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GLUTAMATE BIOSYNTHESIS IN CELL-FREE EXTRACTS OF *RHODOSPIRILLUM RUBRUM*

BY

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ABSTRACT

Glutamate formation from 1-^{14}C-acetate was studied with cell-free extracts of *Rhodospirillum rubrum*. The activity of glutamate synthesis required potassium bicarbonate and C_3-compound such as phosphoenolpyruvate and pyruvate, indicating an involvement of CO_2-fixation reaction. Phosphoenolpyruvate was more active than pyruvate as an acceptor of CO_2 fixation. The radioactivity in glutamate synthesized from 1-^{14}C-acetate was found to be diluted by the addition of any intermediate from citrate to α-ketoglutarate in the tricarboxylic acid cycle. The isotope distribution in glutamate synthesized from 1-^{14}C-acetate and from ^{14}C-bicarbonate also supported the involvement of the mechanism via the tricarboxylic acid cycle.

Discussion was made on the discrepancy between the results with cell-free extracts and those with intact cells.

Introduction

Athiorhodaceae is known to be able to grow either anaerobically in light or aerobically in dark on media containing an adequate organic compound as a carbon source. Because of its adaptability to such a wide variety of growth conditions, it has been utilized as a favorable organism for the study of the mechanism of metabolic control. In this connection, the current informations on glutamate biosynthesis in *Rhodospirillum rubrum* are of great interest. Under aerobic-dark conditions, it has been established that glutamate is synthesized via the TCA cycle* in this organism as in most aerobic organisms (1). However, as to the mechanism of glutamate formation under anaerobic-light conditions, two theories, incompatible with each other, have been proposed. Some workers have claimed the involvement of the mechanism via the TCA cycle. On the other hand, there have been a number of reports which suggest the occurence of a novel mechanism which cannot be explained by the operation of the TCA cycle. The former mechanism was first proposed by Cutinelli *et al.* (2) based on the labeling pattern of glutamate isolated from the cells grown on the medium containing 1-^{13}C, 2-^{14}C-acetate or $NaH^{14}CO_3$. As a support for

* The abbreviations used are: TCA cycle, tricarboxylic acid cycle; PEP, phosphoenolpyruvate; α-KGA, α-ketoglutarate; G-6-P, glucose-6-phosphate.

this proposal, the presence of all the enzymes related to the TCA cycle was demonstrated by Eisenberg (3) in the cell-free extracts of this organism. Moreover, Benedict and Rinne (4) presented some evidence supporting that the radioactivity of ^{14}C-acetate and of $NaH^{14}CO_3$ was incorporated into glutamate via the TCA cycle in the cell-free extracts. As to the latter mechanism, Hoare (5) reported a unique labeling pattern of glutamate in the experiment with resting cell suspensions using 1-^{14}C-acetate, 2-^{14}C-acetate or $NaH^{14}CO_3$ as a labeled substrate. Similar results were also obtained by Shigesada *et al.* (6) using 1, 4-^{14}C-succinate. More recently, Evans *et al.* (7) demonstrated an activity of α-ketoglutarate synthetase in the cell-free extracts and suggested the possibility that this enzyme is involved in glutamate biosynthesis.

Although these observations are apparently incompatible with each other, a unifying explanation of them is possible if we assume that more than two mechanisms are involved in glutamate biosynthesis in *R. rubrum*. At present, however, detailed informations on the reaction mechanism of the novel pathway are lacking and much remains to be clarified for the verification of this hypothesis.

The present report deals with the demonstration of the mechanism via the TCA cycle in glutamate synthesis with the cell-free extracts as an attempt to elucidate the control mechanism of glutamate synthesis under various conditions.

EXPERIMENTAL

Materials

1-^{14}C-Acetate (25 μCi/μmole) was obtained from Daiichi Chemicals Company. $Ba^{14}CO_3$ (2 μCi/μmole), supplied by the Radiochemical Center (England), was converted to $KH^{14}CO_3$. *cis*-Aconitic anhydride and nucleotides such as ATP and NADP were purchased from Sigma Chemical Company. PEP was prepared by a modification of the method of Yoshikawa *et al.* (8). All the other organic acids and inorganic chemicals were obtained from Nakarai Chemicals Company.

