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THE PHOTOSYNTHETIC CYCLE: RELATIONSHIPS OF GLUTAMIC ACID AND GLUTAMINE

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The experiments which are described below were performed to find out more about the inhibitory effect of diazo-oxo-norleucine (DON) on the amino acids and other acids of the Krebs cycle. Specifically, the relationship between glutamic acid, glutamine and α-ketoglutaric acid was investigated. Barker, et al. (1) in 1956 had already found that during photosynthesis with Scenedesmus azaserine acts to produce a marked increase in the levels of glutamine and the Krebs cycle acids, accompanied by a corresponding depletion of the amino acid reservoirs.

Van der Meulen (2, 3, 4) has studied the influence of DON and azaserine on photosynthesis and on the amino acid and dicarboxylic acid pools of Chlorella and Scenedesmus. The effects of azaserine and of DON are very much the same on Scenedesmus; no inhibition on Chlorella was found with azaserine.

Long-time photosynthetic experiments with DON showed a very sharp increase in the amount of glutamic acid which is formed during five minutes. A very pronounced maximum is observed after 20 minutes of DON inhibition

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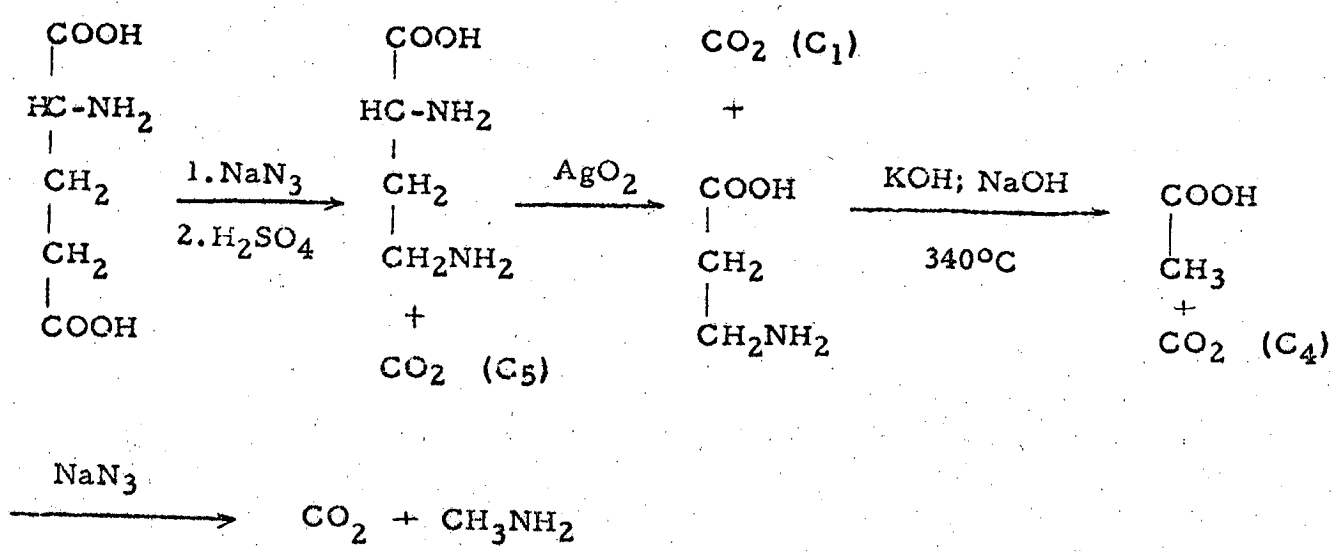
at a suitable concentration. However, after this time a very sharp drop occurs, and after 50 minutes the amount of glutamic acid drops to 20% of the amount present after 20 minutes and then remains nearly constant. The amount of α-ketoglutaric acid and glutamine continues to rise during these experiments.

