2% aqueous phosphomolybdic acid spray, followed by 1:2 ammonia–ethanol).

Reaction of 3,5-Dibromophloretylglycine (XIX) with NBS.—To a solution of 200 mg. (0.0053 mole) of XIX in 30 ml. of 20% acetonitrile–acetate buffer (0.16 *M*, *p*H 4.6) was added a solution of 93 mg. (0.00053 mole) of NBS in 10 ml. of the same solvent. Colorless needles began to separate within 5 minutes. After 2.5 hours, the reaction mixture was chilled briefly and the dienone-lactone VII was collected by filtration (84 mg., 49%, m.p. 173–175°).

No melting point depression was observed on admixture with a specimen of VII prepared from 3,5-dibromophloretic acid (VI). The ultraviolet spectra of the two substances were identical. Paper chromatography of a sample of the reaction mixture on Whatman #1 paper in 1-butanol–acetic acid–water (4:1:5) showed the presence of a single ninhydrin-positive spot, corresponding in R_f to a glycine standard. Quantitative ninhydrin assay[21] of an aliquot of the reaction mixture gave a value of 76% cleavage, based on the color yield of a glycine standard.

Reaction of Phloretylglycine (XVIII) with NBS. A. Preparative Experiment.—To a solution of 334 mg. (0.0015 mole) of phloretylglycine in 25 ml. of 20% acetonitrile–acetate buffer was added, in 5 equal portions at one-minute intervals, a solution of 820 mg. (0.0046 mole) of NBS in the same solvent. Within 10 minutes, a crystalline solid had begun to separate. After 2 hours, the product was collected by filtration (230 mg., 48%, m.p. 166–173°). Recrystallization from acetonitrile–water yielded colorless needles melting at 172–174°. The product was shown to be identical to the dienone-lactone VII by ultraviolet spectrum and mixed melting point.

B. **Analytical Experiment.**—A series of reaction mixtures containing phloretylglycine (10^{-3} *M*) and NBS (0–3.6×10^{-3} *M*) in the same solvent as above were allowed to stand at room temperature for 30 minutes. At the end of this time, no NBS remained, as determined by iodometric titration. From each solution, aliquots were removed for spectrophotometric determination of the dienone-lactone VII (optical density at 260 mμ), and ninhydrin assay for glycine.[21] The results of this experiment are shown in Fig. 1.

In a control run, it was noted that glycine reacted rapidly with NBS under the conditions of the cleavage reaction. Using a solution containing glycine and NBS each at 10^{-3} *M*, it was found that less than half of the NBS remained after two minutes (iodometric titration). The decrease in ninhydrin color occurred more slowly.

Reaction of N-Carbobenzyloxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine (XX) with NBS.—A solution of N-carbobenzyloxy-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine[11] at 4×10^{-4} *M* was allowed to react with 3 equivalents of NBS or of bromine under the conditions described above for phloretylglycine (XVIII). After 30 minutes, aliquots were removed for ninhydrin assay, which indicated a cleavage yield of 40%. Paper chromatography on Whatman #1 paper in 1-butanol–acetic acid–water (4:1:5) gave a single ninhydrin-positive spot of R_f corresponding to that of a standard sample of isoleucine. Spectral examination of a sample of the reaction mixture showed the presence of an intense maximum at 260 mμ.

Acknowledgments.—The authors thank Dr. W. C. Alford and his associates of this Institute for carrying out the microanalyses and determining optical rotations, and Mr. Wm. M. Jones for measuring the infrared spectra. We are indebted to Dr. A. Patchornik for valuable discussions. We are grateful to Prof. E. J. Corey for informing us of his results in a similar study prior to publication.

(21) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

BETHESDA, MD.

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Study of Inhibition of Azaserine and Diazo-oxo-norleucine (DON) on the Algae *Scenedesmus* and *Chlorella*[1]

BY PETRONELLA Y. F. VAN DER MEULEN[2] AND JAMES A. BASSHAM

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The effects of azaserine and DON (diazo-oxo-norleucine) on the metabolism of the algae *Scenedesmus* and *Chlorella* during photosynthesis with $C^{14}O_2$ are presented and found to be largely the same for both inhibitors. DON appears to inhibit the formation of α-N-formylglycinamidine ribotide from α-N-formylglycinamide ribotide, as is known for azaserine. α-N-Formylglycinamide ribotide and glutamine accumulate with both inhibitors, being absent from the control experiment. A proposal is presented that a possible site of inhibition is the synthesis of glutamic acid from α-ketoglutaric acid. The relationship between glutamine and glutamic acid is discussed and the suggestion made that there may be a biosynthetic route to glutamine not involving glutamic acid as an intermediate. Indications for a still wider interference of the inhibitors are seen.

Introduction

In studies of the effect of azaserine on *Scenedesmus* during photosynthesis with $C^{14}O_2$ the inhibitor was found to cause many changes in the labeling of metabolic intermediates with C^{14}.[3] The transamination reactions were given as possible sites of inhibition. This work has been extended, and in this report a study on the inhibition effects of both azaserine and diazo-oxo-norleucine (DON) on the metabolism of the algae *Scenedesmus* and *Chlorella* is reported.

