# ARGININE METABOLISM IN *CHLAMYDOMONAS REINHARDI*. A NEW TYPE OF CITRULLINE DEGRADATION

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## 1. Introduction

The study of the regulation of the arginine biosynthesis in *Chlamydomonas* [1, 2] is complicated by the degradation of arginine. As we reported earlier [3], the first enzyme of the catabolic pathway is arginine deiminase (L-arginine imino hydrolase, EC 3.5.3.6.). This enzyme causes the breakdown of arginine to citrulline and ammonia. Citrulline is then further degraded. Two pathways for the degradation of citrulline have been reported. This communication describes a third hitherto unknown route in *Chlamydomonas*. The enzyme involved in the reaction exhibits a pH optimum of 8.5, needs no cofactors, and is inhibited by orthophosphate. Formation of ornithine and ammonia are not observed, thus excluding the possibility that this enzyme is identical with citrulline hydrolase. Thin-layer chromatography of radioactive reaction products shows the formation of a product, lacking intact ureido and α-amino groups, but still containing all the carbon atoms of citrulline.

## 2. Experimental procedures

The organism used throughout this study is *Chlamydomonas reinhardi* strain 137 C wild type, mating type minus, kindly supplied by Dr. R.P.Levine. Conditions of growth and the preparations of cell-free extract have been described earlier [1]. Normally the cell cultures contained 200 μg/ml arginine as sole nitrogen source unless otherwise stated. The crude cell extracts were dialyzed against 0.2 M tris buffer, pH 8.5, for 18 hr at 4°. Citrulline was determined according to the method of Archibald [6], ornithine according to Chinard [7], and ammonia according to Sigma Technical Bulletin 108. Phosphate was determined by the method of Chen et al. [8], and protein by Lowry's method [9]. Thin-layer chromatography was performed on silica gel plates with chloroform-methanol-17% $NH_4OH$ (2: 2 : 1). Citrulline [$^{14}$C-ureido] was obtained from the Radiochemical Centre, Amersham, while $^3$H-citrulline was prepared from $^3$H-ornithine (uniformly labelled) and unlabelled carbamyl-phosphate with the help of ornithine transcarbamylase (OTCase) from *Chlamydomonas*. $CO_2$ was determined manometrically.

## 3. Results and discussion

Two types of reactions leading to degradation of citrulline have hitherto been known:

1. citrulline + orthophosphate → ornithine + carbamoylphosphate, catalyzed by a catabolic OTCase [4], and
2. citrulline → ornithine + $NH_3$ + $CO_2$, catalyzed by citrulline hydrolase [5].

Our first attempts to detect the breakdown of citrulline were performed in a way that would reveal degradation by either route. Citrulline (0.6 μmoles) was incubated in 0.2 M tris buffer, pH 8.5, in the presence of 2 mM phosphate, 5 mM $MgCl_2$, and dialyzed crude extract of wild type. The whole reaction mixture (1 ml) was incubated for 1 hr at 37°, after which the decrease of citrulline was determined. Under these circumstances a typical experiment showed the disappearance of 0.18 μmoles citrulline. To ascertain whether phosphate was needed for the reaction, the same experiment was performed in the absence of phosphate.


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In these conditions the degradation of citrulline was improved, and 0.47 μmoles were found to have disappeared. In the latter case, the residual amount of phosphate was measured according to Chen et al. [8]; it appeared to be less than 0.05 μmoles. These results show clearly that cells grown on arginine as the sole source of nitrogen possess an enzyme which is able to attack citrulline. Almost no activity was found in cultures grown on normal amounts of $NH_4Cl$ (400 mg/l), which indicates that this enzyme is involved in the catabolic pathway of arginine. Phosphate is not needed; on the contrary, it causes inhibition. The enzyme preparations can be stored at $-20°$, and the activity is destroyed by heating. The degradation of citrulline can be achieved in tris, glycinate, and borate buffers. The pH curve was determined in tris; it showed an optimum at pH 8.5 (fig. 1).

