Single-atom Tracing in a Model Network of Carbohydrate Metabolism and Pathway Selection

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Abstract: Studies on computation of pathways connecting two metabolites have been reported. However, they did not intend to find pathways containing cycling, although there are biologically important cycles such as citric acid cycle (CAC). Whilst computation of pathways connecting two atoms, single-atom tracing, would contribute to finding pathways which include those containing cycling, it produces too many pathways to examine. The present article proposes a strategy to select pathways from those obtained by single-atom tracing, where coexistence of reactions on each pathway, specifically coexistence of a reaction and its reverse reaction forming a futile cycle together or reactions regulated in a reciprocal manner, is checked to select pathways based on biochemical meaning of the pathway. Using this strategy, 121 pathways were selected from total 7876 pathways from carbon atoms of glucose to CO\textsubscript{2} in a model network of carbohydrate metabolism. The selected pathways included pathways using reactions or metabolites of CAC or pentose phosphate pathway multiple times. These results indicate that the proposed strategy can contribute to listing a limited number of pathways which include those containing cycling as possibly biochemically meaningful pathways.

Keywords: metabolic networks, carbohydrate metabolism, glycolysis, gluconeogenesis, citric acid cycle, pentose phosphate pathway, connectivity matrix method

1. Introduction

To know source metabolites suitable for production of a specific target metabolite is important in metabolic engineering. To contribute to this end, \textsuperscript{13}C Metabolic Flux Analysis is conducted to determine the distribution of metabolic fluxes in an organism\cite{1}, \cite{2}, \cite{3}, \cite{4} and methods for computing the pathways to connect potential source metabolites with the target metabolite in a given metabolic network have been reported, where atomic tracing is used to judge which pathways are biochemically meaningful\cite{5}, \cite{6}, \cite{7}, \cite{8}.

In most of those pathway-computing studies\cite{5}, \cite{6}, \cite{7}, metabolites are considered as nodes in the network, although enzymatic transformations of metabolites are traced at the atom level. There, because the purpose of computation is to find the pathways connecting two metabolites, metabolic pathways in which the same compound is encountered multiple times are not output\cite{5} or, whilst other methods such as Refs.\cite{6}, \cite{7} have a feature that they find pathways based on some optimal criteria, it is not described whether such pathways are considered or not. In one of those approaches, atoms are considered as nodes, but there is no description about multiple appearances of the same compound on one pathway\cite{8}. Pathways with multiple appearances of the same compound are pathways containing cycle(s). Thus, approaches to compute both linear and cycle-containing pathways have not been considered.

In the case of metabolism of glucose to CO\textsubscript{2}, both linear and cycle-containing pathways are important. Glucose is metabolized to the acetyl portion of acetyl-CoA and CO\textsubscript{2} through glycolysis and subsequent oxidative decarboxylation of pyruvate, where CO\textsubscript{2} is formed from glucose by a linear pathway. Then, the acetyl portion of acetyl-CoA is condensed with oxaloacetate, incorporated to citrate, and metabolized to CO\textsubscript{2} through citric acid cycle (CAC), where none of the two carbon atoms derived from acetyl-CoA are released as CO\textsubscript{2} during the first round of CAC, but they are released in the second or later round(s). In this pathway from glucose to CO\textsubscript{2} through acetyl-CoA, intermediates of CAC appear multiple times. To compute such non-linear pathways as well as linear pathways, multiple appearances of the same compound on a pathway should be allowed.

I proposed concept of metabolic network as atom network\cite{9}, \cite{10}, where nodes are atoms included in metabolites instead of metabolites, and edges are chemical bonds which define the structure of metabolites (intra-metabolite connectivity) and atom-to-atom correspondences via reaction between metabolites (inter-metabolite connectivity). Atom-network structure, a mathematical object, describes almost all information concerning structure of a metabolic network including chemical structure of metabolites and enzymatic transformation. In the study when I got the idea of atom network, I calculated all the pathways connecting the aldehyde carbon, C1, of glucose with CO\textsubscript{2} in a model network of carbohydrate metabolism and found 253 pathways\cite{11}. In the study, pathways from a specific single atom to another specific single atom were computed instead of those from a specific metabolite to another specific metabolite. In the present article,
such an approach is called single-atom tracing. In the single-atom tracing, multiple appearances of the same atom are avoided instead of multiple appearances of the same compound.

The above 253 pathways should include both linear and cycle-containing pathways, but seem to include ones with less biochemical meaning. Because 253 is a large number, it is a huge labor to judge which is important among those pathways. This problem seems to be specific for single-atom tracing and not to be encountered in computation of pathways where metabolites are considered as nodes. In the present article, I propose a strategy to select biochemically meaningful pathways computationally from those obtained through single-atom tracing and present its application to pathways from each carbon atom of glucose to CO$_2$ in a model network of carbohydrate metabolism composed of reactions for glycolysis, oxidative decarboxylation of pyruvate, CAC, pentose phosphate pathway (PPP) and gluconeogenesis where the same atom-mapping information as in Ref. [11] is used for single-atom tracing. The model network is shown in Fig. 1.

Please understand that description in the present paper is based on the following principle concerning the term ‘cycle’. The name of CAC contains ‘cycle’. The set of reactions included in PPP can be understood as a cycle and PPP is sometimes called pentose cycle. However, it may happen that parts of CAC or PPP form a linear pathway where the same compound or the same reaction appears less than twice. In the present article, I strictly distinguish such use of reactions in CAC or in PPP as a linear pathway from use as a cycle.

2. Results and Discussion

2.1 A Strategy for Selecting Pathways

To select biochemically meaningful pathways, coexistence of two reactions on a pathway was considered. There seem to be two cases where coexistence of two reactions $R_1$ and $R_2$ on a pathway is related to biochemical meaning of the pathway potentially. One is the case that reaction $R_1$ is the reverse reaction of $R_2$, where coexistence of $R_1$ and $R_2$ results in futile cycling. Here, because a reaction and its reverse reaction are considered as separate steps on a pathway, the term futile cycling is used. The other is the case that reactions $R_1$ and $R_2$ are regulated in a reciprocal manner, where coexistence of $R_1$ and $R_2$ seems to be against the purpose of the coordinated regulation. Thus, the strategy I propose here is to select pathways to exclude such cases. The rationale of this strategy will be discussed later. To apply this strategy to pathways in general metabolic networks, I set the following three simple criteria for pathway selection: Filters 1, 2 and 3.

