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The effects of different nitrogen sources on the primary and secondary pathways of Azotobacter vinelandii cells were studied by means of the radiorespirometric method. C^{14} labeled glucose and several key amino acids related to the tricarboxylic acid cycle, such as glutamic acid, aspartic acid, alanine, serine and glycine were used as tracing substrates. It is known that in Azotobacter vinelandii 80 percent of the substrate glucose is catabolized via the Entner-Doudoroff pathway, 20 percent by way of the pentose phosphate pathway. Operation of the tricarboxylic acid cycle has also been demonstrated. The present results indicate that the cells grown in different nitrogen sources such as molecular nitrogen, ammonium nitrate, aminoid and nitrate nitrogen metabolized glucose in the same manner without a noticeable change in the catabolic patterns.

Azotobacter vinelandii cells utilized the l isomer of glutamic acid preferentially to the d isomer. The latter is metabolized only

after the l isomer is exhausted. The l and d isomers of alanine are utilized concurrently and apparently at the same rate. L-aspartic acid was extensively converted to CO_2 whereas the d isomer is not utilized. The l and d isomers of serine were both metabolized.

Alanine is utilized to a significant extent by resting cells as well as under proliferating conditions; glutamic acid is metabolized to an appreciable extent only under proliferating conditions i.e. in the presence of an energy source.

The kinetics of $C^{14}O_2$ evolution for Azotobacter vinelandii cells metabolizing specifically labeled glutamic acid, aspartic acid, alanine and glycine revealed two phases of utilization. 1. An initial slow phase which probably reflects an adaptation period; 2. A later phase at a relatively faster rate of utilization.

The rates and extents of $C^{14}O_2$ production for cells metabolizing labeled glutamic acid, aspartic acid, alanine and serine confirmed the operation of tricarboxylic acid cycle in intact Azotobacter vinelandii cells.

THE UTILIZATION OF SOME AMINO ACIDS BY AZOTOBACTER VINELANDII

by

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TO MY PARENTS

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THE UTILIZATION OF SOME AMINO ACIDS BY AZOTOBACTER VINELANDII

INTRODUCTION

The metabolism of A. vinelandii is of great interest because this organism is unique in its versatility to oxidize extensively carbonecous compounds and its ability to assimilate molecular nitrogen. A. vinelandii belongs to genus Azotobacter which is an easily recognizable group of bacteria, widely distributed in soil and water (19, p. 195-213). The Azotobacter are characterized morphologically by the large size of their cells, by their strictly aerobic mode of life and their capability of non-symbiotic nitrogen fixation.

The mechanism of carbohydrate dissimilation in A. vinelandii has been elucidated to a great extent within the last decade. The work of Mortenson and Wilson (29, p. 425-435; 30, p. 713-721) showed the operation of pentose phosphate (PP) pathway in cell free extracts of this organism. Soon after, the operation of Entner-Doudoroff (ED) pathway was suggested by Mortenson et al. (28, p. 238-244) who found that the 6-phosphogluconate was split into pyruvate and glyceraldehyde-3-phosphate by cell free extracts of A. vinelandii. However the direct demonstration of the two key enzymes associated with ED pathway i.e. 6-phosphogluconic dehydrase and 2-keto-3-deoxy-6-phosphogluconic aldolase has not yet been reported. On the other hand all the enzymes of Embden-Meyerhof-Parnas (EMP) pathway except phosphohexokinase have been found in

the cell free extracts (29, p. 425-435). Recently Still and Wang (34), using radiorespirometric method (42, p. 1869-1872; 43, p. 207-216; 45, p. 3614-3622) as well as incorporation studies, obtained direct evidence for the concurrent operation of ED and PP pathways in proliferating A. vinelandii cultures. In the radiorespirometric experiments, the rates and extents of $C^{14}O_2$ production from the carbon atoms of glucose-1-, -2-, -3-, -3, 4-, and -6-C^{14} were in the order of C-1 > C-2 > C-3 ≥ C-4 > C-6. In the incorporation experiments C-1, C-2 and C-6 of glucose were found to be preferentially incorporated respectively into C-1, C-2 and C-3 of alanine isolated from proliferating cells, metabolizing labeled glucose. The authors concluded that in this organism glucose is catabolized mainly by way of the ED pathway, operation of the PP pathway may not exceed 20% of the total catabolized glucose.

The utilization of pyruvate, a key intermediate in glucose catabolism has been studied to a great extent (35, p. 605-617; 36, p. 619-622; 37, p. 221-225). Stone and Wilson (37, p. 221-225) have demonstrated the operation of the TCA cycle in cell free extracts of A. vinelandii, by using acetate-1-C^{14} as substrate which was rapidly incorporated into the cellular TCA cycle intermediates.

The assimilation of molecular and combined nitrogen by A. vinelandii has been subject of a great number of studies because of the widespread agronomic interest and the role of combined nitrogen compounds as possible intermediates in biological nitrogen

fixation. Direct studies of amino acid utilization are relatively few and in many cases contradictory.

Due to a lack of knowledge of the trace element requirements of this organism, along with inadequate buffering of incubation medium and doubtful cultural purity, a great deal of literature on the nutrition of this organism contained conflicting and uninterpretable information up until 1930. The literature in this regard, prior to 1930 has been reviewed by Fuller and Rettger (14, p. 219-234) and Thompson (40, p. 149-161). From their own experiments, Fuller and Rettger reported that Azotobacter can utilize ammonium salts, nitrates, urea and creatine. Later Thompson confirmed these findings; in addition he found that asparagine and glutamic acid, but little if any alanine, dl-valine, dl-α-amino-n-butyric acid, dl-α-amino-valeric acid, dl-phenylalanine, peptone, egg albumen or casein were used. Nitrogen fixation was usually depressed by the utilizable nitrogen containing compounds. Burk and Horner (5, p. 213-214) found that both ammonium and urea nitrogen were superior to elemental nitrogen as source of nitrogen for A. vinelandii.

Greaves et al. (15,p.9-19) reported varying degrees of stimulation of nitrogen fixation in Azotobacter chroococcum by various amino acids and proteins but no evidence of utilization of these compounds was observed. Later Lind and Wilson (22, p. 59-72; 51, p. 219-232) found that aspartate and glutamate were poor nitrogen sources,

but they could be utilized to some extent. Arginine, N_2O and alanine were found to be little or not assimilated. Glycine and NH_2OH were toxic to this organism. Horner and Allison (16, p. 1-14) tested 35 organic nitrogen compounds in the absence of nitrogen gas to see whether any of these compounds could be utilized as nitrogen source. Among these nitrogen compounds tested, they found that only l-aspartic acid, asparagine, d-glutamic acid and adenine were definitely assimilated. All of these were utilized at a slower rate than that of ammonia, nitrate, nitrite and urea. It was concluded that very few nitrogenous compounds would serve as nitrogen sources for A. vinelandii. Among the amino acids only the dicarboxylic acids were found to be assimilated; two, three and four carbon monocarboxylic acids, such as glycine, alanine and 2-amino-n-butyric acid were found to be not significantly utilized. However the Kjeldahl technique, used for the nitrogen determinations in this work is not reliable. This aspect will be discussed in a later section.

A more sensitive and reliable technique for this type of studies was developed by Wilson et al. (52, p. 289-294). These investigators used N^{15} (8, p. 114-115) to determine the effect of nitrogen compounds on nitrogen fixation by A. vinelandii. The growing cultures were supplied simultaneously with nitrogen compounds containing normal nitrogen and molecular nitrogen enriched with N^{15}. Any labeling in the cells at the end of experiment would be

due to the fixation of molecular nitrogen. They found that only ammonia and urea (which is promptly broken down to ammonia in the presence of the urease of the cells) were able to inhibit nitrogen fixation. On the other hand cells had to be adapted to nitrate before nitrate can be used as the sole source of nitrogen, to the exclusion of molecular nitrogen. Aspartate, glutamate or casein hydrolysate did not appear to inhibit nitrogen fixation effectively nor to compete with molecular nitrogen in A. vinelandii metabolism. Asparagine was utilized better than any of the amino acids studied probably due to the ready conversion of the amide group to ammonia. Newton et al. (32, p. 445-451) using concentrated cell suspensions, hence slower biosynthetic processes in the cells, demonstrated that added ammonia seems to be a definite intermediate in the nitrogen fixation process. Allison and Burris exposed growing A. vinelandii cells to N_2^{15} for short periods (1, p. 351-364). They analyzed the N^{15} accumulated in the cells and in the incubation medium. The amide nitrogen of the cells (the fraction which was recovered as NH_3 after acid hydrolysis of the cells) and ammonia of the medium rose initially most rapidly in N^{15} concentration. Glutamic acid also accumulated N^{15} rapidly. The authors concluded that ammonia and the products with which it equilibrates rapidly, such as amides and glutamic acid, were the first

demonstrable products of nitrogen fixation. The rate of labeling of alanine was slow, whereas serine and glycine became labeled relatively faster. Burma and Burris exposed growing cells to $N^{15}H_4^+$ (6, p. 287-295). The results showed that ammonia was incorporated into the cellular amino acids after a short but detectable lag period. However there was a longer lag in N^{15} accumulation into proteins. Among the few amino acids that were in free state in the cells, glutamic acid had by far the highest N^{15} concentration. This rapid labeling of glutamic acid suggested that this amino acid is a primary intermediate in the utilization of either ammonia or molecular nitrogen as nitrogen source. This contention was supported by the demonstration of the presence of an active glutamic dehydrogenase system in the cell free preparations of A. vinelandii (23, p. 635-643). Burma and Burris (7, p. 723-733) investigated the metabolism of nitrogen compounds mainly N^{15} labeled ammonia and glutamic acid by cell free preparations of A. vinelandii. Their results are summarized as follows:

1. $N^{15}H_4^+$ was metabolized very quickly by cell free extracts, the rate of $N^{15}H_4^+$ uptake being stimulated about five fold by the addition of α-ketoglutarate. Among N^{15} labeled amino acids isolated from $N^{15}H_4^+$ metabolizing cell free system, glutamic acid had the highest N^{15} concentration in both the presence and the absence of α-ketoglutarate.

2. There was no lag in the ammonia utilization by cell free

system, in contrast to the intact cells which showed a short but detectable lag period.

3. The cell free system also contained active transaminases, since N^{15} in the glutamic acid was directly or indirectly transferred to 15 amino acids as well as to purines and pyrimidines.

More direct studies on amino acid utilization of A. vinelandii are few in number and somewhat contradictory. Lichstein and Cohen (21, p. 85-91) showed the presence of a very active glutamic-aspartic transaminase in intact cells of A. vinelandii which catalyzed the following reaction:

$$\text{l-glutamate} + \text{oxalacetate} \longrightarrow \alpha\text{-ketoglutarate} + \text{l-aspartate}$$

Alanine-aspartate and alanine-glutamate transaminations were found to be very slow both in intact cells of A. vinelandii (11, p. 143-146) and cell free extracts of A. chroococcum (12, p. 160-162). Magee (23, p. 635-643) reported an active l-glutamic dehydrogenase in cell free system of A. vinelandii. On the other hand Sobek and Clifton (33, p. 408-411) using manometric techniques could not detect any glutamate and glycine oxidation by intact Azotobacter agilis cells. Suto (39, p. 257-261) reported that intact A. vinelandii cells did not oxidize glutamate, but in cell free system glutamate gave aspartate with concomitant liberation of ammonia. He also found (38, p. 894-898) very little aspartate and glycine utilization by intact cells.

Inasmuch as the metabolism of A. vinelandii has been studied quite extensively as separate areas, little attempts were made to examine the interrelationships between the metabolism of carbohydrate and nitrogen. It therefore seemed advantageous to study the effects of different nitrogen sources on the primary and secondary pathways of carbohydrate metabolism in A. vinelandii. In the present work a common carbohydrate, glucose and the key amino acids of TCA cycle, glutamic acid, aspartic acid, alanine glycine and serine were used as substrates in radiorespirometric and limited incorporation experiments to study the metabolic behavior of A. vinelandii cells grown on different nitrogen sources, such as molecular nitrogen, ammonium nitrate and amino acids.

