O-Methylthreonine, a New Bleaching Agent for *Euglena gracilis*

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SUMMARY

O-Methylthreonine (OM) inhibits multiplication and photosynthetic pigment synthesis in *Euglena gracilis* and *Ochromonas danica*; it does not inhibit *Chlamydomonas reinhardtii* or *Rhodospseudomonas palustris*. Inhibition of multiplication and pigment synthesis may be prevented by L-isoleucine. OM also causes the permanent loss of chlorophyll and appreciable loss of carotenoids in *E. gracilis* and this may be prevented by L-isoleucine, α -aminobutyric acid, α -ketobutyric acid, L-threonine, L-homoserine, or L-methionine. α -Ketobutyric acid is most effective on a molar basis and is therefore postulated to be the target of OM inhibition.

Isoleucine plays a role in the biosynthesis of photosynthetic pigments and lipids in *Euglena gracilis*, for the incorporation of ^{14}C -L-isoleucine radioactivity into both the pigments and lipids is markedly diminished by bleaching agents which prevent pigment synthesis without affecting multiplication, i.e. O-methylthreonine or streptomycin. When pigment synthesis is blocked amino acids accumulate in the culture supernate.

INTRODUCTION

Many chlorophyll-containing organisms have been bleached temporarily by an assortment of chemical and physical agents (Scher & Aaronson, 1958; Aaronson & Scher, 1960) but only the phytoflagellate, *Euglena gracilis*, has been rendered permanently apochlorotic—that is, free of chlorophyll and capable of multiplication—by chemical agents such as streptomycin (Provasoli, Hutner & Schatz, 1948; Jirovec, 1949) and antihistamines (Gross, Jahn & Bernstein, 1955) and physical agents such as heat (Pringsheim & Pringsheim, 1952) and ultraviolet irradiation (Pringsheim, 1958). This phenomenon of apochlorosis is one of the few instances where a change in the environment results in a specific, permanent, inherited change in the phenotype of an organism. Other specific environmentally induced and heritable changes such as bacterial transformation, transduction, or killer factor in *Paramecium*, unlike *Euglena* bleaching, involve the transfer of nucleic acids or particles such as a bacteriophage or killer factor containing nucleic acids. In apochlorosis in *E. gracilis* diverse agents operating in unknown ways affect the same site—the synthesis of chlorophyll, of the plastid or both. We do not know how any of these agents function to produce apochlorosis.

Recently we were fortunate in obtaining DL-O-methylthreonine (OM, also called β -methoxythreonine) which inhibited multiplication and pigment synthesis of *Euglena gracilis* and several metaphyta; this inhibition was annulled by isoleucine

(Gray & Hendlin, 1956). We have confirmed these observations and have shown that OM renders *E. gracilis* permanently apochlorotic and that α -ketobutyrate or amino acids that can be metabolized to α -ketobutyrate prevent bleaching by OM. We also have shown that isoleucine is involved in the synthesis of photosynthetic pigments and lipids.

METHODS

The organisms used were the phytoflagellates *Euglena gracilis* var. *Z*, *Ochromonas danica*, *Chlamydomonas reinhardtii*, and the photosynthetic bacterium *Rhodospseudomonas palustris*.

The chemically defined media and the procedures for the growth of these organisms were described elsewhere (Aaronson & Scher, 1960). Multiplication was measured as optical density (OD) with a Welch Densichron (Chicago, Illinois, U.S.A.). Photosynthetic pigments were routinely determined by the extraction of organisms with 3–5.0 ml. portions of methanol; these were pooled and the concentration of pigments assayed by reading the OD with Klett–Summerson colorimeter using the 42 (400–500 m μ) and 66 (640–700 m μ) filters.

