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METABOLISM OF THIOCTIC ACID IN ALGAE

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METABOLISM OF THIOCTIC ACID IN ALGAE

Hans Grisebach, R. C. Fuller, and M. Calvin

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University of California, Berkeley 4, California

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ABSTRACT

Thioctic acid labeled with sulfur-35 has been prepared and its metabolism by algae has been studied. It is converted by the algae into a number of forms, all of which upon hydrolysis yield either the disulfide or its sulfoxide. One of these constituted the major portion of the labeled material in the chloroplasts. Aerobic metabolism for some minutes is required to produce this form. Preliminary studies of the chemical nature of this form suggest it to be esterified on the carboxyl group with a moiety of very high lipid solubility.

## METABOLISM OF THIOCTIC ACID IN ALGAE\*

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6-Thioctic acid (pyruvic oxidase, lipoic acid) has been proposed as a compound that may take part in the light reaction of photosynthesis.<sup>1, 2, 3</sup> It was therefore of interest to study the metabolism of 6-thioctic acid in algae.

So far the metabolism of 6-thioctic acid, in respect to its role as a cofactor for the oxidation of pyruvic acid and other  $\alpha$ -keto acids,<sup>4</sup> has been studied only in microorganisms and animal tissues. Gunsalus<sup>5</sup> has shown that 6-thioctic acid exists in *S. Faecalis* in at least five forms: (a) a bound form, (b) water-soluble, solvent-insoluble form, (c) "weak" acid, (d) "strong" acid, and (e) "neutral" form. The "weak" and "strong" acids were later identified as the 6-thioctic acid and 6-thioctic acid sulfoxide, whereas the other forms of 6-thioctic acid were not further characterized. The water-soluble forms of 6-thioctic acid seem to be protein complexes.<sup>6, 7</sup>

Chromatographic separation of extracts from various photosynthetic organisms have shown several compounds that have the biological activity of 6-thioctic acid. The criterion for biological activity of these compounds was the response of propionate-inhibited *S. faecalis* grown on an acetate-free medium.<sup>8</sup> Chromatography in a mixture of butanol-ethanol-water gave the major biologically active compounds at  $R_f$ 's of 0.4, 0.7, and 0.9. The compounds of  $R_f$  0.4 and 0.7 were identified as 6-thioctic sulfoxide and 6-thioctic acid, respectively. The more lipid-soluble compound with  $R_f$  0.9 was not identified. In order to try to identify this and to find other forms of thioctic which might not be active in the bacterial growth response test, the metabolic fate of  $S^{35}$ -labeled thioctic acid was investigated.

Since the natural concentration of 6-thioctic in *Scenedesmus* is only at the most 5  $\gamma$  of 6-thioctic/1 g of wet cells,  $S^{35}$ -labeled 6-thioctic acid with an activity of  $\sim 40$  mc/mmole<sup>9</sup> was used for the experiments. *Scenedesmus* was fed with  $S^{35}$ -6-thioctic at a concentration of 0.5 mg  $S^{35}$  6-thioctic/1 g of wet cells under conditions described in the experimental part.<sup>10</sup> The

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6-thioctic acid was rapidly taken up, and the distribution of the 6-thioctic acid between algae and medium changed with time as follows: After 1 min, 16% in the cells; 10 min, 25%; 30 min, 42%; and 1 hour, 47%. After one hour's incubation with S<sup>35</sup> 6-thioctic, cells were extracted with ethanol and water and chromatographed on paper in one dimension in a solvent system of butanol saturated with 0.5 N ammonia. Five radioactive compounds were observed in this solvent system at the R<sub>f</sub> values 0.98, 0.51, 0.33, 0.17, 0.1. The majority of activity was observed at R<sub>f</sub> 0.98, 0.51, and 0.16. The latter two spots were identified as 6-thioctic acid and 6-thioctic sulfoxide by cochromatography with authentic samples. In a butanol-ethanol-water solvent system (80:20:saturation) in which the 6-thioctic acid and its sulfoxide ran close to the solvent front, at least seven radioactive compounds with R<sub>f</sub> values between 0 and 0.5 were present. Two-dimensional chromatography (phenol-water; butanol-propionic acid) gave 95% of the radioactivity on the front of the chromatogram where the 6-thioctic acid, the 6-thioctic sulfoxide, and the more lipid-soluble compounds ran together. Three percent of the activity was at R<sub>f</sub> 0.9 x 0.4, and a very weak spot (~0.4%) appeared at R<sub>f</sub> 0.2 x 0.16.

