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 cystine 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.

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INFLUENCE OF pH ON 2,4-DICHLOROPHENOXYACETIC AND ACETIC ACID ACTIVITY IN CHLORELLA [1,2]

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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 condition 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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Results indicated that below the pK of the acid being tested the concentration of undissociated acid required for a standard response did not vary greatly, but at progressively higher pH levels it appeared that the concentration of undissociated molecules required in the bathing medium was progressively less. These data were interpreted as indicating that the whole response could not be attributed to the undissociated molecules, alone, but that part of the response must be due to the ions.

In a previous publication on the inhibition of photosynthesis in *Chlorella pyrenoidosa* by 2,4-dichlorophenoxyacetic acid (2,4-D) (25) data were presented in which almost the complete range of inhibition of photosynthesis was achieved with a single concentration of 2,4-D by varying the pH of the solution or by selecting a single pH and varying the concentration of 2,4-D. However, insufficient data were available to plot the concentration of undissociated molecules required for a standard response over a wide pH range. Such data were subsequently obtained and are presented here. Because of the limited solubility of 2,4-D it was not possible to extend the study over the desired pH range. Additional studies were made using acetic acid. This acid was suitable because of its high solubility in water and its inhibitory effects on photosynthesis.

Materials and Methods

Chlorella pyrenoidosa Chick (Emerson's strain, kindly provided by Dr. David Appleman) was used as the test plant. Cells were produced in a glass tube 1.25 meters long and 37 mm in diameter, using a nutrient solution containing 0.025 M KNO_3, 0.02 M $MgSO_4$, 0.018 M $KHPO_4$, 0.001 M $CaCl_2$, and the following microelements: B, Mn, Zn, Cu, Mo, and Fe as tartrate. Air scrubbed through an activated carbon filter and water was bubbled through the solution to provide CO_2 and agitate the solution, keeping the cells in suspension. Illumination was provided by two 30-watt fluorescent lamps, one mounted on each side of the culture tube at a distance of about 25 mm. Aluminum reflectors were used to increase the light intensity. Cold air was passed between the fluorescent tubes and the culture tube to keep the solution between 25° and 30° C. The solution was inoculated with cells grown in pure culture on agar. After 3 days of growth in the nutrient solution under these conditions the density of cells was sufficient for determinations of photosynthesis and respiration in an illuminated Warburg respirometer which provided a light intensity of about 1200 fc at the level of the flasks (25).

Cell suspensions were centrifuged to separate the cells from the nutrient solution. A 10-ml aliquot of cell suspension containing approximately 20 mg (dry weight) of Chlorella cells was centrifuged for photosynthesis and 30 or 50 ml aliquots providing approximately five times as many cells were used for respiration measurements. The cells were resuspended in 3 ml of potassium citrate buffer (0.04 M) containing the necessary amounts of 2,4-D or acetic acid partially neutralized to the desired pH levels with KOH. These suspensions were transferred to the Warburg flasks.

Citric acid was previously tried for inhibition of photosynthesis and found to be without effect at the concentration used in the buffer. Eny (9, 10) showed that citric was one of the least effective of the organic acids tried for promoting growth or altering respiration of Chlorella.

Diethanolamine-carbonate buffer (14) was used in the two side arms and center well of the Warburg flasks to maintain a constant supply of CO_2 at about 1 % for photosynthesis. This buffer was composed of 60 ml diethanolamine, 45 ml 6 *N* HCl, 60 ml water, and 30 gm $KHCO_3$.

The bath was maintained at 25° C. After 30 minutes of equilibration the oxygen evolution during the ensuing hour was determined. This was followed by 30 minutes equilibration in the dark before measuring oxygen uptake. The oxygen evolution during the light period and the oxygen uptake during an equal dark period were combined to give a measure of true photosynthesis. Where respiration studies were made separately, the oxygen uptake during a 2-hour dark period was determined. Larger amounts of Chlorella were used to adjust the total oxygen uptake in the controls to an amount between 75 and 800 μl per

