OXIDATION OF 2,3',6-TRICHLOROINDOPHENOL BY THE LIPOXIDASE SYSTEM\textsuperscript{1,2}

GEORGE FRITZ AND HARRY BEEVERS

DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, WEST LAFAYETTE, INDIANA

In 1949 Smith and Stotz (11) found that homogenates from several plant sources were able to catalyze directly the oxidation of the leuco (reduced) form of the dye 2,3',6-trichloroindophenol (2,6-dichlorobenzeneindolo-3'-chlorophenol) to the blue form, a reaction involving the removal of hydrogen, and the enzyme system was called "dye oxidase." The nature of the enzyme was not completely elucidated, but it was concluded (11) that "dye oxidase" was distinct from peroxidase, catechol oxidase and cytochrome oxidase, although each of these enzymes in the presence of its substrate was shown to be able to oxidize the leuco dye (10, 11, 12). Balls, Axelrod and Kies (1) had previously shown that a closely related indophenol dye could be oxidized through the lipoxidase system; in fact it has been known for some time that a variety of dyes and other substances may be oxidized by a coupled reaction if they are present when lipoxidase is catalyzing the oxidation of its substrates—linoleic, linolenic and arachidonic acids and their esters—by atmospheric oxygen (see (3) for a review). This suggested the possibility to us that such a system might contribute to "dye oxidase" activity and an investigation was undertaken with the results described below.

MATERIALS AND METHODS

The procedure of Smith and Stotz (11) was followed to obtain the dye solutions; the dye was reduced with gaseous hydrogen in the presence of palladium, and was used immediately after reduction.

Linoleic acid was emulsified with gum ghatti (2). The gum ghatti solution, in the quantities used, did not affect the rate of autooxidation of the reduced dye, nor did the blue dye fade in its presence. Soybean lipoxidase prepared according to Cosby and Sumner (1a), was purchased from the Worthington Biochemical Corporation, Freehold, New Jersey; the solid was dissolved in water (5 to 10 mg/ml) as enzyme solution. Catalase was obtained from Bios Laboratories, New York. Reactions were performed at room temperature in cuvettes of one cm light path, and a Beckman Model B spectrophotometer was used to follow changes in the optical density of the dye at 645 nm. (For ultra-violet measurements, silica cuvettes were used with a Beckman Model DU spectrophotometer.)

RESULTS

The leuco dye was found to be oxidized by soybean lipoxidase plus linoleic acid, but neither was effective alone. Figure 1 shows that when lipoxidase was added to the reduced dye containing 0.3 ml of linoleic acid emulsion, a rapid production of the blue form of the dye occurred (curve 6); the rate of the reaction was proportionately lower with smaller amounts of linoleic acid (curves 4 and 5). However, after the first two to three minutes of the reaction, the dye began to fade (curves 4, 5, and 6), and this loss of color was more rapid with increasing amounts of linoleic acid. This fading (which was distinct from the reduction of the blue dye, since the dye was permanently bleached and the color could not be regenerated with the use of mild oxidizing agents), suggested that a secondary reaction was superimposed upon the initial oxidation of the leuco dye. By employing the blue (oxidized) form of the dye as the starting material, it was found that neither linoleic

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acids nor soybean lipoxidase alone had any effect upon the color, but that the addition of both of these together to the blue dye caused a rapid and permanent fading. By the use of Thunberg technique, it was found that oxygen was required for both the enzymatic oxidation of the leuco dye and for the enzymatic fading of the blue form.

Although the fading was a complicating feature of the reaction, it was clear that lipoxidase and its substrate could act as "dye oxidase." Therefore it was of interest to find if the lipoxidase system existed in the tissue extracts used by Smith and Stotz. For this purpose potato was selected, since this exhibited especially high "dye oxidase" activity (11). Potato homogenates which were shown to possess thermolabile "dye oxidase" activity, catalyzed the oxygen absorption of linoleic acid, and the optical density at 234 m$\mu$ of methanol extracts of the reaction mixture (prepared according to the procedure of Holman (2)) increased during the reaction and paralleled the oxygen consumption; this increase in optical density has been shown to be associated with the enzymatic oxidation of lipoxidase substrates (2, 13). The presence of substrates of lipoxidase in the potato was suggested in an experiment in which the acidified homogenate was extracted with ether; the residue remaining after evaporation of the ether was emulsified with phosphate buffer and was found to absorb oxygen in the presence of soybean lipoxidase. This demonstration of the lipoxidase system in potato confirms the results of Kirsanova (4), who found "carotene oxidase" in homogenates from this tissue. ("Carotene oxidase" was later renamed lipoxidase, since the oxidation of carotene was shown to be due to a coupled reaction brought about by lipoxidase and its substrates—for a review see Holman and Bergström (3).)