EXPERIMENTAL METHODS

Cultural Conditions

Cells of A. vinelandii ATCC 9104 were preserved on glucose-agar medium and transferred monthly. Prior to each experiment cells were transferred to a new glucose-agar slant and permitted to grow for two to three days at room temperature. The cells grew in colonies surrounded by a greyish slime and if kept long enough at room temperature a light brown pigment is produced. At the end of this period a loopful inoculum was transferred to 250 cc Erlenmeyer flasks, each containing 50 cc of liquid medium. The flasks were shaken in a rotary shaker at 28-30°C until cells reached logarithmic phase. Growth was followed by turbidimetric analysis with a Klett-Summerson photoelectric colorimeter. After another transfer, the cells at the logarithmic stage of growth were used in radiorespirometric studies.

Media. In the present work four types of media with different nitrogen sources were used. The composition of each of these media is given in Table I. In the first medium, the nitrogen source is atmospheric nitrogen, therefore Mo^{+6} and Fe^{++} concentrations were increased ten-fold and two and a half fold respectively, to facilitate the nitrogen fixation process (10, p. 121-124; 13, p. 564-567). The second medium contains NH_4NO_3 as nitrogen source with NH_4^+ nitrogen being 100 ppm, NO_3^- nitrogen 50 ppm. The total nitrogen

TABLE I

Growth Media Providing Different Nitrogen Sources for Azotobacter vinelandii

Type of Medium	Conc. of Salts gr per liter	Nitrogen Source Conc.		Molybdenum Conc.		Iron Conc.	
		Compound gr per liter	ppm N	Compound gr per liter	Mo^{+6} µg per ml	Compound gr per liter	Fe^{+2} µg per ml
Nitrogen free medium	K_2HPO_4, 0.8 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.2 $CaSO_4 \cdot 2H_2O$, 0.1	----	---	$Na_2MoO_4 \cdot 2H_2O$ 0.0025	1	$FeSO_4 \cdot 7H_2O$ 0.015	3
NH_4NO_3 medium	K_2HPO_4, 0.8 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.2 $CaSO_4 \cdot 2H_2O$, 0.1	NH_4Cl, 0.2 NH_4NO_3, 0.3	150	$Na_2MoO_4 \cdot 2H_2O$ 0.00025	0.1	$FeSO_4 \cdot 7H_2O$ 0.006	1.2
Amino Acids medium	K_2HPO_4, 0.8 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.2 $CaSO_4 \cdot 2H_2O$, 0.1	Bacto* casamino acids 1	100	$Na_2MoO_4 \cdot 2H_2O$ 0.0025	1	$FeSO_4 \cdot 7H_2O$ 0.015	3
NO_3^- medium	K_2HPO_4, 0.8 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.2 $CaSO_4 \cdot 2H_2O$, 0.1	$NaNO_3$ 0.98	161	$Na_2MoO_4 \cdot 2H_2O$ 0.0025	1	$FeSO_4 \cdot 7H_2O$ 0.015	3

* The % composition of Bacto casamino acids is as follows: Arginine 4%, aspartic acid 0.5%, glutamic acid 5%, glycine 1%, histidine 2%, isoleucine 4.5%, leucine 10%, lysine 7%, methionine 2%, phenylalanine 4%, threonine 4%, tyrosine 2%, valine 7%.

All media contained 1.2% glucose

provides approximately 13 mg nitrogen per gram of the sugar substrate which is necessary for optimal growth of A. vinelandii. The third medium contains 100 ppm nitrogen in the form of amino nitrogen. This medium has the same Mo^{+6} and Fe^{+2} concentrations as nitrogen free medium because it has been shown that amino acids can only slightly inhibit nitrogen fixation (52, p. 289-294). Nitrate medium also contains a relatively higher concentration of Mo^{+6}, since Mo^{+6} was shown to be necessary for nitrate utilization by Azotobacter (24, p. 178-183).

Prior to the experiments the cells were adapted to the above media by two daily transfers. Nitrogen free, NH_4NO_3 and nitrate media gave good cell growth whereas in amino acids medium cells grew relatively slower, with lower cell yield and considerable slime production.

Purity of the Culture. It is well known that Azotobacter may easily be contaminated with certain contaminants which may be very difficult to remove. The purity of the culture was checked monthly by incubation in a peptone medium (3, p. 587-618). In a filtered, clear, sugar-free mineral medium containing 1% bactopeptone and 0.1% meat extract, Azotobacter would grow very little, whereas common contaminants would show abundant growth. Microscopic analysis and Gram tests were made often, particularly at the beginning and at the end of each of the radiorespirometric experiments.

C^{14}-labeled Substrates

Glucose-1-, -2- and -6-C^{14}, dl-glutamate-3,4-C^{14}, dl-serine-1- and -3-C^{14}, glycine-1- and -2-C^{14} and dl-aspartate-4-C^{14} were obtained from New England Nuclear Corporation. DL-Alanine-1-, -2- and -3-C^{14}, dl-glutamate-1-, -2- and -5-C^{14} and dl-aspartate-3-C^{14} were purchased from Nichem, Inc. L-Alanine-1-C^{14}, l-glutamate-1-C^{14} and l-aspartate-4-C^{14} were obtained from California Corporation for Biochemical Research.

Radiorespirometric Experiments and Radioactivity Measurements

The radiorespirometric studies with labeled amino acids and glucose were carried out according to the method of Wang et al. (42, p. 1869-1872; 43, p. 207-216; 45, p. 3614-3622). The medium used in each tracer experiment was identical to that used in obtaining the cell crop, except that C^{14}-labeled amino acids were used in addition to freshly added unlabeled carbohydrate. Respiratory $C^{14}O_2$ was trapped periodically in 2-amino ethanol-absolute ethanol (1:2) solution, mixed with 10 cc of toluene containing terphenyl (3 g/l) and 1,4-bis-2(5-phenyloxazolyl)-benzene (30 mg/l) in a 20 cc glass counting vial. Countings were carried out with a Packard Tricarb Scintillation spectrometer with the photomultiplier voltage set at 1175 volts and pulse discriminator at 10-100 volts in the red channel. Countings were usually carried out to a standard deviation of no greater than 2%. Details of counting and sample preparation are

described elsewhere (41, p. 274-290; 45, p. 3614-3622).

At the end of experiments, the cells and incubation media were separated by centrifugation and aliquots of each were counted in thixotropic gel according to the method of White and Helf (46, p. 46-48). The efficiency of liquid scintillation counting with respect to each type of counting samples was determined by the use of internal standards.

Incorporation Experiments

The incorporation experiments were carried out in a similar manner as that described under radiorespirometric experiments except a higher radiochemical level was used for the labeled substrate. At the end of radiorespirometric experiments the cells were harvested by centrifugation. An aliquot of medium and cells were assayed for radioactivity as mentioned previously. The rest of the cells were washed in glucose free mineral medium and 5 ml of the cell suspension containing about 12.5 mg cells (dry weight) were dried over P_2O_5 in a vacuum desiccator The dried cells were then hydrolyzed with 20% HCl in sealed pyrex tubes for 16 hours, under 15 pounds of pressure. At the end of hydrolysis, the cells were filtered to remove humin. The hydrolysate was evaporated to dryness in vacuo and taken up in a defined amount of water. A small aliquot of the hydrolysate was counted directly to determine the total activity; another aliquot was subjected to paper chromatography to

separate the individual amino acids. The solvents used for the paper chromatography were 80% phenol-water and butanol: acetic acid: water, 40:10:40. The radioactivity of the amino acids was measured by a windowless paper chromatogram scanning counter and also by means of a liquid scintillation counting technique described by Wang and Jones (44, p. 203-205).

RESULTS

The radiorespirometric pattern of A. vinelandii metabolizing specifically labeled glucose in NH_4NO_3 medium was reported previously (34). It is therefore of interest to examine whether different sources of nitrogen provided for this organism such as molecular nitrogen, nitrate, ammonia, or amino nitrogen would make any difference in its catabolic mechanisms particularly with respect to catabolic pathways of glucose and several key amino acids. With this in mind, a set of radiorespirometric experiments were carried out. It was found that the cells adapted to different nitrogen sources metabolized glucose in the same manner, without an apparent change in the catabolic patterns. The radiorespirometric patterns presented in the following work are plotted on the basis of time in hours against hourly percent recoveries of substrate radioactivity in CO_2. The radiochemical inventories of substrate activity in CO_2, cells and media at the end of each experiment are shown in corresponding Tables. The inventories for the above experiments are given in Table II.

Insofar as the catabolic pathways for amino acids are concerned the first amino acid studied was alanine. The following experiments are carried out with NH_4NO_3 grown cells, unless otherwise stated. A preliminary experiment was carried out to see if the utilization of this amino acid required an accompanying energy source, e.g. glucose. For this experiment the l isomer of alanine

TABLE II

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Specifically Labeled Glucose, in Presence of Four Different Types of Nitrogen Sources

Cell History	Substrate	Level		Radiochemical Recovery of Substrate percent			
		μc	mg	CO_2	Cell	Medium	Total
Grown on NO_3^-	Glucose-1-C^{14}	0.28	30	86	4	10	100
	Glucose-2-C^{14}	0.25	30	74	16	12	102
	Glucose-6-C^{14}	0.25	30	59	24	16	99
Grown on N_2	Glucose-1-C^{14}	0.28	30	90	3	7	100
	Glucose-2-C^{14}	0.25	30	82	13	4	99
	Glucose-6-C^{14}	0.25	30	71	22	6	99
Grown on NH_4NO_3	Glucose-1-C^{14}	0.28	30	89	4	8	101
	Glucose-2-C^{14}	0.25	30	78	16	7	101
	Glucose-6-C^{14}	0.25	30	63	26	10	99
Grown on amino acids	Glucose-1-C^{14}	0.28	30	90	2	8	100
	Glucose-2-C^{14}	0.25	30	77	10	5	92
	Glucose-6-C^{14}	0.25	30	75	20	5	100

Experimental Conditions - incubation temperature, 29°C; cell age, 16 hours; pH of medium for radiorespirometry, 7.3; initial cell weight (dry), 5 mg; medium volume, 11 ml; aeration rate, 61 ml per min ; duration of the experiment, 5 hours.

was used. The radiorespirometric pattern of 1-alanine-1-C^{14} utilization with and without glucose is shown in Figure 1. The radiochemical recoveries are given in Table III. It can be seen from the comparison of cell weights before and after the experiment, that glucose was necessary to maintain proliferating conditions. Consequently subsequent amino acid experiments were carried out in the presence of 300 mg glucose to ensure the presence of a proliferating environment for this organism. The kinetics of utilization of alanine by A. vinelandii cells are shown in Figure 2 for cells metabolizing different concentrations of l-alanine. Table IV shows the effect of alanine concentration on the distribution of substrate radioactivity in CO_2, cells and media.