Mass culture of organisms permanently bleached by DL-O-methylthreonine (OM), streptomycin, or an incubation temperature of 35.5° were grown in chemically defined media at 25° in the light. Pigments were extracted by a variation of the aforementioned method and the supernatant fluids prepared by centrifuging the organisms, filtering the supernatant fluids and concentrating it 10–40-fold by reduced pressure distillation. The concentrated supernatant fluids were examined for porphyrins and nucleic acid derivatives with the Beckman DU spectrophotometer. The supernatant fluids were also examined for nucleic acid derivatives by ascending paper chromatography on Whatman No. 1 paper with a butanol + acetic acid + water (4 + 1 + 5, v/v) solvent system. Amino acids were assayed by adding 1.0 ml. of culture supernatant fluid to a mixture of 1.0 ml. of a solution of 0.5 g. ninhydrin/100 ml. acetone, and 3.0 ml. water. This mixture was heated for 5 min. at 90° and the OD read on the Klett–Summerson colorimeter with the 54 (520–580 m μ) filter.

Supernatant fluids were examined for amino acids by both ascending and descending paper chromatography on Whatman No. 1 paper with the following solvent systems: isopropanol + acetic acid + water (7 + 2 + 1, v/v) *n*-butanol + acetic acid + water (12 + 3 + 5, v/v) or methanol + pyridine + water (80 + 4 + 20, v/v). Amino acids were developed by dipping the paper in a solution of 0.2 g. ninhydrin/100 ml. acetone, air-drying the paper, and heating 5 min. at 90–100°. Photosynthetic pigments were separated by the method of Strain (1953), with Whatman No. 1 paper soaked in 10% (v/v) glycerol in methanol, air-drying the paper, and developing pigments in 0.5 g. *n*-propanol in 100 ml. of light petroleum. Pigment spots were identified by their fluorescence or absorption when exposed to Wood's light.

Radioactivity was determined with a Nuclear-Chicago scaler with a model D47 gas flow Micromil window counter (Nuclear-Chicago, Inc., Chicago 45, Illinois, U.S.A.).

Uniformly-labelled ¹⁴C-L-isoleucine was purchased from Schwarz Laboratories, Inc. (Mount Vernon, New York, U.S.A.).

We are indebted to Dr D. Hendlin of the Research Laboratories of Merck Sharp and Dohme, Inc. (Rahway, New Jersey, U.S.A.) for calling our attention to the effect of OM on *Euglena* and a generous gift of this compound.

The bleached and normal organisms were examined with (i) a Zeiss Lumipan microscope equipped for phase, darkfield, and polarized light microscopy through the kind co-operation of Dr J. J. Lee (Dept. of Micropaleontology, American Museum of Natural History, New York, New York, U.S.A.); (ii) a Zeiss standard microscope GFL equipped for fluorescent microscopy with a Reichert 'Fluorex' maximum-pressure mercury-vapour arc-light source, type HBO 200, through the courtesy of Messrs H. A. Fischer and D. Amsterdam of the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn, New York, U.S.A.

RESULTS

Effect of OM on multiplication

OM inhibited multiplication of several photosynthetic flagellates (Tables 1, 2). *Ochromonas danica* was at least ten times more sensitive than *Euglena gracilis*. The photosynthetic bacterium, *Rhodospseudomonas palustris*, and the phytoflagellate, *Chlamydomonas reinhardtii*, were not inhibited by OM up to concentrations of 30.0 mg./100 ml. The inhibition of multiplication of *O. danica* and *E. gracilis* was annulled by isoleucine; this prevention of inhibition was competitive. The partial inhibition of *E. gracilis* multiplication by OM was also annulled by several other amino acids, aliphatic acids, and certain steroids and their precursors. These were not examined in detail because of the limited supply of OM.

