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Hexabromoiridate IV as an electron acceptor: comparison with hexachloroiridate IV and hexacyanoferrate III

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Three artificial electron acceptors potassium hexacyanoferrate ($K_3Fe(CN)_6$) 'ferricyanide' (HCF III), potassium hexachloroiridate (K_2IrCl_6) (HCI IV), and potassium hexabromoiridate (K_2IrBr_6) (HBI IV), have been compared with respect to their reduction by roots, membrane permeability, phytotoxicity and their absorption spectra. In this paper it will be shown that HBI IV as well as HCI IV are more effective electron acceptors than HCF III in regard to their lower charge, redox potential and toxicity. The toxicity of these compounds is negligible when used in micromolar concentrations. Both do not permeate the plasma membrane of root cells. Low concentrations of all three electron acceptors stimulates elongation growth of *Lepidium sativum* L. roots. The reduction of HBI IV by maize roots was characterized and the effect of artificial auxins on the reduction rate of HBI IV has been investigated.

Introduction

Two different types of plasmalemma redox systems have been characterized by several groups. One of those systems, the so-called 'turbo-system', is induced by iron deficiency in dicots and non-grass monocots. The second system ('standard system') appears to be present in all plants including grasses. The natural electron acceptor of the plasma membrane 'standard system' is still unknown. Oxygen [1–3], and semidehydroascorbate [4] have been proposed. The redox mechanisms of plant plasma membranes have been investigated using different artificial electron acceptors [2]. The most widely used electron acceptor is potassium hexacyanoferrate (HCF III). Both the reduced and oxidized form of hexacyanoferrate do not permeate biological membranes. The redox reaction is not linked to a pure chemical release (or binding) of protons like the redox reaction of most other electron acceptors, e.g., DCPIP. The different colors of the reduced and oxidized form makes the redox reaction easy to detect. Unfortunately, HCF III is not stable, especially in light where it releases iron and cyanide. The latter may inhibit the cyanide-sensitive respiration while iron interacts with the turbo-system, which is linked to iron uptake. Recently, hexachloroiridate (HCI IV) has been introduced as a new electron acceptor [5]. HCI IV and HCF III both act as impermeable electron acceptors and share the type of redox reaction mentioned above. The high molar extinction coefficient of HCI IV of 3680 $M^{-1} \cdot cm^{-2}$ [5], which exceeds that of HCF III (1000 $M^{-1} \cdot cm^{-2}$ [6]) provides a significant advantage for measurement of the reaction.

Unfortunately, the redox potential of HCI IV ($E_0' =$ 870 mV) is higher than that of the water-oxygen couple ($E_0' = 816$ mV), so HCI IV is not only reduced by water but also by many organic molecules (e.g., proteins and some buffers) at a very slow rate.

The aim of the present work was to compare HCF III with HCI IV and a third electron acceptor potassium hexabromoiridate (HBI IV) with regard to (a) membrane permeability, (b) phytotoxicity, (c) absorption spectra and (d) reduction by maize roots.

Materials and Methods

Plant material. Seeds of *Zea mays* L. cv. Goldprinz (C. Sperling, Lüneburg, F.R.G.) were soaked in tap

Abbreviations: HCF III, hexacyanoferrate ($K_3Fe(CN)_6$) 'ferricyanide', HCI IV, hexachloroiridate (K_2IrCl_6; HBI IV, hexabromoiridate (K_2IrBr_6); DCPIP, 2,6-dichlorophenolindophenol; ED_{50}, effective dose 50% (elongation growth 50% inhibition); Mes, morpholinoethane sulfonic acid; oxone, potassium monopersulfate ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$); AAS, atomic absorption spectroscopy; α-NAA, α-naphtyl acetic acid; 2,4-D, 2,4-dichlorophenoxy acetic acid; LIAC, light-inducable absorbance change.

