STUDIES ON THE PHOTOSYNTHETIC REACTION. III. THE EFFECTS OF VARIOUS INHIBITORS UPON GROWTH AND CARBONATE-FIXATION IN CHLORELLA PYRENOIDOSA [1,2]

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The chromatographic-radioautographic technique for the analysis of soluble, intracellular metabolites has been eminently successful in its original application—that of determining the mechanism of carbon dioxide fixation in green plants (1). To the extent that the absence of light can be considered an inhibitor, the technique is thus shown to be valid for the study of inhibition mechanisms.

The first application of the technique to inhibition by a chemical agent (2) appears in the negative, though apparently valid, result of adding malonate to photosynthesizing Scenedesmus. Various extensions of this technique have appeared (3, 6, 10, 14). The work of Havinga et al (6) on the effects of antibiotics upon Scenedesmus was the first systematic attempt to apply the technique to chemical inhibitions. Except for some small but essential modifications of procedure, this work is the model for the present paper.

The present study is negative with regard to its ultimate purpose of finding specific inhibitors of photosynthesis, but there remains a body of data on the effects of various inhibitors upon growth and carbon dioxide fixation in Chlorella.

MATERIALS AND METHODS

ALGAE AND MEDIA: *Chlorella pyrenoidosa* Chick (American Type Culture Collection No. 7516) were grown in pure culture, either on agar slants (4) or in continuous culture shake flasks, using the major nutrients of Myers (7) (except for supplying the iron as the ethylenediamine-tetraacetic acid complex), the modified micronutrients of Arnon (8), and 5 % CO_2 in air.

For growth tests, algae were scraped from agar slants and diluted with sterile water to a concentration of 3 to 5×10^7 cells/ml. For measurements of radiobicarbonate fixation and for the preparation of extracts for chromatography, the shake flask harvests were centrifuged (30 min, 800 rcf at tube tip) and resuspended to 2 % concentration by volume in the experimental medium.

This experimental medium contained: 5×10^{-3} M KNO_3, 2×10^{-3} M $MgSO_4$, 0.25×10^{-3} M $Ca(NO_3)_2$, 2.3×10^{-6} M $Na_2B_4O_7$, 1.9×10^{-6} M $MnCl_2$, 1.0×10^{-6} M Na_2MoO_4, 0.17×10^{-6} M $CoCl_2$, 0.16×10^{-6} M $CuCl_2$, 0.15×10^{-6} M $ZnCl_2$, 1.6×10^{-8} M $Fe(NH_4)_2(SO_4)_2$, and 0.02 M potassium phosphate buffer. The iron at 1000-fold concentration was autoclaved separately in acidic solution. The phosphate buffers used were (a) K_2HPO_4 and KH_2PO_4 in the molar ratio 1:19, (b) the same salts in the ratio 3:17, and (c) K_3PO_4. In a complete growth mixture, after equilibration with 5 % CO_2, the pH's were 5.1, 6.0 and 7.4. The first of these solutions is rather poorly buffered and variations of as much as 0.4 pH unit were sometimes observed.

GROWTH TESTS: A 24-tube aeration apparatus was built according to the general plan of Myers (9). The tubes were autoclaved with 5.0 ml of double strength experimental medium plus 3.5 ml of water. While still warm, the tubes containing alkaline medium were aerated with pure CO_2 to redissolve precipitated material. All tubes were equilibrated with 5 % CO_2 in air before adding the 0.1 ml of iron solution, 1 ml of pH-adjusted, appropriately sterilized inhibitor solution (or water) and 0.5 ml of the aqueous algae suspension. To prevent evaporation loss during the 48-hr growth test, the aerating gas was first passed through water. After temperature equilibration with the light sources the suspensions inside the tubes had a temperature of 25° C. Optical densities were measured twice a day at 610 mμ by means of a Lumetron colorimeter. For non-inhibited control runs the optical densities were initially in the range 0.10 to 0.15, and after 48 hours they were in the range 0.55 to 1.30. Separate non-inhibited controls were used at each pH. In the first run with any inhibitor a control without algae was included to note possible interference with colorimetric readings by either the inihibitor itself or its possible decomposition products. Tubes were always run in duplicate, and their optical readings stated in terms of percentages rarely differed by more than three or four units in either initial or final readings.

The inhibition data of table I are stated as percentages (compared to the non-inhibited controls) based on optical density increments from the start of the experiment to 40 hours. The 40-hr period is arbitrary for the sake of the table. Actually, day to day comparisons of results are improved by using either the 40 or the 48-hr period, according to which happens to give control run optical densities nearest an arbitrary density. This is true because the day to day differences in non-inhibited growth curves are primarily in the lag period.

