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STUDIES ON THE INCORPORATION OF $^{14}CO_2$ INTO GLUTAMIC ACID IN *CHLORELLA PYRENOIDOSA*

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Abstract—The incorporation of carbon-14 into glutamate from $^{14}CO_2$ in light and dark and from glucose-U-^{14}C has been investigated in *Chlorella*. In all cases the rate of glutamate labelling exceeds that of the citrate. The sequence of formation of the products of dark $^{14}CO_2$ fixation and the position of the carbon-14 within aspartic acid (C_4 = 60–70%) and glutamic acid (C_1 = > 95%) is consistent with a carboxylation of phosphoenolpyruvate giving oxaloacetate which is metabolized via the TCA cycle to give glutamate, citrate behaving as an asymmetric molecule. Addition of fluoroacetate resulted in all cases in an inhibition of glutamate labelling and in an accumulation of radioactive citrate. It was concluded that in both light and dark the activity in glutamate derived from $^{14}CO_2$ passes through citrate and the TCA cycle.

IN AUTOTROPHICALLY grown *Chlorella*, under steady state conditions in dark[1] or light[2], glutamic acid is not a primary carboxylation product but with time contains an increasing proportion of the total incorporated $^{14}CO_2$. Moses *et al.*[1] concluded that $^{14}CO_2$ was incorporated in the dark by a carboxylation of phosphoenolpyruvate into oxaloacetate and the activity transferred to glutamate via the TCA cycle. In the light in the presence of ammonium ions $^{14}CO_2$ is incorporated into glutamate 10 times more rapidly than into citrate and Bassham and Kirk[3] suggested that this might indicate an important light-stimulated pathway of glutamate synthesis other than through the TCA cycle. Unpublished dark $^{14}CO_2$ fixation experiments of the present author however indicated that this phenomenon was not necessarily light specific, and a reinvestigation of glutamate labelling from $^{14}CO_2$ was undertaken.

RESULTS

In Fig. 1 are shown the results of a typical dark $^{14}CO_2$ fixation experiment; the main labelled products during the first minutes were aspartate and malate, subsequently glutamate and citrate become radioactive. After 8 min, activity was also found in sugar phosphates, serine and alanine. Glutamate was more rapidly labelled than citrate and in this respect similar to the results of Bassham and Kirk obtained in the light. Degradation of the radioactive aspartate using an aspartic decarboxylase preparation showed (Fig. 2) that 60–70% of its activity was located in the carbon 4 (the β carboxyl) at all times between 4 and 60 min. Removal of both carboxyls by ninhydrin showed the activity to be confined to carbons 1 and 4 of the aspartate. Degradation of the radioactive glutamate showed the carbon 14 to be confined to the C_1 position. The above results are consistent with carboxylation of pyruvate or phosphoenolpyruvate to give oxaloacetate and hence aspartate by transamination and

[1] V. MOSES, O. HOLM-HANSEN and M. CALVIN, *J. Bacteriol.* 77, 70 (1959).
[2] A. A. BENSON and M. CALVIN, *J. Exp. Botany* 1, 63 (1950).
[3] J. A. BASSHAM and M. KIRK, *Biochim. Biophys. Acta* 43, 447 (1960).

malate by reduction. The oxaloacetate is metabolized via the TCA cycle to give α-ketoglutarate and hence glutamate. For C_4 labelled aspartate to give rise to C_1 labelled glutamate citrate must behave as an asymmetric molecule (as has been shown in animal tissues[4]) the hydroxyl in isocitrate being formed on that part of the molecule contributed by oxaloacetate. The C_1 of oxaloacetate is then lost in the decarboxylation of isocitrate to give α-ketoglutarate.

