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FORMATION OF RADIOACTIVE CITRULLINE DURING PHOTOSYNTHETIC $C^{14}O_2$ - FIXATION BY BLUE-GREEN ALGAE

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ABSTRACT

Citrulline has been isolated and identified from extracts of Nostoc muscorum. All members of the Cyanophyceae hitherto investigated show a relatively large amount of the CO_2 fixed during photosynthesis in citrulline (ranging as high as 20% in Nostoc) when compared to the trace amounts found in the Chlorophyceae. Nostoc also has the ability to fix C^{14} in citrulline during dark fixation, but at a rate slower than in light.

As no free urea or arginine was found in Nostoc, it is likely that citrulline is functioning in reactions other than those leading to arginine and urea synthesis. Other possible functions for citrulline are briefly discussed.

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* Fulbright and ASLA research fellow, 1955-56.

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INTRODUCTION

Norris and co-workers[1] found that during photosynthetic $C^{14}O_2$ fixation by blue-green algae a significant part of the radioactivity was incorporated into an unknown compound chromatographically close to alanine (Fig. 1).[2] The amount of the radioactivity in this compound formed by Nostoc muscorum after five minutes' photosynthesis with radioactive carbon dioxide was reported as high as 20.9% of the total radioactivity soluble in 80% ethanol. In the other blue-green algae examined the radioactivity of the unknown compound was somewhat lower, but always higher than in the green algae. Since the radioactivity of this compound was so high in Nostoc muscorum it seemed possible that this substance might have an important role in the early stages of carbon dioxide metabolism.

This compound has now been isolated from Nostoc muscorum and identified as citrulline.

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1 Norris, Norris, and Calvin, J. Exptl. Botany 6, 64-74 (1955).
2 Benson, Bassham, Calvin, Goodale, Haas, and Stepka, J. Am. Chem. Soc. 72, 1710 (1950).

