



UNCLASSIFIED

HW-30252

Experimental Biology  
and Medicine

This document consists  
of 12 pages. Copy No. 13  
of 97 copies. Series A

THE EFFECT OF TRITIUM OXIDE ON SOME  
SYNTHETIC PROCESSES OF CHLORELLA PYRENOIDOSA

By

J. W. Porter and H. J. Knauss

Biology Section  
Radiological Sciences Department

December 29, 1953

HANFORD ATOMIC PRODUCTS OPERATION  
RICHLAND, WASHINGTON

Operated for the Atomic Energy Commission by the  
General Electric Company under Contract #W-31-109-Eng-52

Route To	Read By	Date	Route To	Read By	Date
<i>Biology Library</i>					
<i>Elizabeth Greff</i>	<i>3269</i>				

UNCLASSIFIED

1144568

UNCLASSIFIED

2

HW-30252

Experimental Biology  
and Medicine

INTERNAL DISTRIBUTION

Copy Number

1	H. M. Parker - M. L. Mickelson
2	A. B. Greninger - O. H. Greager
3	J. E. Maider - W. K. MacCready
4	W. D. Norwood - P. A. Fuqua
5	D. W. Pearce - Z. E. Carey
6	W. A. McAdams
7	J. M. Smith - J. W. Healy
8	D. E. Warner - R. F. Foster
9	R. C. Thompson
10	J. W. Porter - H. J. Knauss
11	L. K. Bustad - F. P. Hungate
12	H. A. Kornberg
13	Biology Library - Elizabeth Groff
14	W. A. Watts
15	300 File
16	700 File
17	Yellow File
18 - 20	Extra Copies

UNCLASSIFIED

1144569

EXTERNAL DISTRIBUTION

Copy Number

21	Aeromedical Laboratory (WADC)
22 - 23	Argonne National Laboratory
24	Armed Forces Special Weapons Project, Washington
25	Army Chemical Center
26 - 28	Atomic Energy Commission, Washington
29 - 30	Brookhaven National Laboratory
31	Bureau of Medicine and Surgery
32	Carbide and Carbon Chemicals Company (K-25 Plant)
33 - 38	Carbide and Carbon Chemicals Company (ORNL)
39 - 40	Carbide and Carbon Chemicals Company (Y-12 Plant)
41	Chicago Patent Group
42	Chief of Naval Research
43	Columbia University (Failla)
44 - 46	duPont Company, Augusta
47 - 48	General Electric Company (ANPP)
49	Goodyear Atomic Corporation
50	Hanford Operations Office
51	Iowa State College
52	Kirtland Air Force Base
53	Knolls Atomic Power Laboratory
54 - 55	Los Alamos Scientific Laboratory
56	Massachusetts Institute of Technology (Evans)
57 - 59	Mound Laboratory
60	Naval Medical Research Institute
61 - 63	New York Operations Office
64	Patent Branch, Washington
65	Public Health Service
66	RAND Corporation
67	The Surgeon General
68	USAF Radiation Laboratory
69	U. S. Naval Radiological Defense Laboratory
70	UCLA Medical Research Laboratory (Warren)
71 - 74	University of California Radiation Laboratory, Berkeley
75 - 76	University of Rochester
77	University of Tennessee (Comar)
78	University of Washington
79 - 82	Western Reserve University (Friedell)
83 - 97	Technical Information Service, Oak Ridge

ABSTRACT

Chlorella were grown for 3 days in the presence of 20 mc/ml of tritium oxide. Then the cells were grown for 30 hours in non-radioactive inorganic nutrient solution in the presence of tracer quantities of C<sup>14</sup> bicarbonate.

The cells were fractionated into several gross fractions and aliquots were plated and counted for radioactivity. C<sup>14</sup> contents in each fraction were compared with those in identical fractions of unirradiated control cells.

Irradiated cells synthesized 2 - 8 times as much triglycerides as control cells. The irradiated cells also incorporated more C<sup>14</sup> into the nucleic acid fraction, and less into the chlorophyll a fraction, than did unirradiated control cells.

Implications of the results are discussed.

THE EFFECT OF TRITIUM OXIDE ON SOME  
SYNTHETIC PROCESSES OF CHLORELLA PYRENOIDOSA

INTRODUCTION

Previous papers<sup>(1, 2)</sup> reported on the effect of several concentrations of tritium oxide on growth, division, average cell size and viability of Chlorella. These papers also reported on the action of tritium oxide on the genetic system of Chlorella and on the ineffectiveness of glucose, bacto peptone and yeast extract in increasing the survival of irradiated (tritium oxide exposed) cells.