Van der Meulen also compared the specific activities of glutamic acid and glutamine. During the first 25 minutes after DON injection, glutamine seems to have a higher specific activity than glutamic acid. These facts lead one to suspect that there may be a way by which glutamine can be formed other than through glutamic acid. One method of getting an answer to this question would be to make a comparison of the distribution of the radioactivity of the glutamic acid, α-ketoglutaric acid and the glutamine after a photosynthetic experiment with carbon-14 and the following experiments were done for this purpose.

EXPERIMENTAL

Counting techniques and degradation studies on glutamic acid: All the radioactivity measurements for the determination of the distribution of label were made by measuring the $C^{14}O_2$ in ionization chambers, and the organic material was combusted in sealed tubes with copper oxide according to the procedure of Wilzbach (5). Carbon dioxide from the degradations was trapped in sodium hydroxide solution and converted to barium carbonate. The CO_2 from the barium carbonate was liberated by copper sulfate at 650° in a sealed tube and transferred to an ionization chamber (6).

For the degradation of glutamic acid, the following procedure was tried:



The Schmidt degradation was checked with glutamic-5-C^{14} acid and glutamic-1-C^{14} acid and this general procedure has been described by Phares (8).

Sulfuric acid (1.2 ml, 100%) was added, at 10°, to a mixture of 2 mmoles of glutamic acid (294.3 mg) and 200 mg of sodium azide. Over a period of 10 minutes the temperature was raised to 70° and maintained at that point for 30 minutes. The usual precautions were taken to avoid any atmospheric CO_2. The yield of carbon dioxide is about 60-70%. Three determinations of the radioactivity of the glutamic-5-C^{14} acid (expressed in mV/min/mmole) and the barium carbonate derived from it by the Schmidt reaction are tabulated below:

glutamic acid:	sample 1	1939 mV/min/mmole
	2	1946
	3	1952
	average	1946
barium carbonate:	sample 1	1860
	2	1839
	3	1853
	average	1871

The barium carbonate has only 95.1% of the activity of the glutamic acid. There were three possible reasons for this result: (1) a blank of $BaCO_3$; (2) an inconplete conversion of $BaCO_3$ to CO_2 in the sealed tubes; and (3) the possibility that at least in the case of the glutamic acid some of the C_1 is split off by the Schmidt degradation procedure. Therefore, a Schmidt degradation was performed with glutamic-1-C^{14} acid. The results of this latter experiment showed that the barium carbonate had about 6% of the radioactivity of the glutamic-1-C^{14} acid which clearly shows that some of the resulting carbon dioxide comes from the C_1 of glutamic acid.

Another experiment was done to check the oxidative decarboxylation of α,γ-diaminobutyric-1-C^{14} acid to β-alanine. It turned out that about 40% of the CO_2 is derived from further oxidations, and the specific activity of the carbon dioxide which was trapped during the reaction of the α,γ-diaminobutyric-1-C^{14} acid with silver oxide was only 58.4% of the theoretical amount. Therefore, the C_1 was determined by ninhydrin oxidation; the yields of the reaction from β-alanine to acetic acid were generally poor, being only 5-10%.

<u>Isolation of glutamic acid and glutamine from photosynthesis or dark fixation experiments:</u> The algae were killed by pouring the suspension into four parts of boiling ethanol and this suspension was immediately cooled to 5°. The insoluble fractions were separated by centrifugation and extracted with cold 20% ethanol. The combined extracts were concentrated by freeze drying. The concentrated extracts of 200λ of wet-packed cells were placed on a sheet of Whatman No. 3 filter paper for two-dimension chromatographic separation. For the first direction a water-saturated phenol was used; the pH was adjusted to

5.5 with 0.1 N sodium hydoxide. For the second direction, butanol-propionic acid-water was used. In the box there was a 0.3% ammonia solution.

