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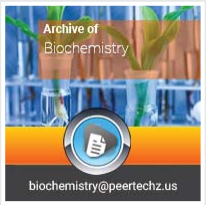

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Review Article

The use of Intramolecular carbon isotope distributions ($^{13}c/^{12}c$) of biomolecules to study temporal organization of post-photosynthetic metabolism in a plant cell

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It was found that the pyruvate decarboxylation reaction (PDR) plays a key role in post-photosynthetic metabolism, and PDR products are structural units involved in the synthesis of almost all its metabolites [1,2]. Carbon atoms of PDR products (marker atoms) make up the carbon skeletons of metabolites. Here we show that analysis of isotope fractionation in PDR makes it possible to estimate the isotopic composition of each marker atom and its dependence on two factors: 1) on the kinetic isotopic effect of C – C bond cleavage and 2) on the isotopic effect when the pyruvate pool is depleted (Raleigh isotope effect). It is also shown that marker atoms can be divided into three groups. The first group includes atoms of methyl groups of acetate (C2-) fragments and atoms of pyruvate (C3 fragments) subjected to decarboxylation. Their carbon isotope composition does not change during decarboxylation and can serve as an internal standard. The second group includes the carbon atom of CO_2 and the acyl-carbon atom of C2 – fragments. According to the kinetic isotope effect theory, they may be either enriched in ^{12}C. relative to the atoms of the first group, when pyruvate pool depletion is less than 50% or enriched in ^{13}C when pyruvate pool depletion is more than 50%. The third group includes the acyl and adjacent carboxyl atoms of the residual pyruvate, located at the ends of the cleaved C - C bond. These atoms are always enriched in ^{13}C as compared with the atoms of the first and second groups at the same extent of pyruvate pool depletion. The comparison of the theoretically expected distribution of marker atoms and the experimentally measured isotope distribution of carbon atoms in the metabolites allows for studying the structural and temporal organization of post-photosynthetic metabolism.

Some preliminary notes are necessary to study the temporary organization of post-photosynthetic metabolism

Nowadays, studies and applications of the non-statistical carbon isotopes distribution in biomolecules have increasingly attracted the attention of researchers, since this technique, despite the difficulties of experimental determination of atoms' position in the molecules [3], appears to be a delicate and very effective tool for solving many problems of modern biochemistry, biology and physiology of organisms [1]. First of all, this is bound to the task of identifying metabolic pathways in organisms of various types. Genetically similar organisms, due to their high adaptive capacity, even minor changes in their habitat causes changes in their metabolism [4]. The method can be applied to solve the practical tasks in biotechnology to identify new and search for alternative metabolic pathways in bacteria exposed to radiation [2].

A complex, but no less important task is the use of ^{13}C distribution for identification in metabolomics and natural products, including a wide range of biochemical

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and physiological tasks, which arise when it is necessary to investigate the metabolism of an unexplored organism or to identify the pathway of synthesis of an unknown compound [5,6]. The applications of the ^{13}C distribution technique to various geochemical tasks, including distinguishing biogenic or abiogenic life [7] the investigations of organic compounds associated with extraterrestrial life or with life in extreme conditions and other studies in related fields of knowledge [8,9].

Below we will consider a method for determining the spatial and temporal organization of metabolism in organisms based on the analysis of the relationship between the metabolic pathway and carbon isotope distribution in biomolecules.

It is known that in the cells of living organisms there are biological rhythms underlying the mechanism of the "biological" clock [10]. They reflect the adaptation of the cells of organisms to the rotation of the Earth around the Sun, in other words, to the change of day and night. Biological rhythms are accompanied by the rearrangement of carbon fluxes and associated metabolic processes [11].

It has been shown (Ivlev, 2008) that during the phase of glycolysis in the glycolytic chain of the cell, the reaction of enzymatic decarboxylation of pyruvate (PDR) occurs, accompanied by fractionation of carbon isotopes. Fractionation depends on two reasons. The first is caused by the different rates of carbon-carbon bond destruction, at the ends of which one of the atoms is the isotope ^{13}C. Such bonds are destroyed at a lower rate than the ^{12}C - ^{12}C bonds. As a result, a kinetic isotopic effect arises.