- **Filter 1**: Coexistence of a reaction and its reverse reaction is not allowed.
- **Filter 2**: Coexistence of any reaction included in A and any reaction included in B is not allowed, where each of A and B is a group of different reactions and, when reactions included in A are upregulated, reactions included in B are downregulated and vice versa.
- **Filter 3**: Both the coexistences excluded in Filters 1 and 2 are not allowed.

When these filters were applied to the present model network, A was defined as a group of irreversible pathways which work mainly for glycolysis and B was defined as a group of irreversible reactions which work mainly for gluconeogenesis, where, more concretely, A consisted of hexokinase including glucokinase, phosphofructokinase, and pyruvate kinase and B consisted of glucose-6-phosphatase, fructose-1,6-bisphosphatase, and phos-
in Filter 2, but the exclusion rate for pathways from C2, C3, C5, and C6 of glucose was lower in Filter 1 than in Filter 2, indicating exclusion rates in Filters 1 and 2 depended on start point carbons. On the other hand, the exclusion rate in Filter 3 was always higher than those for Filters 1 and 2, which did not depend on start point carbons. Therefore, Filters 1 and 2 seem to be complementary to each other and Filter 3, which is the combination of Filters 1 and 2, is recommended for effective selection of possibly biochemically meaningful pathways.

### 2.3 Pathways Selected by Filter 3

I overview some features of pathways selected by Filter 3. As described in Introduction, pathways obtained by single-atom tracing may include linear pathways and cycle-containing pathways where the same compound or reaction appears multiple times, and pathways from carbons of glucose to CO₂ are assumed to inevitably include cycle-containing pathways. Therefore, it was examined if cycles are seen on the pathways selected by Filter 3. Existence of cycles was judged computationally by multiple use of the same compound or reaction.

As described in Table 3, cycles were found in no less than 85% of the pathways from C1, C2, C3, C5, and C6 of glucose to CO₂ when existence of cycles was judged by multiple use of the same compound. The pathways from C4 did not include any cycles.

As for the pathways from C1, the number of pathways with multiple use of the same reaction was different from that with multiple use of the same metabolite. Among the 6 pathways with multiple use of the same metabolite, 4 pathways were found to use citrate synthase reaction, a reaction in CAC to form citrate, two or three times. Two pathways were found to use fructose-6-phosphate (F6P) twice, forming a cycle in PPP, but to use glucose-6-phosphate dehydrogenase (G6PD) reaction, a reaction in PPP, less than twice. Pathway No.9 in Table 4 is an example of the former 4 pathways and Pathway No.2 is an example of the latter two pathways.

As for the pathways from C2, the 22 pathways containing cycle(s) were found to use citrate synthase two or three times or G6PD twice. There were no pathways where F6P was used multiple times without multiple use of G6PD.

As for the pathways from C3, the 36 pathways containing cycle(s) were found to use citrate synthase or G6PD two or three times. There were 4 pathways where F6P was used multiple times (twice) without multiple use of G6PD. These were found to use citrate synthase multiple times. Pathway No.5 and Pathway No.20 in Table 5 are examples of multiple use of G6PD. Several reactions of glycolysis are reversible and their reverse reactions are used for gluconeogenesis. In Pathway No.20, a part of those reactions (the 15th, 16th and 18th to 21st reactions) are used in the direction of glycolysis and another part in the direction of gluconeogenesis (the 8th reaction). This pathway cannot be understood from the viewpoint of glycolysis and gluconeogenesis. It is of interest if this pathway works in vivo.

Whilst there were no cycles in the 10 pathways from C4 of glucose to CO₂, all the 20 pathways from each of C5 and C6 to CO₂ were found to use citrate synthase two or three times, but to use both G6PD and F6P less than twice. It was found
that there were interesting one-to-one correspondences between the pathways from C5 to CO2 and those from C6 to CO2. Each reaction sequence of the shorter half of the pathways from C5 contained one pyruvate dehydrogenase complex (PDC) reaction to form acetyl-CoA from pyruvate. When the PDC reaction was replaced by pyruvate carboxylase (PC) reaction in those reaction sequences, reaction sequences of the shorter half of the pathways from C6 were obtained. An example is shown in Table 6. On the other hand, each reaction sequence of the longer half of the pathways from C5 contained one PC reaction to form oxaloacetate from pyruvate. When the PC reaction was replaced by PDC reaction in those reaction sequences, reaction sequences of the longer
Table 3  
Number of pathways from carbon atoms of D-glucose to CO$_2$ selected using Filter 3 and estimation of cycle occurrence. CAC and PPP indicate citric acid cycle and pentose phosphate pathway, respectively.

| Start point | Number of pathways | Pathways selected using Filter 3 | Pathways with CAC or PPP reactions used as a cycle |
|-------------|--------------------|----------------------------------|-----------------------------------------------|
|             | Total              | Pathways accompanied by multiple appearance of the same metabolite(s) or reaction(s) | None | CAC only | PPP only | Both |
|             |                    | Metabolite(s) | Reaction(s) | 3 | 4 | 0 | 0 |
| C1          | 7                  | 6              | 4            | 2 | 12 | 2 | 8 |
| C2          | 24                 | 22             | 22           | 4 | 12 | 8 | 16 |
| C3          | 40                 | 36             | 36           | 10 | 0 | 0 | 0 |
| C4          | 10                 | 0              | 0            | 0 | 20 | 0 | 0 |
| C5          | 20                 | 20             | 20           | 0 | 20 | 0 | 0 |
| C6          | 20                 | 20             | 20           | 0 | 20 | 0 | 0 |

half of the pathways from C6 were obtained.