(1) The work described in this paper was sponsored, in part, by the U. S. Atomic Energy Commission and, in part, by a grant from the Rockefeller Foundation to Professor Melvin Calvin.

(2) Centraal Laboratorium, Toegepast Naturrwetenschappelijk Onderzoek (T.N.O.) Delft, the Netherlands. Netherlands-America Foundation Fellow, 1956–1957.

(3) S. A. Barker, J. A. Bassham, M. Calvin and U. C. Quarck, THIS JOURNAL, **78**, 4362 (1956).

Experimental Procedures

The experiments were of two types: type A, in which first inhibitor and then, after 5 minutes, $C^{14}O_2$ is added to the algae suspension; and type B in which the algae are continuously in contact with $C^{14}O_2$, before, during and after the addition of inhibitor.

Experiments of type A show the effect of the presence of inhibitor for a fixed time on the distribution of radioactivity among the metabolic intermediates after 5 minutes photosynthesis. In experiments of type B the distribution of radioactivity among metabolites following introduction of the inhibitor can be followed over a longer period of time.

A brief description of experiments of type A and B with experimental details is given below, followed by tables and charts of the results of those experiments. Any deviation from the standard procedure is noted under the experiment concerned. Further experimental details have been published elsewhere.[4]

(4) P. Y. F. van der Meulen, Rijks Universiteit, Leiden, The Netherlands, Thesis, 1958.

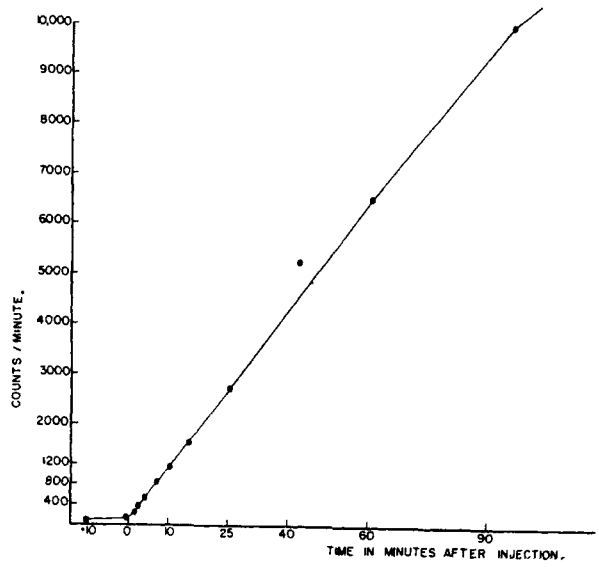

Fig. 1.—Radioactivity in glutamine before and after injection of azaserine in *Scenedesmus* photosynthesizing in the presence of $C^{14}O_2$ (expt. B-1). Time of injection of inhibitor is given as time zero.

Experiment of Type A.—Freshly harvested algae (*Scenedesmus obliquus* or *Chlorella pyrenoidosa*) were centrifuged at 2000 r.p.m. for 7 minutes, washed with distilled water and centrifuged again. They were then suspended to a 1% suspension (1 ml. of wet-packed algae per 100 ml.) in distilled water, and 1 ml. of KH_2PO_4 buffer (*p*H 6.8, 2.5×10^{-3} *M*) was added per 100 ml. of suspension. Of this suspension, 20 ml. was placed in each of two thin illumination vessels (lollipops), arranged next to each other and immersed in a water-bath to keep them at the same temperature of 20 to 22° and to prevent heating by the lights. A stream of 2% or 4% CO_2-in-air was introduced into the suspension through a capillary. The lights (reflector flood, 150 watt, on each side at a distance of 20 cm. from the lollipops) were turned on, and the algae were allowed to photosynthesize for 15 minutes. Then a known amount of inhibitor, dissolved in a small amount of water, was added to one of the lollipops, the same volume of distilled water being added to the other. After the time chosen for the inhibitor to effect its influence, 500 μl. of bicarbonate-C^{14} (0.036 *N*, 400 μc. per ml.) was added at the same time to each lollipop, and the lollipops were immediately closed and shaken in the light for 5 minutes. The suspension was then poured rapidly into 80 ml. of boiling alcohol. The resulting 80% ethanol suspension was again brought momentarily to the boiling point and, after cooling, centrifuged at 2000 r.p.m. for 15 minutes. The precipitate was re-extracted with 20% ethanol, and the extracts combined. By evaporating small aliquots of the 80% ethanol suspension and of the combined extracts on aluminum plates, removing excess bicarbonate with acetic acid, and measuring the radiation from the plates, the total radioactivity fixed by the algae, as well as the radioactivity in the soluble compounds, was calculated.

The combined extracts then were concentrated under vacuum and analyzed by the usual methods of paper chromatography and radioautography.[5,6] Labeling with C^{14} of individual compounds was determined by direct counting on the paper chromatogram.

