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SITES OF AZASERINE INHIBITION DURING PHOTOSYNTHESIS BY SCENEDESMUS

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ABSTRACT

L-azaserine has been found to have a profound effect on the reservoir sizes of many of the metabolic intermediates produced during photosynthesis by Scenedesmus. Marked increases in levels of glutamine and the acids of the Krebs cycle were accompanied by a corresponding depletion of the amino acid reservoirs, indicating that one of the major sites of azaserine action is in reactions involving transamination. In contrast, the photosynthetic carbon cycle is virtually unaffected and the rate of formation of sucrose is increased.

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INTRODUCTION

The success attending the use of azaserine[1, 2] as a specific inhibitor of one stage in the metabolic pathway leading to the synthesis of inosinic acid in pigeon liver prompted us to use this antibiotic in a similar attack on purine synthesis in Scenedesmus. However, investigation of the products produced during photosynthesis by suspensions of these algae in the presence of azaserine showed that a more widespread interference with metabolism had occurred. The purpose of this communication is to describe the nature of these effects and to attempt to assess their importance in a general picture of the metabolic effects of azserine.

EXPERIMENTAL

Experimental Procedure

Two suspensions, each containing washed Scenedesmus cells (packed volume, 0.2 cc) and KH_2PO_4 solution (0.4 cc; 3.2×10^{-6} M) in 21 cc and one with added L-azaserine (4 mg), were left for 1 hour in thin glass containers illuminated on each side by a 150-watt light (reflector flood) to achieve steady states with 4% CO_2 in air. Each suspension was then allowed to photosynthesize for 5 minutes with $NaHC^{14}O_3$ solution (0.9 cc; 360 μc) and then flushed with air for 1 minute. The cells were killed by pouring them into boiling ethanol (88 cc), and the resulting 80% ethanol extract was separated from insoluble material which was then reextracted with 20% ethanol (100 cc). The total fixation of radioactivity was determined in each case by uniformly distributing and drying 50-μl aliquots of the 80% ethanol suspensions plus

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* Rockefeller Research Fellow, 1955-56.

1 Hartman, Levenberg, and Buchanan, J. Am. Chem. Soc. 77, 501 (1955).

2 B. Levenberg and J. M. Buchanan, J. Am. Chem. Soc. 78, 504 (1956).

50 μl 6 N acetic acid on aluminum discs, and counting the radioactivity with a Scott large-window Geiger-Mueller tube. The radioactivities extracted from each suspension with 80% ethanol and with 20% ethanol were determined in a similar manner. After the combined extracts of each suspension were concentrated to 3 to 4 cc, aliquots (calculated to contain 1 x 10^6 counts/min. each) were applied to several washed Whatman No. 4 papers and separated first in phenol-water and then in butanol-propionic acid in the manner described by Wilson and Calvin.[3] After radioautographs of the chromatograms had been made, the various components detected on the papers were counted (Table I). Since the correction for self-absorption of radiation would be the same for each compound, no correction was applied. The results of a duplicate experiment, in which 1 mg of azaserine was used, are also presented (Table I).

Other experiments carried out in a manner identical with those described above were (1) a repetition of the 1-mg-azaserine experiment in which an intense photospot light was substituted for one of the reflector floods for 50 minutes of the 1-hour steady-state period (Table II), and (2) an experiment in which one suspension contained 4 mg azaserine and the other contained both azaserine (4 mg) and glutamine (5 mg) (Table III).

Chromatograms from each of the above experiments were sprayed with ninhydrin to obtain a qualitative estimate of the concentrations of amino acids present. Glutamine obtained from azaserine chromatograms, as well as the glutamic acid produced from it by hydrolysis with 1 N HCl at 100° for 2 hours, were characterized by cochromatography on two-way paper chromatograms and by ionophoresis on paper for 3 hours at 600 v in 0.1 M propionate, pH 5.6. Sucrose, amino acids, and carboxylic acids mentioned in Table I were likewise characterized by cochromatography on two-way paper chromatograms and most of them also by ionophoretic separations. The lipids, phospholipids, and pigments all were moved at a similar distance in phenol-water but were separated in butanol-propionic acid into two mobile areas designated lipid (containing some pigment), phospholipid (containing no visible pigment), and a stationary area designated X (containing some pigment). No radioactivity could be extracted from these areas by water elution, but most of the activity was extractable with a mixture of petroleum ether and ethanol. The compounds in Area X ran faster than diphosphopyridine nucleotide in phenol-water, but showed the same lack of mobility in butanol-propionic acid.

[3] A. T. Wilson and M. Calvin, J. Am. Chem. Soc. 77, 5948 (1955).

