Lipid Synthesis in the Presence of Nitrogenous Compounds in Chlorella pyrenoidosa[1]

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Summary. Acetate was incorporated by *Chlorella pyrenoidosa* into nonvolatile water-soluble compounds (amino acids, organic acids and carbohydrates) with pH optimum between 4 and 5. Incorporation into lipid was maximal at pH 7.5. The proportion of incorporated acetate in lipid was not significantly affected by acetate concentration and chlorophyll concentration in the ranges tested. Illumination of *Chlorella* during acetate metabolism increased the synthesis of lipid with concomitant decrease in the synthesis of water-soluble compounds. Nitrate and ammonium ions had essentially no effect on acetate metabolism. Inhibition by nitrite was greater on the synthesis of lipid. Illumination reversed the gross inhibition by nitrite, but altered the distribution of incorporated label in favor of the water-soluble compounds.

The relationship of syntheses of lipid and water soluble acids in chloroplast preparations has been reported (6). For further study on the effects of nitrogen assimilation on the pathway of carbon metabolism in plants, *Chlorella pyrenoidosa* has been used because of its relative ease of manipulation, and because results obtained with the alga may be of more physiological consequence than studies with isolated chloroplasts.

Relationship of lipid synthesis and nitrogen metabolism is indicated by the changes in chemical composition of algae supplied with different sources of nitrogen (9). Since illumination stimulates both nitrate reduction (4), and fatty acid synthesis (5, 10), one may suspect that under certain conditions the 2 processes compete for reductant. The work reported in this paper was designed to assess the degree of competition under photosynthetic and nonphotosynthetic conditions.

Materials and Methods

Culture of Chlorella. The culture of *Chlorella pyrenoidosa* was maintained under continuous illumination on a medium of 2 % agar in modified Hoagland's nutrient solution. Cells were grown in a tube 120 x 5 cm containing modified Hoagland's nutrient solution innoculated with cells from the stock culture (3). The culture was continuously aerated at approximately 4 liters per minute with air which had passed through activated charcoal and then redistilled water. Illumination was provided by 2 30-w daylight fluorescent lamps mounted 2.5 cm from the culture tube at each side. During cell multiplication, the culture was maintained below absorbancy of 1.0 at 525 mμ by addition of nutrient solution. Cells were harvested for experimental work 4 to 7 days after innoculation.

Reaction Mixtures. Approximately 100 ml of the *Chlorella* culture was centrifuged, and the nutrient solution discarded. The cells were washed by resuspension in distilled water followed by centrifugation, and the washed cells were then resuspended in 5 ml distilled water. The chlorophyll concentration was determined by a modification of Arnon's method (1). The aliquot of *Chlorella* suspension was shaken with 1 ml methanol:chloroform (2:1, v/v) to break the cells, 19 ml acetone was then added and water to make a final volume of 25 ml. Chlorophyll concentration was then calculated from the OD reading at 652 mμ. Reaction mixtures consisted of 0.5 ml 10^{-1} M buffer (phosphate, pH 6.5 unless otherwise stated), 0.5 ml *Chlorella* suspension, radioactive substrate, and water to make a final volume of 1.50 ml. The reaction mixtures, in glass stoppered centrifuge tubes, were maintained at 30° in a water bath.

Extraction and Assay. When acetate was used as a substrate, 4.5 ml of methanol:chloroform (2:1, v/v) was added at the end of the reaction period. After vigorous shaking, 1.5 ml chloroform was added, the mixture shaken again and then centrifuged to form a chloroform layer and an aqueous layer (2). The layers were separated by use of dropping pipets. The residue was extracted with the chloroform:methanol, and water. The aqueous fraction was washed with 2 ml chloroform and the chloroform fraction was washed with water. The chloroform fractions were combined, as were the aqueous fractions. The water soluble fraction was acidified with 0.2 ml 0.2 N HCl. Aliquots of the fractions were dried on planchets and counted with a Geiger Muller counter.

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When glucose, malonate or mevalonate was used as substrate, the incubation mixture was centrifuged at the end of the experimental period and the supernatant fraction containing the unused substrate was discarded. The cells were washed by resuspension in 1.0 ml distilled water followed by centrifugation. The washed cells were then extracted as described above except that the aqueous phase was not acidified.

The water soluble fraction was fractionated in some cases by chromatography on a column of Dowex 50 (H^+) (10 × 0.9 cm). The water eluate containing organic acids and carbohydrates was chromatographed on Dowex 7 (formate) (10 × 0.9 cm). The water eluate from the latter column contained carbohydrates. Amino acids were eluted from the Dowex 50 with 2 N NH_4OH. Organic acids were eluted from the Dowex 1 with 8 N formic acid. Radioactivity of the fractions was assayed by counting a dried aliquot with a Geiger Muller counter.

