Biochem. J. (1974) **142**, 65–74
Printed in Great Britain

Inhibition and Uncoupling of Photophosphorylation in Isolated Chloroplasts by Organotin, Organomercury and Diphenyleneiodonium Compounds

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(*Received* 18 *January* 1974)

1. Trialkyltin, triphenyltin and diphenyleneiodonium compounds inhibited ADP-stimulated O_2 evolution by isolated pea chloroplasts in the presence of phosphate or arsenate. Tributyltin and triphenyltin were the most effective inhibitors, which suggests a highly hydrophobic site of action. Phenylmercuric acetate was a poor inhibitor of photophosphorylation, which suggests that thiol groups are not involved. 2. Triethyltin was a potent uncoupler of photophosphorylation by isolated chloroplasts in media containing Cl^-, but had little uncoupling activity when Cl^- was replaced by NO_3^- or SO_4^{2-}, which are inactive in the anion–hydroxide exchange. It is suggested that uncoupling by triethyltin is a result of the Cl^-–OH^- exchange together with a natural uniport of Cl^-. Tributyltin, triphenyltin and phenylmercuric acetate had low uncoupling activity, probably because in these compounds the uncoupling activity is partially masked by inhibitory effects. 3. At high concentrations the organotin compounds caused inhibition of electron transport uncoupled by carbonyl cyanide *m*-chlorophenylhydrazone or NH_4Cl. At these high concentrations the organotin compounds may be producing a detergent-like disorganization of the membrane structure. In contrast, diphenyleneiodonium sulphate inhibited uncoupled electron transport at low concentrations; however, this inhibition is less than the inhibition of photophosphorylation, which suggests that the compound also inhibits the phosphorylation reactions as well as electron transport. 4. The effects of these compounds on basal electron transport were complex and depended on the pH of the reaction media. However, they can be explained on the basis of three actions: inhibition of the phosphorylation reactions, uncoupling and direct inhibition of electron transport. 5. The inhibition of cyclic photophosphorylation in the presence of phenazine methosulphate by diphenyleneiodonium sulphate shows that it inhibits in the region of photosystem 1.

Early observations on the actions of trialkyltin compounds showed that they were inhibitors of mitochondrial oxidative phosphorylation (Aldridge, 1958; Aldridge & Street, 1964) and also weak uncouplers of oxidative phosphorylation and inhibitors of uncoupled respiration. Sone & Hagihara (1964) confirmed the inhibition of phosphorylation by tributyltin and emphasized the similarity of its action to that of oligomycin, but failed to observe significant inhibition of the 2,4-dinitrophenol-stimulated respiration. Selwyn *et al.* (1970) demonstrated that the trialkyltin compounds mediated Cl^- transport via a compulsory exchange for OH^- across mitochondrial, erythrocyte and liposome membranes. Also exchange was a catalytic effect of organotin compounds and not modification of an existing carrier. By using this knowledge Stockdale *et al.* (1970) re-examined the action of organotin compounds on mitochondrial respiration and proposed that they have two actions: first a direct action on the phosphorylation reactions resembling that of oligomycin, and secondly, in media containing Cl^-, a weak uncoupling effect. Stockdale *et al.* (1970) ascribed the inhibition of 2,4-dinitrophenol-stimulated respiration to structural damage consequent on swelling in a medium which contained Cl^-. The long-chain trialkyl- and triphenyl-tin compounds were reported to have a detergent-like action at high concentrations. The role of the anion–hydroxide exchange in uncoupling has been confirmed by Rose & Aldridge (1972).

The importance of pH was shown by Coleman & Palmer (1971), who found that triethyltin was a potent inhibitor of uncoupler-stimulated respiration in Cl^--containing media at pH 6.9, but was ineffective at pH 7.1 and above. This work has been extended by Dawson & Selwyn (1974), who ascribe the inhibition to the Cl^-–OH^- exchange causing collapse of a pH gradient across the mitochondrial membrane, thereby lowering the intramitochondrial pH to that of the medium or below, and when the medium is at pH 6.8 the respiratory enzymes have a very low activity.

Harris *et al.* (1973) have reported that trialkyltin compounds also inhibit the adenine-nucleotide transporter.

Diphenyleneiodonium compounds were shown by Holland & Sherratt (1972) to mediate Cl^-–OH^- exchange reactions across mitochondrial membranes, but were shown to be relatively ineffective as inhibitors of oxidative phosphorylation. However, Holland *et al.* (1973) proposed that the inhibition by diphenyleneiodonium compounds of mitochondrial oxidation reactions *in vivo* was by a direct inhibition of the oxidation of NADH-linked substrates rather than by Cl^-–OH^- exchange.

Tributyltin chloride was shown to inhibit photophosphorylation and coupled electron transport in isolated chloroplasts from spinach and *Euglena* by Kahn (1968), but the inhibition of electron flow to ferricyanide was abolished by addition of NH_4Cl. The ionophoretic activities of organotin and organomercury compounds on isolated chloroplasts have been demonstrated (Watling & Selwyn, 1970), but the relative effectiveness of the various organotins was different from the observed effectiveness on mitochondrial systems.