Both glutamine and glutamic acid were eluted. The glutamine was hydrolyzed by heating with dilute hydrochloric acid for five hours and rechromatographed in one direction with phenol. After dilution the glutamic acid was recrystallized twice. From both samples of glutamic acid (one derived from glutamine) the specific activity per mmole was carefully determined and compared with the value obtained for the barium carbonate after the Schmidt reaction and the ninhydrin oxidation.

a. Photosynthesis experiment: To 40 ml of a 2% suspension of Chlorella (from shake flask culture) was added 0.8 ml 2.5×10^{-3} M K_2HPO_4/KH_2PO_4 buffer* followed by 15-minute flushing with a mixture of 3.7% CO_2-in-air. The sample was illuminated with two photoflood lamps. Then a solution of 10 μg/ml of DON was injected and 1.5 ml $NaHC^{14}O_3$ (0.026 M, 400 μc/ml) was added in the light with carbon dioxide. The reaction vessel ("lollipop") was shaken for 15 minutes in the light and the algae were then killed. The radioactivity determinations from the degradation studies showed:

Glutamic Acid	C_1	21.2% of total activity
	C_5	22.7% of total activity
Glutamine	C_1	19.6% of total activity
	C_5	20.2% of total activity

The ratio of glutamic acid to glutamine on the paper was about 1:1.

In order to learn whether glutamic acid and glutamine are derived from different sources it was necessary to shorten the photosynthesis times to

* In all the later experiments, a volume of this buffer was added equal to the volume of the wet-packed algae cells used.

avoid a uniform distribution of radioactivity. Therefore, the activity of the C_1 and C_5 of glutamic acid from ordinary photosynthesis experiments of varying times was determined; these experiments were performed in the usual manner.

A Chlorella suspension was maintained for 15 minutes in the light and flushed with a 0.6% CO_2-in-air; then the $NaHC^{14}O_3$ was injected. The results of the glutamic acid degradations are as follows:

Photosynthesis time minutes	Glutamic acid C_1 % of total activity	Glutamic acid C_5 % of total activity
10	19.7	22.5
6	21.3	23.0
3	21.7	24.2
1	32	12

The values of the one-minute photosynthesis experiment are not very accurate. The activities were very low and the rror may be as high as 15-20%.

It thus appears, as a result of these experiments, that the glutamic acid seems to be very nearly uniformly labeled, even in as short a time as three minutes. Nevertheless, the total amount of radioactivity in the glutamic acid is rather small, about 0.3% of the total fixation.

It is not surprising that glutamic acid formed in the light is uniformly labeled very soon, if one assumes that it is formed via the Krebs cycle from acetate derived from the same source as the C_1-C_2 and C_5-C_6 pairs of hexose. Quite another route of glutamic acid formation is expexted from dark fixation. The glutamic acid from Scenedesmus, after a 10-minute dark fixation experi-

ment, showed the following distribution of activity: C_1, 96% and C_5, 8%. (Five percent of the C_1 activity is found in the C_5, as mentioned above.)

Experiments with photosynthesis together with dark fixation: The conditions of the various experiments are described below.

Preliminary experiment: 25 ml of 1% Chlorella suspension plus 0.5 ml phosphate buffer; 2 hours in complete darkness, the last 10 minutes with 25 μg DON/ml suspension, 3 minuteslight and 0.5% CO_2-in-air, 10 minutes dark fixation with $NaHC^{14}O_3$, 1 minute light and again 5 minutes dark fixation. Glutamic acid contained only about 16% of the total activity fixed, but there was none, or very little, in the glutamine. This may be explained by the fact that the ATP pool for amidation of the carboxyl group is too small. This same observation was made in a further series of experiments (see Table I).

The procedure and conditions of the six experiments whose results are summarized in Table I are as follows:

Experiment 1: 10 ml of 1.5% Chlorella suspension, 120 minutes in the dark in distilled water, 15.0 μg DON/ml suspension added, 20 minutes light flushed with 0.5% CO_2-in-air, 6 minutes photosynthesis with 200 λ $NaHC^{14}O_3$.

Experiment 2: Same as experiment 1 without DON. Algae were kept 150 minutes in the dark.

Experiment 3: 10 ml of 1.5% Chlorella suspension, 180 minutes in the dark in distilled water, 15.0 μg DON/ml suspension added, 20 minutes dark, 2 minutes light, 15 minutes dark fixation with 200 λ $NaHC^{14}O_3$.