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water for 12 h and sterilized for 10 min with 15% H_2O_2. The seedlings were germinated for 48 h on wet filter paper in the dark at 26 °C. *Lepidium sativum* L. cv. Mega (C. Sperling, Lüneburg, F.R.G.) and *Nicotiana sylvestris* L. (from R. Kappler, University of Hamburg, F.R.G.) were used for toxicity tests. The experimental procedures are described below.

Chemicals. HCI IV was purchased from Ventron (Heidelberg, F.R.G.). Oxone was obtained from Aldrich (Steinheim, F.R.G.). HBI IV was prepared as described by Gutbier and Ries [7]. HCF III and all other chemicals were obtained from Merck (Darmstadt, F.R.G.).

Determination of the toxicity of the electron acceptors. (1) Inhibition of growth and germination rate was measured using *Lepidium sativum* L. cv. Mega. Seeds were germinated in petri dishes for 24 h on fibre glass filters type no 6 (Schleicher & Schuell, Dassel, F.R.G.) moisted with solutions of the electron acceptors between 0.05 mM and 10 mM in 10 mM KCl and 1 mM $CaCl_2$ (pH 5.5). (2) Determination of the ED_{50} was performed with frozen (−20 °C) pollen of *N. sylvestris* L. using the pollen-tube-growth-test [8]. The pollen were suspended in culture medium with 10% sucrose, 3 mM $Ca(NO_3)_2$, and 0.001% boric acid in aqua destilata, 10 mM Mes [KOH] ad pH 5.6. For measurement 100 µl solution of the electron acceptors in aqua destilata were added to 900 µl of the pollen culture and incubated for 18 h in the dark at 25 °C. Quantification of cell wall growth was performed using alican blue 8 GX (Sigma, Deisenhofen, F.R.G.) as described by Kappler and Kristen [9].

Estimation of HBI IV uptake by roots. (1) In order to estimate the amount of HBI IV taken up by the plants, a sample of the medium was taken after 15 min and 30 min and oxidized with oxone. The re-oxidation was started by addition of 60 µl oxone (10 mg/ml) to 2 ml assay volume. The absorption was measured at 585 nm until the maximum of absorbance was reached. The incubation medium was buffered with 0.1 mM Mes (pH 5.5) [KOH/HCl]. (2) The iron content of root segments was measured by AAS as described by Qiu et al. [10], the iridium content was determined using the same method. The incubation was performed in 10 mM KCl and 1 mM $CaCl_2$ in aqua bidestilata (pH 5.5). For measurements of iridium the material was dissolved in concentrated HCl/HNO_3 (3/1, v/v) for 4 h at 100 °C. The AAS was carried out with a Perkin-Elmer 5000 (compensation by continuum lamp). For the determination of iron the roots were dissolved in perchloric acid 70% using a temperature program starting at 70 °C and ending at 180 °C after 13 h. The spectrometry was carried out with a Perkin-Elmer with Zeeman compensator.

Measurement of the reduction of HBI IV by maize roots. The reduction of HBI IV was measured using a computer-controlled pH redoxstat. One of the most important features of a pH-redoxstat is that there is no change of pH during the experiment. This allows measurement in unbuffered solutions. The pH of the incubation medium was measured by a pH electrode connected to an Apple II microcomputer via AD-converters (type pH 530 D WTW, Weilheim, F.R.G.) and readjusted by continuous titration with KOH using a computer-controlled dilutor (Microlab M, Hamilton, Bonaduz, Switzerland). Similarly, the concentration of the oxidized form of HBI (HCI or HCF, respectively) was held constant. The concentration of HBI IV was determined with a flow-through cuvette by a photometer (LKB Ultrospec) at $E_{585-800\,nm}$ and kept constant by continuous titration with HBI IV stock solution via a second dilutor. Net proton efflux and HBI IV reduction were calculated from the amount of titrator added. Further details about this system were published by Böttger and Hilgendorf [11].