Organometallic compounds of tin and mercury could interfere with the photophosphorylation system in several ways: first by binding with one or more of its components and thereby inhibiting electron transport or energy coupling directly, secondly by affecting the electrochemical potential gradient, or thirdly by a general disruptive detergent-like action. In the present study a range of different organometallic compounds and the ionophoretic diphenyleneiodonium ion have been used to provide information about the relations between, and perhaps mechanisms of, ion exchange, uncoupling and inhibitory activities. The use of an oxygen electrode with a recorder and chloroplasts that exhibit photosynthetic control allows continuous monitoring of the effects of the organometals on chloroplasts in a variety of photosynthetic states. In addition the incorporation of $[^{32}P]P_i$ into ATP under the same conditions as those used for measurement of O_2 evolution is used to distinguish between stimulating and uncoupling activities and, with diphenyleneiodonium sulphate, to provide information as to its site of action in the electron-transport chain. Simultaneous measurements of the light scattered at narrow angles and 90° (which may relate to changes occurring in the whole plastids and the thylakoids respectively) are used to provide a continuous indication of changes in volume, shape and conformation of the chloroplasts produced by organometallic compounds.

Materials and Methods

Preparation of chloroplasts

Chloroplasts were isolated from leaves of pea seedlings *Pisum sativum* var. Meteor grown in moist vermiculite for approx. 20 days at 16°C. Chloroplasts were prepared in sorbitol media by the procedure of Kalberer *et al.* (1967) with the following modifications. Whole leaves were homogenized with 70ml of preparation medium in an AtoMix blender and the slurry was filtered through Nytal 25T (11–20 mesh) (Henry Simon Ltd., Stockport, U.K.). After centrifugation the sedimented chloroplasts were resuspended in a reaction medium containing 300mM-sorbitol and 25mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] (adjusted with NaOH to pH7.6) for 'class 1' chloroplasts. For 'class 2' chloroplasts the pellet was resuspended in 20ml of medium containing 30mM-sorbitol and 25mM-Hepes, adjusted to pH7.6 with NaOH, and centrifuged at 2500*g* for 1min. Finally the chloroplasts were taken up in reaction medium at a concentration of approx. 1mg of chlorophyll/ml. Chlorophyll was measured spectrophotometrically by the method of Arnon (1949).

Measurement of oxygen evolution

Oxygen evolution was measured with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) incorporated into a Perspex reaction vessel with a jacket through which water at 18°C was circulated. Oxygen partial pressure was lowered to 25% of the air saturation value by flushing with O_2-free N_2, the chloroplasts were then added and the Perspex lid lowered. The lid was especially designed to facilitate the addition of organometals through a small aperture of sufficient dimensions for the needle of a Hamilton syringe and with a long diffusion path to minimize oxygen entry. White light was provided at a saturating intensity (1.764×10^5lx at the vessel surface) by a Rank Aldiss 2000 projector and the chloroplast suspension was stirred at all times with a magnetic follower. The electrode current was recorded on a Rikadenki potentiometric recorder (KA series; Rikadenki Kogyo Co. Ltd., Tokyo, Japan) with a pre-amplifier incorporating variable gain controls.

Measurement of ATP formation

Experiments involving incorporation of $[^{32}P]P_i$ into ATP were carried out in stoppered 1.5ml Eppendorf micro-centrifuge tubes maintained at 18°C in a water bath supplied with horizontal illumination of 1.764×10^5lx at the water-bath surface by a Rank Aldiss 2000 projector. After termination of the reaction by addition of trichloroacetic acid to a final concentration of 167mM the contents of the tubes were centrifuged for 2min at 15000*g* in an Eppendorf 3200 micro-centrifuge, and a 1ml sample removed for determination of charcoal-adsorbable radioactivity by the method of Hind & Jagendorf (1963).

Measurement of light scattering

Observations of the light scattered at narrow

angles (less than 15°) from chloroplast suspensions in the absence of actinic illumination were carried out as described previously (Watling & Selwyn, 1970). Simultaneous measurements of light-scattering at narrow and 90° angles were performed on chloroplast suspensions in a 1.5cm light-path cuvette with four optical faces. An improved amplifier system was used in which one outlet carried the signal light and the other outlet activated a photocell driving the demodulator for a twin-channel phase-locked amplifier, incorporating variable gain and back-off controls on each channel. This was used for amplification of the signals given by the two photomultipliers set at 90° and 180° to the signal light. As in the method for narrow-angle scattering used previously (Watling & Selwyn, 1970), a small stop to block off unscattered light was placed in front of the 180° photomultiplier tube. The outputs from the amplifier were fed into a Rikadenki multipen recorder. For the light-scattering measurements the amplified signals from the photomultipliers at a narrow angle and 90° were backed off to give 10–100% and 50–150% respectively of the amounts of light scattered. Actinic illumination was provided by a 30W bulb connected to a stabilized 6V d.c. power supply. The lamp was mounted directly above the cuvette housing and the light-beam focused and masked to illuminate the whole cuvette by a Wratten 70 filter.

Materials

Reagents were obtained as described previously (Watling & Selwyn, 1970) and in addition Hepes was purchased from Hopkin and Williams Ltd., Romford, Essex, U.K., carbonyl cyanide *m*-chlorophenylhydrazone from Calbiochem Ltd., London W.1, U.K., and triethyltin as the sulphate was prepared from triethyltin hydroxide obtained from the Tin Research Institute, Perivale, Greenford, Middx., U.K. All reaction media and grinding media were made up in deionized distilled water; triethyltin and diphenyleneiodonium sulphate were dissolved in aq. 50% (v/v) ethanol; phenylmercuric acetate at concentrations greater than 10mM was dissolved in NaOH and then neutralized with Hepes. All other organometals were in ethanolic solution. $[^{32}P]P_i$ in sterilized HCl solution, pH2–3, was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. No measurable change in pH was observed on the addition of 0.5μCi of KH_2PO_4/ml to the reaction medium.

