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Oscillatory Nature of Metabolism and Carbon Isotope Distribution in Photosynthesizing Cells

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1. Introduction

A study of carbon isotopic characteristics of plants and animals, such as, shifts in carbon isotope ratio of plant biomass relative to environmental CO_2, $\delta^{13}C$ values of biochemical fractions and individual metabolites, different isotopic patterns of biomolecules and diurnal isotopic changes of respired CO_2, evidences that in a living cell carbon isotope fractionation takes place.

The above characteristics might be the source of valuable information on cell metabolism and regulation of metabolic processes, on assimilate transport, and different aspects of "organism – habitat" interactions. The efficiency of the involving this information in living organism studies greatly depends on the validity of carbon isotope fractionation model used for the interpretation. The validity of the model first of all is determined by the adopted view on the nature of isotope effect origin.

Two alternative points of view have been suggested in the literature. One of them asserts (Galimov, 1985; Schmidt, 2003) that carbon isotope effect and isotope distribution in biomolecules are of thermodynamic order. It means that isotope distribution of metabolites doesn't depend on biosynthesis pathway but is determined by the properties of the molecules themselves, i.e. by their structure and energy characteristics. According to the second point, supported by most of the researchers, the metabolic isotope effects are of the kinetic nature and carbon isotope distributions in metabolites are determined by mechanisms and pathways of their formation.

A lot of facts accumulated till now allow saying with confidence that thermodynamic concept is erroneous and the rare casual coincidences only simulate thermodynamic equilibrium (O'Leary & Yapp, 1978; Monson & Hayes, 1982; Ivlev, 2004). In some publications it was shown that the "thermodynamic" idea is "incompatible with the concept of life as a fundamental phenomenon" (Varshavski, 1988; Buchachenko, 2003). So we'll concentrate on the kinetic concept.

Within the frame of the "kinetic" concept two different approaches have been developed. The first is the steady-state model assumes that all the processes in a living cell during photosynthesis proceed simultaneously in stationary conditions. It also means that carbon isotope fractionation proceeds in stationary conditions too. The approach was put forward by Park and Epstein (Park & Epstein, 1960, 1961) and was developed by Farquhar et al.

(1982), Vogel (1993) and others (Gillon & Griffiths, 1997). Hayes (2001) has extended this approach to the common case, including secondary metabolism (metabolism in glycolytic chain).

According to the steady-state model, carbon isotope fractionation in photosynthesis can be presented as follows:

$$\Delta = a + (b - a)\, p_i / p_a \tag{1}$$

where Δ is a carbon isotope discrimination, equal to the difference between $\delta^{13}C$ of environmental CO_2 and that of biomass carbon; a is a carbon isotope effect of CO_2 diffusion from the space into a photosynthesizing cell; b is a carbon isotope effect of ribuloso-1,5-bisphosphate (RuBP) carboxylation appearing in CO_2 fixation; p_a and p_i are the CO_2 partial pressures in the atmosphere and in the leaf space.

This simple steady-state balance model was rather convenient to explain the coherence between physiological response of plants to changing environmental conditions that impact stomatal conductance and net photosynthesis. Especially positive results were obtained in the field of carbon – water relations (Farquhar et al., 1989). Nevertheless even such a simple expression (1) turned to be contradicting. According to (1), isotope discrimination Δ approaches a or b values dependent on what is rate controlling stage – diffusion either biochemical. Direct measurements of activation energy of mesophyll cell conductance (Laisk, 1977) showed that diffusion is a rate-limiting stage in CO_2 assimilation. Hence, according to model, most of C_3-plants should be "heavier" than they are and Δ values should approach 4 - 5‰, i.e. a, whereas in fact they are close to 29‰, i.e. b. Other discrepancies were described in (Ivlev, 2003). The more the equation (1) was used, the more inconsistencies were found. Numerous corrections were introduced into expression (1) to take into account other processes, where carbon isotope fractionation might be, and to remove inconsistencies. Entirely the expression (1) was transformed into expression like the following (Farquhar & Lloid, 1993):

$$\Delta = a\,\frac{p_a - p_i}{p_a} + a_i\,\frac{p_i - p_c}{p_a} + b\,\frac{p_c}{p_a} - \frac{1}{p_a}\left(\frac{eR_d}{k} + f \cdot \Gamma^*\right) \tag{2}$$

where p_a, p_i and p_c refer to the partial pressure of CO_2 in the atmosphere, substomatal cavity and chloroplast, respectively, a is the fractionation during the diffusion in air, a_i is the combined fractionation during dissolution and diffusion through the liquid phase , b is the assumed net fractionation during carboxylation by ribuloso-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and by phosphoenolpyruvate carboxylase (PEPC), k is the carboxylation efficiency, R_d is the day respiration rate, Γ^* is the CO_2 compensation point in the absence of day respiration, e and f are the fractionation during dark respiration and photorespiration, respectively.

The expression (2), unlike to (1), is inconvenient for isotope fractionation analysis in photosynthesis since contains too many parameters to be determined. Even more complex expressions are obtained when it is required to describe intramolecular isotope distribution (Tcherkez et al., 2004). Using theoretical analysis of carbon isotope fractionation in metabolic chain under stationary conditions Hayes (2001) have shown that it was impossible to predict isotope composition of metabolites and their isotopic patterns since they depend not only on the isotope characteristics of the prior metabolites in the chain, but on the partitioning of

carbon fluxes at the down-stream cross-points. Thus the integrative steady-state approach is insufficient for the explanation of short-term or intramolecular carbon isotope fractionation processes.

Another approach of the kinetic concept is presented in the works of Ivlev and colleagues (Ivlev, 1989, 1993, 2008; Igamberdiev et al., 2001; Ivlev et al. 2004; Roussel et al, 2007). Opposite to steady-state idea, the authors put forward and developed the idea that metabolic processes are discrete and periodic ones. Periodicity of metabolic processes allows concluding that substrate pools in cells are periodically filled and depleted. It is well known fact that isotope fractionation accompanying the metabolic processes in case of depletion is followed by Raleigh effect (Melander&Sauders,1983). This effect establishes the dependence between isotope ratio of initial substrate ($\delta^{13}C_{init.substrate}$), reaction product ($\delta^{13}C_{product}$), isotope fractionation coefficient (α) and the extent of pool depletion F in accordance with the equation:

$$[\delta^{13}C_{product} \cdot 10^{-3} + 1] = [\delta^{13}C_{init.substrate} \cdot 10^{-3} + 1]\frac{1}{F}[1-(1-F)^{1/\alpha}], \quad (3)$$

where $\alpha = {}^{12}k/{}^{13}k$, ${}^{12}k$ and ${}^{13}k$ are the rate constants of isotopic species of the molecules.

The Raleigh effect is the most essential feature of carbon metabolism in a living cell. It closely relates to filling/depletion regime of cell functioning and to oscillatory character of metabolic reactions. Another fundamental feature related to Raleigh effect is the strict temporal sequence of metabolic reactions, i.e. temporal organization in a cell. Using equation (3) and carbon isotopic composition of metabolites, it is possible to distinguish the temporal sequence of many metabolic events.

Kinetic nature of carbon isotope effect and participation of polyatomic carbon molecules in metabolic reactions give the evidences that most of the biomolecules in metabolic chains inherit their isotope distributions from the precursors thus proving there is no isotope exchange between carbon atoms within the carbon skeletons. Most frequent cases where isotopic shifts emerge are linked with C – C bond cleavage, especially at the cross-points of metabolic pathways. The kinetic nature of isotope effect is manifested by the fact that only those carbon atoms of skeleton disposed at the ends of broken bonds undergo isotopic shifts. These and specificity of enzymatic interactions determine individual isotopic pattern of the biomolecules. Taking into account the above factors in combination with Raleigh effect and the putative pathways of the metabolite synthesis allows reconstructing of isotopic patterns of the molecules and gives a fine tool for metabolism study.

Finally, the known regularities of inter- and intramolecular carbon isotope distribution in a cell indicate that metabolic oscillations are undamped and in-phase. Otherwise these isotopic regularities couldn't exist. The existence of the regularities on account of the Raleigh effect means, that at a given functional state of a cell, the metabolite syntheses within the repeated cell cycles occur at a certain level of substrate pool depletion. Moreover the functioning of different cells is synchronized.

2. Carbon isotope fractionation in photosynthesis and photosynthetic oscillation concept

The first step to the oscillation model was the emergence of the discrete model based on the experimentally observed data on ^{12}C enrichment of plant and photosynthesizing

microorganism biomass relative to ambient CO_2 at different conditions. The model assumed that CO_2 assimilation is a discrete process and CO_2 enters the cells by separate batches (Ivlev, 1989), but not by continuous flow like in a steady-state model (Farquhar et al., 1982). On account of isotope effect in RuBP carboxylation discrete model explained different levels of ^{12}C enrichment of photosynthetic biomass by the Raliegh effect accepting that only part of the CO_2 batches is fixed. The observed isotopic difference between C_3 and C_4 plants (Smith & Epstein, 1971) was explained by the same manner. Indeed, due to anatomical peculiarities of C_4- plants (Edwards & Walker, 1983) they are capable to re-assimilate almost all respired CO_2 thus increasing the extent of CO_2 batches depletion (*F* in expression 3)

The question was - what's the reason making CO_2 flux to be discrete? It was assumed that CO_2 assimilation flux periodically is interrupted by the reverse flux of the respired CO_2 directed from the cell to the environments. It was also assumed that such a "ping -pong" mechanism is due to double function of the key photosynthetic enzyme – Rubisco, which is capable to function as carboxylase or oxygenase depending on CO_2/O_2 concentration ratio in a cell (Ivlev, 1992). Switching mechanism splits CO_2 flux entering and leaving the cell into separate batches. This hypothesis got strong support when new carbon isotope effect of photorespiration has been discovered (Ivlev, 1993).

