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ACTION OF CYANIDE ON THE PHOTOSYNTHETIC WATER-SPLITTING PROCESS

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KCN inhibits O_2 evolution from inside-out chloroplast thylakoid vesicles suspended in a low chloride-containing medium. Evidence is presented to suggest that the inhibition is due to CN^- and that this anion blocks electron flow close to the water-splitting process by interacting with a photo-oxidised species.

It is well known that cyanide can interfere with the activity of a wide range of enzymes [1] and it was Warburg [2] who demonstrated that this compound inhibits photosynthesis of intact organisms. Some years later, Whittingham [3] reported evidence to suggest the primary site of cyanide inhibition in photosynthetic tissue was the enzymic processes giving rise to CO_2 fixation. However, in recent years it was found that higher concentrations of this compound could also inhibit the light-induced electron-transport reactions which take place in and on the chloroplast thylakoid membrane [4,5]. The site of action was the copper-containing protein, plastocyanin, which acts as an electron carrier between PS I and PS II. In this report we show that under certain circumstances cyanide can also inhibit photosynthetic O_2 evolution by blocking electron flow on the oxidising side of PS II. This new finding is particularly important, since it has implications in terms of elucidating the mechanism of the water-splitting process. It has been possible to detect the inhibitory effect of cyanide by using inside-out chloroplast thylakoid vesicles which expose the components of the water-oxidation process to the external medium.

Thylakoid vesicles of normal and inverted membrane topography were prepared from *Pisum sativum* (Feltham First) using the procedures described elsewhere [6,7]. Both vesicle types were pelleted by centrifugation (30 min at $35000 \times g$) before being suspended in 100 mM potassium phosphate buffer, pH 7.4, and at an anion concentration and composition specified in the figure legends. The resuspended thylakoid vesicles were pre-illuminated with white light (intensity of less than 2000 $J \cdot m^{-2} \cdot s^{-1}$) for 20 min before enzymatic activities of PS II were recorded. PS II activity was monitored by light-induced O_2 evolution and by DCIP reduction. The initial rates of photosynthetic oxygen evolution, with benzoquinone or potassium ferricyanide as the acceptor pool for the reducing equivalents, were measured using a Rank oxygen electrode (Rank Brothers Ltd., Bottisham, Cambridge) and with saturating white light. The photo-induced reduction of DCIP was monitored optically using a Perkin-Elmer 557 double-beam spectrophotometer. Chlorophyll concentrations were calculated using the method of Arnon [8].

As shown in Fig. 1A at low chloride concentrations, the incubation of inside-out thylakoid

Abbreviations: Chl, chlorophyll; PS, photosystem; DCIP, 2,6-dichlorophenolindophenol; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

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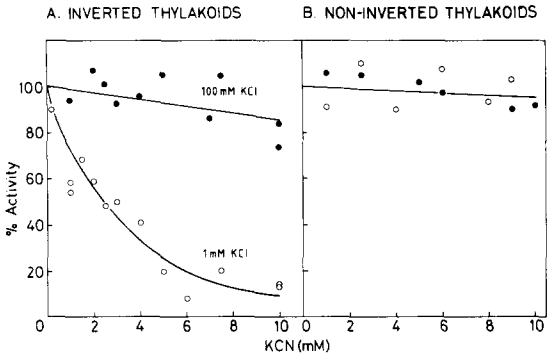

Fig. 1. KCN inhibition of photosynthetic oxygen evolution: the effect of the thylakoid membrane topography and chloride. Pelleted thylakoid vesicles were resuspended in 100 mM potassium phosphate, pH 7.4, containing either 1 mM or 100 mM KCl and supplemented with KCN. The suspensions were adjusted to 200 μg Chl/ml and pre-illuminated for 20 min under white light while on ice. Initial rates of light-induced oxygen evolution were recorded with 0.5 mM benzoquinone as the electron acceptor. The oxygen-evolution rates were expressed as % control activity. The measured control rates were: (A) 'Inverted' membranes: 36 and 20 μmol O_2/mg Chl per h for the 100 mM and 1 mM KCl suspensions, respectively. (B) Normal non-inverted membranes: 180 μmol O_2/mg Chl per h for both 100 mM and 1 mM KCl suspensions.

