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# Biochemistry of Nitrate Respiration in *Pseudomonas stutzeri*

## I. Aerobic and Nitrate Respiration Routes of Carbohydrate Catabolism

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ABSTRACT

SPANGLER, W. J. (Oregon State University, Corvallis), AND C. M. GILMOUR. Biochemistry of nitrate respiration in *Pseudomonas stutzeri*. I. Aerobic and nitrate respiration routes of carbohydrate catabolism. J. Bacteriol. **91**:245–250. 1966.—The metabolic pathways of glucose catabolism were studied in *Pseudomonas stutzeri* under aerobic conditions and under conditions of nitrate respiration. Studies on both glucose and gluconate catabolism, by the radiorespirometric method, indicated that these substrates are degraded in the same manner, i.e., the Entner-Doudoroff and pentose phosphate pathways. There appeared to be no major shift in primary metabolic pathways when nitrate was used as the terminal hydrogen acceptor in nitrate respiration as opposed to aerobic respiration with free molecular oxygen. It was shown that glucose is not degraded to any appreciable extent under anaerobic conditions in the absence of nitrate. Tentative evidence suggests that the tricarboxylic acid cycle functions under both conditions of oxygen relationships and that the rate of carbon oxidation via the tricarboxylic acid cycle is slower with nitrate respiration than under aerobic conditions.

Nitrate respiration, as currently defined (3, 7), involves the reduction of nitrate to nitrogen gas or nitrogen oxides by a respiratory-type mechanism. A stoichiometric relationship exists between organic carbon oxidation and nitrate reduction whereby nitrate becomes the terminal hydrogen acceptor, as compared with aerobic respiration in which free molecular oxygen is the terminal hydrogen acceptor. The respiratory mechanism utilizes an electron transport system (2) in contrast to nitrate reduction or "nitrate fermentation" at the substrate level (4).

The mechanism of nitrate respiration apparently differs from that of aerobic respiration involving free molecular oxygen in that, although nitrate-oxygen does accept hydrogen ions to form water, it is the nitrate-nitrogen which becomes reduced.

A limited number of denitrifying bacteria, such as *Pseudomonas denitrificans*, *P. aeruginosa*, *P. stutzeri*, and *Micrococcus denitrificans*, are capable of respiration with nitrate under anaerobic conditions. One of these cultures, *P. stutzeri*, was selected for the present study. Previous studies on the *Pseudomonadaceae* have shown that the Entner-Doudoroff (ED) and pentose phosphate (PP) pathways predominate for the catabolism of glucose under aerobic conditions (6). However, a review of the literature revealed no information on the metabolic pathways of an organism grown under anaerobic conditions with nitrate as the terminal hydrogen acceptor (nitrate respiration). Thus, we were prompted to determine the pathways of glucose dissimilation which may function, in *P. stutzeri*, under conditions of nitrate respiration and to compare these results with those obtained under aerobic conditions with no nitrate. These results should indicate whether the same or different pathways are functional when free molecular oxygen or nitrate is used as the terminal hydrogen acceptor in *P. stutzeri*.

The findings provide information regarding the participation of the ED and PP pathways in this organism under aerobic conditions and conditions of nitrate respiration.

### MATERIALS AND METHODS

*Test organism and media*. The culture used in this study was a strain of *P. stutzeri* isolated from soil.

Aerobic cells were grown in a medium containing (in grams): yeast extract (Difco), 1.0; $(NH_4)_2PO_4$, 0.5; $KH_2PO_4$, 1.14; $K_2HPO_4$, 1.45; $MgSO_4 \cdot 7H_2O$, 0.1; $MoO_3$, 0.001; $FeSO_4$, 0.001; glucose, 5.0; and 1,000 ml of distilled water. Cells for nitrate respiration studies were grown in the above medium containing, in addition, 7.3 g of $KNO_3$ per liter. The *p*H of the media was adjusted to 6.9, and the media were autoclaved at 15 psi for 15 min. The glucose was sterilized separately by filtration and added aseptically to each medium after autoclaving.

*Growth of cells.* Aerobic cells were grown in air with agitation, at 30 C, and were harvested by centrifugation. Cells to be used in nitrate respiration experiments were incubated anaerobically under a 100% helium atmosphere, with agitation at 30 C.