The present paper reports data on the comparative ability of irradiated (tritium oxide) and unirradiated Chlorella to synthesize some cellular components. In these studies cells were grown for 24 - 30 hours in the presence of C<sup>14</sup> bicarbonate and then fractionated. Counts were then made of the C<sup>14</sup> in each fraction. Differences in C<sup>14</sup> incorporation, in per cent of the total C<sup>14</sup> incorporated into the cells, were considered indicative of changes in cellular metabolism effected by the tritium oxide.

METHODS

Chlorella were grown for 3 days in inorganic nutrient solution that contained 20 mc/ml of tritium oxide<sup>(1, 2)</sup>. The cells were centrifuged from the nutrient solution, suspended in fresh inorganic nutrient solution and stored in the refrigerator until subcultured. Control cells were treated identically, except that they were grown in the absence of tritium.

Irradiated and unirradiated Chlorella were subcultured for 24 - 30 hours in glass stoppered tubes by the following procedure. Ten milliliters of Myer's nutrient solution, pH 5.5, were added to each of 4 tubes. One

milliliter of  $\text{NaHCO}_3$  solution (5 mg/ml) was added to each tube and the tubes were inoculated with equal masses of algae. (Final optical densities of the solutions were in the range 0.100 - 0.300). One-tenth milliliter (approximately  $10 \mu\text{c}$ )<sup>1</sup> of  $\text{NaHC}^{14}\text{O}_3$  was added to each tube and the tubes were stoppered and placed upon an electrically-driven rocker platform. The final volume of solution was 11.5 ml and the final pH was 6.3 - 6.5. Daylight fluorescent light incident on the tubes during the growth period was approximately 250 f. c.

The suspensions of Chlorella were removed from each tube at the end of the growth period, made to volume, and then light absorption readings were made. Equal quantities of carrier cells (non-radioactive) were added to the tubes and the cells were centrifuged for 2 minutes at 8500 rpm in a Servall centrifuge. The cells were resuspended, centrifuged again and then made to a volume of 5 ml with cold distilled water. Aliquots were plated, and counts of radioactivity were made.

The cells were again centrifuged and then slurried with a minimum of water (approximately 1.0 ml) into 40 ml of boiling methanol. Extraction of pigments was complete after about 15 minutes of refluxing. Methanol insolubles were centrifuged, washed with hot methanol, centrifuged, washed with ether, centrifuged and then suspended in 10 ml of water. An aliquot of this fraction was plated and counts of radioactivity were made. The remainder of the fraction was stored in the refrigerator and later separated into protein, nucleic acid, starch and cellulose fractions.

The methanol soluble fraction was evaporated to 10 ml with a stream of nitrogen and an aliquot was plated and counted. The remainder was diluted to 40% with water and then extracted twice with 10 ml portions of ethyl ether. The ether extract was washed 3 times with water and the

---

<sup>1</sup> $\text{NaHC}^{14}\text{O}_3$  was obtained from Tracerlab, Boston, Massachusetts.

washes were combined with the ether insoluble fraction. The ether soluble and insoluble fractions were made to volume and aliquots were plated and counted. Further separations of the methanol soluble, ether insoluble fractions were not made.

The ether soluble fraction was evaporated to dryness with a stream of nitrogen and then taken up in 70:30 petroleum ether-ethyl ether. The solution of lipids was then chromatographed on powdered sucrose (1.7 x 25. cms). Two fractions, designated  $\beta$ -carotene and luteol fractions, were taken through the column and collected separately. The substances remaining on the column were divided into 3 fractions and designated as chlorophyll a, chlorophyll b and above chlorophyll b. Separations of pigments were excellent, and divisions were made just below luteol, chlorophyll a, chlorophyll b and a yellow band above chlorophyll b. The three fractions remaining adsorbed on the sucrose column were transferred quantitatively to clean chromatographic tubes and each fraction was eluted with ethyl ether. Each of the five fractions was evaporated to dryness with nitrogen and then made to a volume of 2.0 ml. Aliquots of each fraction were plated and counted.

The  $\beta$ -carotene-triglyceride fraction was refluxed for 30 minutes with 10 ml of 20% KOH in methanol. The solution was cooled, diluted with an equal volume of water, and then extracted twice with petroleum ether. The petroleum ether solution (non-saponifiables) was washed once with water and then made to volume. An aliquot was plated and counted.

