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Light *versus* Dark Carbon Metabolism in Cherry Tomato Fruits

I. OCCURRENCE OF PHOTOSYNTHESIS. STUDY OF THE INTERMEDIATES

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ABSTRACT

The photosynthetic properties of the internal and peripheral tissues of the cherry tomato fruit (*Lycopersicum esculentum* var. cerasiforme Dun A. Gray) were investigated. Whole fruit and their isolated tissues evolve large amounts of CO_2 in darkness. In the light, this evolution decreases but nevertheless remains a net evolution; 3-(3,4-dichlorophenyl)-1,1-dimethylurea abolishes the effects of light.

Incorporation of $^{14}CO_2$ by leaves and fruit tissues demonstrates that the outer region of the fruit has the highest photosynthetic efficiency on a chlorophyll basis; the internal fruit tissue, richer in chlorophyll, has a much lower efficiency. The identification of intermediates following short term incubations with $^{14}CO_2$ shows that in darkness the fruit accumulates the majority of label in malate. In the light, leaf tissue exhibits a pattern of incorporation characteristic of C-3 metabolism, whereas fruit tissue exhibits a decreased labeling of malate with a concomitant appearance of label in Calvin cycle intermediates. This is in agreement with the levels and types of carboxylating activities demonstrated *in vitro*; especially noteworthy is the very low ribulose diphosphate carboxylase activity in the internal fruit tissue.

The photosynthetic potential, phosphoenolpyruvate carboxylase activity, and quantities of malate accumulated by fruit tissues are parallel to their chlorophyll content during growth and maturation.

Relatively few studies have dealt with the physiology of fruit chloroplasts and chromoplasts including two recent reports on the photosynthetic potential of green apple fruit (2, 9). One of us previously showed that the internal and external regions of the cherry tomato fruit pericarp undergo different pigmentary (8) and ultrastructural (5) evolutions during growth and maturation. We were interested in knowing if the physiological properties of these two pericarp regions also differed.

The experiments reported here extend certain of our previous studies (6, 7) and involve the CO_2 metabolism and photosynthetic properties of growing and maturing fruits. In addition, labeled metabolic intermediates were isolated following incubation of tissues in darkness and in the light in the presence of labeled precursors. The carbon metabolism of fruit tissues is shown to be unlike that of the leaf, which is typically C-3.

MATERIALS AND METHODS

Lycopersicum esculentum var. cerasiforme Dun A. Gray were cultivated in a greenhouse under a regime of 16 hr light (sunlight supplemented with artificial light) at 24 C and 8 hr darkness at 17 C in an atmosphere of 50% relative humidity. The vermiculite in which the plants were grown was infiltrated with the following nutrient solution: (in mg/l) KNO_3, 274; KH_2PO_4, 137; $MgSO_4 \cdot 7H_2O$, 274; $(NH_4)_2SO_4$, 137; $Ca(NO_3)_2 \cdot 4H_2O$, 1,095; $Na_2EDTAFe$, 41; KCl, 2.74; H_3BO_3, 3; $MnSO_4 \cdot H_2O$, 1.7; $ZnSO_4 \cdot 7H_2O$, 0,274; $(NH_4)_2Mo_7O_{24} \cdot H_2O$, 0.274; $CuSO_4 \cdot 5H_2O$, 0.137 · H_2SO_4 (0.137 ml/1,000 l) was also incorporated.

The diameters of green growing fruits which were utilized varied between 4 and 15 mm. Maturing fruits, the diameters of which varied between 15 and 20 mm, were classified in stages "a" through "g" as a function of their color. Thus, stages "a" through "c" represent green to pink and "d" through "g" represent pink to red (*cf.* refs. 7 and 8 for a more complete description of this system). In most of the experiments presented, the fruit was first cut in two and the two regions of the pericarp were mechanically separated with a spatula. The internal fluidic portion is referred to here as the pulp and the relatively firm external portion is called the flesh. The majority of experiments presented here were performed on green adult fruits of 15 mm diameter. When photosynthetic controls were desired, adult leaves 15 × 25 cm were picked from the same plants as the fruits. All experimental material was harvested between 9 and 11 AM unless otherwise indicated.