Growth of Bacteria

Rhodospirillum rubrum was grown on malate-$(NH_4)_2SO_4$ medium devised by Ormerod *et al.* (9). Incubation was carried out anaerobically under the illumination with 250 watt lamp for 48 hr at 28°C. The yield of cells from 1.5 liters media was 12-13 g in wet weight. The medium used for the stock culture consisted of the following constituents per liter of distilled water: Ehrlich beef extracts, 10 g; Peptone, 10 g; KH_2PO_4, 6.4 g; $Na_2HPO_4 \cdot 12H_2O$, 3.8 g; and agar, 20 g.

Preparation of Cell-free Extracts

The cells, which were harvested by centrifugation, were washed twice with 0.05 *M* potassium phosphate buffer, pH 7.0. Then they were suspended in the same buffer, and were disrupted with a sonic disintegrator (Kubota KMS-100) at 10 kc for 15 min. The mixture was centrifuged in a cold at 100,000$\times g$ for 2 hr to remove cell debris and chromatophores. The soluble extracts obtained

were dialyzed against the phosphate buffer overnight at 4°C.

Reaction with Cell-free Extracts

Typical reaction mixture contained the following components in a final volume of 2.0 ml; ATP, 10 μmoles; NADP, 1 μmole; GSH, 2 μmoles; yeast concentrate, 1 mg; $(NH_4)_2SO_4$, 1 μmole; $MgSO_4 \cdot 7H_2O$, 2 μmoles; $MnSO_4 \cdot 4H_2O$, 0.2 μmole; potassium phosphate buffer, pH 7.0, 100 μmoles; 1.0 ml of cell-free extracts; and 1-^{14}C-acetate or $KH^{14}CO_3$ as indicated. The quantities of substrates used were indicated in each experiment. Reactions were carried out in test tubes with a side bulb under hydrogen atmosphere at 30°C for 30 min. Then the mixture was deproteinized by adding 1 ml of 10 % perchloric acid and the precipitate was centrifuged off. The excess of perchloric acid in a supernatant was precipitated as potassium salt at 0°C by adding 1 *N* KOH. The products in the supernatant were analyzed according to the methods described below.

Analytical Procedure

The supernatant obtained was passed through a column (1×5 cm) of Dowex 50 (H^+ form). The effluent was collected as the organic-acid fraction. The amino-acid fraction was obtained by eluting the column with 4 *N* NH_4OH. Both fractions were chromatographed on filter paper (Toyo Roshi No. 50). The solvent for the separation of organic acids was ether-acetic acid-water (13 : 3 : 1, v/v) (10) and that for amino acids was phenol-acetic acid-water (65 : 10 : 25) (11). Each area of the paper corresponding to glutamate, aspartate, citrate, succinate and citramalate was cut out and it was eluted with 0.1 *N* NH_4OH.

Estimation of α-KGA was carried out as 2, 4-dinitrophenylhydrazone derivative according to the method of Katsuki *et al.* (12). The derivatives were subjected to paper chromatograpy using a solvent system of butanol-ethanol-0.1 *N* $NaHCO_3$ (10 : 3 : 10). The area corresponding to α-KGA was eluted with 0.1 *N* $NaHCO_3$.

The radioactivity was counted with a Nuclear Chicago gas flow counter.

Degradation of Labeled Glutamic Acid

In general, carbon atoms of the compounds to be considered were converted into CO_2 and their specific radioactivities were measured in a form of $BaCO_3$. For the determination of the specific radioactivity of ^{14}C-glutamate as a whole, it was submitted to the wet combustion by the procedure of Knight (13), after the addition of non-labeled glutamate as a carrier.