Seedlings of *Zea mays* L. cv. Goldprinz were grown as described above. Before measurement the seedlings were preincubated at 25 °C for 12 h in an aerated medium containing 10 mM KCl and 1 mM $CaCl_2$ in aqua bidestilata. The experiments were carried out in a climate chamber at 25 °C and a light intensity of 71 $\mu Es \cdot m^{-2} \cdot s^{-1}$, the pH was 5.5. α-NAA and 2,4-D were dissolved in 0.1 M KOH and adjusted to pH 5.5 (HCl).

Results and Discussion

One difference between the three acceptors is their redox potential, which of HCI IV ($E_0' = 870$ mV) and HBI IV ($E_0' = 815$ mV [12]) exceeds that of HCF III ($E_0' = 360$ mV) more than twice. The two-fold negative charge of the iridium complexes allows easier diffusion through the surface-charge barrier than the three-fold negative charge of HCF III [13]. Because of the lower charge of HCI IV and HBI IV we expect better access to the membrane, and higher reduction rates. Form and size of the molecules as well as the higher redox potential may also influence the reduction rate.

All three complexes did not inhibit the germination rate of *Lepidium sativum* L. in a concentration range from 0.05 mM to 10.0 mM. Growth of *Lepidium* roots, however, was slightly stimulated by concentrations up to 0.5 mM of HCF III, up to 1.0 mM for HBI IV, and up to 10.0 mM of HCI IV (Fig. 1), higher concentrations inhibited the elongation growth. HCF II, HBI III, and HCI III have no significant effect on root growth (data not shown).

Similar results were obtained using the pollen-tube-growth-test for the determination of the ED_{50}-value. This test is highly sensitive for toxicity and was used to compare the toxicity of the three electron acceptors. Low ED_{50} values indicate high toxicity. HBI IV has an ED_{50} of 266 ± 7 ppm (0.4 ± 0.04 mM), closely followed