Results

Inhibition of photophosphorylation

Inhibition of ADP-stimulated oxygen evolution. On illumination isolated chloroplasts suspended in buffered sorbitol medium gave a slow constant rate of O_2 evolution, which was stimulated by ADP in the presence of either phosphate (Fig. 1*a*) or arsenate (Fig. 1*b*). In the presence of phosphate the rate decreased to a slow rate when the ADP was consumed, but with arsenate the rate remained constant. This is in agreement with the work of Avron & Jagendorf (1959), who proposed an unstable

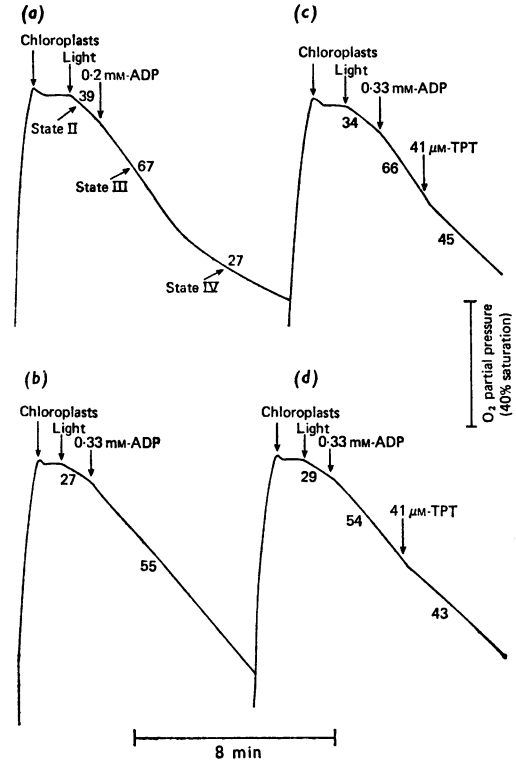


Fig. 1. *Inhibition of ADP-stimulated oxygen evolution by tripropyltin (TPT)*

Broken chloroplasts at a concentration of 60μg of chlorophyll/ml were suspended in 300mM-sorbitol, 25mM-Hepes adjusted to pH7.6 with NaOH, 2.2mM-$K_3Fe(CN)_6$, 3.3mM-$MgCl_2$, at a temperature of 18°C in a total volume of 4.5ml. (*a*) Photosynthetic control of the rate of evolved O_2 in the presence of 10mM-KH_2PO_4 adjusted to pH7.6 with KOH. (*b*) Stimulation of O_2 evolution when ADP is added in the presence of 10mM-KH_2AsO_4 adjusted to pH7.6 with KOH. (*c*) Inhibition by tripropyltin of ADP-stimulated O_2 evolution in the presence of 10mM-KH_2PO_4 adjusted to pH7.6 with KOH. (*d*) Inhibition by tripropyltin of ADP-stimulated O_2 evolution in the presence of 10mM-KH_2AsO_4 adjusted to pH7.6 with KOH. Numbers along the traces refer to rates of O_2 evolution expressed as μg-atoms of oxygen/h per mg of chlorophyll.

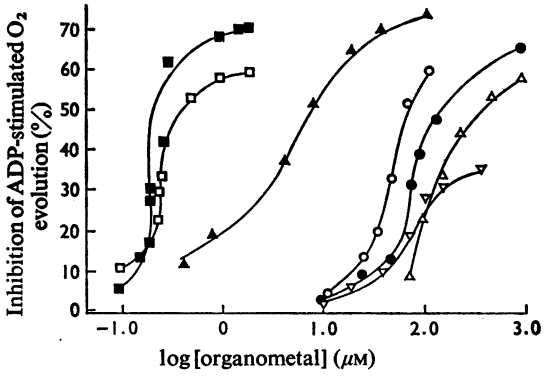


Fig. 2. *Inhibition by organometals of ADP-stimulated oxygen evolution*

Reaction conditions were the same as those described for Fig. 1, with the addition of 10mM-KH_2PO_4 adjusted to pH 7.6 with KOH and 0.333mM-ADP adjusted to pH 7.6 with NaOH. ■, Tributyltin; □, triphenyltin; ▲, tripropyltin; ○, diphenyleneiodonium sulphate; ●, trimethyltin; △, triethyltin; ▽, phenylmercuric acetate.

ADP–arsenate complex. Following the convention of Chance & Williams (1955) applied to chloroplasts by West & Wiskich (1968), the ADP-stimulated rate is referred to as state III and the slower rate, after phosphorylation of ADP, is referred to as state IV. The chloroplast preparations used in the work reported here exhibited photosynthetic control (with state III/state IV rate ratios greater than 3). In the study of inhibition, by organometals, of ADP-stimulated O_2 evolution, sufficient ADP was added to maintain state III for the duration of the experiment. Organometals were found to inhibit photosynthetic-O_2 evolution stimulated by either ADP plus phosphate or ADP plus arsenate, for example, tripropyltin (41μM) inhibited the ADP–phosphate system by 31% and the ADP–arsenate system by 23% (Figs. 1*c* and 1*d* respectively). The different degrees of inhibition of the two systems may signify that tripropyltin inhibits at or after the step where phosphate or arsenate is taken up. Other organometallic compounds and diphenyleneiodonium sulphate also inhibited ADP-stimulated O_2 evolution. The relative effectiveness of these compounds as inhibitors is summarized in Fig. 2; at 50% inhibition the order is tributyltin > triphenyltin > tripropyltin > diphenyleneiodonium sulphate > trimethyltin > triethyltin, the concentration ratios being 1:1.8:40:339:646:2104. The maximum inhibition produced by these compounds is 70% of the rate of O_2 production in the presence of ADP and phosphate. However, this inhibition decreases the rate to approximately that in the absence of ADP, i.e. approx. 100% inhibition of the extra O_2 production caused by the addition of ADP. Since the state II rate (before addition of ADP) is different from the state IV rate (after utilization of the added ADP) it is not possible to quantify a correction, and further, since the involvement of phosphorylation and ATP breakdown in the state II and state IV rates is not known, it is not possible to justify making such a correction. With phenylmercuric acetate the very low maximal inhibition (35%) indicates some additional effects, possibly the phenylmercuric acetate was binding to thiol groups not in the active centre of the phosphorylation system and thiol groups may therefore be absent from the active centre.