The water wash was added to the alkaline methanol solution, and the solution was acidified with HCl. The solution was extracted twice with ethyl ether. The ether solution (fatty acids) was washed once, made to volume, and an aliquot was plated and counted.

The water wash was added to the acidified alcohol-water solution. The solution was evaporated to dryness, and the residue was extracted with butyl alcohol. An aliquot of the butanol solution was plated and counted.

The fatty acids of the ether extract were separated into saturated and unsaturated fatty acids by the method of Hilditch<sup>(3)</sup>. Palmitic, stearic, caprylic, oleic and linolenic acids were added as carriers. Aliquots of the saturated and unsaturated fatty acid fractions were plated and counted. Further separations of the lipids were not made.

The methanol insoluble fraction was centrifuged again and then extracted by shaking with 1 M NaCl for 30 minutes at room temperature. The NaCl insoluble fraction was centrifuged and then suspended in 10 ml of water. Aliquots of each fraction (NaCl solubles and NaCl insolubles) were plated and counted.

The protein fraction was solubilized by digestion with crystalline trypsin. Ten milliliters of phosphate buffer, of pH 7.0, were added to the NaCl insoluble fraction. Two milliliters of a solution of crystalline trypsin were then added and the tubes were incubated for 3 hours at 35 - 40°C. The non-digestible residue was centrifuged from the trypsin digest and each fraction was made to volume. An aliquot of each fraction was plated and counted.

The insolubles remaining after trypsin digestion were heated with 1% HCl for 30 minutes. The acid solubles (mainly starch) and insolubles (mainly cellulose) were separated by centrifuging and then each fraction was made to volume. Aliquots of each fraction were plated and counted.

All counts of C<sup>14</sup> in samples were made with a thin mica window beta counter. (The tritium of the various fractions did not interfere with C<sup>14</sup> counts, for the beta particle given off when tritium disintegrates is of too weak an energy to pass the mica window). Samples of irradiated and

unirradiated cells and fractions thereof were counted on the same counter and on the same shelf. Quantities plated were of weights which gave negligible self absorption, with one or two exceptions. Appropriate self-absorption correction factors were used in these cases. All results were calculated to d/m/sample, with appropriate correction factors, so that  $C^{14}$  recovery in each fractionation step could be determined.

## RESULTS

The quantities of  $C^{14}$  incorporated into cellular components of irradiated and unirradiated Chlorella during a 30-hour growth period are reported in Table I. Values given are those obtained in a single experiment; but similar results were obtained in three other experiments.

It will be noted (Table I) that irradiated Chlorella incorporated more  $C^{14}$  into the nucleic acid and fatty acid (triglyceride) fractions and less  $C^{14}$  into the chlorophyll a fraction than did unirradiated Chlorella. The most striking difference is found in the quantities of  $C^{14}$  incorporated into the triglyceride fractions of the two algae. It will be noted also (Table I) that irradiated Chlorella incorporated more  $C^{14}$  into both saturated and unsaturated fatty acid fractions than did unirradiated Chlorella.

## DISCUSSION

Previously we found<sup>(2)</sup> that after 3 days of growth in 20 mc/ml of tritium oxide (approximately 6000 rep/day, calculated) approximately 70 per cent of Chlorella cells had been changed such that death ensued on subsequent growth in non-radioactive solution. Death was not immediate, but rather gradual over at least a 3 day growth period; and death of the cells was not due to selective action of the tritium oxide on the processes of photosynthesis or the synthesis of the substances present in bacto peptone and yeast extract.

TABLE I  
THE INCORPORATION OF C<sup>14</sup> INTO IRRADIATED AND UNIRRADIATED  
CHLORELLA DURING A 30-HOUR GROWTH PERIOD