Utilization of l and d isomers of alanine was studied with the use of 1-alanine-1-C^{14} and the racemic mixture, dl-alanine-1-C^{14} as the respective substrates. The radiorespirometric patterns of cells metabolizing the l-isomer (curve a) and the racemic mixture of alanine (curve b) are shown in Figure 3. Distribution of substrate radioactivity in CO_2, cells and media is shown in Table V and the kinetic data of CO_2 production are presented in Figure 3. These data suggest that both l and d isomers of alanine are metabolized concurrently, apparently at the same rate by A. vinelandii. The data on the utilization of d-alanine-1-C^{14} were calculated by taking the difference between the $C^{14}O_2$ production data in the dl-alanine-1-C^{14} experiment and that in the 1-alanine-1-C^{14} experiment. The

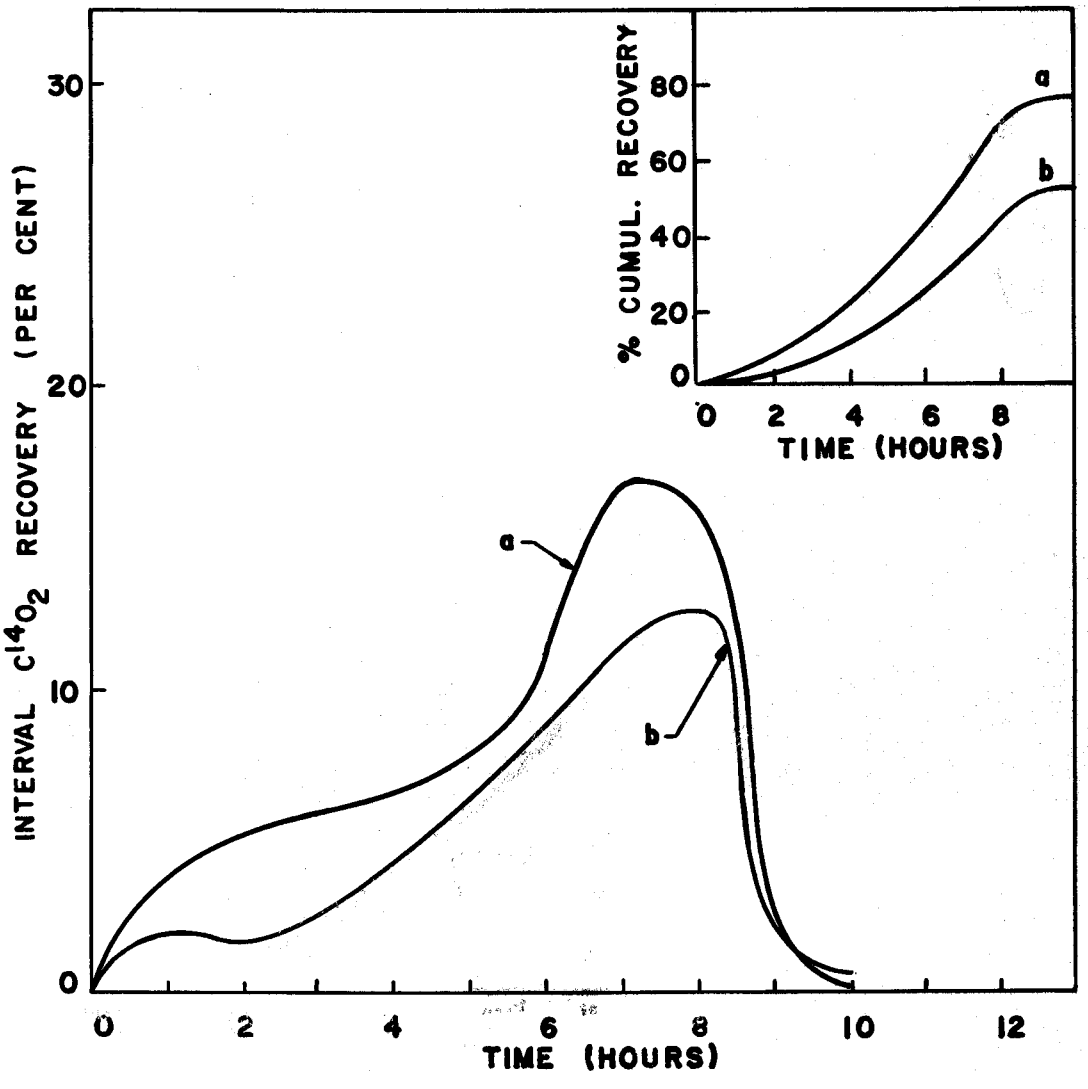

FIGURE 1. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing labeled l-alanine with and without glucose.

Legend: a, without glucose; b, 300 mg glucose.

TABLE III

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Labeled L-Alanine with and without Glucose

Glucose Added mg	Radiochemical Recovery of Substrate percent				Cell weight (dry) before the experiment mg	Cell weight (dry) after the experiment mg
	CO_2	Cells	Medium	Total		
0	76	16	5	97	8.5	10
25	68	23	9	100	8.5	18
50	52	33	15	100	8.5	29
300	53	35	12	100	8.5	35
600	58	33	12	103	8.5	35

Experimental Conditions - incubation temperature, 29°C; cell age, 16 hours; pH of medium for radiorespirometry, 7.3; initial cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; radioactive substrate, 1-alanine-1-C^{14}; radiochemical level, 0.20 μc; chemical level, 1.25 mg 1-alanine.

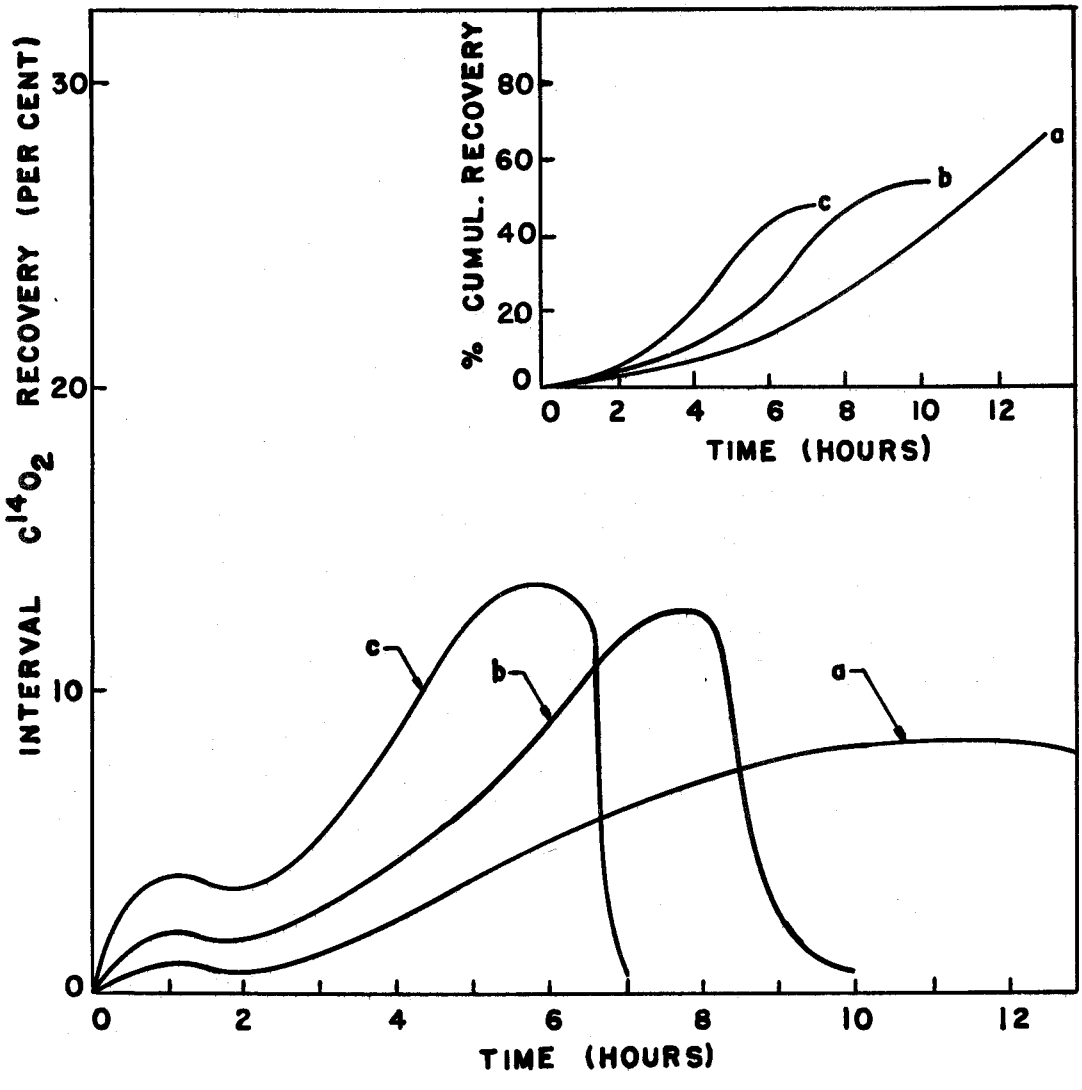

FIGURE 2. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing different concentrations of l-alanine-1-C^{14}

Legend: a, 2.5 mg L-Alanine; b, 1.25 mg L-Alanine; c, 0.625 mg L-Alanine.

TABLE IV

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Different Concentrations of Labeled L-Alanine

Experiment	Substrate	Level		Radiochemical Recovery of Substrates percent			
		μc	mg	CO_2	Cells	Medium	Total
a	L-Alanine-1-C^{14}	0.20	2.5	68	21	10	99
b	L-Alanine-1-C^{14}	0.20	1.25	55	34	11	100
c	L-Alanine-1-C^{14}	0.20	0.625	43	40	14	97

Experimental Conditions - incubation temperature, 29°C; cell age, 16 hours; pH of medium for radiorespirometry, 7.3; initial cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min; glucose, 300 mg.

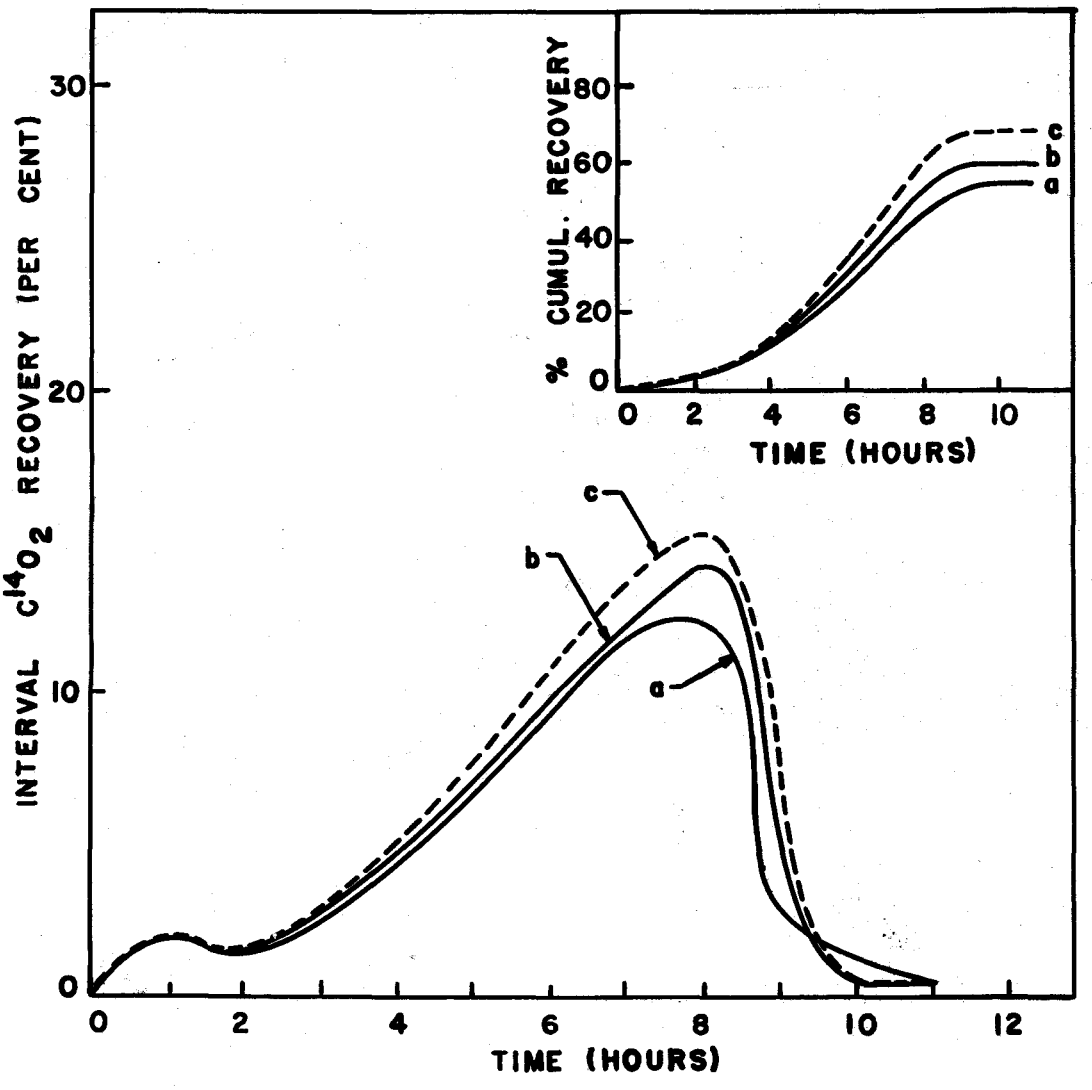

FIGURE 3. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing labeled l-, dl- and d-alanine,

Legend: a, L-Alanine; b, DL-Alanine; c, D-Alanine (calculated).