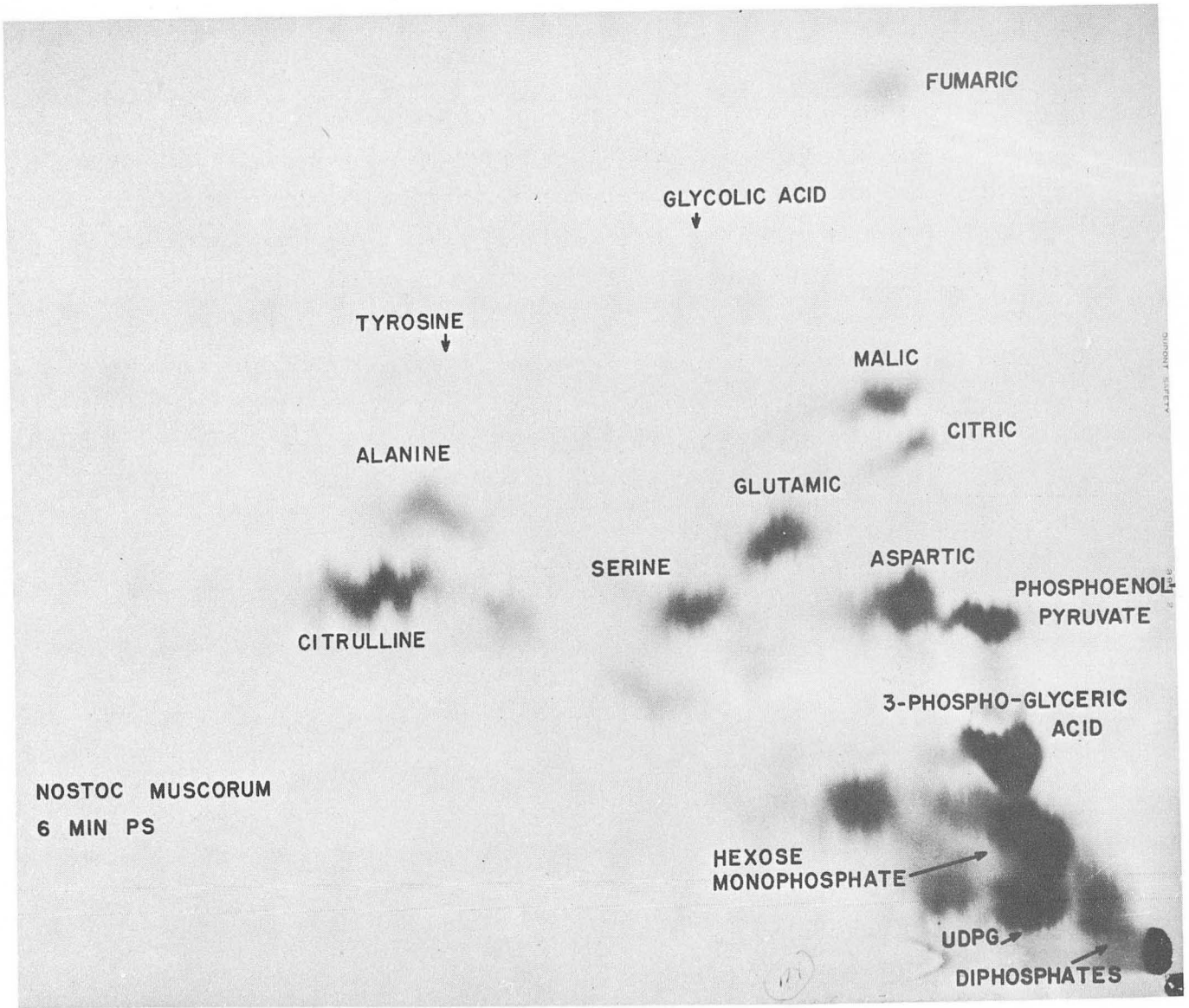

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Fig. 1. Six-minute photosynthesis in Nostoc muscorum, showing distribution of C^{14}-labeled compounds in the fraction soluble in alcohol-water.

EXPERIMENTAL

Isolation

Nostoc muscorum was grown on a shaker apparatus[3] in 2500-ml flasks containing about 1100 ml of algal suspension. In most cases the flasks were equipped with a draining apparatus to permit the use of one algal culture for a long period of time. Approximately 900 ml of the suspension was drained each time. The temperature was maintained at 24°–25° C, and continuous lighting of slightly more than 1000 foot-candles was supplied by fluorescent lamps. All flasks were flushed with air containing approximately 4% carbon dioxide. The following nutrient solution was used: 0.001 M magnesium sulphate, 0.012 M potassium nitrate, 0.0056 M dibasic potassium phosphate, 0.0009 M ammonium chloride, 0.0001 M calcium nitrate, 1.0 ml. Fe-EDTA solution,[4] 1.0 ml. A_5 + Co micro-element solution.[5]

Immediately after removal from the shaker apparatus the cells were centrifuged, rinsed once with distilled water, and resuspended into distilled water to form a 10–15% suspension. The suspension was then poured into a flat, circular vessel ("lollipop") of about 5 mm internal thickness,[6] in a water bath between two incandescent lamps (photospot RS P-2) equipped with infrared filters. The suspension was allowed to become adapted to the light conditions for 15 minutes, during which time a stream of nitrogen was bubbled through. After this time, one ml of standard solution of radioactive sodium bicarbonate (0.025 M, 400 μC/ml) was added and the lollipop was shaken by hand for 6 minutes. The suspension was then poured immediately into an amount of boiling ethanol sufficient to make an 80% aqueous ethanol suspension. The mixture was brought to boiling again and cooled, and the amount of fixed radioactivity was measured. The ethanol extracts from many such experiments were combined and stored in the cold room at 4° C. All together 150 ml of tightly packed cells were used, re-

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3 A. A. Benson et al., Photosynthesis in Plants, Iowa State College Press, Ames, Iowa, 1949, p. 381.
4 59.0 g versen-Ol and 24.9 g $FeSO_4 \cdot 7\ H_2O$ in one liter of distilled water.
5 D. I. Arnon, Am. J. Botany 25, 322-25 (1938).
6 C. Ouellet and A. A. Benson, J. Exptl. Botany 3, 238 (1952).

sulting in a final extract of 5400 ml. The soluble fraction of this extract obtained by centrifugation contained 0.3×10^6 dis/min/ml of C^{14} radioactivity. (Total activity 1.6×10^9 dis/min.)