CARBONATE-FIXATION TESTS: The constant temperature bath (25° C) was a flat vase connected to a constant temperature reservoir by a circulating pump. Light from a 300 w reflector spot lamp had a 7-inch path including 5 inches through water. The reaction vessels were flat centrifuge tubes (Corning No. 8520) calibrated at the 5.0 ml volume.

All tests were carried out in the pH 6.0 experimental medium. To insure an aerating gas of uniform composition for any one series of experiments, the 5 %

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CO_2 in air was necessarily supplied from a single cylinder of compressed mixture. The resuspended algae were stored in a test tube under experimental conditions, aliquots being withdrawn as needed. Inhibitor solutions were made up with the experimental medium and readjusted to pH 6.0. By means of long hypodermic syringes this inhibitor solution and plain experimental medium were added to each tube to a total of 0.50 ml. Experiments were run consecutively along with interspersed controls. Using a sharp-tipped pipette each tube received 0.50 ml of the algal suspension. After 5 minutes preexperimental time, the aeration jet was removed and 0.10 ml (0.5 μc) of sodium bicarbonate-C^{14} was injected by syringe. The tube was shaken without removing from the water-bath or from the light beam. After exactly two minutes the experiment was ended by placing the tube in a beaker of boiling alcohol and quickly adding into the tube 4 ml of boiling alcohol. After 5 minutes in the boiling alcohol each tube was adjusted to 5.0 ml and centrifuged. The supernatants were used for triplicate 1-ml planchets for determination of C^{14}-activity. Where necessary, corrections were applied for the self-absorption effect of inhibitors upon the observed radioactivity. In table II the radioactivities for in-

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE GROWTH OF CHLORELLA PYRENOIDOSA

INHIBITOR	CONC	% INHIBITION		
		pH 5.1	pH 6.0	pH 7.4
Antibiotics				
Azaserine (o-diazoacetyl-L-serine) (Parke, Davis)	0.001 M	58	19	0
	0.002 M	81	79	28
	0.01 M	90	83	74
Bacitracin * (61 U/mg)	500 μgm/ml	11	...	0
	1000 μgm/ml	53	...	0
	5000 μgm/ml	100	...	19
Carbomycin (Magnamycin, Pfizer) (1075 μg/mg)	100 μgm/ml	92	98	100
(-)-4-Oxo-2-thiazolidine-hexanoic acid (actithiazic acid, Pfizer) (crystalline)	10 mg/ml	0	0	0
Erythromycin (Ilotycin, Lilly)	36 μgm/ml	0	0	2
	145 μgm/ml	...	15	37
	725 μgm/ml **	63	78	87
Gliotoxin	2 μgm/ml	93	92	95
	2 μgm/ml †	75 †	82 †	59 †
Neothiolutin (Pfizer)	2.5 μgm/ml	21	48	100
	5 μgm/ml	37	75	100
	10 μgm/ml	100	100	100
Netropsin	5 μgm/ml	67	71	70
	10 μgm/ml	100	100	100
Oxytetracycline (Terramycin, Pfizer) (900 μgm/mg)	1 μgm/ml	S ††	S	S
	10 μgm/ml	S	S	S
	50 μgm/ml ‡	...	...	...
Pleocidin	1 μgm/ml	75	75	30
	5 μgm/ml	100	100	100
Polymyxin B (6850 U/mg)	0.5 μgm/ml	30	0	0
	5 μgm/ml	100	100	93
Puromycin (Stylomycin, Lederle)	10^{-4} M	-27 ‡‡	-9	-21
	10^{-3} M	- 5	30	30
	10^{-2} M	100	100	100
Rimocidin Sulfate (Pfizer) (1000 μgm/mg)	100 μgm/ml [a]	11	13	31
Streptomycin	10^{-4} M	77	85	88
	10^{-3} M	87	100	100
Tetracycline (Achromycin, Lederle)	Unstable under conditions of the test.			
Thiolutin (Acetopyrrothine, Pfizer)	2 μgm/ml	82	77	95
	5 μgm/ml	100	100	100
Uracil analogs				
5-Bromouracil	10^{-5} M	- 1	- 4	-14
	10^{-3} M	35	31	65
	10^{-2} M	97	99	95
5-Nitrouracil	10^{-3} M	-15	-17	7
Orotic Acid	10^{-3} M	- 3	- 6	- 8
2-Thiouracil	10^{-3} M	31	19	35
Thymine	10^{-3} M	6	-14	8
Plant growth regulators				
2,4-Dichlorophenoxyacetic acid	1 mg/ml	0	0	0
Indole-3-acetic acid	10 μgm/ml	42	30	32
	100 μgm/ml	71	77	68
Sulfonic acids				
2,3-Dihydroxypropane-1-sulfonic acid	10^{-2} M	0	0	0
Isethionic acid	10^{-2} M	0	0	0
Taurine	10^{-2} M	0	0	0
Taurineamide	10^{-2} M	24	15	...