Potassium nitrate was added to the medium to be used in anaerobic experiments at a level of 7.3 g per liter. Anaerobic conditions were maintained throughout the incubation period by means of a mercury trap attached to the culture flask. This arrangement maintained a slight positive pressure in the flask, allowing for the escape of excess metabolic $CO_2$ through the mercury trap while simultaneously excluding the entry of atmospheric oxygen.

After harvesting, the aerobically and anaerobically grown cells were washed twice with their respective growth media, without glucose added, and were adjusted to a concentration of 20 mg per 30 ml for the radiorespirometric experiments.

*Radioisotopes.* Glucose-*1-C*$^{14}$, *-2-C*$^{14}$, *-3,4-C*$^{14}$, *-6-C*$^{14}$, and gluconate-*1-C*$^{14}$ were obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Glucose-*3-C*$^{14}$, gluconate-*2-C*$^{14}$, *-3,4-C*$^{14}$, and *-6-C*$^{14}$ were obtained through the kind cooperation of C. H. Wang of this University. Gluconate-*2-C*$^{14}$, *-3,4-C*$^{14}$, and *-6-C*$^{14}$ were prepared from correspondingly labeled glucose by the method of Moore and Link (5).

*Radiorespirometric method.* The radiorespirometric experiments were performed according to the method of Wang et al. (8). A series of incubation flasks was placed in a Warburg apparatus at 30 C. Each flask contained 20 mg of cells per 30 ml of medium without glucose or gluconate. To the side arm, 10 mg of specifically labeled glucose or gluconate (0.25 $\mu$c) was added for aerobic experiments, and 5.0 mg of specifically labeled glucose or gluconate was added for nitrate respiration experiments. In the case of the aerobic experiments, the flasks were sparged with air at a flow rate of 50 cc/min with shaking; for nitrate respiration experiments, the flasks were sparged with 100% helium at a flow rate of 30 cc/min with shaking. After a 15-min equilibration period, the substrates were tipped in, and metabolic $C^{14}O_2$ from each flask was trapped in 10 ml of absolute ethyl alcohol-ethanolamine (2:1). Samples were taken at hourly intervals and diluted to 15 ml with absolute ethyl alcohol. A 5-ml portion of each sample was placed in 10 ml of toluene containing 2,5-diphenyloxazole (6 g per liter) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (100 mg per liter) in a 20-ml glass counting vial.

*Liquid scintillation counting.* The scintillation samples were counted by means of a Packard Tri-Carb model 314 E liquid scintillation spectrometer. All counting was done under flat spectrum conditions, with the exception of samples derived from glucose-*3-C*$^{14}$ and gluconate-*3,4-C*$^{14}$ which were counted under balance point conditions. Because of the scarcity of these labels, they were added at lower levels of activity (0.01 and 0.007 $\mu$c/flask, respectively) than the remaining labels. Counting was usually carried out at a standard deviation no greater than 2%.

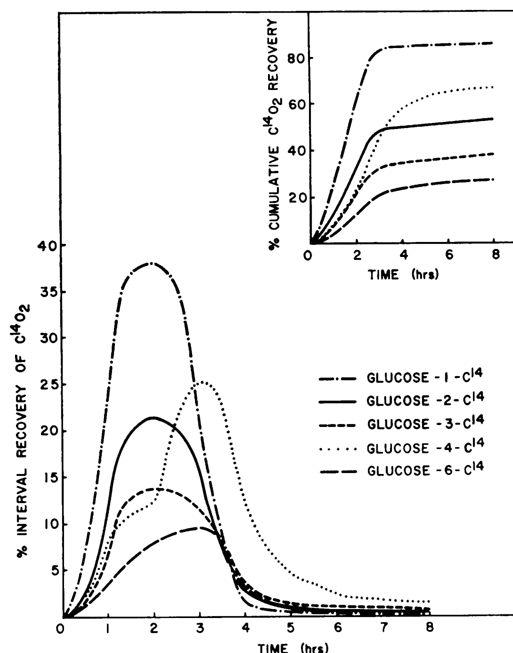


FIG. 1. *Aerobic radiorespirometric patterns for the utilization of glucose.*

At the termination of each experiment, the cells and media were separated by centrifugation, and portions of each were counted in thixotropic gel. Gel samples were counted under the same counting conditions as the liquid $C^{14}O_2$ samples. The efficiency of liquid scintillation counting for each type of sample was determined by means of appropriate internal standards.