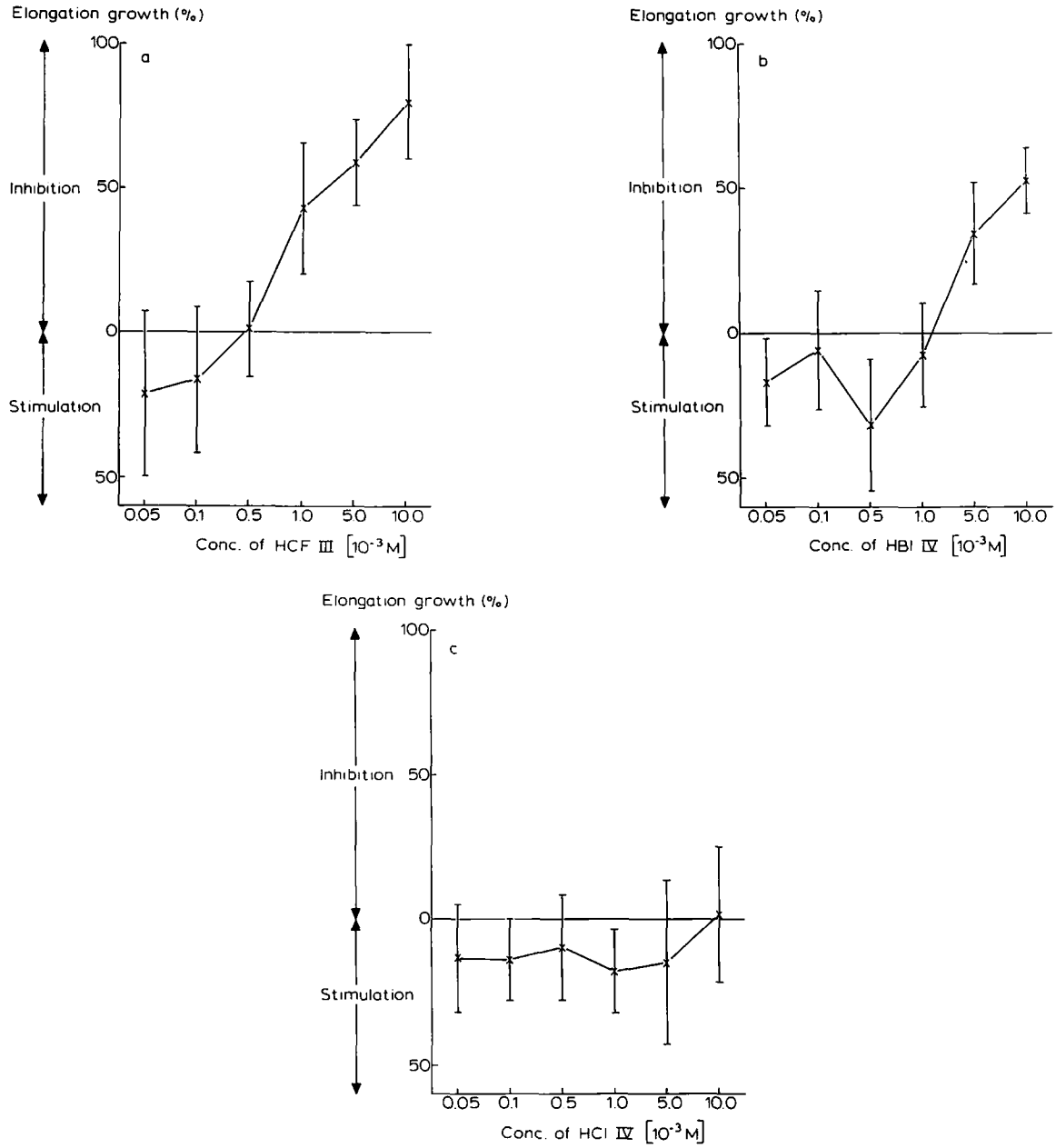


Fig. 1. Inhibition of the root growth of *Lepidium sativum* L. after treatment with HCF III, HBI IV, and HCI IV. The control is set to 0%. Assays were carried out in 10 mM KCl and 1 mM $CaCl_2$ (pH 5.5) without buffer. Ten plants were used per assay, each assay was replicated three times. The incubation time was 24 h.

by HCF III with 325 ± 34 ppm (0.9 ± 0.1 mM), and HCI IV with an ED_{50} of 950 ± 49 ppm (1.9 ± 0.1 mM).

The toxicities of all three complexes are very low. In the concentration range used in electron-transport experiments, however, the iridium complexes are not toxic compared to HCF III, which is normally used in concentrations close to the ED_{50} we found in the pollen-tube experiments. In physiological experiments both HBI IV and HCI IV can be used in concentrations far below their ED_{50}, because their higher extinction coefficient allows photometric determination 4 times below the detection limit of HCF III and their lower charge makes it possible to measure electron-transport processes even at micromolar concentrations. Growth of *Lepidium* roots was slightly enhanced by addition of micromolar concentrations of all three electron acceptors. Furthermore, low concentrations have the advantage that the ionic composition of the incubation medium is only slightly altered.

A possible uptake of HBI IV was measured by two different approaches: the electron acceptor was re-oxidized after reduction by plants and the recovery in the bathing medium determined by spectrophotometry. Secondly, the content of iron and iridium in roots was detected by AAS. (1) Craig and Crane [14] have shown that 98% of the HCF III could be re-oxidized by oxone

TABLE I

Measurement of the recovery of HBI IV by re-oxidation with oxone (60 µl of 0.1 g/10 ml solution added to 2 ml assay volume) after 15 min and after 30 min incubation time

The assay was carried out in 10 mM KCl, 1 mM $CaCl_2$, 1 mM Mes buffer (pH 5.5). The reduction of the control was caused by reaction with the buffer.