* Due to frothing, the aeration rate in this case was very low.

** Added as a non-sterile suspension.

† Gliotoxin solution aerated for 24 hours before start of experiment.

†† S = Stimulation.

‡ Decomposition products prevent turbidity readings.

‡‡ Negative percentages indicate degree of stimulation.

[a] Complete inhibition for about 24 hours, followed by rapid growth.

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON THE FIXATION OF CARBONATE BY CHLORELLA PYRENOIDOSA

INHIBITOR	CONC	RADIOACTIVITY OF SOLUBLE EXTRACTS COMPARED TO CONTROLS *
Azaserine	0.05 M	0.95
Bacitracin	1 mg/ml	1.3
	2 mg/ml	2.0
	6 mg/ml	2.7
	38 mg/ml	3.6
Erythromycin	100 μgm/ml	1.18
	500 μgm/ml	1.35
	1000 μgm/ml	1.46
Gliotoxin	3 to 150 μgm/ml **	0.64 to 0.75 **
Netropsin	25 μgm/ml	1.00
	250 μgm/ml	0.96
Pleocidin	5 μgm/ml	0.98
Polymyxin B	100 μgm/ml	1.10
	300 μgm/ml	0.39
	500 μgm/ml	0.00
Streptomycin	10^{-3} M	0.8
	10^{-2} M	0.8
Thiolutin	4 μgm/ml	0.95
	10 μgm/ml	0.80
5-Bromouracil	10^{-3} M	0.8
	10^{-2} M	1.0
2-Thiouracil	10^{-3} M	0.9
	10^{-2} M	1.0

* Controls taken as 1.00.
** The 150 μgm/ml is a suspension. The literature value of solubility is 70 μgm/ml. Within the concentration range tested, inhibitor concentration is not a limiting factor.

hibited experiments are stated as ratios compared to the non-inhibited controls.

CHROMATOGRAPHIC TESTS: The procedure was the same as that for the carbonate-fixation tests, except that the quantities used were ten times as large, and that the reaction medium itself was excluded from the alcoholic extract. This exclusion was accomplished by passing the reaction mixture through a Celite filter bed on a Büchner funnel connected to an adapter with two receivers (13). Within two or three seconds, the filtrate could be collected, the other receiver rotated into position, and algae cells on the filter killed by the addition of boiling 80 % alcohol.

Two-dimensional chromatography of concentrated alcoholic extracts was carried out on Whatman No. 1 paper, oxalate-washed (6) and unwashed, first in phenol-water (72:28 w/w), then in butanol-propionic acid-water (equal volumes of 95 % v/v aq *n*-butanol and 44 % v/v aq propionic acid) (1). Figure 3 of reference 1 is an example of the chromatographic pattern.

Radioactivities were calculated in terms of the percentages of a chromatogram's total radioactivity to be found in each of its spots.

RESULTS

The growth inhibition tests (table I) served to eliminate from further consideration the unstable compounds (rimocidin, tetracycline, and oxytetracycline) and the ineffective compounds (thiazolidonehexanoic acid, nitrouracil, orotic acid, thymine, dichlorophenoxyacetic acid, and all the sulfonic compounds).

By comparison of the effective inhibitor concentrations in the growth test and in the carbonate-fixation test (table II), the growth-inhibiting concentrations of azaserine, bacitracin, erythromycin, netropsin, pleocidin, 5-bromouracil, and 2-thiouracil were found to be ineffective in the carbonate-fixation test. At growth-inhibiting concentrations gliotoxin was a partial inhibitor of carbonate-fixation, but the inhibition of carbonate-fixation could not be increased by increasing inhibitor concentration. Polymyxin B, streptomycin, and thiolutin were fair to good inhibitors of carbonate-fixation, but only at concentrations well in excess of those required for comparable growth-inhibition.