Table 1. *Effect of OM on multiplication and pigment synthesis of O. danica*

Concentration (mg. %, w/v) <i>O</i> -Methylthreonine (OM)	Optical density (as % of normal)					
	No isoleucine			DL-isoleucine 3.0 mg. %, w/v		
	Cell	660 m μ	420 m μ	Cell	660 m μ	420 m μ
0.6	103	86	80	100	107	100
1.0	66	29	23	100	105	100
2.0	20	8	6	103	100	100
4.0	5	—	—	103	82	100

Effect of OM on photosynthetic pigment formation in Euglena gracilis

When grown in OM at concentrations between about 1.0 and 10.0 mg./100 ml., there was no marked inhibition of multiplication but the resulting suspension of organisms was white and the organisms contained little if any carotenoids and no chlorophyll (Table 2). When samples of these organisms were plated on inhibitor-free agar media the resulting colonies were white and successive weekly transfers of the progeny of these colonies (33rd at this writing) in the light remained white, i.e. permanently apochlorotic. Extraction of the photosynthetic pigments of these white organisms revealed no chlorophyll; however, in the absence of OM, carotenoids were synthesized but only 4% of the normal quantity. Similar low quantities of carotenoids were found in dark-grown organisms or organisms bleached by streptomycin or high temperature (35.5°) incubation.

Bleaching of Euglena gracilis

Bleaching by OM was competitively annulled by L-isoleucine, L-threonine, L-homoserine, α -aminobutyric acid or α -ketobutyric acid; α -ketobutyric acid was most

Table 2. *Effect of OM on multiplication and pigment synthesis by E. gracilis and its prevention by isoleucine*

Concentration (mg. %, w/v) <i>O</i> -methylthreonine	Concentration of <i>L</i> -isoleucine (mg. %, w/v)											
	0				1.0				10.0			
	Cell O.D.	Colour of culture	O.D. 660 m μ	O.D. 420 m μ	Cell O.D.	Colour of culture	O.D. 660 m μ	O.D. 420 m μ	Cell O.D.	Colour of culture	O.D. 660 m μ	O.D. 420 m μ
0	1.54	G*	92	333	1.49	G	83	323	1.58	G	88	365
0.1	1.94	G	83	337	1.52	G	70	294	1.46	G	73	299
0.3	2.84	G	47	222	1.64	G	70	290	1.30	G	60	260
0.6	2.22	W	1	25	2.36	G	53	258	1.26	G	54	242
1.0	1.14	W	1	6	2.58	G	34	177	1.40	G	66	300
3.0	1.54	W	0	0	1.94	W	0	12	1.30	G	45	222
6.0	1.28	W	—	—	1.90	W	0	0	2.12	G	61	320
10.0	1.38	W	—	—	1.43	W	—	—	2.55	W	3	93
30.0	0.02	—	—	—	0.13	W	—	—	1.52	W	0	8
50.0	0	—	—	—	0.02	—	—	—	0.54	W	0	0

* G, Green; W, white.

Table 3. Compounds preventing the bleaching of *E. gracilis* by DL-O-methylthreonine (OM)

Compound	Conc. (in mM) needed to prevent bleaching by 0.23 mM of OM
$\begin{array}{c} \text{OCH}_3 \\ \\ \text{CH}_3\text{-CH-CH-COOH} \\ \\ \text{NH}_2 \end{array}$ DL-O-methylthreonine	
$\begin{array}{c} \text{CH}_3\text{-CH}_2\text{-CH-CH-COOH} \\ \quad \\ \text{CH}_2\text{NH}_2 \end{array}$ L-isoleucine	0.23
$\begin{array}{c} \text{OH} \\ \\ \text{CH}_3\text{-CH-CH-COOH} \\ \\ \text{NH}_2 \end{array}$ L-threonine	0.25
$\begin{array}{c} \text{CH}_3\text{OH-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$ L-homoserine	0.50
$\begin{array}{c} \text{CH}_3\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$ α-aminobutyric acid	0.29
$\begin{array}{c} \text{CH}_3\text{-CH}_2\text{-C-COOH} \\ \\ \text{O} \end{array}$ α-ketobutyric acid	0.1

