Metabolic Connection between Oxalacetate and Glutamate in *Rhodospirillum rubrum*

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In studies of the acetate metabolism of *Rhodospirillum rubrum* Cutinelli *et al.*[1] investigated the incorporation of acetate and CO_2 into various amino acids, *e.g.* alanine, aspartic acid, and glutamic acid. It was found that the C-structure of alanine arose through a carboxylation of acetate and that the α and β carbon atoms of aspartate mainly originated from the carboxyl and the methyl groups of acetate, respectively, whereas the carboxyl groups of aspartate were derived from CO_2, with oxalacetate probably being a likely intermediate: The isotope distribution in glutamate indicated a formation of this compound by a condensation of acetate with oxalacetate, the latter being derived *via* the citric acid cycle or by stepwise carboxylation of acetate. In their experiments acetate and CO_2 were the sole sources of carbon in the growth medium.

In a later publication on the same subject Hoare [2] reported an isotope distribution in glutamate from acetate-1-^{14}C and acetate-2-^{14}C which could not be explained in terms of citric acid cycle activity because the carboxyl group of acetate appeared in C-2 and C-5 of glutamate and the methyl group in C-3 and C-4. These results certainly would lead to the consequence that a novel, and interesting variant of glutamate synthesis might be involved, one possibility being a carboxylation of succinylcoenzyme A, in analogy with the ferredoxin dependent pyruvate synthesis from acetylcoenzyme A and CO_2.[3] In order to test this hypothesis of a succinylcoenzyme A carboxylation we have exposed *R. rubrum* cells grown on acetate-bicarbonate medium to succinate-1,4-^{14}C for 20 min. If the above hypothesis were valid the ketoglutarate formed should have a predominant ^{14}C-labelling in positions 2 and 5. Our analysis of the labelling pattern of glutamic acid isolated from this experiment showed, however, that practically all ^{14}C from the carboxyl groups of succinate was localized in C-1 of glutamate. The only possible conclusion from this result is that succinate carboxylation is nil, and that the succinate has passed through the citric acid cycle in the ordinary way thus generating oxalacetate-1,4-^{14}C in passing as coupling partner for unlabelled acetylcoenzyme A.

Since the experiments of Hoare were performed with cells grown on malate medium, we repeated the procedure under these conditions using acetate-1-^{14}C and unlabelled CO_2, with exposure of the cells to the labelled acetate for 4 min. Glutamic acid (6.1×10^4 cpm/mmole) was isolated and degraded by ninhydrin decarboxylation of C-1 and a Schmidt decarboxylation of C-5.[4] The radioactivity of C-5 was 5.2×10^4 cpm/mmole and that of C-1 0.3×10^4 cpm/mmole. Thus, 85 % of the total radioactivity of glutamic acid were localized to the γ-carboxyl group and about 5 % to the α-carboxyl group and only about 10 % in the remaining C atoms. The labelling in C-1 is easily explained as the result of re-cycling of acetate-1-^{14}C in the system. The insignificant labelling in positions 2, 3, and 4 could be the result of the experimental conditions, where intracellulary stored unlabelled malate caused a dilution of any oxalacetate that could be labelled from double carboxylation of acetate *via* pyruvate. However, under conditions where acetate and bicarbonate are the sole sources of carbon the radioactivity of acetate-1-^{14}C should substantially appear in position 2 of oxalacetate and consequently in C-3 of glutamic acid. The earlier experiments of Cutinelli *et al.* carried out over a ten hour period of growth are in agreement with this view.

It thus appears that the glutamic acid synthesis in *R. rubrum* follows the regular way from ketoglutarate derived from the citric acid cycle but that the oxalacetate, besides being a cycle intermediate, is continuously formed in considerable amounts from double carboxylation of acetate. The contradictory results of Hoare emanate from an ambiguous degradation series in which C-2, C-5 and C-3, C-4 of glutamic acid are randominized during the degradation reactions involving decarboxylation of symmetrical succinic acid representing C_5C_2-C_3C_4-C_3C_4-C_2C_5 of glutamate.

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2. Hoare, D. S. *Biochem. J.* 87 (1963) 284.
3. Buchanan, B. B., Bachofen, R. and Arnon, D. J. *Proc. Natl. Acad. Sci. U. S.* 52 (1964) 239.
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Ozonolysis of Phenols

IV. 2,3-Dihydroxynaphthalene

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In connection with our studies on the ozonolysis of phenols,[1-3] 2,3-dihydroxynaphthalene has been ozonised in ethyl acetate at 3–4°C. The ozonolysis of *o*-diphenols is of interest in view of the remarkable results of Woodward with the ozonolysis of a veratrol derivative as a step in his strychnine synthesis.[4] Catechol has been ozonized by one of the present authors,[2] but yielded small fragments only, resulting from a complete destruction of the aromatic ring.

Dihydroxynaphthalene readily absorbed ozone, at least five moles being taken up. This, however, leads to a far-reaching degradation of the naphthalene ring-system, and the present investigation is concerned with the reaction with two moles of ozone only.

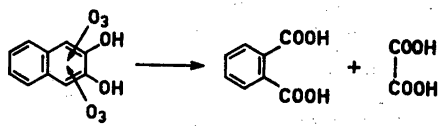
The ozonation could be followed visually as a brownish-yellow colour developed in the reaction mixture. Maximum intensity of the coloration was obtained after the absorption of one mole of ozone. Thereafter it diminished until the solution was colourless after the absorption of two moles. At this point the ozonation mixture contained active oxygen in an amount roughly corresponding to half the absorbed ozone. After ozonation the solution was treated with water until the active oxygen had disappeared. The quantitative determinations of reaction products are summarised in Table 1.

Table 1. Reaction products from ozonolysis of 2,3-dihydroxynaphthalene with 2 moles of ozone.

Reaction product	Moles	% recovered C
Carbon monoxide	0.03	0.3
Carbon dioxide	0.83	8.3
Formic acid	0.14	1.4
Oxalic acid	0.35	7.0
Phthalic acid	0.93	74.2

It will be seen that about 91 % of the initial carbon content has been recovered in the reaction products. The deficit is partly due to some intractable, dark tarry matter, which defied purification and identification.

Formation of phthalic acid can be caused by ozone-attack on the bonds adjacent to the hydroxyl groups with rearrangement of the intermediate zwitterion or peroxide:



This reaction requires equimolecular amounts of phthalic and oxalic acids in the products, while the found ratio was 0.93:0.35. Obviously a major part of phthalic acid has been formed through a different reaction path. Evidence for this is found in the fact that, if hydrolysis of the ozonation mixture was omitted, phthalaldehydic acid was isolated in an amount of 0.49 mole per mole of starting material. The same mode of attack as before can be envisaged, but followed by a different rearrangement: