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CONCENTRATION-DEPENDENT EFFECTS OF SALICYLALDOXIME ON CHLOROPLAST REACTIONS

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SUMMARY

Salicylaldoxime has been found to have a variety of concentration-dependent effects on chloroplast activities. At low concentrations (< 10 mM), salicylaldoxime reversibly inhibits all reactions which involve Photosystem II. Since the DCMU-insensitive silicomolybdate Hill reaction is also inhibited, one site of inhibition is definitely located before the DCMU-sensitive site, possibly before the photoact. The inhibition kinetics and the response of chloroplast fluorescence may indicate another site in the DCMU-sensitive region. At almost exactly the same concentrations (< 10 mM), salicylaldoxime uncouples phosphorylation reversibly, whether it is supported by Photosystem II or by Photosystem I. At higher concentrations (approx. 20 mM) salicylaldoxime inhibits Photosystem II irreversibly, uncouples irreversibly, and begins to cause changes in chloroplast light scattering which could be manifestations of membrane damage. At very high concentrations (approx. 45 mM) salicylaldoxime irreversibly inhibits Photosystem I activity in the region of plastocyanin. This is indicated by the ability of salicylaldoxime to inhibit the photooxidation of cytochrome *f* but not the photooxidation of *P*-700.

INTRODUCTION

The inhibitory effects of salicylaldoxime on photosynthesis were first studied by Green et al. [1]. Since that time salicylaldoxime has been widely used as an inhibitor in various algal and chloroplast preparations.

The effects of salicylaldoxime on chloroplast reactions were first studied by Trebst and his associates [2, 3] and the inhibitions of oxygen evolution and coupled ATP formation in the Hill reaction were reported. Because of the reputation of salicylaldoxime as a copper chelator, and because salicylaldoxime did not inhibit electron transfer between reduced DCIP and NADP, it was suggested that salicylaldoxime inhibited electron transfer between the water splitting reaction and cyto-

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DAD, diaminodurene; DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenylindophenol; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

chrome *f*, probably at the copper containing protein, plastocyanin [2]. However, later studies by Katoh and San Pietro [4] showed that plastocyanin is a highly unlikely site of inhibition since long incubations of the isolated protein with salicylaldoxime do not damage the protein. They also presented evidence that salicyladoxime inhibition occurs near Photosystem II. More recently, Katoh [5] and Kimimura and Katoh [6] concluded from inhibition and fluorescence studies that the salicylaldoxime inhibition site is located on the water-splitting side of Photosystem II.

Spectroscopic studies with algae, however, seem to place a site of inhibition between the two photosystems. In a *Chlamydomonas* mutant, Hildreth [7] found that salicylaldoxime inhibits cytochrome *b* (b_{664}) oxidation and placed a salicylaldoxime sensitive site between cytochrome *b* and cytochrome *f*. Fork and Urbach [8] studied the effects of salicylaldoxime on light-induced spectral changes in *Ulva* and concluded that the inhibition occurred at plastocyanin and before cytochrome *f*.

Urbach and Simonis [9] found that salicylaldoxime inhibited phosphate uptake in *Ankistrodesmus*. This phosphate uptake is often correlated with DCMU-insensitive photophosphorylation. The inhibition of this "phosphorylation", implies a site of salicylaldoxime action after that proposed by Kimimura and Katoh [5, 6]. In algal chloroplasts, salicylaldoxime inhibits Photosystem I cyclic photophosphorylation, stimulates Photosystem I hydrogen production and stimulates methyl red reduction according to Stuart and Gaffron [10–12]. Recent studies by Rosen et al. [13] with chloroplasts, indicate that some Photosystem I reactions can be inhibited with salicylaldoxime, but other Photosystem I reactions are insensitive to the inhibitor.

In this study, we attempt to resolve the various and seemingly contradictory effects of salicylaldoxime which have been reported. We conclude from experiments that salicylaldoxime has multiple concentration-dependent effects on chloroplasts including: (a) low concentration inhibition at Photosystem II, (b) low concentration uncoupling of photophosphorylation, (c) high concentration inhibition of Photosystem I in the region of plastocyanin, and (d) high concentration membrane effects.

MATERIALS AND METHODS

Chloroplasts were prepared by grinding fresh market spinach (*Spinacia oleracea*) in a 0.3 M NaCl solution containing 30 mM Tricine/NaOH (pH 7.7) and 3 mM $MgCl_2$ in a Waring Blendor at 4 °C. The resulting suspension was filtered through cheesecloth and centrifuged at 2500 × g for 4 min. The chloroplasts were gently resuspended with a small paint brush into a 0.2 M sucrose solution containing 20 mM Tricine/NaOH (pH 7.5), 3 mM $MgCl_2$, and 10 mM KCl. The resulting suspension was centrifuged at 1000 × g for 20 s to remove cell debris, followed by a centrifugation at 1600 × g to pellet the chloroplasts. This pellet was resuspended as before in the same medium, except that bovine serum albumin (1 mg/ml) was added to the final suspension (0.5 mg chlorophyll/ml). Hydroxylamine chloroplasts were prepared as has been previously described [16].

Salicylaldoxime was purchased from ICN Pharmaceutical Inc. and was used without further purification. Stock solutions of 100 mM salicylaldoxime were prepared by dissolving the reagent in 60 °C water and maintaining the stock solution at room temperature. Silicomolybdic acid was a generous gift of Dr. Rita Barr of Purdue University. Aqueous solutions of silicomolybdate were centrifuged to remove insoluble

material. The silicomolybdate reaction is known to be DCMU insensitive [14, 15].

Most of electron transport reactions were measured using a Clark-type oxygen electrode, either as O_2 production or as methylviologen-mediated O_2 uptake. Electron transport from H_2O to DBMIB or to 2,5-dimethylquinone was measured spectrophotometrically using ferricyanide as the terminal acceptor [17]. Electron transport from NH_2OH to DCIP was also measured as absorbance changes (560 nm). The actinic light used was a rate-saturating red light (> 640 nm; approximately 600 kergs · s^{-1} · cm^{-2}). The reaction cuvettes were thermostated at 20 °C. Photophosphorylation was assayed as the incorporation of ^{32}P-labeled orthophosphate into ATP by the method detailed elsewhere [18]. The compositions of the reaction mixtures are given in Fig. 1 and Table I. Unless detailed elsewhere, incubations with salicylaldoxime were for 2 to 3 min, a period which was adequate to produce maximal inhibition.

Oxidation-reduction of cytochrome *f* and *P*-700 was observed with an Aminco-Chance Dual Wavelength Spectrophotometer. The actinic light was a monochromatic red light (640 nm, half-band width 10 nm; light intensity at the cell approximately 5 kergs · s^{-1} · cm^{-2}). The oxidation-reduction of *P*-700 was observed at 703 nm (reference, 730 nm). The oxidation-reduction of cytochrome *f* was followed by observing the absorbance change of the *α*-band at 554 nm (reference, 540 nm or 565 nm).

Chloroplast fluorescence was measured using a recording fluorimeter built on the basis of a Beckman DU spectrophotometer, whose cuvette compartment was modified so as to allow actinic illumination (at right angles to the photomultiplier) of a

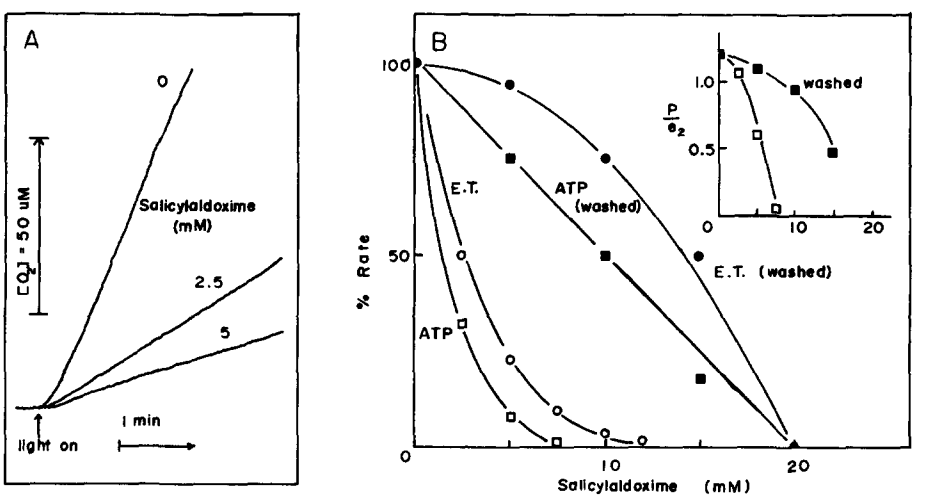

Fig. 1. Partially reversible effects of salicylaldoxime on the ferricyanide Hill reaction and on associated phosphorylation. (A), oxygen production traces in the presence of 0, 2.5 and 5 mM salicylaldoxime. (B), the effects of increasing salicylaldoxime concentration and their partial reversal by chloroplast washing. The control rates of electron transport and ATP synthesis were 625 μequiv./h per mg chlorophyll and 370 μmol ATP/h per mg chlorophyll. In wash experiments, chloroplasts were first exposed to the indicated concentrations of salicylaldoxime in the reaction mixture (minus ferricyanide, ADP, P_i) for 5 min at 20 °C, washed twice at 4 °C by centrifugation with the wash medium used for chloroplast preparation (see Methods), and then assayed for their activity. The reaction mixture (2 ml) contained 0.1 M sucrose, 30 mM Tricine/NaOH buffer (pH 8.0), 10 mM KCl, 3 mM $MgCl_2$, 0.75 mM ADP, 5 mM [^{32}P]Na_2HPO_4, 0.4 mM potassium ferricyanide and chloroplasts equivalent to 50 mg chlorophyll. For other conditions, see Methods.

thermostated cuvette (20 °C). A weak blue actinic light was obtained by filtering a beam from a tungsten lamp through a layer of saturated $CuSO_4$ solution and an interference filter (470 nm) plus a Corning color filter (No. 556). The intensity of actinic light was approximately 0.5 kerg $\cdot s^{-1} \cdot cm^{-2}$ at the cuvette. A red cut-off filter ($T > 660$ nm) protected the photomultiplier from the actinic illumination.

RESULTS

Differential inhibition of Photosystem I and Photosystem II by salicylaldoxime

Salicylaldoxime inhibits a variety of Photosystem I and Photosystem II partial reactions as well as the sequential electron transfer through both photosystems. Table I compares the sensitivity of several reaction groups toward salicylaldoxime. Reactions which involve electron transfer through both photosystems are most sensitive to salicylaldoxime. These reactions include electron transfer from water to methylviologen, water to ferricyanide, and catechol to methylviologen [19]. They are 90 % inhibited by salicylaldoxime at about 8 mM. As a group, with the exception of silicomolybdate reduction [14, 15], the reactions supported or largely supported by Photosystem II alone, such as electron transfer from hydroxylamine to DCIP, water to DBMIB, or water to dimethylquinone [17] are slightly less sensitive to salicylaldoxime.

The Photosystem I-mediated partial reactions with electron flow from DAD to methylviologen and TMPD to methylviologen, were the most insensitive of the reactions tested, with 90 % inhibition requiring a salicylaldoxime concentration of about 45 mM. In the following, some of the details of these inhibition experiments are described together with results from phosphorylation experiments.

The effects of salicylaldoxime on reactions involving Photosystem II

Fig. 1A shows that inhibition of Photosystem II (measured as electron transport from water to ferricyanide) by salicylaldoxime is essentially linear and non-progressive over the 3-min inhibition period. Fig. 1B shows the rapid diminution of the rate of ferricyanide reduction and associated phosphorylation with the increase in salicylaldoxime concentration. Complete inhibition is achieved with approximately 10 mM salicylaldoxime. The inhibition by this range of salicylaldoxime concentrations (< 10 mM) is largely reversed when the inhibited chloroplasts are washed free of salicylaldoxime (Fig. 1, "washed" curves). The inhibition becomes completely irreversible, however, when the salicylaldoxime concentration is increased to 20 mM. As clearly indicated by the decreasing P/e_2 ratio (Fig. 1B, inset) the chloroplasts are completely uncoupled in the presence of salicylaldoxime, over the same range as that which causes the inhibition of electron transport (approx. 10 mM). The uncoupling effect is, like the electron transport inhibition, largely reversible (Fig. 1B, inset).

The effect of salicylaldoxime on chloroplast fluorescence

Fig. 2 shows the effects of salicylaldoxime on chloroplast fluorescence as compared with the effects of CCCP. The concentration ranges used cover the ranges of inhibition concentrations of both inhibitors. Salicylaldoxime and CCCP inhibit reactions involving Photosystem II at concentrations around 10 mM and 100 μM, respectively. (Inhibition data for CCCP not shown, but see ref. 20.)

As the upper traces show the induction of the variable part of the fluorescence

TABLE I

SALICYLALDOXIME INHIBITION OF VARIOUS ELECTRON TRANSPORT REACTIONS

The basic ingredients of the reaction mixture (2 ml) were 0.1 M sucrose, 30 mM HEPES/NaOH buffer (pH 7.5), 30 mM KCl and chloroplasts equivalent to 50 μg chlorophyll (except Photosystem I reactions had 25 μg chlorophyll); ferricyanide, 0.4 mM; catechol, 1 mM (with 5 mM ascorbate); DBMIB, 20 mM; DMQ (2,5-dimethylbenzoquinone), 0.5 mM; NH_2OH, 20 mM; DCIP, 40 μM; diaminodurene, 1 mM (with 5 mM ascorbate); TMPD, 1 mM (with 5 mM ascorbate); silicomolybdate, 0.3 mM, with 5 μM DCMU. For details of reaction conditions see Methods. References given are for the reaction systems, not for the inhibition data, which are ours.

Reaction	Salicylaldoxime concentration (mM)		Photosystem	References
	50 % inhibition	90 % inhibition		
$H_2O \rightarrow$ methylviologen*	2	8	II+I	
$H_2O \rightarrow$ ferricyanide*	2	8	II+I	
Catechol $\rightarrow$ methylviologen*,**	1	8	II+I	[19]
$H_2O \rightarrow$ silicomolybdate (ferricyanide)	1	8	II	[15]
$H_2O \rightarrow$ DBMIB (ferricyanide)	8	15	II	[17]
$H_2O \rightarrow$ DMQ (ferricyanide)†	7	13	II	[17]
$NH_2OH \rightarrow$ DCIP	5	13	II•(+I)	[34]
Diaminodurene $\rightarrow$ methylviologen††	40	45	I	[35]
TMPD $\rightarrow$ methylviologen	40	45	I	[35]

* 10 mM methylamine (HCl) was present as uncoupler.

** NH_2OH-washed, non-water splitting chloroplasts [16] were used.

† 1 μM DBMIB was present to block photosystem I reduction.

†† Photosystem II was blocked by 2.5 μM DCMU.

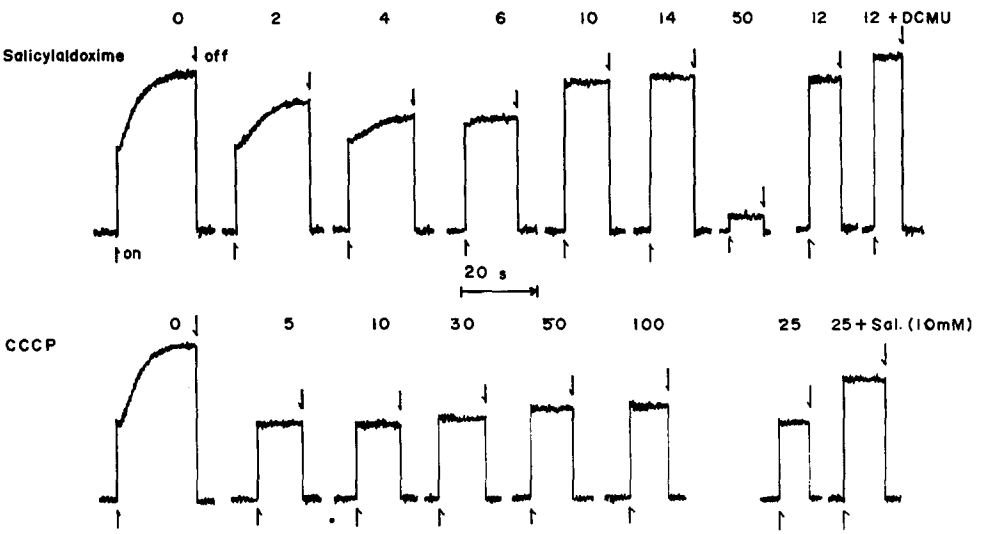

Fig. 2. The effects of salicylaldoxime and CCCP on chloroplast fluorescence. The composition of the reaction mixture was as in Table I, except that the chlorophyll concentration was 10 μg/ml and no electron acceptor was present. Concentrated solutions of salicylaldoxime and CCCP were added in small increments to yield the indicated mM (salicylaldoxime) or μM (CCCP) concentrations. Time intervals between additions were 30–40 s.

is slowed down by 2–6 mM salicylaldoxime. These concentrations of salicylaldoxime are already strongly inhibitory (see Table I). However, as the concentration of salicylaldoxime is raised further, the overall fluorescence yield increases. At 10 mM salicylaldoxime (which almost completely inhibits electron flow through Photosystem II), the fluorescence yield reaches the steady-state level of control chloroplasts. Thereafter, the fluorescence yield declines again and is almost completely abolished by 50 mM salicylaldoxime. This final concentration of salicylaldoxime irreversibly inhibits both photosystems and causes drastic membrane changes (see next section). The effect of salicylaldoxime on chloroplast fluorescence was previously studied by Katoh [5, 6], who observed only the steady decline in fluorescence yield between 3.3 and 10 mM salicylaldoxime, which is in contrast to the data above showing a transient fluorescence increase. The cause of this discrepancy is not known.