(1) Decarboxylation with sodium azide···The method of Stoppani *et al.* (14) was slightly modified: The sample of radioactive glutamate was combined with an appropriate amount of nonlabeled glutamate and about 100 μmoles of the mixture were dissolved in 0.25 ml of 100 % H_2SO_4 placed in a reaction flask. The reaction flask was connected to a scrubber filled with a solution of 5 % potassium permanganate in 1 *N* H_2SO_4 and the scrubber was further connected to a trap which contained 5 ml of CO_2-free 0.5 *N* NaOH. After cooling on ice, 50 mg of sodium azide were added to the glutamate solution. The flask was placed in a water bath, the temperature of which was raised from 35°C to 80°C in 40-50 min, then from 80°C to 100°C in 10 min, and finally kept at 100°C for 15

min. Then the system was swept with CO_2-free air for 15 min. The $^{14}CO_2$, which was trapped in NaOH solution, was precipitated by adding 1 ml of 5 % $BaCl_2$. $Ba^{14}CO_3$ obtained, after drying in a desiccator, was weighed and counted for its radioactivity. The weight of $BaCO_3$ was corrected for the blank value which was obtained according to the same procedure as above except for the omission of glutamate. The radioactivity was corrected for the self absorption. By this method, the extent of location of ^{14}C at C-5 in glutamate molecule could be determined.

(2) Decarboxylation with Chloramine T···This procedure was carried out according to Hoare's method (5) with a slight modification. A round-bottom flask with a side bulb was used in this experiment. The sample containing about 200 μmoles of glutamate was dissolved in 0.2 ml of water and it was placed in a side bulb. Three ml of 15 % Chloramine T and 1 ml of 1 *M* citrate buffer (pH 2.5) were placed in the main compartment. This flask was connected to another round-bottom flask containing 5 ml of CO_2-free 0.5 *N* NaOH. The contents of both flasks were frozen. After the evacuation of the system, the contents of the side bulb were tipped into the main compartment. The flask containing NaOH solution was cooled in an ice-bath and the reaction flask was kept at 35°C in a water bath. After 3 hr $^{14}CO_2$ produced was counted in a form of $Ba^{14}CO_3$ as described above. This method provided an information on the location of ^{14}C at C-1 in glutamate.

When the isolation of succinate, the reaction product, was required, the reaction was carried out using 1 *M* acetate buffer (pH 4.7) instead of citrate buffer. After the reaction, succinic acid was extracted with ether using a Kutcher-Steudel extractor, and was decarboxylated with sodium azide by the same procedure as described above. By this treatment, both carboxyl-carbons of succinate, which correspond to C-2 and C-5 of glutamate, were degraded to CO_2. The location of ^{14}C at C-2 in glutamate was calculated from the data obtained by these two experiments.

RESULTS

Incorporation of Radioactivity into Glutamate from 1-^{14}C-Acetate

To elucidate the pathway of glutamate formation with the cell-free extracts, following experiments were carried out using 1-^{14}C-acetate as a labeled substrate. Benedict and Rinne (4) suggested that acetate is condensed with oxalacetate which is formed by the fixation of CO_2 with pyruvate. For the reexamination of their results the effects of pyruvate and of $KHCO_3$ on the conversion of 1-^{14}C-acetate into glutamate were investigated. As shown in Fig. 1, the incorporation of radioactivity into glutamate from 1-^{14}C-acetate was enhanced with increasing concentrations of $KHCO_3$ up to 40 m*M*. Most of the subsequent experiments were carried out at the concentration of 40 m*M* of $KHCO_3$ unless otherwise indicated. From these results it was suggested that $KHCO_3$ was necessary for the conversion of acetate to glutamate. However, it is not clear whether pyruvate is a true substrate for CO_2 fixation. There exists the possibility of the action of PEP in place of pyruvate as substrate for CO_2 fixation. Thus the effect of varying the concentration of pyruvate and PEP on the incorporation of 1-^{14}C-acetate into glutamate was investigated. The results are

