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Metabolism of tartaric and malic acids in *Vitis*: A review — Part B

by

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The physiology of malic acid

Of the two quantitatively most important acid components in the grape leaf and berry — tartaric and malic acids — the former displays the essential characteristics of a secondary product, a phenomenon which has been reviewed in the first section of this article (84). Malic acid, on the other hand, is known to be a very active intermediate in grape metabolism (71, 97, 111) and seems to play a significant role in anabolic reactions, such as dark fixation of carbon dioxide (66, 67, 96) as well as in the acid catabolizing processes of fruit ripening (55, 99, 104). In accordance with this twofold physiological function, malic acid is accumulated in young tissues, particularly the fruit, in concentrations ranging up to 15 mg/g fresh material in green berries. After the onset of ripening, a rapid fall in malate content to 2—3 mg/g FW takes place within a time period of little more than a week (1, 45, 85), which amounts to a net malic acid decrease of between 5 and 10 µmoles per day and berry. Almost simultaneously an even more drastic increase in sugar concentration, mainly glucose and fructose, occurs (2, 15). No comparable changes in acid content are detectable in leaf samples, where malate starts to accumulate steadily after an initial lagphase in very young tissue, reaching its final level when the lamina has attained about one fourth of its full size (46, 65). There is no indication of a standstill in malic acid synthesis or a change to intensified malate remetabolization as long as the leaf is photosynthetically active. This lack of variation in the acid content of maturing leaves, with virtually constant malate levels of approximately 2 mg/g FW, may be attributed to a balanced activity of synthesizing, dissimilatory, and perhaps transport mechanisms (59, 86). In late autumn an export from the senesceing leaf into the lignifying shoot was observed, as also seen for tartaric acid (46).

Although the capacity for β-carboxylation is undoubtedly present in grape material (34, 60, 67, 87), and malate was found to be the predominant radioactive compound after dark fixation of $^{14}CO_2$ in grape berries and leaves (61, 66, 83, 96), no acidification of the tissue was detectable in the dark (55), which precludes the functioning of a crassulacean acid type metabolism. Still, the coincidental timing of acid disappearance and sugar accumulation has prompted an intensive investigation of the correlationship between these two counter-current pathways (23, 24, 82) and it has been demonstrated in experiments with specificly labelled tracer that a reversal of carbon flow occurs during berry ripening (88). However, the quantitative contribution of the gluconeogenic reaction to sugar content at maturity, is believed to be rather limited (85).

1. The role of malate in C_3-metabolism

The physiological function of malate is well defined in plants assimilating CO_2 by the C_4-dicarboxylic acid pathway of photosynthesis, where malic acid is a primary product, which has been shown to serve as a transport vehicle, shuttling CO_2 from the outer (mesophyll) to the inner (bundle sheath) compartments of the leaf (40, 80, 106). Its role in the transitory binding and storage of carbon dioxide, as reflected by a diurnal acidification/deacidification rhythm in the mostly succulent tissues performing crassulacean acid metabolism, is equally evident (50, 51, 106).

In contrast to this, the relevance of abundant dicarboxylic acid production in C_3 -plants (6, 7, 64, 93) is poorly understood and even the physiological background for the striking pattern of malate accumulation and remetabolization, which takes place during fruit ripening, has received comparatively little attention (37, 38, 61, 85, 110, 112). Our comprehension of the phenomenon of malic acid formation in the C_3-group of plants is consequently confined to the knowledge of the biochemical transformation directly involving the acid, which are believed to be analogous to the enzymic reactions in C_4- and CAM-plants.

The metabolic origin of the CO_2-acceptor molecule, presumably phosphoenol pyruvate, in the β-carboxylative step of C_3-plants, still remains uncertain. Only recently BOECHER and KLUGE (6, 7) have presented evidence that in spinach leaves the availability of the acceptor molecule, rather than that of the appropriate CO_2-species, controls the rate of this assimilatory reaction and thus ultimately the extent of malate synthesis. Their data seem to favour the hypothesis of a direct derivation of the C_{1-3} -moiety of malate from phosphoglyceric acid originating in the Calvin cycle. However, carbon flow via a photosynthetic and transitory hexose or starch pool to malic acid within the leaf (74) cannot be excluded from the results at hand. In analogy to the path of carbon in CAM-plants, remetabolization of starch is considered an alternative in C_3 -plants when the supply of phosphoglyceric acid is limited (7). Apart from these two possibilities, serine was proposed as the ultimate C_3-precursor of dicarboxylic acids on the basis of identical labelling patterns in the amino acid and the C_{1-3} moiety of malate (41). However, the biochemical nature of the necessary transformations is unknown and, moreover, KENT compared the labelling of pool-forming substances such as malate, alanine and serine with that of a short-lived intermediate (oxaloacetic acid) after a metabolic time of 10 min, which would inevitably lead to extensive random distribution of label in the first case, thus making comparisons difficult.