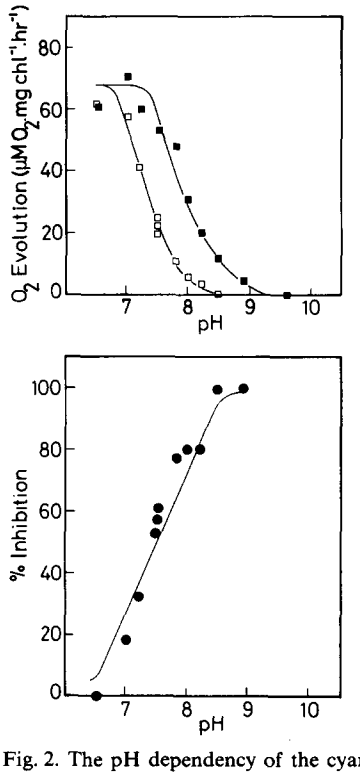

Fig. 2. The pH dependency of the cyanide inhibition. Pelleted 'inside-out' thylakoid vesicles were resuspended in 10 mM Hepes, 10 mM Tris, 10 mM glycylglycine, 1 mM KCl at various pH values. For each pH value, the effect of added 5 mM KCN on rate of O_2 evolution was observed after a 20 min pre-illumination, as in Fig. 1. Electron acceptor was 0.1 mM ferricyanide. (A) Rates of O_2 evolution expressed in μmol O_2/mg Chl per h; ■ control samples devoid of added KCN, □ samples supplemented with 5 mM KCN. (B) % inhibition caused by KCN at each pH value. The data fit an acid-base titration curve with a pK of 7.5.

vesicles with KCN resulted in the inhibition of photosynthetic O_2 evolution. Such an inhibition was not observed with vesicles with normal membrane topography or with inside-out vesicles bathed in a high chloride-containing medium. The inhibition required that the membranes were pre-illuminated and could be reversed by giving a subsequent dark treatment or by the addition of mild reducing agents such as 2 mM hydroquinone and 2 mM ascorbate. These observations suggest that the action of this inhibitor was dependent on the presence of a photo-oxidised species. The experiment in Fig. 1 was performed at pH 7.4 where the cyanide (pK 9.3) was present predominantly in the undissociated HCN form. We show in Fig. 2 that the relative extent of inhibition caused by cyanide is increased at alkaline pH. Although this result is complicated by the inhibition of the control rate at high pH it suggests that CN^-, rather than HCN, is the active species. This conclusion, however, may not be entirely correct since the effect of pH does not match that expected for a pK of 9.3 and there is always the explanation that the cyanide-sensitive site becomes more accessible to the poison at higher pH.

The sensitivity of the KCN effect to chloride levels could be due to the requirement of the water-splitting process for this anion [9,10]. In the absence of chloride other anions were not as effective at protecting against cyanide inhibition. The relationship, however, between chloride and cyanide was not competitive as shown by the double-reciprocal plot in Fig. 3. This plot indicates a K_i at pH 7.4 of $13.6 \cdot 10^{-3}$ M for the effect but assuming that the active species is the cyanide anion this value drops to about 10^{-4} M. It seems likely that the expression of the cyanide action at

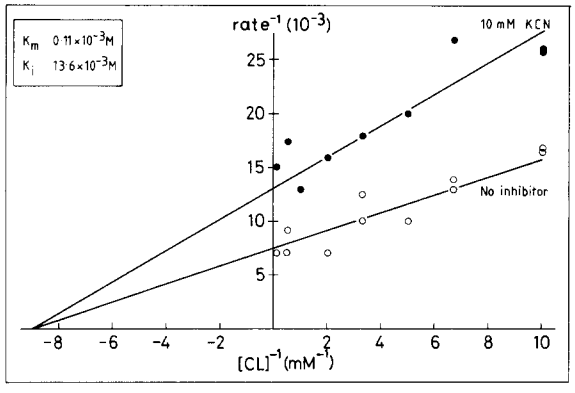

Fig. 3. Non-competitive inhibition by cyanide of the chloride-dependent photosynthetic oxygen evolution. Pelleted 'inside-out' thylakoid vesicles were resuspended in 100 mM potassium phosphate, pH 7.4, at different chloride concentrations and were either supplemented with or devoid of 10 mM KCN. The initial rates of photosynthetic oxygen evolution were recorded after a 20 min pre-illumination period, as in Fig. 1. The computed K_m of 0.11 mM for the chloride effect in the inverted thylakoid vesicles matches the earlier reported K_m value of 0.9 mM for thylakoid vesicles of normal membrane topography [10].

low chloride levels is due to impaired electron flow between H_2O and the PS II reaction centre (P-680) which will tend to promote the formation of oxidised species in this part of the electron-transport chain.