In addition, it was found that selected pathways contained traditional, well-known pathways to metabolize the 6 carbons of glucose to CO$_2$ through PDC reaction and CAC. For details, please see Supplementary Materials, where information of all the pathways selected using Filter 3 is described.

Whereas single-atom tracing in the model network produced a lot of pathways, the number of the selected pathways was small enough to allow careful examination of individual pathways. Such pathways as Pathway No. 20 from C3 would be overlooked without careful examination of individual pathways. In general, Filter 3 worked well. Further, aided by computation, it was found that the selected pathways include pathways containing cycling in PPP or CAC as well as linear pathways. In spite of well-known importance of cycles such as CAC and urea cycles, previous studies did not focus on computation of pathways containing cycling as mentioned in Introduction, probably because the purpose of computation was to find out biosynthetic pathway of useful products. In the present approach, both linear and cycle-containing pathways can be computed. This may mean that the present approach covers all the pathways should be computed. The present approach would at least supplement those previous approaches through finding cycles.

The above description that reducing the number of candidate pathways by the present pathway selection makes careful examination of each pathway possible could be certainly applicable to pathway calculation in relatively small metabolic networks composed mainly of central metabolic pathways. In large networks where the number of pathways computed by single-atom tracing is more than 100,000, the situation is different. In such a case, 1,000 pathways would remain to be examined after pathway selection by Filter 3 even if the exclusion rate is assumed to be 99%. One thousand is a huge number to examine each carefully and another method may be necessary for further selection of pathways. However, the present approach for pathway selection remains to be useful because it is expected to exclude a lot of pathways.

2.4 Rationale of Pathway Selection Strategy

So far I have claimed that pathways selected by Filter 3 are possibly biochemically meaningful because they are accompanied neither by futile cycling resulting from coexistence of a reaction and its reverse reaction nor by coexistence of reactions regulated in a reciprocal manner which will be against the purpose of the coordinated regulation. However, I have not discussed the rationale of this pathway selection strategy in detail and the following question remains to exist: Are excluded pathways biochemically meaningless? Here I will show excluded pathways are exceptional through considering the features of typical pathways for producing a target metabolite from a source metabolite, posulating function of a pathway can be expressed as stoichiometric balance of the entire pathway and considering purposiveness of pathways. In most pathways, called ‘typical pathways’ here, for producing a target metabolite from a source metabolite, the following two rules are kept.

- Rule 1: One or several atoms of the source metabolite are traced to atoms of the target metabolite.
- Rule 2: There are one-to-one correspondences between individual steps each of which is a reaction and individual enzymes responsible for those steps.

Rule 1 is essential to declare a metabolite works as a source metabolite of a target metabolite. Rule 2 is about correspondences between steps constituting a pathway and enzymes responsible for those steps. As for Rule 2, the following two types of exceptions can be considered theoretically.

- X: It happens that multiple steps are catalyzed by a single enzyme.
- Y: It happens that a single step is catalyzed by different enzymes.

X is classified into the following three.

- X1: It happens that a single enzyme catalyzes both a reaction and its reverse reaction each of which is a step on the pathway.
- X2: It happens that a single enzyme catalyzes the same reaction multiple times as different steps on the pathway.
- X3: It happens that a single enzyme catalyzes different reactions each of which is a step on the pathway.

On pathways where X1 or X2 occurs, the same metabolite may appear multiple times. Those pathways include pathways which have not been considered in previous studies as described in Introduction.
Table 4  Examples of pathways from C1 of D-glucose to CO₂ selected using Filter 3. Letters in parentheses, 'c' and 'm' indicate compartmentation of the metabolites: c, cytosolic; m, mitochondrial. '>' and '<' indicate direction of reactions or transport processes. GA3PD: glyceraldehyde-3-phosphate dehydrogenase

| Pathway No. 9 | Pathway No. 2 |
|---------------|---------------|
| [1] C1 D-Glucose | [1] C1 D-Glucose |
| > Hexokinase | (c) > Hexokinase |
| [2] C1 D-Glucose 6-phosphate | (c) [2] C1 D-Glucose 6-phosphate |
| > Phosphohexose isomerase | (c) > Phosphohexose isomerase |
| [3] C1 D-Fructose 6-phosphate | (c) [3] C1 D-Fructose 6-phosphate |
| < Transketolase | (c) > Phosphofructokinase |
| [4] C1 D-Xylose 5-phosphate | (c) [4] C1 D-Fructose 1,6-bisphosphate |
| < Ribulose-5-phosphate 3-epimerase | (c) > Aldolase |
| [5] C1 D-Ribulose 5-phosphate | (c) [5] C1 Dihydroxyacetone phosphate |
| > Ribose-5-phosphate ketoisomerase | (c) > Triose phosphate isomerase |
| [6] C1 D-Ribose 5-phosphate | (c) [6] C3 D-Glyceraldehyde 3-phosphate |
| > Transketolase | (c) > GA3PD |
| [7] C3 Sedoheptulose 7-phosphate | (c) [7] C3 1,3-Bisphospho-D-glycerate |
| > Transaldolase | (c) > Phosphoglycerate kinase |
| [8] C3 D-Fructose 6-phosphate | (c) [8] C3 3-Phospho-D-glycerate |
| > Phosphofructokinase | (c) > Phosphoglycerate mutase |
| [9] C3 D-Fructose 1,6-bisphosphate | (c) [9] C3 2-Phospho-D-glycerate |
| > Aldolase | (c) > Enolase |
| [10] C3 Dihydroxyacetone phosphate | (c) [10] C3 Phosphoenolpyruvate |
| > Triose phosphate isomerase | (c) > Pyruvate kinase |
| [11] C1 D-Glyceraldehyde 3-phosphate | (c) [11] C3 Pyruvate |
| > GA3PD | (c) > Pyruvate carboxylase |
| [12] C1 1,3-Bisphospho-D-glycerate | (c) [12] C3 Oxaloacetate |
| > Phosphoglycerate kinase | (m) > Citrate synthase |
| [13] C1 3-Phospho-D-glycerate | (c) [13] C2 Citrate |
| > Phosphoglycerate mutase | (m) > Aconitase |
| [14] C1 2-Phospho-D-glycerate | (c) [14] C2 cis-Aconitate |
| > Enolase | (m) > Aconitase |
| [15] C1 Phosphoenolpyruvate | (c) [15] C2 Isocitrate |
| > Pyruvate kinase | (m) > Isocitrate dehydrogenase |
| [16] C1 Pyruvate | (c, m) [16] C2 2-Oxoglutarate |
| > Pyruvate dehydrogenase complex | (m) > 2-Oxoglutarate dehydrogenase complex |
| [17] C1 CO₂ including HCO₃⁻ | (c, m) [17] C1 Succinyl-CoA |
| (c, m) > Succinyl-CoA synthetase |
| [18] C1 Succinate | (m) > Succinate dehydrogenase |
| [19] C1 Fumarate | (m) > Fumarase |
| > Fumarase | (m) | |
| [20] C1 L-Malate | (m) | |
| > Malate dehydrogenase | (m) | |
| [21] C1 Oxaloacetate | (m) | |
| > Citrate synthase | (m) | |
| [22] C6 Citrate | (m) | |
| > Aconitase | (m) | |
| [23] C6 cis-Aconitate | (m) | |
| > Aconitase | (m) | |
| [24] C6 Isocitrate | (m) | |
| > Isocitrate dehydrogenase | (m) | |
| [25] C1 CO₂ including HCO₃⁻ | (c, m) | |
Table 5  Examples of pathways from C3 of D-glucose to CO₂ selected using Filter 3. Letters in parentheses, 'c' and 'm' indicate compartmentation of the metabolites: c, cytosolic; m, mitochondrial. '>' and '<' indicate direction of reactions or transport processes. GA3PD: glyceraldehyde-3-phosphate dehydrogenase