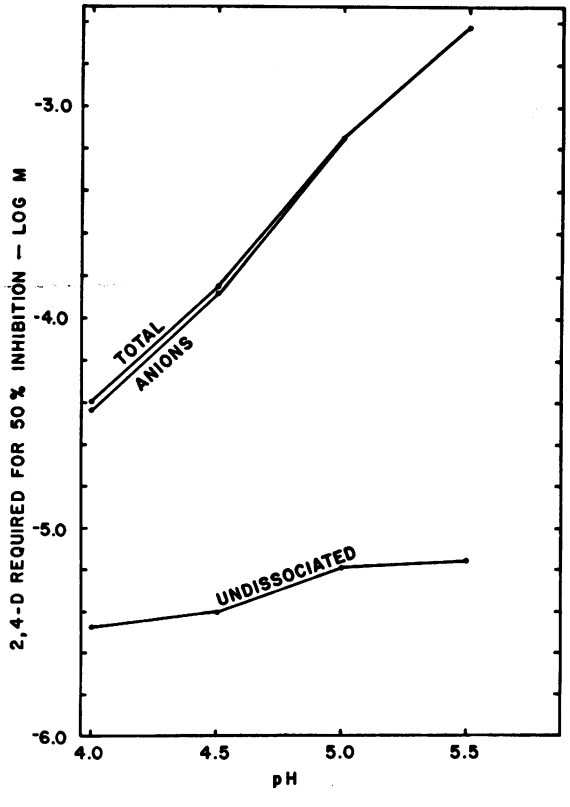

Fig. 1. Amount of 2,4-D required in bathing medium at various pH levels for 50 % inhibition of photosynthesis in *Chlorella pyrenoidosa*.

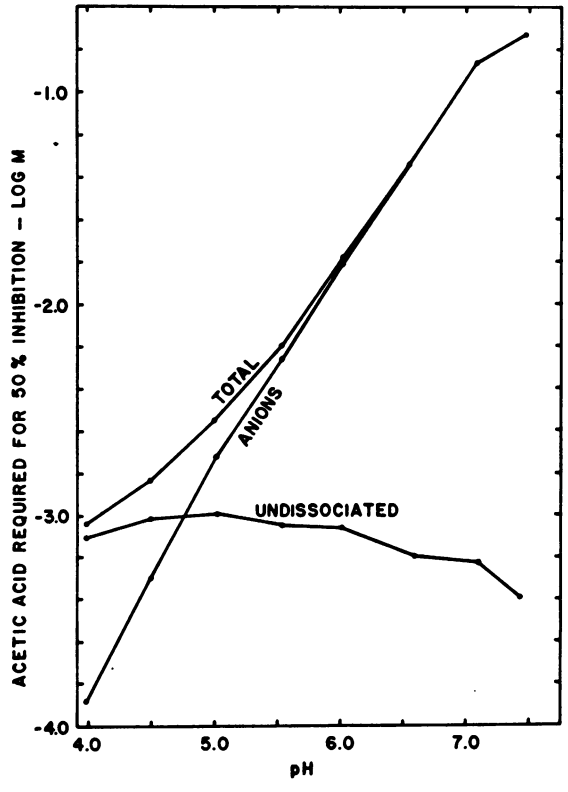

FIG. 2. Amount of acetic acid required in bathing medium at various pH levels for 50 % inhibition of photosynthesis in *Chlorella pyrenoidosa*.

hour. In such runs 10 % KOH was used in the side arms and center well to remove CO_2. In all cases the surface area of the solution in the center well was increased by insertion of a piece of pleated filter paper.

Calculations of undissociated acid were made by employing pH values determined after the runs, and pK 2.96 for 2,4-D (25), and pK 4.76 for acetic acid (8). The 2,4-D (provided by the Dow Chemical Co.) used was first recrystallized 3 times from hot water.

RESULTS

As in previous trials (25), the percentage inhibition of photosynthesis by 2,4-D over the middle range of inhibition fits straight lines when plotted against molar concentrations of undissociated 2,4-D better than it fits log molar concentrations. By using the regression equation calculated from the concentration series at each pH, the amount of undissociated 2,4-D required for 50 % inhibition of photosynthesis was determined for each pH studied. The latter ranged from pH 4.0 to pH 5.5 with points at half pH intervals. The total, the dissociated, and the undissociated 2,4-D required for 50 % inhibition at each pH are shown in figure 1. It is interesting to note that the amount of undissociated 2,4-D required for 50 % inhibition of photosynthesis in the Chlorella increased slightly over the range from pH 4.0 to pH 5.5.

The amount of undissociated acetic acid required for 50 % inhibition of photosynthesis in Chlorella was determined over the range from pH 3.97 to pH 7.42 (fig 2). Up to pH 5 there was a slight increase in the amount of undissociated acid required as the pH increased. Above pH 5.0 the amount of undissociated acid required for 50 % inhibition decreased slightly with increasing pH.