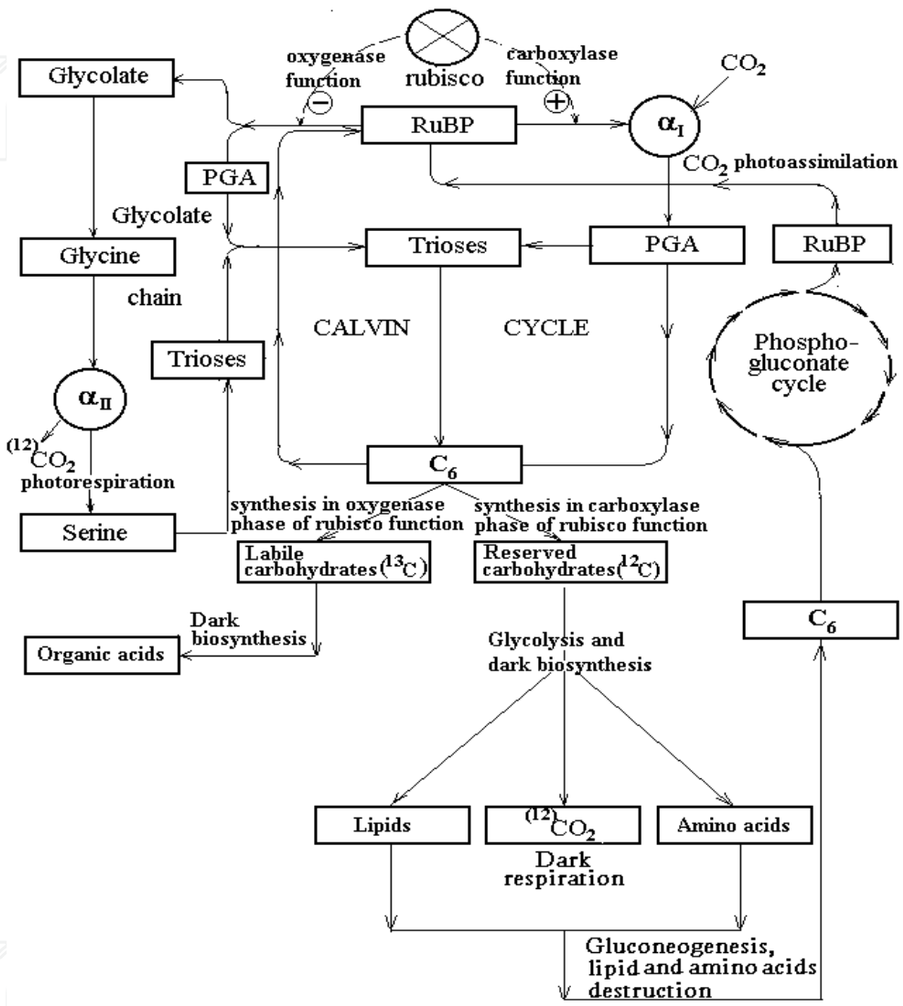
Some facts known from the literature the traditional steady-state model failed to explain. In gas exchange experiments with the use of CO_2 enriched in ^{13}C the advantageous fixation of "heavy" molecules instead of "light" ones by leaves of different plants was observed (Sanadze et al., 1978). The similar results with the use of $^{14}CO_2$ were obtained in the experiments with alga (Voznesenskii et al., 1982). Moreover the primary assimilates turned to be isotopically "heavier" relative to the ambient CO_2. In the experiments with photosynthesizing bacteria *Ectothiorhodospira shaposhnikovii* there was a change in the sign of isotope discrimination linked with the growth of ^{13}C content in the ambient CO_2 (Ivanov et al.,1978). To explain these facts it was assumed an existence of the new isotope effect related to photorespiration, and having the opposite sign to that in CO_2 assimilation. The analysis of the tentative points in photorespiration loop, where such an effect might emerge, showed that the most plausible point for its origin was glycine dehydrogenase reaction (Ivlev, 1993) (Fig.1), where decarboxylation of glycine occurs.

The following study of Calvin cycle and photorespiration biochemistry in virtue of carbon isotope composition of the primary assimilates allowed concluding on two phases of Calvin cycle functioning. In the first phase Calvin cycle produces glucose-6-phosphate (G6P) and other products from the fixed CO_2. During this phase the derived products are used to accumulate the reserve pool of starch to feed glycolytic chain in the dark and provide substrates for lipid, protein and lignin components syntheses. Carbon isotope fractionation in RuBP carboxylation results in ^{12}C enrichment of the cycle metabolites and finally the biomass as a whole relative to the ambient CO_2.

It takes some time to substantiate the experimental validity of the hypothesis and to prove that glycine decarboxylation is the very point *in vivo* where carbon isotope fractionation results in ^{13}C enrichment of biomass (Ivlev et al., 1996; 1999; Igamberdiev et al., 2001; 2004).

In the second phase Calvin cycle forms in combination with glycolate cycle the photorespiratory loop. The residual part of G6P produced in the previous phase converts into pentoses and then in form of phosphoglycolate leaves Calvin cycle and enters glycolate cycle where oxidative glycine decarboxylation occurs (Fig.1). After some transformations carbon flux in form of trioses returns back to Calvin cycle. Carbon isotope fractionation in

glycine decarboxylation produces CO_2 enriched in ^{12}C evolving from the cell, whereas carbon substrates spinning in the loop are enriched in ^{13}C and result in corresponding enrichment of photorespiratory products and biomass. The level of ^{13}C enrichment depends on what how many turns carbon substrate flux makes in the loop, or what the extent of photorespiratory pool depletion is achieved (Raleigh effect).



α_1 – a point of carbon isotope fractionation in CO_2 assimilation (carbon isotope effect in RuBP carboxylation), α_{11} – a point of carbon isotope fractionation in photorespiration (carbon isotope effect in glycine decarboxylation)

Fig. 1. Oscillating model of carbon isotope fractionation in photosynthesis.

2.1 Experimental facts support the presence of photosynthetic oscillations

The experimental data presented in Tables 1-3 show distinct differences in isotope composition of metabolites derived in the both phases of Calvin cycle oscillations. Table 1 illustrates ^{13}C enrichment of leaf oxalates of different C_3 and CAM-plants (Rivera & Smith, 1979; Raven et al., 1982). Their synthesis is mainly bound to glycolate cycle of photorespiratory loop.

Plant species	Plant type	Whole leaf	Oxalates	Reference
Spinaceae oleracea	C_3	- 27.5	- 11.9	Rivera&Smith, 1979
Pelagronium	C_3	- 31.0	- 12.4	Rivera&Smith, 1979
Mereurialis perennis	C_3	- 27.9	- 13.7	Rivera&Smith, 1979
Spinaceae oleracea	C_3	- 25.7	- 19.9	Raven et al., 1982
Echinomastus intertextus	CAM	- 13.4	- 7.3	Raven et al., 1982
Echinomastus horizonthalomus	CAM	- 13.0	- 7.8	Raven et al., 1982
Escobaria ruberoulosa	CAM	- 12.3	- 8.3	Raven et al., 1982
Opuntia euglemannii	CAM	- 13.3	- 8,5	Raven et al., 1982
Opuntia imbricata	CAM	- 14.1	- 8.7	Raven et al., 1982

Table 1. Carbon isotope ratio of leaf biomass and oxalates of some oxalate accumulating plants.

^{13}C Distribution in protein fraction of some photosynthesizing microorganisms (Abelson & Hoering, 1961) gives more evidences in favor of the oscillation model (Table 2). Amino acids like serine, glycine, alanine and aspartic acid, whose pools, at least, in part are supplied from photorespiratory loop, have appeared more enriched in ^{13}C as compared with those whose synthesis predominantly bound to glycolytic chain and Krebs cycle, like glutamic acid, leucine and lysine (Igambediev, 1988; 1991).

Microorganism Amino acid	*Chlorella* total carbon	*Anacystis* total carbon	*Gracilaria* Total carbon	*Euglena* total carbon
Serine	- 5,7	-	- 14,1	- 8,3
Glycine	- 14.3	- 10,0	- 10,2	- 10,0
Alanin	- 10,3	- 9,8	- 15,2	-14,3
Aspartic acid	- 6,6	- 9,7	- 14,4	-9,7
Glytamic acid	- 18,7	- 11,1	- 17,2	-17,3
Leucine	- 22,7	- 17,3	- 22,5	-23,5
Lysine	- 17,0		-	-22,8

Table 2. Carbon isotope distribution in amino acids from protein fraction from biomass of some photosynthesizing microorganisms. Isotopic shifts are given relative to nutrient CO_2 having $\delta^{13}C = 0‰$. Extract from Table 3 in (Abelson & Hoering, 1961).

Index	Concentration of NaCl in medium, mM		
	0	425	595
$\delta^{13}C$ of dry matter, ‰	- 61.6	- 59.0	- 64.5
$\delta^{13}C$ of lipids, ‰	- 66.0	- 65.0	- 63.8
$\delta^{13}C$ of proteins, ‰	- 42.1	- 40.9	- 47.3
$\delta^{3}C$ of labile sugars‰	- 30.0	-	- 30.5
$\delta^{13}C$ of proline, ‰	- 29.0	-	- 31.5

Table 3. Distribution of ^{13}C in biomass and biochemical fractions of marine alga *Chorella stigmatophora*, grown under effect of different environmental factors. $\delta^{13}C$ of ambient CO_2 is -21%o (Ivlev & Kalinkina, 2001; Kalinkina & Udel'nova, 1990)

The same picture illustrated by the data on ^{13}C distribution in biomass of marine alga *Chlorella stigmatophora* grown under different environmental conditions on the CO_2 of the known carbon isotope ratio is presented in Table 3.

Lipids and proteins distinctly differ in carbon isotope ratio as compared with labile sugars and organic acids. In special experimental studies (Kalinkina &Udel'nova, 1991; Kalinkina & Naumova, 1992) the authors have proven that these components were the products of photorespiration pathway whereas most of lipids and proteins are synthesized via glycolytic chain and Krebs cycle (Metun, 1963; Strikland, 1963).

Quite another object, C_3-CAM tropical plant *Clusia minor*, grown under different environmental conditions is given in Table 4. Soluble sugars and organic acids, whose origin is linked with photorespiratory carbon flux, are enriched in ^{13}C as compared with amino acid and lipid fractions. Notably the latter fraction, besides lipids, contains pigments some of which, like chlorophyll (Ivlev, 1993), at least partially are formed at the expense of photorespiratory flux. It makes lipid fraction isotopically "heavier" than amino acid fraction is. Thus all the presented data confirmed the idea on two phases of Calvin cycle functioning and drew to the conclusion that the phases are alternating, i.e. are separated in time. In fact, if the processes proceeded simultaneously the isotopically different carbon fluxes couldn't arise. Passing the same pieces of Calvin cycle they would inevitably mix. Bearing in mind that the first phase of cycle functioning corresponds to carboxylase function of Rubisco, while the second – to oxygenase one, we called the first as carboxylase phase and the second as oxygenase. To confirm the oscillating idea we tried to get more independent arguments and examined isotopic patterns of metabolites derived in different phases of Calvin cycle.