When the concentrations of trialkyltins were increased beyond those which gave maximal inhibition of ADP-stimulated O_2 evolution certain anomalies were observed. For example, tributyltin or triphenyltin (approx. 2μM) caused a short initial period of inhibition of ADP-stimulated O_2 evolution, but this was followed by a time-dependent increase in the rate of O_2 evolution, which with tributyltin was to a rate greater than that observed before the addition of ADP. In general the inhibition of photophosphorylation by organometals occurred at relatively low concentrations, and higher concentrations caused stimulation of O_2 evolution, possibly by uncoupling. However the situation was reversed with triethyltin, stimulation of ADP-enhanced O_2 evolution was observed at lower concentrations than those producing inhibition.

Inhibition of ATP formation. The effects of selected concentrations of tripropyltin, diphenyleneiodonium sulphate and triethyltin on [^{32}P]P_i incorporation into ATP are given in Table 1, together with the effects of the same concentrations on ADP-stimulated, basal and uncoupled O_2 evolution. In the case of tripropyltin the inhibition of phosphorylation was slightly greater than the inhibition of ADP-stimulated O_2 evolution, but the lack of inhibition of the uncoupled system and the lesser inhibition of the basal rate of O_2 evolution indicates that the inhibition was on the energy-conserving steps rather than directly on electron transfer. With triethyltin there was a decrease in [^{32}P]P_i incorporation but stimulation of state III O_2 evolution and massive stimulation of state II O_2 evolution (basal rate) which suggest uncoupling by triethyltin. With diphenyleneiodonium sulphate there was inhibition of O_2 evolution in the uncoupled system which indicates a direct effect on the electron-transfer processes. The greater inhibition of state III rate of O_2 evolution and even greater inhibition of [^{32}P]P_i incorporation suggest that diphenyleneiodonium sulphate also inhibits energy-conservation steps and has uncoupling activity. Cyclic phosphorylation in the presence of phenazine methosulphate was inhibited by diphenyleneiodonium sulphate to a lesser extent than non-cyclic

Table 1. *Inhibition of ATP formation by organometallic and diphenyleneiodonium compounds*

The inhibition of ATP formation by these compounds is compared with their effects on state III, state II and uncoupled O_2 evolution (results taken from Figs. 2, 4 and 5 respectively). Broken chloroplasts at a concentration of 60 μg of chlorophyll/ml were suspended in 300 mM-sorbitol, 25 mM-Hepes adjusted to pH 7.6 with KOH, 2.2 mM-$K_3Fe(CN)_6$, 3.3 mM-$MgCl_2$, 0.5 mM-ADP adjusted to pH 7.6 with NaOH, 3.4 mM-KH_2PO_4 adjusted to pH 7.6 with KOH and 0.5 μCi of $[^{32}P]P_i$ in a total volume of 1.0 ml at 18°C. Values marked * are the results of experiments where 0.3 mM-phenazine methosulphate was used as electron-transfer cofactor, and in those experiments $K_3Fe(CN)_6$ was omitted.

	[^{32}P]ATP formation		Oxygen evolution		
	(μmol of phosphate esterified/h per mg of chlorophyll)	Inhibition (% of control)	State III (ADP stimulated)	State II (basal)	Carbonyl cyanide *m*-chlorophenylhydrazone uncoupled
Tripropyltin (22.7 μM)	8.6	74.3	60% inhibition	30% inhibition	No effect
Diphenyleneiodonium sulphate (80 μM)	6.0	82.1	60% inhibition	20% inhibition	50% inhibition
	*16.8	*62.2			
Triethyltin (28 μM)	26.2	21.6	30% stimulation	240% stimulation	No effect
Control	33.4	—	—	—	—
	*44.4	—	—	—	—

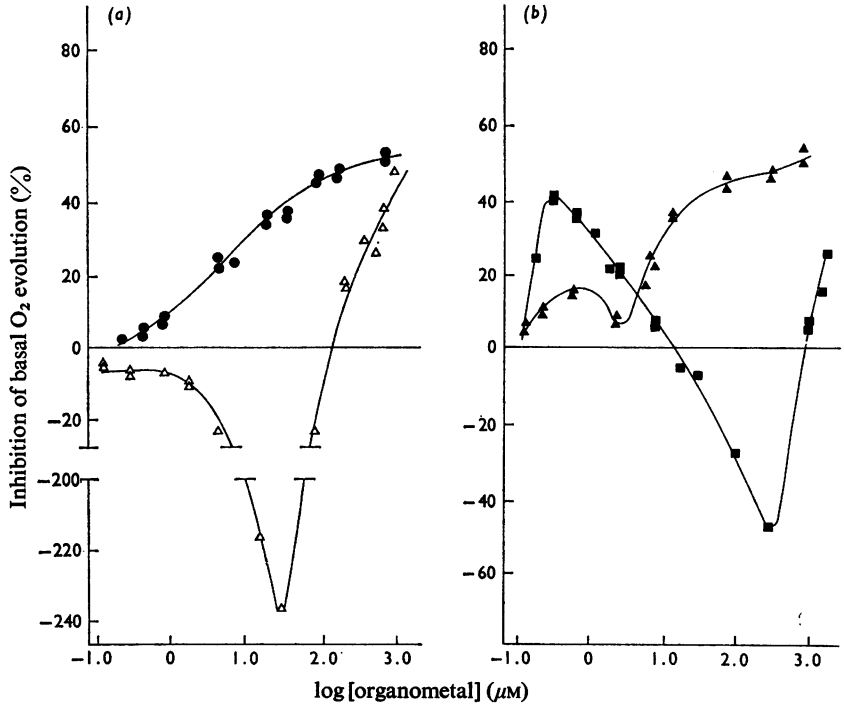


Fig. 3. *Inhibition and stimulation of basal electron transport by organotin compounds*