Carbon Dioxide Gas Exchange Experiments. The tissue to be tested was placed in a thermostated reaction chamber (25 C). White saturating light (400 $w \cdot m^{-2}$, 400–700 nm) was provided by a xenon lamp (Osram 1001), filtered through 15 cm of water + an anticaloric filter, Balzer Calflex B1/K1. Air slightly enriched in CO_2 (430 μl/l) was supplied continuously at a constant rate of 9 l/hr and the CO_2 concentration in the gas mixture leaving the reaction chamber was determined with an IR gas analyzer.

Short Term $^{14}CO_2$ Incorporations. The plant tissues were physically fragmented as follows: flesh tissue (approximately 200 mg) was cut into about 25 pieces (4 × 4 × 1 mm); the corresponding pulp (approximately 300 mg) was divided into about 15 pieces (4.5 × 3 × 1 mm); half of a foliole was cut into about 100 pieces (4 × 2 × 0.1 mm). The tissue fragments were placed in small Erlenmeyer flasks which were sealed with rubber serum caps and then immersed in a temperature-controlled water bath at 25 C. The tissues were equilibrated in darkness for 5 min and then under 140 w/m² for 2 min. At the end of this equilibration, 0.5 ml of the following solution was added to each flask with a hypodermic syringe and needle: 30 mM tris (pH 7.8), 3 mM $MgCl_2$, 60 mM $NaH^{14}CO_3$ (specific radioactivity 4.1 μCi/μmol). Dark controls consisted of identical flasks

wrapped in several thicknesses of aluminum foil. The reactions were stopped after the appropriate times by the addition of 1 ml of 2 N HCl.

Extraction of Labeled Compounds. Following the incubation with radioactive precursor, the tissue fragments were rapidly frozen in liquid N_2. The frozen tissues were then ground in melting isopentane with a mortar and pestle, lyophilized and the powder mixed with 1 ml of formamide. Preparatory high voltage paper electrophoresis was performed at pH 4.5 (buffer = 8.5 ml of pyridine, 12.5 ml of glacial acetic acid, 70 mg of EDTA/g·80 ml of H_2O [4]). After autoradiographically locating radioactivity, the various bands were eluted with water overnight. The neutral amino acids sugars and starch remaining at the origin were separated by extracting the formamide with diethyl ether and then eluting the neutral soluble compounds with water for 12 hr. The sugars and amino acids thus obtained were separated by an additional paper electrophoresis in acidic buffer (formic acid-acetic acid-water, 1:3:16, v/v). Starch was estimated by digesting the insoluble material at the origin with a mixture of α- and β-amylases (Sigma) at 37 C for 24 hr. Soluble radioactivity appearing in the incubation medium was determined.

The compounds migrating during the preparative electrophoresis were pooled and an aliquot of this mixture was separated with electrophoresis in the first dimension and chromatography in the second dimension (3).

Assays of Carboxylase Activities. The activities of ribulose diphosphate and phosphoenolpyruvate carboxylases (EC 4.1.1.39 and 4.1.1.31, respectively) were assayed as previously described (10). Cell-free extracts of the various tissues were prepared as previously described (7) in an atmosphere of N_2 at 0 C in the presence of 14 mM β-mercaptoethanol to prevent oxidation by phenols. RuDP[1] carboxylase activity was assayed in the supernatant obtained after centrifugation of the crude homogenate at 20,000g for 15 min. PEP carboxylase activity was determined in the uncentrifuged extract. Acid-stable radioactivity was determined by transferring aliquots to filter paper circles, drying under a heat lamp, and then counting in a toluene fluor (0.4% PPO + 0.01% POPOP [w/v]) in an Intertechnique SL 40 scintillation counter.