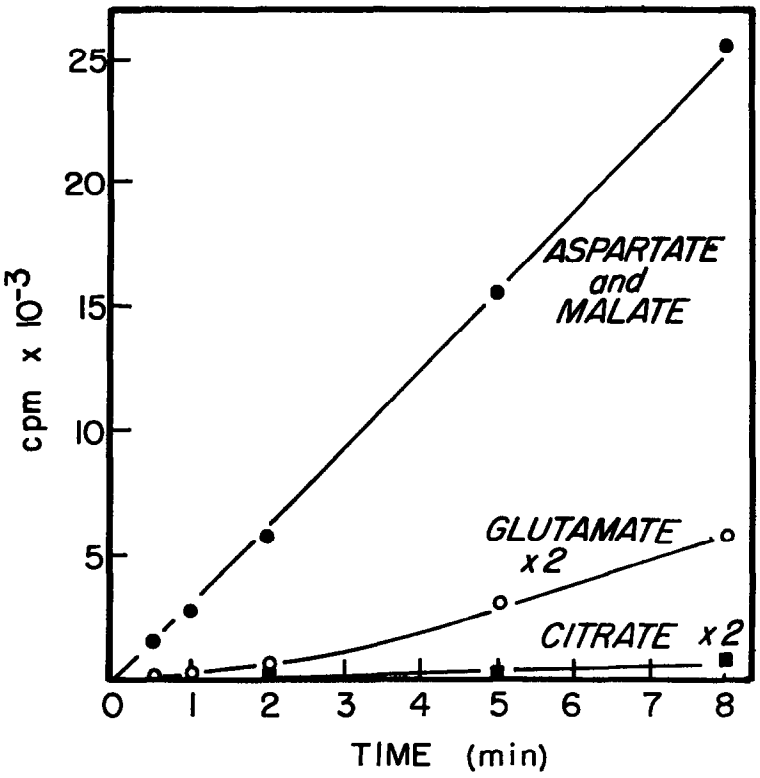

FIG. 1. TIME COURSE OF DARK $^{14}CO_2$ FIXATION INTO GLUTAMATE, CITRATE ASPARTATE AND MALATE.

Formation of C_1 labelled glutamate would also result from a carboxylation of γ-aminobutyrate[5,6] but no evidence for this reaction was found in the present experiments. When the $^{14}CO_2$ was replaced by $^{12}CO_2$ in an experiment like that in Fig. 1, the activity in aspartate and malate fell subsequent to the change over, while the activity in glutamate continued to rise.

Further evidence for the derivation of labelled glutamate from the aspartate via citrate was obtained by use of the inhibitor fluoroacetic acid.[7] In the presence of this inhibitor (10^{-2} M) a more rapid initial labelling of citrate than glutamate was observed from $^{14}CO_2$ in the dark (Fig. 3). In several experiments with fluoroacetate a marked stimulation of total $^{14}CO_2$ uptake was also observed.

[4] V. R. POTTER and C. HEIDELBERGER, *Nature* **190**, 553 (1949).
[5] O. WARBURG, H. KLOTZSCH and G. KRIPPAHL, *Naturwiss.* **44**, 235 (1957).
[6] W. VISHNIAC and R. C. FULLER, *Fed. Proc.* **17**, 328 (1958).
[7] R. A. PETERS, *Proc. Roy. Soc. (London) B.* **139**, 143 (1952).

When NH_4^+ is added to a *Chlorella* suspension the rate of $^{14}CO_2$ fixation into both TCA cycle compounds and glutamate is greatly increased.[8] This NH_4^+ stimulated glutamate labelling was also found to be inhibited by fluoroacetate.

Similar results were obtained when glucose-U-^{14}C was metabolized by *Chlorella* in the dark in the presence of fluoroacetate (Table 1). The sequence of labelling of the ethanol soluble compounds from glucose is consistent with its metabolism via the EMP pathway to pyruvate and then by the TCA cycle to α-ketoglutarate and glutamate. Glutamate becomes