Table 4. Effect of isomers of threonine on OM inhibition of *Euglena*

Compound concentration (mg. % w/v)		Concentration of OM (mg. % w/v)	
		1.0	3.0
0		optical density	
D-threonine		2.18 W*	1.42 W*
1.0		2.42 W	1.66 W
3.0		2.62 W	1.94 W
6.0		2.32 W	1.60 W
10.0		2.40 W	1.68 W
L-threonine		2.12 W	1.28 W
1.0		2.60 G	2.08 W
3.0		2.48 G	2.26 G
6.0		2.54 G	2.58 G
10.0			

W, White colour of cell suspension; G, green colour of cell suspension.

effective on a molar basis (Table 3). Bleaching by OM was also annulled by L-methionine and tiglic acid (an intermediate in isoleucine synthesis) and by crotonic acid; these also suppress multiplication at higher concentrations and are therefore not very effective as anti-bleaching agents. The D-isomers of the above amino acids did not prevent the bleaching by OM, e.g. D-threonine (Table 4). They did, however, partially prevent the inhibition of multiplication by OM (Table 5). Mixtures of

Table 5. *Effect of some chemicals on the multiplication and ability to prevent OM bleaching of E. gracilis*

Compound	Highest conc. studied (mg. % w/v or v/v)	Effect on multiplication	Ability to prevent bleaching by OM
Organic acids			
Acetic acid	100	—	—
3-Acetyl-butyric acid	10	—	—
cis-Aconitic acid	10	—	—
Adipic acid	10	—	—
α -Amino-adipic acid	10	—	—
α -Amino-butyric acid	10	—	+
DL- β -Amino-n-butyric acid	10	—	—
γ -Amino-butyric acid	10	—	—
δ -Amino-levulinic acid	10	—	—
δ -Amino-n-valeric acid	10	—	—
Butyric acid	10	—	—
2-Carboxy-butyric acid	10	—	—
Citric acid	10	—	—
Crotonic acid	10	+(10)	+
2,4-Diamino-butyric acid	10	—	—
DL-Diethylmalate	10	—	—
Dimethylmalonate	100	P (80)	—
Ethyl-2-hydroxy-butyric acid	10	—	—
Ethyl-lactate	10	—	—
Fumaric acid	10	—	—
3-Hydroxy-butyric acid	10	—	—
2-Hydroxy-isobutyric acid	10	—	—
α -Hydroxy- α -methyl-butyric acid	10	—	—
β -Hydroxy- β -methyl-glutaric acid	30	—	—
3-Hydroxypropionic acid	10	—	—
Isobutyric acid	10	—	—
L-Isocitric acid	10	—	—
Isovaleric acid	10	—	—
α -Keto-n-butyric acid	10	—	+
α -Ketoglutaric acid	10	—	—
β -Ketoglutaric acid	10	—	—
β -Ketoglutaric acid, dimethyl ester	10	—	—
α -Keto-isovaleric acid	100	—	—
Lactic acid	10	—	—
Malic acid	10	—	—
3-Methoxybutyric acid	100	P (100)	—
DL- α -Methyl-n-butyric acid	10	—	—
β -Methyl-glutaric acid	10	—	—
Methylmalonic acid	30	—	—
α -Methyl-n-valeric acid	10	—	—
β -Methyl-n-valeric acid	10	—	—
γ -Methyl-n-valeric acid	50	—	—
Oxalacetic acid	10	—	—
Propionic acid	100	—	—
Succinic acid	10	—	—
Tiglic acid	10	+(10)	+

Table 5 (cont.)