It was found that the unknown compound could be separated with the amino acids from other compounds by means of a cation-exchange resin. Hence, the centrifuged ethanol extract was passed through an Amberlite IR-120 (H^+-form) column (16 x 350 mm) with a flow rate 1 ml/min. The radioactivity of the solution that came through the column was about 0.22×10^6 dis/min/ml. (Total activity 1.2×10^9 dis/min.) That which remained on the column was 430×10^6 dis/min in total. The column was washed with distilled water (15 ml/ml of resin) and the amino compounds were displaced with 1 N ammonium hydroxide. The ammonia was distilled from the eluate in vacuo until the pH of the solution was 6.8. Qualitative observation of a chromatogram showed that citrulline and aspartic acid had most of the radioactivity, while serine, alanine, and glutamine each had smaller amounts. Compared with other amino acids present, glutamic acid was only very slightly radioactive, but the ninhydrin spray showed that the total amount of glutamic acid was at least equal to the sum of all other ninhydrin-positive compounds.

An attempt was then made to remove aspartic and glutamic acids by means of anion-exchange resin Amberlite IR-4 B (column 16 x 350 mm). While most of the aspartic acid stayed on the column, only part of the glutamic acid was retained. Figure 2 shows a two-dimensional chromatogram of the effluent solution purified with Amberlite IR-4 B. The solution was concentrated to a small volume in vacuo at 40°C. The distribution of the radioactivity is shown below:

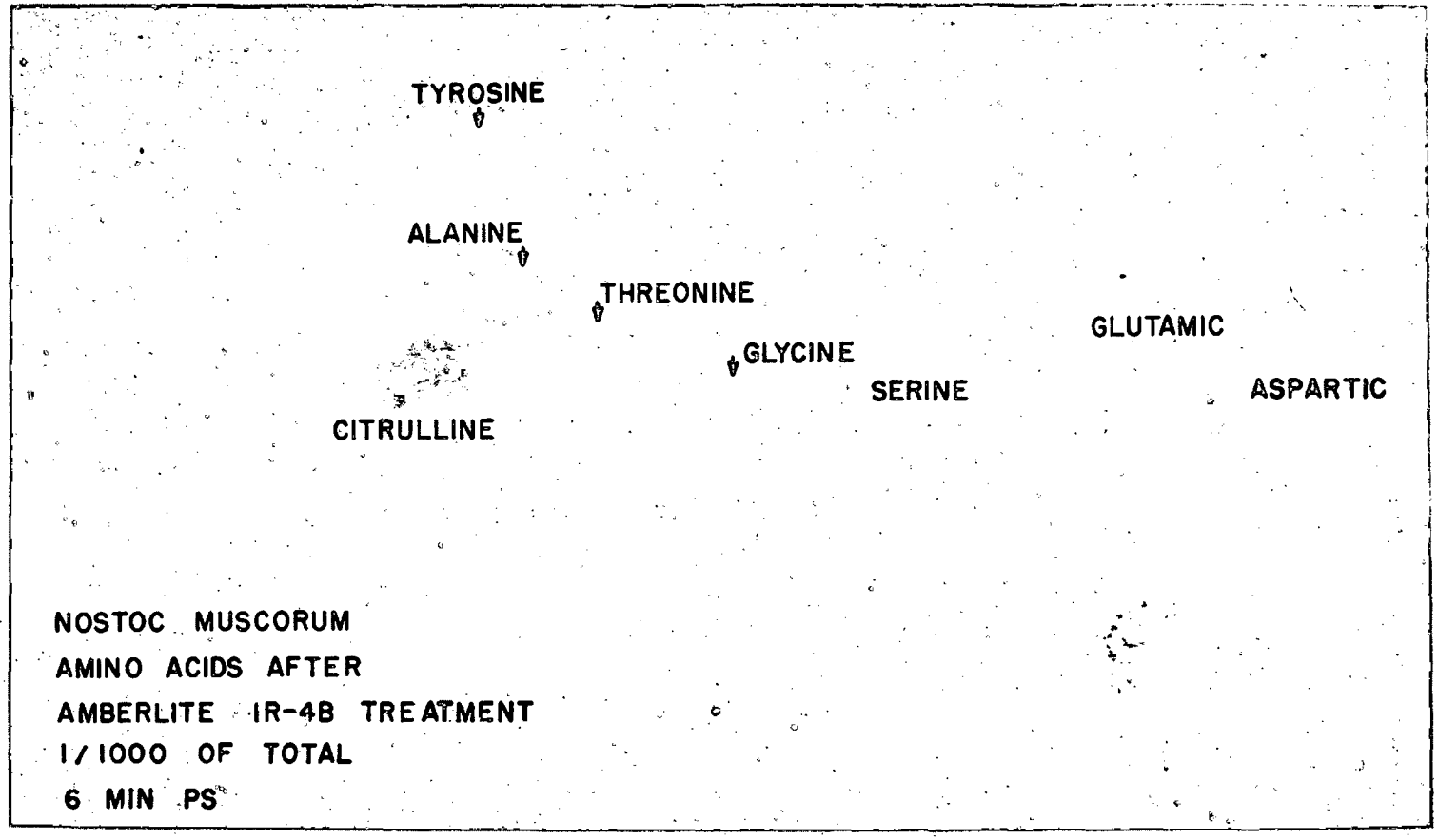

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Fig. 2. Composition of the ethanol extract of Nostoc muscorum after treatment with amberlites IR-120 and IR-4B.

Compound	Activity (x 10^6 dpm)
Glutamic acid (left)	7.8
Aspartic acid (left)	9.5
Serine	28.6
Glycine	6.1
Threonine	6.5
Alanine	19.1
Tyrosine	11.4
Citrulline	112.0
Others	39.0

The radioactivity of citrulline thus was about 25.8% of the radioactivity retained by the IR-120 cation-exchange resin and about 6.9% of the total ethanol-soluble radioactivity.

The hydrogen ion concentration was made 1.5 N with concentrated hydrochloric acid to form a final volume of 15 ml and passed very slowly into a Dowex-50-cation-exchange resin (H^+ form) column (25 x 500 mm, 200–500 mesh). The amino compounds were eluted by means of hydrochloric acid according to the following scheme:

No. of fractions	Size of fractions (ml)	Normality of HCl
1- 43	3.5	1.5
44-147	7.5	1.5
148-239	7.5	2.5
240-290	7.5	4.0
291-351	10.0	4.0

One-dimensional chromatograms were made with 50 λ of each fraction and the locations of the amino compounds were determined by ninhydrin spray. The radioactivity of each of the fractions was measured. Figure 3 shows the elution curve based on these measurements. The citrulline appeared in fractions Nos. 319-334.