2. CO_2 -assimilation and malic acid synthesis in grape material

Although in grape leaves the malic acid content is never depleted to zero level and even after 14 days in the dark, concentrations in the range of 0.6 mg/g FW were determined in young material (POSSNER, pers. comm.), no indication of a correlation between malic acid accumulation and the absence of light was detectable. In immature Sultana berries there was even a suggestion of an increased acid titre following illumination, which is not compatible with the function of CAM (55). In accordance with this observation it has been unequivocally shown that the C_3 photosynthetic pathway operates in grape leaves with phosphoglyceric acid and hexose mono- and diphosphates as primary products (22), while ^{14}C-incorporation from $^{14}CO_2$ into malic acid in short-term experiments emphasized the intermediary or end-product characteristics of this compound (9). On the other hand it has been demonstrated that green berries already after a 10 s exposure to $^{14}CO_2$ in the light incorporate approximately 40 % of radioactive label into malate and aspartate, thus exhibiting a trend towards C_4-metabolism (81, 91). Determinations of enzyme activities and analyses of primary assimilation products in tomato leaves and fruits indicate that these tissues also differ considerably in their assimilation characteristics (110). While the leaves perform straightforward C_3-metabolism, malate was found to be the main labelled product in the fruit after $^{14}CO_2$-assimilation in the light as well as in the dark. Still, the CO_2-compensation points and the lack of diurnal fluctuations in acidity rule out the presence of an active C_4- or crassulacean acid metabolism. These results are in agreement with mass-spectrometric data on grape material. The overall carbon isotope ratio ($\delta^{13}C$: 103) in leaves and berries of *Vitis vinifera* was found to range between -24.6 ‰ and 26.3 ‰ (21), the mean value for C_3-plants being -27.8 ± 2.75 ‰. This indicates that ribulose-1,5-diphosphate carboxylase is the predominant enzyme of CO_2-fixation in the investigated tissues, because PEP-carboxylase discriminates less against ^{13}C, which results in values around -11 ‰ in plants performing C_4-metabolism (20). It seems noteworthy in this context that a discrimination of -23.5 ‰, as determined in malic acid isolated from grape berries, correlates perfectly with the values predicted for this intermediate in C_3-plants (-22 ‰ to -25 ‰: 109). That discrimination of ^{13}C in tartaric acid was the same, may consequently not be considered as an indication of an inadequate shift of malic acid values towards the PEP-carboxylase value to account for a malate synthesis via this enzyme, as has been suggested (21). Rather, a higher rate of discrimination would be expected in tartaric acid, which is believed to originate, exclusively from the carbohydrate pool (84), and thus in a C_3-plant as the grapevine should be subject to the full discriminating power of ribulose-1,5-diphosphate carboxylase (-28.3 ‰: 103 and lit. cited therein).

It is therefore concluded that the grapevine, in agreement with its habitus and ecological characteristics (5), assimilates carbon dioxide by the C_3-mechanism. The labelling pattern of malate obtained after $^{14}CO_2$-fixation in the dark by grape leaves and berries, with the bulk of radioactivity concentrated in the carboxyl (mainly C_4) atoms (66, 96), unequivocally proves that β-carboxylation is an important step in the acid synthesis. The enzyme responsible for this reaction, PEP-carboxylase (E.C. 4.1.1.31) (34, 59, 61, 67, 87), was found to be more active in grape leaves cultivated under suboptimal growth-conditions. However, no concomitant increase in malate content of the tissue was detectable, which was taken as evidence that also in this material PEP-carboxylase is not the pace-setting enzyme for malic acid synthesis (9). A time-course study of ^{14}C-incorporation into the C_1-C_3 and C_4 carbon atoms of malate further showed that after as little as 10 seconds 44 % of the label appeared in the C_3-moiety in the light, which suggests a direct metabolic relationship between photosynthetic formation of C_3 -units and malate biogenesis (9). The involvement of serine (41) in this biochemical sequence is difficult to evaluate, but it must be noted that a mere coincidence of labelling falls short to prove any precursor-product relationship, when no pertinent kinetic data are available, which allow an assessment of the rate and direction of carbon flow. Moreover, the distribution of label in oxaloacetate (41) and aspartate (42) does not coincide with that of serine and malic acid, which supports the hypothesis that randomization had occurred in the malate pool(s). Also, progressive favouring of the glycolate pathway by increasing the oxygen concentration in assimilatory experiments with $^{14}CO_2$ application to grape leaves, resulted in the expected doubling of ^{14}C-incorporation into the glycine/serine pool, but the effect on malic acid synthesis was not in a comparable range (65). Conclusive evidence for the proposed pathway of malate formation via serine (42) is thus lacking, however , the possibility of an interrelationship between photorespiratory components on one side, and glycolytic C_3-compounds on the other, cannot be dismissed entirely at this stage.