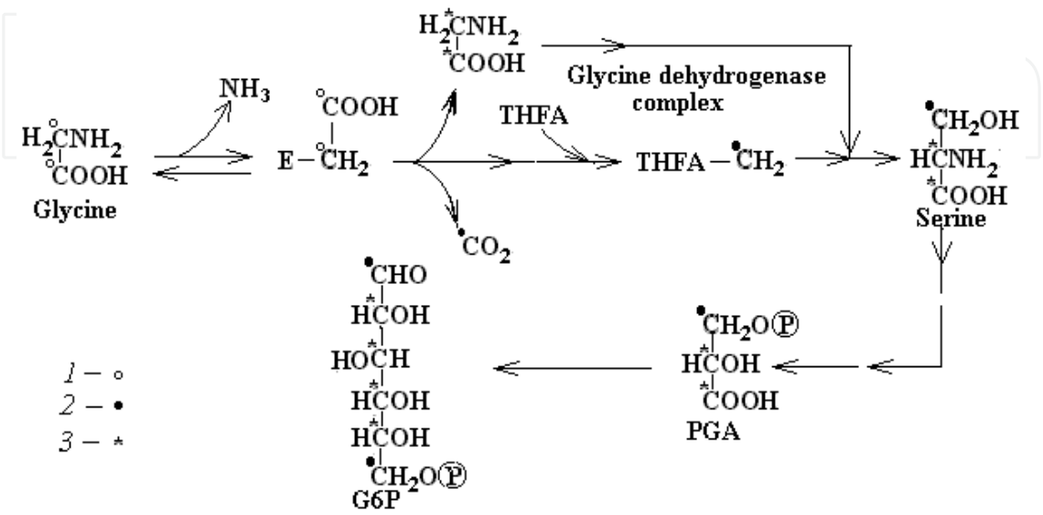
N	FRACTION	WET SEASON		DRY SEASON	
		Exposed leaf	Shaded leaf	Exposed leaf	Shaded leaf
		Dawn	Dusk	Dawn	Dusk
1	Total carbon	-25.7	-30.3	-24.6	-29.1
2	Lipids and pigments	-28.7	-32.2	-27.7	-30.8
3	Amino acids	-31.7	-32.6	-31.3	-32.7
4	Soluble sugars	-21.2	-29.2	-17.9	-21.9
5	Organic Organic acids	-22.3	-27.7	-21.1	-24.5

Table 4. Carbon isotope composition of biochemical fractions isolated from leaves of *Clusia minor* under different environmental conditions (Borland et al., 1994). Samples were taken at dawn and dusk. $\delta^{13}C$ Values are given in per mille relative to PDB standard.

2.2 Intramolecular isotopic patterns of glucose, anomalous isotope composition of CO_2 evolved in light enhanced dark respiration, and some non-isotopic arguments support the oscillation hypothesis

As noted above, kinetic nature of isotope effects and specificity of enzymatic interactions provide specific carbon isotope distribution of many metabolites. Having compared isotopic patterns of G6P, formed in carboxylase and oxygenase phases of Calvin cycle we found they should be quite different. According to the theoretical estimates the synthesis of G6P in carboxylase phase results in practically uniform ^{13}C distribution along the molecule skeleton due to transaldolase and transketolase cycle reactions which randomize atoms with cycle

turns growth. Carbon isotope distribution of G6P synthesized in photorespiration loop is characterized by ^{13}C enrichment of carbon atoms in C-3 and C-4 positions of glucose skeleton, slight ^{13}C enrichment in C-2 and C-5 positions, while atoms C-1 and C-6 are enriched in ^{12}C (Fig. 2). To understand this specific distribution let's follow what isotope fractionation in glycine dehydrogenase complex (GDC) occurs.



Empty circles (1) denote isotope composition of carbon atoms in the initial substrate, filled circles (2) denote atoms get enriched in ^{12}C, asterisks (3)

Fig. 2. The emergence of the isotope inhomogeneity in G6P as a result of kinetic carbon isotope effect in GDC.

As shown on Fig. 2, isotope distribution in G6P is determined by isotope distributions in glycine and C_2-fragment derived in decarboxylation. In glycine decarboxylation both atoms of residual glycine get enriched in ^{13}C while methylene carbon atom as well as CO_2 located at the ends of the cleaved C – C bond relative to the atoms in the initial substrate get enriched in ^{12}C (Melander & Saunders, 1983). In GDC the methylene fragment linked with the cofactor, tetrahydrofolic acid (THFA), is transferred to the residual glycine molecule thereby forming the serine (Oliver et al., 1990). Following transformations result in the specific isotopic pattern of G6P shown on Fig.2. Moreover at each turn of the carbon flux, spinning in photorespiratory loop, isotope distribution not only retains, but is reproduced again and again. So ^{13}C enrichment of G6P as well as intramolecular isotopic discrepancies increase with the number of turns (with the growth of photorespiration intensity) (Ivlev et al., 2010). Isotope pattern of G6P synthesized in carboxylase phase is not studied yet. But glucose from the starch of storage organs of some plants has been investigated (Table 5).

Bearing in mind that G6P is the main structural unit used for glucose synthesis and comparing data in Table 5 with the results of G6P modeling (Ivlev, 2005; Ivlev et al., 2010), it is easy to conclude they are strongly resembled. Hence the starch glucose is of photorespiratory origin. The assertion is supported by the fact that storage organs are formed in the period of ontogenesis when oxidative processes related to intensification of photorespiration sharply increase (Abdurachmanova et al., 1990; Igamberdiev, 1991). This fact correlates with the observed ^{13}C enrichment of seeds, fruits, and edible roots of plants as

compared with the carbon isotopic composition of other plant organs (leaf, stem) (Lerman et al., 1974; White, 1993; Saranga et al., 1999; Ivlev et al., 1999).

Object	$\delta^{13}C$ of glucose	$\Delta^{13}C = \delta^{13}C - \delta^{13}C_{glucose}$, i - atom number					
		$OCH_{(1)}$-$HC_{(2)}OH$-$OHC_{(3)}H$-$HC_{(4)}OH$-$HC_{(5)}OH$-$C_{(6)}H_2OH$					
		C_1	C_2	C_3	C_4	C_5	C_6
Beta vulgaris, tuber (Rossman et al., 1991)	-25.2	-1.6	-0.4	+2.1	+6.3	-1.7	-5.1
Zea mays, seeds (Rossman et al., 1991)	-10.8	-1.7	-0.1	+ 1.1	+3.6	-0.2	-3.6
Zea mays, seeds (Ivlev et al., 1987)	-12.5	-3.1	+ 1.9				-1.9
Triticum aestivum, seeds (Galimov et al., 1977)	-23.1	-7.1	+3.5*				-7.1
Solanum tuberosum, tuber (Galimov et al., 1977)	-24.9	-9.1	+4.5*				-9.1
Oryza sativum, seeds (Galimov et al., 1977)	-26.1	-6.9	+3.5*				-6.9
Pisum sativum, seeds (Galimov et al., 1977)	-24.9	-4.1	+2.1*				-4.1

Note: The isotopic shifts of the carbon atoms $\Delta^{13}C$ are given relative to total glucose carbon. $\delta^{13}C$ values of glucose are given in PDB units. * The $\delta^{13}C$ values of C-3 and C-4 atoms were calculated according to Galimov et al. (1977) assuming that the isotopic composition of the other carbon atoms equals to that of the C – 1 and C – 6.

Table 5. Intramolecular carbon isotope distribution in the starch glucose of storage organs of various plants.

The uneven carbon isotope distribution in oxygenase G6P explains the recently established fact of anomalous ^{13}C enrichment of light enhanced dark respiration CO_2 relative to labile carbohydrates from phloem sup, the supposed respiratory substrate. Indeed, the consideration of labile carbohydrates (oxygenase G6P), accumulated in the light, as substrate for dark synthesis of organic acids (Borland et al, 1994), allows concluding the following way of the conversion (Ivlev & Dubinsky, 2011). At first glucose splits into two triose molecules. Then the latter are subjected to decarboxylation and derived C_2-fragments are used to form organic acid skeletons while the evolved CO_2 forms LEDR CO_2 which inherits atoms from the C-3 and C-4 positions. The atoms as shown above are enriched in ^{13}C. The level of ^{13}C enrichment depends on light intensity and confirms the existence of Raliegh effect in photorespiration. The increase in illumination intensifies photorespiration, thus implying the increase in number of turns of carbon flux in photorespiratory loop. This in turn leads to photorespiratory pool depletion and to ^{13}C enrichment of the respired CO_2. Barbour et al. (2007) have noticed the relationship of light intensity and LEDR CO_2 ^{13}C enrichment in the experiment.

The oscillatory model suggests a coherent explanation of the relative ^{13}C enrichment of heterotrophic tissue of plants (seeds, stem, roots) comparing with autothrophic ones (leaves)

(Cernusak et al, 2009). In fact, labile carbohydrates are the main carbon source for heterotrophic growth (Kursanov, 1976). On the other hand, labile carbohydrates, being photorespiratory products, are enriched with ^{13}C. Gessler et. al. (2008) has confirmed this assertion experimentally. The authors found that water soluble fraction of leaf organic matter mainly consisting of the labile carbohydrates is enriched with ^{13}C unlike to the insoluble fraction mainly consisting of proteins and lipids whose origin relates to starch formed in carboxylase phase. Similarly, the model explains the resemblance in $\delta^{13}C$ values of the leaf water soluble organic matter and that of the phloem sap. Hence the above isotopic data firmly support the oscillation hypothesis.