The electron-transfer pathway between the oxygen-evolving complex and P-680 contains at least one, as yet unidentified, component [11]. This component, designated Z, generates the EPR-detectable Signal II when oxidised and is the site to which artificial electron donors, such as diphenylcarbazide, feed electrons to the photo-oxidised P-680 when the oxygen-evolving complex is blocked. In Fig. 4, we have monitored the reduction of DCIP in an attempt to determine the site of cyanide inhibition. As shown, after cyanide treatment the rate of reduction of the artificial receptor was significantly inhibited. The degree of inhibition matched the inhibition of O_2 evolution measured with the same preparation. When diphenylcarbazide was added to the cyanide-treated membranes a rapid rate of DCIP reduction was recorded which was identical with that observed by adding diphenylcarbazide to untreated membranes. As is also shown in Fig. 4, when hydroxylamine was

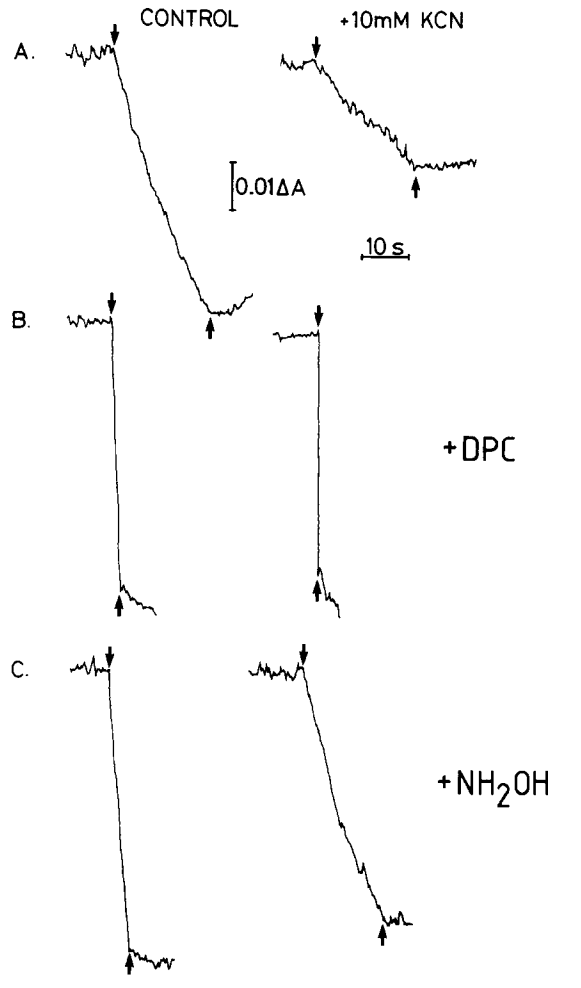

Fig. 4. The photo-induced DCIP reduction. Conditions as for the 1 mM KCl thylakoid suspension of Fig. 1A. The pre-illuminated vesicles were diluted to 50 μg Chl with the incubation buffer and 50 μM DCIP added. (A) No further additions, (B) +0.2 mM diphenylcarbazide (DPC), (C) +5 mM NH_2OH. The upward and downward directed arrows represent the onset and the cessation of illumination, respectively.

used as a PS II electron donor instead of diphenylcarbazide, the result was less clear with only a partial restoration of the inhibited system.

The results presented in this report indicate that there is an inhibitory action of cyanide on the oxidising side of PS II close to the water-splitting process. The requirement for pre-illumination and for low concentrations of chloride seems to suggest the necessity of a photo-oxidised species for full expression of cyanide inhibition, a notion sup-

ported by the protective nature of reducing conditions. The precise site of action of cyanide is less clear especially because hydroxylamine did not mimic the effect of diphenylcarbazide. Nevertheless, it is certain that cyanide does not block electron flow from Z to P-680$^+$ but acts on a component more closely associated with water oxidation. Exactly why the cyanide effect is expressed with the inverted membrane systems of inside-out vesicles will require further clarification but presumably reflects the exposure of the water-splitting process to the suspension medium.

The detection of a cyanide-sensitive site on the donor side of PS II may provide new insight into the mechanisms of photosynthetic oxygen evolution. It is possible that cyanide is binding to an oxidised metalloprotein such as a haem. Two haems which could be involved in the electron-transport process on the oxidising side of PS II are the high-potential cytochrome *b*-559 [12] and the catalase-type haem [13,14]. We carried out experiments to test whether cyanide inhibited the photo-oxidation of cytochrome $b\text{-}559_{HP}$ and found that this cytochrome is not the site of action although the haem protein still remains a possibility. However, it is also worth noting that cyanide can inhibit quinone proteins [15] and that this type of complex may function in the transport of electrons from water to P-680$^+$ [16,17].

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