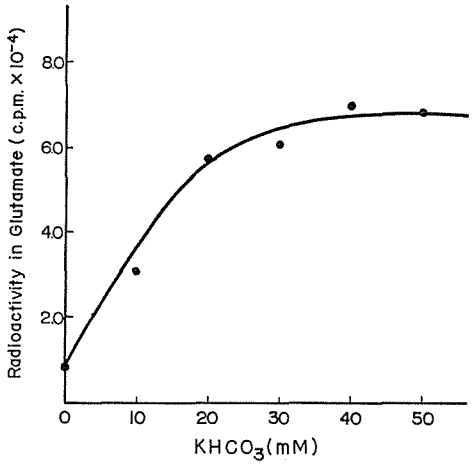

Fig. 1. Effect of bicarbonate on the incorporation of the radioactivity into glutamate from 1-^{14}C-acetate. The reaction mixture in a total volume of 2.0 ml contained 10 μmoles of ATP, 1 μmole of NADP, 2 μmoles of GSH, 1 mg of yeast concentrate, 10 μmoles of $(NH_4)_2SO_4$, 2 μmoles of $MgSO_4 \cdot 7H_2O$, 0.2 μmole of $MnSO_4 \cdot 4H_2O$, 100 μmoles of potassium phosphate buffer, pH 7.0, the enzyme (19 mg of protein), 0.5 μmole of pyruvate, 5.5×10^5 c.p.m. (0.03 μmole) of $CH_3{}^{14}COONa$, and indicated amounts of $KHCO_3$. The reaction was carried out as described in the text.

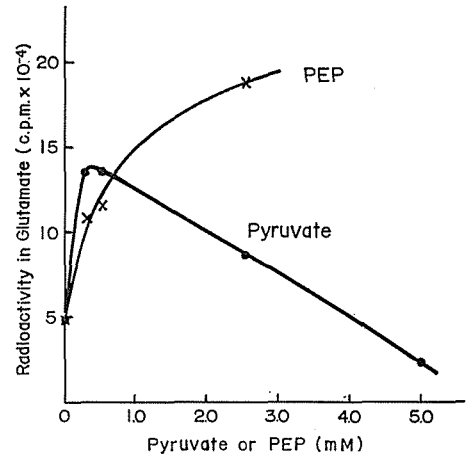

Fig. 2. Effect of pyruvate or PEP on the incorporation of the radioactivity into glutamate from 1-^{14}C-acetate. The reaction mixture was the same as described in Fig. 1 except for the use of 80 μmoles of $KHCO_3$ and indicated amounts of pyruvate or PEP. The other conditions were described in the text.

shown in Fig. 2. When pyruvate was used as an acceptor for CO_2 fixation, the maximum incorporation of radioactivity was observed at the concentration of 0.25 m*M*. Above this concentration, however, it decreased inversely with increasing concentrations of pyruvate. On the contrary, the incorporation into organic-acid fraction icreased linearly with increasing concentrations of pyruvate. In this case the radioactivity was found to be incorporated into citramalate. These observations suggested that at higher concentrations of pyruvate, condensation reaction of pyruvate with acetyl-CoA becomes predominant over its reaction with CO_2, and that citramalate was not metabolized further to glutamate. On the other hand, such a decrease of the incorporation into glutamate was not seen in the case of PEP.

Incorporation of Radioactivity into Glutamate from $KH^{14}CO_3$

From the results shown in Figs. 1 and 2, it was expected that glutamate was synthesized from C_3-compound, bicarbonate and acetate. Therefore, the following experiments were carried out to confirm this idea. The incorporation of ^{14}C into glutamate and organic acids from ^{14}C-bicarbonate was examined when pyruvate or PEP was used as an acceptor. The results are shown in Fig. 3. It is evident that C_3-compound such as pyruvate or PEP was necessary for CO_2 fixation and that the latter was more active than the former. These results suggest an involvement of PEP carboxylase [EC 4.1.1.31] rather than pyruvate carboxylase [EC 6.4.1.1] in the fixation reaction.