*Calculation of values for glucose-4-C*$^{14}$. The following formula was used for the calculation of values for glucose-*4-C*$^{14}$: $G_4 = 2(G_{3,4}) - G_3$. The calculated values obtained for glucose-*4-C*$^{14}$ are considered valid, since the total recoveries for glucose-*3-C*$^{14}$ and *-3,4-C*$^{14}$ approached 100% in these experiments. The curves for glucose-*4-C*$^{14}$ (Fig. 1 and 3) may, however, show some irregularities, since each hourly recovery was calculated from hourly recoveries of two glucose labels. Small differences in either specific label may produce irregular interval recoveries used to plot the calculated glucose-*4-C*$^{14}$ curves. However, the total recoveries obtained for glucose-*4-C*$^{14}$ are based on total recoveries of $C^{14}O_2$ from glucose-*3-C*$^{14}$ and *-3,4-C*$^{14}$, rather than on hourly recoveries.

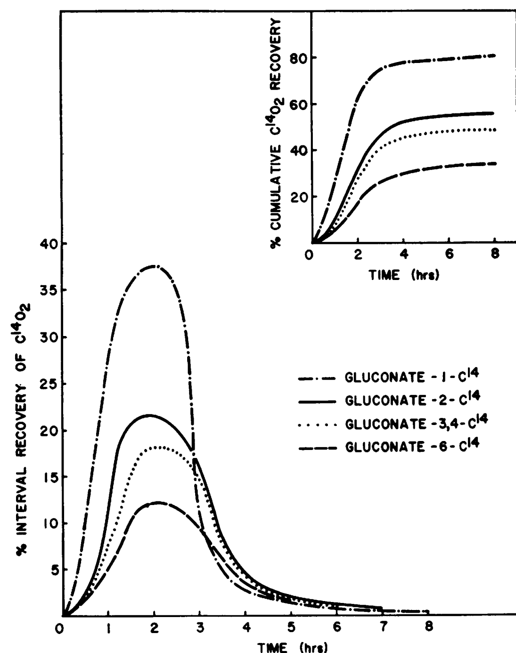


FIG. 2. *Aerobic radiorespirometric patterns for the utilization of gluconate.*

TABLE 1. *Aerobic incorporation of $C^{14}$ label into cells, medium, and $CO_2$ by Pseudomonas stutzeri cells*

| Substrate | Isotope recoveries | | | |
|---|---|---|---|---|
| | $CO_2$ | Cells | Medium | Total |
| | % | % | % | % |
| Glucose-*1*-$C^{14}$ | 86 | 13 | 6 | 105 |
| Glucose-*2*-$C^{14}$ | 53 | 42 | 6 | 101 |
| Glucose-*3*-$C^{14}$ | 38 | 57 | 5 | 100 |
| Glucose-*3,4*-$C^{14}$ | 53 | 42 | 5 | 100 |
| Glucose-*4*-$C^{14}$* | 68 | 27 | 5 | 100 |
| Glucose-*6*-$C^{14}$ | 27 | 59 | 9 | 95 |
| Gluconate-*1*-$C^{14}$ | 81 | 6 | 9 | 96 |
| Gluconate-*2*-$C^{14}$ | 56 | 37 | 6 | 99 |
| Gluconate-*3,4*-$C^{14}$ | 49 | 43 | 4 | 96 |
| Gluconate-*6*-$C^{14}$ | 34 | 57 | 5 | 96 |

* Calculated from glucose-*3*-$C^{14}$ and glucose-*3,4*-$C^{14}$ recoveries.

## RESULTS AND DISCUSSION

*Aerobic respiration.* Figures 1 and 2 show the aerobic radiorespirometric patterns when glucose and gluconate were utilized as the respective carbon sources. Total $C^{14}O_2$ recoveries, as well as cell incorporation, the per cent activity remaining in the medium, and the per cent recovery of total added label, are given in Table 1 for glucose and gluconate under aerobic conditions.