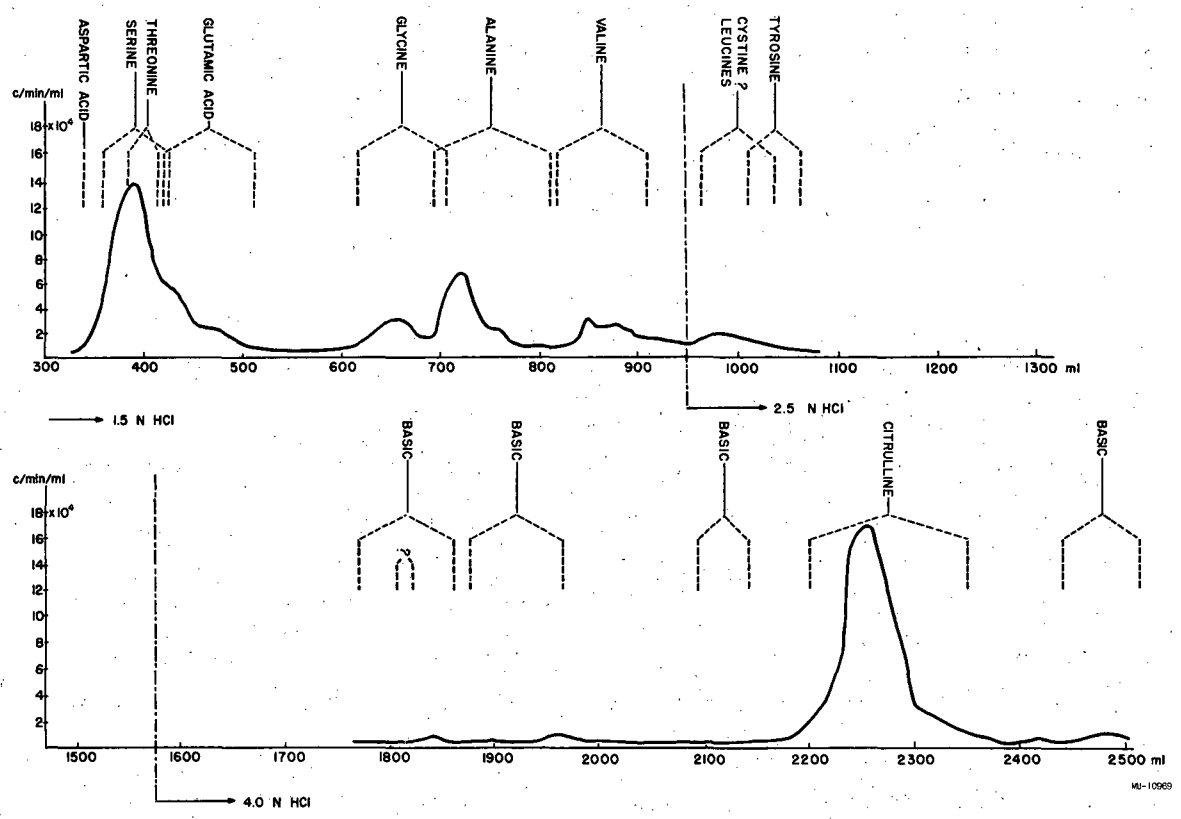

Fig. 3. Elution radioactivity curve for the partially purified ethanol extract from Nostoc muscorum.

The fractions containing citrulline were practically free of other radioactive compounds. However, some ninhydrin-positive inactive impurities could be found. Fractions 319-334 were combined and taken to dryness in vacuo at 40° C and the resulting yellowish mass was dissolved in 5 ml of water. The radioactivity in the solution was 55×10^6 dpm. The citrulline could be freed from impurities having an absorption peak at 2350 Å by repeatedly evaporating to 0.1 ml with a nitrogen stream and precipitating the impurities by means of 2.0 ml absolute ethanol. A yellowish syrupy mass of 12.1 mg was finally obtained. This substance solidified after a small volume of absolute ethanol was added and the container was scratched with a metal spatula; it had 45.7×10^6 dpm in total. Several unsuccessful attempts were made to recrystallize the material, which was found to be rather hygroscopic.

Identification

The isolated material showed a positive ninhydrin reaction. The compound also developed a yellow color typical for a $-NH-\overset{O}{\overset{\|}{C}}-NH_2$ group when treated with p-dimethylaminobenzaldehyde.[7] Aniline-hydrogenphthalate,[8] 1,2-naphthoquinone-4-sodium sulfonate,[9] isatin,[10] and p-dimethylaminobenzaldehyde over isatin[11] sprays gave negative results.

The ion-exchange behavior provided good evidence that the compound was amphoteric. The compound also behaved like alanine and glucose in both pH 2.5 and 6.7 on paper electrophoresis. In 0.2 N sodium hydroxide the compound appeared to split into two spots. However, about 90% of the radioactivity was in the spot moving more towards the anode than glucose, but far less than alanine. The minor spot moved farther than alanine but less far than aspartic acid.

Treatment of a small amount of the compound with either phenylhydrazine or 2,4-dinitrophenylhydrazine followed by two-dimensional chromatography showed that no reactive aldehyde or ketone was present.