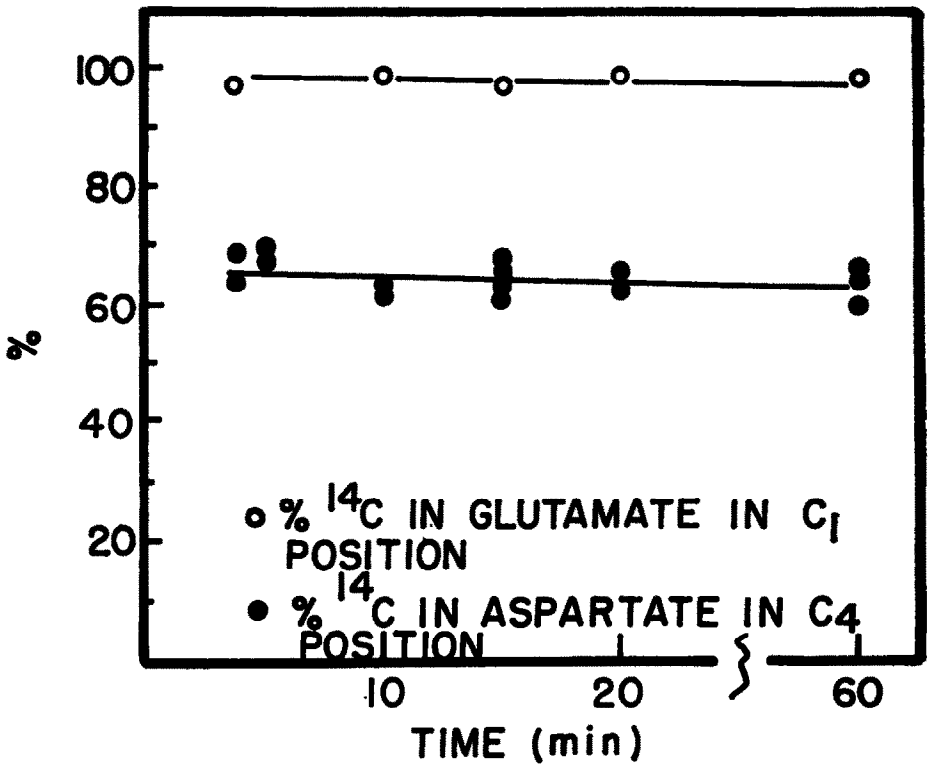

FIG. 2. PROPORTION OF THE ^{14}C IN ASPARTATE ● IN CARBON 4 AND IN GLUTAMATE ○ IN CARBON 1 AFTER DIFFERENT TIMES OF EXPOSURE TO $^{14}CO_2$ IN THE DARK.

radioactive more rapidly than citrate in the control but the reverse is the case in the presence of fluoroacetate. The inhibitor also increased the rate of flow of carbon through the sugar phosphates to alanine and compounds of the TCA cycle. Unpublished experiments have shown that 10^{-2} M acetate has similar effects.

In experiments to investigate the effect of fluoroacetate on glutamate labelling in the light, *Chlorella* cells in the presence of 10^{-3} M NH_4Cl were exposed to $^{14}CO_2$ for 1 min, then flushed with $^{12}CO_2$ and the levels of ^{14}C in citrate and glutamate followed for the next 15 min. Fluoroacetate (10^{-2} M) decreased the activity in glutamate and increased that in citrate, whereas the rates of citrate and glutamate labelling in the control were initially equal. In a

[8] R. G. HILLER, *J. Exp. Botany* **15**, 15 (1964).