TABLE V

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Labeled L-, DL- and D-Alanine

Curve	Substrate	Level		Radiochemical Recovery of Substrates, percent			
		μc	mg	CO_2	Cell	Medium	Total
a	L-Alanine-1-C^{14}	0.20	1.25 L*	55	34	11	100
b	DL-Alanine-1-C^{14}	0.26	1.25 DL*	60	28	12	100
c	D-Alanine-1-C^{14} (calculated)	---	1.25 D*	65	22	13	100

* DL, racemic mixture; L, L isomer; D, D isomer.

Experimental Conditions - incubation temperature, 29°C; cell age, 16 hours; pH of medium for radiorespirometry, 7.3; initial cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

calculated data are represented in Figure 3, by curve c.

The metabolism of individual carbon atoms of alanine by A. vinelandii was studied with the specifically labeled alanine in the form of racemic mixtures i.e dl-alanine-1-, -2-, and -3-C^{14} as substrates. The radiorespirometric patterns representing the utilization of the above substrates are shown in Figure 4. The radiochemical inventories of substrate activity in CO_2, cells and media are given in Table VI. The heavy incorporation of the labeling of dl-alanine-1-C^{14} into cellular constituents of this highly oxidative organism appeared to be unusual (Table VI). One would expect that alanine can be readily converted to pyruvic acid which is in turn decarboxylated oxidatively to acetate (35, p. 605-617). Hence an incorporation experiment was carried out to examine the fate of dl-alanine-1-C^{14} in cells. The results are given in Table VII.

The radiorespirometric patterns of serine and glycine utilization are given in Figures 5 and 6 respectively. A set of experiments were carried out to compare the metabolism of serine and glycine by A. vinelandii cells adapted to three different sources of nitrogen: molecular, NH_4NO_3 and amino nitrogen. No apparent difference was noted in the overall utilization of these amino acids by cells adapted to three different sources of nitrogen. The radiorespirometric patterns with cells grown in molecular nitrogen and amino nitrogen showed close resemblance to that grown in NH_4NO_3 (Figures 5 and 6). The rate of $C^{14}O_2$ evolution from cells

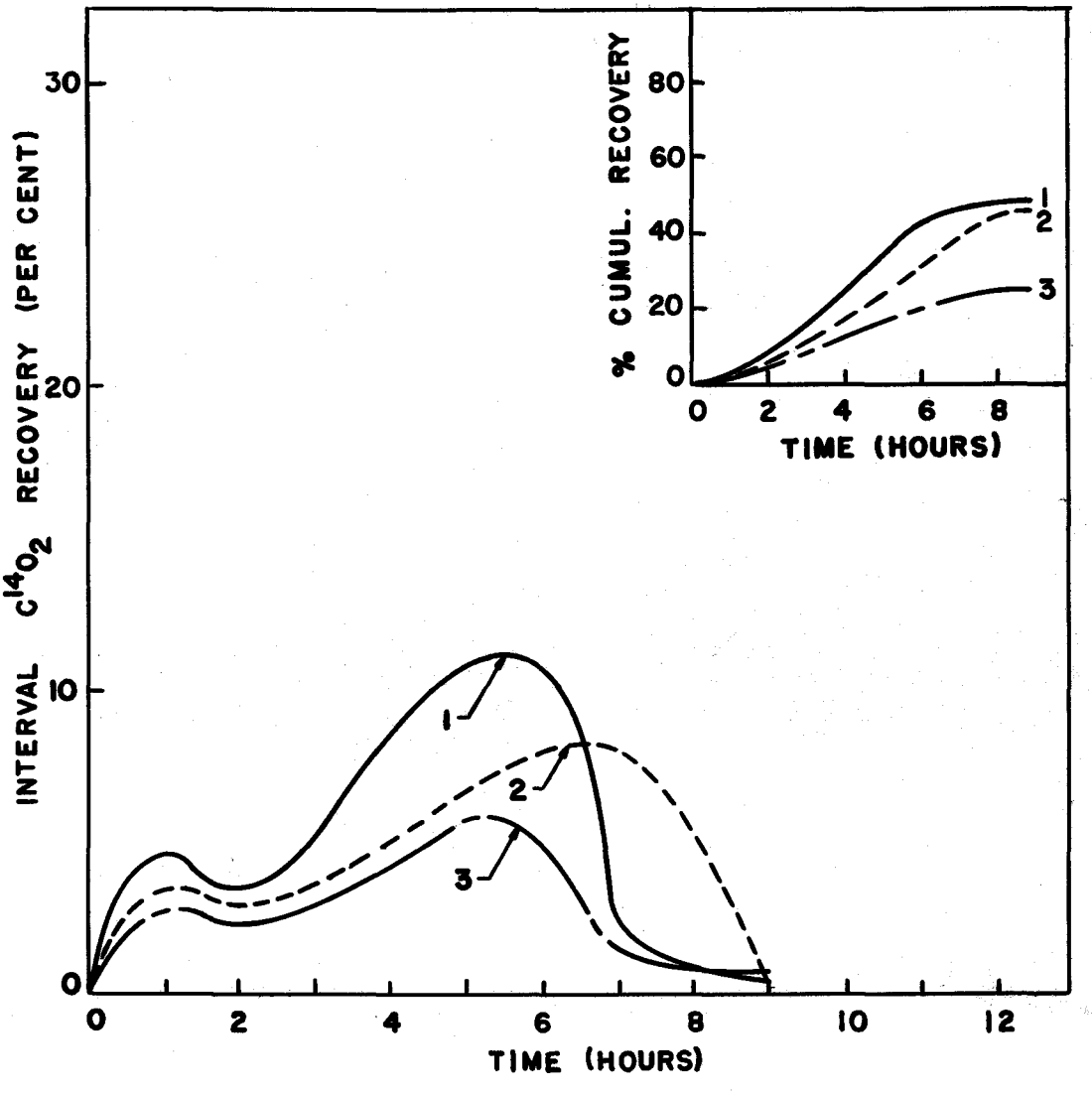

FIGURE 4. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing specifically labeled dl-alanine.

Legend: DL-Alanine-1-C^{14} ————————
DL-Alanine-2-C^{14} – – – – – – –
DL-Alanine-3-C^{14} —— – —— –

TABLE VI

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Specifically Labeled DL-Alanine

Substrate	Level		Radiochemical Recovery of Substrate, percent			
	μc	mg*	CO_2	Cells	Medium	Total
DL-Alanine-1-C^{14}	0.13	0.625	48	40	11	99
DL-Alanine-2-C^{14}	0.12	0.625	45	46	8	99
DL-Alanine-3-C^{14}	0.12	0.625	28	45	26	99

* Racemic mixture

Experimental Conditions - incubation temperature, 29°C; cell age, 16 hours; pH of medium for radiorespirometry, 7.2; initial cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

TABLE VII

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing DL-Alanine-1-C^{14}

Substrate	Level		Radiochemical Recovery of Substrate, percent			
	μc	mg	CO_2	Cells	Medium	Total
DL-Alanine-1-C^{14}	5.29	0.625*	53	28	12	93

* Racemic mixture

Incorporation of DL-Alanine-1-C^{14} into Cell Amino Acids

Amino Acid	Radiochemical Recovery of Substrate, percent
Alanine	95
Glutamic acid	2
Unidentified	2

Experimental Conditions - incubation temperature, 29°C; cell age, 15 hours; pH of medium for radiorespirometry, 7.2; initial cell weight (dry), 9 mg; final cell weight (dry), 31 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

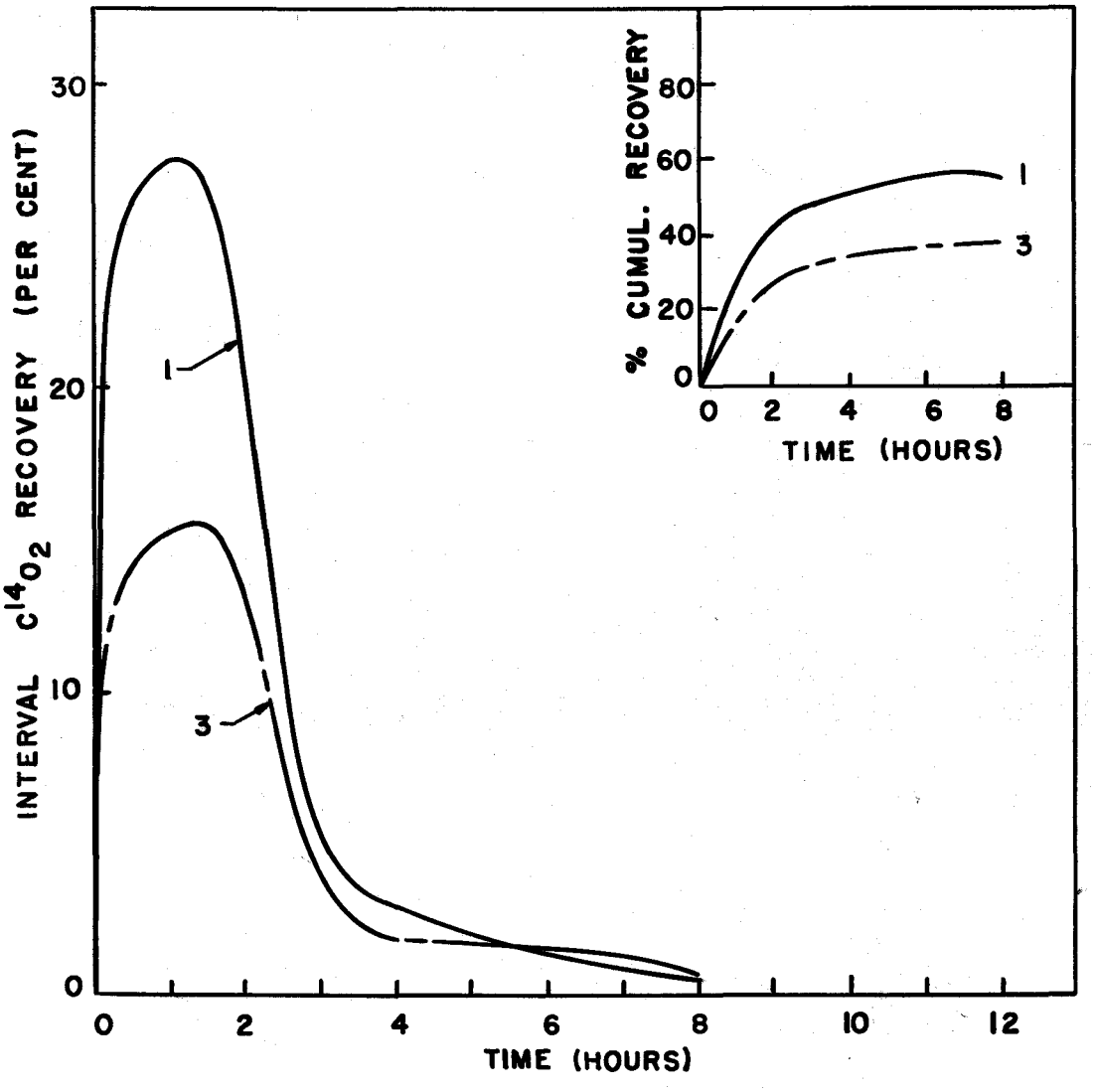

FIGURE 5. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing specifically labeled serine.