	<u>Control-1</u>	<u>Control-2</u>	<u>Irradiated-1</u>	<u>Irradiated-2</u>
Original optical density	.206	.207	.207	.202
Final optical density	.960	1.10	.840	.840
Cell mass doublings	2.17	2.33	2.01	2.04
<u>Cell Fraction</u>				
			<u>d/m x 10<sup>-6</sup></u>	
Whole cells	17.0	16.0	17.0	17.0
Methanol extract	4.9	--	4.6	4.4
Methanol insolubles	11.0	11.0	10.0	10.0
Ether extract	2.9	2.1	2.5	2.3
Methanol soluble, ether insoluble	1.4	1.6	1.7	1.4
$\beta$ -carotene and triglycerides	.15	.13	.29	.29
Luteol	.14	.16	.16	.12
Chlorophyll a	.38	.34	.29	.30
Chlorophyll b	.11	.09	.09	.11
Above chlorophyll b	.50	.38	.39	.44
Non-saponifiables				
( $\beta$ -carotene)	.084	.064	.066	.069
Fatty acids	.081	.039	.17	.18
Butanol solubles	.008	.010	.013	.011
Unsaturated fatty acids	.0037	.0072	.019	.019
Saturated fatty acids	.0009	.0009	.006	.007
NaCl extract (nucleic acids)	.28	.28	.34	.33
NaCl insolubles	10.0	9.6	10.0	9.9
Trypsin digest (protein)	2.3	1.9	2.0	2.0
Residue	4.4	4.6	4.3	4.3
Acid soluble (starch)	1.9	2.0	1.9	1.9
Acid insoluble (cellulose)	1.6	2.0	1.6	1.6

The results reported in this paper give additional information on the changes that do or do not take place in Chlorella exposed to ionizing radiations. Surprisingly, many processes are not affected, at least at the gross level of analysis we are reporting, by concentrations of tritium oxide which inhibit growth and cell division. Thus our results indicate that protein, starch and carotenoid synthesis are unaffected, in relation to other synthetic processes in the cell, by growth inhibiting levels of tritium oxide. It is possible, however, and perhaps even probable, that more detailed analysis of these fractions (amino acid separations, for example) will show some effect of ionizing radiations on these synthetic processes.

Tritium oxide, at growth inhibiting concentrations, does affect the biosynthesis of triglycerides, and probably also chlorophyll a and nucleic acids, in Chlorella. Cells grown 3 days in the presence of 20 mc/ml of tritium oxide synthesized 2 - 8 times as much triglycerides (in each of 4 experiments) as control cells during a 30-hour growth period in the presence of tracer  $C^{14}O_2$ . (Values are normalized to represent equal total quantities of  $C^{14}$  incorporated into experimental and control cells). The irradiated cells also incorporated more  $C^{14}$  into the nucleic acid fraction, and less into the chlorophyll a fraction, than did unirradiated control cells.

The increase in triglyceride content of irradiated Chlorella furnishes a lead as to one of the possible actions of the tritium oxide. For an increase in triglyceride content to occur, an increase in acetate (acetyl CoA) must have occurred in the irradiated Chlorella, or the enzymes for fat synthesis in the irradiated Chlorella must have become more efficient in relation to other enzymes in the cell. An increase in acetate could be brought about by either of three conditions. (1) A block, or a partial block, could have occurred in a reaction or reactions, that competes for acetate, or (2) more acetate could have been formed in the irradiated cell by a block in a reaction or reactions, that competes for the precursor, or precursors, of acetate,

or (3) more acetate could have been formed through the addition of hydrogen, normally oxidized by terminal oxidases to water, to a two carbon acceptor and precursor of acetate. Selective damage to terminal oxidases would be required for a shunting of hydrogen to the two carbon acceptor.

It seems improbable that the efficiency of fat synthesizing enzymes, relative to other enzymes within the cell, would be increased through the action of tritium oxide. These enzymes, in other species, are known to be associated with the mitochondria, and they are soluble in water under appropriate conditions<sup>(4)</sup>. Presumably they would be subjected to as much radiation (indirect action produced by the ionization of water) as would other water soluble enzymes, and unless they are extremely resistant to ionizing radiations, they would be damaged as extensively as other water soluble enzymes.

#### ACKNOWLEDGMENTS

The authors are indebted to the Biology Control Unit for the preparation and calibration of the  $\text{NaHC}^{14}\text{O}_3$  solution, and for the counting of  $\text{C}^{14}$  samples plated from the various cellular fractions.

#### BIBLIOGRAPHY

1. Porter, J. W. and H. J. Knauss, "Inhibition of growth of Chlorella pyrenoidosa by beta-emitting radioisotopes," Document HW-29666 (1953) (UNCLASSIFIED).
2. Porter, J. W. and M. S. Watson, "Gross effects of growth inhibiting levels of tritium oxide on Chlorella pyrenoidosa," Document HW-30056 (1953) (UNCLASSIFIED).
3. Hilditch, T. P., "The separation of saturated from unsaturated acids," Chem. Products 3 (9), 78, 81 (1940).
4. Brady, R. O. and S. Gurin, "Biosynthesis of fatty acids by cell-free or water soluble enzyme systems," J. Biol. Chem. 199, 421-431 (1952).