# ARGINOSUCCINIC ACID FROM CHLORELLA

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Artificially induced enzyme blocks are invaluable in elucidating metabolic pathways. Lyophilization or desiccation with acetone was found to inactivate some of the enzyme systems of *Chlorella pyrenoidosa* cells; consequently it was hoped that a systematic study of the inhibited reactions and the alterations of metabolism resulting from such treatment might furnish a better understanding of the intermediary metabolism of algae. Out of this study has developed the isolation and characterization of arginosuccinic acid, a compound of unusual properties and significance for intermediary metabolism.

*Experimental.—Chlorella* cells were grown photoheterotrophically at room temperature in 1-liter cotton-stoppered Erlenmeyer flasks on a mechanical shaker, with 4% glucose as the carbon source and 0.4% urea as the nitrogen source. Cells were harvested by centrifugation, washed twice, acetone-dried or lyophilized, and stored in the refrigerator; 6 to 8 g. of dried cells were obtained per flask.

When a 5% aqueous suspension of acetone-dried cells was shaken at room temperature on a mechanical shaker with fumarate or malate (final concentration 0.1 *M*), an amino acid was produced and excreted into the medium in quantities readily detectable within twenty minutes, although production continued for several hours. This compound could be distinguished from all common amino acids by two-dimensional paper chromatograms, employing as solvents water-saturated phenol, ammonia atmosphere ($R_f$ 0.27), followed by a 4:1:1 *n*-butanol-acetic acid-water mixture ($R_f$ 0.08). A purple spot was obtained when the papergrams were sprayed with ninhydrin, while a more specific but less sensitive color reagent, alkaline picrate, produced an orange spot.

Similarly treated cells from cultures grown with nitrate instead of urea as the sole nitrogen source did not produce this substance unless arginine, in addition to fumarate or malate, was added to the suspension of acetone-dried cells. Acetone-dried cells prepared from cultures grown in a urea medium have a much higher arginine content and did not require exogenous arginine to produce the new compound (arginosuccinic acid), but arginine addition did increase the rate of formation, as well as the final yield. Arginine, when added singly to a suspension of acetone-dried cells grown on either urea or nitrate, was transformed into citrulline, as in the work with bacteria recently reported by Schmidt, *et al.*[1] and Knivett,[2]

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but when sufficient malate or fumarate was also added, arginosuccinic acid was formed instead of citrulline.

In arginosuccinate production arginine could not be replaced by citrulline, ornithine, creatine, or guanidine, while the role of fumarate-malate could not be played by succinate, aspartate, glutamate, citrate, lactate, pyruvate, acetate, butyrate, glycolate, phosphoglycerate, tartrate, maleate, ethanol, etc.; oxalacetate had a small but definite amount of activity. Preliminary time course experiments with cyclohexanol as a partial fumarase inhibitor indicated that fumarate rather than malate may be the immediate precursor, because with equal substrate concentrations the arginosuccinate ninhydrin spot was less intense with malate than with fumarate. When one reviews known reactions in which fumarate will act but malate will not, the addition of ammonia to fumarate to form aspartic acid comes to mind; in an analogous manner arginine might add to fumarate to form arginosuccinic acid.

To produce sufficient quantities of arginosuccinate so that it could be isolated, acetone-dried cells (cultured in urea) were shaken under toluene with arginine and malate for 10 hours, after which time the cells were centrifuged off and recharged with fresh substrates; recharging at intervals was continued until 40 hours had elapsed, after which time the cells were discarded. The combined supernatants were concentrated, deproteinized, and passed through a Dowex-50 column with 4 *N* HCl as eluant. Removal of the HCl *in vacuo* from the fractions containing arginosuccinic acid left a mixture of the latter, large amounts of KCl, and some arginine. An alcohol extract of this residue was next passed through another Dowex-50 column and eluted with 2.5 *N* HCl. Removal of the HCl as before gave a very hygroscopic white solid, which papergrams showed contained only an estimated 5% ninhydrin reactive impurity (arginine). The solid was dissolved in alcohol and filtered; slow addition of a dilute alcoholic solution of aniline to the filtrate gave a white precipitate, which was washed several times with alcohol and immediately dried in a vacuum desiccator. This precipitate was relatively non-hygroscopic and free from other ninhydrin reactive compounds; its temperature of decomposition was approximately 270°C. Although insoluble in alcohol, it dissolved readily on addition of HCl; from aqueous solution it gave a white precipitate with silver nitrate in the presence of nitric acid.

Three-day hydrolysis of arginosuccinic acid with 6 *N* HCl at 100°C. gave arginine as the only ninhydrin reactive product, while a 12-hour 4 *N* barium hydroxide hydrolysate showed two ninhydrin spots corresponding to ornithine and aspartate.