Legend: DL-Serine-1-C^{14} ————————
DL-Serine-3-C^{14} —— – ——— –

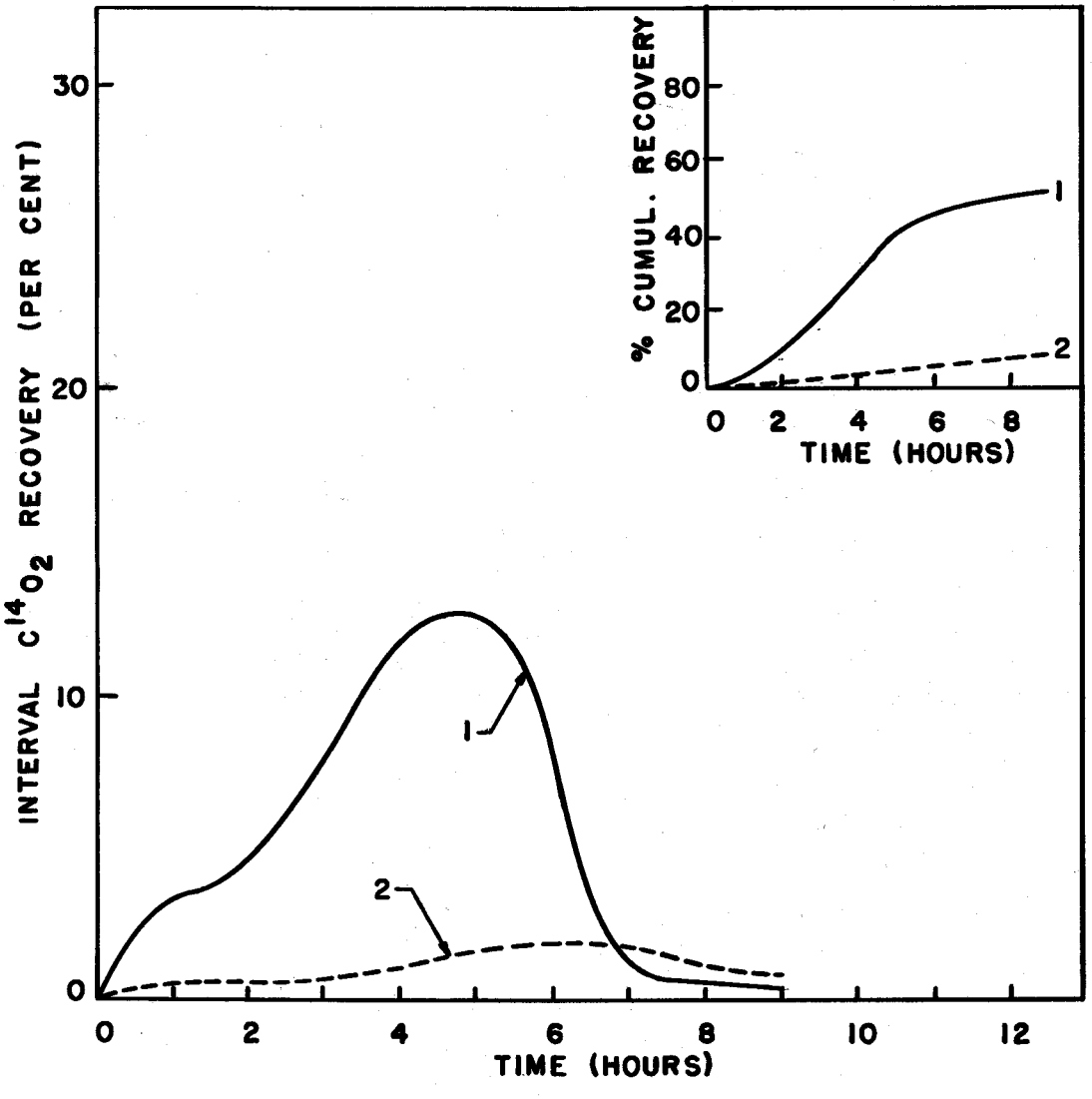

FIGURE 6. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing specifically labeled glycine.

Legend: Glycine-1-C^{14} ————————
Glycine-2-C^{14} – – – – – – – –

metabolizing the respective labeled substrates was relatively slower in the experiment with amino acids medium, possibly due to the dilution of the labeled substrates by amino acids in the medium and the slower rate of cell growth in this medium (See Experimental Methods). The radiochemical recoveries of substrate activity in CO_2, cells and media for the above mentioned cells metabolizing serine and glycine, are given in Tables VIII and IX respectively.

In the case of aspartic acid some preliminary experiments were carried out to examine the relative utilization of l and d isomers of this amino acid, by A. vinelandii cells. In these experiments l-aspartate-4- and dl-aspartate-4-C^{14} were used as the respective substrates. The radiorespirometric patterns and radiochemical recoveries in CO_2, cells and media are shown in Figure 7 and Table X respectively. It is apparent that the d isomer of aspartic acid is not metabolized at all by the A. vinelandii cells. More than 50% of the substrate radioactivity was recovered in the medium when labeled dl-aspartic acid was used as the substrate. Moreover hourly and total $C^{14}O_2$ recoveries of l-aspartate-4-C^{14} were exactly twice as much as that of dl-aspartate-4-C^{14} (Table XI). Consequently the experiments where dl-aspartate-3 and -4-C^{14} were used as the respective substrates, data are presented on the basis that l-aspartate being the actual substrate. The radiorespirometric patterns of the cells metabolizing l-aspartate-3- and -4-C^{14} are given in Figure 8.

TABLE VIII

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Specifically Labeled Serine, in Presence of Three Different Types of Nitrogen Sources

Cells History	Substrate	Level		Radiochemical Recovery of Substrate, percent			
		μc	mg**	CO_2	Cells	Medium	Total
Grown on molecular nitrogen	DL-Serine-1-C^{14}	0.11	0.625	60	37	4	101
	DL-Serine-3-C^{14}	0.08	0.625	45	37	14	96
Grown on NH_4NO_3*	DL-Serine-1-C^{14}	0.11	0.625	57	34	9	100
	DL-Serine-3-C^{14}	0.08	0.625	38	39	22	99
Grown on amino acids	DL-Serine-1-C^{14}	0.11	0.625	56	24	21	101
	DL-Serine-3-C^{14}	0.08	0.625	40	23	37	100

* Shown in Figure 5.
** L isomer.

Experimental Conditions - incubation temperature, 29°C; pH of media for radiorespirometry, 7.2; initial cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg; cell age (grown on N_2 and NH_4NO_3), 15 hours, (grown on amino acids), 17 hours.

TABLE IX

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Specifically Labeled Glycine, in Presence of Three Different Types of Nitrogen Sources

Cell History	Substrate	Level		Radiochemical Recovery of Substrate percent			
		μc	mg	CO_2	Cells	Medium	Total
Grown on molecular nitrogen	Glycine-1-C^{14}	0.09	0.625	55	37	5	97
	Glycine-2-C^{14}	0.10	0.625	13	78	9	100
Grown on NH_4NO_3 *	Glycine-1-C^{14}	0.09	0.625	50	46	5	101
	Glycine-2-C^{14}	0.10	0.625	9	74	16	99
Grown on amino acids	Glycine-1-C^{14}	0.09	0.380	55	41	5	101
	Glycine-2-C^{14}	0.10	0.380	7	77	13	97

* Shown in Figure 6.

Experimental Conditions - incubation temperature, 29°C; pH of medium for radiorespirometry, 7.2; initial cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg; cell age, (grown on N_2 and NH_4NO_3) 15 hours, (grown on amino acids) 17 hours.

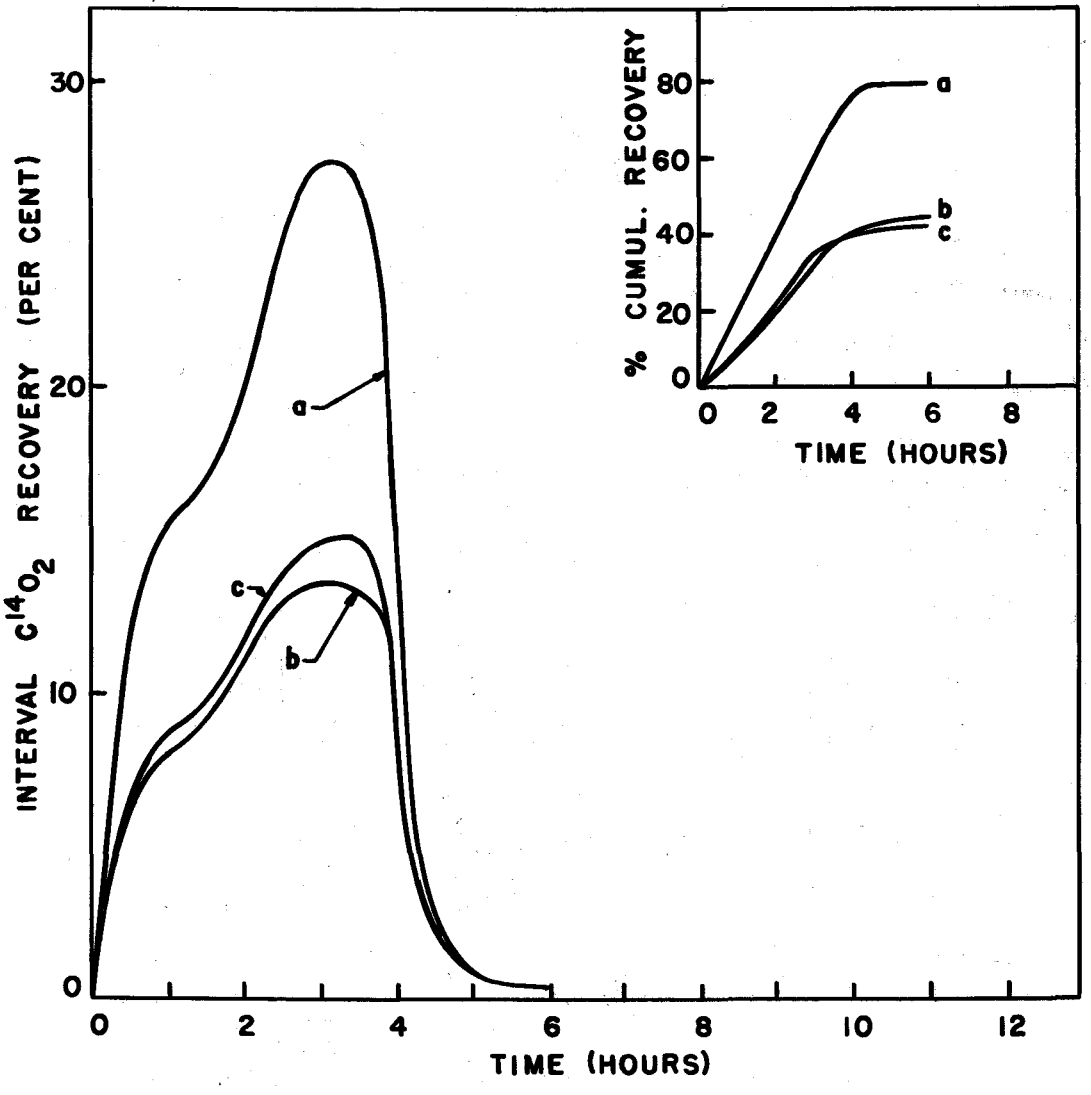

FIGURE 7. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing labeled l- and dl-aspartic acid.

Legend: a, L-Aspartate-4-C^{14} + 0.625 mg L-Aspartate
b, DL-Aspartate-4-C^{14} + 0.625 mg L-Aspartate + 0.625 mg D-Aspartate
c, DL-Aspartate-4-C^{14} + 0.625 mg L-Aspartate

TABLE X

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Labeled L- and DL-Aspartic Acid

Experiment	Substrate	Level		Radiochemical Recovery of Substrates percent			
		μc	mg	CO_2	Cells	Medium	Total
a	L-Aspartate-4-C^{14}	0.37	0.625 L*	80	13	7	100
b	DL-Aspartate-4-C^{14}	0.17	1.25 DL*	43	7	50	100
c	DL-Aspartate-4-C^{14}	0.17	0.625 L*	43	7	52	102

* L, L isomer, DL, racemic mixture.

Experimental Conditions - incubation temperature, 29°C; cell age, 16 hours; pH of medium for radiorespirometry, 7.2; cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

TABLE XI

Hourly and Cumulative Recoveries of $C^{14}O_2$ from Azotobacter vinelandii Cells Metabolizing Labeled L- and DL-Aspartic Acid

Hours	Hourly Recoveries		
	L-Aspartate-4-C^{14} + 0.625 mg L-Aspartate (a)	DL-Aspartate-4-C^{14} + 1.25 mg DL-Aspartate (b)	DL-Aspartate-4-C^{14} + 0.625 mg L-Aspartate (c)
1	16	8	9
2	19	11	10
3	28	14	15
4	16	9	8
5	1	1	1

Hours	Cumulative Recoveries		
	L-Aspartate-4-C^{14} + 0.625 mg L-Aspartate (a)	DL-Aspartate-4-C^{14} + 1.25 mg DL-Aspartate (b)	DL-Aspartate-4-C^{14} + 0.625 mg L-Aspartate (c)
1	16	8	9
2	35	19	19
3	63	33	34
4	79	42	42
5	80	43	43

Experimental Conditions - similar to those of Table X.