Broken chloroplasts, 60 μg of chlorophyll/ml final volume, were suspended in 300 mM-sorbitol, 25 mM-Hepes adjusted to pH 7.6 with NaOH, 3.3 mM-$MgCl_2$, 4.4 mM-$K_3Fe(CN)_6$ at 18°C in a total volume of 4.5 ml. (*a*) ●, Trimethyltin; △, triethyltin; (*b*) ▲, tripropyltin; ■, tributyltin. Note the break in the ordinate for the triethyltin curve.

phosphorylation; however photosystem 2 was not blocked and could possibly have contributed electrons to photosystem 1. The observed inhibition of cyclic phosphorylation shows that there was inhibition in the region of photosystem 1, but does not rule out inhibition of phosphorylation and/or electron transport in photosystem 2 and other parts of the electron-transport chain. It is clear from these results that in the case of tripropyltin the major effect at the concentration used and under these

conditions is inhibition of the energy-conservation (phosphorylation) steps. In addition tributyltin may have some uncoupling activity and with triethyltin uncoupling appears to be the major effect. Diphenyleneiodonium sulphate appears to be an effective inhibitor of the electron-transport system.

Uncoupling action of organometallic compounds

Stimulation of basal electron transport. Basal electron transport (state II) was measured by the continuous monitoring of O_2 evolution in the absence of ADP and phosphate. The type of effect of organometals on electron transport depended not only on the concentration of the compound but also on the time of incubation with it and on the pH of the medium. Fig. 3 shows the major types of effect. In general three phases of action can be considered, inhibition of O_2 evolution at low and high concentrations of organometal with a phase of decreased inhibition or even stimulation of O_2 evolution at intermediate concentrations. The inhibitory effects are discussed below. Of the group of compounds studied only triethyltin (at low concentrations) produced significant stimulation of O_2 evolution (Fig. 4*a*). Tributyltin and phenylmercuric acetate also caused stimulation of O_2 evolution but the effects were of smaller magnitude, required higher concencentrations and changed to inhibition in a time-dependent fashion.

Stimulation of state IV oxygen evolution. The rate of state IV O_2 evolution is slower than the rate of state III O_2 evolution from isolated chloroplasts (Fig. 1); this is thought to be due to a restraint imposed on the system by the building up of non-phosphorylated intermediates or the high-energy state. Addition of tripropyltin to an illuminated chloroplast suspension in state IV caused a stimulation of the rate of O_2 evolution; this was produced by concentrations of tripropyltin in the range 0.1–730 μM, the maximum stimulation was 60% with 400 μM-tripropyltin.

The stimulation of both basal and state IV O_2 evolution may be accounted for by uncoupling. A mechanism is proposed for uncoupling (Scheme 1), which is an extension of the scheme proposed by Watling & Selwyn (1970) and considers a trialkyltin mediated coupled exchange of Cl^- for OH^-; such an exchange would proceed in response to and by its action dissipate the pH component of the electrochemical potential gradient. The electrogenic move-

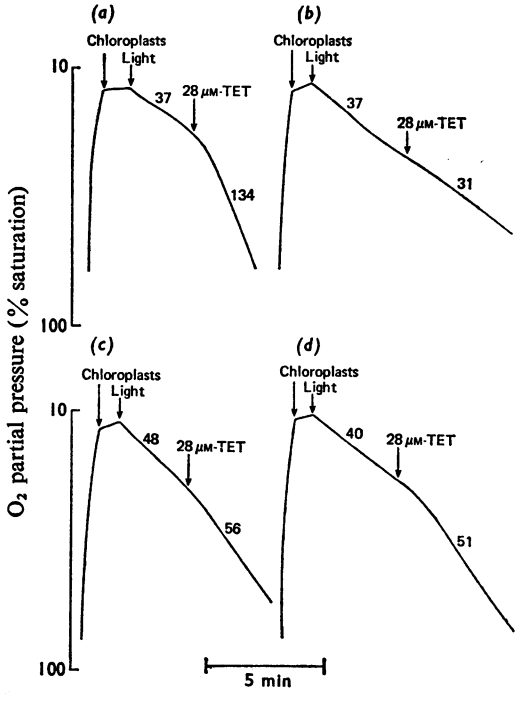
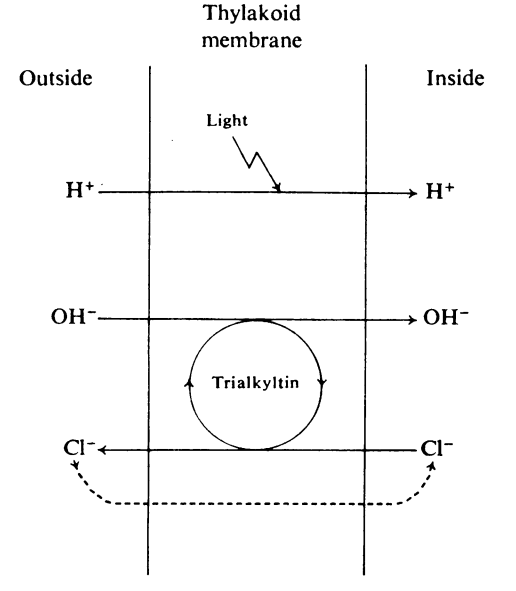


Fig. 4. *Effect of anions on the stimulation of basal oxygen evolution by triethyltin (TET)*

Conditions were as described for Fig. 3 with the following modifications: (*a*) no change; (*b*) $MgCl_2$ omitted; (*c*) $MgCl_2$ replaced with $Mg(NO_3)_2$; (*d*) $MgCl_2$ replaced with $MgSO_4$. Numbers along the traces refer to rates of O_2 evolution expressed as μg-atoms of oxygen/h per mg of chlorophyll.