Table I

The effect of azaserine on Scenedesmus

	Control I	Azaserine I (4 mg)	Control II	Azaserine II (1 mg)
Determination on aluminum disc (counts/min)				
Total fixation	22×10^6	27×10^6	29.2×10^6	33×10^6
80% ethanol extract	45.5%	47%	47.3%	47.3%
20% ethanol extract	5.9%	7.5%	7.5%	8.5%
Determination on paper (counts/min equal total radioactivity placed on each paper)				
Glutamine	332	1192	390	1312
Glutamic acid	2066	1227	2632	1139
Aspartic acid	4436	989	7020	762
Serine	1839	849	2411	990
Alanine	1166	272	2000	314
Glycine / Threonine	400	574	1045	908
Tyrosine	261	171	444	295
Valine	1051	787	1468	1053
Sucrose	923 x 9.5[a]	2380 x 9.5	937	2072
Malic Acid	3922 x 9.5	5814 x 9.5	2691 x 9.5	4263 x 9.5
Citric acid	466 x 9.5	1118 x 9.5	145 x 9.5	400 x 9.5
α-Ketoglutaric acid	40	200	270	1054
Fumaric acid	269	108	381	344
Succinic acid	1117	1689	822	922
Glyceric acid	576	966	1349	845
Glycolic acid	200	3182	240	880
Lipids	1650 x 9.5	1259 x 9.5	2540 x 9.5	1923 x 9.5
Phospholipids	1229 x 9.5	1071 x 9.5	1350 x 9.5	853 x 9.5
Area X	1001 x 9.5	751 x 9.5	621 x 9.5	538 x 9.5
Sugar phosphates, PGA and origin	14,040 x 9.5	16,379 x 9.5	15,071 x 9.5	14,424 x 9.5

[a] The factor 9.5 was used whenever the radioactivity was counted through aluminum foil.

Table II

The effect of azaserine and strong light on Scenedesmus

	Control III	Azaserine III (1 mg)
Determination on aluminum disc (counts/min)		
Total fixation	20.9×10^6	14×10^6
80% ethanol extract	47.8%	57.8%
20% ethanol extract	2.5%	4.4%
Determination on paper (counts/min)		
Glutamine	529	4964
Glutamic acid	4951	2945
Aspartic acid	9089	2262
Serine	2834	1591
Alanine	2345	951
Glycine } Threonine }	949	929
Sucrose	1357 x 9.5	1907 x 9.5
Malic acid	3743 x 9.5	6479 x 9.5
Citric acid	254 x 9.5	1919 x 9.5
α-Ketoglutaric acid	381	1851
Fumaric acid	344	540
Succinic acid	1621	3180
Glyceric acid	1553	1312
Glycolic acid	285	5 06
Lipids	3582 x 9.5	2491 x 9.5
Phospholipids	2259 x 9.5	1665 x 9.5
Area X	725 x 9.5	506 x 9.5
Sugar Phosphates, PGA and origin	11,800 x 9.5	9312 x 9.5

Table III

Attempted reversal of azaserine inhibition with glutamine

	Azaserine (4 mg) alone	Azaserine (4 mg)+ glutamine (5 mg)
Determination on aluminum disc (counts/min)		
Total fixation	33.6×10^6	35.8×10^6
80% ethanol extract	41.2%	52.9%
20% ethanol extract	1.15%	2.22%
Determination on paper (counts/min)		
Glutamine	2182	6572
Glutamic acid	1353	2704
Aspartic acid	666	939
Serine	735	690
Alanine	393	473
Glycine / Threonine	503	527
Sucrose	1496 x 9.5	1111 x 9.5
Malic acid	3697 x 9.5	4631 x 9.5
Citric acid	586 x 9.5	446 x 9.5
α-Ketoglutaric acid	696	1052
Fumaric acid	242	249
Succinic acid	870	1308
Glycolic acid	3424	3138
Lipids	1639 x 9.5	2192 x 9.5
Phospholipids	541 x 9.5	1310 x 9.5
Area X	797 x 9.5	1370 x 9.5
Sugar Phosphates PGA and origin	11, 970 x 9.5	12, 383 x 9.5

The triose phosphate, pentose monophosphate, glucose cyclic 1, 2-phosphate, hexose monophosphate, phosphoglyceric acid, and phosphoenolpyruvate were eluted from control and azaserine chromatograms, and treated with purified "Polidase" phosphatase, and the products were analyzed by paper chromatography and radioautography.[3]

The effect of azaserine (4 mg) on Chlorella was also determined in two experiments (Table IV) identical with that described for the Scenedesmus model experiment except that in one case the time of flushing with air was 30 sec and in the other, 60 sec.