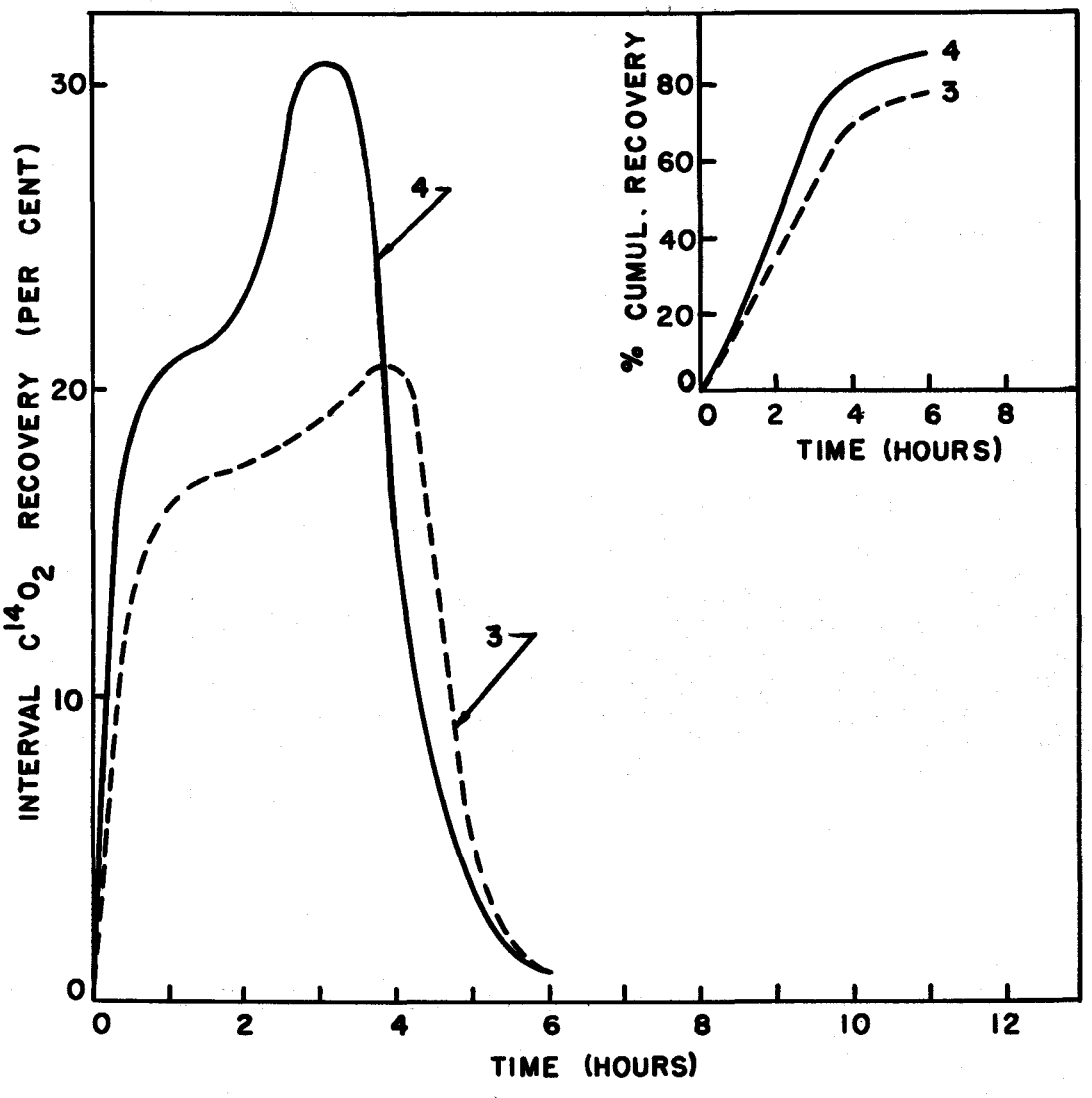

FIGURE 8. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing specifically labeled aspartic acid.

Legend: L-Aspartate-4-C^{14} ————————
L-Aspartate-3-C^{14} – – – – – – – –

TABLE XII

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Specifically Labeled Aspartic Acid

Substrate	Level		Radiochemical Recovery of Substrates, percent			
	μc	mg**	CO_2	Cell	Medium	Total
L-Aspartate-4-C^{14}	0.045	0.625	89	0	13	102
L-Aspartate-3-C^{14}	0.055	0.625	80	7	13	100

* In the experiments dl-aspartate-3- and -4-C^{14} were used as substrates; since d-aspartate was not utilized, calculations are made on the basis of l isomer being the actual substrate.

** L isomer

Experimental Conditions - incubation temperature, 29°C; cell age, 17 hours; pH of medium for radiorespirometry, 7.2; cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

Glutamic acid utilization by A. vinelandii cells was studied essentially in the same fashion described for other amino acids. Results in the preliminary experiments demonstrated that glucose was necessary for the utilization of glutamic acid, at a reasonably good rate. The radiorespirometric patterns of labeled glutamic acid utilization in presence of 5 and 300 mg glucose are shown in Figure 9. The distribution of substrate radioactivity in CO_2, cells and media is given in Table XIII. The relative rates of utilization of l and d isomers of glutamic acid was studied with the use of l-glutamate-1- and dl-glutamate-1-C^{14} as the respective substrates. The radiorespirometric patterns and substrate inventories of A. vinelandii cells metabolizing l-glutamate-1- and dl-glutamate-1-C^{14} are shown in Figure 10 and Tables XIV and XV respectively. It is evident from these data that l isomer was utilized preferentially as compared to the d isomer.

Radiorespirometric experiments employing glutamate-1-, -2-, -3, 4- and -5-C^{14} as substrates indicated the operation of a very active TCA cycle in this organism in line with previous findings (35, p. 605-617; 37, p. 221-225). Since the specifically labeled l-isomers of glutamic acid were not available, the above experiments were carried out with the specifically labeled racemic mixtures. As stated previously, l-glutamate was used preferentially to d-glutamate, furthermore the first five hours of $C^{14}O_2$ production in the radiorespirometric experiment presented in Figure 10, was

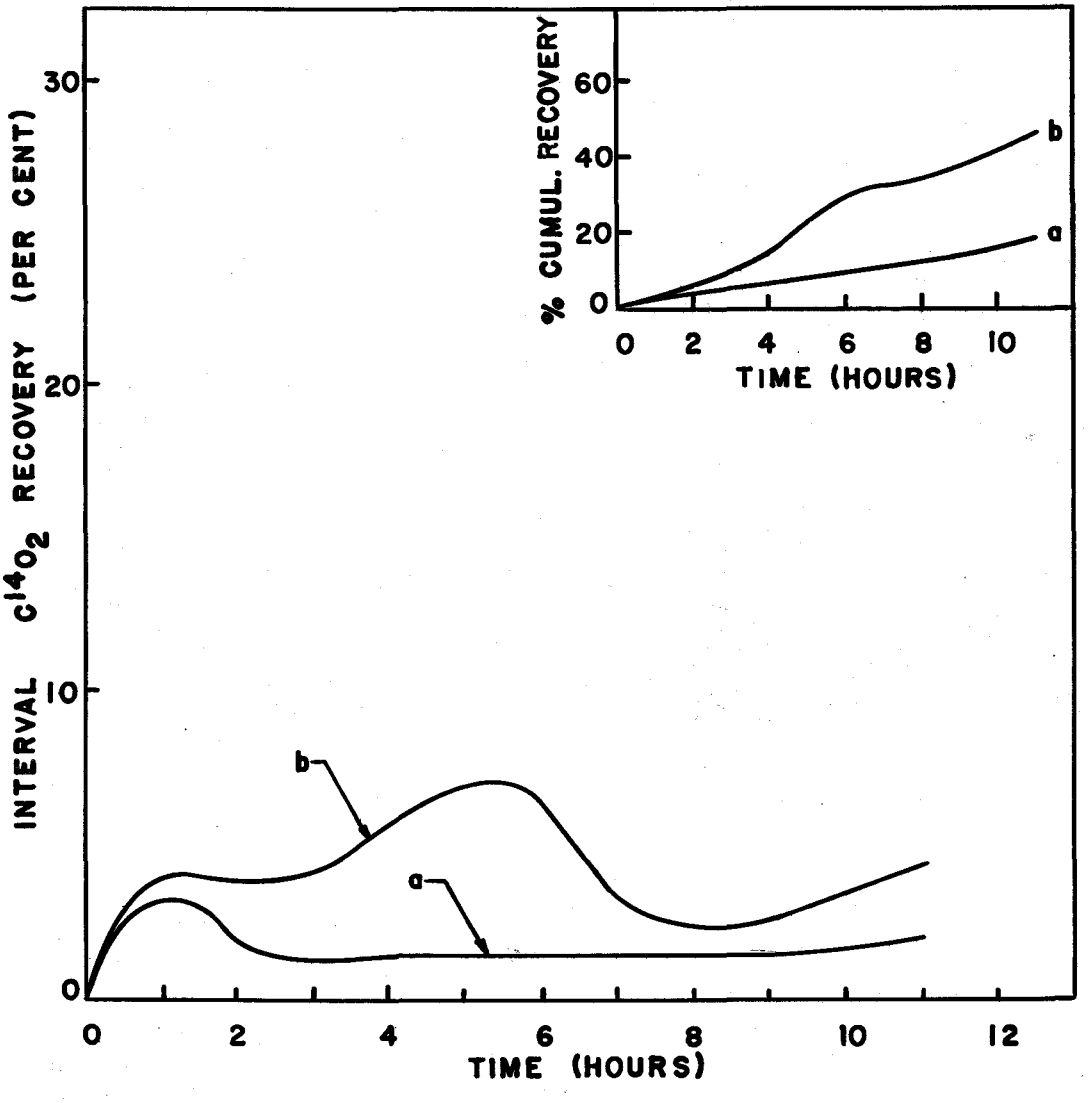

FIGURE 9. Radiorespirometric Patterns.
Azotobacter vinelandii cells metabolizing labeled glutamic acid with and without glucose.

Legend: a, without glucose; b, 300 mg glucose.

TABLE XIII

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Labeled Glutamic Acid with and without Glucose

Experiment	Glucose added to the medium mg	Substrate	Level		Radiochemical Recovery of Substrate percent			
			μc	mg*	CO_2	Cells	Medium	Total
a	5	DL-Glutamate-1-C^{14}	0.30	2.5	19	0	80	99
b	300	DL-Glutamate-1-C^{14}	0.26	2.5	47	6	45	98

* L isomer

Experimental Conditions - incubation temperature, 29°C; cell age, 15 hours; pH of medium for radiorespirometry, 7.3; initial cell weight, 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min.