Extraction and Determination of Malate. Tissues were killed by placing in boiling water in the ratio of 2 g of tissue/10 ml of H_2O. They were then disrupted with a Potter-type tissue homogenizer and exhaustively extracted with 10 ml of H_2O. The homogenate was centrifuged and the supernatant was used for enzymic determination of malate content (3).

RESULTS

Incorporation of $NaH^{14}CO_3$ throughout Growth and Maturation of Fruit. At each of the defined stages of growth and maturation, the Chl content of each of the tissues was determined and their capacity to fix $^{14}CO_2$ (as $NaH\ ^{14}CO_3$) in saturating light and in darkness was measured. Figure 1 shows that in both tissues of the fruit, fixation of CO_2 in the light and in darkness is almost parallel to the Chl content of the tissue. At all stages examined, the pulp was less active than the flesh on a per fruit basis, being 2.5 times less active in the dark and 10 times less active in the light. Since the pulp has a higher Chl content than the flesh, these differences would be more pronounced if the data were expressed on a Chl basis. In spite of this relatively higher Chl content, the pulp exhibits virtually no photosynthesis, as conventionally defined by the light-dark fixation. In fact, toward the end of maturation, there appears to be an increase in dark fixation in the absence of any detectable light-stimulated fixation by the pulp.

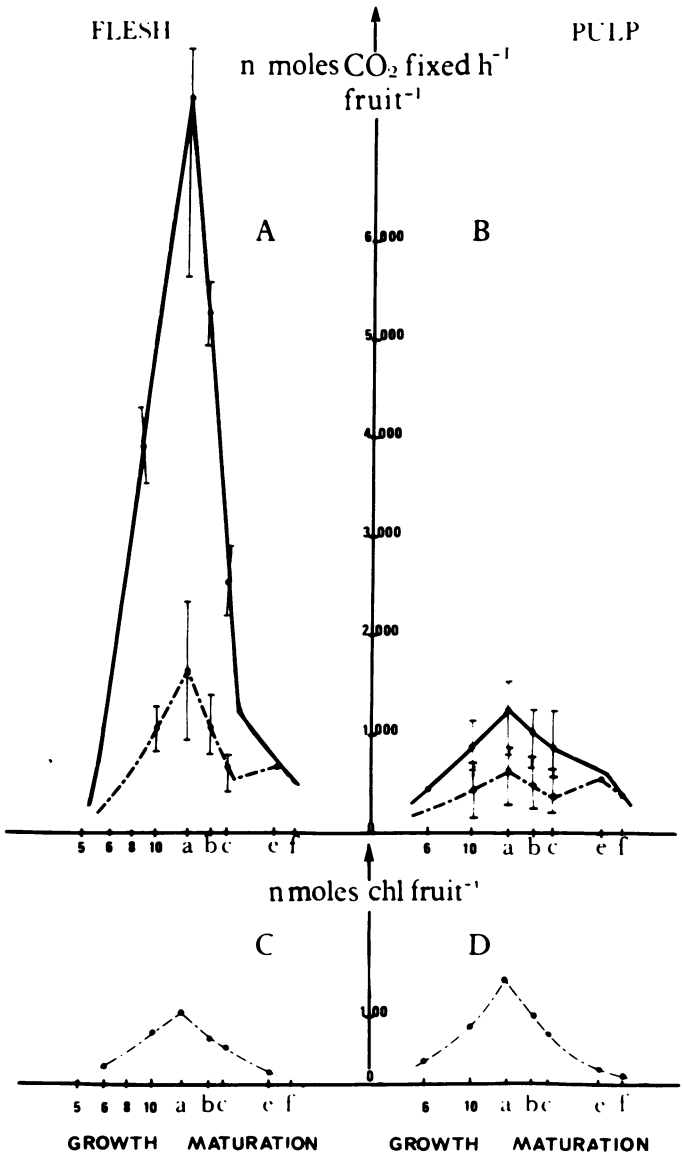

FIG. 1. $^{14}CO_2$ fixation and Chl accumulation by cherry tomato tissues throughout their growth (stages 5-10) and maturation (stages a-g). Tissues were preincubated for 5 min at 25 C in darkness (flasks wrapped with aluminum foil) or under 140 $w \cdot m^{-2}$ of incandescent light. At zero time, 0.5 ml of the following solution was added: 32 mM tris-HCl (pH 7.8), 3.2 mM $MgCl_2$, 60 mM $NaHCO_3$; $NaH^{14}CO_3$ was added to the stock solution to obtain 250 μCi/0.5 ml. The reaction was stopped after 45 min (reaction still linear) by the addition of 3.5 ml of NCS Tissue Solubilizer (Amersham/Searle, Amersham, U.K.). The tissues were digested for 17 hr at 37 C, whereupon 0.5 ml of glacial acetic acid was added to each flask in order to obtain an acid pH. Aliquots of 50 μl were transferred in triplicate to filter paper circles which were dried under a heat lamp, thereby driving off any unfixed $^{14}CO_2$ and residual acid. Scintillation counting was performed as for carboxylase assays. A and B: CO_2 fixation by flesh and pulp; (- - - -): darkness; (——): light. C and D: accumulation of Chl; C: flesh; D: pulp.

This rather unexpected behavior prompted a more thorough investigation of the CO_2 fixation properties of these tissues at the fully green adult stage a.