	Time (min)	HBI IV before re-oxidation (µM)	HBI IV after re-oxidation (µM)	Recovery (%)
Control	0	66.25	66.25	100.0
(without plants,	15	33.75	67.75	102.3
with HBI IV)	30	24.17	66.25	100.0
Experiments	0	66.25	66.25	100.0
(with plants,	15	24.17	67.50	101.9
with HBI IV)	30	12.33	66.00	99.6

in the culture-medium after a 10 min incubation with carrot cells. The results of Lüthen and Böttger [5] have shown the same for HCI IV using maize roots. The data shown in Table I are very similar to the results for HCF III [14] and for HCI IV [5], respectively, indicating that there is no or at least negligible uptake of HBI IV. The HBI IV content of the medium was determined after incubation and re-oxidation with oxone. After 15 min we detected 101.9%, and after 30 min incubation 99.6% of the HBI IV content (Table I). Fig. 2 indicates decomposition of the complex: after an initial oxidation of HBI III to HBI IV by oxone, the complex is destroyed by excess of the oxidant; probably a conversion to IrO_2 takes place. Since there also is a time-dependence of the amount of HBI IV found after re-oxidation, it seems to be necessary to measure both time and concentration

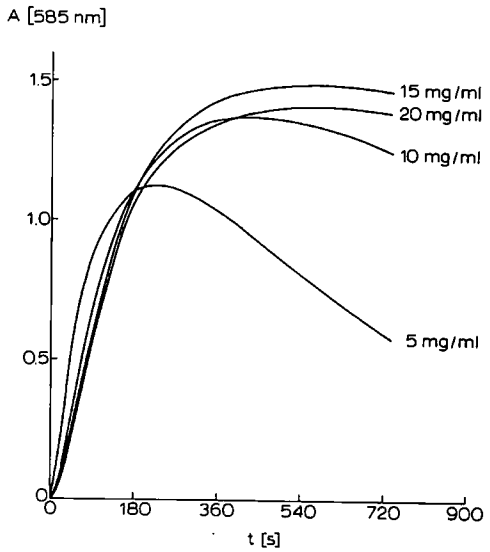


Fig. 2. Time and concentration dependence of the oxidation of HBI III with oxone. The HBI IV concentration was determined photometrically at 585 nm. Different amounts of oxone were added at zero time to 2 ml assay volume, the reaction was monitored continously.

TABLE II

Determination of iron and iridium in maize root segments by AAS after 15 min of incubation in 0.8 mM HCF III, HCI IV or HBI IV (dissolved in 10 mM KCl, 1 mM $CaCl_2$, pH 5.5)

	ng/mg DW	nM/g DW	nM/g DW minus control
Control	85.0	1522 Fe	–
	0.0 Ir	0.0 Ir	–
HCF III	91.1 Fe	1631 Fe	109 Fe
HCI IV	82.4 Ir	429 Ir	429 Ir
HBI IV	76.2 Ir	396 Ir	396 Ir

dependences to get interpretable results. (2) The amount of iron determined after incubation with HCF III using AAS is comparable with the results published by Qiu et al. [10]. The iridium content found after incubation with HBI IV and HCI IV is similar to the iron content (Table II). It remains unknown whether the iron and iridium found by AAS is inside the cell or bound to the cell wall.

Despite the problems which arise using the re-oxidation method with oxone, the results obtained with this method are in conformity with the results found by AAS. Both methods indicate that the permeability of the iridate complexes is equal or lower than the permeability of HCF III, at least for maize root plasma membranes.

HCF III is a widely used electron acceptor for the examination of the 'standard system' of electron transfer by plasma membranes [15]. Previous investigations have reported an inhibition of the proton secretion at very low levels of HCF III [11] and HCI IV [5], respectively, and an enhancement at higher concentrations.

The dependence of HBI IV reduction and H^+-efflux on the iridate concentration was measured simultaneously. Both, HBI IV reduction and proton secretion, increased with higher concentrations of HBI IV (Fig. 3). We neither found an inhibition of the proton secretion at HBI IV concentrations down to 1 µM, nor a saturation of the reduction rate and the proton secretion up to 100 µM.