The second reason that causes the permanent sequential enrichment of the isotopic composition of carbon atoms in PDR products in the ^{13}C isotope is the depletion of the substrate (pyruvate) pool associated with ^{13}C accumulation in the pool. This ^{13}C enrichment is called the isotope Rayleigh depletion effect.

Marker atoms and their use in the studies of intramolecular carbon isotopic distributions in metabolites of the glycolytic chain. The temporal organization of metabolite synthesis

From the formal scheme of enzymatic decarboxylation of pyruvate, shown below one can see that the reaction products include not only acetate fragments and CO_2, but also residual pyruvate since as a result of the reaction, the isotopic composition of carbon atoms in it also changes. The empty circles indicate the carbon atoms in the PDR products (carbon atoms of methyl groups in the "active" acetate (C2 - fragments) and in the residual pyruvate (C3 -fragments), whose isotopic composition does not change during PDR and remains the same as in the initial pyruvate. These atoms make up a special group in which the isotopic composition of the atoms does not depend on either the kinetic isotopic effect of the C - C bond cleavage or the Rayleigh effect. Therefore, the isotope composition of these atoms can serve as an internal standard for the isotopic composition of the carbon of the remaining atoms of the metabolites of the glycolytic chain.

The carbon atom in CO_2 and the acyl-carbon atom in C_2 - fragments are marked with filled circles. According to the kinetic isotope effect concept [12], the isotopic composition of the above carbon atoms at the pyruvate pool depletion of less than 50% is enriched in ^{12}C relative to the carbon atoms of the internal standard (atoms of the first group), whereas at the pyruvate pool depletion more than 50%, the above carbon atoms are enriched in isotope ^{13}C.

Asterisks indicate the carbon atoms of the third group, which includes the acyl and carboxyl carbon atoms of the residual pyruvate, located at the ends of the cleaved C - C bond. According to the theory, these atoms are always enriched in ^{13}C as compared with the atoms of the second group if they are formed at a level of more than 50% of the pyruvate pool depletion.

The features of carbon isotope fractionation in metabolic processes, as shown [13], are the fact that the isotope effect arises only in a small number of reactions occurring at points where metabolic processes converge or diverge. In the vast majority of cases, the carbon isotope composition of atoms or fragments of molecules is completely inherited in metabolic chains from their predecessors.

The analysis of the metabolism of the glycolytic chain allows concluding that PDR products are the structural units for the synthesis of almost all metabolites. But if it is the case, then the carbon atoms that make up the above-mentioned PDR products can be used as markers to determine the source of their origin in the studied metabolites. Considering that the carbon atoms of the second and third groups are associated with the extent of the pyruvate pool depletion in the presence of an internal standard (the carbon atoms of the first group), the isotopic composition of carbon atoms can be used to study the temporal organization of metabolism.

It should be stressed that the isotope composition of carbon atoms in PDR products, and in all other metabolites of post-photosynthetic metabolism depends on the extent of pyruvate pool depletion. It is the result of the periodicity of the functioning of the glycolytic chain and means the chain operates in the regime of filling /depletion of the substrate pool. The information becomes available while comparing marker distribution with the experimentally studied patterns of ^{13}C distribution in the metabolites under consideration.

It is obvious that the Rayleigh isotope effect occurs in the phase of glycolysis when the pool is depleted. In such a case, the Rayleigh isotope effect is superimposed on the isotopic heterogeneity of the molecules caused by the kinetic isotopic effect of pyruvate decarboxylation. Examples of using the distribution of marker atoms to study the temporal organization of metabolism are given below.