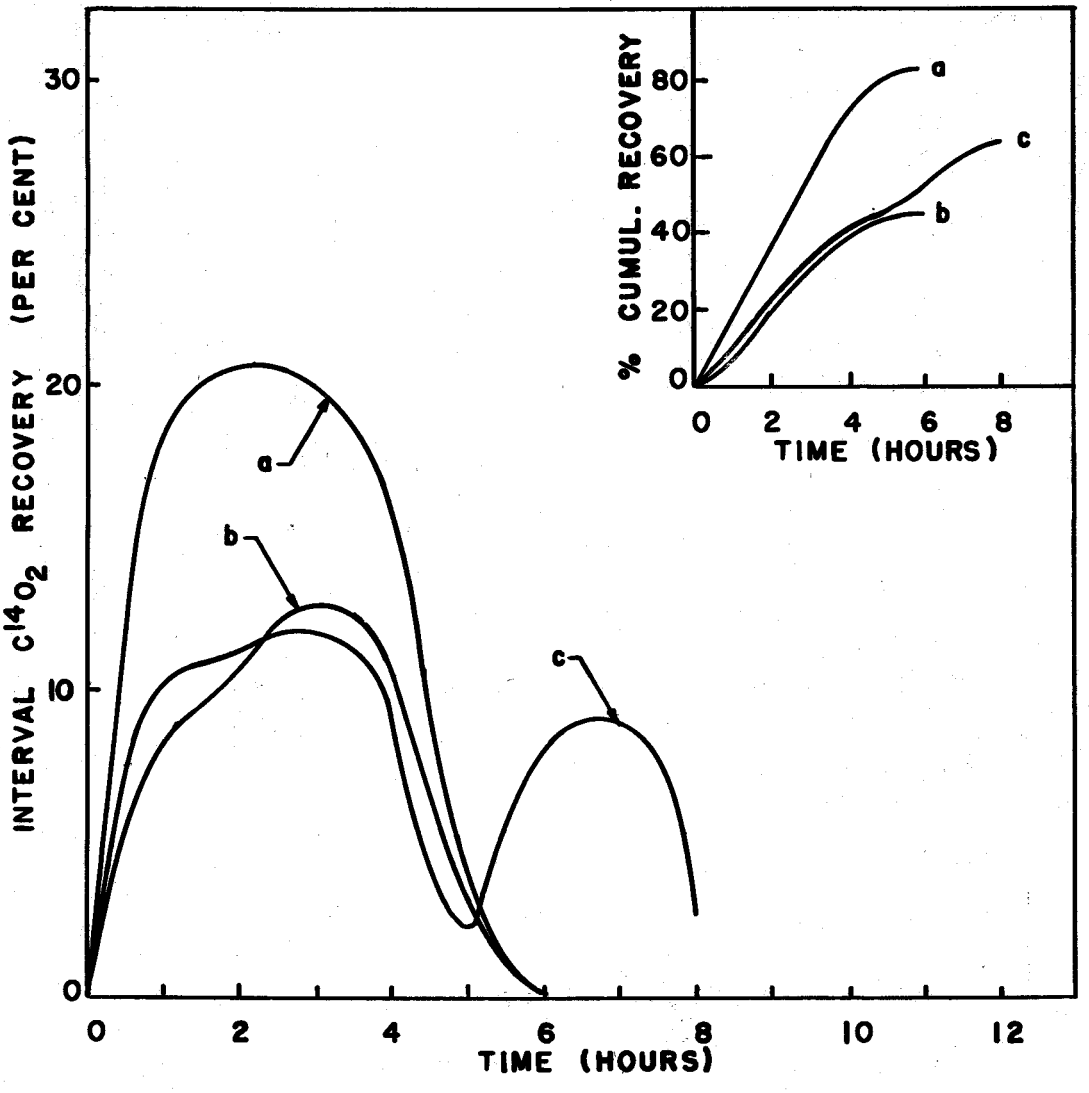

FIGURE 10. Radiorespirometric Patterns. Azotobacter vinelandii cells metabolizing labeled l- and dl-glutamatic acid.

Legend: a, L-Glutamate-1-C^{14} + 1.25 mg L-Glutamate; b, DL-Glutamate-1-C^{14} + 1.25 mg L-Glutamate + 1.25 mg D-Glutamate; c, DL-Glutamate-1-C^{14} + 1.25 mg L-Glutamate.

TABLE XIV

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Labeled L- and DL-Glutamatic Acid

Experiment	Substrate	Level		Radiochemical Recovery of Substrates percent			
		μc	mg**	CO_2	Cells	Medium	Total
a	L-Glutamate-1-C^{14}	0.26	1.25 L	85	9	4	98
b	DL-Glutamate-1-C^{14}	0.26	2.5 DL	43	7	45	95
c	DL-Glutamate-1-C^{14}	0.26	1.25 L	66 (45*)	28	6	100

* Completion of L-isomer utilization.
** DL racemic mixture, L, L isomer.

Experimental Conditions - incubation temperature, 29°C; cell age, 17 hours; pH of medium for radiorespirometry, 7.3; cell weight (dry), 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

TABLE XV

Hourly and Cumulative Recoveries of $C^{14}O_2$ from Azotobacter vinelandii Cells Metabolizing Labeled L- and DL-Glutamic Acid

Hours	Hourly Recoveries		
	L-Glutamate-1-C^{14} + 1.25 mg L-Glutamate (a)	DL-Glutamate-1-C^{14} + 2.5 mg DL-Glutamate (b)	DL-Glutamate-1-C^{14} + 1.25 mg L Glutamate (c)
1	19	8	10
2	21	10	11
3	19	12	12
4	18	10	10
5	7	2	2*
6	1	1	9
7			10
8			2

Hours	Cumulative Recoveries		
	L-Glutamate-1-C^{14} + 1.25 mg L-Glutamate (a)	DL-Glutamate-1-C^{14} + 2.5 mg DL-Glutamate (b)	DL-Glutamate-1-C^{14} + 1.25 mg L-Glutamate (c)
1	19	8	10
2	40	18	21
3	59	30	33
4	77	40	43
5	84	42	45*
6	85	43	54
7			64
8			66

* Completion of L isomer utilization.

Experimental Conditions - similar to those of Table XIV.

shown to be due to the utilization of l-glutamic acid exclusively (Table XV). Therefore the radiorespirometric patterns shown in Figure 11, are those calculated, on the basis of l-glutamate utilization only, by doubling the $C^{14}O_2$ yields observed in the dl-glutamate experiments. The distribution of substrate radioactivity in CO_2, cells and media for A. vinelandii cells metabolizing specifically labeled glutamate-1-, -2-, -3, 4- and -5-C^{14} is shown in Table XVI.

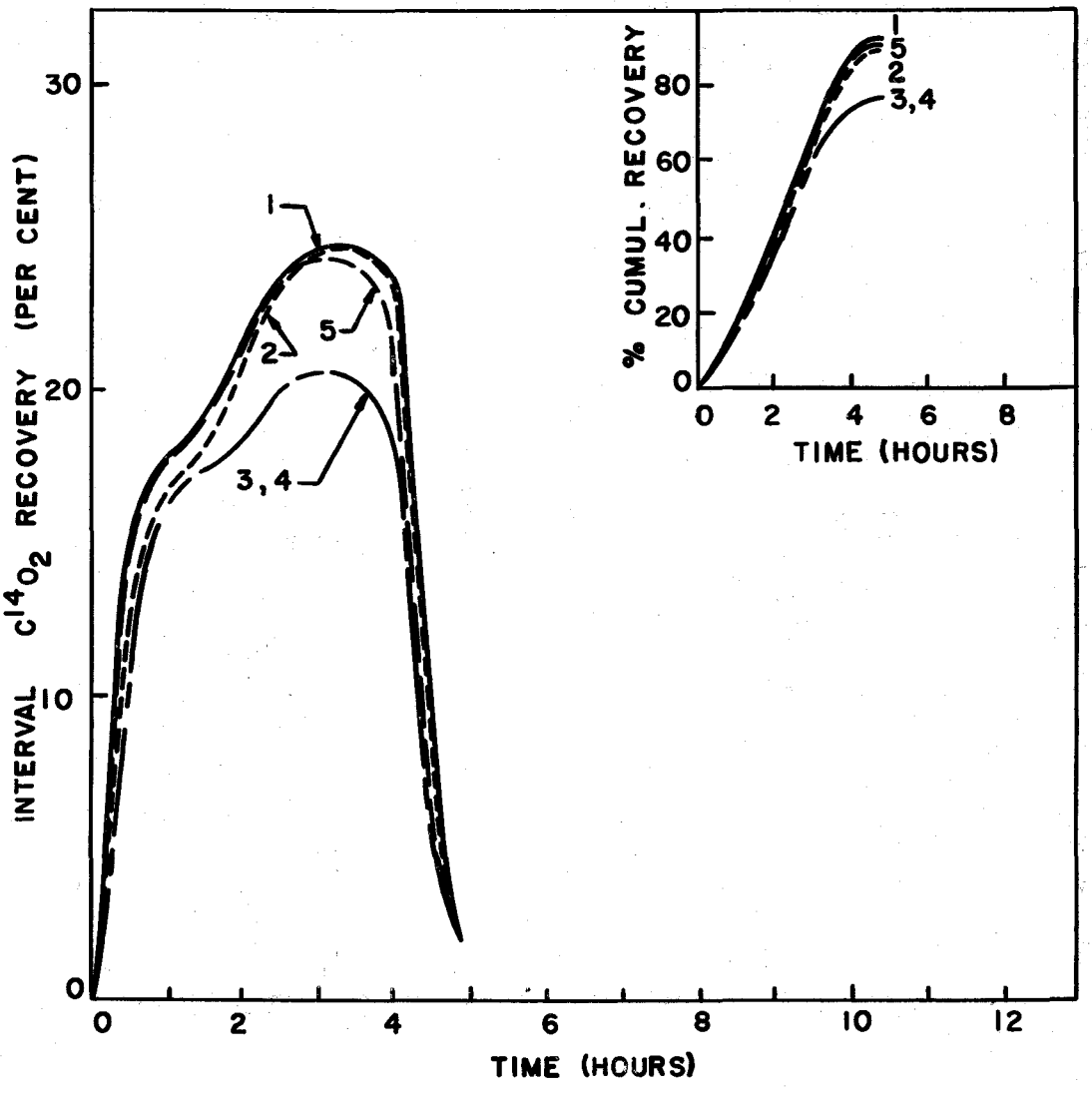

FIGURE 11. Radiorespirometric Patterns. Azotobacter vinelandii cells metabolizing specifically labeled glutamic acid.

Legend: Glutamate-1-C^{14} ——————
Glutamate-2-C^{14} - - - - - - - - - - - - -
Glutamate-3,4-C^{14} —— — ——— —
Glutamate-5-C^{14} — — — — — — — — —

TABLE XVI

Distribution of Substrate Radioactivity for Azotobacter vinelandii Cells Metabolizing Specifically Labeled Glutamic Acid

Substrate	Level		Radiochemical Recovery of Substrates percent			
	c	mg*	CO_2	Cells	Medium	Total
DL-Glutamate-1-C^{14}	0.09	0.625	45	29	24	98
DL-Glutamate-2-C^{14}	0.08	0.625	44	33	23	100
DL-Glutamate-3-4-C^{14}	0.07	0.625	38	38	25	101
DL-Glutamate-5-C^{14}	0.08	0.625	44	30	21	96
Calculations on the basis of L-Glutamate Utilization						
L-Glutamate-1-C^{14}	0.045	0.625*	90			
L-Glutamate-2-C^{14}	0.04	0.625	88			
L-Glutamate-3-4-C^{14}	0.035	0.625	76			
L-Glutamate-5-C^{14}	0.04	0.625	88			

* L isomer

Experimental Conditions - incubation temperature, 29°C; cell age, 17 hours; pH of medium for radiorespirometry, 7.3; initial cell weight, 8.5 mg; medium, 12 ml; aeration rate, 61 ml per min ; glucose, 300 mg.

DISCUSSION

The present study is aimed at the elucidation of the interrelations between the carbohydrate pathways and nitrogen metabolism in growing A. vinelandii cells. C^{14} labeled glucose and several key amino acids, related to TCA cycle intermediates were used respectively as substrates in the radiorespirometric and in the incorporation experiments.

C^{14} specifically labeled amino acids such as glutamic acid, aspartic acid, alanine, serine and glycine has been shown to be quite useful to elucidate the processes of intermediary metabolism in intact bacterial cell. Important considerations underlying the use of such amino acids can be summarized as follows:

1. The amino acids which are related to the key members of TCA cycle are relatively permeable to cells whereas the di- and tricarboxylic acids of TCA cycle are usually impermeable. Because of this characteristic, the metabolic fate of these amino acids can be used as evidence demonstrating the occurrence of TCA cycle in intact microbial cells. This point can be illustrated by the following example: In 1948, Karlsson and Barker (20, p. 913-921) using intact A. agilis cells found that citrate was not oxidized and other members of TCA cycle were utilized after prolonged periods of adaptation. The authors concluded that TCA cycle does not function in this organism. Later Stone and Wilson (35, p. 605-617; 36, p. 619-622; 37, p. 221-225) showed definitely the operation of TCA

cycle by cell free extracts of A. vinelandii.

2. The radiorespirometric technique (42, p. 1869-1872; 43, p. 207-216; 45, p. 3614-3622) provides data on the hourly and total $C^{14}O_2$ recoveries from microorganisms metabolizing C^{14} labeled substrates. This method has some advantage to the conventional Warburg technique in certain points. The radiorespirometric method enables one to follow the kinetics of $C^{14}O_2$ production from specifically labeled amino acids in the presence of other unlabeled carbon compounds. This point is important in amino acid utilization, since in some cases the amino acid in question is metabolized slowly or not at all without an energy source added to the medium e.g. glucose. In radiorespirometry, it is also possible to trace the complete utilization of administered substrate with respect to all catabolic pathways under proliferating conditions. Furthermore the analysis of the radioactivity of the incubation medium and cellular constituents, derived from the labeled substrate at the completion of the radiorespirometric experiment gives further insight to the metabolic mechanism of the organism in question.