The effects of CCCP on chloroplasts can be seen in the lower traces. The variable portion of the fluorescence is extremely sensitive to CCCP and is abolished completely by concentrations of CCCP which are only mildly inhibitory (< 5 μM). This confirms an earlier report [20] but our results are somewhat at variance, in that the increasing concentration of CCCP slightly increased the fluorescence yield as did salicylaldoxime. However, the increased level of fluorescence, which reaches maximum at about 100 μM (where Photosystem II is completely inhibited), never reaches the level of the control steady state level. It starts to decline as the CCCP concentration exceeds 100 μM. Significantly, the addition of salicylaldoxime markedly increases the CCCP-suppressed fluorescence.

The effects of salicylaldoxime on Photosystem I reactions

In these experiments, two Photosystem-I electron transport reactions (DAD to methylviologen and TMPD to methylviologen) and two phosphorylating systems (DAD to methylviologen and DAD-mediated cyclic photophosphorylation) were measured in the presence of various concentrations of salicylaldoxime.

As the salicylaldoxime concentration exceeds 10 mM, it begins to affect both Photosystem I reactions and the conformation of chloroplasts (Fig. 3A). The latter effect can be observed as an increase in the turbidity of chloroplast suspension. The DAD → methylviologen reaction is stimulated by intermediate concentrations of salicylaldoxime (20 mM) before a precipitous inhibition sets in at about 30 mM, where the rate of TMPD-methylviologen reaction is also sharply decreasing. However, at no salicylaldoxime concentrations is the TMPD system stimulated. Since the DAD system is coupled to phosphorylation whereas the TMPD system is not, we first suspected that the increase in the DAD mediated rate might be due to uncoupling, but this rate increase was found to correlate better with the increase in light scattering (turbidity). The uncoupling by salicylaldoxime occurs at much lower concentrations: both the noncyclic and cyclic photophosphorylation reactions are abolished by 10 mM salicylaldoxime (Fig. 3A, B). These data correlate very well with the uncoupling of ferricyanide reduction seen in Fig. 1 (inset), where the P/O_2 ratio was diminished in a similar fashion over the same concentration ranges. The light scattering reaches its maximum at about the same concentration,where Photosystem I electron transport

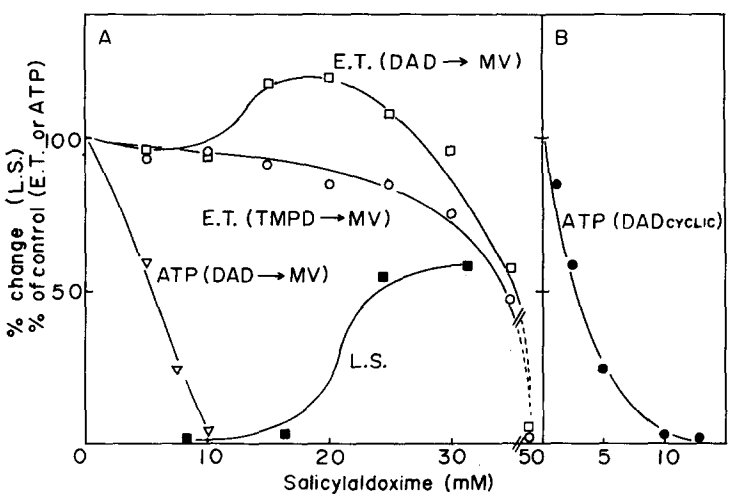

Fig. 3. The effects of salicylaldoxime on Photosystem I reactions. Diaminodurene (DAD)- and N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD)-mediated electron transport (E.T.) to methylviologen (MV) was measured as oxygen uptake. In (A), the basic ingredients of the reaction mixture were as in Fig. 1, except that ferricyanide was replaced by 50 μM methylviologen and the artificial electron donor system 1 mM DAD plus 5 mM ascorbate or 1 mM TMPD plus 5 mM ascorbate was added. In (B), 1 mM DAD was the only electron carrier. The control rates of the DAD and TMPD-mediated electron transport were 665 and 610 μmol O_2/h per mg chlorophyll. The control rates of the DAD-mediated noncyclic and cyclic phosphorylation were 180 and 186 μmol ATP/h per mg chlorophyll, respectively. Light-scattering changes (L.S.) were measured as absorbance changes (increase) at 540 nm.