Some examples of using marker atoms and experimentally observed ^{13}C distributions to study the temporal organization of the synthesis of metabolites

There are dicarboxylic oxalic acid and tricarboxylic citric acid in the Krebs cycle, whose ^{13}C distribution along the carbon

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skeleton of the molecules has been fully studied. It is possible to compare marker atoms and ^{13}C distributions in such cases to conclude how they correspond to each other. Figure 1 shows the synthesis of the polycarbonic acids under consideration formed from reaction intermediates. The corresponding markers are denoted with the symbols adopted above. Marker atoms distribution was constructed and compared with the experimentally observed distribution of ^{13}C in these acids. For convenience, numbers next to carbon atoms indicate the positions of marker atoms that they occupy in the intermediates.

^{13}C distributions of polycarbonic (malic and citric) acids formed in the Krebs cycle. The pathways of synthesis of polycarbonic acids are shown. Figure 2. Indicates the isotopic shifts relative to the average carbon isotope composition of the molecules given in VPDB units.

Markers of the first group denoted with empty circles, whose carbon isotope composition does not change during pyruvate decarboxylation, fall into different positions of oxalacetic and citric acid. As one can see from Figure 2, mentioned marker atoms, in accordance with the theory, retain approximately equal values of $\delta^{13}C$. For the molecules under study, they ranged from – 11.2% to -12%.

Marker atoms of the second group, representing acyl-atoms of C2-fragments, participate in syntheses of both polycarbonic acids (Figure 2). According to $\delta^{13}C$ values, they all fall into a narrow range with $\delta^{13}C$ varying from +2.3% to +4.9%. Notably, despite a narrow range, the marker atoms, included in the synthesis of different polycarbonic acids differ markedly by $\delta^{13}C$ values. For citric acid, the differences of markers included

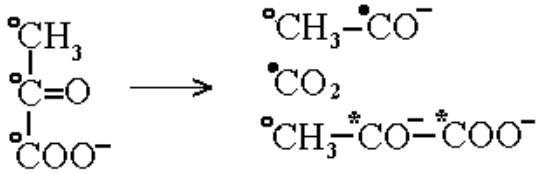

Figure 1: The scheme of enzymatic decarboxylation of pyruvate. All symbols are explained in the text below.

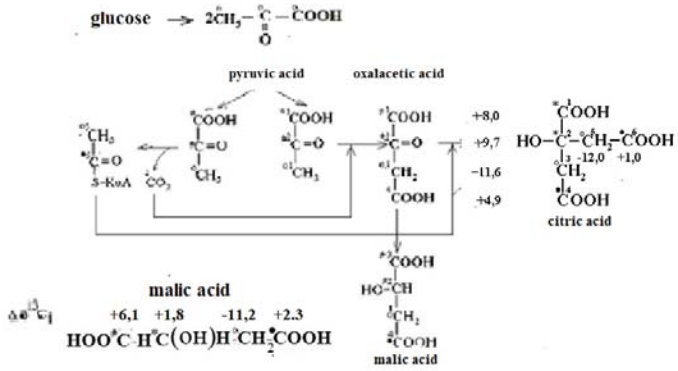

Figure 2: The comparison of marker atoms and experimentally observed. ^{13}C distributions of polycarbonic (malic and citric) acids formed in the Krebs cycle. The pathways of synthesis of polycarbonic acids are shown. Figure 2. Indicates the isotopic shifts relative to the average carbon isotope composition of the molecules given in VPDB units.

in different C2-fragments are +1,0 and +4,9 respectively. The explanation is that two C2 fragments, containing designated atoms are formed at different pyruvate pool depletion.

Marker atoms of the third type, representing acyl and adjacent carboxylic atoms from the residual pyruvate fall into a narrow range of $\delta^{13}C$ variations, which turned out to be shifted to the area with positive values of $\delta^{13}C$ relative to the range occupied by markers of the second type (Figure 2). They participate in the syntheses of both polycarbonic acids. In accordance with kinetic isotope theory [12], the isotopic shift is explained by the fact that pyruvate is used to form C2 fragments containing marker atoms of the second group. Hence both marker groups are bound to the Raleigh isotope effect and depend on the extent of the pyruvate pool depletion. From Figure 2 one can see that the marker atom of the third group comprises the range from +6.1% to +1.8% for malic acid) and the range (+8.0% and +9.7% for citric acid. Hence, the experimental data confirm the theoretical assertion.

