Atractyloside inhibits competitively by displacing ADP from the active site of the translocase, the affinity of the mammalian translocase being 50–100 times higher for atractyloside than for adenine nucleotide (Klingenberg, 1970). Bongkrekic acid, on the other hand, inhibits by increasing the binding of ADP to the translocase (Erdelt et al., 1972).

In plant mitochondria, bongkrekic acid inhibits coupled respiration in the presence of ADP (State 3) and ATPase activity (Passam et al., 1973). The present studies show that the concentration of bongkrekic acid required for maximal inhibition of State 3 respiration is 0.2nmol/mg of protein in Jerusalem-arthichoke (Helianthus tuberosus) mitochondria and 0.4nmol/mg of protein in cauliflower (Brassica oleracea) mitochondria. Similar to its action in mammalian mitochondria, bongkrekic acid exhibits a time-lag before inhibiting O₂ uptake in plant mitochondria, and a 2min preincubation is used as an experimental procedure. Inhibition is critically dependent on the pH of the incubation medium. Maximal inhibition occurs below pH7.0; above pH7.5 no inhibition occurs. Inhibition at pH7.0 may be reversed by raising the pH to above 7.5. The concentration of bongkrekic acid required for maximal inhibition is decreased by the inclusion of ADP or ATP in the incubation medium. Measurements of oxidative and substrate-level phosphorylation show that bongkrekic acid inhibits at the translocase. In contrast, atractyloside fails to inhibit either the state 3 respiration or the adenosine triphosphatase activity of Jerusalem-arthichoke or cauliflower mitochondria, unless added at extremely high concentrations (1–3mm). Carboxyatractyloside (gummiferin) is also without effect on plant mitochondria.

These results are interpreted as indicating that either (a) the plant and animal translocases differ in affinity towards atractyloside, or (b) a factor antagonistic to the action of atractyloside is present within the plant mitochondria.

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Valinomycin Sensitivity of Cytochrome c Oxidase Vesicles
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It is now possible to demonstrate ion transport and respiratory control in vesicles formed from phospholipids and purified cytochrome c oxidase (Hinkle et al., 1972; Racker, 1973; Hunter & Capaldi, 1974). The reconstituted systems can be shown to generate a membrane potential (Jasaitis et al., 1972) and under suitable conditions, show respiration-dependent movements of protons and K⁺ ions (Hinkle et al., 1972). The energy-dependent processes depend on the asymmetric location of substrate (reduced cytochrome c) and are abolished by uncouplers of oxidative phosphorylation.

These reconstituted systems, however, differ from mitochondria and submitochondrial particles in their response to uncoupling agents. Valinomycin is required in addition to a proton porter (carbonyl cyanide trifluoromethoxyphenylhydrazone) for full release of respiratory control. Hinkle et al. (1972) found that carbonyl cyanide trifluoromethoxyphenylhydrazone alone stimulated respiration in vesicles of cytochrome c oxidase and partially purified soya-bean phospholipid by a factor of 4.0. This could be raised to 4.5
times the control respiration rate by the further addition of valinomycin to the carbonyl cyanide trifluoromethoxyphenylhydrazone-stimulated system. The combination of valinomycin and nigericin in the presence of K⁺ ions gave the largest respiratory stimulation. Hunter & Capaldi (1974), using a reconstituted system of cytochrome c oxidase plus a phospholipid mixture extracted from ox heart mitochondria, found an even more marked response of carbonyl cyanide trifluoromethoxyphenylhydrazone-stimulated respiration to valinomycin. An increase in respiratory rate of 2.6 induced by carbonyl cyanide trifluoromethoxyphenylhydrazone alone was raised to 6.7 when carbonyl cyanide trifluoromethoxyphenylhydrazone and valinomycin were used in combination. Valinomycin alone caused only a slight increase in the control respiratory rate.

In this communication we report differences in valinomycin sensitivity between cytochrome c oxidase vesicles prepared by using (i) purified phospholipids from egg yolk and (ii) phospholipids extracted from ox heart mitochondria. Partial inhibition of the oxidase by NaN₃ abolishes the observed differences and induces an inhibitory effect of valinomycin on respiration.

Cytochrome aa₃ was purified from Keilin-Hartree-type ox heart submitochondrial particles by the method of Fowler et al. (1962) as modified by van Buuren (1972). The final preparation had a haem a:protein ratio of 10-12 and a turnover number of 40-70 s⁻¹ in 50mM-potassium phosphate plus 0.5% Tween. Phosphatidylcholine (egg yolk), phosphatidylethanolamine (egg yolk) and cardiolipin (ox heart) were purchased from Lipid Products, Nutfield Nurseries, South Nutfield, Surrey, U.K. Cytochrome c oxidase vesicles (phospholipid: protein ratio of approx. 30) were prepared by sonication essential

![Diagram](image)

**Fig. 1. Changes in aerobic steady-state amounts of cytochrome c reduction on the addition of valinomycin (0.4 µg/ml) and carbonyl cyanide trifluoromethoxyphenylhydrazone (1 µM) to a suspension of cytochrome c oxidase vesicles**

The complete system (3 ml) contained 67mM-potassium phosphate, pH 7.4, 33 µM-cytochrome c, 5 mM-potassium ascorbate and 30 µl of cytochrome c oxidase vesicles (approx. 100 µM-cytochrome aa₃). Increased oxidation of cytochrome c is used as a measure of increased electron flux. (a) Type I vesicles; (b) type II vesicles. FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone.

