been found to be associated with PEPC. However, the $K_m$, ($HCO_3^-$) is larger for carbonic anhydrase than for PEPC, i.e. PEPC is more efficient at the same concentration of HCC>3~ (Blanke and Notton, 1991). In the absence of any reports on carbonic anhydrase in fruits, $CO_2$-$HCO_3^-$ equilibrium seems so far determined by physical and chemical parameters rather than by enzyme reactions. Given the large concentrations of $CO_2$ in 'Fuerte' avocado fruit (Table 1), supply of substrate $HCO_3$ from intercellular $CO_2$ is not a limiting factor to the PEPC reaction.

Effects of $CO_2$ shock and CA storage on PEPC in pre-climacteric avocado fruits. The enzyme PEPC is located in the cytosol in the mesocarp of the avocado fruit and has two substrates, $HCO_3$/$CO_2$ and PEP. Effects of $CO_2$ shock and CA storage were simulated by assaying for PEPC activity under the equivalent $HCO_3$ concentrations at pH 7 (Table 1). The Michaelis-Menton constant ($K_m$), i.e. the substrate concentration which gives half maximum enzyme activity, of PEPC was calculated to be 14 $\mu$M $HCO_3$, taking into account 63 $\mu$M $HCO_3$ from the 450 ppm ambient $CO_2$ in our laboratory. Maximum PEPC activity was observed between 0.2 and 125 mM $HCO_3$. These bicarbonate concentrations are equivalent to 0.14% and 87% $CO_2$ in the intercellular space which indicates that the $CO_2$ refixation by PEPC appears not inhibited by the high intercellular $CO_2$ concentration in the avocado fruit under $CO_2$ shock or in CA storage.

Interaction between salt concentration and the two substrates of PEPC. At a constant salt concentration adjusted to 1 50 mM (buffer containing endogenous $HCO_3$ plus added $HCO_3$), no $CO_2$/$HCO_3$ inhibition was observed at pH 7.0 and concentrations up to 125 mM $HCO_3$ (Fig. 4). Under saturating PEP concentrations, PEPC activity showed a non-competitive relationship between both substrates, $HCO_3$ and PEP, a result similar to that found for apple fruit (Blanke *et al*., 1987). This was as expected given the proposed $CO_2$ concentrating mechanism of PEPC in fruit (Blanke, 1992).

## Conclusions

Much of the fascination in studies with avocado fruit lies in (a) attempting its photosynthetic classification, (b) in the potential to contribute to its carbon economy and (c) to explain differences in response of cultivars to $CO_2$ under $CO_2$ shock and in CA storage. Further research is needed to identify the role of PEPC in the carbon metabolism of avocado fruit and to investigate effects of $CO_2$ shock or CA storage on respiration and fruit metabolism.

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Table 1. Effect of postharvest treatment of 'Fuerte' avocado fruit on equilibria between intercellular $CO_2$ concentration and solute bicarbonate ($HCO_3$) at pH 7 in the cytosol, compared to the $CO_2/HCO_3$ range for maximal PEPC activity in the mesocarp. [z]

| Treatment | $CO_2$concentration [y] (%) | $HCO_3^-$ concentration (mM) |
|---|---|---|
| Harvest | 1-3 | 1.4-4.2 |
| $CO_2$ shock | 20 | 28 |
| $CO_2$ shock | 25 | 35 |
| $CO_2$ shock | 30 | 42 |
| CA storage | 10 | 14 |
| Maximal PEPC activity ranges | | |
| From | 0.14% | 0.2 mM |
| To | 87% | 125 mM |

[z] Data calculated from Henderson-Hasselbach equation.
[y] The ambient $CO_2$ concentration is ca. 0.034% $CO_2$.

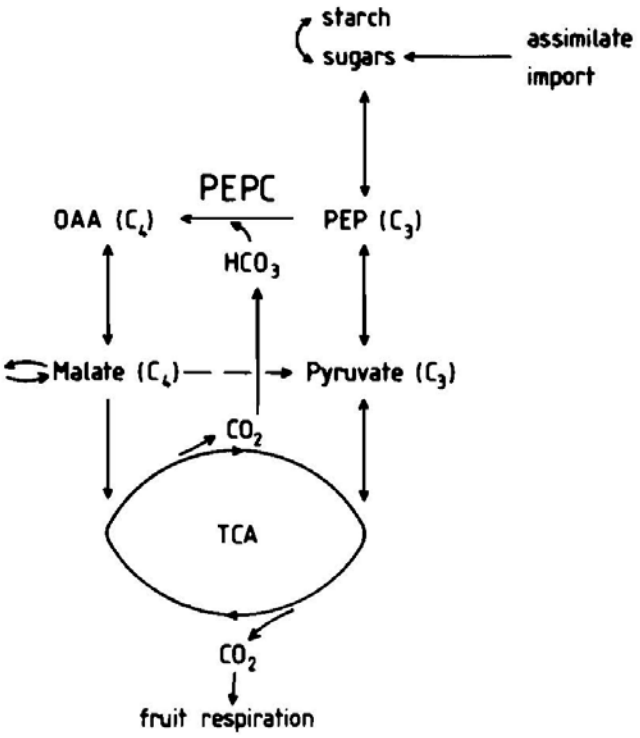


Fig. 1. Schematic of the biochemical reactions involved in respiration and $CO_2$ refixation. Avocado fruit tissue respires assimilates in the tricarboxylic (TCA) cycle, thereby producing $CO_2$. This $CO_2$ partly accumulates in the intercellular spaces, is partly refixed as soluble bicarbonate ($HCO_3$) by the action of the enzyme PEPC or is respired through the fruit peel.