The observation that phosphate appears not to be needed means that no catabolic OTCase is involved, but that citrulline hydrolase may be present. Citrulline hydrolase causes formation of ornithine, $CO_2$ and $NH_3$. We therefore attempted to detect the presence of these compounds in the reaction mixture (see experimental procedures). Numerous experiments, in which sometimes more than 90% of the citrulline disappeared, revealed that neither ornithine nor $NH_3$ was formed. Moreover, Warburg experiments showed that no $CO_2$ was formed. Experiments in which citrulline [$^{14}$C-ureido] was used, showed also that in general no radioactive $CO_2$ was liberated. However, in a few experiments some $CO_2$ formation was observed, corresponding at the most to 10% of the citrulline that disappeared. When using enzyme preparations which had been subjected to 10-fold purification by chromatography of the dialyzed crude extracts on Sephadex G-200 (column size 45 X 3, and 0.2 M tris (pH 8.5) as effluent) we were never able to observe $CO_2$ formation. This makes it likely that the residual formation of $CO_2$ is due to a side or subsequent reaction. The absence of $CO_2$, $NH_3$ and ornithine is a strong indication that no citrulline hydrolase is involved, but that a hitherto unknown enzyme is present. To identify the reaction products we used thin-layer chromatography of reaction mixtures in which radioactive citrulline had been degraded. With citrulline $^{14}$C-ureido one reaction product was observed on silica gel plates with $R_f$ 0.7, while the $R_f$-value of citrulline was 0.6. In contrast to citrulline this product did not show colour with ninhydrin. The use of $^{3}$H-labelled citrulline, made from uniformly labelled ornithine and unlabelled carbamoylphosphate, and of $^{14}$C-5 labelled citrulline both resulted in the formation of one product at $R_f$ 0.7. This means that the C-skeleton of citrulline remains intact. Since the reaction product does not show colour formation either with diacetylmonoxime (as citrulline and other ureido compounds do) or with ninhydrin, the free α-amino group as well as ureido must have been modified. We therefore believe that ring formation or dimerization has occurred. The same product is formed in the inhibited reaction in the presence of phosphate, which means that both in the presence and in the absence of phosphate the same reaction proceeds, which does not require the presence of phosphate. Since arginine-dependant strains, which are unable to make ornithine from glutamate, contain ornithine after growth on arginine [1, 2], and since moreover the catabolic enzyme ornithine transaminase has been observed in *Chlamydomonas* [10], it is likely that the unknown product is a precursor of ornithine in the catabolic pathway. We therefore expect that another enzyme converts product $R_f$ 0.7 into ornithine. We do not want to exclude the possibility that the enzyme described in this report is also able to cause ornithine formation if the right cofactors are added. Under these circumstances the reaction leading to product $R_f$ 0.7 would be a part reaction, and the enzyme would differ extensively from the

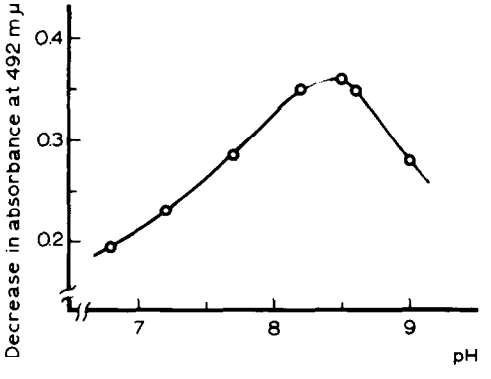


Fig. 1. pH Optimum of citrulline degrading enzyme. The reaction mixture contained, in a final volume of 1 ml, 0.6 μmoles citrulline, 5 μmoles $MgCl_2$, 200 μmoles tris buffer, and cell extract. The mixture was incubated for 1 hr at 37° after which citrulline was determined [6].

known citrulline degrading enzymes. Evidence that this citrulline degrading enzyme is involved in the catabolic pathway is obtained from the observations that the enzyme is present in cultures grown on arginine as the source of nitrogen, and absent if grown on minimal medium. This is identical to arginine deiminase [3]. The enzyme is also found in cultures grown on less than 1/10 of the normal amount of $NH_4$. In this case, also, ammonia represses the formation of the catabolic enzyme. For a further characterization of product $R_f$ 0.7 large-scale preparations are in progress.

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