There was an endeavor to find a direct evidence of the photosynthetic oscillations (Roussel et al., 2007). By using a fast response CO_2 gas exchange system the authors measured CO_2 concentration fluctuations in the subcellular space in tobacco leaves at low CO_2 concentrations nearby the compensation point. The chosen condition provided an easier way to discover the assumed oscillations. Because of a background noise, a special mathematical procedure was required to isolate the periodic component in the temporal sequence and to build an attractor proving the existence of the real oscillatory regime. The CO_2 concentration pulses with a period of the order of a few seconds were explained by the feedback interactions between CO_2 assimilation and photorespiration.

Fig.3 shows the principal interactions between the main participants of photosynthesis process and key enzyme Rubisco having dual function. Since the process occurs in different compartments: CO_2 assimilation in chloroplasts, photorespiratory CO_2 release in mitochondria, a certain time interval is needed for the CO_2 depletion near Rubisco. The delay in CO_2 release, following RuBP oxygenation, and competition between CO_2 and O_2 provide the conditions for oscillations (Roussel & Igamberdiev, 2011).

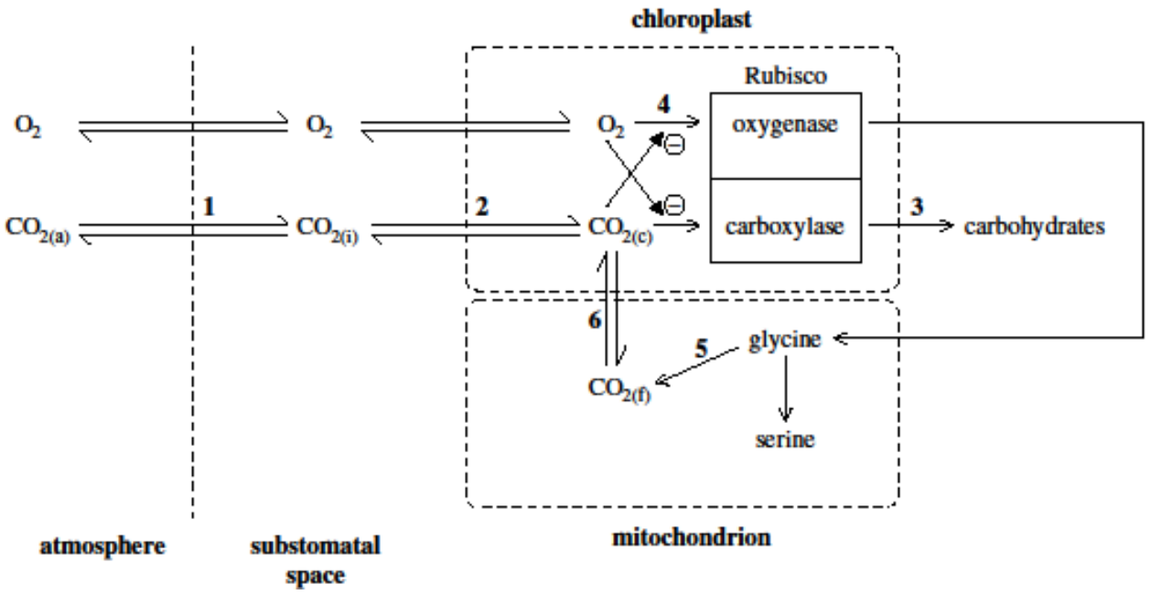


Fig. 3. Simplified scheme for carbon assimilation and photorespiration.

Dashed lines indicate compartmental boundaries. Carbon dioxide in the atmosphere passes through the stomata and enters the substomatal space (step 1). Eventually, it reaches the chloroplasts (transport step 2) where carbon is fixed (3). Under normal conditions, the leaf

interior is well ventilated, leading to a reasonably uniform distribution of oxygen. Oxygen may participate in photorespiration (4), eventually leading to the appearance of glycine in the mitochondria and thus to photorespiration (5). The carbon dioxide produced by photorespiration is free to diffuse through the cytoplasm to the chloroplasts (6). Also shown is the inhibition of photorespiration by carbon dioxide and of carbon fixation by oxygen.
To check up this possibility we carried out the computational analysis of the scheme (Dubinsky & Ivlev, 2011). It can be presented as follows (Fig. 4). According to the scheme (Fig. 4), RuBP binds to the enzyme which is activated by Mg^{2+} and CO_2 (this is not considered here for simplification) and a quasi-equilibrium of the RuBP with the enzyme E is attained first (Tapia et al., 1995; Mauser et al., 2001). Then RuBP–enzyme complex reacts either with CO_2 or O_2 and the formation of the assimilation products occurs. The products are used either for further transformations in the cycle (the carboxylase phase) or for utilization in the photorespiration loop, comprising the Calvin cycle coupled with the glycolate cycle (initiated by the oxygenase phase).

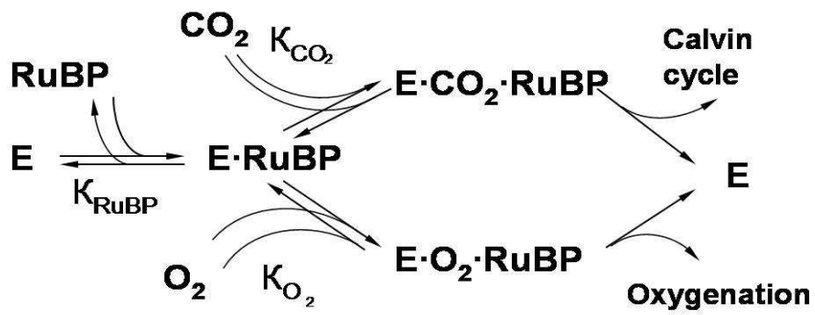


Fig. 4. The principal scheme of photosynthesis considering carboxylase and oxygenase functions of Rubisco.

The scheme on Fig. 4 is convenient for mathematical description and computational analysis. It was described by three differential equations:

$$\frac{dx}{dt} = \frac{1}{5}V_c \cdot \frac{(x/K_{RuBP})\cdot(y/K_{CO_2})}{1+(x/K_{RuBP})+(x/K_{RuBP})\cdot(y/K_{CO_2})+(x/K_{RuBP})\cdot(z/K_{O_2})} - \frac{1}{10}V_{ox} \cdot \frac{(x/K_{RuBP})\cdot(z/K_{O_2})}{1+(x/K_{RuBP})+(x/K_{RuBP})\cdot(y/K_{CO_2})+(x/K_{RuBP})\cdot(z/K_{O_2})} - V_{out}\frac{(x/K_{out})}{1+(x/K_{out})}$$

$$\frac{dy}{dt} = -V_c \cdot \frac{(x/K_{RuBP})\cdot(y/K_{CO_2})}{1+(x/K_{RuBP})+(x/K_{RuBP})\cdot(y/K_{CO_2})+(x/K_{RuBP})\cdot(z/K_{O_2})} + \frac{V_{ox}}{2} \cdot \frac{(x/K_{RuBP})\cdot(z/K_{O_2})}{1+(x/K_{RuBP})+(x/K_{RuBP})\cdot(y/K_{CO_2})+(x/K_{RuBP})\cdot(z/K_{O_2})} + k_{CO_2}(CO_{2out}-y)$$

$$\frac{dz}{dt} = V_c \cdot \frac{(x/K_{RuBP})\cdot(y/K_{CO_2})}{1+(x/K_{RuBP})+(x/K_{RuBP})\cdot(y/K_{CO_2})+(x/K_{RuBP})\cdot(z/K_{O_2})} - \frac{3}{4}V_{ox} \cdot \frac{(x/K_{RuBP})\cdot(z/K_{O_2})}{1+(x/K_{RuBP})+(x/K_{RuBP})\cdot(y/K_{CO_2})+(x/K_{RuBP})\cdot(z/K_{O_2})} + k_{O_2}(O_{2out}-z) + J_{O_2}$$

where following notations are used: x, y and z are the RuBP, CO_2 and O_2 concentrations respectively, K_{CO2} , K_{O2} and K_{RuBP} are the equilibrium constants of the reactions E ·RuBP ·CO_2→E ·RuBP + CO_2, E ·RuBP ·O_2→E ·RuBP + O_2 and E ·RuBP→E + RuBP, respectively. Two first equations in their simplified form (Dubinsky et al., 2010) describe sugar (x) and CO_2 (y) concentration variations. The third describes variations of O_2 (z) concentration. In the set of equations V_c is the maximum rate of RuBP carboxylation, V_{ox} is the maximum rate of RuBP oxygenation. V_{out} is the maximum rate of sugar efflux, K_{out} is the Michaelis constant of the pseudoenzyme by means of which sugars are removed from the system (the real mechanism of sugars removal is certainly more complicated but it is the simplest way to describe the effect of sugar efflux saturation). k_{CO2} is the CO_2 diffusion coefficient from the surrounding medium into a cell, CO_{2out} is the CO_2 concentration in the medium, k_{O2} is the O_2 diffusion coefficient from the medium into the cell, O_{2out} is the O_2 concentration in the medium.

The solution of the system with cell parameters, taken from the literature (Dubinsky & Ivlev, 2011), results in establishing of counter-phase undamped oscillations with the period of 1 – 3 sec for CO_2 and O_2 and in respective oscillations of CO_2/O_2 ratio (Fig. 5). The oscillations could switch over Rubisco from carboxylase function to oxygenase and back.

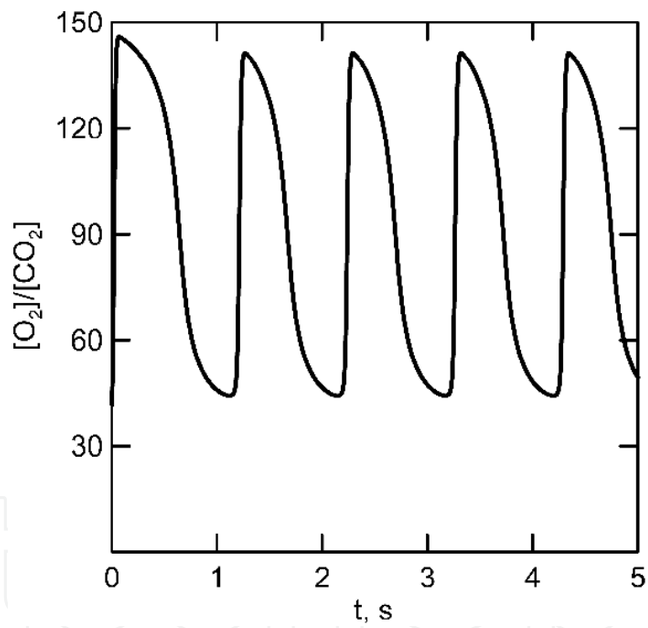


Fig. 5. The calculated photosynthetic oscillations of CO_2/O_2 concentration ratio according to the model described in the text.