Reagents. Acetate-1-^{14}C and glucose-U-^{14}C were obtained from Volk Radiochemical Company.

Results

Distribution of Incorporated Substrate. The experiments to be described measured the proportions of incorporated substrate found in lipid and nonvolatile water-soluble compounds. It was established that variations in the amount of *Chlorella,* in the range 0.02 to 0.18 mg chlorophyll per ml reaction mixture, did not affect the distribution of incorporated acetate. Variations in the concentration of acetate, from 4.3 to 107 mμmoles per ml reaction mixture, showed that the percentage of incorporated label in lipid was constant up to 60 mμmoles/ml with a slight decline at higher concentrations. The time elapsing between transfer of the *Chlorella* from agar to the nutrient solution and harvesting the cells affected the proportion of label in the 2 classes of compounds. In 1 experiment where cells were harvested 3, 5, 7, and 10 days after innoculation, the percentages of incorporated acetate found in lipid were 78, 75, 55, and 38 respectively. Consequently, the data obtained are comparable only within a given experiment.

Illumination invariably stimulated lipid synthesis. The distribution of incorporated label in the water-soluble fraction was 60 to 70 % in amino acids, 25 to 35 % in organic acids, and 5 to 15 % in carbohydrates.

Effect of pH on Carbon Metabolism. When acetate was used as radioactive substrate, the total incorporation was maximal between pHs 4 and 5 (fig 1). In this range the incorporation into the water-soluble compounds was approximately 10 times that of the incorporation into lipid. The pH most suitable for incorporation of acetate into lipid was 7.5, when the incorporation of acetate into non-volatile water-soluble compounds was only twice that into lipid. When glucose was used as radioactive substrate, the pH optima for incorporation into water-soluble compounds and lipid were 8.0 and 7.5, respectively. For

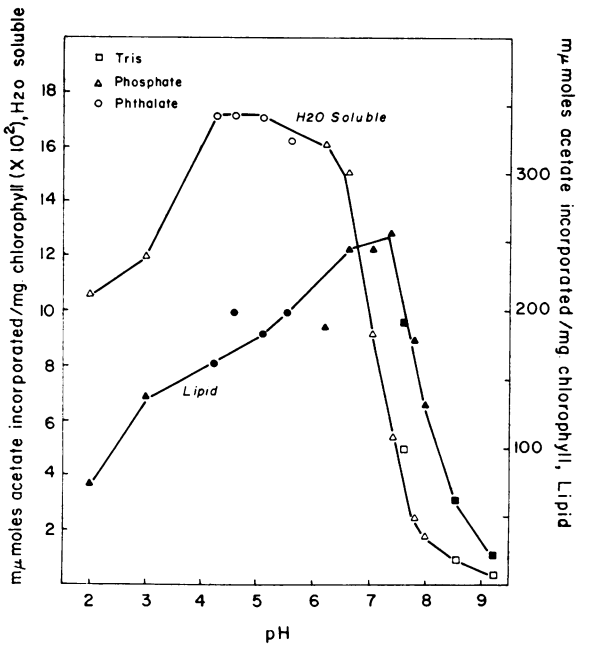

FIG. 1. Effect of pH on acetate metabolism. The reaction mixtures contained 50 μmoles buffer, 143 mμmoles acetate-1-^{14}C, and 0.5 ml *Chlorella* suspension (0.08 mg chlorophyll) in a final volume of 1.50 ml. Incubation was for 25 minutes at 30° under laboratory light conditions.

further experiments, pH 6.5 (phosphate buffer) was chosen as standard since at this pH both lipid and nonvolatile water-soluble compounds were significantly labeled.

Neither malonate nor mevalonate was significantly incorporated into lipid at any pH tested.

Carbon Metabolism in the Presence of Nitrogenous Compounds. The influence of nitrogenous compounds on the metabolism of acetate and glucose is shown in table I. Ammonium ions consistently caused a slight stimulation of incorporation into the water-soluble fraction, primarily amino acids, but had essentially no effect on lipid synthesis. Nitrate affected neither the metabolism of acetate nor glucose. The concentration of nitrite used was inhibitory when the reaction vessels were not illuminated. Inhibition of acetate metabolism was observed at concentrations of 10^{-4} M nitrite and above in darkness. Although inhibition of incorporation into both water-soluble and lipid fractions was observed, inhibition of lipid synthesis was invariably more severe. At nitrite concentrations higher than 10^{-4} M, the inhibition observed with reaction mixtures in darkness could be reversed by illumination. The presence of nitrite during acetate metabolism in the light was always accompanied by a change in the distribution of incorporated acetate. As compared to controls, the incorporation into lipid was less. Inhibition of acetate metabolism by hydroxylamine was observed at concentrations of 10^{-5} M and higher.