The distribution of the metabolic products of 6-thioctic acid in the cell varies with the time of the contact of the 6-thioctic with the cells. Tables I and II, as well as Fig. 1, show the distribution of the activity after various lengths of time of incubation in two separate experiments. The amount of the more lipid-soluble compound(s) increases with time, but the relative amount seems to decrease after a longer time because the amount of the "insoluble" (origin of the chromatogram) increases considerably. One can see clearly that the 6-thioctic lipid(s) is formed only inside the cells. The medium does not contain any detectable amount of this compound. To demonstrate that the 6-thioctic acid lipid is a true metabolic product and not an artifact of the killing of the cells, extraction procedure, or chromatography, the S<sup>35</sup> 6-thioctic acid was added to living algae which were then killed immediately and chromatographed. The results shown in Table III indicate clearly that this compound is a product of metabolism in the cell. Furthermore, heat-killed algae incubated with thioctic acid do not form any considerable amount of the 6-thioctic acid lipid.

Because of the previously mentioned relationship between 6-thioctic and photosynthesis, and in order to see where the different forms of the 6-thioctic acid are located in the cells, the cells were fractionated. After three and four hours' contact of Chlorella\* with S<sup>35</sup>-labeled 6-thioctic acid the algae were centrifuged, washed with water, resuspended in phosphate buffer, and ruptured by treatment in a 9-kc oscillator. Plastid material containing chlorophyll was isolated by ultracentrifugation at 100,000 x G for 20 min. This plastid material was then extracted with hot 95% ethanol and the extract

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\* It is difficult to obtain good plastid preparations from Scenedesmus, but the total thioctic acid distribution in the two organisms is very similar.

Table I

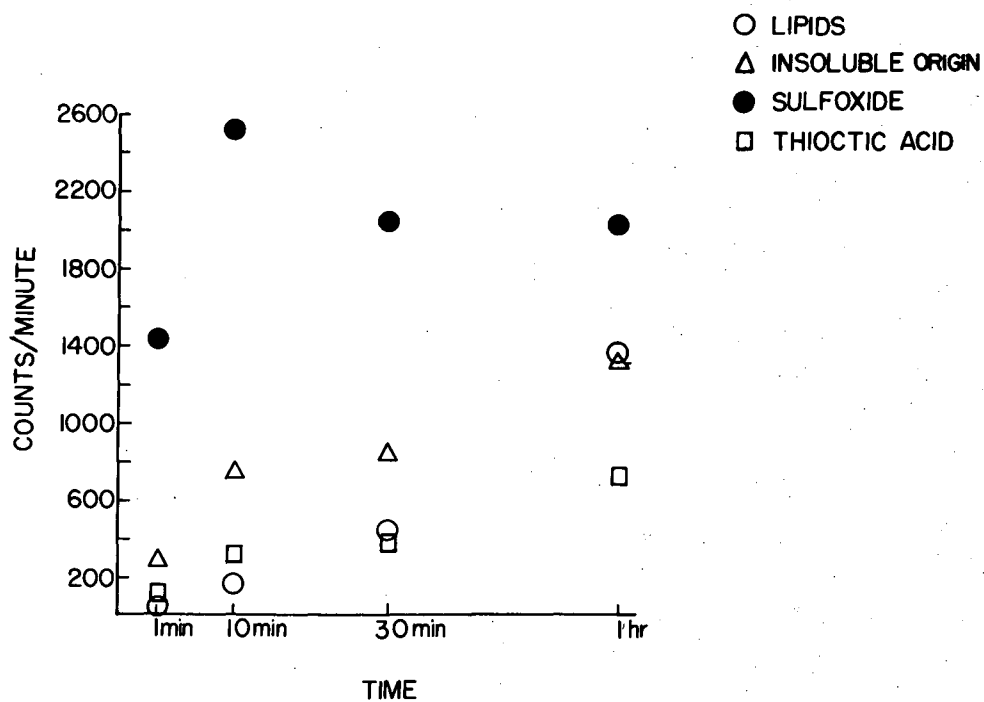
Radioactivity distribution as % of total S <sup>35</sup> on chromatogram								
Time	Origin		6-Thioctic Sulfoxide		6-Thioctic Acid		Chromatogram Front. (lipids)	
	Extract	Medium	Extract	Medium	Extract	Medium	Extract	Medium
1 min	14.0	4.7	79.5	24.0	4.8	73.0	2.0	0
10 min	19.5	5.0	68.0	37.0	8.2	56.0	4.0	0
30 min	22.0	5.5	57.0	27.0	10.0	39.0	12.0	0
1 hr	24.0	11.0	37.0	35.0	13.5	53.0	25.0	0
4 hr from other experiment	59.2	--	13.6	--	8.9	--	14.5	--
1 hr with dead algae	16.5	--	50.9		27.5		1.5	
Control (6-thioctic extracted together with algae)	5.3		4.8		85.0		2.0	