The radiorespirometric patterns obtained for glucose and gluconate are typical of cells which use a major ED pathway and a minor PP pathway (6). This is shown primarily by the recovery patterns of $C^{14}O_2$ from C-1, C-2, and C-4 of glucose. An organism which uses an ED pathway in conjunction with the tricarboxylic acid cycle for the catabolism of glucose should give the following radiorespirometric pattern for glucose: $1 = 4 > 2 = 5 > 3 = 6$. This is fully in agreement with the ED scheme as it was originally described (1). When the ED scheme is operative, glucose is converted to 2-keto-3-deoxy-6-phosphogluconate. The keto-deoxy compound is then split into pyruvic acid and glyceraldehyde-3-phosphate which, in turn, can be converted to pyruvate if the requisite enzymes are present. The carboxyl groups of the pyruvate, so derived, originate from the C-1 and C-4 positions of glucose. These positions are then decarboxylated at the highest rate when pyruvate is decarboxylated in the formation of acetyl-coenzyme A (CoA), which is dissimilated via the tricarboxylic acid cycle. This idealized description clearly does not fit the cases depicted in Fig. 1 and 2; however, the yields from C-1 and C-4 (86 and 68%) are clearly higher than those from C-2, C-3, and C-6 (53, 38, and 27%, respectively). The curves for C-3 and C-6 of glucose do fall close enough to indicate an ED pattern. Recoveries from C-1 and C-4 of glucose do not approach coincidence of the two curves. However, the $C^{14}O_2$ yields from C-1 and C-4 of glucose are clearly the highest of all labels used. If a PP pathway were also functioning in this organism concurrently with the ED pathway, one would expect a pronounced yield from C-1 as compared with C-4 because of decarboxylation of the C-1 position of gluconate to form a $C_5$ compound. This $C_5$ compound would be further degraded or rearranged by the PP pathway, or would be incorporated into the cell directly as a pentose. Since part of the gluconate derived from glucose would be utilized via the pentose phosphate pathway with a consequent loss of this fraction of C-1 as $C^{14}O_2$, one would expect the $C^{14}O_2$ yield from the C-1 position to be higher than that of the C-4 position, i.e., 86 and 68%, respectively.

The diverse and ill-defined nature of the PP route makes it difficult to trace the fate of substrate carbon via the radiorespirometric method, especially if the overall scheme is confused by the concurrent operation of another pathway, as is apparent in this case. That both of these

TABLE 2. *Incorporation of $C^{14}$ label into cells, medium, and $CO_2$ by Pseudomonas stutzeri under anaerobic conditions without nitrate*

| Substrate | Isotope recoveries | | | |
|---|---|---|---|---|
| | $CO_2$ | Cells | Medium | Total |
| | % | % | % | % |
| Glucose -*1*-$C^{14}$...... | 31 | 4 | 52 | 85 |
| Glucose-*2*-$C^{14}$....... | 4 | 3 | 89 | 96 |
| Glucose-*3,4*-$C^{14}$..... | 2 | 2 | 76 | 80 |
| Glucose-*6*-$C^{14}$....... | 1 | 3 | 99 | 103 |

pathways are operative, however, may be further supported by comparing the radiorespirometric patterns for glucose and gluconate. The patterns for each specific label of glucose coincide very well with the corresponding gluconate pattern. Since glucose must be converted to gluconate before either the ED or PP pathways will function, and since the equilibrium between glucose and gluconate is essentially irreversible, one would expect these patterns rather than patterns typical of the Embden-Meyerhof (EM) pathway. The relatively low yields from the 3,4 positions of glucose and gluconate (53 and 49%), as well as the apparent ease of conversion of glucose to gluconate and subsequent extensive breakdown of gluconate, rule out any major role for the EM scheme in this organism.

*Anaerobic respiration.* As a test for fermentation under anaerobic conditions rather than conditions of true nitrate respiration, a series of flasks containing specifically labeled glucose was run under anaerobic conditions without nitrate. Labeled glucose and carrier glucose were added at the same concentration as for the anaerobic experiments with nitrate. $C^{14}O_2$ samples were taken at hourly intervals for 12 hr, and the cells and medium were counted at the end of each experiment. The results of these experiments are shown in Table 2. The high yields of $C^{14}O_2$ from glucose-*1*-$C^{14}$ (31%) as compared with the very low yields from glucose-*2*-$C^{14}$, -*3,4*-$C^{14}$, and -*6*-$C^{14}$ (4, 2, and 1%, respectively) are quite indicative that glycolysis does not function in *P. stutzeri* under anaerobic conditions. This is especially evident in the low yield from glucose-*3,4*-$C^{14}$. If glycolysis were functioning, one would expect a very high $C^{14}O_2$ yield from this label, not 2% as obtained in these experiments. Although a total recovery of 31% was obtained from glucose-*1*-$C^{14}$, the interval recoveries were in the order of 2 to 3% per hour. Since 2 moles of reduced pyridine nucleotides are formed per mole of glucose converted to pentose via gluconate, it is obvious that these reactions could not proceed at a rapid rate in the absence of a hydrogen acceptor. Indeed, these reactions could only proceed as a result of slow reoxidation of reduced coenzymes, presumably through some synthetic reaction or reactions of endogenous metabolism which require reduced coenzymes. Consequently, no peak value for $CO_2$ production was obtained. The high total yields from C-1 (31%) indicate that the initial decarboxylation of gluconate, in the PP pathway, is functioning, but that terminal respiration has ceased, as evidenced by the low recoveries from all other labeled positions of glucose.

