A LIGHT ACTIVATION PHENOMENON IN THE ENZYMATIC AND NONENZYMATIC REDUCTION OF TETRAZOLIUM SALTS*

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Communicated by S. A. Waksman, July 9, 1953

Photoreduction of 2,3,5-triphenyltetrazolium chloride (TTC) has been reported by a number of investigators.[1-5] According to Gierlach and Krebs,[4] photoreduction of TTC occurs at wave-lengths shorter than 3650 Å. Anaerobic conditions have been reported to facilitate reduction of TTC in the dark.[3] We have found that the photoreduction of TTC solutions occurs more rapidly under alkaline conditions.

In studies on the mechanism of reduction of TTC by washed cell suspensions of yeasts,[6] it was noted that certain cultures did not reduce the tetrazolium in the dark. It was found that the ability to reduce TTC in the dark was related to the age of the cultures. Cells from cultures of yeasts incubated 12 to 14 hrs. in liquid media with continuous agitation reduced TTC equally well in the light or in the dark, whereas, cells from cultures incubated for 72 hrs. had lost most of their ability to reduce TTC in the dark (table 1). It was also noted that various inhibitors of TTC reduction were more effective in the dark than in the light. In particular, the inhibitory effect of monoiodoacetate and of zinc on 72-hr. cultures was pronounced in the dark and negligible in the light.

Although ultra-violet radiations can, under certain conditions, cause reduction of TTC, it can be seen from the table that the microbial reduction described in the present studies is an enzymatic reaction. From studies with inhibitors, it appears that hydrogen donating systems within the cell are affected by the photoactivation. Attempts to activate the TTC solutions and the yeast suspensions by irradiation, before they were mixed, were unsuccessful.

Brodie and Gots[7] have reported that tetrazolium is reduced by a flavoprotein enzyme. It therefore appears as if the light activation is directed at this type of enzyme system. It also appears that this system has been altered by aging the yeast cultures. Evidence for the involvement of a flavin-type substance in the light-activated reaction has been obtained.

The properties of a non-enzymatic light-activated system for the reduction of tetrazolium salts have been studied (table 2). The system consisted of the tetrazolium salt (6×10^{-3} M), riboflavin or riboflavin phosphate (10^{-4} M), cysteine·HCl (2.5×10^{-3} M) and pH adjusted within the range 7.3–8.5. The photoreductions were carried out using 10 ml. volumes in uncovered 94-mm. petri dishes. A 375-watt Sylvania super

flood light was used for the illumination at a distance of 30–50 cm., with a $CuSO_4$ solution ($M/100$) of 7 mm. thickness acting as a heat filter.

Both TTC and blue tetrazolium[8] (BT) were studied in the system and it was found that under optimum conditions, TTC was more easily reduced than BT. Earlier work[6] had indicated that increased tetrazolreductase activity could be obtained by adding various metal-chelating agents to resting cell suspensions of yeasts. It was therefore of interest to find that the disodium salt of ethylenediaminetetraacetic acid (Na_2-

TABLE 1

PHOTOACTIVATION OF ENZYMATIC TETRAZOLIUM REDUCTION BY WASHED RESTING CELLS ON *Candida albicans*

	TETRAZOLIUM REDUCTION AFTER 2-HOUR INCUBATION					
	12-HOUR CULTURES				72-HOUR CULTURES	
	PARENT STRAIN		FILAMENTOUS MUTANT		FILAMENTOUS MUTANT	
SYSTEM WITH TTC	LIGHT	DARK	LIGHT	DARK	LIGHT	DARK
Boiled cells	None	None	None	None	None	None
Boiled cells + NaCN (0.005M)	None	None	None	None	None	None
Washed cells	Trace	Trace	+++	+++	+++	Trace
Washed cells + Zn^{++} (0.005M)	Trace	Trace	Trace	Trace	++	None
Washed cells + Cu^{++} (0.005M)	None	None	None	None	None	None
Washed cells + NaCN (0.005M)	++++	++++	++++	++++	++++	++
Washed cells + Na_2EDTA	++++	+++	...	...	++++	++
Washed cells + iodoacetate	...	...	...	...	+++	Trace

The yeasts were grown in 100 ml. volumes of a 1 per cent peptone and 0.5 per cent glucose medium at 28°C. with continuous agitation. The cells were harvested by centrifugation and washed twice with distilled water. Suspensions with the appropriate concentration of substrate or inhibitor were made up to 5 ml. in Pyrex test tubes. One set of tubes was exposed to indirect, diffused sunlight and the other set was placed in a dark cabinet. After two hours the tubes were examined for reduction of TTC (original concentration = 0.02 per cent) and scored on a comparative bases, i.e., trace = faint reduction, + = visible reduction, etc.

EDTA) could replace cysteine in the photoactivated system for the reduction of BT. In the case of TTC it was found that the addition of Na_2EDTA alone, at a pH on the alkaline side, permitted the photochemical reduction to occur. Riboflavin was not necessary for the photoreduction of TTC but greatly stimulated the reduction.

These findings led to the obvious conclusion that a metal ion(s) was associated with the tetrazolium salts and stabilizes them against photoreduction. Spectrographic analyses of samples of BT and TTC revealed the presence of large amounts of metals in BT, whereas TTC was found

to be relatively free of metals (table 3). One of the reaction components for the synthesis of BT is obtained commercially as a stabilized powder with 5 per cent zinc chloride and 20 per cent aluminum sulfate.[8]

TABLE 2

RIBOFLAVIN SENSITIZED NON-ENZYMATIC PHOTOCHEMICAL REDUCTION OF TETRAZOLIUM DYES

SYSTEM	REDUCTION AFTER % MINUTES ILLUMINATION
BT	None
BT + glucose + glycine	None
BT + cysteine (pH 8.8)	None
BT + R + cysteine (pH 8.0)	++++
BT + R + cysteine (pH 6.9)	None
BT + RP (pH 9.0)	None
BT + RP (pH 7.4)	None
BT + RP + Na_2EDTA (pH 7.3)	++++
BT + RP + cysteine (pH 7.8)	+

R = riboflavin.
RP = riboflavin phosphate.