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7 C. E. Dent, Biochem. J. 43, 169 (1948).
8 S. M. Partridge, Nature, 164, 443 (1949).
9 D. Müting, Naturwissenschaften 39, 303 (1952).
10 Acher, Fromageot, and Jutisz, Biochim. et Biophys. Acta 5, 81 (1950).
11 J. B. Jepson and I. Smith, Nature 172, 1100 (1953).

The compound was readily deaminated by means of nitrous acid,[12] indicating that it had a primary α-amino group. The most interesting thing found during this reaction was the almost complete loss of radioactivity. Only one new faintly radioactive spot was formed having R_f-values close to those of proline. The loss of the radioactivity was probably due to the reaction of the nitrous acid with the carbamyl group. The degradation experiments showed that an average of 67% of the radioactivity was lost, all as carbon dioxide. This amount varied greatly with different reaction conditions. It seems to be quite possible that the radioactivity of the carbamyl group might be even higher (near 100%), because of several possible side reactions that might lead to ring closure, etc.

Treatment with ninhydrin split off only 2 to 3% of the radioactivity as carbon dioxide. Some was lost as other products, which were not identified. The degradations were carried out under the conditions as described by Linko.[13] It was also found that the radioactivity of citrulline on a paper chromatogram did not decrease appreciably after the ninhydrin spray, even after several months.

On acid hydrolysis with 1 N and 6 N hydrochloric acid (20 hours, 105° C, sealed tube) 40% and 60% of the compound, respectively, were decomposed to inactive ornithine, as would be expected with citrulline.[14] The compound was relatively unstable to alkaline hydrolysis.

The isolated compound was readily acetylated with acetic anhydride at room temperature. Only one major product was formed. This was eluted from the chromatogram and hydrolyzed with 1 N hydrochloric acid under the same conditions as described above. Three slightly radioactive compounds were formed, one of which was identical with the original unknown compound. Again about 70% of the radioactivity was lost; this must have been because of removal of the carbamyl group, for a large amount of inactive ornithine was formed.

Hydrogenation with Adams platinum oxide catalyst at room temperature and normal pressure for 2 hours left the compound largely unchanged. This was taken to indicate the absence of carbon-carbon double bond. After about 3 hours' hydrogenation, however, a few new compounds were

12 Consden, Gordon, and Martin, Biochem. J. 41, 594 (1947).
13 P. Linko, Suomen Kemistilehti B 28, 96 (1955).
14 J. K. Miettinen and A. I. Virtanen, Physiologica Plantarum 5, 540 (1952).

formed containing 1% to 10% of the radioactivity. The major reaction product was slightly above valine on a two-dimensional chromatogram and has been identified as urea. After 12 hours' hydrogenation about 35% of the radioactivity was in urea.

Cochromatography with inactive citrulline constituted the final proof of identity (Fig. 4).

Biological Control of Citrulline Formation

From data available on the distribution of citrulline in algae, it appears that relatively high concentrations of citrulline is a peculiarity of the Cyanophyceae. Thus, Norris[1] reported that in 5-minute photosynthesis experiments with radioactive carbon dioxide, the percentage of the total alcohol-soluble activity found in citrulline was as follows: Phormidium, 2.6; Nostoc, 20.9; Synechococcus, 6.7; Scenedesmus, 0.2; Chlorella, none found; Spirogyra, 0.5; Euglena, 0.4; Porphyridium, 1.3; Funaria, 0.6. Citrulline has now also been found in Anabaens (0.6%), in addition to confirming the general distribution above, although there were fluctuations in the individual values whose origin is as yet unknown.

Citrulline becomes very rapidly labeled with radioactive carbon, both during photosynthetic CO_2 fixation and during dark fixation of radioactive bicarbonate. For the dark-fixation experiments, Nostoc was grown and harvested in the usual way, and adapted in a lollipop in total darkness for 20 minutes, during which time the suspension was flushed with nitrogen. Reference to Fig. 5, in which both the light and dark fixation of radioactive carbon in citrulline are shown, indicates that in the light the formation of radioactive citrulline is almost linear with time for the first 6 to 7 minutes and then diminishes, while in the dark there is a slow but continuous increase up to 30 minutes' fixation time. It would appear that, within the experimental error, the asymptotic value is the same for both. In one experiment, a suspension of algae was divided into two parts, one of which was adapted with air and the other with N_2. Comparison of 10-minute C^{14} fixation into citrulline showed no significant difference. However, this time of C^{14} exposure may have been too long (see Fig. 5), and further work is required to clarify this point.