Experiments of Type B.—A suspension of algae (0.5 or 1.0%) was prepared as in experiment A. Sixty ml. of this suspension was placed in a thin illumination vessel in a "steady state" apparatus similar to that described in previous studies.[6,7] After about 15 minutes illumination of the algae, a check was made of the rate of photosynthesis. The system was then filled with air and 0.7 to 0.8% $C^{14}O_2$ containing 2.4 mc. of C^{14}. At a given time, after 20 to 40 minutes of photosynthesis with $C^{14}O_2$ the inhibitor dissolved in a small amount of water was injected into the suspension by means of a needle through a rubber cap on an opening of the vessel. Samples of the suspension were taken at dif-

TABLE I

RADIOACTIVITY IN VARIOUS COMPOUNDS IN *Scenedesmus* EXTRACTS AFTER 5 MINUTES OF PHOTOSYNTHESIS WITH BICARBONATE-C^{14} WITH DON

Conditions: 1% suspension of *Scenedesmus;* inhibitor DON, 100 μg. per ml. of suspension. One hour after adding inhibitor the algae suspension was flushed with air for 1 minute, then 300 μl. of bicarbonate-C^{14} (120 μc.) was added and photosynthesis with $HC^{14}O_3$ allowed to proceed for 5 minutes. Equal amounts of radioactivity were chromatographed, requiring, respectively, 1/17 and 1/18th of the extracts.

Compound	Counts per minute (sensitivity about 1 c.p.m. per 7 d.p.m.). Control	Inhibited	Ratio: I/C
Aspartic acid	5582	485	0.087
Serine	3458	384	.11
Glycine	1151	383	.33
Threonine	408	204	.50
Alanine	1689	531	.317
Glutamine	192	2284	12
Glutamic acid	1975	777	0.39
α-Ketoglutaric acid	219	1055	4.8
Citric acid	1250	1715	1.4
Malic acid	24500	25200	1.0
Sucrose	4014	8093	2.0
Diphosphates	5000	2400	0.48
Total fixation	23.1×10^6	21.7×10^6	
Total C^{14} in extract	18.4×10^6	17.1×10^6	

TABLE II

RADIOACTIVITY OF VARIOUS COMPOUNDS IN *Chlorella* EXTRACTS AFTER 5 MINUTES PHOTOSYNTHESIS WITH BICARBONATE-C^{14} WITH DON

Conditions: 1% suspension of *Chlorella;* inhibitor DON, 200 μg. per ml. of suspension; after one hour in the presence of inhibitor, 1 minute of air flushing followed by 5 minutes of photosynthesis with bicarbonate-C^{14}. Equal amounts of radioactivity were chromatographed, corresponding to 1/26th and 1/16th of the extracts (which were the extracts of 8 mg. and 12.5 mg. wet-packed algae, respectively).

Compound	Counts per minute Control	Inhibited	Ratio: I/C
Aspartic acid	1648	760	0.46
Serine-glycine[a]	5811	6613	..
Alanine	21375	9479	0.45
Glutamine	602	1385	2.27
Glutamic acid	9662	9468	..
α-Ketoglutaric acid[a]			
Citric acid	406	648	1.6
Malic acid	1172	2140	1.8
Glyceric acid	1519	578	0.38
Glycolic acid	140	261	1.9
Fumaric acid	326	426	1.3
Lactic acid	410	1911	4.7
Citrulline	154	489	3.2
Sucrose	57568	32056	0.56
Unknown spot 1	719	3142	4.3
Unknown spot 2	1464	2978	2.0
Total fixation in c.p.m.	23.4×10^6	40.5×10^6	
C.p.m. in extract	16.5×10^6	26×10^6	

[a] These compounds do not separate well and have been counted together.

(5) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, THIS JOURNAL, **72**, 1710 (1950).

(6) A. T. Wilson and M. Calvin, *ibid.*, **77**, 5948 (1955).

(7) J. A. Bassham, K. Shibata, K. Steenberg, J. Bourdon and M. Calvin, *ibid.*, **78**, 4120 (1956).