Crane et al. [16] reported a decrease of the reduction rate of HCF III after application of 2.4-D in carrot cells. An inhibition of electron transfer and simultaneously of proton secretion after application of auxins has been reported for maize roots [11]. The same behavior was observed for HCI IV [5].

As shown in Fig. 4 α-NAA and 2,4-D also inhibit HBI IV reduction and proton secretion to the same extent as for HCF III and HCI IV. Within the limit of our investigation we are able to report that the regulation of the HBI IV reduction rate and HBI IV stimulated proton secretion appears to be the same as it is for HCI IV.

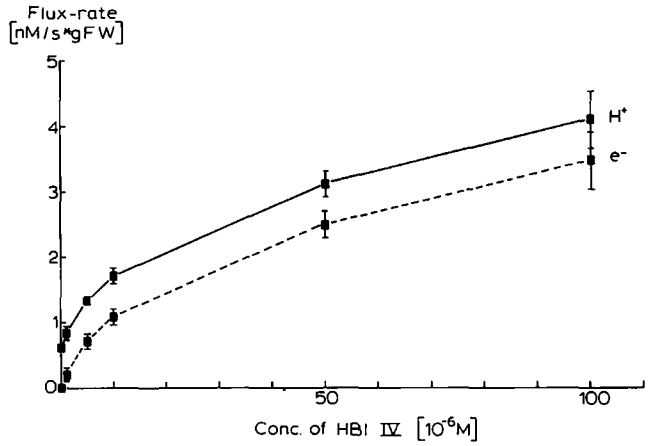
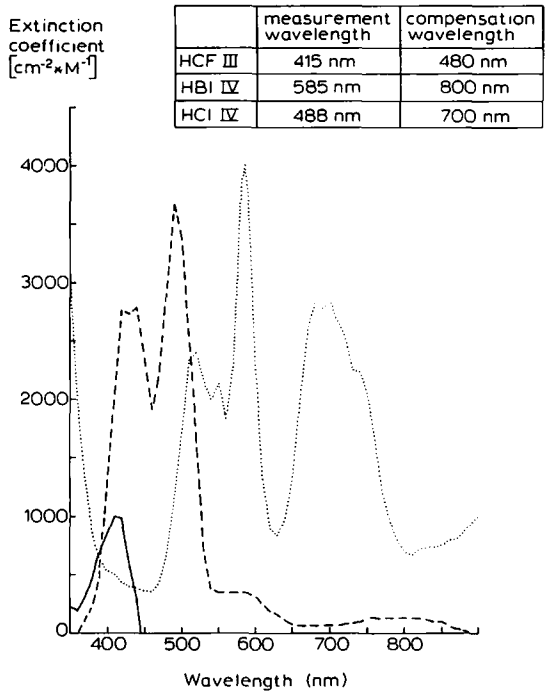


Fig. 3. Dependence of HBI IV reduction on the concentration and corresponding enhancement of the proton secretion of maize roots. The incubation medium was 10 mM KCl and 1 mM $CaCl_2$ (pH 5.5).

The spectra of HCF III, HCI IV and HBI IV (Fig. 5 demonstrate the much higher extinction coefficient of HCI IV and HBI IV (4000 $M^{-1} \cdot cm^{-2}$ at 585 nm (pH 5.5), in incubation medium) compared with HCF III. HBI IV has a violet color, and its extinction coefficient allows the photometric determination of micromolar amounts in aqueous solution. The reduced forms of each of the three complexes (HCF II up to 1 mM, HCI III, and HBI III up to 100 μM) are almost colorless or very pale yellow for the latter two. The inset to Fig. 5 shows the absorption maxima (measurement wavelength) and minima (compensation wavelength) of the three electron acceptors. These measurement and compensation wavelengths have been used for the determination of the reduction rate of the three acceptors by maize roots.



	measurement wavelength	compensation wavelength
HCF III	415 nm	480 nm
HBI IV	585 nm	800 nm
HCI IV	488 nm	700 nm

Fig. 5. Spectra of HCF III (———), HCI IV (— — —) and HBI IV (·····). The reduced form of each of the three complexes (HCF II, HCI III, and HBI III) is colorless or very pale yellowish for the latter two (Spectra not shown). Inset: The absorption maxima (measurement wavelength) and minima (compensation wavelength) of the three electron acceptors used for the determination of the reduction rate of the three acceptors in a pH-redoxstat of maize roots.