| Pathway No. 5          | Pathway No. 20          |
|------------------------|------------------------|
| [1] C3 D-Glucose       | [1] C3 D-Glucose       |
| > Hexokinase           | > Hexokinase           |
| [2] C3 D-Glucose 6-phosphate | > Glucose-6-phosphate dehydrogenase |
| > Glucose-6-phosphate dehydrogenase | |
| [3] C3 6-Phospho-D-glucono-1,5-lactone | > 6-Phosphogluconolactonase |
| > 6-Phosphogluconolactonase | |
| [4] C3 6-Phospho-D-gluconate | > 6-Phosphogluconate dehydrogenase |
| > 6-Phosphogluconate dehydrogenase | |
| [5] C2 D-Ribulose 5-phosphate | > Ribulose-5-phosphate 3-epimerase |
| > Ribulose-5-phosphate 3-epimerase | |
| [6] C2 D-Xylose 5-phosphate | > Transketolase |
| > Transketolase | |
| [7] C2 D-Fructose 6-phosphate | > Sedoheptulose 7-phosphate |
| < Phosphohexose isomerase | > Transaldolase |
| [8] C2 D-Glucose 6-phosphate | > Phosphohexose isomerase |
| > Glucose-6-phosphate dehydrogenase | |
| [9] C2 D-Glucose 6-phosphate | > Glucose-6-phosphate dehydrogenase |
| > 6-Phosphogluconolactonase | |
| [10] C2 6-Phospho-D-gluconate | > 6-Phosphogluconolactonase |
| > 6-Phosphogluconolactonase | |
| [11] C1 D-Ribulose 5-phosphate | > 6-Phosphoglucone dehydrogenase |
| > Ribulose-5-phosphate 3-epimerase | |
| [12] C1 D-Xylose 5-phosphate | > Transketolase |
| > Transketolase | |
| [13] C1 D-Fructose 6-phosphate | > Sedoheptulose 7-phosphate |
| < Phosphohexose isomerase | > Transaldolase |
| [14] C1 D-Glucose 6-phosphate | > Glucose-6-phosphate dehydrogenase |
| > Glucose-6-phosphate dehydrogenase | |
| [15] C1 6-Phospho-D-glucono-1,5-lactone | > 6-Phosphogluconolactonase |
| > 6-Phosphogluconolactonase | |
| [16] C1 6-Phospho-D-gluconate | > 6-Phosphogluconate dehydrogenase |
| > 6-Phosphogluconate dehydrogenase | |
| [17] C1 CO₂ including HCO₃⁻ | > Dihydroxyacetone phosphate |
| (c, m) | > Triose phosphate isomerase |
| [18] C1 D-Glyceraldehyde 3-phosphate | > GA3PD |
| > GA3PD | |
| [19] C1 1,3-Bisphospho-D-glycerate | > Phosphoglycerate kinase |
| > Phosphoglycerate kinase | |
| [20] C1 3-Phospho-D-glycerate | > Phosphoglycerate mutase |
| > Phosphoglycerate mutase | |
| [21] C1 2-Phospho-D-glycerate | > Enolase |
| > Enolase | |
| [22] C1 Phosphoenolpyruvate | > Pyruvate kinase |
| > Pyruvate kinase | |
| [23] C1 Pyruvate | > Pyruvate dehydrogenase complex |
| (c, m) | |
| [24] C1 CO₂ including HCO₃⁻ | > Pyruvate dehydrogenase complex |
| (c, m) | |