According to the theory of the kinetic isotope and taking into account the effect of neighboring atoms isotopic shifts of atoms at the ends of the broken bond should be approximately the same. However, this correspondence is observed only for citric acid (+8.0% and +9.7%). In the case of malic acid, the isotopic shifts are very different (+6.1% and +1.8%). To explain it additional experimental confirmation is needed.

The distribution of ^{13}C has been fully studied for the acetoin molecule (3-hydroxy-2-butanone). The synthesis of the molecule is associated with the metabolism of the glycolytic chain as well [14]. Figure 3 shows the pathway of acetoin synthesis. One can see that this molecule is synthesized from two C2 fragments, which, in turn, are formed from pyruvate. The distribution of marker atoms in the acetoin molecule in which the acyl atoms of C2 fragments (the second marker group) are associated with the carbon atoms of methyl groups (the first marker group) fully coincide with the experimentally studied ^{13}C distribution (Figure 2).

As said before, the markers of the first group practically do not change their carbon isotope composition (-33.3% and - 35.5%). Carbonyl atoms (markers of the second group) differ significantly in their isotopic composition (-29.1% and -18.5%). The difference between the second and the third atoms of the carbon skeleton inherited from the acyl carbon atoms of C2 -fragments indicates that they are formed at different extents of pyruvate pool depletion.

Arguments, indicating the temporal organization of synthesis of some of the lipid components

As known, the synthesis of fatty acids in most plant cells occurs by condensation of C2-fragments according to the "head to tail" principle, while the formation of C2-fragments is associated with the cleavage of C3-fragments (with decarboxylation of pyruvate molecules). It means the same isotopic ratios between marker and ^{13}C distributions, as in the above examples, should be observed in fatty acids as well. Let's consider the results of studies on carbon isotope fractionation

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in glucose fermentation by E. *coli* using a substrate with a known isotope composition [15]. The results obtained are given in Table 1.

Bacteria, feeding on glucose (δ ^{13}C= -9.0), evolved acetic acid into the medium. Part of the isolated acid was used to determine the isotopic composition of its total carbon. The other part of the acid was subjected to fragmentation to study the isotope composition of its methyl and carboxylic carbon. A lipid fraction was isolated from the bacterial biomass, in which the isotopic composition of the total carbon of fatty acids was determined. It turned out that the carbon of the methyl group of acetate fragments has the same isotope composition as the carbon of the initial glucose. It means that the methyl carbon belongs to the markers of the first group. The isotopic composition of carboxylic carbon is significantly enriched in ^{12}C relative to methyl carbon. From this, it follows that the above marker atoms belong to the second group, which depends on the extent of pyruvate pool depletion.

The close results were obtained by Hayes and Monson [16], who investigated the intramolecular distribution of ^{13}C in fatty acids isolated from E. *coli* biomass grown on glucose with a known carbon isotopic composition (Table 2).

It should be underlined first that fatty acids are formed only via C2 fragments. The even atoms of the fatty acid molecules correspond to marker atoms of the second type whose carbon isotope composition doesn't depend on pyruvate pool depletion and, hence, is the same for all the fatty acids irrespective of their synthesis pathway. This is proved by the proximity of carbon isotope composition of even atoms and feeding glucose carbon.

Note, that, as in the previous cases, the range of δ ^{13}C values for markers of the first and second type are absolutely different. The odd atoms, which are acyl-atoms of C2 fragments, belong to marker atoms of the second type, depending on the extent of pyruvate pool depletion. As one can see from Table 2 that odd atoms noticeably differ from each other, which confirms their dependence. The even atoms, which are methyl atoms of C2 fragments practically have the same carbon isotope composition which confirms their belonging to the markers of the first group.