Scheme 1. *Postulated mechanism for trialkyltin-mediated uncoupling via Cl^-–OH^- exchange*

ment of Cl^- to the inside of the thylakoids would release the electrical potential difference generated by proton movements. In view of the low permeability of Cl^- at pH7.6 (Packer *et al.*, 1966; Schuldiner & Avron, 1971) the movements of these ions to the inside of the thylakoids is likely to be the rate-limiting step of the uncoupling process.

Actions of organometals in media devoid of Cl^-. According to the proposed mechanism of uncoupling, organometals should not produce uncoupling of chloroplasts suspended in media devoid of anions capable of participating in anion–hydroxide exchange reactions. Cl^- should be excluded from such a medium, however, Hind *et al.* (1969) have reported a definite requirement for Cl^- in the photosystem 2 reactions especially with broken 'class 2' chloroplasts. Sulphate, a possible alternative to Cl^- as anions for the Mg^{2+}, has been reported by Ryrie & Jagendorf (1971) to inhibit the coupled phosphorylation reactions. Passive permeability studies on chloroplasts suspended in ammonium salts and recorded by changes in light scattering show that NO_3^- and SO_4^{2-} are not active in trialkyltin-mediated anion–hydroxide exchange reactions. Total absence of $MgCl_2$ from the reaction medium used in photophosphorylation studies results in a normal rate of basal O_2 evolution from the chloroplasts, but stimulation of the rate on addition of ADP was not observed and photosynthetic control was abolished. Addition of either $Mg(NO_3)_2$ or $MgSO_4$ resulted in a reduction of the basal rate of O_2 evolution, but in either case addition of ADP-stimulated O_2 evolution and the photosynthetic control ratios were of the same magnitude as those obtained in the presence of $MgCl_2$. The uncoupling by 400μM-tripropyltin of state IV O_2 evolution from chloroplasts was abolished by the substitution of $MgSO_4$ for $MgCl_2$ in the reaction medium, and the uncoupling by the same concentration of tripropyltin was reduced by approx. 44% when $Mg(NO_3)_2$ was substituted for $MgCl_2$ in the reaction medium. Uncoupling of basal O_2 evolution (in the absence of ADP and phosphate) by triethyltin was completely abolished when $MgCl_2$ was excluded from the reaction medium (Fig. 4*b*). The apparent inhibition was due to a time-dependent decrease in the rate of O_2 evolution which was also observed in the absence of triethyltin. In media containing $Mg(NO_3)_2$ (Fig. 4*c*) or $MgSO_4$ (Fig. 4*d*), triethyltin stimulated O_2 evolution by only 16% or 29% respectively. This feeble stimulation when contrasted with the value of more than 200% stimulation in the presence of $MgCl_2$ demonstrates the need for Cl^- in the uncoupling activity of triethyltin.

Inhibition of electron transport

Effect of organometals on uncoupled electron transport. Electron transport was uncoupled in the absence of ADP and phosphate by 2μM-carbonyl cyanide *m*-chlorophenylhydrazone which produced approx. 15-fold stimulation of O_2 evolution. Diphenyleneiodonium sulphate was found to be a more effective inhibitor of uncoupled electron transfer than the organometals, the latter inhibiting at concentrations around 1mM whereas diphenyleneiodonium sulphate inhibited at concentrations of about 50μM (Fig. 5). Trimethyltin at concentrations greater than 1mM caused aggregation of the chloroplasts; the inhibition of O_2 evolution observed with such concentrations of trimethyltin was non-linear and may be due to a non-specific denaturation of chloroplast structure. Stimulation of O_2 evolution by concentrations of tributyltin, tripropyltin and triethyltin below 1mM indicates enhancement of uncoupling since there was a lack of such further stimulation when chloroplasts were maximally uncoupled by 4.4μM-carbonyl cyanide *m*-chlorophenylhydrazone. Since inhibition of uncoupled electron transport by organometallic compounds occurred at higher concentrations than inhibition of phosphorylating electron transport it would appear that the latter effect cannot be explained solely by a direct inhibition of the electron-transport system by these compounds.

Inhibition of basal electron transport. The complexity of effects on basal electron transport is described above and examples are shown in Fig. 3. Trimethyltin exhibited only a monotonic inhibition of basal O_2

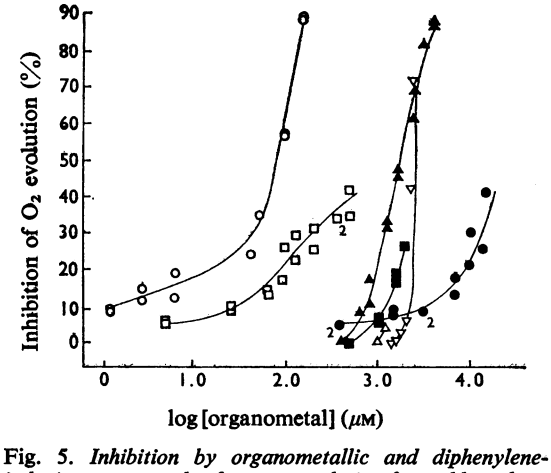


Fig. 5. *Inhibition by organometallic and diphenyleneiodonium compounds of oxygen evolution from chloroplasts uncoupled by 2.2μM-carbonyl cyanide m-chlorophenylhydrazone*

Conditions were as in Fig. 3 except that $K_3Fe(CN)_6$ was at a concentration of 2.2mM, and the uncoupler was present. ○, Diphenyleneiodonium sulphate; □, triphenyltin; ▲, tripropyltin; ■, tributyltin; △, triethyltin; ▽, phenylmercuric acetate; ●, trimethyltin.