By Filter 1, pathways where X1, an exception concerning Rule 2, occurs are excluded from pathways keeping Rule 1. On pathways where X1 occurs, a reaction is canceled by its reverse reaction. This means that those pathways are partially not purposive. Further, it can be postulated that apparent substantial function of a pathway is to result in stoichiometric balance of the entire path-
Table 6  An example of a pair of pathways from C5 and C6 of D-glucose to CO₂ selected using Filter 3. A pathway from C5 is shown left and a pathway from C6 right. Letters in parentheses, 'c' and 'm' indicate compartmentation of the metabolites: c, cytosolic; m, mitochondrial. '>' and '<' indicate direction of reactions or transport processes. GA3PD: glyceraldehyde-3-phosphate dehydrogenase

| Pathway No. 30                                                                 | Pathway No. 17                                                                 |
|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| [1] C5 D-Glucose                                                              | [1] C6 D-Glucose                                                              |
| > Hexokinase                                                                 | > Hexokinase                                                                 |
| [2] C5 D-Glucose 6-phosphate                                                | [2] C6 D-Glucose 6-phosphate                                                |
| > Phosphohexose isomerase                                                   | > Phosphohexose isomerase                                                   |
| [3] C5 D-Fructose 6-phosphate                                               | [3] C6 D-Fructose 6-phosphate                                               |
| > Phosphofructokinase                                                       | > Phosphofructokinase                                                       |
| [4] C5 D-Fructose 1,6-bisphosphate                                         | [4] C6 D-Fructose 1,6-bisphosphate                                         |
| > Aldolase                                                                  | > Aldolase                                                                  |
| [5] C2 D-Glyceraldehyde 3-phosphate                                         | [5] C3 D-Glyceraldehyde 3-phosphate                                         |
| > GA3PD                                                                     | > GA3PD                                                                     |
| [6] C2 1,3-Bisphospho-D-glycerate                                           | [6] C3 1,3-Bisphospho-D-glycerate                                           |
| > Phosphoglycerate kinase                                                   | > Phosphoglycerate kinase                                                   |
| [7] C2 3-Phospho-D-glycerate                                                | [7] C3 3-Phospho-D-glycerate                                                |
| > Phosphoglycerate mutase                                                   | > Phosphoglycerate mutase                                                   |
| [8] C2 2-Phospho-D-glycerate                                                | [8] C3 2-Phospho-D-glycerate                                                |
| > Enolase                                                                   | > Enolase                                                                   |
| [9] C2 Phosphoenolpyruvate                                                   | [9] C3 Phosphoenolpyruvate                                                   |
| > Pyruvate kinase                                                           | > Pyruvate kinase                                                           |
| [10] C2 Pyruvate                                                            | [10] C3 Pyruvate                                                            |
| > Pyruvate dehydrogenase complex                                            | > Pyruvate carboxylase                                                      |
| [11] C1 Acetyl-CoA                                                           | [11] C3 Oxaloacetate                                                        |
| > Citrate synthase                                                          | > Citrate synthase                                                          |
| [12] C5 Citrate                                                             | [12] C2 Citrate                                                             |
| > Aconitase                                                                 | > Aconitase                                                                 |
| [13] C5 cis-Aconitase                                                        | [13] C2 cis-Aconitase                                                        |
| > Aconitase                                                                 | > Aconitase                                                                 |
| [14] C5 Isocitrate                                                           | [14] C2 Isocitrate                                                           |
| > Isocitrate dehydrogenase                                                  | > Isocitrate dehydrogenase                                                  |
| [15] C5 2-Oxoglutarate                                                     | [15] C2 2-Oxoglutarate                                                     |
| > 2-Oxoglutarate dehydrogenase complex                                      | > 2-Oxoglutarate dehydrogenase complex                                      |
| [16] C4 Succinyl-CoA                                                         | [16] C1 Succinyl-CoA                                                         |
| > Succinyl-CoA synthetase                                                    | > Succinyl-CoA synthetase                                                    |
| [17] C1 Succinate                                                           | [17] C1 Succinate                                                           |
| > Succinate dehydrogenase                                                   | > Succinate dehydrogenase                                                   |
| [18] C1 Fumarate                                                            | [18] C1 Fumarate                                                            |
| > Fumarase                                                                  | > Fumarase                                                                  |
| [19] C1 L-Malate                                                            | [19] C1 L-Malate                                                            |
| > Malate dehydrogenase                                                      | > Malate dehydrogenase                                                      |
| [20] C1 Oxaloacetate                                                        | [20] C1 Oxaloacetate                                                        |
| > Citrate synthase                                                          | > Citrate synthase                                                          |
| [21] C6 Citrate                                                             | [21] C6 Citrate                                                             |
| > Aconitase                                                                 | > Aconitase                                                                 |
| [22] C6 cis-Aconitase                                                        | [22] C6 cis-Aconitase                                                        |
| > Aconitase                                                                 | > Aconitase                                                                 |
| [23] C6 Isocitrate                                                           | [23] C6 Isocitrate                                                           |
| > Isocitrate dehydrogenase                                                  | > Isocitrate dehydrogenase                                                  |
| [24] C1 CO₂ including HCO₃                                                  | [24] C1 CO₂ including HCO₃                                                  |

way which means formation of the target metabolite using the source metabolite. Combined action of a reaction and its reverse reaction which coexist on a pathway does not affect final stoichiometric balance of the pathway. Therefore, this pair of a reaction and its reverse reaction can be omitted as meaningless from the entire pathway if keeping Rule 1 is not considered. If the enzyme
The network structure of the metabolic pathway, "M1 \rightarrow M2 \rightarrow M3 \rightarrow M4 \rightarrow M5 \rightarrow M3 \rightarrow M2 \rightarrow M6", which is called Pw1. M1, M2, M3, M4, M5 and M6 are metabolites.

The network structure of the metabolic pathway, "M1 \rightarrow M2 \rightarrow M3 \rightarrow M4 \rightarrow M5 \rightarrow M3 \rightarrow M2 \rightarrow M6", which is called Pw1. M1, M2, M3, M4, M5 and M6 are metabolites.

Fig. 2  The network structure of the metabolic pathway, “M1 \rightarrow M2 \rightarrow M3 \rightarrow M4 \rightarrow M5 \rightarrow M3 \rightarrow M2 \rightarrow M6”, which is called Pw1. M1, M2, M3, M4, M5 and M6 are metabolites.

In conclusion, the problem of single-atom tracing that it produces too many pathways to be examined was overcome by pathway selection or characterization based on the criteria about coexistence of reactions and both linear and cycle-containing pathways which have possible biochemical meaning were found successfully. Single-atom tracing followed by the present pathway selection method would contribute to proposing pathways, especially ones containing cycling, which should have their biochemical meaning examined in wet experiments.