Experiment 4: Same as experiment 3 without DON. Algae were kept 210 minutes in the dark.

Experiment 5: 10 ml of 1.5% Chlorella suspension, 240 minutes in the dark in distilled water, 15.0 μg DON/ml suspension added, 15 minutes dark, 3 minutes light with 0.5% CO_2-in-air, 1 minute photosynthesis, 10 minutes dark fixation with 200 λ $NaHC^{14}O_3$.

Experiment 6: Same as experiment 5, without DON. The algae were maintained for 275 minutes in the dark. The degradation data from mixed photosynthesis and dark fixation experiments were determined, and it turned out that after one minute photosynthesis and 10 minutes dark fixation the label showed almost uniform distribution: glutamic acid: C_1, 25.3%; C_5, 24.6%; glutamine: C_1, 24.5%; C_5, 23.9%. Therefore, the experimental times were shortened still further, and in order to get more glutamine and glutamic acid in short times, small amounts of ammonium nitrate were added before the radioactive bicarbonate.

Experiment 7: 10 ml 2% Chlorella suspension, 11 minutes in the light with 0.6% CO_2-in-air, 35 μg DON/ml suspension added, 15 minutes light with 0.6% CO_2-in-air, 300 λ $NaHC^{14}O_3$, 30 seconds photosynthesis and 6 minutes dark fixation. The degradation data are as follows: glutamic acid: C_1, 40.5%; C_5, 20.4%; glutamine: C_1, 37.4%; C_5, 22.9%.

Experiment 8: 10 ml 2% Chlorella suspension, 15 minutes in the light with 1% CO_2-in-air, NH_4NO_3 solution added to make a 10^{-3} M solution, 5 minutes in light with 1% CO_2-in-air, 15 seconds photosynthesis, 4 minutes dark fixation with 300 λ $NaHC^{14}O_3$/

Experiment 9: 10 ml 2% Chlorella suspension, 5 minutes in the light with 1% CO_2-in-air, 35 μg DON ml suspension added, 10 minutes light with 1% CO_2-in-air, NH_4NO_3 added to make 10^{-3} M solution, 5 minutes light with

1% CO_2-in-air, 15 seconds photosynthesis, 4 minutes dark fixation with 300 λ $NaHC^{14}O_3$.

Experiment 10: 10 ml 2% Chlorella suspension, 20 minutes in the light with 1% CO_2-in-air, 15 seconds photosynthesis, 5 minutes dark fixation with 300λ $NaHC^{14}O_3$.

Experiment 11: 10 ml 2% Chlorella suspension, 5 minutes in the light with 1% CO_2-in-air, 35 μg DON/ml suspension added, 15 minutes in the light with 1% CO_2-in-air, 15 seconds photosynthesis, 4 minutes dark fixation with 300 λ $NaHC^{14}O_3$.

Table II shows the amount of radioactivity fixed in the different compounds and Table II shows some degradation data from these experiments.

RESULTS AND DISCUSSION

The above experiments indicate that glutamic acid, in the light, is uniformly labeled very rapidly which is in accordance with the theory of its formation from hexose (or phosphoglyceric acid) via acetate and the Krebs cycle. In the dark, the glutamic acid may be formed by carboxylation of γ-aminobutyric acid and/or by carboxylation of phosphoenol pyruvate and thence via the Krebs cycle. For instance, labeled γ-aminobutyric acid was found in Experiment 7.