Table II\*

Radioactivity distribution as % of total S <sup>35</sup> on chromatogram								
Time	Origin		6-Thioctic sulfoxide		6-Thioctic acid		Chromatogram front (lipids)	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
10 min	34.6	29.7	33.8	42.7	16.2	16.9	10.8	7.0
1 hr	38.6	24.9	26.0	33.3	10.9	16.6	21.5	21.5
1 hr dead algae		16.5		50.9		27.5		1.5

\* Cells were not separated from medium before extraction as in Table I.



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Fig. 1. Total number of counts on paper. Time experiment with Scenedesmus. Data for "extract" in Table I.

Table III

Percent of 6-Thioctic derivatives with various treatments			
Compound	S <sup>35</sup> 6-Thioctic acid	S <sup>35</sup> 6-Thioctic acid added and cells killed at once	5.25-hr uptake in dark, aerobic
6-Thioctic acid	91.4 <sup>a</sup>	85.0	6
6-Thioctic acid sulfoxide	5.4	5.0	14
Front	0.85	2.2	36
Origin	2.3	5.3	41

<sup>a</sup> Percentage distribution of total activity on chromatogram

chromatographed with butanol-0.5 N ammonia. Table IV shows the distribution of the radioactivity in the cells. Table V shows the distribution of the radioactivity in the plastid extract. One can see that in the ethanol-soluble part of the plastids the 6-thioctic lipid is the major metabolic product. The major part of the radioactivity in the cells is in the ethanol-insoluble fraction of the plastids.

Table IV

$S^{35}$ distribution in $S^{35}$ 6-thioctic-fed chlorella		
	Incubation time	
	3 hours	4 hours
Plastids	7.3%	6.3%
Supernatant	16.8	24.6
Insoluble	76.0	70.0

Table V

Radioactivity distribution as % of total $S^{35}$ on chromatogram of plastid extract	
Origin	8.2
6-Thioctic acid sulfoxide	7.7
6-Thioctic acid	24.0
Chromatogram front (lipids)	60.0

#### Thioctic acid lipids

Further studies were undertaken to determine the nature of the 6-thioctic acid lipid(s) and its relationship to the plastid pigments. The 6-thioctic lipid was eluted from the paper and hydrolyzed with 4 N HCl for one hour at 120°C. Chromatography of the hydrolysis products in butanol-0.5 N ammonia gave two radioactive spots which corresponded to 6-thioctic acid and 6-thioctic acid

sulfoxide, respectively, and the 6-thioctic lipid was destroyed. The results of the hydrolysis with 0.1 N HCl at 110°C at various lengths of time are shown in Table VI.

Table VI

Hydrolysis of 6-thioctic acid lipid with 0.1 N HCl at 110° C	
<u>Time</u>	<u>% Hydrolysis</u>
10 min	3.5
30 min	19.5
1 hr	43.5
3 hr	68.5

Furthermore, the 6-thioctic acid lipid was treated with lipase (steapsin commercial product, Nutritional Biochemicals Corp.) in 0.1 N acetic acid for 24 hours at 37°C. The results in Table VII show that the 6-thioctic lipid is hydrolyzed by the lipase.