Short Term $NaH^{14}CO_3$ Incorporation in Green Adult Fruits and Leaves. The above mentioned experiments, certain of which were performed on maturing fruits, sometimes lasted for 45 min so that a measurable incorporation could be obtained with a reasonable dose of $^{14}CO_2$. We chose green adult fruits for the following experiments since they have the highest Chl

[1] Abbreviations: RuDP: ribulose diphosphate; PEP: phosphoenolpyruvate; PS: photosystem; RPPC: reductive pentose phosphate cycle.

content and manifest the greatest light-stimulated incorporation of $^{14}CO_2$. We also included leaf material from the same plants as a photosynthetic reference. The experiments shown in Table I represent 10-sec incorporations and can be expected to reflect the physiology of the plant more realistically. Expressed on a fresh wt basis, the leaf incorporates significantly more $^{14}CO_2$ than the other tissues, strongly indicating that it is the most important site of photosynthetic carbon assimilation for the plant. As suggested by our initial experiments, the flesh participates actively in a photosynthetic metabolism. Although this tissue is relatively deficient in Chl, its photosynthetic efficiency on a Chl basis is considerably higher than that of the other tissues studied, being five times greater than that of the leaf and 17 times greater than that of the pulp.

Carbon dioxide Gas Exchange Experiments. Since our experiments were performed with mechanically disrupted tissue fragments in a liquid medium exposed to $NaH^{14}CO_3$, we wanted to know the physiological validity of the results before continuing. We studied the photosynthetic properties of our material using a gas exchange technique. Leaves and entire fruits, as well as flesh and pulp, were placed in a chamber flushed with CO_2-enriched air (430 μl/l). The CO_2 concentration in the efflux gas was then measured (Fig. 2). The leaf exhibits a conventional photosynthetic response, evolving some CO_2 in the dark and consuming considerably more in the light. Whole fruits, on the contrary, always exhibit a new evolution of CO_2, although the amount evolved decreases in the light.

When the separated fruit tissues were used, we found that the additive response is in good agreement with the activity of the whole fruit. The pulp which accounts for about 70% of the fresh wt of the fruit also contributes between 60 and 70% of the respiratory CO_2 of the fruit. On the other hand, the flesh accounting for about 30% of the fresh wt of the fruit contributes at least two-thirds (and often more in other experiments) of the light-stimulated CO_2 consumption of the fruit, confirming our previous data (Fig. 1).

The gentle mechanical separation of the two tissues leads to a minimum of injury, at least concerning the responses studied. In addition, when the flesh is cut in small pieces, the response is quite similar to that of the whole tissue. This light-stimulated decrease of CO_2 evolution in the flesh is abolished by 25 μM DCMU (Fig. 2C'), strongly suggesting dependence on the photochemical activity.