In the experiments where DON was added, the glutamic acid seems to be derived from glutamine (Experiments 7 and 9). The reductive amination of α-ketoglutaric acid seems to be blocked (Experiment 9). In experiments where no DON was added, the main route to glutamic acid seems to be via α-ketoglutaric acid, but glutamine, at least in the presence of ammonia, is

TABLE I

Effect of DON upon the distribution of C^{14} in Chlorella after various fixation conditions

	Exper. 1		Exper. 2		Exper. 3		Exper. 4		Exper. 5		Exper. 6	
cpm on paper	1.4×10^6		4.6×10^5		1.4×10^5		7.3×10^5		4.8×10^5		5.5×10^5	
Glycolic acid	3.41×10^5	24%	9.23×10^4	20%								
Glycine & serine	32100	2.3%	29200	6.3%	4430	3.2%	5400	7.4%	19000	3.9%	29000	5.3%
Alanine	6490	0.45%	5890	1.3%	3480	2.5%	1200	1.6%	13800	2.9%	22950	4.1%
Glutamine	3470	0.25%	490	$<$ 0.1%	none		none		1070	0.2%	1570	0.3%
Glutamic acid	3380	0.25%			11270	8%	3800	5.1%	14270	3%		
Aspartic acid	7790	0.55%	7770	1.7%	18400	13%	9200	12.5%	20000	4.1%	25400	4.6%
Malic acid	13740	1%	7116	1.5%	1540	1.1%	1440	2%	4270	0.9%	3270	0.6%

TABLE II*

Effect of DON and/or NH_4NO_3 upon distribution of C^{14} in *Chlorella* after various fixation conditions

	Exper. 8 NH_4NO_3		Exper. 9 NH_4NO_3 & DON		Exper. 10		Exper. 11 DON	
cpm on paper	3×10^5		2.6×10^5		2.5×10^5		1.7×10^5	
α-Ketoglutaric acid			8550	3.3%			386	0.22%
Glutamic acid	35700	12%	24000	9.2%	20000	10%	18600	11.0%
Citric acid	4850	1.6%	11190	4.3%	1130	0.56%	3160	1.9%
Malic acid	26800	8.9%	82000	31.6%	8310	4.1%	28000	6.5%
Fumaric acid	8330	2.8%	21200	8%	2460	1.2%	7000	4.1%
Glycine & serine	11410	3.8%	4460	1.7%	5040	2.5%	4480	2.6%
Aspartic acid	83500	29%	9780	3.8%	58000	29%	36700	21%
Alanine	43300	14.5%	14820	5.7%	40000	20%	34260	20%
Glutamine (citrulline?)	3410	1.1%	8200	3.1%		0.1%		0.1%

* % based upon amount placed on the origin of the chromatogram

TABLE III

Distribution of C^{14} in glutamine, glutaric acid and α-ketoglutaric after short-time fixation

		Exper. 8	Exper. 9
C_1	Glutamic acid	19.9%	54.1%
C_5	Glutamic acid		12.9
C_1	Glutamine	61.5	49.7
C_5	Glutamine		11.0
C_1	α-Ketoglutaric		15.7

formed by another route (compare the results of Experiments 8 and 9). It appears that the inhibitory effect of NH_4 ions and DON is, in general, the same and is quite pronounced; see Table II.

Another effect of the diazo-oxo-norleucine seems to be the blocking of the reductive transamination and amination reactions which are pyridoxal-dependent. Therefore, the ammonia pool is built up and all nonreductive reactions which use ammonia and which are pyridoxal-independent are favored; perhaps an amidation of α-ketoglutaric acid takes place. This amide may form a heterocyclic compound (I, see Figs. 1 and 2).

Another sink for the ammonia would ae the amidation of α-aminobutyric acid (found by the decarboxylation of glutamic acid) to its amide. Both of these amides can lead to glutamine. Compound III by direct (alpha) carboxylation would produce glutamine labeled in the C_1 carbon. Compound I, by reduction and ammonolysis, would lead to glutamine from -ketoglutarate without passing through glutamic acid itself. This latter reaction would also account for the primary entry of nitrogen into the amino group of the glutamine without passing through glutamic acid.

We have thus arrived at two alternative routes to labeled glutamine not involving glutamic acid as a direct precursor. The relative importance of these, together with the direct route through glutamic acid, will be determined by the conditions of the experiment, and the exact relationships must await a more extensive set of degradations.