Table VII

Radioactivity distribution of 6-thioctic acid lipid hydrolyzed with commercial lipase		
	Lipid + Lipase	Control (lipid + 0.1 N CH <sub>3</sub> COOH without lipase)
Origin	16%	4%
6-Thioctic acid sulfoxide	55	
6-Thioctic acid	11	
Front	19	96

The same result was obtained with a pig pancreas lipase preparation. <sup>††</sup> According to the literature<sup>11</sup> pancreas lipase hydrolyzes only esters of fatty

<sup>††</sup> Kindly supplied by Dr. F. Nord, Fordham University, New York, New York.

acids with glycerol or normal alcohols but not the cholesterol esters. There is some doubt if certain lipase preparations are specific for triglycerides. The specificity of the pig pancreas lipase was therefore checked with n-amyl acetate (as a substrate), which was readily hydrolyzed. So no further information about the type of ester in the 6-thioctic acid lipid could be obtained with this lipase preparation. The hydrolytic behavior, with respect to both acid and enzyme, pretty well establishes the 6-thioctic acid lipid(s) as 6-thioctic acid esterified at the carbosyl group with either glycerol or a normal alcohol.

It should be mentioned that in an experiment where the temperature during the hydrolysis of the 6-thioctic acid lipid with 4 N HCl was lower (stream bath) a radioactive spot appeared on the chromatogram in butanol-0.5 N ammonia which had an  $R_f$  higher than 6-thioctic acid but lower than the 6-thioctic acid lipid. This spot could not be seen after more vigorous hydrolysis. A possible explanation may be that if the 6-thioctic acid is esterified with glycerol, under milder conditions the ester is only partly hydrolyzed to a di- or mono-glyceride, and one would expect such a compound to have an  $R_f$  between 6-thioctic acid and 6-thioctic lipid.

On regular paper chromatograms, the 6-thioctic acid lipid always runs with the solvent front together with the chlorophyll and the other plant pigments. On paper impregnated with Quilon<sup>12</sup> and with absolute methanol as the solvent, the 6-thioctic acid lipid separated into two spots with  $R_f$  values 0.7 and 0.8, and some radioactivity was spread out on the front of the chromatogram. There was no definite separation of the 6-thioctic acid lipid from the chlorophyll.

Experiments were therefore undertaken to separate the 6-thioctic acid lipid from the pigments. On an  $Al_2O_3$  column with petroleum ether, petroleum ether + 3% benzene, and petroleum ether + 3% absolute ethanol as eluents, no separation of the radioactivity from the chlorophyll band could be obtained. The recovery of the activity was only 39%. The rest of the activity was strongly absorbed on top of the column. On powdered sugar (commercial product dried for 12 hours at 90°C), a separation of the bulk of the activity from the chlorophyll fraction could be obtained. Two typical experiments are shown in Tables VIII and IX. Fractions 3 and 4 from Table VIII and fraction 3 from Table IX appeared as gray bands on the sugar columns. According to the spectrum this is pheophytin a + b. Fraction 3 from Table IX was rechromatographed on powdered sugar and developed with ligroin-benzene 1:1. The bulk of the radioactivity could be separated in this way from the gray band and the solution showed only very weak absorption in the visible range of the spectrum. These experiments show that the 6-thioctic acid lipid is not bound to any of the plant pigments.