TABLE 3

SPECTROGRAPHIC ANALYSIS OF METAL CONTAMINATION OF TETRAZOLIUM DYES, RIBOFLAVIN, AND DISODIUM VERSENE

METAL	BT	TTC	RIBOFLAVIN	RIBOFLAVIN PHOSPHATE	Na_2EDTA	BLANK
Al	Strong	Weak	Weak	Strong	Medium	...
Ca	Strong	...	Weak	Strong	...	...
Cr	Medium	...	...	...	...	...
Cu	Weak	Trace	Weak	Weak	Trace	...
Fe	Weak	Weak	Very weak	Weak	Trace	Trace
Mg	Strong	Strong	Strong	Very strong	Strong	Trace
Mn	Weak	...	...	...	Trace	...
Na	Strong	...	...	Very strong	Very strong	...
Zn	Very strong	...	...	...	...	...

BT—from Dajac Laboratories
TTC—an Eastman product
Na_2EDTA—Bersworth Chemical Company
Riboflavin and riboflavin phosphate—Hoffman-La Roche

The data presented account for the role of a metal chelating agent in the photochemical reduction, and for the greater sensitivity of TTC to photoreduction.[9]

Riboflavin appears to function in the photochemical reaction as the activated substance which reduces the tetrazolium compound. Although it has an unusual complexing affinity for iron,[10] it does not function merely

as another metal chelating agent. A previously irradiated solution of riboflavin phosphate to which a small amount of Na_2EDTA had been added would reduce TTC in the dark, indicating a relatively long life for the "active riboflavin."

It is interesting to compare our findings with those of Galston[11] who reported a riboflavin-sensitized *photo-oxidation* of indoleacetic acid and related compounds under acidic conditions in which light activated riboflavin was presumed to act as a hydrogen carrier between the substrate and its oxidized product. Our results seem to be analogous, but under the conditions we used the reaction is shifted to the reductive rather than the oxidative side, possibly by the shift in hydrogen-ion concentration. Riboflavin in the present studies seems to be functioning in a manner which has been proposed for chlorophyll:[12]

$$(Me^{2+}C)\cdot H_2O \xrightarrow{h\nu} (Me^{2+}C)^*\cdot H_2O$$
$$(Me^{2+}C)^*\cdot H_2O \rightarrow (Me^{+}C)\cdot H_2O^{+}$$
$$(Me^{+}C) + Ox \rightarrow (Me^{2+}C) + Ox^{-}$$

where $Me^{2+}C$ = metal-chlorophyll complex.

Future experiments should indicate the nature of the "active riboflavin" molecule in this type of photoreduction.

These reactions, interesting in themselves, have several implications. They emphasize the necessity of taking the possible occurrence of photochemical reactions into account when studying enzymatic reduction of tetrazolium compounds, or when studying flavin-catalyzed reductions in general. We have noted that many of our cytochemical studies with TTC have been complicated by photochemical reduction of the tetrazol in wet mounts under illumination from a microscope lamp. Secondly, the effect of Na_2EDTA focuses attention on the importance of metal chelation in maintaining oxidation-reduction balances in intracellular systems.

Acknowledgment.—The authors wish to thank Mr. Niel Shimp and Prof. Arthur Prince of the Soils Department of the Agricultural Experiment Station for making the spectrographic analyses recorded in table 3.

* Journal Series Paper—New Jersey Agricultural Experiment Station, Rutgers University, the State University of New Jersey, Department of Microbiology. This investigation was supported in part by a grant from the National Institute of Health, Public Health Service.

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*THE INTERACTION OF AUXIN AND LIGHT IN THE GROWTH RESPONSES OF PLANTS**

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Communicated July 17, 1953

Introduction.—Many of the growth and developmental responses of higher plants are influenced by light or by the interplay of light and dark. The control of the morphogenetic aspects of plant behavior appears to be exerted through mechanisms which are distinct from and in some cases at least independent of the photosynthetic machinery. Light affects directly, for example, the growth of hypocotyls and mesocotyls, coleoptile elongation, leaf expansion, the germination of certain seeds, and the pathway of bud differentiation. The photochemical control of floral initiation, the phenomenon of photoperiodism, constitutes a particularly well described instance of the morphogenetic role of light. Despite our recognition of light as a dominant factor in plant development, we have however little more than descriptive knowledge of the processes involved.

A second factor which we recognize today as of general significance in the control of plant morphogenesis is the chemical control exerted by hormones of the auxin group. Auxins control many morphogenetic responses, and it so happens in addition, that many of these auxin-controlled responses are further affected by light. That this is so is particularly evident in the much-studied cases of coleoptile growth and of floral induction. In both of these instances light and exogenous auxin supplied in appropriate dosages act in the same direction, promoting the growth of coleoptiles[1] and the flowering of long day plants[2] or on the other hand suppressing the flowering of short day plants.[3] Added light and added auxin can, to a considerable extent, replace one another in the control of these two quite different plant responses.

We may logically inquire whether there may exist some relation between the effects of light and those of auxin. We might ask whether light in some