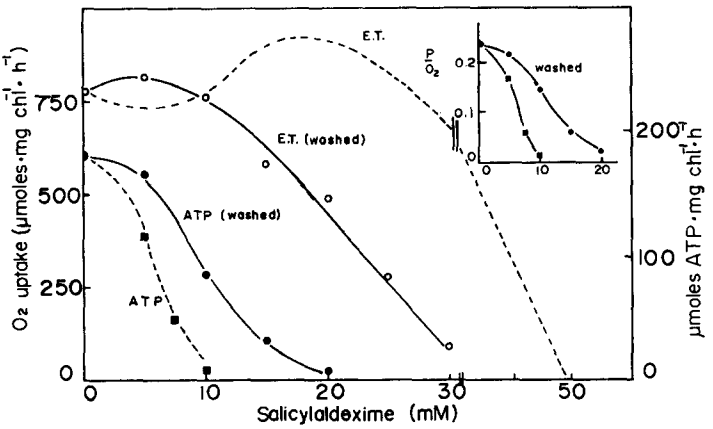

Fig. 4. The effect of chloroplast washing on salicylaldoxime inhibition of Photosystem I electron transport (E.T.) and ATP synthesis. Diaminodurene (DAD)-mediated electron transport was measured as O_2 uptake as in Fig. 3. Chloroplast washing was done as described in Fig. 1. Dashed curves for unwashed chloroplasts are from Fig. 3. Chl, chlorophyll.

is rapidly diminishing (30 mM), suggesting that the Photosystem I inhibition may be related to the membrane conformational changes responsible for the increase in light scattering. Later experiments will further define the redox step of Photosystem I which is primarily affected by the membrane changes.

When washing experiments similar to Fig. 1 were done followed by measurement of electron transfer from DAD to methylviologen, the results seen in Fig. 4 are obtained. When salicylaldoxime is added directly to the reaction mixture, the curve indicated by the dashed line is obtained. (This is the same curve seen in Fig. 4A.) After chloroplasts are treated with salicylaldoxime and then washed free of the inhibitor, the rate of electron transport from DAD to methylviologen is considerably reduced, below that observed when the salicylaldoxime is directly added. Thus the inhibition of Photosystem I is not merely irreversible; the inhibition is enhanced by washings. The rate of ATP generation, however, is higher in the washed chloroplasts than in chloroplasts with the salicylaldoxime present. This amounts to another example of the partial reversibility of the salicylaldoxime uncoupling effects, and this is borne out in Fig. 4 (inset), where part of the P/O_2 ratio is restored by the washing procedure.

The site of Photosystem I inhibition by salicylaldoxime

In an effort to locate the site of Photosystem I inhibition, we were prompted to examine the high concentration effects of salicylaldoxime on oxidation-reduction of cytochrome *f* and *P*-700. In these experiments the effect of salicylaldoxime was examined using chloroplasts which had been treated with 50 mM salicylaldoxime for 5 min and then briefly washed with a salicylaldoxime-free medium. (Washing does not reverse inhibitions caused by 50 mM salicylaldoxime; see Fig. 4.) Results from *P*-700 experiments are shown in Fig. 5A. No *P*-700 photooxidation was observed in control chloroplasts (upper left), but DCMU did elicit photooxidation by blocking electron flow from Photosystem II (upper right). In salicylaldoxime-treated chloroplasts *P*-700