Since it could not be seen clearly from the separation on Quilon paper how many compounds are in the lipid fraction, the purified 6-thioctic acid lipid (chromatographed on powdered sugar) was distributed in a twenty-five-stage countercurrent apparatus between 95% methanol and n-hexane. The distribution curve is shown in Fig. 2. Two compounds seem to be present in the lipid fraction. The tubes 0-6 and 16-24 were combined into two pools, concentrated, hydrolyzed for one hour with 4 N HCl at 110°C, and then chromatographed with butanol-0.5 N ammonia. The 6-thioctic acid sulfoxide was the major

Table VIII

Liquid chromatograph of the 6-thioctic acid lipid on powdered sugar; percent distribution of the initial activity

Fraction No.	Developer solvent	Percent of initial radioactivity
1	benzene/ligroin <sup>a</sup> 1:7	6.2
2	benzene/ligroin 1:4	2.9
3	benzene/ligroin 1:1	24.0
4	benzene/ligroin 4:1	9.0
5	ether/benzene 400:1	4.0
6 (a) before chlorophyll band	ether/benzene 40:1	3.0
6 (b) chlorophyll band	ether/benzene 40:1	1.6

Recovery 51%<sup>b</sup>

<sup>a</sup> Boilling point 65° - 110°

<sup>b</sup> The rest of the activity stays on top of the column and is probably polymerized product.

Table IX

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Liquid chromatogram of the 6-thioctic acid lipid on powdered sugar; % distribution of the initial radioactivity. The chromatogram was developed first with benzene/ligroin (bp 65°-110°) 1:8 and then with benzene/ligroin 1:1.

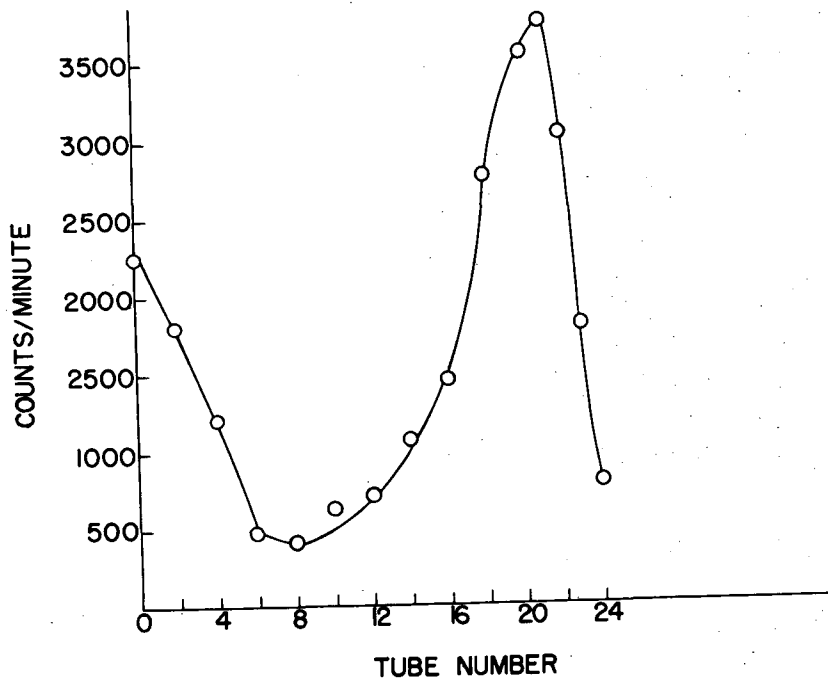
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Fraction 1	strong yellow	11%
Fraction 2	weak yellow	6.5
Fraction 3	weak green	13.4
Fraction 4	strong yellow	19.6
Fraction 5	chlorophyll a	1.4
Fraction 6	chlorophyll b	<u>5.2</u>
		57%

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Fig. 2. Distribution of the 6-thioctic acid lipid after chromatography of powdered sugar between 95% ethanol and n-hexane.

hydrolysis product from Tubes 0-6. In addition, there was some activity on the front, which can be unhydrolyzed or nonhydrolyzable material, and a spot with lower  $R_f$  than the 6-thioctic acid sulfoxide. The first hydrolysis of the Tubes 16-24 with 4 N HCl gave as the major product a compound with higher  $R_f$  than the 6-thioctic acid but lower  $R_f$  than the 6-thioctic lipid. On further hydrolysis with 6 N HCl this spot gave 6-thioctic acid. In addition there was some 6-thioctic acid sulfoxide and a spot with lower  $R_f$  than the 6-thioctic sulfoxide. It seems from these results that the major compound in Tubes 0-6 is an ester of the 6-thioctic acid sulfoxide, and the major compound in Tubes 16-24 is an ester of 6-thioctic acid.