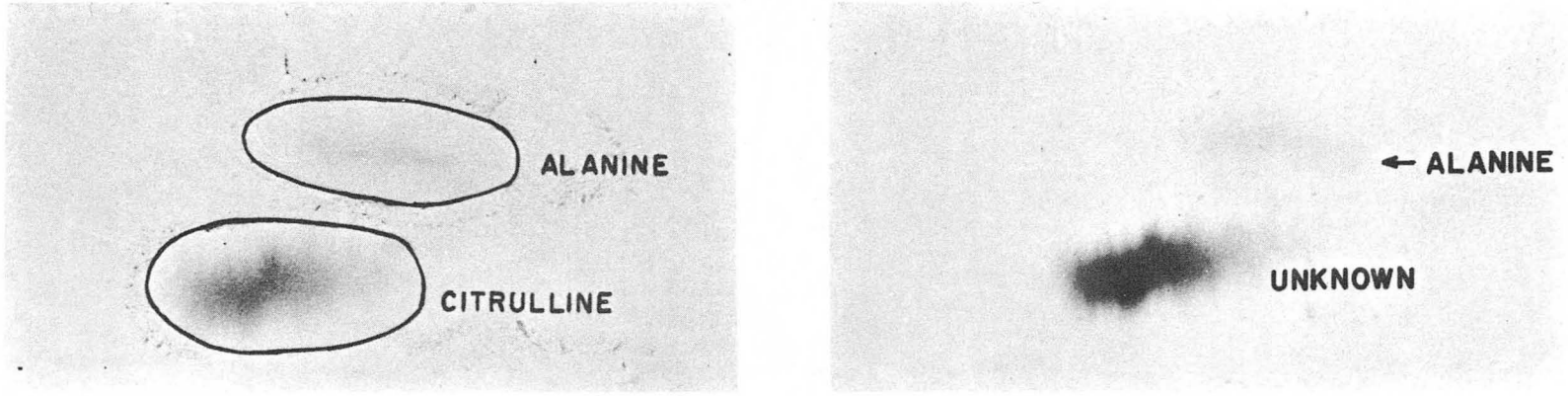

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Fig. 4. Co-chromatography of unknown (5×10^5 dpm) with added unlabeled citrulline (40 μg) and labeled alanine (5×10^4 dpm). Left: ninhydrin-sprayed paper; right: radioautogram.