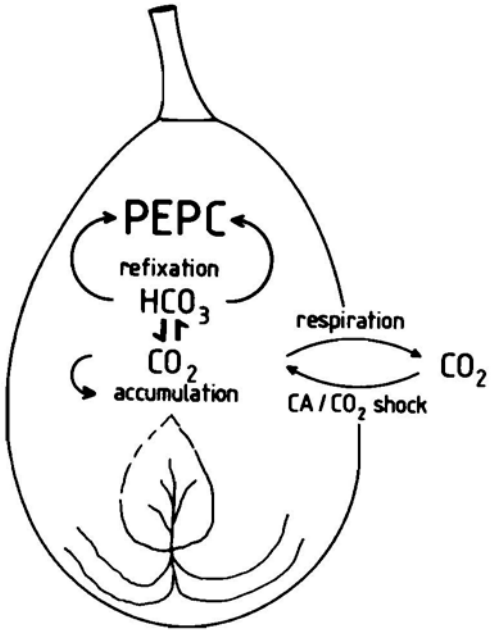


Fig. 2. Schematic of respiration and $CO_2$ refixation in avocado fruit. Fruit tissue respires $CO_2$. The enzyme PEPC refixes $CO_2$ in the soluble form of bicarbonate. The $CO_2$ which is not refixed, accumulates within the fruit or is respired through the peel. In CA storage or under $CO_2$ shock, ambient $CO_2$ penetrates the fruit.

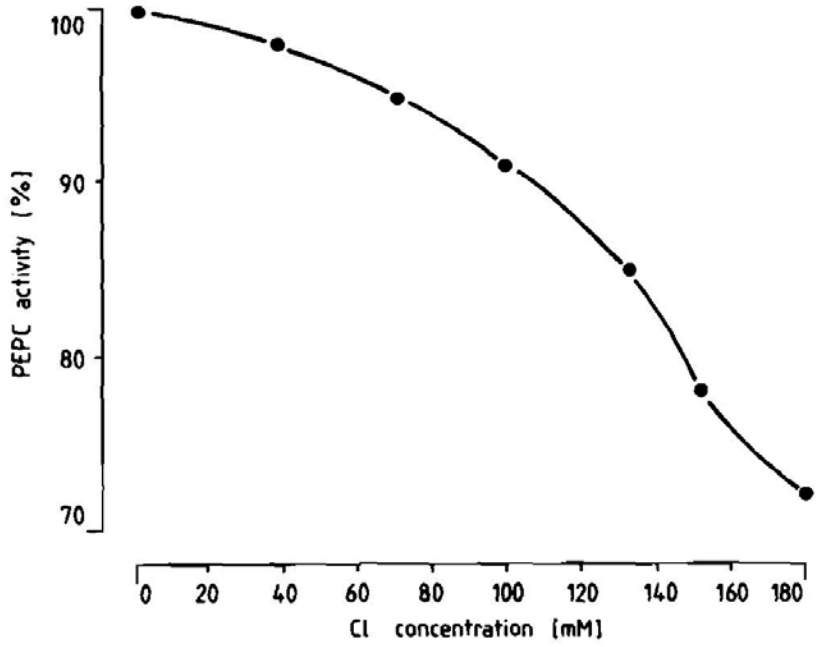


Fig. 3. Effect of salinity on PEPC from 'Fuerte' avocado fruit. PEPC was assayed at pH 7.8 after addition of 0 to 180 mM chloride at saturating (2.2 mM) PEP and (5 mM) $HCO_3$ concentration.

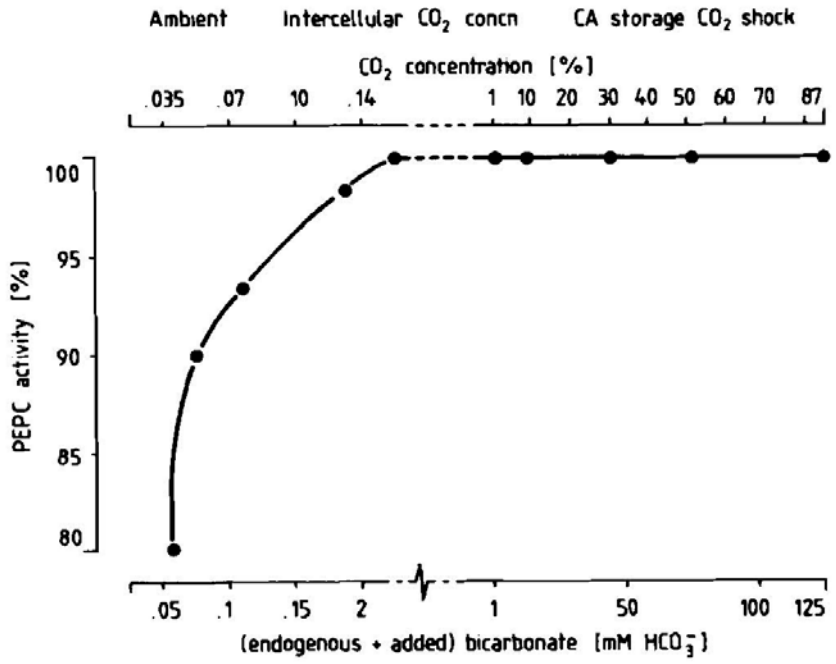


Fig. 4. Effect of $CO_2$/$HCO_3$ on PEPC from pre-climacteric avocado fruit. PEPC was assayed under saturating (2.2 mM) PEP concentration at pH 7.0 in the presence of up to 125 mM $HCO_3$ and taking into account the endogenous ambient $CO_2$ .