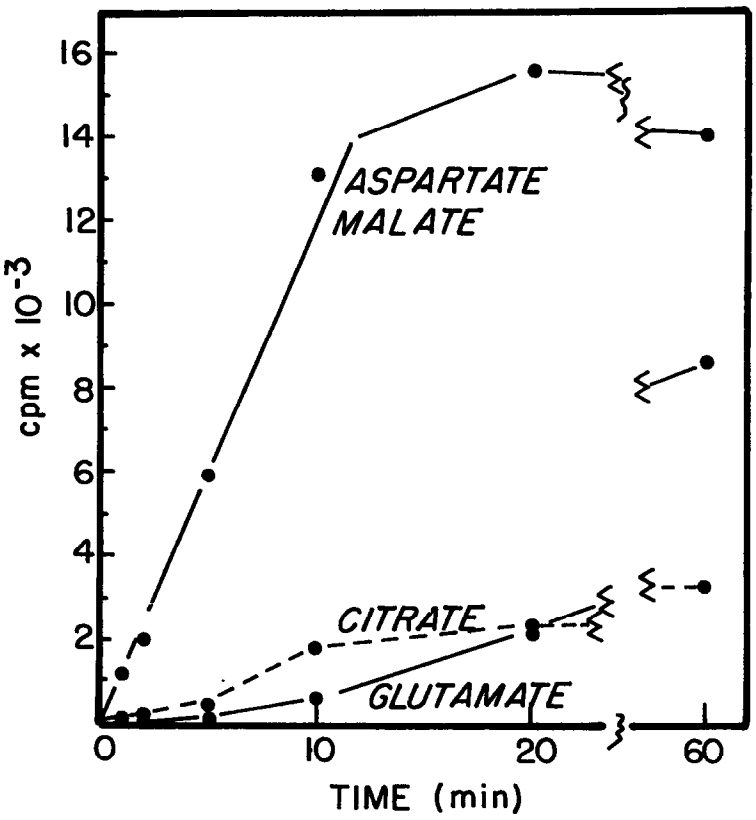

FIG. 3. TIME COURSE OF DARK $^{14}CO_2$ FIXATION IN THE PRESENCE OF 10^{-2}M FLUOROACETIC ACID INTO GLUTAMATE, CITRATE, ASPARTATE AND MALATE.

TABLE 1. EFFECT OF FLUOROACETATE ON GLUCOSE METABOLISM IN *Chlorella* IN THE DARK.

	Control, time (min)			+ Fluoroacetate, time (min)		
Compound	3	8	15	3	8	15
Sugar phosphates	23·9	20·8	9·77	22·3	11·5	4·59
Glutamate	0·48	0·98	2·42	+	0·44	1·30
Citrate	—	+	0·09	0·27	1·80	2·97
Alanine, Malate, Aspartate	1·19	4·21	7·53	4·03	12·57	12·44

Conditions: 1000-μl packed cells in 27·7 ml/10^{-3} M KH_2PO_4 containing fluoroacetate (final conc. 10^{-2} M). The reaction was started by the addition of 3·0 μc (3·0 μg) glucose-U-^{14}C and stopped by taking a 6-ml sample directly into boiling alcohol.

All figures are counts per minute x 10^{-6}; + = detectable.

further experiment the cells were exposed to $^{14}CO_2$ under similar conditions to those of Bassham and Kirk[3] and the effect of two concentrations of fluoroacetate on the rate of citrate and glutamate labelling investigated. The results are shown in Fig. 4. The total $^{14}CO_2$ incorporation, the ethanol soluble radioactivity and the total activity in sugar phosphates is similar in