The conditions of gas exchange are somewhat more physiological than those of $^{14}CO_2$ fixation (tissues fragments immersed in buffer). Nevertheless, the similarities of the results obtained with the two methods suggested that the $^{14}CO_2$ technique could be used in the following series of experiments to yield valid results.

^{14}C Distribution among Metabolic Intermediates. Following a 10-sec pulse with $^{14}CO_2$ in darkness and in the light, dark fixation represents 38 and 86% of the total light fixation of the flesh and pulp, respectively (Table II and ref. 7). Table II shows that malate accounts for the majority of dark fixation and significant amounts of radioactivity are also found in citrate. In the light, the leaf exhibits a distribution pattern characteristic of C-3 carbon metabolism, with 90% of the incorporated radioactivity found in RPCC intermediates. The distribution of radioactivity among the RPCC products of the flesh was appar-

Table I. Rates of fixation of $NaH^{14}CO_3$ by cherry tomato tissues.

Samples were prepared and incubated as described in Materials and Methods. White light (140 W·m^{-2}) was supplied by incandescent lamps. Uptake was for 10 sec.

Tissue	µmol CO_2 fixed per hour				Light / Dark fixation (ratio)
	per g fr wt		per mg Chl		
	Dark	Light	Dark	Light	
Leaf	54	650	15	180	12
Flesh	18.5	48	370	960	2.6
Pulp	10	11.5	103	115	1.1

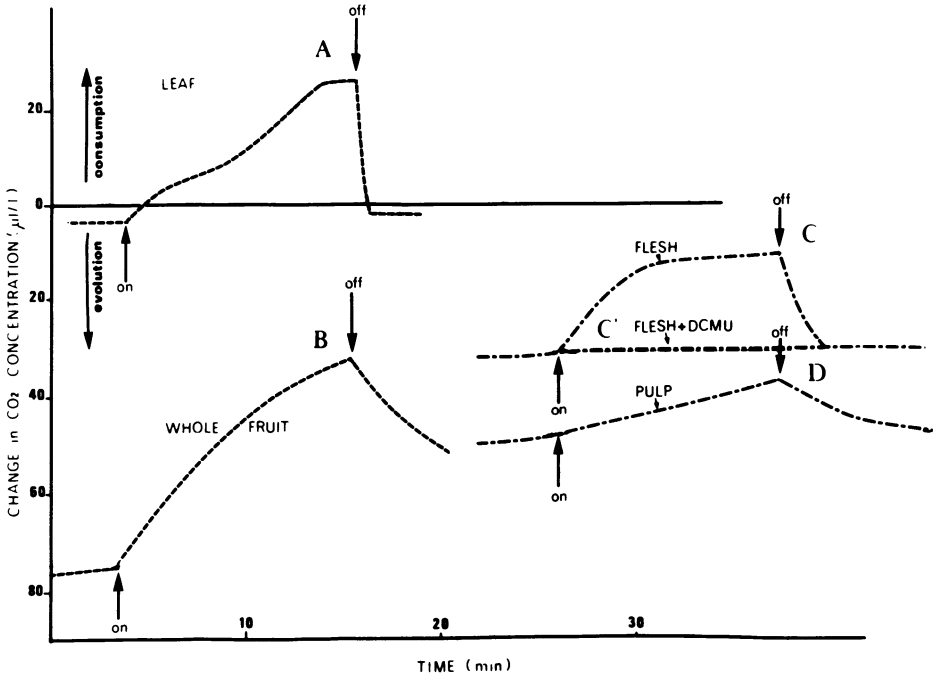

FIG. 2. CO_2 gas exchange by various cherry tomato tissues in darkness and in light. Air containing 430 μl/l CO_2 was introduced into the reaction chamber at a constant flow rate of 9 l/hr in an open circuit (nonrecirculating). The CO_2 concentration in the efflux gas was continuously measured with an IR gas analyzer. A: Leaf, 70 mg fresh wt, containing 252 μg of Chl. B: Two whole fruits, 3 g fresh wt, containing 276 μg of Chl. C and D: Flesh and pulp obtained from the fruit in B; flesh, 875 mg fresh wt, containing 40 μg of Chl; pulp, 2.25 g fresh wt, containing 236 μg of Chl. C': Flesh from C + 25 μM DCMU. Arrows indicate light on and off. The y axis is graduated in relative CO_2 concentrations, *i.e.* the reference line at 0 corresponds to 430 μl/l.