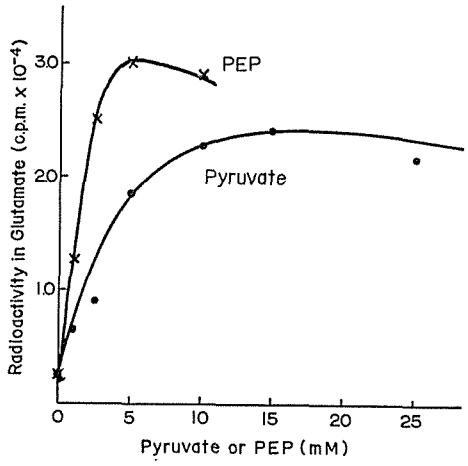

Fig. 3. Effect of pyruvate or PEP on the incorporation of the radioactivity into glutamate from $KH^{14}CO_3$. The reaction mixture was the same as described in Fig. 2 except for the use of 10 μmoles of CH_3COONa and 2×10^6 c.p.m. (10 μmoles) of $KH^{14}CO_3$ instead of $CH_3{}^{14}COONa$ and $KHCO_3$.

Cofactor Requirement for the 1-^{14}C-Acetate Conversion into Glutamate

It was found that the following cofactors were required for the formation of ^{14}C-glutamate: ATP, CoA, NADP, Mg^{++} and Mn^{++}. NAD caused a slight inhibition.

When G-6-P was added to the reaction mixture for the generation of $NADPH_2$, ^{14}C incorporation into glutamate decreased, while that into organic acids increased. The examination of chromatogram of the ^{14}C-organic acids revealed a remarkable increment of radioactivity in citrate. This indicates that G-6-P, catalyzed by the endogenous G-6-P dehydrogenase, took a role in the reduction of NADP which is necessry for isocitrate dehydrogenation to α-KGA.

Dilution of Radioactivity in Glutamate by the Addition of Some Compounds

If the idea that glutamate is synthesized via the TCA cycle is valid, the addition of an intermediate from citrate to α-KGA in the TCA cycle is expected to cause a dilution of the radioactivity of glutamate synthesized. Table I shows the results which were obtained when the reaction was carried out in the presence of α-KGA. As can be seen in the table, the radioactivity of glutamate was lowered by the addition of α-KGA. In addition, the specific radioactivity of α-KGA, after the reaction, was higher than that of glutamate at any concentration of α-KGA added. This means that α-KGA is an intermediate in the bio-

Table I. Dilution of the Radioactivity in Glutamate Synthesized from 1-^{14}C-Acetate by α-KGA.

The reaction mixture contained 80 μmoles of $KHCO_3$ in addition to the components described in Fig. 1.

α-KGA added	Radioactive compound isolated					
	α-KGA			Glutamate		
	Incorporation of ^{14}C	Amounts	Specific radioactivity	Incorporation of ^{14}C	Net synthesis	Specific radioactivity
μmoles	c.p.m. $\times10^{-3}$	μmoles	c.p.m. $\times10^{-3}$ per μmole	c.p.m. $\times10^{-3}$	μmoles	c.p.m. $\times10^{-3}$ per μmole
0	6.42	0.02	321.0	189.0	2.04	92.6
10	92.1	7.06	13.0	20.1	3.45	5.8
20	107.0	12.8	8.4	9.3	3.21	2.9
30	117.0	22.1	5.3	5.8	3.93	1.5
40	128.0	35.1	3.6	3.6	4.09	0.9

synthesis of glutamate. Table II summarizes the effect of the addition of citrate, *cis*-aconitate, isocitrate, citramalate, mesaconate, malate and succinate on the incorporation of the radioactivity into glutamate from 1-^{14}C-acetate. Similarly to α-KGA, the compound such as citrate, *cis*-aconitate and isocitrate caused a dilution of radioactivity of glutamate. But citramalate and mesaconate were ineffective. Succinate and malate stimulated the conversion of 1-^{14}C-acetate to glutamate. It seems that these two acids exerted their actions in supplying oxalacetate to form citrate by the condensation reaction with 1-^{14}C-acetate.

Table II. Effect of Some Compounds on the Radioactivity in Glutamate Synthesized from 1-^{14}C-Acetate.

Ten μmoles of each compound were added to the reaction mixture described in Table I.