These data are best explained by assuming that this compound is that postulated and partially characterized by Ratner[3] as the intermediate in the synthesis of arginine by mammalian liver from citrulline plus aspartate:

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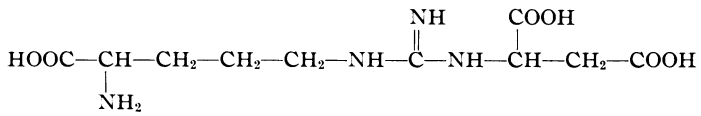

It is likely that arginosuccinic acid is identical also with the unknown compound of Davison and Elliott[4] produced on incubation of various tissues with arginine plus fumarate, since the precursors are identical and the $R_f$ values appear similar.

Microbiological assays were next employed in order to obtain quantitative data which might throw further light on the structure and purity of the isolated arginosuccinic acid. The organisms used were *Leuconostoc mesenteroides* P-60 for arginine and aspartate assays, and *Escherichia coli* B, mutant No. 43, for ornithine and arginine assays. The latter organism[5] was found to require for growth ornithine, citrulline, or arginine, on an equimolar basis, in addition to the glucose-asparagine-salts minimal medium.

Unfortunately, neither of these two organisms nor *Lactobacillus casei* was able to use the intact arginosuccinic acid molecule to supply its aspartate or arginine requirement; thus direct microbiological assay with these organisms was impossible.

Since these organisms fail to utilize arginosuccinate unless it is "predigested" for them, it was necessary to employ chemical hydrolysis and analyze for the fragments. Ten milligrams of the hygroscopic column isolate hydrolyzed three days with 6 *N* HCl gave, by both *E. coli* and *L. mesenteroides* assay, the same amount of L-arginine, 2.1 mg. (calculated as the monohydrochloride). When an aliquot of this acid hydrolysate was further subjected to barium hydroxide hydrolysis no aspartate was formed, but DL-ornithine calculated equivalent to 4.1 mg. of DL-arginine monohydrochloride from the original sample was produced. This when taken in conjunction with the 2.1 mg. of L-arginine monohydrochloride found above indicates that the arginine moiety in arginosuccinic acid had been racemized. The alternative possibility that both arginine and guanidosuccinic acid were formed by acid hydrolysis was ruled out by the lack of aspartate production upon subsequent alkaline hydrolysis. Since alkaline hydrolysis is faster and produces two analyzable fragments instead of one, it was the analytical method of choice for studies of per cent purity.

A sample of purified, vacuum-dried arginosuccinic acid, weighing 18.9 mg. and found by paper chromatography to be free from other amino acids, was hydrolyzed for 20 hours with 4 *N* barium hydroxide. The hydrolysate was found to contain by *E. coli* assay the equivalent of 10.0 mg. of DL-ornithine monohydrochloride and by *L. mesenteroides* assay 5.1 mg. of DL-aspartic acid. Assuming the purified arginosuccinic acid to be in the monohydrochloride form, this assay showed that from each mole of arginosuccinic acid were produced 1.02 moles of ornithine and 0.66 mole of

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aspartic acid. These results can be explained by assuming that 0.34 mole of arginosuccinic acid underwent hydrolytic cleavage to form arginine before subsequent conversion to ornithine, while 0.66 mole was cleaved into aspartic acid, ornithine, carbon dioxide and ammonia, with perhaps citrulline and ureidosuccinate as transitory intermediates in the process. The per cent purity calculated from the moles of ornithine liberated gives a value of 102%.

*Discussion.*—Ratner[3] obtained the barium salt of the intermediate formed by liver from citrulline and aspartate, and she estimated that her sample was 81% pure from its barium content, assuming 1.5 moles of barium per mole of intermediate.

Larger amounts of arginosuccinic acid can be produced with less trouble from acetone-dried *Chlorella* (or *Scenedesmus*), however, and no complicated enzyme isolation is required. The procedure described here leading to the production of the pure compound, together with the chromatographic, microbiological, and colorimetric characterization techniques, should prove helpful for further investigations of the role of arginosuccinic acid in metabolism.

Several puzzling aspects of this problem remain to be worked out, however. Knivett[2] reported that the formation of citrulline from arginine was inhibited by disubstituted guanidines, among other compounds; this would perhaps explain why arginine added singly to acetone-dried cells yields citrulline, while the addition of fumarate or malate along with the arginine inhibits the formation of citrulline. The disubstituted guanidine, arginosuccinate, which is formed might inhibit in higher concentrations an enzyme involved in one of the subsequent transformations usually occurring during arginine metabolism; this could be yet another example of the intricate mechanisms by means of which alternate metabolic pathways are selected as the physiological environment varies.

The failure of the three organisms tested to grow on the intact arginosuccinate molecule does not preclude the possibility that a more determined search will turn up organisms with which a direct assay can be made. Since toxicity was ruled out by suitable controls, the lack of biological activity of the intact molecule might be ascribed to configurational properties, permeability restrictions, or to inter- or intramolecular cyclization. That at least some of the molecules may be cyclized is perhaps indicated by the fact that the color with alkaline picrate is similar to that with creatinine, both on papergrams and in solution; however, color reactions are often unpredictable, and it may be that the disubstituted guanidine structure of arginosuccinate as written could produce such a color with alkaline picrate. The time characteristics of the intensity of the color developed with alkaline picrate are quite critical; the optimum conditions for quantitative determinations of arginosuccinic acid by this method are currently being worked out.