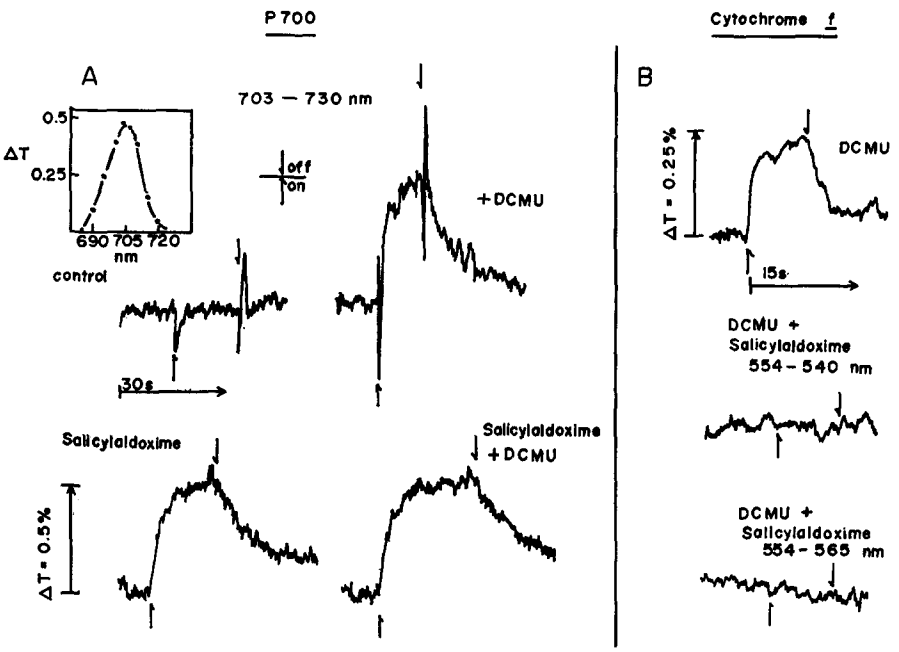

Fig. 5. The effects of salicylaldoxime (high concentration) and of DCMU on *P*-700 and cytochrome *f* in chloroplasts as observed by dual-wavelength spectroscopy. The composition of the reaction mixture was essentially as in Table I, but with 50 μM methylviologen as the electron acceptor and with chloroplasts equivalent to 75 μg chlorophyll/ml. Trace (0.5 mM) ascorbate was added to keep *P*-700 and cytochrome *f* reduced in the dark. In salicylaldoxime experiments, chloroplasts were treated with 50 mM salicylaldoxime, washed, and then subjected to experiments (see text). For other technical details, see Methods. Note that in DCMU-poisoned chloroplasts both *P*-700 and cytochrome *f* are photooxidized, while in salicylaldoxime-treated chloroplasts only *P*-700 is photooxidized.

was similarly photooxidized (lower left). Addition of DCMU to salicylaldoxime-treated chloroplasts did not further stimulate *P*-700 photooxidation (lower right) suggesting that salicylaldoxime had already completely blocked the electron pathway before *P*-700. The inset in Fig. 5A offers assurance that the change being measured in salicylaldoxime-treated chloroplasts was actually *P*-700 photooxidation.

It is interesting to point out that fluorescence spikes which were observed in control and DCMU-poisoned chloroplasts upon turning on and off the light (upper traces) were not seen in salicylaldoxime-treated chloroplasts (lower traces). It was shown earlier (Fig. 2) that high concentrations of salicylaldoxime (50 mM) virtually abolish chloroplast fluorescence. Apparently the high concentration quenching of fluorescence is completely irreversible as is the high concentration inhibition of electron transport.

Fig. 5B shows that the photooxidation of cytochrome *f*, which can be observed clearly in DCMU-poisoned chloroplasts (top trace) is completely inhibited in the salicylaldoxime-treated chloroplasts (middle and bottom). Since *P*-700 is still functional (Fig. 5A), it follows that electron transfer between cytochrome *f* and *P*-700 has been disrupted or, less likely, cytochrome *f* itself may have been rendered nonfunctional by the salicylaldoxime treatment. Almost identical spectroscopic observations have

been made for KCN inhibition [21], which has been demonstrated unequivocally to occur indeed at the plastocyanin site [22].

DISCUSSION

Salicylaldoxime has been widely used in a variety of photosynthetic systems, as has been discussed earlier in this paper. These observations have been fragmentary and contradictory and many aspects of the effects of salicylaldoxime on the various photosynthetic preparations have not been clear. This paper deals only with chloroplasts, but the general trends of salicylaldoxime activity may be mimicked in the algal systems.

We have shown that, at the pH values most frequently used by workers (7.5–8), salicylaldoxime has a variety of concentration-dependent effects on chloroplasts, including: (1) the largely reversible inhibition of Photosystem II at about 10 mM, (2) the largely reversible uncoupling of phosphorylation at the same concentration (10 mM), (3) the irreversible inhibition of Photosystem I at about 45 mM, and (4) the irreversible membrane damage beginning at about 20 mM (as detected by a light scattering increase).

These multiple effects of salicylaldoxime explain some of the inhibitions and stimulations which have been reported in the literature. The fact that inhibition of Photosystem II and uncoupling of phosphorylation are brought about by almost precisely the same concentrations of salicylaldoxime (Figs. 1, 3) will explain the data which seemed to suggest that "blocking" of the photosynthetic chain occurred at a point which was relatively close to Photosystem I and was shared in common by noncyclic electron transport and by phosphorylating cyclic electron transport [3]. The inhibition of DCMU-insensitive photophosphorylation and the stimulation of methyl red reduction [9, 10] can be explained in terms of uncoupling or alteration of the membrane conformation. The inhibition of Photosystem I reactions [13] could be explained as a high concentration effect of salicylaldoxime on the plastocyanin region. There are Photosystem I electron donors which support salicylaldoxime-insensitive electron flow [13], but this could be explained if the substances donated electrons directly to *P*-700 as does reduced DCIP [21].