Thus the theoretical calculations proved the principal possibility of the existence of sustained oscillations in carbon metabolism of a photosynthesizing cell.

Let's consider now how Calvin cycle works in different phases of photosynthetic oscillations from the point of ^{13}C isotope distribution in metabolites. In carboxylase phase of oscillations Calvin cycle works, as shown on Fig. 6. Due to carbon isotope effect in RuBP carboxylase complex all carbon atoms fixed happened to be enriched in ^{12}C relative to ambient CO_2. Transketolase and transaldolase reactions of the cycle randomize carbon atoms along carbon skeletons, i.e. Calvin cycle works as a mixer. It results in practically uniform ^{13}C

distributions within metabolites. The pools of metabolites accumulated in this phase and utilized further in secondary metabolism to provide glycolytic chain, lignin synthesis and other metabolic needs with carbon source form so-called "light" (enriched in ^{12}C) carbon flux (see below).

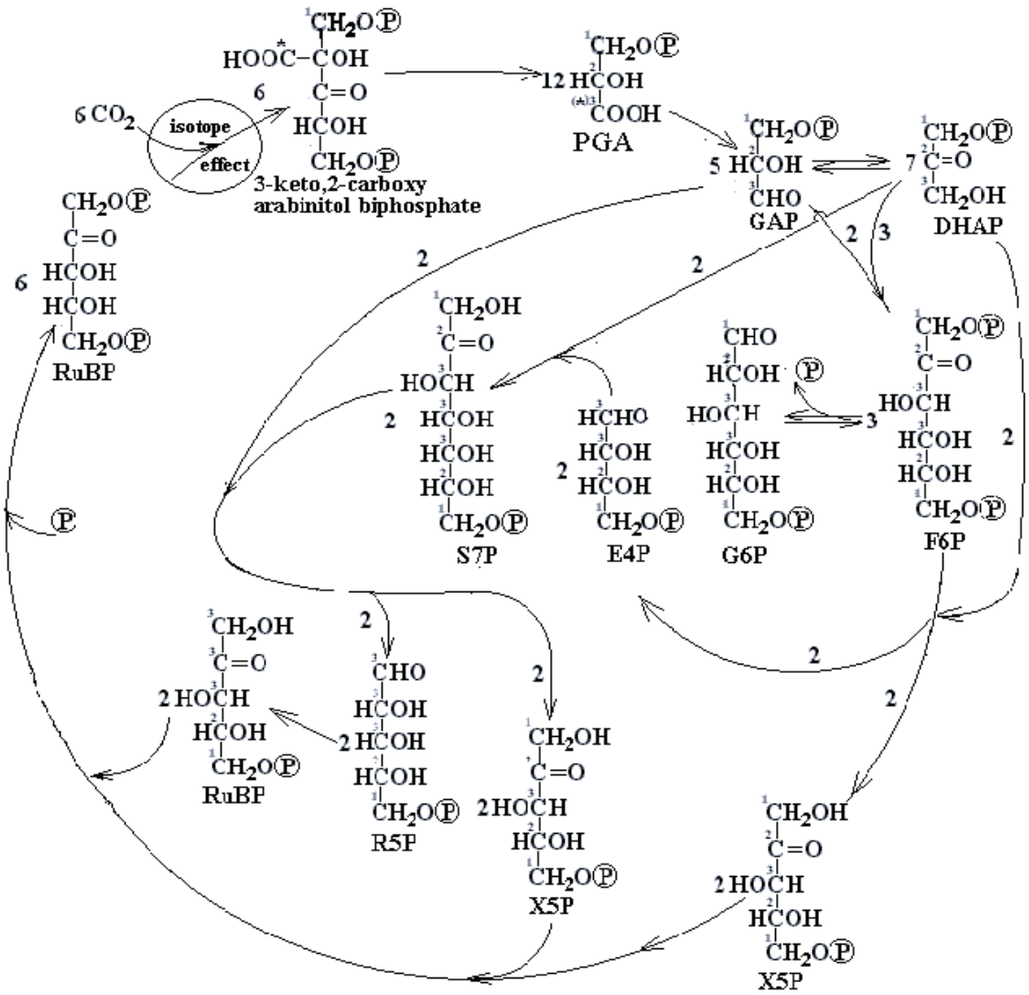


Fig. 6. Calvin cycle in carboxylase phase of Rubisco functioning.

The figures on the arrows and before the molecular formulas denote the number of the molecules involved in transformations of the cycle and formed in them; the figures before the atoms denote the number of carbon atoms in the PGA molecule; asterisks * are the exogenous carbon atoms attached to the carboxyl group of 2-carboxy-3-ketopentite and then to the C-3 position of PGA; P in a circle is the phosphate group in the molecules.

In oxygenase phase Calvin cycle works as shown on Fig. 7. Due to the isotope effect in GDC all metabolites formed in cycle transformations get enriched in ^{13}C relative to G6P, left after carboxylase phase. At that the specific intramolecular ^{13}C distributions determined by kinetic nature of the effect, by the specificity of enzymatic interactions and by the Raleigh effect appear. ^{13}C-Enrichment and heterogeneity of isotope distribution of metabolites becomes greater with the photorespiration intensity. The pools of metabolites mainly labile

carbohydrates, some amino acids (glycine, serine, and related compounds) accumulated in oxygenase phase, like those formed in carboxylase phase, are utilized in secondary metabolism syntheses (organic acid, some parts of complex molecules, etc.)

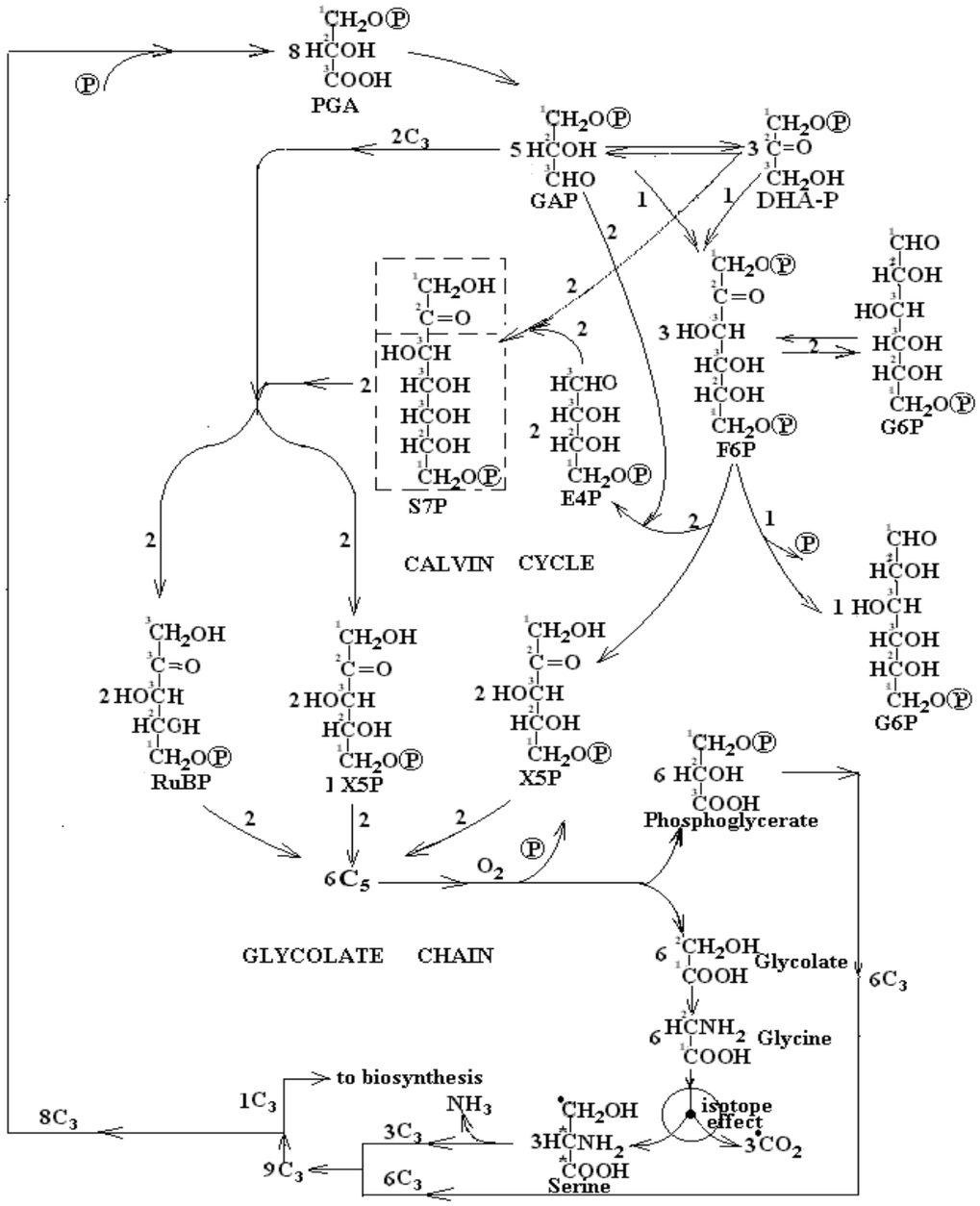


Fig. 7. Calvin cycle in oxygenase phase of Rubisco functioning.

All symbols denote the same as on Fig. 6.

Strict temporal organization of metabolism in a cell prevent from complete mixing of the above carbon fluxes (see below) and allows to use isotopic characteristics to investigate metabolic relations, pathways, assimilate transport, etc. More arguments evidencing in favor of photosynthetic oscillations were given in the work of Ivlev (2010).