Compound	Highest conc. studied (mg. % w/v or v/v)	Effect on multiplication	Ability to prevent bleaching by OM
Amino acids			
D-Alloisoleucine	165	—	—
DL-Allothreonine	10	—	—
Asparagine	10	—	—
Aspartic acid	10	—	—
L-Cysteine	10	—	—
L-Glutamic acid	50	—	—
L-Glutamine	50	—	—
Glycine	100	P (100)	—
L-Homoserine	10	—	+
L-Isoleucine	110	P (66)	+
D-Isoleucine	10	—	—
L-Leucine	110	P (110)	—
D-Leucine	110	P (66)	—
DL-Methionine	10	P (10)	+
β -Methylaspartic acid	10	—	—
O-Methylthreonine	50	+(80)	—
DL-Norleucine	330	P (11)	—
DL-Norvaline	150	P (20)	—
DL-Serine	10	—	—
L-Threonine	100	P (30)	+
D-Threonine	100	P (30)	—
L-Valine	100	—	—
D-Valine	100	—	—
Alcohols and amines			
1-Amino-2-propanol	10	—	—
3-Amino-propanol	10	—	—
2-Amino-2-methyl-propanol	10	—	—
Cyclopentylamine	100	—	—
β -Mercaptoethanol	100	+(10)	—
2-Methyl-butylamine	10	—	—
Pyridoxamine	10	—	—
Threamine	10	—	—
Sterols and sterol precursors			
Cholesterol	10	—	—
Ergosterol	10	—	—
Farnesol	10	—	—
Geraniol	10	—	—
Isoprene	10	—	—
Lanosterol	10	—	—
Mevalonic acid lactone	10	—	—
β -Sitosterol	10	—	—
Squalene	10	—	—

* +(10) conc. in mg. which cause complete inhibition. P (10) conc. in mg.% which causes partial inhibition.

amino acids, water-soluble vitamins, NH_4Cl , succinic acid and glycine, alone or together did not prevent the bleaching by OM.

The supernatant fluids of cultures bleached by OM, streptomycin or heat contained large quantities of amino acids. Neither these nor the supernatant fluids from normal cultures grown in the light or in the dark and concentrated 10 times prevented bleaching by OM when used in the range 0.01–10.0 % (v/v). These same supernatant

fluids in the above concentration range as well as the amino acids and keto acids which prevented OM-bleaching failed to re-green OM-bleached organisms.

We examined the bleaching ability of several compounds that resembled the branched-chain amino acids as well as compounds reported to inhibit isoleucine synthesis, e.g. penicillamine inhibition of *Escherichia coli* is prevented by isoleucine, valine or leucine (Aposhian, Blair, Morris & Smithson, 1959). Mercaptoethanol was included because Brachet (1958) reported that it decreased the size of algal chloroplasts. None of these compounds prevented bleaching by OM nor did they act as bleaching agents themselves; some inhibited multiplication (Table 5).

Microscopic examination of permanently bleached Euglena gracilis

Organisms from cultures of *Euglena gracilis* permanently bleached by OM, streptomycin, or elevated incubation temperature (85.5°) were compared with normal green organisms by ordinary light, phase, dark field, fluorescent and polarized light microscopy. No differences in number or appearance of intracellular bodies in any of the bleached organisms were noted; the 'eye spot' was present in all. Pringsheim (1941) noted that incubation for extended periods in the dark was necessary for the disappearance of the eye spot in bleached organisms; our cultures were maintained in the light.

The sole difference noted between bleached organisms and normal green ones was the disappearance of the green colour and absence of the red fluorescence of chlorophyll. Some of the colourless bodies seen in the bleached organisms contained oriented internal structures when examined under polarized light.

Table 6. *Effect of OM on cell and pigment synthesis and the accumulation of amino acids*

Cells were grown for this experiment in 100 ml. volumes in 500 ml. screw-top flasks in light.

Compound (mg./100 ml. water)	Cell O.D.	Colour of culture	O.D. at		Approx. conc. of amino acids in supernate (mg./ml.)
			660 m μ	420 m μ	
0	2.22	Green	303	1000	0*
OM 1	1.20†	White	6	57	0.008
10	1.18†	White	5	69	0.02
OM 1 + L-isoleucine 10	2.46	Green	300	1000	0
10 + L-isoleucine 10	0.92†	White	7	91	0.166

* Cells grown in presence of amino acids incorporate them almost completely.