3. Materials and Methods

3.1 Model Network of Carbohydrate Metabolism

The model carbohydrate metabolic network used in the present study is a revision of the network reported previously [11], where reversibility of reactions were modified to conform more to general text-book description of carbohydrate metabolism. The network used was composed of reactions for glycolysis, oxidative decarboxylation of pyruvate, CAC, PPP and gluconeogenesis as described in Introduction. The model network consisted of 46 metabolites and 35 reactions or membrane transport. Individual metabolite and reaction numbers were assigned in an indexing process as described below. As for several metabolites and reactions, the number for mitochondrial occurrence was differentiated from the number for the cytosolic occurrence. For details, please see Fig. 1. Tables 7, 8 and 9, and Supplementary Materials. Table 7 describes indexing of metabolites of glucose and carbon atoms. Table 8 describes indexing of currency metabolites. Table 9 describes indexing of processes which include reactions and cur or such coexistence is exceptional, if any. Thus, use of Filter 2 in the present study is explained. Here, Rule 3, which should be kept in ‘typical pathways’, is set as follows:

- Rule 3: Any two reactions on a pathway are not regulated in a reciprocal manner.

It happens that cycles such as a cycle resulting from coexistence of phosphofructokinase and fructose-1,6-bisphosphatase reactions are called ‘substrate cycle’ instead of ‘futile cycle’, where it is described that it seems likely that substrate cycles are biologically important in amplifying metabolic signals and that substrate cycles have a potential biological role in generation of heat produced by the hydrolysis of ATP [14]. Therefore, as far as pathways excluded by Filter 2 keep Rule 1, it may not be concluded that they are biochemically meaningless. However, they remain to be exceptional because coexistence of reactions regulated in a reciprocal manner is against the purpose of regulation or not purposive. There seems to be a sound rationale of distinguishing pathways in the present model network by Filter 2 and use of Filter 2 can be extended to use of appropriate Filter 2 in other metabolic networks. The reasons why pathways excluded by Filters 1 and 2 are exceptional have been explained here. Filter 3 is the combination of Filters 1 and 2 and there seems to be a sound rationale of distinguishing pathways by each of Filters 1 and 2 as described above. Thus, there seems to be a sound rationale of distinguishing pathways by Filter 3. Pathways selected by Filter 3 are pathways which are judged to be not exceptional by both Filters 1 and 2. In this sense they are possibly biochemically meaningful.

In conclusion, the problem of single-atom tracing that it produces too many pathways to be examined was overcome by pathway selection or characterization based on the criteria about coexistence of reactions and both linear and cycle-containing pathways which have possible biochemical meaning were found successfully. Single-atom tracing followed by the present pathway selection method would contribute to proposing pathways, especially ones containing cycling, which should have their biochemical meaning examined in wet experiments.
### Table 7  List of metabolites of glucose and their carbon species.\(^a\)

| No. and Name (compartment) | Index of carbon atoms |
|-----------------------------|-----------------------|
| 1  D-Glucose               | (c) (1,1) (1,2) (1,3) (1,4) (1,5) (1,6) |
| 2  D-Glucose 6-phosphate   | (c) (2,1) (2,2) (2,3) (2,4) (2,5) (2,6) |
| 3  D-Fructose 6-phosphate  | (c) (3,1) (3,2) (3,3) (3,4) (3,5) (3,6) |
| 4  D-Fructose 1,6-bisphosphate | (c) (4,1) (4,2) (4,3) (4,4) (4,5) (4,6) |
| 5  D-Glyceraldehyde 3-phosphate | (c) (5,1) (5,2) (5,3) |
| 6  Dihydroxyacetone phosphate | (c) (6,1) (6,2) (6,3) |
| 7  1,3-Bisphospho-D-glycerate | (c) (7,1) (7,2) (7,3) |
| 8  3- Phospho-D-glycerate  | (c) (8,1) (8,2) (8,3) |
| 9  2- Phospho-D-glycerate  | (c) (9,1) (9,2) (9,3) |
| 10 Phosphoenolpyruvate     | (c) (10,1) (10,2) (10,3) |
| 11 Pyruvate                | (c,m) (11,1) (11,2) (11,3) |
| 12 L-Lactate               | (c) (12,1) (12,2) (12,3) |
| 13 Acetyl-CoA              | (m) (13,1) (13,2) |
| 14 Oxaloacetate            | (m) (14,1) (14,2) (14,3) (14,4) |
| 15 CO\(_2\) including HCO\(_3\) \(^-\) | (c,m) (15,1) |
| 16 Citrate                 | (m) (16,1) (16,2) (16,3) (16,4) (16,5) (16,6) |
| 17 cis-Aconitate           | (m) (17,1) (17,2) (17,3) (17,4) (17,5) (17,6) |
| 18 Isocitrate              | (m) (18,1) (18,2) (18,3) (18,4) (18,5) (18,6) |
| 19 2-Oxoglutarate         | (m) (19,1) (19,2) (19,3) (19,4) (19,5) |
| 20 Succinyl-CoA            | (m) (20,1) (20,2) (20,3) (20,4) |
| 21 Succinate               | (m) (21,1) (21,2) |
| 22 Fumarate                | (m) (22,1) (22,2) |
| 23 L-Malate                | (m) (23,1) (23,2) (23,3) (23,4) |
| 24 L-Malate                | (c) (24,1) (24,2) (24,3) (24,4) |
| 25 Oxaloacetate            | (c) (25,1) (25,2) (25,3) (25,4) |
| 26 6-Phospho-D-glucono-1,5-lactone | (c) (26,1) (26,2) (26,3) (26,4) (26,5) (26,6) |
| 27 6-Phospho-D-gluconate  | (c) (27,1) (27,2) (27,3) (27,4) (27,5) (27,6) |
| 28 D-Ribulose 5-phosphate | (c) (28,1) (28,2) (28,3) (28,4) (28,5) |
| 29 D-Ribose 5-phosphate   | (c) (29,1) (29,2) (29,3) (29,4) (29,5) |
| 30 Sedoheptulose 7-phosphate | (c) (30,1) (30,2) (30,3) (30,4) (30,5) (30,6) (30,7) |
| 31 D-Erythrose 4-phosphate | (c) (31,1) (31,2) (31,3) (31,4) |
| 32 D-Xylosulose 5-phosphate | (c) (32,1) (32,2) (32,3) (32,4) (32,5) |

\(^a\) Modified and reproduced, with permission, from http://www.cc.okayama-u.ac.jp/~jo25/imac/btk2006. Letters in parentheses, "c" and "m" indicate compartmentation of the metabolites: c, cytosolic; m, mitochondrial.