The possibility that enzyme systems other than lipoxidase can act as "dye oxidase" in any particular extract is not excluded by the above experiments. Smith, Robinson and Stotz (10) have shown that peroxidase in the presence of its substrate oxidized the leuco dye and demonstrated the existence of this enzyme in potato homogenates. In considering the identity of "dye oxidase," Smith and Stotz (11) suggested that peroxidase was not involved; however, Dr. F. G. Smith (personal communication) indicated that "dye oxidase" activity could be inhibited by cyanide and azide, and might be attributed to peroxidase, if a source of hydrogen peroxide were present. In the present investigation, it was found that when leuco dye oxidation occurred in the presence of soybean lipoxidase and linoleic acid, as shown in figure 1, addition of catalase or 0.01 M cyanide or azide had virtually no effect. This eliminated the possibility that a very feeble peroxidase activity, shown by colorimetric methods to be present in the soybean lipoxidase preparation, was involved in the oxidation. On the other hand, when leuco dye oxidation was catalyzed by potato homogenate ("dye oxidase" action), application of 0.01 M cyanide or azide inhibited the development of the blue color by 60 to 80%, and catalase reduced the activity by 40%. Thus although the lipoxidase system is present in potato, it appears that peroxidase plays a major role in "dye oxidase" activity of homogenates from this tissue. In addition, the operation of a distinct "dye oxidase" is not ruled out. The elucidation of the relative importance of the different oxidizing systems in extracts from other tissues would require further work.

The fading of the blue (oxidized) dye, observed with the soybean lipoxidase system was also observed when potato homogenates were used as the source of lipoxidase. The addition of both potato homogenate and linoleic acid together to the blue dye caused a rapid and permanent fading, which was unaffected by the addition of cyanide, azide or catalase.

**DISCUSSION**

Although both the lipoxidase and peroxidase systems may act as "dye oxidase," only lipoxidase in the presence of added substrate was found to cause the fading of the blue (oxidized) dye. It is therefore apparent that if lipoxidase and its substrate are present in any given tissue extract, they would interfere in an enzyme assay in which the dye was used. This factor would seem to weigh against the use of the dye in colorimetric enzyme assays.

The dye has no metabolic significance per se, but it is interesting to note that the reduction of the di-
chloro derivative (the trichloro derivative was used here) by succinate and succinate dehydrogenase has been developed as an assay method for the dehydrogenase (7). The present investigation of the oxidation of the reduced dye through the lipoxidase system indicates that the dye might serve in a model enzyme system as a carrier in the oxidation of succinate by atmospheric oxygen through the lipoxidase system. The fact that both cysteine and reduced glutathione may be oxidized to the corresponding disulfides through the lipoxidase system from pea seeds (5) focuses attention upon the possible importance of lipoxidase as a terminal oxidase in plant respiration. Thus, with glutathione reductase (6), reduced triphosphopyridine nucleotide might be oxidized by oxygen through the lipoxidase system; a similar sequence of reactions may be visualized for the oxidation of reduced diphosphopyridine nucleotide by atmospheric oxygen through cysteine reductase (8), and the lipoxidase system.

**Summary**

The lipoxidase enzyme system from plant sources is able to catalyze the oxidation of the leuco (reduced) form of the dye 2,3',6-trichloroindophenol to the blue form and may thus contribute to the activity called "dye oxidase" by Smith and Stotz. It appears that in potato extracts peroxidase systems also contribute to the "dye oxidase." A property peculiar to the lipoxidase system is that it causes the permanent oxidative fading of the blue form of the dye.

**Literature Cited**


9. Smith, F. G. Personal communication.


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**INFLUENCE OF pH ON 2,4-DICHLOROPHENOXACETIC AND ACETIC ACID ACTIVITY IN CHLORELLA**

L. C. Erickson, R. T. Wedding and B. L. Brannaman

Department of Plant Biochemistry, University of California

Citrus Experiment Station, Riverside, California

The principal effect of external pH on the activity of auxins and other weak organic acids is to regulate the degree of their dissociation (6, 20, 22). The importance of this effect lies in the fact that only the undissociated molecules of such acids appear to readily penetrate the plasma membranes of plant cells and to reach an equilibrium concentration in which the concentration within the cell approximates that of the external bathing medium (6). It would therefore appear that the effects of any compound of this type on the functioning of a living cell should be directly related to the concentration of undissociated molecules in the bathing medium. However, Smith et al (21) demonstrated that the concentration of undissociated molecules producing a standard effect (LD 50 for spore germination of Colletotrichum circinans) was not constant over a wide pH range.

The question of the relative importance of the undissociated acid molecules and the ions of these compounds in the physiological responses of plants has recently been the subject of investigations by Simon and Blackman (20), Simon and Beevers (18, 19), and Blackman and Robertson-Cuninghame (3). They performed experiments and recalculated data of other workers to determine the concentration of undissociated molecules in the external medium that would give a standard response at several pH levels.

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