† The number of cells here represents a lag in multiplication rate rather than true inhibition and would reach full growth if allowed to incubate longer.

Supernatant fluids of bleached Euglena cultures

The supernatant fluids of experimental cultures in which OM had induced bleaching contained large quantities of amino acids. There were no amino acids in the supernatant fluids of cultures grown without OM or not bleached by OM. Supernatant fluids of cultures in which isoleucine had prevented the bleaching by OM and whose organisms remained green contained only small quantities of isoleucine (Table 6). When organisms permanently bleached by OM, streptomycin, or heat were

grown in a chemically defined medium in the absence of bleaching agents and the supernatant fluids concentrated and chromatographed, large quantities of amino acids also were found in the culture fluids; more amino acids appeared in the culture fluids of streptomycin and heat-bleached than in OM-bleached supernatant fluids.

That some of this accumulated amino-acid material reflects lack of isoleucine utilization is shown by the appearance of radioactivity in amino acids other than isoleucine that accumulate when pigment synthesis is blocked (Table 7).

Table 7. Effect of bleaching agents on the incorporation of ^{14}C -isoleucine

Concentration (mg./100 ml. water)	% total radioactivity supplied incorporated into	
	Cells	Methanol extract
^{14}C -L-Isoleucine 10.0	94	42*
^{14}C + O-Methylthreonine 3.0	90	15
^{14}C + O-Methylthreonine 6.0	89	25
^{14}C + O-Methylthreonine 10.0	83†	16
^{14}C + Streptomycin 150.0	80	20

* 22 % of total radioactivity in methanol extract appeared in pigments (carotenoids and chlorophyll).

† 11 % of total radioactivity in supernate appeared in amino acids other than isoleucine.

This amino-acid accumulation presumably results from the cessation of protein synthesis in the chloroplast or the cessation of pigment synthesis. To decide which, organisms were grown in uniformly labelled ^{14}C -L-isoleucine, with and without bleaching agents (Table 7). Usually about 45 % of the radioactivity incorporated from isoleucine appeared in the methanol extract containing photosynthetic pigments and other lipids. Bleaching concentrations of agents that bleach, i.e. OM or streptomycin, decreased the incorporation of ^{14}C -isoleucine into the organisms, 12 % for OM and 15 % for streptomycin. Incorporation of ^{14}C -isoleucine into the methanol extract, which we assume to include photosynthetic pigments as well as many lipids, was reduced 62 % by OM and 50 % by streptomycin.

Multiplication and hence protein synthesis continued in the presence of bleaching concentrations of OM or streptomycin and incorporation of ^{14}C -isoleucine into the cells was relatively slightly depressed while there was a 50 % or greater decrease in the incorporation of ^{14}C -isoleucine into the pigments and lipids of bleached organisms (Table 7).

Chromatography of the methanol extract of green cells revealed that about 22 % of the total radioactivity was in chlorophylls and carotenoids.

Where synthesis of chlorophyll is prevented, several workers (Lascelles, 1955; Larsen, 1952) have reported the appearance of porphyrins in the supernatant fluids; none were detected in the supernatant fluids of any of the bleached organisms studied here.

Escherichia coli partially inhibited by streptomycin accumulated quantities of nucleic acid derivatives (Roth, Amos & Davis, 1960), and it has been suggested that streptomycin impaired the permeability of the cell membrane in this organism (Anand & Davis, 1960). Examination of the supernatant fluids of cultures of normal dark, light-grown, and permanently bleached *Euglena* revealed 9.0 $\mu\text{g./ml.}$ 260 $\text{m}\mu$ -

absorbing material in light-grown, 11 $\mu\text{g./ml.}$ in dark-grown, 15 $\mu\text{g./ml.}$ in OM-bleached, 15 $\mu\text{g./ml.}$ in streptomycin-bleached, and 18 $\mu\text{g./ml.}$ in heat-bleached *Euglena*. There was no marked impairment of permeability to nucleic acids or their derivatives in bleached organisms.