ently similar to that of the leaf (not shown). There is a difference between the two tissues in that about 20% of the radioactivity incorporated by the flesh is localized in malate + citrate, intermediates which are characteristic of dark CO_2 fixation (12). Considerable label continues to accumulate in malate in the light (about half that found in darkness). In the pulp, which is photosynthetically inefficient for the light-stimulated labeling of RPPC compounds, dark metabolism seems to be retained to an even greater extent in the light: more than half of the label which is incorporated following a 10-sec pulse is still found in malate.

The rather unexpected influence of light on the distribution of radioactive compounds in the fruit tissues led us to ask if this differential distribution of radioactivity between malate (representative of dark metabolism) and the RPPC intermediates is dependent on the light reactions of photosynthesis.

The flesh, which exhibits the greatest light-induced fixation of $^{14}CO_2$, was incubated with $NaH^{14}CO_3$ for 10 and 40 sec in the presence and absence of DCMU. The results (Table III) show that approximately four times more label is incorporated into RPPC compounds after 40 sec than after 10 sec in the absence of DCMU. When DCMU is present, incorporation into Calvin cycle intermediates is two times greater after 40 sec than it is after 10 sec. This less than total inhibition by DCMU may be due to an incomplete penetration of the inhibitor into the interior of the tissue fragments. Alternatively, a substantial pool of reducing power might exist, perhaps maintained by dark phenomena. ATP would be formed by cyclic photophosphorylation, accounting for the continued partial functioning of the Calvin cycle.

In the presence of DCMU, there is a greater accumulation of label in malate and a lesser accumulation in RPPC intermediates (Table III). The incorporation of label into malate after 40 sec is only about 50% greater than that observed after 10 sec. This apparent plateau suggests the rapid filling of a malate pool after incubation with $^{14}CO_2$, approaching the isotopic equilibrium of this pool.

The preponderance of labeled malate in darkness and its persistence in the light in the two fruit tissues prompted us to regard the carboxylating activities of cell-free extract of leaves, flesh and pulp. The results from three independent experiments are presented in Table IV with other published results for comparison. The PEP carboxylase activities of the fruit tissues are 20 to 40 times higher than those of the leaf, while RuDP carboxylase activity in flesh and leaves is comparable. The extremely low RuDP carboxylase activity in the pulp is not the result of the presence of an endogenous inhibitor since mixing experiments with active leaf extracts led to no inhibition of the latter activity. When two varieties of *L. esculentum* are compared, the various carboxylating activities are remarkably similar in leaf tissue. Although the corresponding data for fruit tissues are somewhat divergent (13) it appears that very high PEP carboxylase activities in fruit tissues may be a general phenomenon in this species.

We assayed both PEP carboxylase activity and the size of the malate pool in the pulp and flesh at various stages of the growth and maturation of the fruit. The results (Fig. 3) show that PEP carboxylase activity is very high in the green fruit, progressively decreasing thereafter until extremely low levels are found at the end of maturation. When the experimental results are expressed on a per fruit basis, the curve of malate accumulation is similar to these of Chl accumulation and light-induced $^{14}CO_2$ fixation. When these results are expressed on a per fruit basis, the pulp accumulates twice as much malate as the flesh. If the relative importance of the two parts is considered, we find the same level of malate accumulation on a fresh wt basis.

Table II. Incorporation of $NaH^{14}CO_3$ by cherry tomato tissues and the distribution of radioactivity among metabolic intermediates.