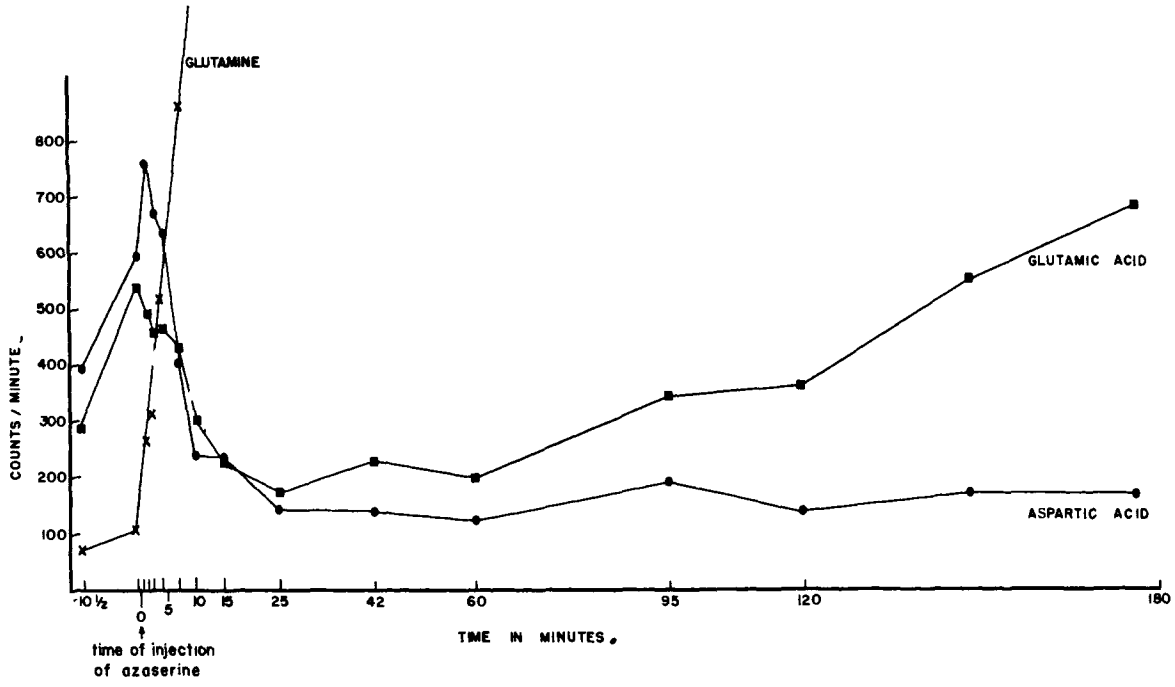

Fig. 2.—Radiocarbon found in aspartic and glutamic acids per extract of 1 ml. suspension of 0.5% *Scenedesmus* suspension during "steady state" $C^{14}O_2$ photosynthesis with azaserine inhibition (exp. B-1).

ferent times before and after addition of inhibitor, from 0.5 minutes up to 3 hours.

During the entire period of the experiment, the CO_2 content of the gas changed from 0.8 to between 0.3 and 0.4%, and no significant change in the rate of photosynthesis could be detected.

The samples, each about 1 ml., were run into 5 ml. of cold methanol and their sizes were determined by weighing. When necessary, more methanol was added to make an 80% suspension. After boiling for 1 minute the aliquots were centrifuged, the residue re-extracted with 20% methanol, concentrated under vacuum, and the total extract analyzed by paper chromatography and radioautography as in experiments of type A. The different compounds were counted on the paper and the activity (in c.p.m. on paper) per extract of 1 ml. of suspension was calculated.[6]

heating for 22 minutes at 65°. The spots were cut out, cut into small pieces, and then placed in a tube with 3 ml. of 71% ethanol and shaken a few minutes. The paper was then removed by centrifugation and the absorption at 575 mμ of the supernatant solution was determined.[8]

A standard curve was made by chromatographing known amounts of amino acids and determining the absorption at 575 mμ in the same way. It was found that with amounts between 2 and 10 μg., an accuracy of 10% could be obtained.

Identification of α-N-Formylglycinamide Ribotide.—With both inhibitors an apparently new radioactive compound, absent in the control, was found on the chromatogram. This compound was found in the area of the chromatogram where glucose cyclic phosphate often is found. When eluted from the paper, treated with a phosphatase preparation, and then

TABLE III

COUNTS PER MINUTE ON PAPER OF CHROMATOGRAMS OF *Chlorella* EXTRACTS AFTER 5 MINUTES OF PHOTOSYNTHESIS WITH BICARBONATE-C^{14} AND ONE HOUR OF INHIBITORY ACTION OF DON

Compound	C.p.m. Control	C.p.m. Inhibitor	Ratio: I/C	μg. in C	μg. in I	μg. I / μg. C	Sp. act. C in c.p.m./μg.	Sp. act. I in c.p.m./μg.
Aspartic acid	2010	110	0.055					
Serine	3540	1850	.54					
Alanine	6130	505	.082					
Glutamine	..	1380			4.4			310
Glutamic acid	1430	1710	1.2	8.1	0.4	0.05	180	4200
C.p.m. in extract	18×10^6	20×10^6						

Conditions: 1% suspension of *Chlorella*; inhibitor DON, 10 μg. per ml. of suspension; one hour of photosynthesis in presence of inhibitor, then 5 minutes of photosynthesis with bicarbonate-C^{14}. Of each, an amount of extract containing 10^6 c.p.m. was chromatographed and the specific activity of the amino acids determined.

Whereas the photosynthetic intermediates reach a steady state with respect to radioactivity in about 10 minutes, and their radiocarbon content is a measure of their concentration, the amino acids and keto acids are still gaining in radioactivity even after 40 minutes. Thus, the amount of radioactivity is not a measure of the concentration in the case of the latter substances.

Estimation of Amino Acids.—In some of the experiments a semi-quantitative determination of the amino acids, especially glutamic acid and glutamine, was made. This was accomplished by spraying the papers with ninhydrin solution (0.5 g. of ninhydrin in 100 ml. of 71% ethanol) and

rechromatographed, the compound then moved a little faster than ribose in phenol–water and a little slower than ribose in butanol–propionic acid. This derived spot co-chromatographed exactly with the compound obtained by treatment with phosphatase of formylglycinamide ribotide which Professor J. M. Buchanan[9] kindly provided.

(8) R. E. Kay, D. C. Harris and C. Entenman, *Arch. Biochem. and Biophys.*, **63**, 14 (1956).

(9) Dr. J. M. Buchanan, Department of Biochemistry, Massachusetts Institute of Technology, Cambridge, Mass.