3. Degradation of malic acid

Due to the predominant role of malic acid in determining fruit quality, practical as well as scientific interest has mainly focused on the phenomenon of acid disappearance after véraison (3, 14, 28, 45, 71, 111, 112), that is the series of physiological events at the onset of ripening. As a consequence of the publications of MEYNHARDT (67) and HAWKER (33, 35), who introduced the techniques for reliable determinations of enzymic activities in grape material, an explanation for the known pattern of acid accumulation and decrease was sought by assessing the *in vitro* activities of the relevant enzymes. However, unlike in apple fruits (39), no correlation between phosphoenolpyruvate carboxylase and malate accumulation, or malic enzyme and acid decrease was detected in grape berries (34). As for malate dehydrogenase (E.C. 1.1.1.37), reports concerning changes in activity throughout maturation are contradictory (18, 34), but a shift in the isoenzyme pattern (18) may indicate that MDH in some compartments (i.e. the mitochondria) is synthesized *de novo* after véraison and although this change may have no influence on the overall MDH-activity, the enzyme could be locally more active than before.

Because of the lack of a massive increase in respiratory CO_2-evolution at the beginning of ripening (28, 54, 55) the grape is classified as a non-climacteric fruit. Still, the sudden change in the respiratory quotient from values close to 1 in the green berry to approximately 2 in the fruit immediately after véraison (54, 55) and the concomitant loss of malic acid, suggest a difference between immature and mature berries in their ability to metabolize malic acid. In accordance with this supposition, $^{14}CO_2$-evolution from green berries in response to added ^{14}C-malate was considerably lower than from ripening material (32, 55, 99).

Besides berry softening, colour change and the start of acid dissipation, véraison embodies the beginning of a massive sugar incorporation (16) into the berry. The simultaneous onset of malate disappearance and of hexose accumulation, has raised the question of a metabolic interrelationship between these two countercurrent sequences. The occurrence of net carbon flow from the acids to the sugar pool in ripening grape berries could indeed be demonstrated by introducing ^{14}C-acids, followed by determination of radioactive label within the isolated fraction of neutral substances (23, 24, 25, 82, 97). Evidence for the biochemical pathway of this transformation was sought by administering biogenic malate via fumarate-2,3-^{14}C for relatively short metabolic periods (6 h), which minimizes randomization of label and the danger of decarboxylation with subsequent refixation of radiocarbon (88). The distribution of ^{14}C-activity within glucose from this experiment revealed that the sugar was mainly (80 %) labelled in the carbons 1,2 and 5,6. This pattern precludes chance reassimilation of $^{14}CO_2$ after decarboxylation, because this would be expected to result in preferential ^{14}C-incorporation into C-atoms 3 and 4 of glucose. The observed distribution of label presents strong evidence that gluconeogenic metabolism of malic acid occurs via oxidation to oxalacetic acid, decarboxylation and subsequent formal reversion of glycolysis to yiel hexose.

Although it has been unequivocally shown that temperature is the predominant factor mediating grape malate content at maturity (13, 30, 43, 44, 48, 49, 76, 107, 108), no direct correlation between changes in temperature and the rate of gluconeogenesis has been found. Instead, ^{14}C-incorporation from radioactive fumarate was determined to be highest at 20 °C, while only about half maximal activity was observed, both at 10 °C and at 30 °C. In agreement with these findings STEFFAN *et al.* (99), after monitoring the fate of malic acid-U-^{14}C in the course of berry development, found that the influence of the ripening stage on gluconeogenesis was more pronounced than that of temperature.