Fruits were picked during the month of April and were prepared and incubated as described in Materials and Methods. Incorporation was for 10 sec. Results are expressed as nmol CO_2 fixed per mg Chl.

Compounds	Flesh		Pulp		Leaf	
	Dark	Light	Dark	Light	Dark	Light
RPCC		1823		90		419
Sugars	7.2	25	2.2	2.4		3.4
Starch		50		1.4		1.2
PEP		136		3.5		13.2
Malate	788	384	207	186	31	8.6
Aspartate	28.4		4.1		2.2	
Citrate	123	92	81	65	7	3.2
Other solubles	19	29	10	4	0.7	27.5
Total	965.6	2539	304.3	352.3	40.9	476.1

Table III. Incorporation of $NaH^{14}CO_3$ by the flesh of cherry tomatoes.

Fruit tissue was prepared as described in Materials and Methods. Where indicated, DCMU was vacuum infiltrated. Fruits were picked in the month of December and fixed approximately 50 μmol $CO_2 \cdot mg\ Chl^{-1} \cdot hr^{-1}$. Results are expressed as nmol CO_2 fixed per mg Chl. Numbers in parentheses are percentages of total radioactivity fixed.

Incubation time (sec)	DCMU	Compounds							
		RPCC		Citrate		Malate		Sugars +Starch	
10	-	52	(15.0)	25	(7.5)	251	(75.5)	5	(1.5)
10	+	34	(7.6)	29	(6.7)	377	(85.4)	1.7	(0.4)
40	-	184	(28.5)	37	(5.7)	407	(63.1)	17	(2.7)
40	+	64.5	(10.6)	63	(10.5)	468	(77.2)	11	(1.8)

Table IV. Ribulose diphosphate and phosphoenolpyruvate carboxylase activities in cell-free extracts from cherry tomato tissues.

Extracts were prepared and assayed as described in Materials and Methods. Results are expressed as μmol substrate transformed per hour and per mg Chl in the extract. Published values corresponding to another variety of tomato are shown for the purpose of comparison.

Organism	RuDP C'ase	PEP C'ase	$\frac{\text{RuDP}}{\text{PEP}}$ C'ase (ratio)
Lycopersicum esculentum var. *cerasiforme* DUN A. GRAY			
Flesh	149	320	0.47
Pulp	3.6	161	0.02
Leaf	123	7.8	15.8
Lycopersicum esculentum Mill.[1]			
Epidermis + mesocarp	302	3147	0.09
Leaf	187	12	15.6

[1] Willmer and Johnston (1976)

DISCUSSION

The present results show that $^{14}CO_2$ (as $NaH^{14}CO_3$) fixation by tomato fruit is enhanced in the light. A parallelism is demonstrated between this ability and the Chl content of fruits throughout their growth and maturation.

Gas exchange experiments showed that the fruit has a high rate of CO_2 evolution in darkness which decreases in the light; similar findings have been reported for apple tissues (9). CO_2 exchange by the fruit remains a net evolution in the light, compared to the leaf which has a net CO_2 fixation in the light. The decrease in CO_2 evolution by fruit tissues in the light is abolished by DCMU, implicating the participation of PSII in this process. The physiology of the fruit is apparently such that relatively large quantities of CO_2 are normally liberated as a result of intense respiration and that some of this CO_2 is refixed by a light-driven process. Fruit photosynthesis might serve to limit CO_2 losses by the climacteric fruit. Thus, atmospheric CO_2