Respiration determinations on the Chlorella treated with 2,4-D were made only in conjunction with photosynthesis. Although the oxygen uptake under these conditions was relatively small, it showed that a stimulation of respiration was produced in the lower concentrations of 2,4-D at each pH.

A separate and more extensive measurement of respiratory stimulation was made using acetic acid. At each pH, concentrations of acid were chosen to permit calculation of the amount of acid required to produce 20 % stimulation of respiration.

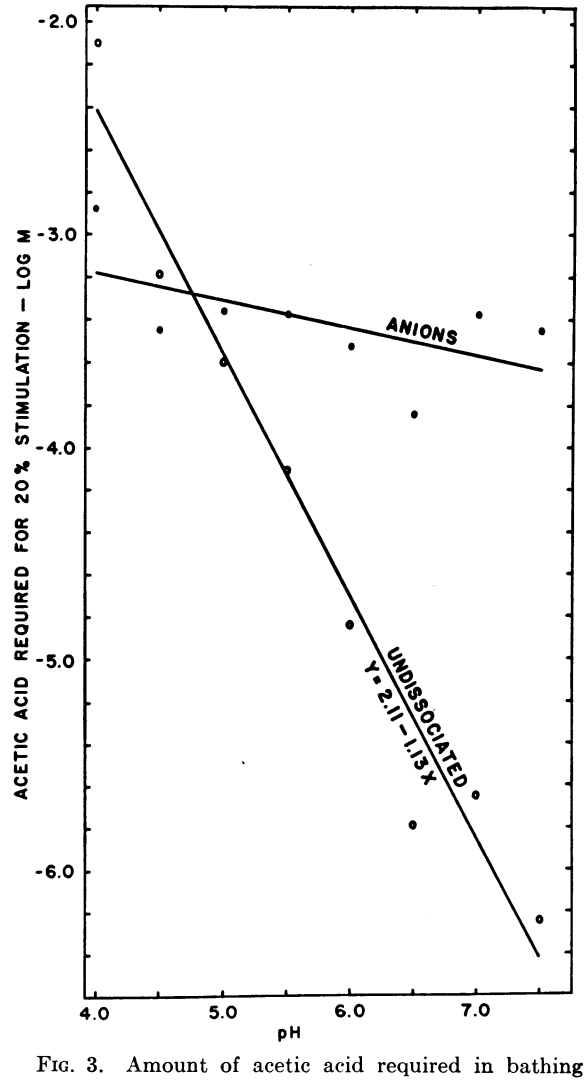

FIG. 3. Amount of acetic acid required in bathing medium at various pH levels for 20 % stimulation of respiration in *Chlorella pyrenoidosa*. For regression equation of anions, Y = – 2.66 – 0.128 X.

Instead of a nearly constant amount of undissociated acetic acid producing the 20 % stimulation at each pH, it was found that with each unit increase in pH the amount of undissociated acetic required was approximately one-tenth as much (fig 3). In other words, the standard 20 % stimulation of respiration was produced by a nearly constant anion concentration of acetate, regardless of pH.

Discussion

More than 200 times as much undissociated acetic acid as undissociated 2,4-D in the bathing medium was required to inhibit by 50 % the photosynthesis of *Chlorella pyrenoidosa*. Simon and Blackman (20) have suggested that a comparison of toxicities of different weak organic acids be made at pH values below the pK of the compounds concerned, so that there would not be a variation of toxicity with change in pH. In the present comparison pH 4 is below the pK of acetic acid (pK 4.76) but well above that of 2,4-D (pK 2.96). However, extrapolation of the lines in figure 1 to the pK of 2,4-D would not appreciably alter the relationship based on the undissociated molecules at pH 4.

The present data do not permit an explanation of the nature of the inhibition of photosynthesis, but they contribute to the information on the subject. Myers (13) found that at pH 4.5, 0.004 M acetic acid, or 0.0012 M butyric acid, or 0.0005 M caproic acid completely blocked photosynthesis in *Chlorella pyrenoidosa*. Since pH 4.5 is below the pK of each acid (n-butyric, pK 4.83; n-caproic, pK 4.85) (15), these values may be considered to be near the maximum toxicity (20). In the present study it was determined that the total acid estimated to produce complete inhibition of photosynthesis at pH 4.49 would be 3.02×10^{-3} M which agrees with the value given by Myers, particularly since he did not determine the least amount of acid that would produce complete inhibition.