Table I. *Comparison of Acetate and Glucose Metabolism in the Presence of Nitrogenous Compounds*

The reaction mixtures contained 50 μmoles phosphate buffer pH 6.5, 0.5 ml *Chlorella* suspension (0.09 mg chlorophyll), either 32 mμmoles acetate-1-^{14}C or 89 mμmoles glucose-U-^{14}C, and nitrogenous compounds as indicated at final concentrations of 2.5×10^{-2} M, in a final volume of 1.50 ml. Incubation was for 25 minutes at 30° under laboratory light conditions.

	mμmoles incorporated/mg chlorophyll					
	Acetate			Glucose		
	H_2O Soluble	Lipid	Total	H_2O Soluble	Lipid	Total
Control	58.5	162	220.5	595	67.3	662.3
NO_3^-	54.3	165	219.3	608	70.9	678.9
NO_2^-	8.7	13.6	22.3	317	6.9	323.9
NH_2OH	15.2	35.7	40.9	283	44.9	326.9
NH_4^+	70.0	164	234	651	68.1	719.1

Table II. *Acetate Metabolism in the Presence of Nitrogenous Compounds and Illumination*

The reaction mixtures contained 50 μmoles phosphate buffer pH 6.5, 32 mμmoles acetate-1-^{14}C, 0.5 ml *Chlorella* suspension (0.23 mg chlorophyll), and nitrogen compounds as indicated at a final concentration of 2.5×10^{-2} M. The final reaction volume was 1.50 ml. Illumination was provided by 2 300-w spot lamps, dark vessels being protected by aluminum caps. Incubation was 25 minutes at 30°.

	Acetate incorporated, mμmoles/mg chlorophyll					
	Dark			Light		
	H_2O Soluble	Lipid	Total	H_2O Soluble	Lipid	Total
Control	14.6	32.8	47.4	10.2	70	80.2
NO_3^-	14.5	30.4	44.9	13.2	66.6	79.8
NO_2^-	6.3	7.0	13.3	22.6	51.6	74.2
NH_2OH	5.2	11.1	16.3	8.1	22.3	30.4
NH_4^+	19.5	31.7	51.2	13.5	64.5	78.0

Effects of concurrent illumination and supply of nitrogenous compounds are shown in table II. In the control, nitrate, or ammonium treatments, the results are consistent with observations made when the physiological variations were made separately. Nitrite inhibition in the absence of illumination is typical in that lipid synthesis was inhibited more than the synthesis of water-soluble compounds (79 % as opposed to 57 %). Under illumination, the inhibition by nitrite strikingly changed. Total incorporation was inhibited only 8 %, whereas the inhibition was 72 % in the unilluminated sample. Illumination reversed the gross inhibition of acetate metabolism but without restoring the balance between lipid synthesis and the synthesis of water-soluble compounds. Hydroxylamine inhibition of acetate incorporation was observed both in darkness and light.

Discussion

The effects of pH on incorporation of acetate by *Chlorella* may be explained by the fact that the undissociated acid penetrates more rapidly than the acetate ion (3). Of the substrate penetrating, a greater proportion is incorporated into lipid as the pH is raised to 7.5 possibly because of greater availability of bicarbonate for the carboxylation reaction in fatty acid synthesis.

The knowledge that illumination of photosynthetic tissue stimulates nitrate reduction (3), and lipid synthesis (5, 10), and that lipid synthesis is stimulated under nitrogen starvation indicate that photosynthetically produced reductant may be used either for lipid synthesis or the several steps of nitrate reduction. Reduction of nitrite and hydroxylamine in reconstituted chloroplast systems was mediated by ferredoxin (7). On the other hand, reduction of nitrate required FMN or FAD, and these cofactors could not be replaced by ferredoxin (8).

In the experiments reported in this paper, the small effect of NH_4^+ on the incorporation of acetate into lipid is to be expected since there is little stimulation of amino acid synthesis and therefore little requirement for reductant in its assimilation by glutamic dehydrogenase. The negligible effect of nitrate on acetate metabolism is surprising. Apparently any assimilation of nitrate has not diverted reductant from the synthesis of lipid. Although assimilation of nitrate was not measured during the course of the experiment, its assimiliation has been assumed since it was present in the reaction mixtures at the concentration used in growing the cells. The effect of nitrite on acetate metabolism was pronounced. Both in the light and in the dark, lipid synthesis was inhibited more than the synthesis of water-soluble compounds, as would be expected from the diversion of reductant. The toxicity of nitrite is almost completely relieved by illumination, pre-

sumably due to its more rapid assimilation in the presence of reductant produced photosynthetically. The large difference in the effects of nitrite and nitrate may be related to the different mechanisms for reduction (7, 8).

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