CITRULLINE UTILIZATION IN CRITHIDIA*

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It was early noted in growth studies (Kidder and Dutta, 1958) that the trypanosomid flagellate Crithidia fasciculata could utilize citrulline, but not ornithine, as a replacement for arginine. A somewhat more efficient use of citrulline than arginine suggested that citrulline might be contributing directly to reactions other than just the production of arginine for protein synthesis.

This report presents evidence for the production of argininosuccinic acid as an intermediate in arginine production from citrulline and for the contribution of the ureide carbon of citrulline, presumably as carbamyl phosphate, for pyrimidine ring synthesis.

Incorporation studies were made with growing cultures and with cell-free sonicates of Crithidia fasciculata. Citrulline-ureido-^{14}C (0.25 μ curies/ml) replaced arginine in the medium of Kidder and Dutta (1958) and Triton WR1339 replaced Tween. After 40 hrs growth (500 ml culture) the cells were harvested by centrifugation, washed three times in cold 0.02 M potassium phosphate buffer (pH 7.4) and the protein precipitated with cold 10% trichloracetic acid (TCA). The precipitate was washed successively with hot TCA, cold TCA, 95% ethanol, hot 95% ethanol:ether (3:1) and ether. Hydrolysis of the protein was accomplished by refluxing with 6 N HCl for 24 hrs. After filtration the filtrate was dried over NaOH in vacuo, the amino acids redissolved and dried twice and finally taken up in 25 ml of water. Aliquots were applied to Whatman No. 1 paper

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and chromatogrammed with phenol:water (9:1), along with authentic samples of L-arginine and L-citrulline. The radioactivity was determined by use of a Tracerlab 4 TT scanner and the position was compared with the reference amino acids, visualized with ninhydrin. Fig. 1 shows that labeled arginine occurred in the protein when organisms were grown on labeled citrulline. This observation was expected, but the pathway of arginine synthesis was unknown.

To test for the possibility that argininosuccinate might be the intermediate, cells from a 300 ml culture containing L-citrulline (310 μg/ml) and no arginine were harvested and washed twice in cold 0.02 M phosphate buffer (pH 7.4). The cells were then suspended in 5 ml of the buffer containing the following: L-citrulline (15 μ moles); L-citrulline-ureido-^{14}C (2.5 μ curies); L-aspartic acid (15 μ moles); ATP (2 μ moles); phosphorylcreatine (20 μ moles). The phosphoryl-creatine was added for ATP generation, as Ratner (1954) has shown that high concentrations of ATP inhibit argininosuccinic acid synthetase activity. The suspension of cells in the above mixture was subjected to sonication, which resulted in a cell-free system. Incubation at 23° was carried out for three hrs, after which the reaction was stopped by the addition of 0.5 ml of 10% perchloric acid. The precipitated protein was removed by filtration and the supernatant was subjected to column

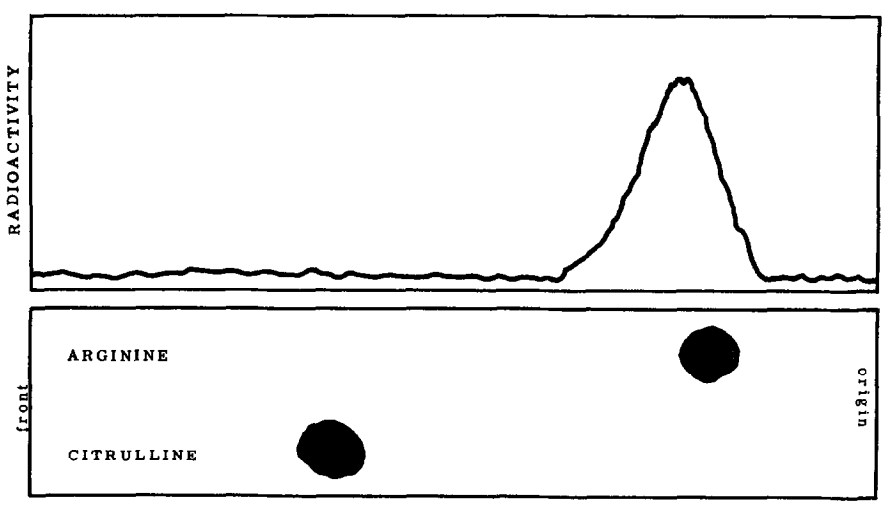

Fig. 1 Radioactivity trace of chromatogram (phenol:water-9:1) of protein hydrolysate of Crithidia grown with citrulline-ureido-^{14}C. Bottom strip contained authentic L-arginine and L-citrulline.

chromatography on Dowex 50-X4, essentially according to the method of Moore and Stein (1954), after the addition of carrier L-citrulline (4 mg), argininosuccinic acid (2 mg) and L-arginine (8 mg). In this system (after the column had been equilibrated with pH 2.2 buffer) citrulline was eluted after approximately 280 ml of pH 3.1 buffer had been collected. The buffer was then changed to pH 5.1 and the argininosuccinic acid was eluted after another 100 ml and arginine after an additional 170 ml. Radioactivities of the peak tubes for the three amino acids under consideration were determined by use of a Tracerlab ultra-thin-window flow counter. As can be seen from Table I, argininosuccinic acid was labeled, indicating that argininosuccinate synthetase was present. The low label in the arginine indicates that the argininosuccinase had been largely inactivated in this type of preparation. As a further demonstration that argininosuccinate had been produced from citrulline in the reaction mixture, 0.1 ml of the argininosuccinate

Table I. Radioactivity of 1 ml aliquots of peak tubes from ion exchange column of cell-free mixture containing citrulline-ureido-^{14}C. (cpm/ml)

Reaction mixture	Citrulline peak	Argininosuccinate peak	Arginine peak
45,000	2,690	522	46

peak tube was chromatogrammed on paper, together with authentic argininosuccinic acid. Figure 2 shows that the radioactivity peak corresponds exactly to the position of the authentic sample.