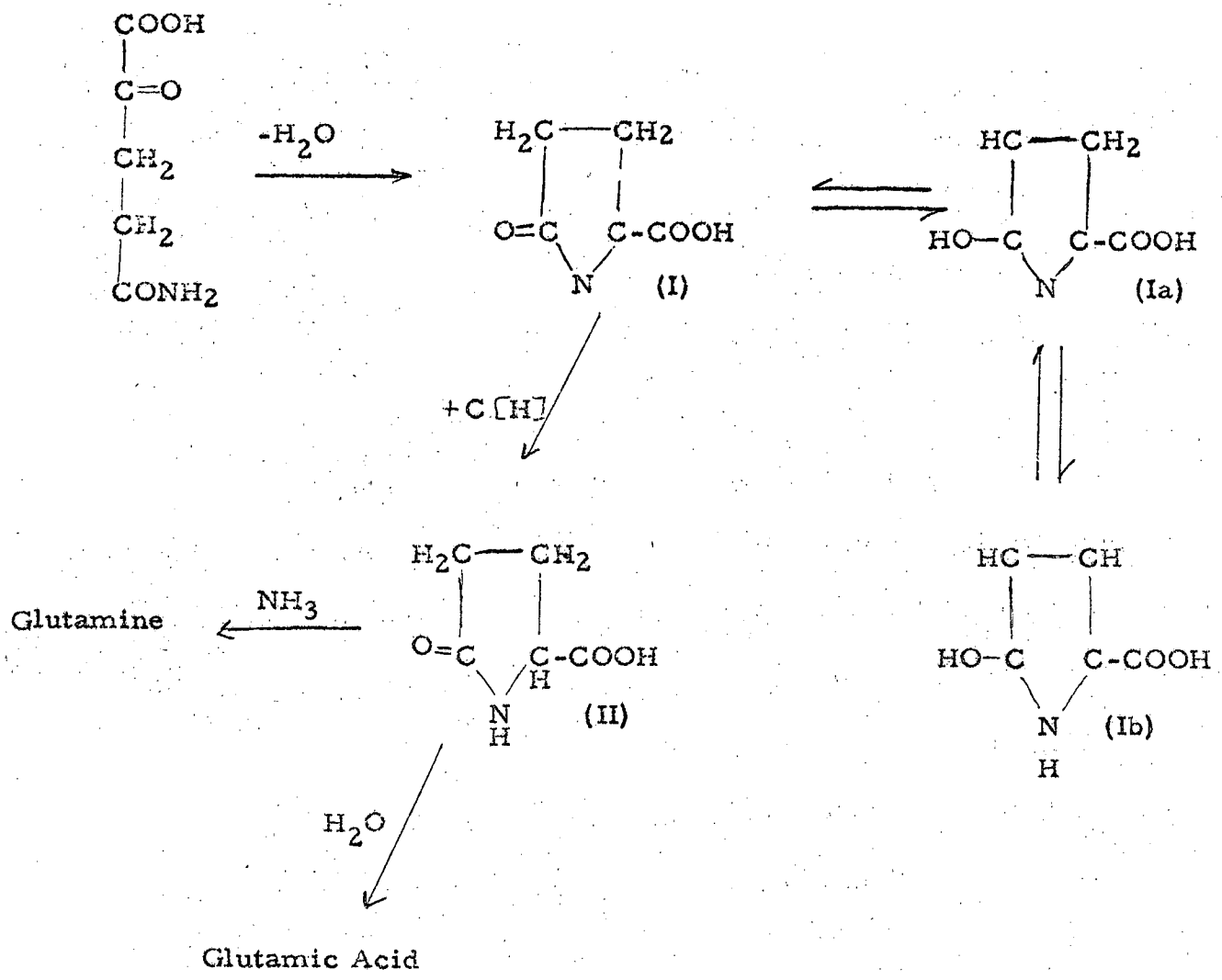


Fig. 1 Possible route to glutamine not involving glutamic acid as an intermediate