Addition	Radioactivity in Glutamate
	c.p.m.$\times 10^{-3}$
none	57.0
Citrate	3.9
cis-Aconitate	2.7
DL-Isocitrate	3.5
DL-Citramalate	56.9
Mesaconate	57.2
DL-Malate	138.0
Succinate	94.1

Labeling Pattern of Glutamate Synthesized

The results described above suggest an involvement of the TCA cycle in the synthesis of glutamate in the cell-free extracts. To confirm this conclusion, the location of ^{14}C in glutamate formed from 1-^{14}C-acetate or ^{14}C-bicarbonate was examined. As shown in Table III, partial degradation of ^{14}C-glutamate formed from ^{14}C-bicarbonate with Chloramine T revealed that 100% of total radioactivity was located at C-1, and when 1-^{14}C-acetate was used as substrate 95% of total radioactivity was present at C-5.

Table III. Location of Label in Glutamate Synthesized from 1-^{14}C-Acetate and $KH^{14}CO_3$.

In Expt. 1 and 2, glutamate was degraded with sodium azide, and in Expt. 3, 4, 5 and 6, with Chloramine T.

Expt. No.	Labeled substrate	Specific activity of Glutamate	Specific activity of $BaCO_3$	Percent of radioactivity at	
				C-5	C-1
		c.p.m./μmole	c.p.m./μmole	%	%
1	$CH_3{}^{14}COONa$	270.0	250.0	92.6	
2	,,	209.5	196.8	94.0	
3	,,	272.4	0.5		0.2
4	,,	247.0	1.1		0.5
5	$KH^{14}CO_3$	210.7	207.8		98.6
6	,,	80.0	82.5		103.2

DISCUSSION

The observations described in this paper suggest that glutamate is synthesized from acetate via the TCA cycle in the cell-free extracts of *R. rubrum* grown even under anaerobic conditions. As summarized in Fig. 4, acetyl-CoA is condensed with oxalacetate to form citrate, which is converted to glutamate

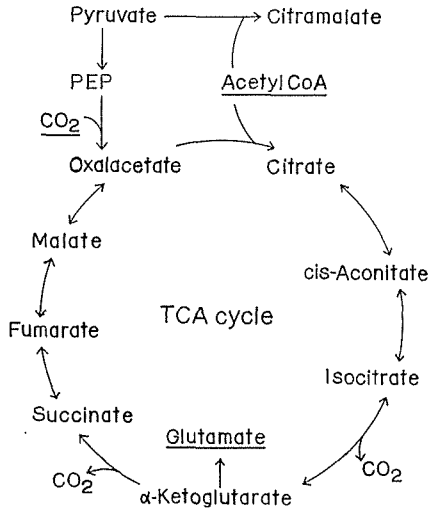

Fig. 4. Pathway for glutamate biosynthesis in the cell-free extracts of *R. rubrum*.

via *cis*-aconitate, isocitrate and α-KGA, succesively.

Cheldelin *et al.*(15) proposed that in *Acetobactor suboxydans* glutamate is synthesized via citramalate and mesaconate from acetate. Benedict (16) also proposed the possibility of the involvement of the citramalate pathway with *R. rubrum* based on the observation that acetyl-CoA was condensed with pyruvate to form citramalate. Although we also observed that 1-^{14}C-acetate was incorporated into citramalate in the presence of pyruvate, we did not obtain any evidence that citramalate was metabolized further to glutamate.

Furthermore, labeling pattern of the glutamate molecule provided an additional information on this problem. The following scheme represents the labeling patterns of glutamate which were synthesized by various mechanisms:

$$\underset{\mathbf{c}\qquad\qquad\mathbf{b}}{HOOCCH_2CH_2CH(NH_2)COOH} \qquad \text{(found)}$$

$$\underset{\mathbf{c}\qquad\qquad\mathbf{b}}{HOOCCH_2CH_2CH(NH_2)COOH} \qquad \text{(via the TCA cycle)}$$

$$\underset{\mathbf{b}\qquad\qquad\mathbf{c}}{HOOCCH_2CH_2CH(NH_2)COOH} \qquad \text{(via citramalate)}$$

where **b** indicates the carbon atom derived from bicarbonate, and **c** from carboxyl carbon of acetate. The observed pattern was compatible with the mechanism via the TCA cycle but not with the one via the citramalate pathway. It is concluded from these results that the citramalate pathway is of little physiological significance, if any, for glutamate biosynthesis with the cell-free extracts.