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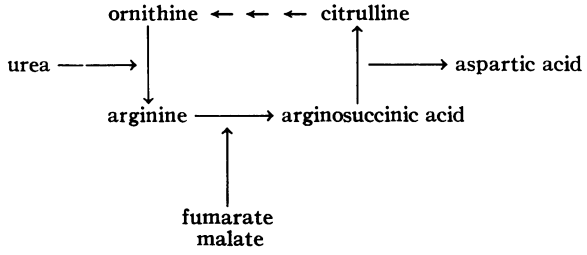
The uniqueness of arginosuccinate in intermediary metabolism is readily apparent when one examines the many possible products of known biochemical importance which might arise from degradation of this compound. Such products include arginine, citrulline, ornithine, fumarate, malate, ureidosuccinate and aspartate. By controlling the available metabolic pathways leading to and from this compound alone, the organism might display a variety of responses.

From contemplation of its structure, arginosuccinic acid appears to be a logical pyrimidine precursor; the work of Wright, *et al.*,[6] establishing ureidosuccinate as a pyrimidine precursor furnishes experimental support for such speculation. Organisms requiring both arginine and a pyrimidine for growth might be used to test this hypothesis. Should arginosuccinate indeed be found to be a pyrimidine precursor, investigations directed toward finding specific inhibitors of that metabolic pathway might prove fruitful as a means of controlling cell division. Inhibition of pyrimidine synthesis near the arginosuccinate level should be less likely to lead to the accumulation of toxic products, since this compound could be readily diverted into other pathways.

Another possible function of arginosuccinate with widespread implications is as an intermediate in urea nitrogen assimulation in cells lacking urease. No measurable urease activity could be found in *Chlorella*, and measurements and calculations showed that non-enzymatic hydrolysis under the experimental conditions employed was not appreciable. In view of the large production of arginine by cells grown with urea as the nitrogen source, it is attractive to suppose that urea might enter the metabolic pool via a reversal of the mammalian-mold urea cycle.




If such a mechanism exists, the initial coupling of urea with ornithine, by analogy with that of citrulline with aspartate,[3] would presumably require ATP.

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[1] Schmidt, G. C., Logan, M. A., and Tytell, A. A., *Federation Proc.*, **11**, 283 (1952).
[2] Knivett, V. A., *Biochem. J.*, **50**, xxx–xxxi (1952).

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[3] Ratner, S., and Petrack, B., *J. Biol. Chem.*, **191**, 693–705 (1951).

[4] Davison, D. C., and Elliott, W. H., *Nature*, **169**, 313–314 (1952).

[5] This mutant was kindly supplied by Miss Barbara Powell of the Department of Zoology.

[6] Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L., and Wilson, D. W., *J. Am. Chem. Soc.*, **73**[2], 1898–1899 (1951).

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# THE MECHANISM OF POLYMERIZATION OF FIBRINOGEN*

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*Introduction.*—Fibrinogen, a rod-like protein of length about 600 Å and molecular weight about 350,000, polymerizes under the catalytic action of thrombin to form fibrin, a three-dimensional network structure. A network strand, as seen under the electron microscope, often exhibits uniform width and little curvature over lengths many hundred times that of fibrinogen; the strand width may range from double to many times that of fibrinogen (about 30 Å), depending on the pH and ionic strength and other conditions. There is good evidence that the fibrinogen units retain their identity within the strands and that their long axes are aligned in the strand direction.

Two intermediates in the polymerization process have been recently identified. The first (**f**) accumulates when thrombin activates fibrinogen at abnormally low pH (about 5),[1] or at abnormally high ionic strength; it has the same gross size and shape as fibrinogen itself, though it has probably suffered the loss of one or more small fragments in the course of activation;[2] it polymerizes rapidly when returned to the normal pH and ionic strength. A similar molecule is formed by dissolving fibrin in 3.5 *M* urea[3] or 2 *M* lithium or sodium bromide.[4] The second intermediate ($\mathbf{f}_n$) accumulates when thrombin activates fibrinogen in the presence of an inhibitor such as 0.5 *M* hexamethylene glycol,[5] 0.5 *M* lithium bromide, or 1 *M* urea.[6] It appears to be a partial polymer of **f** and is so symbolized; **f** can be converted to $\mathbf{f}_n$ and vice versa by changing the solvent composition;[6] and $\mathbf{f}_n$ dissociates to form **f** simply upon dilution.[5,7] The conversion of fibrinogen (*F*) to fibrin is thus pictured as follows:

$$F \xrightarrow{T} \mathbf{f} \rightleftarrows \mathbf{f}_n \rightarrow \text{Fibrin}$$

The intermediate polymer $\mathbf{f}_n$ may be regarded as a sort of prototype of a fibrin strand. Its size and shape have been studied by viscosity and sedimentation,[5,6] flow birefringence,[8,9] and light scattering.[7] In 0.5 *M*

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