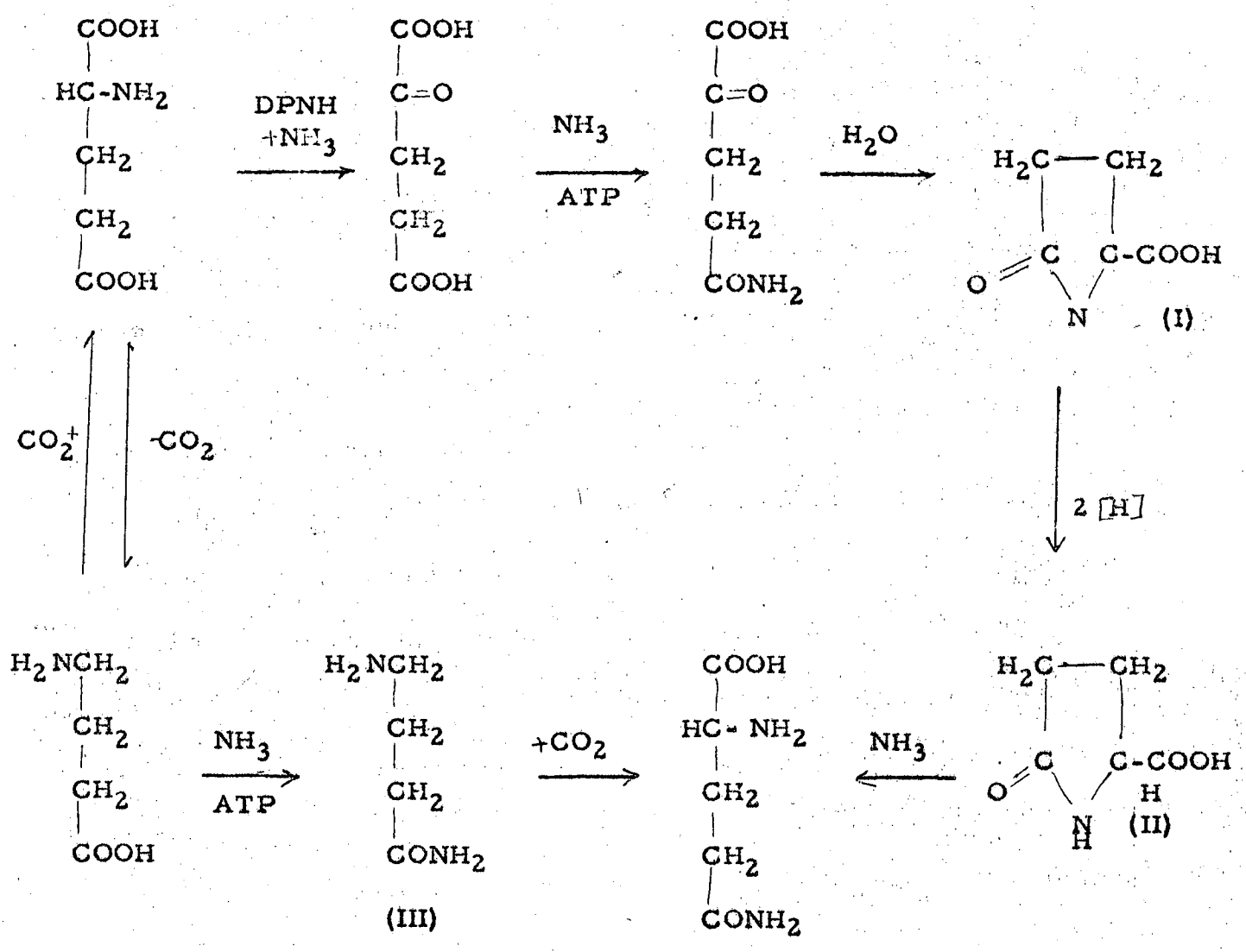


Fig. 2. Interrelations of glutamine and glutamic acid

SUMMARY

From a study of the labeling of a number of products in photosynthesizing algae using $C^{14}O_2$ in the presence of diazo-oxo-norleucine (DON),, as well as in its absence, it is deduced that there exists a route to glutamine which does not pass through glutamic acid.

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