### Table 8  List of currency metabolites.\(^a\)

| No. and Name (compartment) | No. and Name (compartment) |
|-----------------------------|-----------------------------|
| 33  ATP (c,m)               | 40  NADH (c,m) |
| 34  ADP (c,m)               | 41  NADP\(^*\) (c,m) |
| 35  GTP (c,m)               | 42  NADPH (c,m) |
| 36  GDP (c,m)               | 43  CoA (c,m) |
| 37  FAD (c,m)               | 44  Phosphate (c,m) |
| 38  FADH\(_2\) (c,m)        | 45  H\(_2\)O (c,m) |
| 39  NAD\(^*\) (c,m)         | 46  H\(^+\) (c,m) |

\(^a\) Modified and reproduced, with permission, from http://www.cc.okayama-u.ac.jp/~jo25/imac/btk2006. As for letters in parentheses, see Table 7. Carbon atoms of currency metabolites were not numbered.
Table 9  List of processes (reactions and transport processes).a

| No. and Name of       | Processes (compartment) | Substrate                          | Product                                      |
|-----------------------|-------------------------|------------------------------------|----------------------------------------------|
| 1 Hexokinase          | (c)                     | D-Glucose + ATP                    | D-Glucose 6-P + ADP                          |
| 2 Phosphohexose isomerase | (c)                           | D-Glucose 6-P                      | D-Fructose 6-P                               |
| 3 Phosphofructokinase | (c)                     | D-Fructose 6-P + ATP               | D-Fructose 1,6-P + ADP                       |
| 4 Aldolase            | (c)                     | D-Fructose 1,6-P₂                 | Dihydroxyacetone P + Glyceroldehyde 3-P      |
| 5 Triose phosphate isomerase | (c)                         | Dihydroxyacetone P                | Glyceroldehyde 3-P                           |
| 6 Glyceroldehyde-3-P dehydrogenase | (c)                     | Glyceroldehyde 3-P + P₁           | 1,3-Bisphospho-D-glycerate + NADH + H⁺       |
| 7 Phosphoglycerate kinase | (c)                          | 1,3-Bisphospho-D-glycerate + ADP  | 3-Phospho-D-glycerate + ATP                  |
| 8 Phosphoglycerate mutase | (c)                           | 3-Phospho-D-glycerate              | 2-Phospho-D-glycerate                        |
| 9 Enolase             | (c)                     | 2-Phospho-D-glycerate              | Phosphoenolpyruvate + H₂O                    |
| 10 Pyruvate kinase    | (c)                     | Phosphoenolpyruvate + ADP          | Pyruvate + ATP                               |
| 11 L-Lactate dehydrogenase | (c)                          | Pyruvate + NADH + H⁺              | Lactate + NADH⁺                              |
| 12 Pyruvate dehydrogenase complex | (m)                     | Pyruvate + CoA + NAD⁺            | Acetyl-CoA + CO₂ + NADH + H⁺                 |
| 13 Pyruvate carboxylase | (m)                       | Pyruvate + HCO₃⁻ + ATP           | Oxaloacetate + ADP + P₁                      |
| 14 Citrate synthase   | (m)                     | Acetyl-CoA + H₂O                   | Citrate + CoA + Oxaloacetate                |
| 15 Aconitase          | (m)                     | Citrate                            | cis-Aconitate + H₂O                          |
| 16 Aconitase          | (m)                     | cis-Aconitate + H₂O                | Isocitrate                                   |
| 17 Isocitrate dehydrogenase | (m)                           | Isocitrate + NAD⁺                 | 2-Oxoglutarate + CO₂ + NADH + H⁺             |
| 18 2-Oxoglutarate dehydrogenase complex | (m)                     | 2-Oxoglutarate + CoA + NAD⁺    | Succinyl-CoA + CO₂ + NADH + H⁺               |
| 19 Succinyl-CoA synthetase | (m)                        | Succinyl-CoA + GDP + P₁           | Succinate + CoA + GTP                        |
| 20 Succinate dehydrogenase | (m)                       | Succinate + FAD                   | Fumarate + FADH₂                            |
| 21 Fumarase           | (m)                     | Fumarate + H₂O                     | Malate                                       |
| 22 Malate dehydrogenase | (m)                          | Malate + NAD⁺                     | Oxaloacetate + NADH + H⁺                     |
| 23 Glucose-6-phosphate dehydrogenase | (c)                       | D-Glucose 6-P + NADP⁺           | D-Glucono-1,5-lactone 6-P + NADPH + H⁺       |
| 24 6-Phosphogluconolactonase | (c)                        | D-Glucono-1,5-lactone 6-P + H₂O   | 6-Phospho-D-gluconate                        |
| 25 6-Phosphogluconate dehydrogenase | (c)                          | 6-Phospho-D-gluconate + NADP⁺   | D-Ribulose 5-P + CO₂ + NADPH + H⁺             |
| 26 Ribulose-5-phosphate 3-epimerase | (c)                          | D-Ribulose 5-P                    | D-Xylose 5-P                                 |
| 27 Ribose-5-phosphate ketoisomerase | (c)                          | D-Ribulose 5-P                    | D-Ribose 5-P                                 |
| 28 Transketolase      | (c)                     | D-Ribose 5-P + D-Xylose 5-P       | Sedoheptulose 7-P + Glyceroldehyde 3-P       |
| 29 Transaldolase      | (c)                     | Sedoheptulose 7-P + Glyceroldehyde 3-P | D-Erythrose 4-P + D-Fructose 6-P              |
| 30 Transketolase      | (c)                     | D-Erythrose 4-P                   | D-Fructose 6-P + Glyceroldehyde 3-P          |
| 31 Transport          | -                       | Malate mitochondrial             | Malate cytosolic                             |
| 32 Malate dehydrogenase | (c)                          | Malate + NAD⁺                     | Oxaloacetate + NADH + H⁺                     |
| 33 Phosphoenolpyruvate carboxykinase | (c)                          | Oxaloacetate + GTP                | Phosphoenolpyruvate + CO₂ + GDP              |
| 34 Glucose-6-phosphatase | (c)                        | D-Glucose 6-P + H₂O               | D-Glucose + P₁                               |
| 35 D-Fructose-1,6-bisphosphatase | (c)                          | D-Fructose 1,6-P₂ + H₂O          | D-Fructose 6-P + P₁                          |