Enzymatic tests for glucose at the end of each experiment indicated that all activity present in the medium was in the form of glucose. This rules out the possibility of accumulation of fermentation products arising from glycolysis and further indicates that nitrate respiration is necessary for glucose utilization under anaerobic conditions.

*Nitrate respiration.* The nitrate respiration experiments were carried out under anaerobic conditions with $NO_3^-$ rather than free molecular oxygen as the terminal hydrogen acceptor. The respective patterns for glucose and gluconate (Fig. 3 and 4) show that the radiorespirometric patterns for nitrate respiration are quite similar to those obtained under aerobic conditions for both glucose and gluconate. If glucose or gluco-

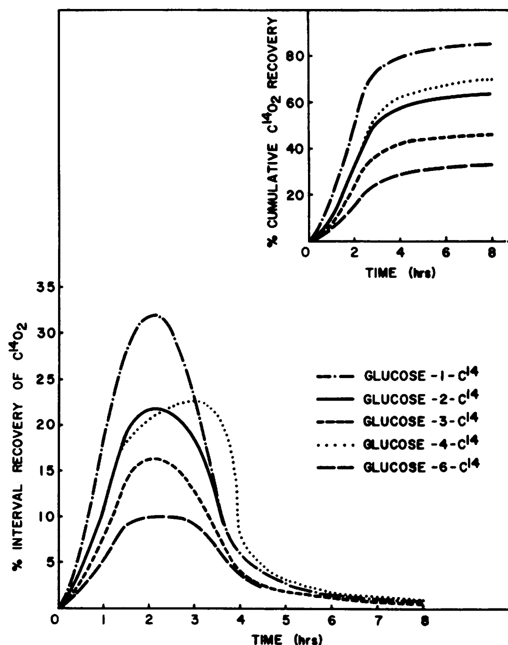


FIG. 3. *Radiorespirometric patterns for the utilization of glucose under conditions of nitrate respiration.*

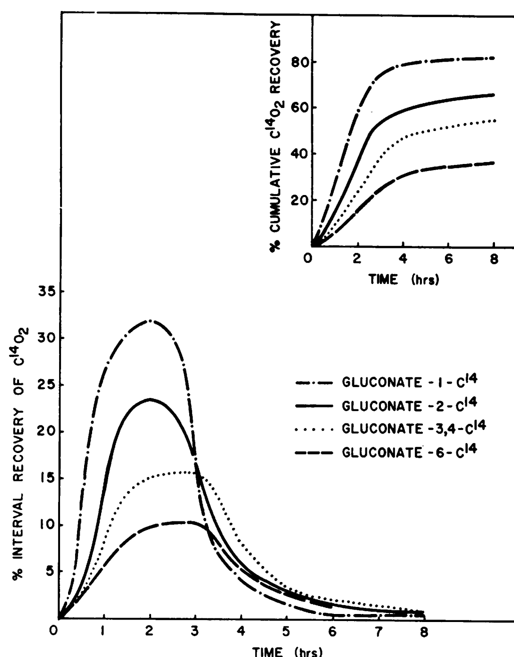


FIG. 4. *Radiorespirometric patterns for the utilization of gluconate under conditions of nitrate respiration.*

nate, or both, were dissimilated differently under conditions of nitrate respiration, such as a marked change in the ED and PP or a shift to the glycolytic pathway (EM pathway), one would expect a significant change in the overall radiorespirometric patterns. No such major differences are evident, which indicates that the same primary pathways are operative under aerobic conditions and under conditions of nitrate respiration. This conclusion is drawn through comparison of the aerobic data depicted in Fig. 1 and 2 and Table 1 with those of Fig. 3 and 4 and Table 3 for nitrate respiration.