The spectrum of HBI IV led us to the idea of a possible application of this complex. Leong et al. [17] and Caubergs et al. [18,19] found a blue light induceable redox system (LIAC) in plant plasma membranes

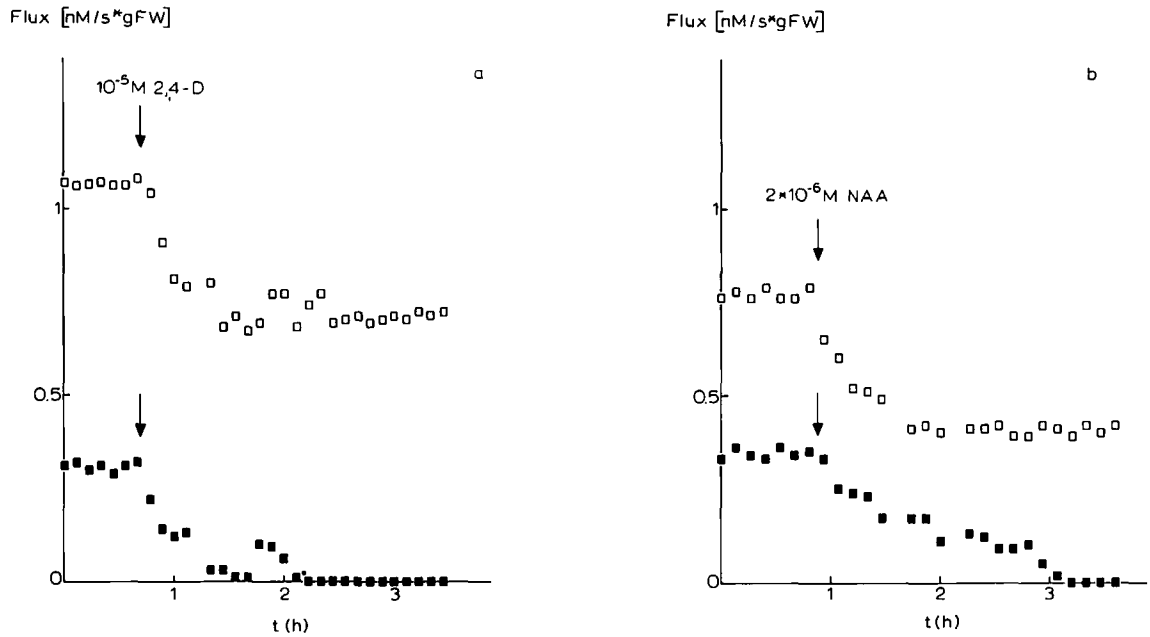


Fig. 4. Effect of artificial auxins: 2,4-D (10^{-5} M) (a) and ($2 \cdot 10^{-6}$ M) (b) α-NAA on proton secretion (□) and HBI IV reduction (■). The incubation medium was 10 mM KCl (pH 5.5), without $CaCl_2$.

which plays an important role in the regulation of some metabolic pathways [20]. Since HCF III absorbs blue light (Fig. 5) the investigation of this system has been done using DCPIP or methylene blue [21]. The redox reaction of methylene blue as well as DCPIP involves a chemical release (uptake) of protons, so there may be some advantages in using HBI IV for these investigations.

We found HBI IV to be an electron acceptor with features similar to HCF III and HCI IV in many ways, some of these properties (e.g., its higher stability compared with HCI IV, the lower charge compared to HCF III, its redox potential close to the water-oxygen couple, and the lack of absorption of blue light) might be suitable to focus special attention on HBI IV.

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