Also, biochemical characterization of the presumptive key enzyme in the gluconeogenic sequence, PEP-carboxykinase (E.C. 4.1.1.32), made clear that although the enzyme displays very distinctive regulatory properties, temperatures up to 40 °C did not affect its activity (87). These results indicate that at elevated temperatures malate catabolizing processes other than gluconeogenesis, notably respiration, must prevail in the grape berry (78, 99).

4. Factors influencing grape malic acid concentrations

Investigations concerning the influence of temperature on acid levels in grapes for practical reasons mainly concentrated on assessing the extent and rate of the decrease in titratable acidity after the beginning of ripening. Because these determinations did not routinely stretch over the phase of malate accumulation (47, 48, 53, 88), the fact went unnoticed that the onset of acid disappearance occurs earlier under warm conditions (13, 44, 86), resulting in a prolonged phase of sugar incorporation with concomitant lower acid contents at harvest (77). From the available data it was concluded that malic acid accumulation was favoured at relatively cool temperatures, and a control mechanism based on the differences in temperature susceptibility of the relevant malate synthesizing and catabolizing enzymes was suggested (58, 60). However, no reflection of the expected maximum tendency for malic acid accumulation at 20 °C was observed in the temperature response curve of dark CO_2 fixation, although the latter reaction was found to be intrinsically related to malate synthesis (61). In addition, the calculated effective turnover rate of grape malic enzyme, extracted from fruits grown under warm or cool conditions was the same, which indicates that temperature has no direct bearing on the activity of this enzyme (86). Subsequent biochemical characterization of malic enzyme from grape berries showed an atypical dependence of enzyme activity on malate levels. The mandatory accessibility of a "relatively high threshold substrate concentration", needed to trigger malate decarboxylation at slightly alkaline pH (19, 26, 27), is alleviated by marginally lowering the pH from 7.4 to 7.0, where an approximation to Michaeli-Menten-kinetics is observed (73). Supplementing the assay mixture with various divalent cations not only affected turnover rates (8, 57) but also enzymic affinity for malate (75), the grape enzyme displaying highest *in vitro* activity as well as maximum affinity for malic acid with Mn^{++} as cofactor (73). On the basis of energetic considerations and of the differences in the pH-optima between the forward (decarboxylating) and reverse reaction, it is concluded that a physiological function of malic enzyme in malate formation is rather unlikely (73, 102, 105). The purified enzyme is further characterized by a very pronounced sensitivity towards changing NADP/NADPH ratios in the assay mixture, a regulating system which is known to exert control on hexose monophosphate shunt activity (62, 63, 70).

A different procedure for the isolation of malic enzyme from grape berries, yielding virtually the same final specific activity was described recently (94, 95). However, no allosteric properties were detected in the purified enzyme. It is not clear from the presented data, whether the difference is due to the use of mature instead of green berries as source material, or whether the high concentrations of NADP and/or NaCl used to desorb the protein from the affinity gel, may possibly have caused this dramatic change in enzyme characteristics.

As mentioned previously, PEP-carboxylase appears not to be the pace-setting enzyme in malate synthesis, however, its activity is inhibited in the presence of malic acid (60), probably as a feedback mechanism preventing excessive malate accumulation in the cytoplasm (61).

The very high respiratory turnover rates of ^{14}C-malate (60—95 %/15 h), observed after administration of uniformly labelled tracer through the pedicel of ripening grapes (78, 99), which is not paralleled by the *in vivo* decrease in malic acid in maturing fruits (98), indicates that compartmentalization, rather than differences in total enzymic activity may regulate malic acid levels during the ripening process. Metabolization of radioactive malic acid injected into the berries to simulate the fate of stored malate, occurred at an appreciably slower rate (ca. 45 %/15 h) irrespective of the developmental stage of the grapes (98). It was thus concluded that two metabolically different malate pools are present in the grape berry. The existence of a virtually inert malate storage pool and that of a rapidly metabolizing pool was also reported by LAKSO (58) and LAKSO and KLIEWER (61). Based on the incorporation kinetics of ^{14}C-activity into malate during continuous feeding of $^{14}CO_2$ to exised green berries at different temperatures, it was demonstrated that the relative size of the metabolic pool decreases with increasing temperature. This could possibly account for the observed temperature dependence of the gluconeogenetic sequence, with optimum turnover rates between 10 and 20 °C (88, 99), because within this range both, substrate availability and enzymic activity are comparatively high. While the metabolic pool most probably contains cytoplasmic as well as mitochondrial malic acid, the vacuole is believed to be the site of malate storage, and indeed considerable amounts of malate were determined in vacuolar extracts from grape subepidermal cells (68).