3. Carbon isotope fractionation in secondary metabolism of photosynthesizing cell

The idea on the existence of energy and carbon oscillations in glycolytic chain was firstly proved in respect to heterotrophic organisms (Sel'kov, 1975, 1978). We have accepted this

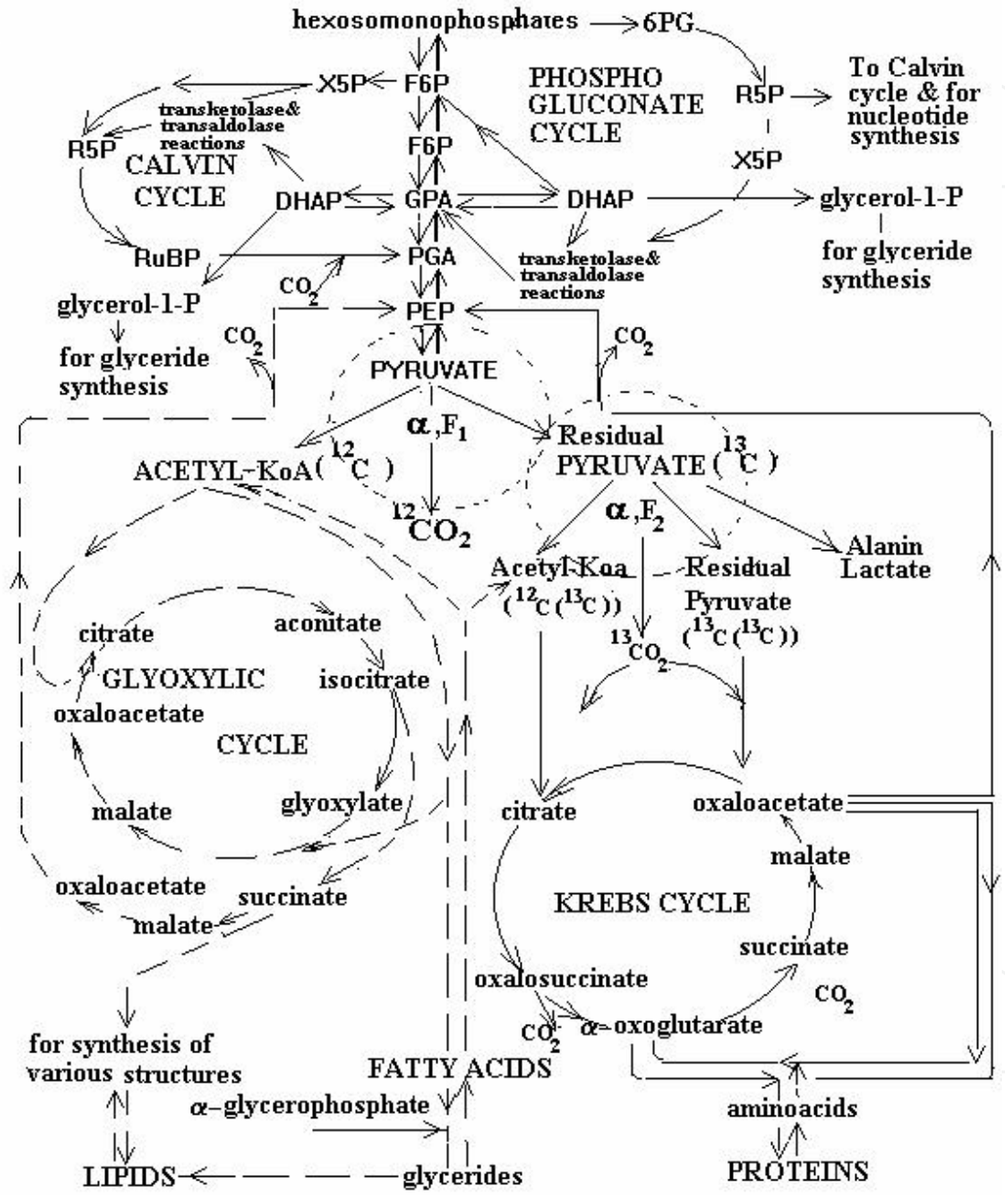


Fig. 8. The simplified diagram depicting temporal organization of secondary metabolism in glycolytic chain (see the text). Dotted lines denote the enzymatic pyruvate decarboxylase complex where carbon isotope fractionation occurs. Abbreviations: X5P, xylose-5-phosphate; R5P, ribose-5-phophate; RuBP, ribulose-1,5-bisphosphate; 6PG, 6-phosphogluconate; F6P, fructose 6-phosphate; FBP, fructose-1,5-bisphosphate; PGA, phosphoglyceric acid; DHA-P, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; α, carbon isotope fractionation coefficient; F, the extent of pyruvate pool depletion.

idea suitable for autotrophic organisms taking into consideration that heterotrophic organisms have originated first in the course of evolution while autotrophs emerged later adding pentose phosphate reductive cycle (Calvin cycle) to glycolytic chain to feed it with substrates (Ivlev, 2009).

Hence photosynthesizing organisms have inherited glycolytic chain from the precursors with all its functions, regulation and temporal organization and with carbon isotope fractionation mechanism as well. Following studies of ^{13}C distribution regularities in autotrophic and heterotrophic biomass showed they have much in common and confirmed this assumption. Unlike to photosynthetic oscillations, glycolytic ones were found to be long-term. According to Sel'kov (1975, 1978), glycolytic oscillations consist of two phases: glycolysis and gluconeogenesis. In glycolysis, which correlates with dark period of photosynthesis, carbon flux goes "down" the chain (Fig. 8). It means carbohydrates (starch) accumulated in carboxylase phase of photosynthetic oscillations transform into lipids and proteins. In gluconeogenesis, which correlates with light period of photosynthesis, carbon flux goes "up". It means that pools of lipids and proteins accumulated in the dark partly destroy and form the reverse substrate flux directed to carbohydrates. "Up" and "down" indicate only general direction of transformations, since glycolytic and gluconeogenetic pathways do not coincide entirely. The fructose-1,6-bisphosphate futile cycle, is the main regulator of glycolytic oscillations, capable to work in opposite directions (hydrolysis/phosphorilation) depending on concentration ratio of hexosomonophosphates to fructose-1,6-bisphophate (Sel'kov, 1975, 1978).

Carbon isotope fractionation occurs in phase of glycolysis and relates to pyruvate dehydrogenase complex, which is the main cross-point in the chain. Due to pyruvate decarboxylation occurring in this point the pyruvate pool is depleted followed by the Raleigh isotope effect. Glycolysis is organized in such a way that metabolites derived of C_2-fragments (fatty acids, carotenoids, steroids, etc.) referred to lipids, emerge when the extent of the pool depletion is less than a half ($F_1 < 0,5$). It causes lipids in general are enriched in ^{12}C relative to ambient carbohydrates. This piece of glycolysis phase is depicted on the Fig.8 as dotted circle with isotope characteristics α and F_1. The second period of glycolysis mainly corresponds to Krebs cycle functioning and protein synthesis. This piece is depicted on the Fig.8 as dotted circle with isotope characteristics α and F_2. The glycolysis proceeds when the extent of pyruvate pool depletion is more than a half ($F_2 > 0.5$). That is why total proteins are enriched in ^{13}C relative to lipids and to ambient carbohydrates as well. It was adopted that ^{13}C patterns of metabolites related to glycolytic chain are determined solely by isotope fractionation in pyruvate decarboxylation and by the specificity of the following enzymatic interactions since no proofs are available evidencing for carbon isotope fractionation in gluconeogenesis phase.

Now let's see carbon isotope fractionation in pyruvate decarboxylation in a more detail. The important role of the reaction is conditioned at least by two reasons. First, the reaction is located at the cross-point of central metabolic pathways. Hence carbon isotope fractionation is typical to all photosynthesizing organisms. Second, the products of the reaction are used as structural units for the synthesis practically for all secondary metabolites. Taking into account the kinetic nature of isotope effect, metabolic pathways and specificity of enzymatic interactions the intramolecular carbon isotope distributions of many metabolites can be easily predicted to be compared with the experimental data (see below). To get this objective

it is necessary to find out ^{13}C distribution in the structural units produced in pyruvate decarboxylation and their dependence on the Raleigh effect.

Three structural units are produced in the above reaction. They are CO_2, evolved in decarboxylation, (C_1-fragments), acetyl-KoA (C_2-fragments), and residual pyruvate (C_3-fragments). According to the isotope effect theory (Melander & Saunders, 1983) only the atoms located at the ends of the broken bonds are subjected to kinetic isotope effect (Fig.9). It means that the effect results in heterogeneous intramolecular isotope distribution

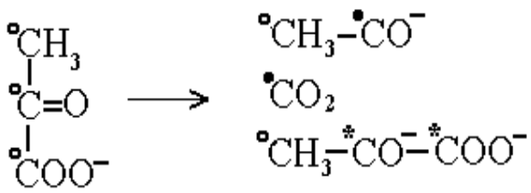


Fig. 9. Three types of carbon atoms resulting in pyruvate decarboxylation.

Empty circles denote atoms with non-changeable isotopic composition in the reaction; filled circles denote atoms getting enriched in ^{12}C relative to the respective atoms of the pyruvate molecules subjected to decarboxylation; asterisks denote atoms getting enriched in ^{13}C to the respective atoms of the initial pyruvate molecules.

Given the isotope composition of the initial pyruvate as that of G6P, derived in carboxylase phase, and taking it as reference level, it is convenient to divide all the atoms in the above fragments into three types. At that one should consider kinetic nature of pyruvate decarboxylation carbon isotope effect and Raleigh effect of pool depletion.

1. Methyl atoms of C_2-fragments and C_3-fragments. Their isotopic compositions during carboxylation remain unchanged and are inherited from the corresponding G6P carbon atoms. It was accepted as an internal standard;
2. Carbonyl carbon atoms of C_2-fragments and CO_2 disposed at the ends of the cleaved C-C bonds. Depending on the extent of pyruvate pool depletion F, their isotope composition can be both enriched in ^{12}C, if F is less than 0,5, or depleted in ^{12}C, if F is more than 0,5.
3. Carboxyl and the neighboring carbonyl atoms of C_3-fragments. Their isotope composition at any F is enriched in ^{13}C relative to atoms of the first and second type.