*Comparison of primary and secondary metabolic pathways.* For the purpose of clarification, the metabolic pathways discussed are described as primary or secondary pathways. The primary pathways functioning in *P. stutzeri* would be the ED and PP pathways, and the secondary pathways would be the tricarboxylic acid and glyoxalate cycles. According to this rationale, the C-1 and C-4 positions of glucose would be the principal labels involved in the primary pathways. $C^{14}O_2$ derived from the C-1 and C-4 positions of glucose would result from the decarboxylation of the C-1 position of pyruvate in the ED scheme. Additional $C^{14}O_2$ from C-1 would arise from the initial decarboxylation of gluconate derived from glucose in the PP pathway. Comparison of the $C^{14}O_2$ yields from the C-1 and C-4 positions of

TABLE 3. *Incorporation of $C^{14}$ label into cells, medium, and $CO_2$ by Pseudomonas stutzeri cells during nitrate respiration**

| Substrate | Isotope recoveries | | | |
|---|---|---|---|---|
| | $CO_2$ | Cells | Medium | Total |
| | % | % | % | % |
| Glucose-*1*-$C^{14}$ | 85 | 8 | 6 | 99 |
| Glucose-*2*-$C^{14}$ | 64 | 27 | 8 | 99 |
| Glucose-*3*-$C^{14}$ | 46 | 39 | 13 | 98 |
| Glucose-*3,4*-$C^{14}$ | 58 | 33 | 9 | 100 |
| Glucose-*4*-$C^{14}$† | 70 | 27 | 5 | 102 |
| Glucose-*6*-$C^{14}$ | 34 | 57 | 11 | 102 |
| Gluconate-*1*-$C^{14}$ | 83 | 1 | 17 | 101 |
| Gluconate-*2*-$C^{14}$ | 66 | 28 | 9 | 103 |
| Gluconate-*3,4*-$C^{14}$ | 55 | 33 | 13 | 101 |
| Gluconate-*6*-$C^{14}$ | 37 | 57 | 9 | 103 |

* Experiments performed in 100% helium atmosphere with $NO_3^-$ as the terminal hydrogen acceptor (1,000 ppm of $NO_3^-$-N).

† Calculated from glucose-*3*-$C^{14}$ and glucose-*3,4*-$C^{14}$ recoveries.

glucose under aerobic conditions (86 and 68%, respectively) and conditions of nitrate respiration (85 and 70%), indicate that the primary pathways function at the same rate under both conditions of oxygen relationship. Secondary pathway activity would then be characterized by $C^{14}O_2$ derived from the C-2, C-3, and C-6 positions of glucose.

Preliminary evidence obtained from nutritional experiments and cell-free enzyme work, in our laboratory, indicates that the tricarboxylic acid and glyoxalate cycles function under both aerobic conditions and conditions of nitrate respiration. Other recent work (Elliott, Ph.D. Thesis, Oregon State University, Corvallis) on oxidative phosphorylation in *P. stutzeri* indicates that, under aerobic conditions, 3 molecules of adenosine triphosphate (ATP) may be formed for each oxygen atom consumed and 2 molecules of ATP per $NO_3^-$ atom consumed under conditions of nitrate respiration. Considering this evidence to be applicable, one would expect that a greater recycling of the tricarboxylic acid cycle would be necessary for an equivalent amount of energy under conditions of nitrate respiration. As a result, more $CO_2$ should be evolved as a result of secondary pathway activity and less substrate carbon would be incorporated into cell constituents under conditions of nitrate respiration as compared with aerobic conditions. As previously indicated, the total $C^{14}O_2$ yields and per cent cell incorporation (Tables 1 and 3) from the C-1 and C-4 positions of glucose are the same under these conditions.

When comparing total $C^{14}O_2$ evolved and per cent cell incorporation derived from the C-2, C-3, and C-6 positions of glucose, it becomes possible to observe a difference in the fate of these carbon atoms. In the tricarboxylic acid cycle, the C-2 and C-5 positions would be converted to $CO_2$ at the greatest rate with less consequent cell incorporation under conditions of nitrate respiration. The C-2 position of glucose does indicate this trend, since 64% of the glucose-*2-C^14^* was converted to $C^{14}O_2$ with 27% cell incorporation, as compared with an aerobic $C^{14}O_2$ yield of 53% with 42% cell incorporation. The C-3 and C-6 positions of glucose would be attacked last and more cell incorporation would be observed under aerobic conditions (57 and 59% aerobically as compared with 39 and 57% under conditions of nitrate respiration). Thus, the general pattern shown by these data indicates that greater secondary or terminal pathway activity occurs under conditions of nitrate respiration than under aerobic conditions. Work currently in progress should help to clarify this point.

## Acknowledgments

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