Assimilation of acetate in plants has been abundantly demonstrated (1, 7, 9, 10, 11, 12, 13, 23, 24). None of these studies on the respiration of acetate related the activity to concentration of substrate. Myers (13) mentioned that concentration of acetate had little effect on oxygen uptake, but since no concentration limits were specified, it must be assumed that he was making determinations near or above the concentration producing maximum stimulation of oxygen uptake. In the present study it was found that, with increasing concentrations of acetate, the oxygen uptake increased rapidly to a maximum and then fell off rather slowly as the concentrations exceeded the optimum.

The problem of the effect of pH on the biological activity of weak acids has received considerable attention without a satisfactory solution. The earlier explanation, that the concentration of undissociated molecules in the external medium governed the activity, has become less acceptable in view of critical data and interpretations presented by Simon and Blackman (20) and by Simon and Beevers (19). Brian and Rideal (5) directed attention to the possible changes in proteins and protein complexes as being more pertinent than the concentration of undissociated acid. Although the proteins are undoubtedly of great importance, it must not be overlooked that the same range in pH has widely different effects on the change in toxicity of substances, depending, in most instances, on the pK of the substances (19).

In the present investigation extreme differences were noted between states of ionization and physiological responses over a wide pH range. Although in the inhibition of photosynthesis by acetic acid a reasonably close relationship existed between 50 % inhibition and the concentration of undissociated molecules in the external solution, the stimulation of respiration, on the other hand, was closely related to the external acetate ion over the whole pH range tested.

Similar extremes are to be found in the presentation of Simon and Beevers (19). One extreme, exemplified by the effect of hydrazoic acid on the inhibition of respiration of yeast (19, fig 3), shows less than a 10-fold change in concentration of undissociated acid required to produce a standard response over a 1,000-fold hydrogen-ion concentration change, whereas the amount of anion required between a 100- and 1,000-fold change for the same hydrogen-ion range. This relationship approaches that of a nearly constant amount of undissociated acid for a given response. The other extreme, exemplified by the effect of β-indoleacetic acid in the pea test and adapted from Bonner's work (4) by Simon and Beevers (19, fig 6), shows between 100- and 1,000-fold change in concentration of undissociated acid required to produce a standard response over a 1,000-fold hydrogen-ion concentration change, whereas the anion change is less than 10-fold. This relationship approaches that of a nearly constant amount of anion for a given response.

The inhibition of yeast respiration by hydrogen fluoride (19, fig 2) appears to be a combination of the relationships described above, in that from pH 3.2, the pK of hydrogen fluoride, to pH 5 the relationship is one of a constant amount of undissociated acid for the response, and above pH 5 the relationship is one of a nearly constant amount of anion.

These data suggest that the modification of functions in plants seems to be related to the external concentration either of undissociated molecules or of anions. Deviations from these relationships might be the result of two or more effects masking or interfering with each other.

How these extremes may be reconciled in the same cells is difficult to understand. One possible explanation is that ions have free entry into the cytoplasm (2). Thus, effects related to the concentration of undissociated molecules would occur in the lipid phase, whereas effects related to the concentration of anions would occur in the aqueous phase.

Another point of view would subscribe to the more usual contention that the cytoplasm is permeable only

to the molecules and that the responses related closely to the concentration of undissociated molecules in the external solution would depend either on undissociated molecules or on anions within the cells. This interpretation leaves the relationship of responses closely related to the external anion concentration dependent on reactions occurring principally at the surface of the protoplast (16).

Although correlations between response and external concentrations of undissociated molecules or of anions are possible, it does not necessarily follow that these relationships reflect activity of the particles within the cells. For example, although the concentration of undissociated acid at various external pH levels remained fairly constant for 50 % inhibition of photosynthesis in response to either 2,4-D or acetic acid at various pH levels, this is not considered evidence that undissociated molecules participated in a reaction within the cells. It could very well have been only the anions within the cytoplasm which brought about the effect. The anions would vary in much the same order as the undissociated molecules, barring changes in the pH of the cytoplasm. The undissociated molecules could have served merely as a form which readily penetrated the cytoplasm.