The ^{13}C distributions in metabolites were analyzed by means of their skeleton reconstruction with allowance of the known pathway, and the specificity of the enzymatic reactions and mixing of atoms in metabolic cycles. The comparison of the theoretically expected and experimentally observed isotope distributions gives the strong arguments in favor of the glycolytic oscillations

3.1 Some examples of ^{13}C distribution in secondary metabolites affirming the oscillatory character of glycolytic chain metabolism

Isotope distributions in lipid components, made of C_2-fragments, are the easiest objects for the isotopic pattern analysis. With allowance for the known fatty acids synthesis pathway (Strickland, 1963), namely the condensation of C_2-fragments according to the head-to-tail principle, there are only odd carbon atoms of skeleton that change their isotope ratios (atoms of the second type). The even atoms (atoms of the first type) remain their isotope composition inherited from the atoms of nutrient carbohydrate. In Table 6 carbon isotope

distributions of some fatty acids isolated from lipid fraction of *E. coli*, grown on glucose with the known isotope ratio are presented.

Fatty acids	$\delta^{1'3}C$, ‰	Odd atoms		Even atoms	
	Total C	N	$\delta^{1'3}C$, ‰	N	$\delta^{1'3}C$, ‰
Myristic 14:0	-13.7	1	-27.1		...
Palmitic 16:0	-12.2	1	-15.2		
Palmitoleic 16:1	-13.0	1	-19.2		
		9	-16.0	10	-9.5
9,10-Methylenepalmitic 17: cycle	-13.7	1	-20.3		
Vaccenic 18:1	-12.6	1	-13.9	12	-9.5
		11	-15.8		

Note: $\delta^{1'3}C$ of nutrient glucose is equal to 9,96‰

Table 6. ^{13}C distribution in some fatty acids from lipid fraction of E. *coli* grown on glucose of the known carbon isotope composition (Monson & Hayes 1982).

As follows from Table 6, isotopic data completely correspond to the known fatty acid synthesis pathway. It confirms that the ^{13}C pattern is determined by isotope effect in pyruvate decarboxylation. Isotope composition of the even atoms (C-10 and C-12) is close to that of the carbon atoms of nutrient glucose, while $\delta^{13}C$ values of the odd atoms (C-1, C-9 and C-11) vary from -13,9 to -27,1. The variations in $\delta^{13}C$ of odd atoms prove they belong to the second type and indirectly evidence on the existence of Raleigh effect accompanying pyruvate pool depletion. The latter in turn indicates the existence of the oscillations. The odd atoms of the fatty acids in all cases are enriched in ^{12}C relative to nutrient glucose ($\delta^{13}C$= -9,96). In the frame of the model, it means the fatty acids are derived at the extent of pool depletion less than 0,5.

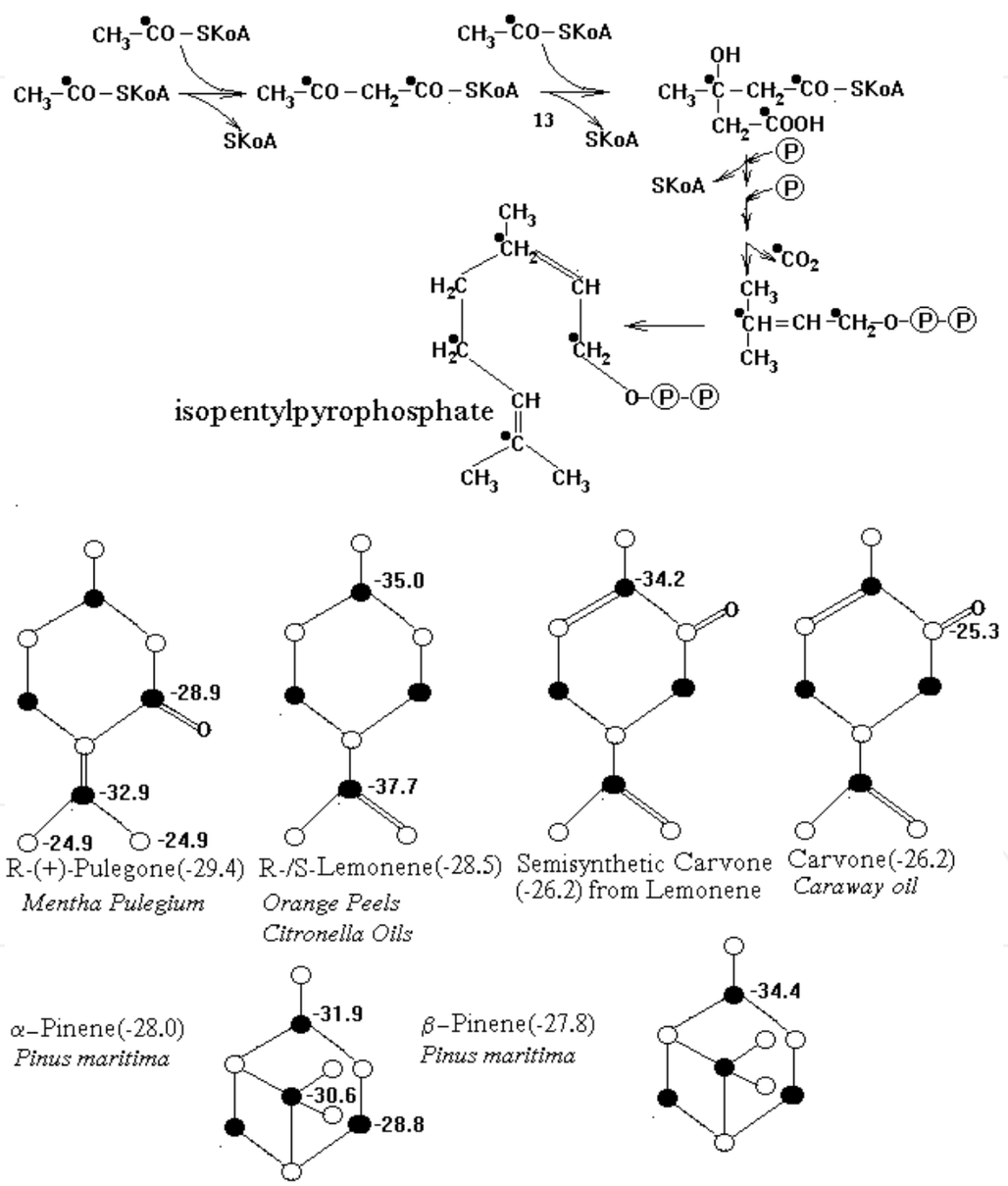
Nutrient glucose	Fatty acids	^{13}C distribution in acetate		
		Total carbon	Carboxyl atom	Methyl atom
-9.0	-12.2	-3.3	+15.0	-8.8

Table 7. ^{13}C distribution in acetate evolved by *E. coli* into the medium in the fermentation of the microorganisms on glucose of the known isotope composition (Blair et al, 1985)

The similar results one can see from Table 7. Acetate, like fatty acids, is made of C_2 units, what is confirmed by its ^{13}C pattern. Carboxyl atom has unusual "heavy" isotope composition ($\delta^{1'3}C$ = + 15‰), while methyl (even) atom has carbon isotope composition close to the nutrient glucose. Such unusual the ^{13}C enrichment of carboxyl atoms supports again its relation to the Raleigh effect and evidences the acetate is formed at high level of pyruvate pool depletion. On contrary, fatty acids have "light" carbon isotope composition ($\delta^{13}C$ = - 12,2‰) evidencing that their carboxyl atom is enriched in ^{12}C relative to glucose and the fatty acid molecules are derived at extent of pool depletion less than 0,5, as in previous example.

Similar conclusions may be done from the analysis of ^{13}C distribution in quite different class of compounds, plant monoterpenes which also made of C_2 structures (Fig. 10). On the top of

Fig.10 the known synthesis pathway of monoterpenes from C_2-fragments is shown (Nicolas, 1963). As before methyl atoms denoted by empty circles (first type atoms) have approximately equal isotope composition and affirm that isotope effect in CO_2 assimilation was about -25‰ for all studied plants. Isotope ratio of carboxyl atoms (second type atoms) denoted by filled circles vary in a wide range what is expected for them and affirm that fatty acids synthesis in cell cycle have some time length.



The empty and filled circles indicate carbon atoms of the first and second type

Fig. 10. Biosynthesis pathway of monoterpenes (Nicolas, 1963) and ^{13}C distribution in some plant compounds (Schmidt et al, 1995).