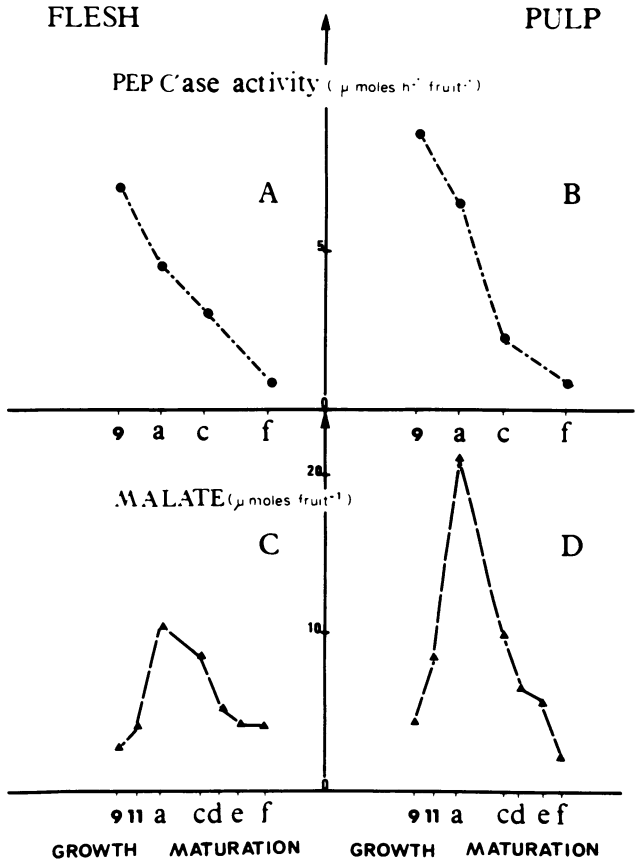

FIG. 3. PEP carboxylase activity (A and B) and malate content (C and D) in cherry tomato fruits during growth and maturation. Before each assay fruits were mechanically separated into flesh (A and C) and pulp (B and D).

fixation does not seem to be necessary for the functioning of the RPPC in the fruit.

Certain qualitative and quantitative differences can be discerned between the flesh and pulp of the fruit. Both are Chl-containing tissues, but the pulp, in contrast to the flesh, is highly inefficient for light-driven CO_2 fixation when expressed on a Chl basis. This is probably due to the low RuDP carboxylase activity found in cell-free extracts from this tissue. Moreover, our previous studies have shown that the Chl in the flesh is organized in efficient PSII units which are capable of intersystem energy transfer, while the pulp is drastically PSII-deficient (7).

^{14}C distribution following short term incorporation (Table III) demonstrates that the dark metabolism, characterized by a preponderant labeling of malate, (+citrate) (1, 12), is apparently retained to varying degrees by fruit tissues incubated in the light. The incorporation of significant quantities of label into malate is consistent with the carboxylation activities of the two regions of the fruit demonstrated *in vitro* (Table IV). Indeed, the flesh accumulates malate and has a high PEP carboxylase activity.

Our experiments show that $^{14}CO_2$ can enter the malate and/or RPPC pools of the fruit. This distribution of $^{14}CO_2$ could be at the level of available CO_2 for the cytoplasmic PEP carboxylase and the chloroplastic RuDP carboxylase, the former having a higher affinity for CO_2 than the latter. This "competition" is best illustrated by the data of Table III, where the presence of DCMU leads to a decreased incorporation of label into RPPC compounds and to an increased incorporation into malate. Another example of this regulation may be seen by comparing the data of Tables II and III. The only significant variable distinguishing these two experiments is that Table II corresponds to fruits picked in April and Table III concerns December fruits. The two fruits fix comparable amounts of label into malate in the light in the absence of DCMU, while fruits picked in April fix 30 times more radioactivity into RPPC compounds than do the winter fruits because of the greater quantity of sunlight.

The climacteric rise of the fruit (11) results in the liberation by the mitochondria of large quantities of CO_2 into the cytoplasm. The high specific activity of PEP carboxylase found in the tomato fruit *in vitro* is entirely consistent with the possibility that a large portion of the CO_2 liberated during the climacteric rise is refixed into malate, allowing some conservation of C; a similar process has been also observed in tissues such as corn roots having nonautotrophic CO_2 fixation (12). In tomato tissues, characterized by a high respiratory activity, the malate formed could enter the mitochondria and thus contribute to the maintenance of the organelles' charge of tricarboxylic acid substrates. The role of malate in these processes has been studied in detail in the companion paper.

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