Assuming that the anion within the cell is the active form or starting point in a series of reactions leading to the inhibition of photosynthesis, another comparison can be made between 2,4-D and acetic acid. Using the concentrations of undissociated 2,4-D and acetic acids determined to be required for 50 % inhibition of photosynthesis at pH 4.0, the anion concentrations in the cytoplasm, which was assumed to be at pH 6.8 (17), were calculated to be 2.35×10^{-2} M and 8.63×10^{-2} M, respectively. This 3- to 4-fold difference is negligible in comparison with the more than 200-fold difference represented by the undissociated molecules. Since there is no reason for believing that the inhibition of photosynthesis in Chlorella is preferentially affected by 2,4-D either as a specific substance or as a plant growth regulator, the nearly complete elimination of difference in activity between the two acids, based on a comparison of anions, is not perplexing.

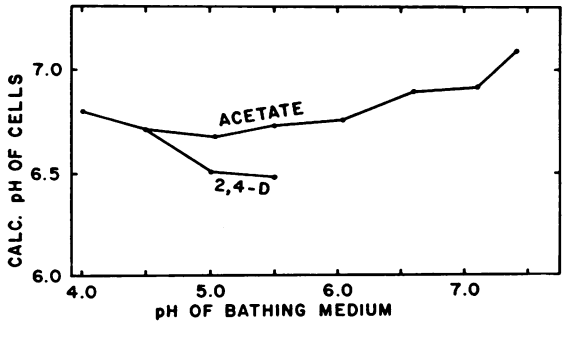

FIG. 4. Variation in pH of cytoplasm of *Chlorella pyrenoidosa* required to maintain a constant anion concentration in the cytoplasm at 50 % inhibition of photosynthesis.

In interpreting the slight deviation of the concentration of undissociated molecules in the external medium from a constant amount for a standard response at several pH levels, there is, in addition to the possibility of a masking or interference previously mentioned, the influence of a change in pH of the cytoplasm. Figure 4 shows the magnitude of change in pH of the cytoplasm required to produce a constant anion concentration in the cytoplasm at the various external pH levels and concentrations of undissociated acid. It may be seen that the maximum change required in tests with acetic acid (0.6 pH unit) would be somewhat higher than that required in tests with 2,4-D (0.3 pH unit). Whether or not such changes in pH in the cytoplasm would be likely is not known.

SUMMARY

The concentration of undissociated 2,4-dichlorophenoxyacetic acid in the bathing medium which produced 50 % inhibition of photosynthesis in *Chlorella pyrenoidosa* increased slightly over the range from pH 4.0 to pH 5.5. Similar determinations for acetic acid showed an increasing amount of undissociated acid required between pH 3.97 and pH 5.0 and a decreasing amount between pH 5.0 and pH 7.42.

The 2,4-D was more than 200 times as effective as acetic acid in inhibiting photosynthesis based on the concentration of undissociated molecules in the bathing medium at pH 4. A comparison of the postulated concentrations of anions in the cytoplasm indicated that 2,4-D was only 3 to 4 times as active as acetic acid in inhibiting photosynthesis.

The amount of acetate anion increasing oxygen uptake by 20 % decreased slightly over the range from pH 4.0 to pH 7.55.

Inhibition of photosynthesis by both 2,4-D and acetic acid appeared to be closely related to the concentration of undissociated molecules in the bathing medium, whereas the stimulation of oxygen uptake by acetic acid appeared to be related to the concentration of the acetate anion in the bathing medium.

Evidence was not available to indicate whether or not the undissociated molecules participated in a reaction within the cells. The role of the undissociated molecule may have been only that of a penetrant.

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CHANGES IN THE ACTIVITIES OF SEVERAL ENZYMES DURING GERMINATION AND SEEDLING DEVELOPMENT IN CORN (ZEA MAYS L.) [1,2,3]

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The literature contains numerous references (e.g., Bonner (1), Van Fleet (18), Weier and Stocking (19)) to the occurrence of catalase, cytochrome oxidase, peroxidase, phosphatase, and polyphenolase activities in higher plants, and an occasional report has appeared dealing with certain of these activities in corn. Thus there have been reports of cytochrome oxidase (4, 9), peroxidase (2), catalase (2, 3, 13), and phosphatase (8) activities in preparations of various corn tissues. In most cases, however, only one stage of development has been considered. The experiments reported here were undertaken when, in connection with other studies in progress at this laboratory, it became necessary to obtain estimates of the five types of activity in preparations of corn embryos, etiolated shoots, and green seedlings. The aim of these experiments was threefold: to adapt published assay methods for use with preparations of corn tissue, to estimate the activities of preparations from corn at the various stages of development listed above, and to study the fractionation of activities effected by high-speed centrifugation.

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