It must be emphasized that C_3-compound and bicarbonate are necessary for the conversion of acetate to glutamate. This suggests that CO_2 fixation with C_3-compound is an important step for the formation of glutamate. Benedict and Rinne (4) proposed that pyruvate carboxylase plays an important role to supply oxalacetate. As mentioned already, however, PEP was a much more effective substrate than pyruvate. It is likely that oxalacetate was formed by the action of PEP carboxylase rather than pyruvate carboxylase. In our system a considerable amount of CO_2 fixation was observed even when pyruvate was used as substrate. This is explainable if PEP is synthesized from pyruvate by the action of PEP synthase. In fact the existence of PEP synthase and its operation for the synthesis of PEP from pyruvate and ATP was demonstrated with *E. coli* (17) and *R. rubrum* (18).

All the observations obtained support the mechanism via the TCA cycle for the synthesis of glutamate with the cell-free extracts and no evidence suggesting

the involvement of other pathway was obtained. In contrast to this conclusion, however, several lines of evidence for the occurence of a novel mechanism of glutamate synthesis have been proposed based on the experiments with intact cells (5, 6). Previously we also observed that glutamate isolated from the cells, which were incubated with 1-^{14}C-acetate or 1, 4-^{14}C-succinate under anaerobic-light conditions, had unique labeling patterns as illustrated in Table IV. The most important point to be noted with these results is that a significant proportion of radioactivity was found at C-2 of glutamate from 1-^{14}C-acetate, and into C-2 and C-5 from 1, 4-^{14}C-succinate, respectively. These labeling patterns are extremely different from those expected with the mechanism via the TCA cycle. These results may be explained if we assume the involvement of either the reductive carboxylation of succinate by α-KGA synthase (18) or the succinate-glycine cycle originaly proposed by Shemin and Russell (19). The preliminary communication suggesting a possible occurence of both reactions in this organism were already demonstrated (7, 20). From these considerations, it is attracting to assume that there exist more than two pathways for glutamate biosynthesis in this organism and that the relative contribution of them is controlled by the environmental conditions under which the cells were grown.

Table IV. Labeling Pattern of Glutamate Synthesized with Whole Cells.

Labeled substrate	Conditions for incubation	Distribution of label at				
		C-1	C-2	C-3	C-4	C-5
		%	%	%	%	%
1-^{14}C-Acetate	[Found]					
	(1) anaerobic-light in the absence of pyruvate	5	16	0	0	70
	(2) anaerobic-light in the presence of pyruvate	5	0	0	0	95
	[Theoretical via TCA cycle]					
	(1) without recycling	0	0	0	0	100
	(2) with extensive recycling	33	0	0	0	67
	(3) coupled with the reaction by pyruvate synthase*	0	0	50	0	50
1, 4-^{14}C-Succinate	[Found]					
	(1) anaerobic-light	30	30	0	0	30
	(2) aerobic-dark	100	0	0	0	0
	[Theoretical via TCA cycle]	100	0	0	0	0

* This indicates that acetate is converted to pyruvate by the action of pyruvate synthase.

One of the reasons for the discrepancy between the results with the cell-free extracts and those with intact cells may be attributed to the inadequate preparation of the cell-free extracts. These experiments were carried out with the preparation from which chromatophores, the photochemical apparatus of this organism, were depleted. There have been some observations suggesting that the operation of the novel mechanism is intimately coupled with the activity of the photochemical reaction system. In addition, it was reported that under anaerobic-light conditions the unique labeling pattern of glutamate was not ob-

tained when the cells were incubated with a substrate at higher level of oxidation such as pyruvate (21). If the above suggestion is the case, it may be important to investigate this problem using the preparation with intact photochemical activity in order to demonstrate the novel reaction with the cell-free extracts. For the elucidation of this interesting problem, further cautious experiments are to be undertaken.

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