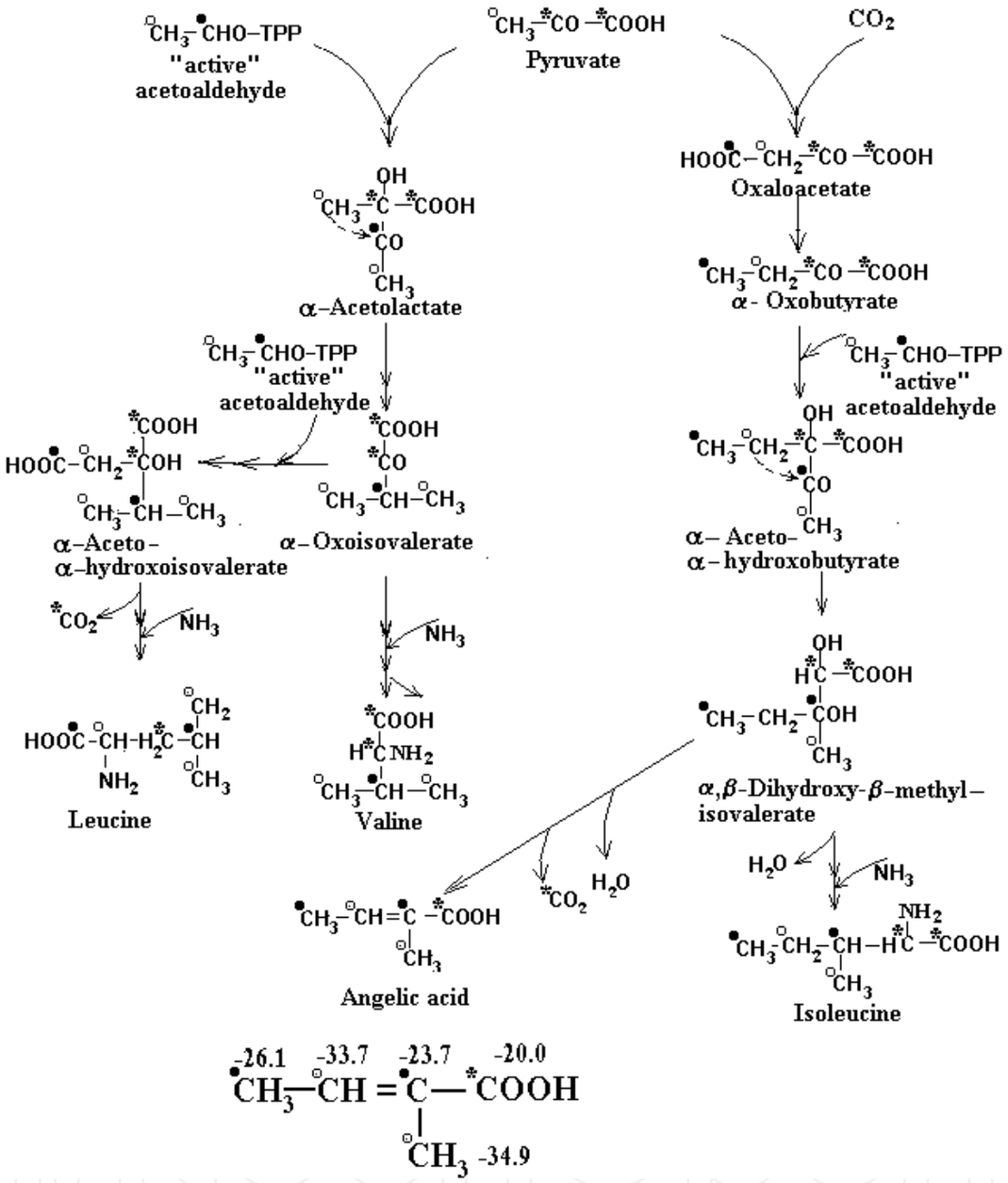


Fig. 11. Biosynthesis pathway of branched aminoacids (Metun, 1963) and ^{13}C distribution in angelic acid precursor of isoleucine, isolated from plant *Angelica Archangelica* (Schmidt et al, 1995).

Two possible synthesis pathways for branched amino acids in photosynthesizing organisms, known from the literature, are shown on Fig.11. One of them leads to leucine (left), another to isoleucine (right). C_2 and C_3 – fragments are the structural units used for their synthesis. Isotopic atoms of all three types, denoted as before, are included in the molecules. Angelic acid, the precursor of isoleucine, was experimentally studied. The entire coincidence with the expected ^{13}C distribution is observed in spite of the internal regrouping of the molecule which occurs at the step of α-aceto-α-hydroxybuterate formation.

^{13}C-Distribution in sinigrin is shown on Fig. 12. It is a glucosinolate that belongs to the family of glucosides found in some plants of the *Brassicaceae* family. At the top of the figure the scheme of its biosynthesis pathway is drawn. The same very good coincidence of the predicted ^{13}C distribution with that of observed takes place.

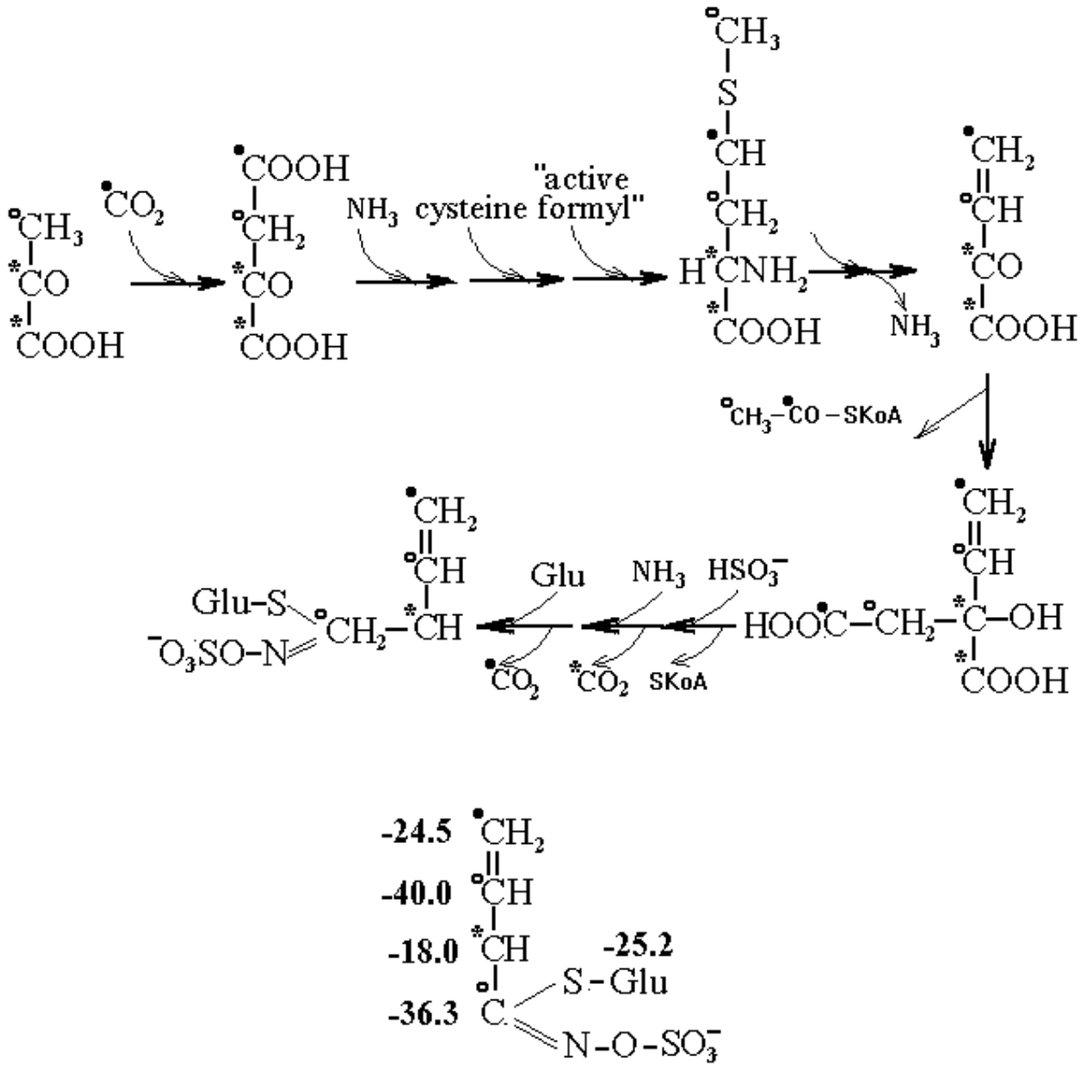


Fig. 12. Biosynthesis pathway and ^{13}C distribution of sinigrin, isolated from plant Angelica Archangelica (Schmidt et al, 1995).

According to the scheme, all three types of isotopic atoms are involved in sinigrin skeleton formation. There are two atoms of the first type which are most enriched in ^{12}C, one atom relating to the third type which was expectedly most enriched in ^{13}C, and one atom of the second type with intermediate isotope composition. The full coincidence of the observed sinigrin isotopic pattern to that expected from the above pathway and the Raliegh law gives one more strong argument in favor of oscillatory character of glycolytic metabolism. Some difference in δ^{13}C values for the first type atoms are the result of two different measurement techniques. Carbon atom of the first type adjacent to double C=C bond was measured by

means of NMR technique which is less précised than mass-spectrometric technique. Nevertheless both atoms are considerably "lighter" than atoms of other types and characterize isotope effect in RuBP carboxylation (or carbon isotope ratio of carbohydrates synthesized in carboxylase phase). Notably, glucose in carbohydrate part of sinigrin (-25.2‰), as compared with atoms of the first type, is much more "heavier". It means that it is originated from the labile carbohydrates formed in oxygenase phase of photosynthetic oscillations. Some other arguments proving the oscillation concept are given in the recent publications (Ivlev, 2010, 2011)

4. Conclusions

The performed analysis of isotopic data proves that primary carbon metabolism in photosynthesis and secondary metabolism in glycolytic chain are of oscillatory character. They discover the existence of Raliegh effect that in turn evidences that cells work in filling/depletion regime and there is strict temporal organization of metabolic events. The examination of isotopic patterns of metabolites allows establishing the sequence of metabolites biosyntheses in cell cycle. It is a very important since it changes the fundamental view on the mechanisms underlying all cell processes. This means that besides metabolic pathways one should consider the parameter of temporal organization (Lloid, 2009).

The existence of regularities in ^{13}C distribution proves the following assertions. 1) cell cycles are rather stable, by other words, cell oscillations are in-phase, i.e. at given functional state conditions temporal sequence of metabolic events weakly depends on the environmental factors; 2) cycle oscillations in different cells are synchronized. This fact is in compliance with the known independence of metabolic clocks on the same factors (Shnol', 1996). It means that oscillatory characteristics are determined by the internal properties of the system itself. By the other words the stable temporal sequence of metabolites syntheses which determine isotopic regularities of ^{13}C distribution in metabolites is formed in the course of evolution (Ivlev, 2009). The changes in the environmental conditions can partly change the sequence of events in metabolic organization to better adaptation of organisms to the environments.

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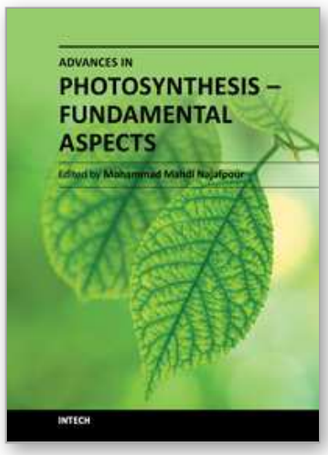

Advances in Photosynthesis - Fundamental Aspects
Edited by Dr Mohammad Najafpour

ISBN 978-953-307-928-8
Hard cover, 588 pages
Publisher InTech
Published online 15, February, 2012
Published in print edition February, 2012

Photosynthesis is one of the most important reactions on Earth. It is a scientific field that is the topic of many research groups. This book is aimed at providing the fundamental aspects of photosynthesis, and the results collected from different research groups. There are three sections in this book: light and photosynthesis, the path of carbon in photosynthesis, and special topics in photosynthesis. In each section important topics in the subject are discussed and (or) reviewed by experts in each book chapter.

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