THE PATHWAY OF ARGININE BIOSYNTHESIS IN *CHLAMYDOMONAS REINHARDI*[1]

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Two arginine requiring mutant strains of the unicellular green alga *Chlamydomonas reinhardi* have been isolated (Ebersold, 1956; Eversole, 1956). They occur in linkage group I (Levine and Ebersold, 1958). One of these strains, *arg-1*, grows on minimal medium (Levine and Ebersold, 1958) supplemented with ornithine, citrulline, or arginine and is thus blocked prior to ornithine in the arginine biosynthetic pathway. The second mutant strain, *arg-2*, grows only on minimal medium supplemented with arginine. Since there is evidence for two distinct arginine pathways in other organisms, it was necessary to determine the pathway of arginine biosynthesis in *C. reinhardi* before the metabolic blocks in these mutants could be specified. In the bacterium *Escherichia coli*, Vogel (1953) has shown that the carbon skeleton of arginine is provided by glutamic acid *via* a series of acetylated intermediates including N-acetyl glutamic acid. Acetylated intermediates are not involved in the biosynthesis of arginine in the fungus *Neurospora crassa* (Srb and Horowitz, 1944). The steps of arginine biosynthesis are identical from ornithine to arginine in these two organisms.

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The algae used in these experiments were wild type, strain 137c, of C. reinhardi and the mutant strains arg-1 and arg-2 described above. Cultures were grown at 25° C under a light intensity of 4000 lux. The liquid high salt medium of Sueoka (1960) supplemented with 10 ug/ml of arginine was used. Cells were harvested after 60 hours of growth.

The arginine biosynthetic pathways in E. coli, strain W, and N. crassa, strain balloon A, B-56 were compared to that in C. reinhardi. The bacteria were grown at 37° C on M-26 basal minimal medium (after Sistrom, see Folsome, 1960) with glucose (0.5 per cent) as the carbon source. Cultures were grown anaerobically since Gorini and Maas (1958) showed that endogenous arginine represses enzymes of the arginine biosynthetic pathway under aerobic conditions. Cultures of N. crassa were grown in the liquid medium of Ryan (1950) at 25° C.

Cell-free preparations were used in all cases. The algae and bacteria were harvested by centrifugation, washed in 0.068 M phosphate buffer at pH 6.8 and disrupted by sonic oscillation. Mycelia of the fungus were collected on a Buchner funnel and disrupted by grinding with dry ice. Unbroken cells and insoluble debris were removed from all preparations by centrifugation at 27,000 x g for 20 minutes at 0° C.

C^{14} glutamic acid was used as a tracer in the experiments reported. Cell preparations which had been exposed to this labelled compound were extracted with cold trichloroacetic acid (TCA) and the TCA soluble material was analyzed for radioactivity. Since material soluble in cold TCA contains most of the metabolic intermediates of the cells, the acetylated intermediates of arginine biosynthesis, if present in any or all of these organisms, can be identified by electrophoretic separation followed by measurement of their radioactivity. The presence of C^{14} label in acetyl glutamic acid in

extracts of C. reinhardi would be suggestive but not conclusive evidence that this compound lies on the arginine pathway of the alga since such acetylation might occur as a side reaction.

To determine whether N-acetyl glutamic acid received label from C^{14} glutamic acid, the cold TCA soluble material was examined by paper strip electrophoresis. The pH_{IEP} of N-acetyl glutamic acid was determined to be 3.8. That of arginosuccinic acid was found to be 3.4, a close agreement with the value of 3.5 found by Ratner et al.(1953). Reactions for each organism were carried out in their respective growth media lacking arginine and were started by the addition of 50 µg/ml of glutamic acid 1-C^{14} (20.0 µc/mg) to the cell-free preparations. After one hour of continuous shaking at 15° C, the reactions were terminated by the addition of 20 per cent cold TCA to a final TCA concentration of 6.7 per cent. Cold TCA extraction was carried out at 4° C with continuous shaking for one hour. The extracts were then centrifuged for 20 minutes at 27,000 x g at 0° C. The supernatant fluid was decanted, washed three times with cold diethyl ether and then analyzed by paper strip electrophoresis.

Electrophoresis was carried out at 4° C on 25 cm strips of 2.5 cm wide Whatman 3MM filter paper. A citric acid-sodium phosphate buffer was used. When the labelling of N-acetyl glutamic was under investigation, a pH of 3.8 was used. For the examination of arginosuccinic acid, a pH of 3.0 was found to separate aspartic acid, glutamic acid, and arginosuccinic acid. About 0.1 ml of the washed TCA extract was spotted on the midline of the paper at one side, and about 0.2 ml of aqueous glucose (two per cent) was placed on the midline at the other side to correct for electro-osmosis. Small amount (ca. 0.05 ml of a one mg/ml solution) of arginine, proline, aspartic acid, glutamic acid, and arginosuccinic acid or N-acetyl glutamic acid were added to the TCA extract to

act as standards in the location of these compounds. A standard map for the seven compounds under the conditions used was established.

Electrophoresis was carried out for 24 hours at a potential of 6v/cm after which the paper strips were dried and sprayed with 0.5 per cent ninhydrin in butanol to develop the amino acids, followed by 25 per cent acetic acid and aniline hydrogen oxalate to locate glucose. The radioactivity in the amino acids was determined with the aid of a Forro Chromatographic Strip Scanner.

The data for the relative activities of the cold TCA soluble intermediates are presented in Table I. In all cases the average of three replicate experiments is given. The data given in Table I show that radioactivity from glutamic acid 1-C^{14} appears in N-acetyl glutamic acid in both C. reinhardi and E. coli but not in N. crassa. On the basis of these results it can be concluded that C. reinhardi is capable of acetylating glutamic acid. While similar results were obtained with E. coli, it is not possible to conclude from these data that N-acetyl glutamic acid lies on the arginine biosynthetic pathway of C. reinhardi. Studies with C. reinhardi such as those of DeDeken (1962) with yeast showing that arginine represses this acetylation would be necessary for a definitive conclusion.

When the cold TCA extracts of wild type and of arg-2 of C. reinhardi are compared for the appearence of radioactivity from glutamic acid 1-C^{14} in arginosuccinic acid, it is found (see Table I) that this compound is labelled in wild type but not in mutant extracts. As will be reported in a later paper, cell-free extracts of arg-2 have arginosuccinase activity comparable to that of extracts of wild type. It seems, therefore, that arg-2 is unable to convert citrulline to arginosuccinic acid.

Table I. Examination of TCA Soluble Material

Compound	Relative Activity[1]				
	C. reinhardi			E. coli	N. crassa
	wild type	arg-1	arg-2		
Glutamic acid	1.2	2.4	3.0	2.8	2.2
N-acetyl glutamic acid	0.24	0.94	0.57	0.31	0
Proline	0.18	0.24	0.61	0.30	0.15
Arginine	0.23	0	0	0.24	0.17
Aspartic acid	0	0	0	0	0
Arginosuccinic acid	0.24	---	0	---	---

[1] A value of 0.25 equals 70 cpm above background.

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