DISCUSSION

It is evident from Table I that with Scenedesmus the azaserine causes a build-up of the acids (e.g., citric, malic, α-ketoglutaric, succinic) of the Krebs tricarboxylic acid cycle and a decrease in radioactivity and amount (revealed by ninhydrin) of the amino acids (glutamic acid, aspartic acid) derived therefrom by amination or transamination. Alanine, serine, valine and tyrosine, which are also known to be formed by transamination of their corresponding keto-acids, were likewise depleted. Because of the low activity of glycine and threonine and the presence of other compounds in this area the effect of azaserine on these amino acids could not be assessed. Glutamine,[4] which is a donor of amino groups during transamination, increases markedly in activity and amount (approximately 1 mg/g cells, as estimated by ninhydrin) in the presence of azaserine. It is therefore suggested that azaserine interferes with transamination reactions, either directly by inhibiting the actual process of transamination or indirectly by interfering with the synthesis of pyridoxal phosphate or pyridoxalamine phosphate, which are necessary as coenzymes of such reactions. The second possibility seems less likely because of the short time available for that inhibition to appear for which these compounds are coenzymes. Figure 1 indicates some of the suggested sites of azaserine inhibition. Kaplan and Stock[5] have also concluded that azaserine is an inhibitor of amino acid synthesis, on the basis of their observation that inhibition of growth in E. coli by azaserine can be overcome by addition of certain amino acids. Two azaserine-inhibited reactions, which have recently been reported, would also fit into this scheme. It has been shown that azaserine inhibits the conversion of α-N-formyl glycinamide ribotide to α-N-formylglycinamidine ribotide[2] and the amination of xanthosine-5'-phosphate to guanosine-5'-phosphate.[6] L-glutamine is the donor of amino groups in both these reactions.

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[4] A. Meister and S. V. Tice, J. Biol. Chem. 187, 173 (1950).

[5] L. Kaplan and C. C. Stock, Federation Proc. 13, 239 (1954).

[6] M. Bentley and R. Adams, Federation Proc. 15, 218 (1956).

Table IV

The Effect of Azaserine on Chlorella

	Control IV	Azaserine IV (4 mg)-air-flushed 30 sec	Control V	Azaserine V (4 mg)-air-flushed 60 sec
Determination on aluminum disc (counts/min)				
Total fixation	25.4×10^6	30.8×10^6	23.4×10^6	34.8×10^6
80% ethanol extract	60.7%	66.4%	62%	62.8%
20% ethanol extract	8.3%	8.25%	10.6%	8.1%
Determination on paper (counts/min)				
Glutamine	250	300	623	530
Glutamic acid	2203	2290	2370	2000
Aspartic acid	2541	2700	1900	2180
Serine	2866	3361	3890	4770
Alanine	9635	8232	10,300	9670
Glycine, Threonine	800	960	780	830
Sucrose	4458 x 9.5	3933 x 9.5	4350 x 9.5	4330 x 9.5
Malic acid	2243	2409	1980	1970
Citric acid	245	250	200	220
Fumaric acid	571	428	380	300
Lipids, Phospholipids	2101 x 9.5	1895 x 9.5	2800 x 9.5	2750 x 9.5
Area X	880 x 9.5	858 x 9.5	630 x 9.5	640 x 9.5
Sugar phosphates, PGA and origin	11,583 x 9.5	12,085 x 9.5	18,690 x 9.5	19,140 x 9.5