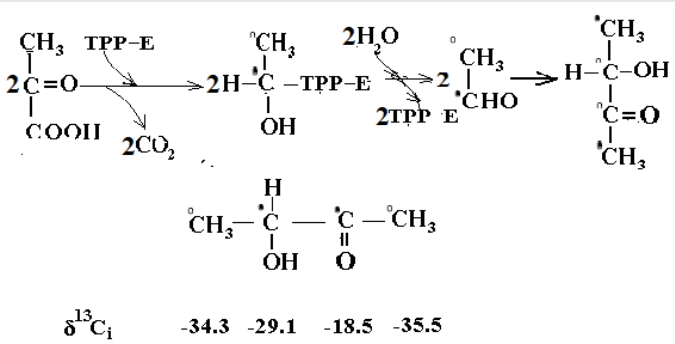

Figure 3: The pathway of acetoin biosynthesis and the distribution of marker atoms in the acetoin molecule are marked with icons. The experimental distribution of ^{13}C along the carbon skeleton [14]. δ ^{13}C values are given in VPDB units.

Table 1: ^{13}C distribution in acetate released by E. coli in the medium during fermentation on glucose with a known isotope composition [15]. The isotopic composition of carbon is given in VPDB units.

Feeding glucose	Fatty Acids	Distribution of ^{13}C in acetate		
		Total carbon	Carboxylic carbon	Methyl carbon
-9.0	-12.2	+3.3	+15.0	-8.8

Table 2: ^{13}C intermolecular isotope distribution of some fatty acids, isolated from lipid fraction of E. *coli*. Grown on glucose with known carbon isotope composition (-9,96%). The data are taken from the work [16].

Fatty acids	δ^{13}C, ‰	Odd atoms		Even atoms	
		N	δ^{13}C, ‰	N	δ^{13}C, ‰
Palmitic 16:0	-12.2		-15.2		...
Palmitoleic 16:1	-13.0	1	-19.2		
		9	-16.0	10	-.9.5
		1	-20.3		
Vaccenic 18:1	-12.6	1	-13.9		
		11	-15.8	12	-9.5

Another example illustrates the intramolecular distribution of ^{13}C in plant terpenes [17,18] (Figure 4). Like fatty acids, terpenes are formed from C2 fragments, but their marker distribution has some peculiarities.

The isotope composition of marker atoms of the first type (methyl atoms) is almost the same and doesn't depend on the position they occupy in the molecule, while the remaining atoms vary markedly, which allows assign them to the second type. By the level of their enrichment in ^{13}C, it is possible to determine the sequence of C2-fragments formation.

Conclusion

The key role of pyruvate decarboxylation reaction (RDP) and the role of its products as the main structural units in the post-photosynthetic metabolism of a cell are shown. Hence the atoms included in the RDP products are considered as the marker showing atom transitions in the metabolic reactions.

In order to use the isotope composition of carbon atoms to study the mechanism and temporal organization of post-photosynthetic metabolism, the factors determining isotope fractionation in RDP were analyzed. They are: 1) the kinetic isotope effect of C - C bond cleavage in pyruvate; and 2) the effect of pyruvate pool depletion (Rayleigh effect).

The selection of atoms in RDP products into three groups, one of which does not depend on the extent of pyruvate pool depletion, allows considering the distribution of marker atoms in any metabolites of post-photosynthetic metabolism. Comparison of the theoretically expected distribution of marker atoms, taking into account their intramolecular carbon isotope distribution, established from the analysis of isotope fractionation in RDP, with the experimentally measured distribution of ^{13}C in the metabolites allows determining the source of carbon atoms and their transitions in metabolic reactions. Considering the dependence of the carbon isotopic composition of marker atoms on pyruvate pool depletion makes it possible to study temporal organization (sequence of inclusion of marker atoms in the synthesis of metabolites. It is the easiest way to study the temporal organization of synthesis of lipid components formed only via C2 -fragments.