a Modified and reproduced, with permission, from http://www.cc.okayama-u.ac.jp/~jo25/imac/bsk2006. As for letters in parentheses, see Table 7. The following processes were treated as reversible: 2,4-9,11,15-17,19-22,23,26-30,31,32.
transport processes. Information described in Fig. 1 and Tables 7, 8 and 9 is also included in Supplementary Materials.

3.2 Indexing of Metabolites, Carbon Atoms and Processes

The procedure reported previously was used [11]. In the following explanation, ‘process’ means reaction or membrane transport. Each metabolite in the model network was given a metabolite number. Thirty-two of the 46 metabolites were metabolites of glucose, each carbon atom of which was given an intra-metabolite carbon number according to the IUPAC nomenclature and indexed as a pair of the corresponding metabolite number and the carbon number. As for the acetyl portion of acetyl-CoA, its carbonyl and methyl carbons were given intra-metabolite carbon numbers 1 and 2, respectively. As for the succinyl portion of succinyl-CoA, its carbon attached to the sulfur atom in thioester linkage was given an intra-metabolite carbon number 1. As for fumarate and succinate, symmetrical molecules, each of a pair of symmetric carbon atoms was given the same carbon number. Fourteen of the 46 metabolites were currency metabolites, which included ATP, NADH, CoA, H2O and so on but did not include acetyl-CoA and succinyl-CoA in the present article. As for the currency metabolites, their carbon atoms were not indexed. Each process was given a process number. The following is a matter related to computation. Assuming that ‘m’ is a metabolite number and that ‘c’ is an intra-metabolite atom number, each atom is indexed by a pair of numbers (m, c). During computation, an atom’s index number is treated as a complex number, m + ci. In the present article, (m, c) also means the complex number, m + ci. Assuming that ‘p’ is a process number, the reverse process of ‘p’ is expressed as ‘-p’.

3.3 Description of Inter-metabolite Atom-level Connectivity in the Metabolic Network

Connectivity matrix (CM) is a general format to describe connectivity in a network, where each of nodes and edges are expressed as a row vector [11]. In the present study, a CM of three columns was prepared to describe all the possible inter-metabolite atom-level connectivities between metabolites of glucose in the above model network of carbohydrate metabolism as described previously [11]. Assuming that ‘m1’ and ‘m2’ are metabolites, that (m1, c1) and (m2, c2) are atom indices and that ‘p1’ is a process number, the row of CM [(m1, c1), (m2, c2), p1] indicates that the atom (m1, c1) in metabolite m1 is converted to atom (m2, c2) in another metabolite m2 through the process p1. In other words, column 3 indicates the relevant process, column 1 indicates an atom in the substrate of the process and column 2 indicates the corresponding atom in the product of the process. The CM obtained had 291 rows. The CM can be seen in btk_connectivity_matrix, a text file contained in Supplementary Materials.

Figure 3 shows a portion of the CM of the model network. In Fig. 3, metabolite numbers 28 and 29 indicate ribulose 5-phosphate and ribose 5-phosphate, respectively. Process number 27 indicates ribose-5-phosphate ketoisomerase reaction. The upper five rows represent conversion of ribulose 5-phosphate to ribose 5-phosphate and the lower three rows represent the reverse reaction, conversion of ribose 5-phosphate to ribulose 5-phosphate.

3.4 Finding all of the Pathways from a Specific Atom to Another Specific Atom Using CM

The procedure reported previously was used [11]. Briefly, all the pathways from a specific atom, ‘s’, to another specific atom, ‘d’, in a given network are formed by the conjugation of two-atom sequences expressed as rows of a CM. Row number sequences of CM corresponding to the pathways are recorded and used for later calculation of metabolite and process sequences. In this procedure, elongation of atom sequences is conducted so that one atom species appears only once in a sequence.

3.5 Selection of Pathways

Assume that p1 and p2 are numbers indicating processes and that processes p1 and p2 cannot exist on the same pathway because of biochemical reasons described under Results and Discussion. I call such a pair of process numbers a not-allowed pair. Information of all the not-allowed pairs of process numbers is stored as a two-column matrix, M, each row of which is composed of two numbers forming a not-allowed pair. After computing pathways as above, process sequences corresponding to each pathway are calculated from the recorded row number sequences of CM. If both numbers forming any not-allowed pair are encountered in a process sequence, the pathway corresponding to the process sequence is excluded. Unless both numbers forming any not-allowed pair are encountered in a process sequence, the pathway is selected as possibly biochemically meaningful.

3.6 Computation

As described above, information necessary for single-atom tracing and pathway selection is expressed as matrices. Using this information, computation in the present study was performed on GNU Octave and Matlab. Information about the m-files used is given in Supplementary Materials.

Supplementary Materials:
At http://www.cc.okayama-u.ac.jp/~jo25/imac/TBIO2018a, the following are available online, 1: README, 2: model_network_metabolites.pdf, 3: model_network_processes.pdf, 4: model_network_map.pdf, 5: btk_connectivity_matrix, 6: btk_connectivity_matrix.mat, 7: comp_name.csv, 8: rxn_name.csv, 9: btk_route.m,
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