Typical Photosystem I reactions such as the transport of electron from DAD or TMPD to methylviologen are totally and irreversibly abolished by 50 mM salicylaldoxime. A site of this Photosystem I inhibition has been resolved by spectroscopic experiments (Fig. 5). Since the photooxidation of *P*-700 is not inhibited, but the photooxidation of cytochrome *f* is inhibited, at least one site of salicylaldoxime inhibition of Photosystem I must lie between *P*-700 and cytochrome *f*. Data of this type are usually interpreted in terms of inhibition at the site of plastocyanin. Since salicylaldoxime, unlike KCN [22], does not affect the copper of isolated plastocyanin [4] it is improbable that the Photosystem I inhibition is due to a direct interaction of salicylaldoxime with plastocyanin per se. It could be that salicylaldoxime in some manner disturbs (or "loosens") the association of plastocyanin with the membrane, and thereby renders the copper protein susceptible to removal. This explanation seems consistent with the peculiar fact that the salicylaldoxime inhibition of Photosystem I reactions is aggravated by chloroplast washing (Fig. 4).

Thus, although at least one of the high concentration effects of salicylaldoxime

appears to be on plastocyanin, it is important to point out that the membrane changes (observed as light scattering increases) probably reflect a far more extensive membrane damage than that discussed above and the case of a site of inhibition at plastocyanin may be trivial.

As already noted, one of the interesting findings that emerged from the present study is that salicylaldoxime uncouples phosphorylation at concentrations which are very similar to those which inhibit Photosystem II. The uncoupling action of salicylaldoxime is presumably due to its phenolic hydroxyl group, rather than to its metal-complexing capability, in which the hydroxyl group plays a part. (The uncoupling actions of phenolic substances are well recognized; see ref. 23.) Of interest is that many of those phenolic or other lipid-soluble anionic uncouplers are known to act as rather potent Photosystem II inhibitors at concentrations not very much higher than their uncoupling concentrations. CCCP [20, 24–26], antimycin [6, 27], tetraphenylboron, desaspidin [28] and certain anilinothiophene derivatives [29] are among those compounds, and it is now apparent that salicylaldoxime also belongs to this group. Primarily based on experiments on chloroplast fluorescence or/and photobleaching, CCCP [20, 25, 26], antimycin and salicylaldoxime [5, 6] have been postulated to impose a "block" between an early photooxidant of Photosystem II and water. If this interpretation were correct, then one would have to make an implausible assumption that the photooxidant is completely inaccessible to any exogenous reducing substance, because so far no single artificial reductant, lipid soluble or water soluble, has ever been shown to induce measurable electron flow through Photosystem II in the presence of these inhibitors. In the present study, we have shown that catechol and hydroxylamine (as a reductant) also completely fail to do so (Table I). In sharp contrast, scores of reductants have been shown to be able to restore electron flow through Photosystem II in chloroplasts in which the pathway between H_2O and the photooxidant of Photosystem II has been interrupted by Tris [30] or hydroxylamine [19, 31].

Homann [28] emphasizes the ability of tetraphenylboron and desaspidin to serve as an efficient electron donor for Photosystem II and attributes the inhibition by these two substances and (also CCCP by implication) to the destruction of Photosystem II by the photooxidation products (see Renger et al. [29] for other views). Homann's interpretation does not seem to apply to salicylaldoxime, however, since (1) we found no evidence of salicylaldoxime being capable of donating electrons to Photosystem II and (2) salicylaldoxime inhibition does not progress in the light (Fig. 1A). Furthermore, the effect of salicylaldoxime on chloroplast fluorescence is different from that of CCCP (Fig. 2). If one is to take a simplistic view that the fluorescence yield is purely a function of the reduction state of the primary electron acceptor of Photosystem II, then a possible interpretation of the biphasic effect of salicylaldoxime on chloroplast fluorescence might be to assume that salicylaldoxime inhibits both sides of Photosystem II at very similar concentrations (< 10 mM). The idea of one site posterior to Photosystem II, however, is not entirely without additional support, since it is a well known fact that the nucleophilic agents 1,10-phenanthroline and related compounds inhibit near the DCMU site (for recent studies, see refs. 32, 33). The fact that salicylaldoxime could stimulate CCCP-suppressed fluorescence yields (Fig. 2) may also be interpreted to suggest this. One site is almost certainly located before the DCMU-sensitive site, since the DCMU-insensitive silicomolybdate Hill reaction [15] is completely inhibited by salicylaldoxime (Table I).

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