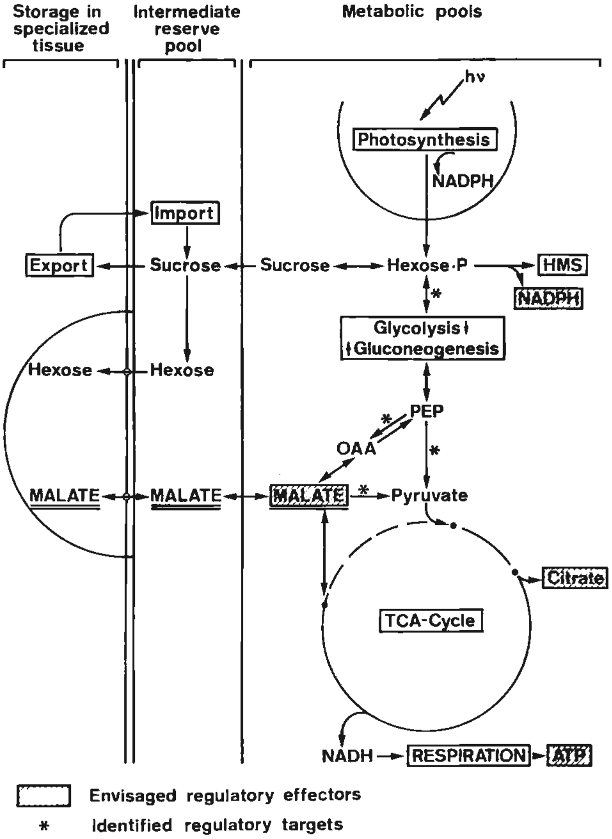
The partitioning of components, in this case malic acid, between active and inactive pools would consequently be controlled by the permeability characteristics of the cellular membranes. Since the decrease in acid at the onset of ripening is accompanied by an impressive accumulation of hexose in grape pericarp cells, the efflux of malic acid from the vacuoles cannot be attributed to a general "leakiness" of the tonoplast membrane (89). The fact that injected ^{14}C-malic acid is translocated from the center towards the periphery in the ripening berry, whereas no transport of label occurs in the green fruit (98), indicates that the changes involve the whole fruit rather than individual cells. It is obvious that malate remetabolization is enhanced in the peripheral tissue during ripening (98), presumably resulting in a depletion of the metabolic malate pool(s), which are replenished from the storage compartments, thus creating a radial malic acid gradient in the berry. The reasons for this polarization of malate catabolism is not clear, but it may be related to the end-user characteristics of the berry since in leaves no comparable pattern of malate accumulation and decrease is observed. However, during extended cultivation in the dark, leaf malate concentrations also decrease to a critical level of approximately 0.6 mg/g FW, which concentration appears to represent a minimal metabolic pool, because upon further etiolation the leaves invariably wilt and die (POSSNER, pers. comm.).

5. Regulation by compartmentation?

The decrease in malic acid concentrations during etiolation of grape leaves indicates that also in leaf cells some potential for malate storage is present. As in other tissues assimilating via the C_3-sequence of photosynthesis, the malate pool appears to be filled in the light and decreases under conditions where energetic demands prevail over the supply of a C_3-compound for ß-carboxylation, presumably phosphoenolpyruvate (7). This mechanism does not preclude malate formation in the dark, provided that the appropriate CO_2-acceptor is available. In agreement with this presupposition, dark fixation rates of grape leaves are considerably lower than photosynthetic CO_2-incorporation, the latter being normally about 40 times more effective (83). However, unlike in green berries, no excessive accumulation of malate is observed in the leaves, probably because the assimilates are translocated towards the metabolic sinks such as fruits, vegetation tips and roots (52). The option to export surplus products correlates logically with the physiological producer function of the adult leaf. With the assimilatory stream being strictly unidirectional (92), this possibility seems not open to the grape, which generally displays the characteristics of a storage organ. Since the berries are reported to lack the enzymes for starch synthesis (36) other means had to be developed to cope with an at least transitory flooding of the symplast with imported assimilates. From the available data it is obvious that the imported sugar is metabolized through the commonly known hexose utilizing pathways (32, 85, 86), notably the hexose monophosphate shunt and glycolysis, as indicated by the high labelling of malate after feeding ^{14}C-sucrose to green grape berries (72). It has further been demonstrated that exogenous malate is not degraded by the berry at this stage of development unless it is supplied through the pedicel (98). This feeding technique was shown to convey the tracer predominantly to the peripheral areas of the berry, which appear to reflect conditions normally found in the leaf with malate concentrations of approximately 2 mg/g FW (17). Since *in vivo* absorption of photosynthetic assimilates also occurs mainly via the peripheral vascular bundles, (56), the relatively high metabolization of malate in green berries, when administered through the pedicel, may be taken as evidence that acids are not physiological transport substances because malate respiration, as indicated by RQ values around 1 at this stage is practically not existent (54). It has to be noted in this context that tartaric acid is also uncharacteristically degraded when supplied through the pedicel of green berries, possibly in a detoxification process (23, 24, 25, 32, 90, 101).