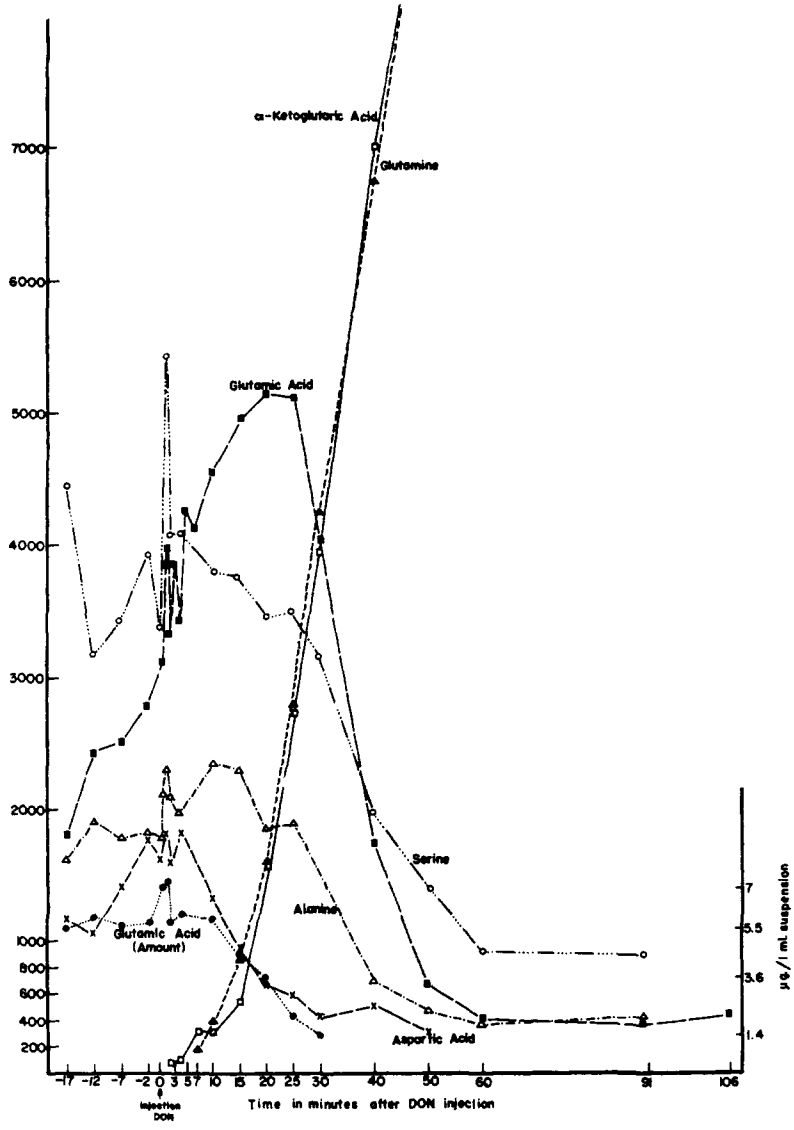

Fig. 3.—Activity in c.p.m. on paper/extract of 1 ml. of suspension of different compounds in samples of 1% *Chlorella* suspension, taken during a long-term $C^{14}O_2$ photosynthesis with DON (15 μg./ml. of suspension) (expt. B-2).

Experiments and Results

A large number of experiments have been carried out.[4] Of these, a few are presented in this report to illustrate the principal results. Results of three experiments of type A are given in Tables I, II and III.

Steady State Scenedesmus—Experiment B-1.—A "steady state" $C^{14}O_2$ photosynthesis experiment with *Scenedesmus* and azaserine (250 μg. per ml. of suspension) as inhibitor was performed. The experiment was carried out as described under B, with a 0.5% suspension of *Scenedesmus*. The radioactivity found in various compounds as a function of time is shown in Figs. 1 and 2.

Steady State Chlorella—Experiment B-2.—A "steady state" $C^{14}O_2$ photosynthesis experiment with *Chlorella* and with DON (15 μg. per ml.) as inhibitor was performed. A 1% *Chlorella* suspension was used. Four samples were taken before injection of the DON and several samples were taken from 0.5 minute up to 2 hours after administration of DON. These samples were analyzed and the radioactivities in individual compounds determined as in experiment B-1. The resulting labeling of various compounds as a function of time before and after injection of inhibitor is shown in Figs. 3 and 4. The specific activities of glutamic acid and glutamine also were determined; see Fig. 5. After a longer time of inhibition some as yet unidentified compounds became labeled, most of them moving quite fast in both directions. They are represented in Fig. 4 by the letters R, S, T, X and Y.

Discussion

The effects of azaserine and DON on *Scenedesmus*, and of DON on *Chlorella* are similar. In each of these cases there occurs a decrease of labeling of amino acids (*e.g.*, aspartic acid, glutamic acid, alanine, serine), and an increase in labeling of glutamine and the acids: α-ketoglutaric, citric, malic, lactic, fumaric and succinic. In addition a new radioactive spot, tentatively identified as α-N-formylglycinamide ribotide, was found in the presence of each inhibitor with *Scenedesmus* but not *Chlorella*.