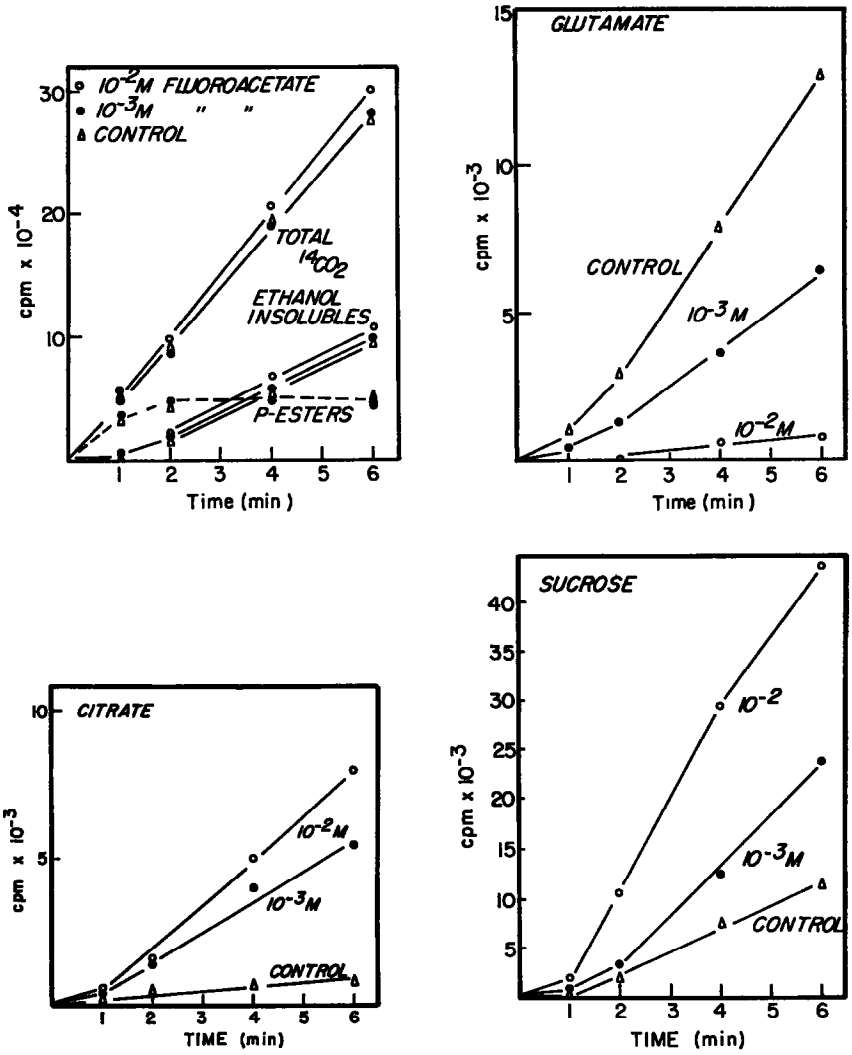

FIG. 4. EFFECT OF TWO CONCENTRATIONS OF FLUOROACETATE ON SOME OF THE PRODUCTS OF PHOTOSYNTHETIC $^{14}CO_2$ FIXATION IN *Chlorella* CELLS SUPPLIED WITH 10^{-3} M NH_4^+.

all the treatments. In the control the activity in glutamate comprises 5–7% of the total radioactivity after 4–6 min and is approximately 5 times as rapidly labelled with ^{14}C as is the citrate; in the presence of 10^{-3} M fluoroacetate the rates are equal; at 10^{-2} M fluoroacetate the rate of citrate labelling greatly exceeds that of the glutamate. At the higher concentration of the inhibitor the inhibition of ^{14}C incorporation into glutamate is not accompanied by a corresponding increase in the activity of citrate. At both levels of the inhibitor there is a marked

increase in the activity of sucrose. The increase in citrate concentration by itself is unlikely to be the explanation of this last result, which may be an indirect effect of a reduced ammonia uptake brought about by inhibition of α-ketoglutarate formation. It has been previously shown[9] that addition of ammonium ions to photosynthesizing *Chlorella* results in a decreased incorporation of ^{14}C into sucrose and an increase into amino acids.

The results described above show that when ^{14}C is supplied to *Chlorella* under aerobic conditions in light or dark as $^{14}CO_2$ or as glucose-U-^{14}C, glutamate is more rapidly labelled than the citrate but the experiments with fluoroacetate indicate that glutamate is formed via the TCA cycle under both conditions. Since, however, even 10^{-2} M fluoroacetate does not completely inhibit ^{14}C incorporation into glutamate this suggests that plant aconitase has a relatively low affinity for fluorocitrate compared to that of the animal enzyme.[10]

In the type of experiment described the rates of labelling quoted are net rates based on levels of activity in radioactive spots on paper chromatograms. If there is a small active pool of citrate which equilibrates slowly with a larger inactive one, and ^{14}C flows rapidly from the active citrate pool to a single large pool of glutamate, then the relative net rates of labelling of the two compounds may be of any magnitude. Furthermore the specific activity of glutamate could exceed that of the citrate in the above case. In *Chlorella* the total citrate concentration on a molar basis is of the order of 1/100 that of the glutamate. Consequently the rate of glutamate labelling is likely to exceed that of the citrate at all but the shortest times. In the photosynthetic experiment (Fig. 4) the specific activity of citrate is, however, approximately 10 times that of the glutamate.