The latter three inhibitors were tested by the chromatographic technique. Considering the distribution of radioactivity upon chromatograms the addition of streptomycin (0.01 M) caused an increase in the radioactivity of sucrose (from 6 to 25 %), and decreases in radioactivity of the phosphate ester area (73 to 55 %), of aspartic acid (7 to 2 %), and of glutamic acid (2 to less than 1 %). Chromatographic patterns in single runs with polymyxin B (400 μgm/ml) and thiolutin (10 μgm/ml) showed no dependable differences from the controls.

DISCUSSION

For various green algae the maximum non-inhibitory dose of bacitracin has been reported as about 1 mg/ml (12). The growth tests of the present studies were essentially in agreement with this, but the short-term measurements of carbonate-fixation at growth-inhibitory levels revealed an unexpected stimulation. The persistence of this stimulation, even at massive dosages of bacitracin (38 mg/ml) was especially remarkable. The explanation of this phenomenon is not apparent.

Erythromycin similarly stimulated short-term carbonate-fixation at growth-inhibitory concentrations, though the effect was not as strong as that of bacitracin. Solubility limitations prevented testing at higher concentrations.

Although the decomposition products of oxytetracycline prevented turbidity measurement at higher antibiotic concentrations, the use of appropriate blanks permitted the qualitative conclusion that oxytetracycline stimulated growth at concentrations of 1 to 10 μgm/ml. Previous reports on the effect of oxytetracycline in growth tests with green algae (5, 12) indicate no inhibition in this concentration range, but they do not report any stimulation. Havinga et al (6) found oxytetracycline and chlortetracycline stimulated the short-term fixation of radiocarbonate at antibiotic concentrations from about 15 to 750 μgm/ml.

The Chlorella used in this study were clearly more

sensitive to gliotoxin than were the Chlamydomonas and Scenedesmus used by Foter et al (5). The unusual aspect of gliotoxin action is its inhibition of carbonate-fixation to the extent of about 30 %, regardless of any gliotoxin concentration in the range 3 to 150 μgm/ml. Since there are many independent mechanisms for the incorporation of radiocarbonate into cellular metabolites, the gliotoxin was probably inhibiting one carbonate-fixation mechanism completely, rather than several mechanisms partially. The data of tables I and II are not sufficient to establish a correlation between growth-inhibition and inhibition of carbonate-fixation, but they do permit the possibility that the primary effect of gliotoxin may be in this inhibited carbonate-fixation mechanism. Even if this were true, it must be pointed out that the term "carbonate-fixation" is meant in its broadest sense: the site of metabolic blockade still could be far removed from any actual carboxylation reaction.

Azaserine, netropsin, and pleocidin had little if any effect on carbonate-assimilation, even at concentrations well is excess of those required for powerful growth inhibition. This was taken as evidence that the inhibition mechanisms were totally unrelated to carbonate-fixation.

Thiolutin and polymyxin B were partially effective as inhibitors of carbonate-fixation; but their clearly greater effect in inhibiting growth was taken as evidence that carbonate-fixation was involved either as a secondary mechanism or as a secondary effect of the primary mechanism. The latter possibility is favored by the failure of the chromatographic tests to reveal any derangement in carbonate metabolism. This is particularly true in the case of polymyxin B, where the quantitative depression of the carbonate metabolism was more obviously at variance with its qualitative integrity.

At streptomycin levels more than sufficient to inhibit growth, the chromatographic study of soluble Chlorella extracts did reveal some derangement in carbonate metabolism. Essentially, streptomycin appeared to have reduced the pool sizes of some major phosphorylated intermediates in photosynthesis, resulting in a more rapid sweep of radioactive carbon into the sucrose pool. A very similar increase of sucrose radioactivity at the expense of radioactivity in the organic phosphate area has been observed in Scenedesmus by Ouellet and Benson (11) as a result of low pH. Unless excess acidity and inhibition by streptomycin are related in some totally unexpected manner, the observed pattern of derangement in carbonate metabolism must be of a rather general and necessarily secondary nature.

Summary

A variety of antibiotics and other possible inhibitors were tested against *Chlorella pyrenoidosa* in both long-term growth tests and in short-term tests of ability to assimilate carbon dioxide in the light. Under conditions of growth inhibition bacitracin, erythromycin, and oxytetracycline appeared to have some stimulatory effect on carbonate-fixation. Gliotoxin, thiolutin, polymyxin B, and streptomycin inhibited the carbonate-fixation.

For the latter three compounds, a study by chromatography-autoradiography provided some evidence that the carbonate-fixation inhibitions were consequences of other, more direct inhibitions.

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