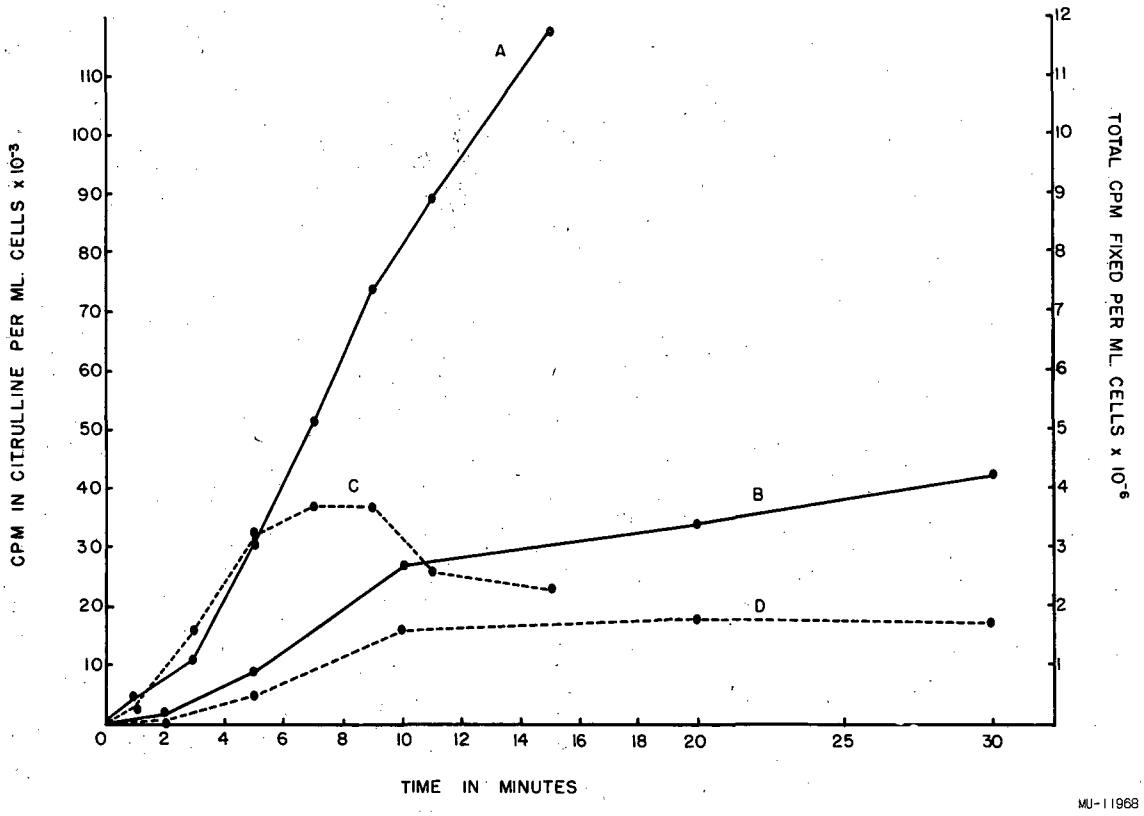

Fig. 5. Curves A and B: Total fixation of C^{14} in light (A) and dark (B) in Nostoc muscorum.
Use right ordinate.

Curves C and D: Incorporation of C^{14} in citrulline in light (C) and dark (D).
Use left ordinate.

DISCUSSION

Though the original crude sample of citrulline isolated from Nostoc was not contaminated by any radioactive compounds, it was contaminated by one or more compounds that reacted weakly with ninhydrin. By spraying a dilution series of the isolated citrulline and a parallel dilution series of pure citrulline with p-dimethylaminobenzaldehyde, it was determined that the original 12.1 mg of isolated material contained only about 500 μg of pure citrulline. The molar specific activity of the isolated citrulline is estimated to be about half of that of the radioactive bicarbonate used. This, coupled with the fact that it is likely that nearly all the activity is located in the carbamyl group while little or no activity is located in the carboxylic group, raises many questions regarding the method of C^{14} incorporation in citrulline and the significance of this compound in the metabolism of these algae.

According to the Krebs-Henseleit cycle,[15] as worked out with liver slices, citrulline plays an integral part in the conversion of ornithine to arginine, which is then hydrolyzed to urea and ornithine. All efforts to detect free urea or arginine in these algae have been negative; the alcohol-insoluble protein and polysaccharide fraction, after photosynthesis with radioactive bicarbonate, was hydrolyzed with HCl of varying concentration in an effort to detect protein-bound arginine, but no large amount of arginine was found. Although it is possible that the carbamyl-labeled citrulline may be formed in an exchange reaction involving a sequence of reversible steps, in at least one of which ATP is involved to account for the light acceleration, a cyclic route for its formation is more attractive, because of the speed with which the specific radioactivity of the carbamyl group approaches that of the CO_2. This would require that citrulline be functioning in other roles in these algae, such as a possible route of transfer of nitrogen from ammonia to amino acids, or as a possible storage pool for the carbamyl group for nucleotide synthesis. In regard to the possible function as a transfer mechanism of ammonia to amino acids, it is interesting to note that the blue-green algae, which characteristically show a very rapid labeling of citrulline with C^{14}, include many species that have the ability to fix atmospheric nitrogen; Nostoc muscorum is one of these.

This work was done under the auspices of the U.S. Atomic Energy Commission.

15 H. A. Krebs and H. Z. Henseleit, Physiol. Chem. 172, 353 (1948).