It can be seen from Table II that there is no significant difference in the formation of the total 6-thioctic acid lipid in light or dark. In another experiment, Scenedesmus was incubated in the presence of the  $S^{35}$ -labeled 6-thioctic acid under aerobic and anaerobic conditions. Table X shows that there is a remarkable decrease of the formation of the 6-thioctic acid lipid under anaerobic conditions.

Table X

Formation of the 6-thioctic acid lipid under aerobic and anaerobic conditions		
Time	% Lipid on Chromatogram <sup>a</sup>	
	Aerobic	Anaerobic
1 hr	25	8
15 min (separate experiment)	5.7	2.1

<sup>a</sup> Corresponding to fraction of total soluble radioactivity.

#### Formation of the 6-Thioctic Acid Sulfoxide in the Dark and in the Light

One of the suggestions<sup>3</sup> as to the role of thioctic acid in photosynthesis involved the photolysis of the 6-thioctic acid in the presence of water, leading to active hydrogen in the thiol form and active oxygen in a sulfenic acid form which, in turn, would give rise to the 6-thioctic acid sulfoxide as an intermediate. If this is the case, the steady-state concentration of the 6-thioctic acid sulfoxide in the algae should be higher under light-saturated conditions than in the dark. Scenedesmus was fed in the dark with  $S^{35}$ -labeled

6-thioctic acid for four hours. One part of the washed and resuspended algae was then kept for ten minutes under conditions for light saturation, the other part in the dark. The extracts (without hydrolysis) contained (as the free compound) 20.5% and 21% sulfoxide in the light experiment and 19% and 21% sulfoxide in the dark experiment. So under these conditions there was no difference in the formation of free sulfoxide in the light and in the dark. These experiments were carried out before much was known about the 6-thioctic acid lipid. As the compound that might take part in the light reaction of photosynthesis is more likely to be a bound form of 6-thioctic acid, such as the 6-thioctic acid lipid that is located in the chloroplasts, than the unbound 6-thioctic acid or its sulfoxide, it is questionable if this result has any significance in respect to the photosynthetic function of the 6-thioctic acid. There are, however, other reasons for rejecting the idea that thioctic acid may be directly involved in the path of oxygen from water to molecular oxygen.<sup>13</sup>

#### Water-Soluble Forms of Thioctic Acid

In the fractionation experiment it was found that most of the radioactivity in the cells was in the insoluble fraction that remains after extraction of the chloroplasts with hot 95% ethanol. This fraction was hydrolyzed with 6 N HCl and chromatographed two-dimensionally (phenol-water; butanol-propionic acid) with the sulfur-containing amino acids added as carriers. Besides the 6-thioctic acid and the 6-thioctic sulfoxide, only one weak radioactive spot (2% of the total activity) appeared on the chromatogram ( $R_f$  0.29 x 0.14), which did not correspond to any of the sulfur-containing amino acids. The insoluble fraction was also treated with hot water. The aqueous extract was strongly radioactive. Chromatography of the water extract in the solvent systems mentioned before, and paper electrophoresis in 0.05 M phosphate buffer pH 6.7, gave in each case only one radioactive compound, which corresponded exactly with 6-thioctic acid. The same result was obtained when the insoluble fraction was extracted with cold water. It seems from this result that this thioctic acid is in a water-soluble, alcohol-insoluble, easily hydrolyzable protein complex of thioctic acid, or that the free acid is very strongly adsorbed onto insoluble protein precipitates.

It was of further interest to see whether the sulfur of the 6-thioctic acid was incorporated into the sulfur-containing amino acids of the soluble proteins. After *Chlorella* had been fed for three hours with  $S^{35}$ -labeled 6-thioctic acid, the soluble proteins were isolated by breaking the cells and precipitating the proteins with ammonium sulfate. The proteins were redissolved in phosphate buffer and dialyzed against  $10^{-4}$  M phosphate buffer. The dialyzed protein solution contained 50% of the radioactivity of the total supernatant (supernatant after removing plastid material by ultracentrifugation). The proteins were hydrolyzed with 6 N HCl and the hydrolysis products chromatographed two-dimensionally (phenol-water; butanol-propionic acid). Besides a very weak spot (1% of the activity,  $R_f$  0.32 x 0.14), which did not correspond to any of the cochromatographed amino acids, only the 6-thioctic acid and the 6-thioctic acid sulfoxide were present.