Examination of the nucleic acids isolated from labeled citrulline-grown cells showed that some of the label was incorporated. Cells from a 200 ml culture containing citrulline-ureido-^{14}C (25 μ curies) plus carrier citrulline (260 μg/ml) were harvested after 96 hrs of growth. Total nucleic acids were isolated and hydrolyzed according to the method of Heinrich, Dewey and Kidder (1953). The hydrolysate was subjected to chromatography, along with authentic samples of CMP, UMP, adenine and guanine. The first chromatogram was on paper with the isopropanol:HCl:water solvent of Wyatt (1955). The 260 mμ absorbing spots were cut out and eluted for radioactivity and

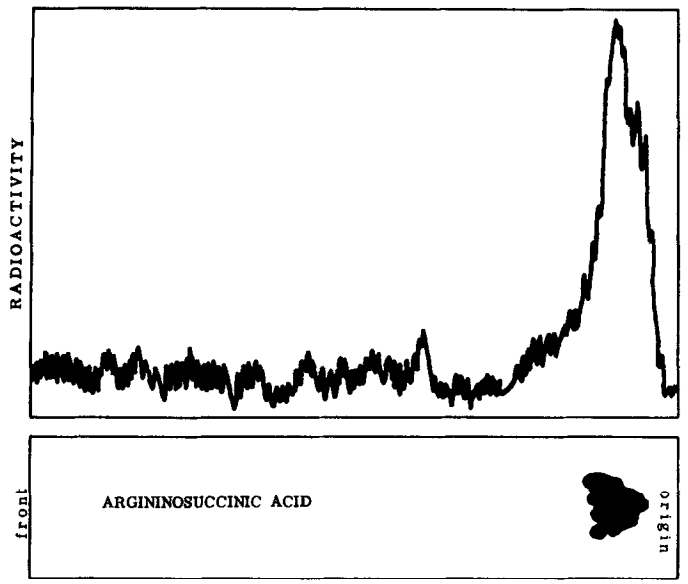

Fig. 2 Radioactivity trace from chromatogram (butanol:acetic acid:water-3:1:1) of fraction from ion exchange column of cell-free sonicate incubated with citrulline-ureido-^{14}C. Bottom strip, authentic argininosuccinic acid.

spectrophotometric determinations. All four nucleic acid derivatives showed radioactivity. Three more chromatographic purifications (TLC on silica gel G with the isobutyric acid: ammonia solvent of Wyatt, 1955) resulted in constant radioactivity for the pyrimidine nucleotides, while the purines contained no label (Table II).

Table II. Citrulline-ureido-^{14}C and $^{14}CO_2$ incorporation into nucleic acid derivatives of Crithidia. (cpm/μ mole)

Addition to growth medium	CMP	UMP	Adenine	Guanine
Citrulline-^{14}C	2,750	2,640	0	0
$NaH^{14}CO_3$	153	75	0	0

An earlier observation in this laboratory (M. R. Heinrich, unpublished) indicated that some pyrimidine labeling from CO_2 occurred in Crithidia, implying weak carbamyl phosphate synthetase activity. We were able to verify this finding by examining the nucleic acid derivatives from cells grown in a medium (in this case yeast extract-glucose-hemin) containing 50 μ curies of $NaH^{14}CO_3$/200 ml culture. Table II shows that low but significant

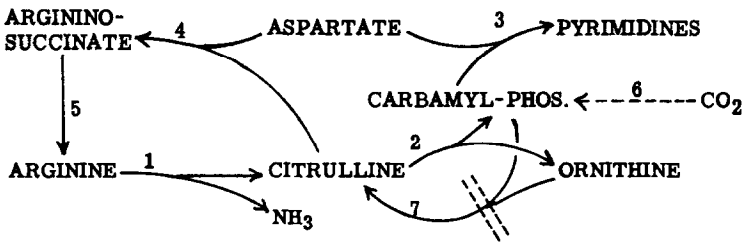

Fig. 3 Scheme of citrulline utilization in Crithidia fasciculata

incorporation of CO_2 occurred in the pyrimidine nucleotides. This level of incorporation of the label can be accounted for both on the basis of dilution by metabolic CO_2 and the relative inefficiency of a carbamyl phosphate synthetase.

These results imply that Crithidia fasciculata possesses an arginine desimidase (Fig. 3, 1), a phosphorolytic citrullinase (2) an aspartic transcarbamylase (3), which has already been demonstrated by Wilson (1964), an argininosuccinate synthetase (4), an argininosuccinase (5) and a weakly functioning carbamyl phosphate synthetase (6). Ornithine transcarbamylase (7), a key urea cycle enzyme, is ruled out for this organism.

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