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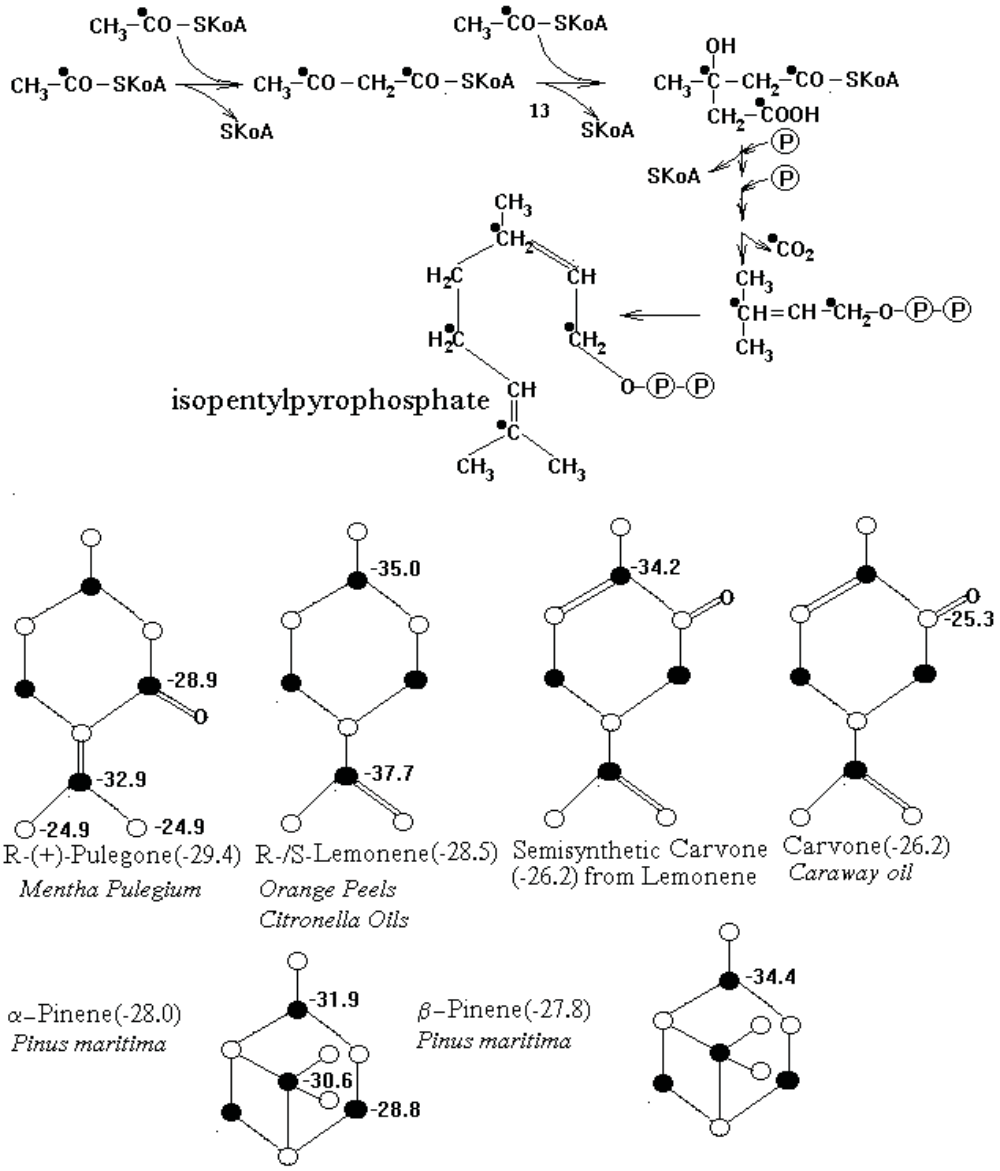

Figure 4: The pathway of synthesis in the post-photosynthetic metabolism of the precursor plant monoterpenes – isopentyl pyrophosphate (top). Distribution of marker atoms from C2 fragments in monoterpenes and isotopic composition of some atoms in the carbon skeleton of molecules (bottom). The empty circles represent the markers of the first group (carbon atoms of methyl groups of C2 fragments) and filled circles denote markers of the second group (acyl atoms of C2 fragments with variable carbon isotope composition).

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