Thioctic acid was also precipitated with the proteins when the 6-thioctic acid was added to the soluble proteins of *Chlorella*, which are then precipitated by adding ammonium sulfate.

## DISCUSSION

The major metabolic products of 6-thioctic acid in Scenedesmus or Chlorella are the 6-thioctic acid ester or its sulfoxide, and possibly a water-soluble 6-thioctic acid protein complex. Both are located in the chloroplasts. These bound forms of 6-thioctic seem to be similar to the metabolic products of 6-thioctic in S. faecalis.<sup>5</sup> The 6-thioctic ester or its sulfoxide might have some connection with the stimulation of the Hill reaction with thioctic acid. Bradley and Calvin<sup>14</sup> have found that the stimulation of the Hill reaction by 6-thioctic acid is quite sensitive to the conditions of the incubation with Scenedesmus. To obtain a stimulation of the Hill reaction, it was necessary to incubate the algae for a time (for example, 50 minutes) in the presence of oxygen in the light or in the dark. In another type of experiment, Shibata<sup>15</sup> found that an inhibition of the Hill reaction by 6-thioctic acid also has an induction period of about 20 minutes. The induction period that is necessary for an effect of the 6-thioctic acid on the Hill reaction cannot be caused by a slow uptake of the 6-thioctic by the cells, as can be seen from the results mentioned above. It is, rather, indicative that a slow rate of formation of a metabolic product of 6-thioctic acid causes the induction period. Because the 6-thioctic ester is formed slowly, as can be seen from Table I, since it is located in the chloroplasts and its formation is decreased under anaerobic conditions but not sensitive to light conditions, it is not unreasonable to suppose that the 6-thioctic acid ester, or its sulfoxide, is the metabolic product of the 6-thioctic that stimulates or -- depending on the conditions -- decreases the oxygen evolution in the Hill reaction.

A number of minor metabolic products of 6-thioctic acid that appear on the chromatogram in butanol-ethanol-water have not yet been further characterized.

## EXPERIMENTAL

Solutions of S<sup>35</sup>-labeled 6-thioctic acid that were used: 0.4 mg/ml in 0.01 M phosphate buffer, pH 6.7, activity 83  $\mu\text{c}/0.41$  mg. Second batch 1.07 mg/ml in 2% sodium bicarbonate, activity 53  $\mu\text{c}/\text{mg}$ .

Spray for thioctic acid: 300 cc acetone; 5 cc 6 N HCl, 1 cc saturated H<sub>2</sub>PtCl<sub>6</sub>, 3-4 cc saturated aqueous sodium iodide, water added to clear the solution.

Time series in light and dark: 50 ml of a 2% suspension of Scenedesmus in phosphated buffer ( $3.2 \times 10^{-6}$  M KH<sub>2</sub>PO<sub>4</sub>, 1 ml for 1 ml of wet cells) was divided into two parts. One part was aerated (air with 4% CO<sub>2</sub>) in the dark for 10 min. 400  $\lambda$  of the S<sup>35</sup>-labeled 6-thioctic acid solution was then added. After 1 min, 10 min, 30 min, 1 hr, and 4 hr 35 min, small samples of cell suspension were withdrawn and filtered rapidly on Supercel, washed two times with distilled water, and extracted with hot 80% and 20% ethanol. The filter had a two-way stopcock so that the medium plus wash water the the cell extract could be collected separately. The extracts were concentrated in vacuo

in the cold and chromatographed in one dimension with butanol-0.5 N ammonia. The other part was kept for 10 min in the light, then 400  $\lambda$   $S^{35}$ -labeled 6-thioctic acid was added and the suspension treated the same as in the dark experiment.