No inhibition was found with azaserine in *Chlorella*.[3] It seems likely that azaserine did not penetrate the cells. The DON apparently penetrates only slowly into the cells of *Chlorella* with the result that a period of time, which depends on the outside concentration, is required before a

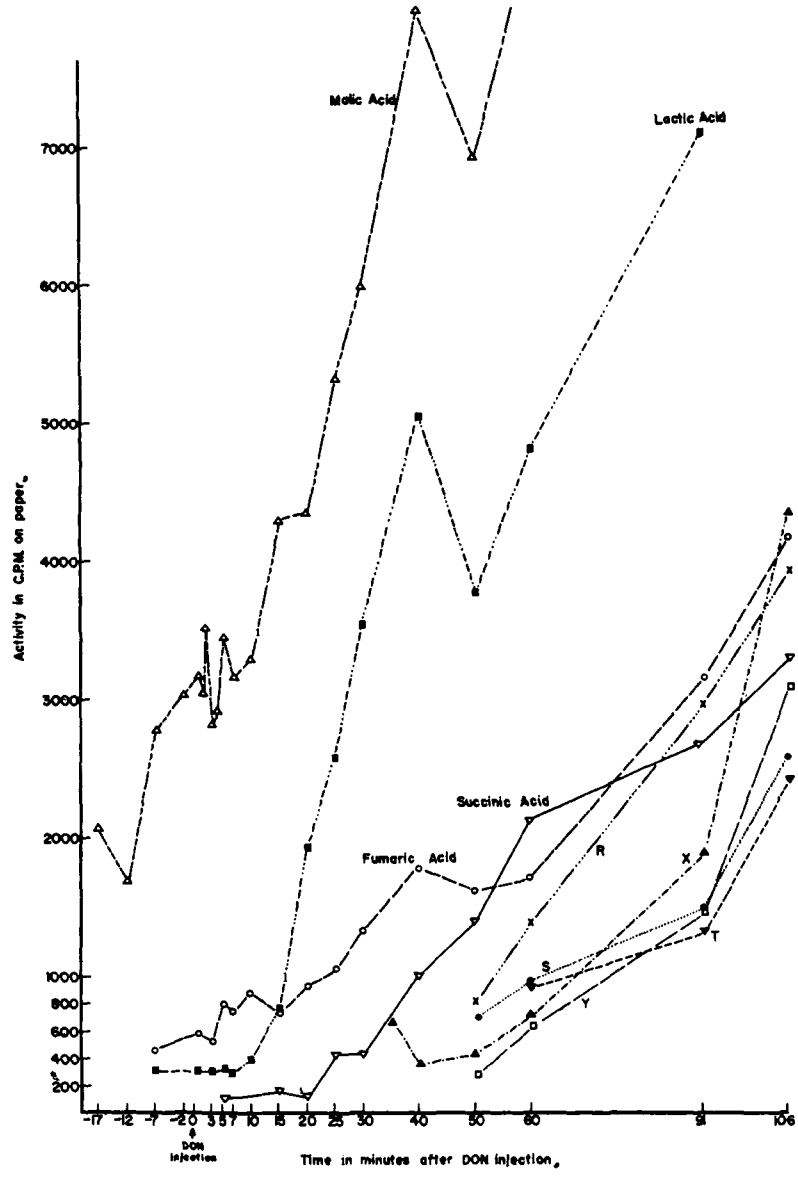

Fig. 4.—Activity in c.p.m. on paper/extract of 1 ml. of suspension of different compounds in samples of 1% *Chlorella* suspension, taken during a long-term $C^{14}O_2$ photosynthesis with DON (15 μg./ml. of suspension). Compounds indicated by letters R, S, T, etc., were not identified (expt. B-2).

sufficiently high level of DON within the cells is reached to effect the inhibition.[4]

Azaserine has been shown[10] to inhibit the formation of α-N-formylglycinamidine ribotide (2-formamido-N-ribosylacetamidine 5′-phosphate) from α-N-formylglycinamide ribotide (2-formamido-N-ribosylacetamide 5′-phosphate). DON appears to inhibit the same reaction, since, with both inhibitors, α-N-formylglycinamide ribotide has been found (in *Scenedesmus*), being absent from the control experiments, and with both inhibitors the glutamine builds up (in both organisms).

Besides inhibition of this reaction and possibly of other transamidations[11,12] the inhibitors appear to affect the metabolism in other points. The more pronounced effects may be summarized as: (1) After there has been time for the inhibitor to penetrate the cells (5 to 10 minutes), total radioactivity in glutamic acid may rise for a few minutes and then fall rapidly (Fig. 3) or may fall immediately (Fig. 2). After a half-hour exposure of the cells to inhibitor, glutamic acid radioactivity may rise slowly (Fig. 2). The concentration of glutamic acid falls from the beginning of inhibition while its specific activity increases continuously (Fig. 5). (2) Aspartic acid, serine and alanine appear to decrease in radioactivity from the beginning of inhibition (Fig. 3). After an hour exposure to inhibitor there is very much less radioactivity in these amino acids and in glycine and threonine than is found with non-inhibited algae (Table I). Inhibition of radiocarbon found in these amino acids (with the possible exception of aspartic acid) ap-