DISCUSSION

O-Methylthreonine killing of leaves of bean, pepper, tobacco, wheat, and tomato was prevented by the simultaneous addition of 2–5 times more isoleucine than OM but not of threonine, serine, leucine, norleucine, or valine. Inhibition of growth and chlorophyll formation in *Euglena gracilis* by OM was prevented by isoleucine and partially prevented by leucine and norleucine (Gray & Hendlin, 1956). We have confirmed their results that inhibition of *E. gracilis* by OM was competitively prevented by isoleucine. Similar results were also obtained with *Ochromonas danica*.

Goodwin & Jamikorn (1954) reported a decrease in multiplication and carotenoid synthesis in dark-grown and streptomycin-containing (bleached) *Euglena gracilis* cultures as compared with light-grown cultures. Gross *et al.* (1955) found little chlorophyll in dark-grown and none in streptomycin- or pyribenzamine-bleached cells while the carotenoid content of dark-grown and streptomycin-bleached was about twice that of pyribenzamine-bleached cells; the colourless euglenid *Astasia longa* had no carotenoids or chlorophyll. Some workers have reported no chlorophyll in dark-adapted *Euglena*; others have reported a small quantity. This discrepancy probably depends on how long the organisms were cultured in the dark and also the amount of light exposure immediately before pigment extraction. Bleaching by OM, as by other agents, results in absence of chlorophyll and decrease in carotenoids.

OM permanently bleached only *Euglena gracilis*, of the several photosynthetic organisms examined by us; this bleaching was prevented by the simultaneous addition of any one of several amino, or keto acids (Tables 3, 5), most effectively by α -ketobutyric acid. We may infer that OM interferes with the utilization of α -ketobutyrate in the synthesis of the photosynthetic pigments and that isoleucine or threonine are on the route to these pigments since both amino acids prevented bleaching by OM (Table 3, Fig. 1). That amino acids, particularly isoleucine, are involved in pigment synthesis may be deduced also from the incorporation of ^{14}C -isoleucine into pigments (Table 7) and inhibition of this incorporation by bleaching.

Other workers noting interrelationships between amino acids and photosynthetic pigments include Huzisige, Terada, Nishimura & Uemura (1957) who found amino acids increasing the chlorophyll content of resting cells of *Euglena*, and Glover, Goodwin & Lijinsky (1951) who reported that leucine stimulated synthesis of carotenoids in *Phycomyces blakesleeanus*.

OM interferes with isoleucine (or more likely, its metabolic product, α -ketobutyrate) in other kinds of organisms. Rabinovitz, Olson & Greenberg (1955) found that OM blocked the incorporation of isoleucine into the proteins of Ehrlich ascites carcinoma, an inhibition prevented by isoleucine. *O*-Methyl-allothreonine did not affect isoleucine incorporation into carcinoma protein.

No other amino acid or organic acid studied prevented bleaching by OM; among those tested were several precursors of chlorophyll such as δ -aminolevulinic acid, and succinate alone or with glycine.

To see whether other inhibitors of isoleucine synthesis had bleaching activity for

Fig. 1. Suggested sites of inhibition of *Euglena gracilis* by O-methylthreonine (OM).

Euglena gracilis we examined penicillamine which inhibited isoleucine synthesis in *Escherichia coli* and certain substituted cyclopentanes, one of which, cyclopentane glycine, prevented isoleucine synthesis in *Escherichia coli* (Harding & Shive, 1954). None bleached.

Since *Euglena* does not reproduce sexually, we attempted to bleach two mating strains of *Chlamydomonas reinhardtii* to see whether mating a bleached with a green offered information on the primary site of bleaching. Unfortunately *Chlamydomonas* was not bleached by OM.