Formation of the thioctic acid sulfoxids in the light and dark: 60 ml of 0.5% suspension of Scenedesmus in phosphate buffer was incubated with 100  $\lambda$  of  $S^{35}$ -labeled 6-thioctic acid solution in the dark for 4 hr. The uptake of the 6-thioctic acid was 35%. The algae were then filtered, washed with distilled water, and resuspended in phosphate buffer. Half of this suspension was then aerated in the dark for 10 min, the algae then killed immediately with hot absolute ethanol, and the extract chromatographed in one dimension with butanol-0.5 N ammonia. The other half of the suspension was kept for 10 min under light-saturated conditions. (The conditions for light saturation were determined in a closed system by measuring the  $CO_2$  uptake with a Beckman  $CO_2$  analyzer.) The algae were then killed and the extract chromatographed as mentioned above.

Fractionation of Chlorella: 20 ml of a 4% Chlorella suspension was incubated under photosynthesis conditions for 3 and 4 hr respectively with  $S^{35}$ -labeled 6-thioctic acid. The cells were then centrifuged, washed with water, resuspended in phosphate buffer, and ruptured by 10-min treatment in a 9-kc oscillator. To remove unbroken cells the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was then centrifuged at 40,000 rpm for 15 min. The chloroplasts were extracted several times with hot 95% ethanol.

Isolation of the proteins from Chlorella: Chlorella was incubated with  $S^{35}$ -labeled 6-thioctic acid and the cells broken in the same way as mentioned in the fractionation experiment. To the 40,000 rpm supernatant, ammonium sulfate was added to 90% of saturation (68.5 g  $(NH_4)_2SO_4$  per 100 ml). The proteins were centrifuged for 10 min at 20,000 rpm, redissolved in 0.05 M phosphate buffer pH 6.8 and dialyzed for 12 hr against  $10^{-4}$  M phosphate buffer.

Hydrolysis of the 6-thioctic acid lipid with lipase: To the alcoholic eluate of the chromatogram 1 ml of distilled water was added and the alcohol evaporated in vacuo. Then 200  $\lambda$  of 0.1 N acetic acid and 1 mg lipase (steapsin commercial product, Nutritional Biochemicals Corp.) were added and the solution kept at 37°C for 12 hr. The experiments with the other lipases were carried out in the same way.

Formation of the 6-thioctic acid lipid under aerobic and anaerobic conditions: A 2% suspension of Scenedesmus was divided into two parts. One part was kept under photosynthesis conditions, the other part was flushed with nitrogen for 15 min.  $S^{35}$ -labeled 6-thioctic acid was added at the same time to both parts and after 1 hr the algae were killed rapidly with boiling absolute ethanol. Chromatography was carried out as described before.

Attempts to prepare "lipothiamid": According to the work of Reed and DeBusk<sup>16</sup> one of the biologically active forms of 6-thioctic acid is the so-called "lipothiamid," an amide of 6-thioctic with vitamin B<sub>1</sub>. Previous work had been done with only very impure samples of 6-thioctic acid (~50% pure). We therefore tried to prepare lipothiamid synthetically with crystalline 6-thioctic acid as a starting material. According to Reed, lipothiamid was prepared by reacting 6-thioctic acid chloride with free thiamine in formamide. In order to prepare the 6-thioctic acid chloride he treated 6-thioctic with oxalylchloride in a sealed tube at 60°-70°C for 90 min. Upon repetition of this experiment with crystalline 6-thioctic acid it was found that even at room temperature the characteristic peak of the 6-thioctic acid at 330 mμ disappeared in benzene solution in the presence of oxalylchloride. This shows that oxalylchloride destroys the 6-thioctic acid even under very mild conditions. With thionylchloride in benzene solution the 6-thioctic acid is destroyed immediately. In another experiment an attempt was made to synthesize lipothiamid by reaction of 6-thioctic acid and thiamin in the presence of dicyclohexylcarbodiimid, which readily forms peptide bonds.<sup>17</sup> As yet, no compounds other than 6-thioctic acid and 6-thioctic acid anhydride have been detected on the chromatogram (butanol-0.5 N ammonia) of the reaction mixture in aqueous tetrahydrofuran.

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