MATERIALS AND METHODS

Chorella pyrenoidosa (Emerson Strain) was grown autotrophically under conditions of constant temperature (25°), CO_2 concentration (4% CO_2 in air v/v) and incident light intensity (two banks of 4×100 W tungsten filament bulbs). After 4 days of growth the cells were harvested by centrifugation and after washing once, resuspended in the experimental medium. This was 5×10^{-4} M KH_2PO_4 for the dark $^{14}CO_2$ fixation and glucose feeding experiments, and phosphate buffer pH 7·3 in which there was a final concentration of 10^{-3} M $NH_4H_2PO_4$ for the photosynthetic experiment. Fluoroacetic acid was added as the potassium salt at pH 6·0 for the glucose-U-^{14}C feeding and dark $^{14}CO_2$ fixation experiments, and at pH 7·3 for the photosynthetic experiment.

Chlorella cells were exposed to $^{14}CO_2$ (i) in the dark by injecting $Na_2{}^{14}CO_2$ through a rubber "subaseal" cap into the cell suspension into a conical flask (7 ml vol.) shaken on a mechanical shaker; (ii) in the light by injecting 25 μc $Na_2{}^{14}CO_3$ into a "lollipop" containing the cells and flushed continuously with 4% CO_2 in air. This flushing was stopped 15 sec after the addition of the $^{14}CO_2$.

The reactions were terminated by injecting a mixture of glacial acetic acid: ethanol (1:4) or passing the cells directly into boiling ethanol. The cells were then extracted with boiling 80% ethanol and subsequently re-extracted with 20% ethanol. Both extracts were combined and taken to dryness *in vacuo*. Subsequent paper chromatographic procedures followed that of Bassham and Calvin[11] except that butanol: acetic acid: water was the second developing

[9] V. MOSES, O. HOLM-HANSEN, J. A. BASSHAM and M. CALVIN, *J. Mol. Biol.* **1**, 21 (1959).
[10] D. H. TREBLE, D. T. A. LAMPORT and R. A. PETERS, *Biochem. J.* **85**, 113 (1962).
[11] J. A. BASSHAM and M. CALVIN, *The Path of Carbon in Photosynthesis*, Prentice Hall, Inc., New Jersey, U.S.A. (1957).

solvent (74:19:50). Radioactive compounds located by radioautography were counted directly on the paper using a mica-end window Geiger–Muller tube.

The proportion of the ^{14}C in aspartate in the C_4 position was determined using an aspartic decarboxylation preparation from *Nocardia*.[12] One ml of eluted aspartate and 2 μmole of carrier aspartate were incubated for 40 min at 30° with 0·1 ml 3 M acetate buffer (pH 4·9), 0·1 ml pyruvate and 0·3 ml lyophilized *Nocardia* cells (≡ 30 mg cells). The pyruvate solution was made by dissolving 0·66 g of sodium pyruvate in 10 ml 0·2 M acetate buffer.

The total activity in both carboxyl groups of aspartate and the carbon 1 (the α-carboxyl) of glutamate were determined by treatment with ninhydrin.[13] The evolved $^{14}CO_2$ was in all cases trapped in KOH and converted into $BaCO_3$ for counting at infinite thinness.

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[12] L. V. Crawford, *Biochem. J.* **68**, 221 (1958).
[13] S. Arnoff, *Techniques in Radiobiochemistry*, Iowa State College Press, Ames, Iowa, U.S.A. (1956).