Table 8. Effect of increasing OM on the incidence of bleached individuals in exposed populations

Compound	Conc. (mg. % w/v)	Colour of culture	Appearance of colonies from sample of culture when plated†			
			No isoleucine		Isoleucine 10.0 mg.	
			White (%)	Green (%)	White (%)	Green (%)
O-Methylthreonine	0.6	White	67	33	—	—
	1.0	White	94	6	—	—
	3.0	White	100	0	—	—
	6.0	White	100	0	—	—
	10.0	White	100	0	67	33
	30.0	White	—	—	100	0
	50.0	White	—	—	100	0

* No. of viable *Euglena* cells/ml. varied from c. 0.5–3.0 × 10⁶.

† Samples taken from cultures shown in Table 2.

As the concentration of OM increased, the proportion of the *Euglena* population which showed bleaching increased to 100 % (Table 8). OM selected neither for OM-resistant nor bleached cells, since the growth-rates of bleached and green cultures were identical.

Unlike most externally induced and inherited changes, OM-bleaching of *Euglena* is a mass phenomenon (Table 8) not clearly dependent on nucleic acid changes as are bacterial transformation, transduction or mutation. Whether this is true of other bleaching agents remains to be seen.

All organisms studied irrespective of bleaching agent have a common feature: they accumulate large amounts of amino acids in the culture fluids, presumably as a consequence of blocked pigment synthesis. The markedly lessened incorporation of ^{14}C -isoleucine into pigment and lipids in OM- and streptomycin-bleached organisms with a much smaller decrease in incorporation into proteins suggests that the amino-acid accumulation resulted from inhibition of the synthesis of plastid pigments and lipids rather than plastid proteins. Anand & Davis (1960) found that normal *Escherichia coli* excreted amino acids; streptomycin treatment caused a fourfold increase in the excretion of amino acids and a greater decrease in intracellular amino acids than in intracellular nucleotides. That isoleucine figures in lipid synthesis may also be inferred from conversion by mouse adipose tissue of isoleucine to fatty acids almost as well as it so converted propionate, acetate, or methylmalonate (Feller & Feist, 1959).

Permanent loss of chlorophyll (apochlorosis or bleaching) as a result of specific environmental changes has been reported by several authors for a few strains of *Euglena gracilis*; it may also occur spontaneously in nature (see review by Pringsheim, 1941 and De Deken-Grenson, 1960). No other photosynthetic organism has been rendered apochlorotic and still able to reproduce. We must note one exception: *Euglena mesnili* kept in the dark lose their chloroplasts permanently (Lwoff & Dusi, 1935).

Provasoli, Hutner & Pintner (1951) claimed the destruction of chloroplasts as well as the inhibition of chlorophyll synthesis. They observed the bleached organisms by fluorescence microscopy which permitted following the fate of the chlorophyll but told nothing of the fate of the plastids. Lwoff & Schaeffer (1949) confirmed bleaching of *Euglena gracilis* by streptomycin but claimed the presence of modified plastids. Vavra (1957) claimed that streptomycin interfered with chloroplast multiplication in *E. gracilis* while De Deken-Grenson & Messen (1958) using light microscopy, said that streptomycin lowered the chloroplast growth-rate so that apochlorotic strains developed as a result of cell multiplication outrunning chloroplast multiplication; this has been withdrawn (De Deken-Grenson & Godts, 1960). Wolken (1956) examined by electron microscopy streptomycin- and heat-treated but no permanently-bleached *Euglena*; he claimed that both streptomycin and heat destroyed the chloroplast structure but his electron micrographs did not exclude a lamina-free body remaining as a vestige of the chloroplast nor do they depict the fate of the chloroplast in permanently bleached strains. The fate of the colourless chloroplast, if such exists, in apochlorotic cells remains unknown.

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