Vol. 3
ally as described by Racker (1973). The vesicles showed respiratory control ratios (valinomycin plus carbonyl cyanide trifluoromethoxyphenylhydrazone) between 1.7 and 2.3 with externally added cytochrome c and ascorbate as substrate. Two types of vesicles were prepared, differing in the type of phospholipids used. Type I vesicles were formed from purified natural phospholipids (phosphatidylycholine, phosphatidylethanolamine and cardiolipin in the proportions of 2:2:1, by wt., respectively). Type II vesicles were formed by using a phospholipid mixture extracted from ox heart mitochondria as described by Rouser & Fleischer (1967), enriched with egg yolk phosphatidylethanolamine (25%, by wt.). Cytochrome c oxidase activity was monitored by following, with time, changes in exogenous cytochrome c steady-state reduction at 550 nm aerobically in the presence of ascorbate (see legend to Fig. 1). This method of measuring oxidase activity was found to give results in good agreement with oxidase activity measurements obtained with an O2 electrode, and had the added advantage of increased sensitivity.

Fig. 1(a) shows the response of cytochrome c oxidase activity in the two types of vesicles to carbonyl cyanide trifluoromethoxyphenylhydrazone and valinomycin. In type I vesicles (purified natural phospholipids), valinomycin added after carbonyl cyanide trifluoromethoxyphenylhydrazone increases the carbonyl cyanide trifluoromethoxyphenylhydrazone-stimulated response by a factor of 1.2. This is a valinomycin effect similar to that observed by Hinkle et al. (1972) in oxidase vesicles prepared by using partially purified soya-bean phospholipids. Valinomycin added to the system before carbonyl cyanide trifluoromethoxyphenylhydrazone has no significant effect on the steady-state cytochrome c reduction value. Type II vesicles (mitochondrial phospholipids) can be seen to show a much greater sensitivity to valinomycin (Fig. 1b). The carbonyl cyanide trifluoromethoxyphenylhydrazone-stimulated response is increased by a factor of 4.0

![Diagram](image)

Fig. 2. Changes in steady-state amounts of cytochrome c reduction on the addition of valinomycin and carbonyl cyanide trifluoromethoxyphenylhydrazone to a suspension of cytochrome c oxidase vesicles in the presence of Na3Azide (167 μM)

Conditions of assay as in Fig. 1 except for the ascorbate concentration which was decreased to 1.67 mM. (a) Type I vesicles; (b) type II vesicles. FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone.
on the addition of valinomycin. This more sensitive effect is similar to that reported by Hunter & Capaldi (1974) in oxidase vesicles prepared by using a phospholipid extract from mitochondria. The stimulation of respiration by valinomycin can also be seen to occur in our vesicles when the ionophore is added before carbonyl cyanide trifluoromethoxyphenylhydrazone. There is no evidence of any significant synergistic effect.

Partial inhibition of respiration with NaN₃ abolishes the differences in valinomycin effect between the two types of vesicles (Fig. 2) and induces an inhibiting effect of valinomycin on oxidase activity. In 166μM-Na₃ (approx. 45% inhibition of respiration). Valinomycin added either before or after carbonyl cyanide trifluoromethoxyphenylhydrazone causes a rise in steady-state cytochrome c reduction values.

The present results confirm the reported differences between the valinomycin sensitivity of cytochrome c oxidase vesicles prepared by using purified natural phospholipids (in this case from egg yolk) and those prepared by using an extract of mitochondrial phospholipids. The latter appear to be particularly sensitive to valinomycin. The partial release of respiratory control by valinomycin indicates a controlling effect of K⁺ on respiration. One way in which this could arise would be if the vesicles possessed a capability for proton-cation exchange, allowing a partial conversion of an electrogenic proton movement into an electrogenic K⁺ translocation. Addition of valinomycin as well as carbonyl cyanide trifluoromethoxyphenylhydrazone would then be required for full release of respiratory control. On this interpretation, the difference in valinomycin sensitivity between the two types of vesicles could result from differences in K⁺/H⁺ exchange capacity. After azide inhibition, respiration is inhibited by valinomycin added either before or after carbonyl cyanide trifluoromethoxyphenylhydrazone in both types of vesicles. This could indicate the partial conversion of membrane potential into an osmotic gradient of a potassium salt, in this case KN₃ (Palmieri & Klingenberg, 1967).

The discharge of the gradient by valinomycin would then induce a diffusion potential inhibitory to the cytochrome c oxidase reaction.

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Stimulation of Electron Transport and Activation of Reduced Nicotinamide-Adenine Dinucleotide Dehydrogenase in Jerusalem-Artichoke Mitochondria

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Isolated plant mitochondria respond in a very complex manner to the addition of adenine nucleotides and weak acid uncoupling agents. Raison et al. (1973) showed that ADP was capable of stimulating the oxidation of citrate in the presence of sufficient oligomycin to completely inhibit oxidative phosphorylation. Laties (1973) reported that ADP was necessary to obtain a maximal response to the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone.

Results obtained in our laboratory confirm these observations and support the suggestion that adenine nucleotides have a role in enhancing electron transport in addition to

Vol. 3