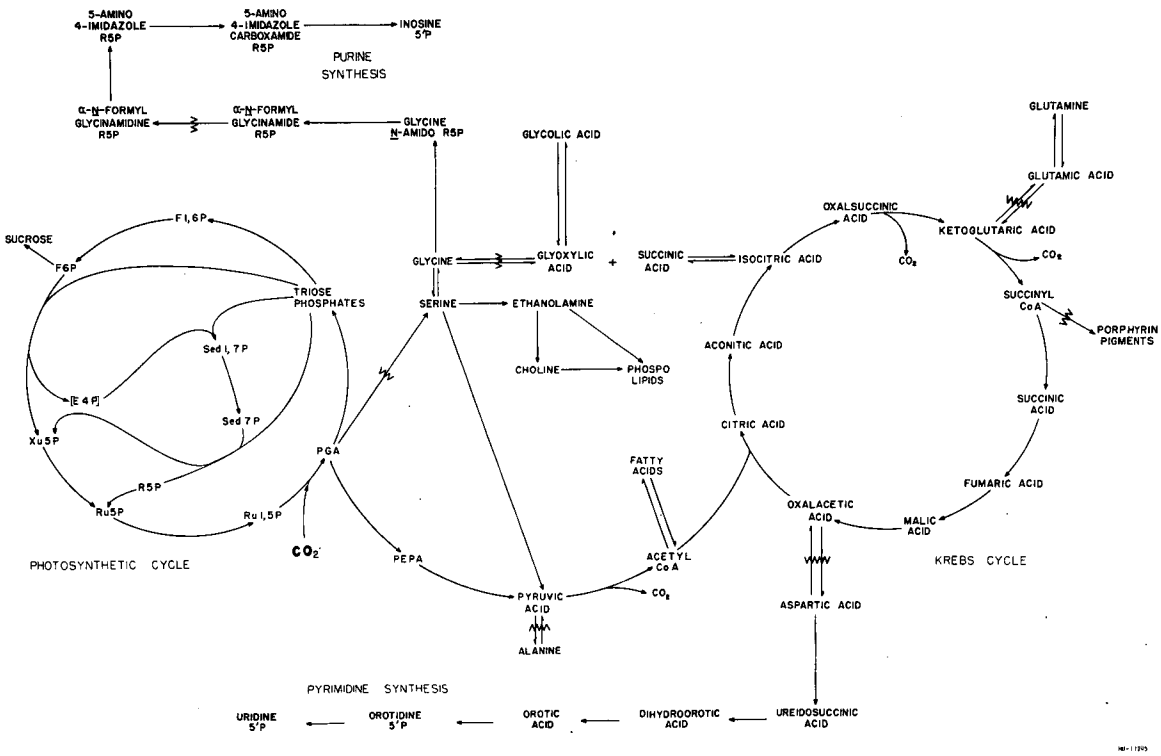

Fig. 1. Possible sites of azaserine inhibition.

An interesting feature is the build-up of glutamine despite the fact that the formation of glutamic acid (its precursor) is partially inhibited by azaserine. It is to be noted, however, that the decrease in glutamic acid is by no means so marked as the decreased formation of aspartic acid in the presence of azaserine. One explanation of this may be that whereas it is known[7] that glutamic acid can be synthesized from NH_4^+ and α-ketoglutaric acid, the synthesis of aspartic acid by a similar reaction of NH_4^+ and oxalacetic acid has not been proved. This alternative pathway of glutamic acid synthesis, not involving transamination, probably operates in Scenedesmus.

Since the formation of serine is blocked partially by azaserine, smaller amounts of ethanalamine and choline (which are derived from it) are available for incorporation into phospholipids, and this may account for the decreased radioactivity found in this region of the chromatograms in the presence of azaserine. The lipid and area X also show decreased activity. This might be accounted for, at least in part, by blockage of a transamination reaction leading to the glycine that is necessary for the synthesis of the heterocyclic rings in the porphyrin pigments.[8]

Glycolic acid, which generally accumulates[3] at low CO_2 pressures, was considerably increased at higher levels of azaserine. This could not be caused by a lower rate of CO_2 fixation, since this is increased by azaserine during photosynthesis. The increased levels of acids in the Krebs cycle may cause an increased formation of glyoxylic acid,[9] which with an inhibited glycine formation would be available for glycolic acid production. The increased rate of CO_2 fixation in the presence of azaserine is probably responsible for the increased level of sucrose drained from the photosynthetic cycle.

Examination of the components of the photosynthetic cycle, by analysis of the products produced by phosphatase action, revealed the presence of glyceric acid and all the usual sugars. Several additional unidentified components were detected in the phosphatased triose phosphate and glucose cyclic 1, 2 phosphate areas eluted from azaserine chromatograms.

The total fixations in the experiments, in which an intense photospot light was substituted for one of the reflector floods for 50 minutes of the 1-hour steady-state period (Table II), indicate that a larger proportion of the algae were killed by the bright light in the presence of azaserine than in

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[7] H. A. Krebs and P. D. Cohen, Biochem. J. 33, 1895 (1939).

[8] Della Rosa, Altman, and Salomon, J. Biol. Chem. 202, 771-9 (1953).

[9] Smith, Stamer, and Gunsalus, Biochem. Biophys. Acta 19, 567 (1956).

its absence. However, control and azaserine chromatograms, each carrying a total activity of 1×10^6 counts/min, showed spots (Table II) with the same order of relative activities as those given in Table I.

An attempt to reverse the effects of azaserine inhibition with glutamine was only partially successful (Table III). Some increase in radioactivity in the lipid, phospholipid, area X, and glutamic acid was observed. The apparent continued build-up of glutamine can be explained by suggesting that while the rate of conversion of C^{14}-labeled precursors to glutamine remains almost the same, the introduction of inactive glutamine causes a dilution of radiocarbon in the glutamine pool, with a consequent lowering in the specific activity of the glutamine being converted to further products and hence an increase in the residual C^{14} glutamine observed. It is interesting to observe that the combined effect of azaserine and added inactive glutamine has caused almost a twentyfold increase of C^{14} glutamine.

Although azaserine caused a marked increase in CO_2 fixation by Chlorella (Table IV), analysis of aliquots containing equal amounts of radioactivity showed that the radioactivity of most of the metabolites relative to one another remained the same.

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