In agreement with previous results (52, 100), sucrose rather than dicarboxylic acids appears to be transported from the leaves to the developing berries, where malic acid is produced and subsequently stored in the mesocarp (17, 29, 54). This tissue, which represents the pulp of the berry, consists of specialized storage containers, adapted to take over and sequester imported components from the peripheral areas (56, 98). It is believed that the "non-storage-type" cells, in addition to the metabolically active compartment, also contains a reserve pool of limited capacity (cf. Fig.) The compounds within this "transitory" or "intermediate pool" are easily accessible for remetabolization and guarantee the sustenance of the vital cellular functions under stress conditions and generally at times when photosynthesis is low. On the other hand, when the supply of assimilates is adequate, the intermediate pool is refilled first and only then the surplus is either exported or, in the case of the peripheral berry tissue, incorporated into the true storage compartment. Accumulation of malic acid in vacuoles has been demonstrated to be a permease catalized, but energy independent reaction in *Bryophyllum daigremontianum* (12). If comparable mechanisms are realized in the grape mesocarp, malate overflow in the cytoplasm of the adjacent peripheral tissue is bound to induce malate transport through the tonoplast of the storage cells, which thus would correspond to export of assimilates by the leaves.

After the onset of ripening, drastic changes in grape metabolism are observed, the most prominent feature being a rigorous reduction of sugar metabolization via glycolysis and consequently malate production (31, 72, 96). Following véraison, imported sucrose in hydrolyzed (79) and, as has been shown recently (10, 11), stored in mesocarp vacuoles by vectorial synthesis of sucrose phosphate during the passage through the tonoplast. The enzymic complex responsible for this transport is thought to catalyze sequential activation of hexose and beyond any doubt energy is required. The ATP demands of this and other biochemical reactions are at this stage of berry development met by respiration of the available intermediate reserves. Due to the severe inhibition of glycolysis, this results in a rapid depletion of intermediate pool substances and vital levels in the metabolic pool must be sustained by an anaplerotic import of malic acid from the storage cells. Depending on the actual energy demands, malic acid in the metabolic compartment is either respired (54) or, as a consequence of the reversal of glycolytic carbon flow, directed towards gluconeogenesis. In agreement with previous reports (88, 99), gluconeogenetic turnover rates would be favoured under cool conditions, when respiratory activity is low. On the basis of this hypothesis, the fluctuations in malic acid concentration during grape berry ripening can be explained by the action of general regulatory mechanisms, for example differential enzyme activity, feedback inhibition and energy charge (4, 69). The presented concept accounts for most of the known characteristics of grape malate metabolism. There appears to be no particular necessity for a separation of leaf and fruit biochemistry, which would imply the existence of fundamental differences in the control of malic acid levels between two organs of the same plant. The fact that the acid fractions of young berries and leaves are practically identical (83), certainly supports the hypothesis of a common and perhaps ubiquitous scheme, mediating the acid content of leaves and fruits.



Biochemistry and compartmentation of malate/sugar metabolism in the grapevine

List of abbreviations

CAM: crassulacean acid metabolism, C_3-metabolism: fixation of CO_2 by Calvin cycle reactions, C_4-metabolism: C_4-dicarboxylic acid metabolism, HMS: hexose monophosphate shunt, TCA-cycle: tricarboxylic acid cycle, FW: fresh weight, RQ: respiratory quotient, MDH: malate dehydrogenase, OAA: oxaloacetic acid, PEP: phosphoenol pyruvate.

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