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(11) M. Bentley and R. Abrams, *Federation Proc.*, **15**, 218 (1956).
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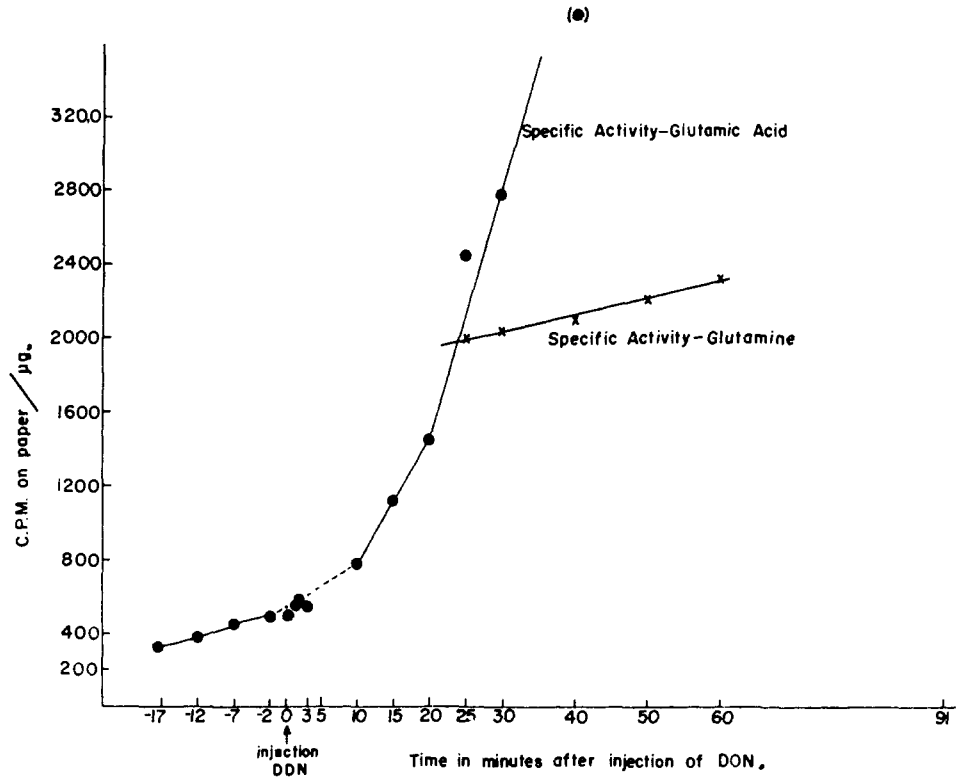

Fig. 5.—Specific activity of glutamine and glutamic acid in samples of a 1% *Chlorella* suspension, taken during a long-term $C^{14}O_2$ photosynthesis with DON (expt. B-2).

pears never to be greater than the decrease in glutamic acid concentration (Table III). (3) Glutamine increases in radioactivity steadily from the beginning of the inhibition (Figs. 1 and 3) and may reach a very high level. Its specific activity, which initially may be higher than that of glutamic acid, increases steadily but falls behind that of glutamic acid. Its concentration increases many-fold during one hour inhibition in *Chlorella*. (4) Acids of the tricarboxylic acid cycle, as well as lactic acid, increase in radioactivity following inhibition (Fig. 4). In particular, C^{14} in α-ketoglutaric acid increases remarkably, especially in the steady-state *Chlorella* experiment (Fig. 3).

It is generally accepted that an important route for the entry of nitrogen into the amino acids in plants involves the conversion of α-ketoglutaric acid to glutamic acid *via* reductive amination with glutamic dehydrogenase.[13-15] Other amino acids then are formed by transamination of the appropriate α-keto acid by glutamic acid.[16] Glutamine is formed from glutamic acid and ammonia *via* a reversible reaction requiring ATP.[17-19]

Glutamine has been found to donate its amide group in several transamidation reactions. In these reactions glutamic acid is formed. Inhibition of such transamidation reactions, such as that already noted in the formation of α-N-formylglycinamidine ribotide, may account for the observed increase in glutamine, and for the decrease in the amount of glutamic acid.

The decrease in radioactive amino acids then may be brought about, as a secondary effect, by a decrease in concentration of glutamic acid. The decrease in radioactive amino acids never appears to be greater than the decrease in glutamic acid (except possibly for aspartic acid) and the extents of both decreases run parallel. This indirect inhibition of transamination may account for the increase in radioactivity of the Krebs cycle acids. The steep increase in labeled lactic acid may have resulted from reduction of pyruvic acid when transamination of the latter to alanine no longer takes place.

So far a reasonable interpretation of the inhibitory effects is possible in terms of known metabolic pathways and may be summarized; On inhibition of some transamidation reaction(s), glutamine will accumulate and glutamic acid (a reaction product of transamidations in which glutamine donates its amide group) will no longer be regenerated. The amount of glutamic acid decreases and consequently the synthesis of amino acids from the corresponding α-keto acids by transamination with glutamic acid may be reduced, which, in its turn, may cause an accumulation of Krebs cycle acids.