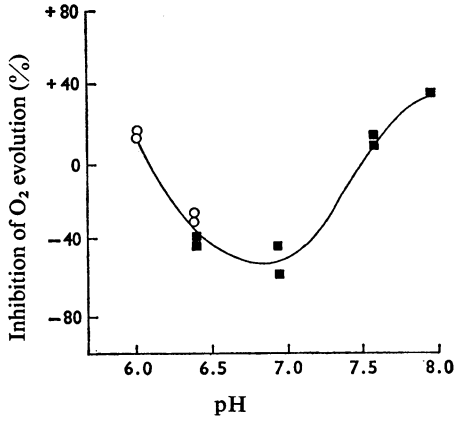


Fig. 6. *Effect of pH on inhibition of electron transport by trimethyltin* (96 μM)

Conditions were as described for Fig. 3 except that at pH 6.5 and below (○), the buffer was 25 mM-Mes [2-(*N*-morpholino)ethanesulphonic acid] and at pH 6.5 and above (■), the buffer was 25 mM-Hepes, the buffers were adjusted to the indicated pH values with NaOH.

evolution and triethyltin showed no inhibition at low concentrations. The other organotin compounds produced inhibition at both low and high concentrations with a range of decreased inhibition or stimulation at intermediate concentrations. Phenylmercuric acetate was not effective as an inhibitor of basal electron transport reactions at concentrations up to 1 mM.

The effect of pH on the inhibition of basal electron transport was investigated by using concentrations of organometals that had given half maximal inhibition of the phosphorylating electron-transport system at pH 7.6; a typical curve is shown in Fig. 6. At pH 6.5 or 7.0 organometals stimulated electron transport, but at higher pH values (7.6 or 8.0) the organometals produced inhibition. At alkaline pH the same concentrations of organometals that inhibited phosphorylation also inhibited the residual electron transport that occurred in the absence of added ADP and phosphate. It is known that very little ATP is formed by chloroplasts suspended in media below pH 7.0 (Uribe, 1970). At pH 6.0, therefore, electron-transport processes may well be uncoupled from phosphorylation reactions. The inhibitory activities of the organometallic compounds at pH 6.0 are a result of either a direct action on one or more of the electron-transport components, or an indirect effect of, for example, pH equilibration across the membrane as has been suggested to be operative in mitochondrial systems by Dawson *et al.* (1972).

Light-scattering changes under photophosphorylation conditions. An earlier report (Watling & Selwyn, 1970) of effects of organometallic compounds on the permeability of chloroplast membranes has shown that the ionophoretic properties of these compounds produced changes in structure and volume of chloroplasts suspended in certain media. Therefore the inhibitory effects of organometals on photophosphorylation could be due to disruption of the chloroplast structure. To investigate this possibility, chloroplasts were suspended in the sorbitol reaction medium (under the conditions used for investigation of inhibition of photophosphorylation) and the effects of adding organometallic compounds were observed by measurements of light scattering. Observations were made on the effects of organometals, at concentrations which caused inhibition of photophosphorylation, on the light scattering by chloroplasts at both 90° and narrow angles with and without actinic illumination. In the absence of organometals actinic illumination of chloroplasts suspended in sorbitol medium caused small changes in the light scattered at both 90° and narrow angles; however, these changes were reversed on cessation of illumination. Addition of 0.14 μM-tributyltin (which produced half maximal inhibition of ADP-stimulated O_2 evolution) with actinic illumination caused only slightly larger changes in the light scattering (8% of the level of scattering at 90°) which were also reversible in darkness. The small degree and reversibility of these changes and the absence of any marked effect of this concentration of tributyltin showed a lack of detergent-like action. A similar lack of effect was observed with corresponding concentrations of other organometals. Therefore the inhibition of coupled electron transport and phosphorylation by organometals is not due to a general disruption of the chloroplasts.

Discussion

From his observations on chloroplasts isolated from spinach and *Euglena* and on a soluble adenosine triphosphatase prepared from chloroplasts Kahn (1968) concluded that the only action of tributyltin on chloroplasts was in inhibition of the energy-coupled reactions. However, Kahn (1968) also observed that tributyltin enhanced phosphorylation and the light-induced pH rise in chloroplasts that had been depleted of the coupling-factor adenosine triphosphatase. The former effect is analogous to the improved P/O ratios sometimes observed on adding oligomycin to submitochondrial particles (Lee & Ernster, 1965). Kahn (1968) also observed, but did not discuss, that tributyltin at approximately 100-fold the concentration required for inhibition of phosphorylation produced slight stimulation of $NADP^+$ reduction by isolated spinach chloroplasts. Further, the inhibition of electron transport in the

phosphorylating system was less than the inhibition of phosphorylation, and significant enhancement of NH_4Cl uncoupling was also produced.