3. The radiorespirometric studies of key amino acids may also reveal specific metabolic characteristics of individual amino acids such as l and d isomer utilization.

The first set of experiments were designed to determine whether A. vinelandii cells grown on different nitrogen sources such

as nitrate, ammonium nitrate, amino acids and molecular nitrogen would show any variation in their overall glucose catabolism. In all four cases, the radiorespirometric patterns of A. vinelandii cells metabolizing specifically labeled glucose substrates, were found to be essentially the same. It can be seen from Table II, the extents of $C^{14}O_2$ production from glucose-1-, -2- and -6-C^{14} are in the order of C-1 > C-2 > C-6 in four types of cells, grown on different nitrogen sources. This implies that concurrent operation of the ED and PP pathways and their relative participation in the overall glucose catabolism (34), function in the same manner regardless of the nitrogen metabolism of the cell.

Insofar as the metabolism of amino acids is concerned, the utilization of alanine by A. vinelandii is an interesting case. Results in a preliminary experiment revealed that l-alanine is used quite extensively by resting cells as well as under conditions which permit proliferation (Figure 1, Table III). This is in contrast to previous reports which show that alanine was not utilized by A. vinelandii (15, p. 9-19; 16, p. 1-14; 22, p. 59-72; 38, p. 894-898; 40, p. 149-161). However the techniques used in these experiments were not very reliable. Thompson (40, p. 149-161) estimated the difference in the amounts of amino nitrogen in the medium before incubation and that after three weeks of incubation. The difference was taken as the amount of amino nitrogen utilized by cells. It is well known that Azotobacter excretes large amounts of nitrogenous compounds into

the incubation medium particularly after prolonged incubation periods (17, p. 168-174). Prior to 1944 most of the nitrogen determinations in nitrogen fixation and in related experiments, mentioned in an earlier section, were done by Kjeldahl procedure. Numerous workers between 1930 and 1940 have shown that various modifications of Kjeldahl procedure do not determine the nitrogen accurately in all types of organic compounds (25, p. 419-421; 47, p. 101-108). Since the absolute amount of nitrogen is determined, the sampling errors become large, particularly in heterogenous systems. If the initial nitrogen content in a system is high and the gains in nitrogen are relatively low, the Kjeldahl method is not sensitive enough to detect small changes of nitrogen content accurately. Moreover long term, stagnant culture experiments that were used by the early workers brought out many problems such as poor aeration, pH drop in the medium, autolysis of the cells, contamination etc. Therefore earlier works on nitrogen fixation and the utilization of nitrogenous compounds cannot be fully accepted.

The kinetics of $C^{14}O_2$ production for cells metabolizing different concentrations of l-alanine revealed two distinct phases of utilization (Figure 2):

1. An initial phase at a slow and constant rate as presented by first small peak in Figure 2.

2. A later phase at a relatively faster rate of utilization represented by the second peak in Figure 2. It is possible that the

initial phase may reflect the barrier derived from substrate permeability. In the short term Warburg experiments reported, (38, p. 894-898) this initial slow phase of utilization, which covered the first two hours of the radiorespirometric experiment, may have left the impression that the utilization of alanine is very slow.

Figure 3 shows that l-and d-alanine are used concurrently and at the same rate by A. vinelandii cells. This suggests the presence of d-alanine oxidase or alanine racemase in the cells. The extent of $C^{14}O_2$ production from l and d isomers of alanine, as well as their incorporation into cellular constituents show an almost complete metabolic equivalence for the two optical isomers (Table V).

Insofar as the catabolic pathways for alanine are concerned radiorespirometric experiments using specifically labeled alanine substrates suggest that alanine is converted to pyruvic acid which is in turn oxidized to CO_2 and H_2O via the TCA cycle. As it can be seen from Figure 4 and Table VI, the rate and extent of $C^{14}O_2$ production from dl-alanine-1-, -2- and -3-C^{14} are in the order of C-1 > C-2 > C-3. It is surprising to find that a considerable amount of the isotope from C-1 of alanine was incorporated into the cellular constituents, almost to the same extent as from C-2 and C-3 of alanine (Table VI). An analysis of the cellular amino acids with regard to the distribution of label from dl-alanine-1-C^{14} revealed that the majority of the radioactivity was in the form of alanine itself, very little radioactivity appearing in glutamic acid and in an

unidentified amino acid (Table VII). This finding implies that there is a rate limiting step in the utilization of alanine. This may be the alanine transamination reaction which was reported to be slow in A. vinelandii and A. chroccum (11, p. 143-146; 12, p. 160-162). The radiorespirometric patterns for serine utilization suggest that serine is readily converted to pyruvate which in turn decarboxylated oxidatively to acetate which is in turn catabolized via the TCA cycle (Figure 5, Table VIII, NH_4NO_3 grown cells). Thus the C-1/C-3 ratio for alanine, with respect to $C^{14}O_2$ recovery, at the completion of the experiment is 5.8, which compares favorably with an analogus value of 6.6 for the ratio of C-1/C-3 of serine (Tables VI and VIII). However the kinetic picture of labeled serine utilization is quite different from that of alanine. In the former case there is only one phase of utilization which is considerably fast (Figure 5) whereas in the latter two distinct phases of utilization are displayed (Figure 6). The fast rate of conversion of dl-serine, apparently, to pyruvate suggest the presence of active l-and d-serine dehydrases.

Recent reports (31, p. 86-90) show that the nitrogen fixation seems to be coupled to carbohydrate metabolism at some steps in the TCA cycle process. Hence C^{14} labeled serine, which is assumed to be converted to pyruvate, was used as substrate in a series of experiments to determine whether or not different nitrogen sources for A. vinelandii would cause an apparent change in the catabolism of serine, hence pyruvate. The results are shown

in Table VIII. The $C^{14}O_2$ recoveries for nitrogen-fixing A. vinelandii cells, metabolizing specifically labeled serine substrates are slightly higher than the corresponding recoveries for cells grown on NH_4NO_3 and amino acids. This may mean either increased serine dehydrase activity or an higher oxidation rate via TCA cycle, or both occur in nitrogen-fixing cells.

According to previous reports, glycine was not metabolized by A. vinelandii cells to a significant extent (16, p. 1-14; 22, p. 59-72; 33, p. 408-411; 38, p. 894-898). However in the present radiorespirometric experiments, proliferating A. vinelandii cells, in the presence of a sufficient amount of energy source i.e. glucose, utilized glycine to a significant extent (Figure 6, Table IX, NH_4NO_3 grown cells). The kinetics of $C^{14}O_2$ production from the glycine substrates are similar to that of alanine, showing two phases of utilization. The dissimilarity of the radiorespirometric patterns for the utilization of serine and glycine further confirms the suggestion that serine is converted to pyruvate rather than metabolized via glycine. Cell permeability, in the case of glycine, does not pose a problem inasmuch as glycine-1-C^{14} and especially glycine-2-C^{14} are heavily incorporated into the cellular constituents of A. vinelandii, and very little activity remained in the medium. The heavy incorporation of glycine-2-C^{14} into the cellular constituents suggests that this amino acid is used very effectively in biosynthetic processes (Table IX, NH_4NO_3 grown cells).

When glycine metabolism was studied with regard to different nitrogen sources for A. vinelandii cells, the results similar to those of serine were obtained (Table IX). One can then conclude that there is no appreciable difference in the secondary carbohydrate pathways of the A. vinelandii cells regardless of the nature of the nitrogen sources.

The experiments with aspartic acid revealed that the d isomer of this amino acid is not utilized at all by A. vinelandii cells (Figure 7, Tables X and XI). Radiorespirometric patterns for the utilization of aspartate-3-C^{14} and aspartate-4-C^{14} were in line with the operation of a very active TCA cycle (Figure 8, Table XII). The kinetics of the $C^{14}O_2$ evolution from labeled aspartic and alanine substrates showed a similar profile in that there were two phases of utilization. However a close analysis of Table XII shows that C-4 and C-3 of aspartic acid are converted extensively to CO_2; the incorporation of these carbon atoms into the cellular constituents was very small in the case of C-3 and none in C-4. This is in line with previous findings which reported the presence of a strong aspartic-glutamic transaminase in Azotobacter (11, p. 143-146; 12, p. 160-162; 21, p. 85-91).

The results obtained in a preliminary experiment demonstrated that glutamic acid is utilized very slowly without an energy source in the medium (Figure 9, Table XIII). Addition of glucose to the incubation medium increased the rate and extent of $C^{14}O_2$

production from labeled glutamic acid. Unlike alanine, glutamic acid is not utilized to any significant extent by resting cells. In previous reports (33, p. 408-411; 39, p. 257-261) in which the Warburg technique and resting cells were used, glutamic acid was found to be not oxidized by A. vinelandii. However under conditions which permit proliferation, i.e., in the presence of sufficient amount of glucose, the cells utilized glutamic acid to a significant extent. The oxidative behavior of proliferating cells compared to that of resting cells is worthy of note. The fact that the cells do not utilize a certain important substrate under resting conditions, does not rule out its utilization in a medium permitting proliferation.

The duration of the experiment is another factor which must be taken into consideration. Intact A. vinelandii cells showed an initial slow phase of utilization in the case of all amino acids, except serine; this phase is probably reflecting an adaptation period, and it is about 2-3 hours in duration. However short term Warburg experiments carried out with intact cells may well give the impression that these amino acids are not utilized to a significant extent. This may be particularly true with alanine and glycine as substrates since (Figures 4 and 6) during the corresponding adaptation periods the rates of utilization are much slower than those of glutamic acid and aspartic acid utilization (Figures 8 and 11).

A. vinelandii cells utilized l-glutamic acid preferentially to d isomer (Figure 10, Tables XIV and XV). In the first five hours

of the radiorespirometric experiment, the hourly $C^{14}O_2$ recoveries of l-glutamate-1-C^{14} were exactly twice as much as that of dl-glutamate-1-C^{14} (Table XV).

The radiorespirometric patterns of cells metabolizing specifically labeled glutamate substrates, again showed the presence of a very active TCA cycle (Figure 11, Table XVI). The rate and extent of $C^{14}O_2$ production from C-2 and C-5 atoms of glutamic acid were almost equal. This suggested the complete randomization of these carbon atoms probably of the succinate stage.

SUMMARY

The interrelationships between the carbohydrate pathways and nitrogen metabolism of growing Azotobacter vinelandii cells have been studied by means of the radiorespirometric method. C^{14} labeled glucose and several key amino acids related to the TCA cycle such as glutamic acid, aspartic acid, alanine, serine and glycine were employed as tracing substrates to study the relation of primary and secondary carbohydrate pathways to nitrogen metabolism in this organism.

The findings indicate that different nitrogen sources for A. vinelandii such as molecular nitrogen, ammonium nitrate, aminoid or nitrate nitrogen do not cause an appreciable change in the primary and secondary carbohydrate pathways.

Insofar as the special metabolic aspects of amino acids were concerned, A. vinelandii cells utilize the l and d isomers of alanine concurrently and apparently at the same rate. The l isomer of glutamic acid is utilized preferentially to the d isomer, which is metabolized only after the l isomer is exhausted. D-aspartic acid is not utilized at all by A. vinelandii cells whereas the l isomer is converted extensively to CO_2. The l and d isomers of serine were both utilized.

The kinetics of $C^{14}O_2$ production for cells metabolizing labeled glutamic acid, aspartic acid, alanine and glycine revealed two phases of utilization: an initial slow phase which probably

reflects an adaptation period and a later phase at a relatively faster rate of utilization.

Alanine is utilized effectively both by resting cells as well as under proliferating conditions, whereas glutamic acid is metabolized to a significant extent only under proliferating conditions i.e. in the presence of an energy source.

The rates and extents of $C^{14}O_2$ production for cells metabolizing labeled glutamic acid, aspartic acid, alanine and serine confirmed the operation of an active TCA cycle in intact cells.

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