Other results obtained in these experiments seem to require an explanation not dependent on synthesis of glutamine from glutamic acid ex-

(13) H. von Euler, E. Adler and T. Steennoff-Erickson, *Z. physiol. Chem.*, **248**, 227 (1937).
(14) M. Damodaran and K. R. Nair, *Biochem. J.*, **32**, 1064 (1938).
(15) J. Berger and G. S. Avery, Jr., *Am. J. Botany*, **30**, 290 (1943); **31**, 11 (1944).
(16) "The Biochemistry of Amino Acids," by Alton Meister, Academic Press, Inc., New York, N. Y., 1957, pp. 192-194.
(17) W. H. Elliott, *J. Biol. Chem.*, **201**, 661 (1953).
(18) G. Denes, *Experientia*, **9**, 24 (1953).
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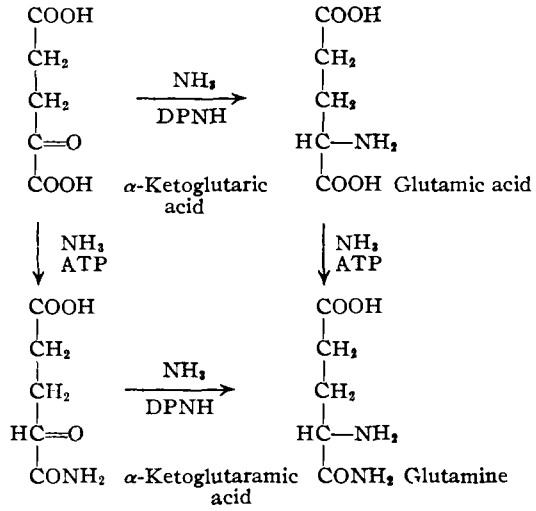
clusively. The increase in total radioactivity in glutamic acid following addition of inhibitor in some experiments is difficult to explain in terms of an inhibition of a transamidation reaction only. Both concentration and total radioactivity of glutamine continue to increase at a constant high rate, even when the level of glutamic acid has fallen from an initial 6 μg. per ml. of 1% suspension to less than 0.2 μg. per ml.

In order to explain these results on the basis of a synthesis of glutamine by way of a reductive amination of α-ketoglutaric acid to glutamic acid followed by amidation to give glutamine, and by no other pathway, it is necessary to call on a separation of the total reservoir of glutamic acid into two more or less isolated reservoirs, capable of possessing different specific activities.

As an alternative explanation of the experimental results, one might invoke the existence of a different route for the synthesis of glutamine, not involving glutamic acid as an intermediate. While no such route has been reported for plants, one might speculate that such a pathway may exist. If, for example, α-ketoglutaric acid were to undergo an amidation in the presence of ammonia and adenosine triphosphate, analogous to the amidation of glutamic acid, the resulting compound would be a α-ketoglutaramic acid, a substance which has been reported as the product of transamination reactions of glutamine in liver.[20-22] Reductive amination of α-ketoglutaramic acid, analogous to the reductive amination of α-ketoglutaric acid, would lead to the formation of glutamine.



Thus, glutamine would have been synthesized by a route not involving glutamic acid. An inhibition of transamidation reactions by which glutamine is converted to glutamic acid would then account for, (1) the continuous increase of radioactivity and concentration of glutamine, (2) the drop in concentration of glutamic acid, (3) a rise in glutamic acid specific activity as more glutamic acid was synthesized directly from α-ketoglutaric acid to replenish its reservoir, and (4) a fluctuation in total glutamic acid radioactivity—first up, then down as its specific activity increased while its concentration was decreasing.

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(20) M. Errera and J. P. Greenstein, *J. Biol. Chem.*, **178**, 495 (1949).

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Reactions of Optically Active Indole Mannich Bases[1]

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Alkylation of diethyl malonate and diethyl acetamidomalonate with optically active 3-(isopropylaminoethylidene)-indole (I) yields racemic diethyl (3-indolyethylidene)-malonate (IIIa) and diethyl (3-indolylethylidene)-acetamidomalonate (IIIb), respectively. The addition of piperidine, ethanol and diethyl malonate to 3-benzylidene-2-methyl-3H-pseudoindole is reported. Optically active 1-methyl-3-(dimethylaminoethylidene)-indole reacts with diethyl acetamidomalonate to give racemic diethyl (3-N-methylindolylethylidene)-acetamidomalonate. A preliminary kinetic study of the reaction of I with diethyl malonate is described.

The usefulness of carbon alkylations with indole Mannich bases and their quaternary salts has been well established.[3] Syntheses of tryptophan and related compounds have been among the most important applications of the method.[4,5] The synthetic aspects of these reactions have been explored extensively,[3] but some questions concerning the mechanisms by which they operate remain unanswered. A 3H-pseudoindole (*e.g.*, II) has been postulated as an intermediate in alkylation reactions by 3-(dimethylaminomethyl)-indole (gramine),[6] and evidence to support its participation has been discovered.[6,7] Information concerning the mechanism of alkylation by simpler quaternary ammonium salts containing benzyl groups has been obtained by a study of reactions of optically active salts containing the α-phenethyl group,[8] and it has long been recognized that a similar study of an op-

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(3) For a critical discussion of the subject of alkylations with amines and their derivatives, see J. H. Brewster and E. L. Eliel, "Organic Reactions," Vol. VII, Chapter 3, John Wiley and Sons, Inc., New York, N. Y., 1953, p. 99.

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