The present observations extend Kahn's (1968) work to other organometals and show that their effects are more complex than suggested by Kahn, and also indicate direct inhibition of electron transport and uncoupling via Cl^-–OH^- exchange. At low concentrations not only tributyltin but also trimethyltin, triethyltin, tripropyltin and triphenyltin have a direct inhibitory action on photophosphorylation at the level of energy conservation. This type of inhibition was not found with the mercurial compounds phenylmercuric acetate and *p*-hydroxymercuriphenylsulphonic acid. However, another synthetic ionophore, diphenyleneiodonium sulphate, has also been found to inhibit the photophosphorylation system, which is in contrast to its lack of inhibition of mitochondrial oxidative phosphorylation (Holland & Sherratt, 1972; Holland *et al.*, 1973). With the organotin compounds the concentrations required to inhibit ADP-stimulated O_2 evolution are very much lower (0.1–100 μM) than those required to inhibit carbonyl cyanide *m*-chlorophenylhydrazone-uncoupled electron transport (2 μM–1.5 mM), which is good evidence that there is a direct effect on the energy-coupling system, as proposed by Kahn (1968). With diphenyleneiodonium sulphate, however, the two effects occur at similar concentrations (10–90 μM), but the observation that ATP formation was inhibited by diphenyleneiodonium sulphate to a greater extent than was uncoupled O_2 evolution suggests either that uncoupling is involved in the inhibition of ATP formation or that there is also direct inhibition of the phosphorylation reactions. Measurements of light-scattering changes and the exclusion of anions effective in the anion–hydroxide exchange have shown that at the concentrations required to inhibit phosphorylation (0.56 μM for tributyltin) neither a detergent-like disruption of the chloroplasts nor the anion–hydroxide exchange (which could produce volume changes and/or pH equilibration) are involved in the inhibition of photophosphorylation by trialkyltin compounds.

At high concentrations the organotin compounds, but not the organomercurial compounds, inhibit O_2 evolution even in the presence of an uncoupler. A similar inhibition at high concentrations is observed in the action of these compounds on basal electron transport. This would appear to be a direct action on the electron-transport system. However, in view of the high concentrations of organometals required this may be a general disruptive effect on the lipoprotein structure rather than a specific inhibition. In contrast with the organometallic compounds, diphenyleneiodonium sulphate inhibits basal and uncoupled electron transport at low concentrations. This inhibition is likely to be a direct action and will contribute to the observed inhibition of phosphorylation reactions, but is of insufficient magnitude to account for all the observed inhibition of ATP formation.

As might be predicted from the observations on mitochondria (Stockdale *et al.*, 1970) and the ability of the chloroplast electron-transport chain to drive protons across the membrane, organotin compounds have been found to act as uncouplers in a medium containing Cl^-. The measurements of phosphate incorporation into ATP have shown that this is true uncoupling, phosphorylation being decreased while electron transport is increased. The extent of release of photosynthetic control was not great; this may be due to slight inhibition of electron transport by the organometals and the low rate of permeation by Cl^-. With triethyltin the great decrease in uncoupling activity on substitution of NO_3^- or SO_4^{2-} for Cl^- demonstrates the role of the anion–hydroxide exchange in uncoupling.

Complex phases of inhibition and stimulation of O_2 evolution were observed with several organometals when their effects on the basal (state II) rate of O_2 evolution and on the controlled (state IV) rate were studied.

It seems that the compounds exhibit three types of action: inhibition of phosphorylation, uncoupling and inhibition of electron transport. These will all cause a decrease in phosphorylation, but electron transport may be either stimulated or inhibited according to the concentration of compound used and the relative effectiveness of the different actions. For example with tributyltin at low concentrations (0.56 μM) inhibition of phosphorylation predominates and inhibition of electron transport is observed. At intermediate concentrations (2 μM) uncoupling is more effective and stimulation of electron transport is seen, and at high concentrations (1 mM) the direct inhibition of electron transport over-rides the uncoupling effect.

The three types of action exhibited by organometallic and diphenyleneiodonium compounds described above change not only with the concentrations used but also with alteration of the pH of the medium in which the chloroplasts are suspended. At pH 7.6 and 8.0 the selected concentrations of organometals inhibited photophosphorylation. At pH 7.0 and 6.5 the same concentrations of organometals produced stimulation of electron transport, presumably by uncoupling. At pH 6.0 no further stimulation could be observed, but inhibition of electron transport was seen. These effects may be accounted for on the following basis. First over the pH range 6.0–8.0 the effectiveness of the trialkyltin compounds as Cl^-–OH^- antiporters increases with lowering pH (Coleman & Palmer, 1971). Secondly, the natural Cl^- permeability increases as the pH is lowered from 8.0 to 6.0 (Packer *et al.*, 1966; Watling, 1972). An additional fact is

that as the pH falls to a value of 7.0 ATP formation decreases to a minimum at that pH (Uribe, 1970).

In chloroplasts tributyltin and triphenyltin are relatively more effective than the other organotin compounds as inhibitors of photophosphorylation, and this suggests a hydrophobic environment for their site of action. The inhibitory action of these compounds may be a result of the readiness with which they form covalent links and the involvement of histidine in the binding of trialkyltins suggested by Rose (1969) and Aldridge & Rose (1969) is in accord with this, although it does not preclude the involvement of other groups. The lack of inhibition by the organomercurial compounds does indicate the absence of a reactive thiol group at the inhibitory sites. Since phenylmercuric acetate, which is effective as a mediator of the Cl^-–OH^- exchange, does not inhibit, the lack of inhibition by organomercurials is unlikely to be due to lack of penetration to the active site.

We thank Dr. A. P. Dawson for discussion, the S.R.C. for financial support and the Tin Research Institute for a sample of triethyltin hydroxide. Much of this work was done during the tenure of an S.R.C. Research Studentship by A.S.W.-P.

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