DINITROPHENOL AND VALINOMYCIN AS UNCOUPLERS IN ISOLATED CHLOROPLASTS

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Received 7 May 1968

The classical uncoupler of oxidative phosphorylation, 2, 4-dinitrophenol (DNP) has generally been observed to inhibit, rather than uncouple [1-3], photosynthetic electron transport and ATP synthesis. High concentrations of DNP, nevertheless, were reported to exert a weak uncoupling effect in chloroplasts under special conditions [4]. The antibiotic valinomycin was observed to be ineffective in chloroplasts [5], although a highly active uncoupler in mitochondria [6].

It has been suggested that uncouplers may function in energy transducing systems by increasing the permeability of membranes to transported ions [7-9]. From studies in both mitochondria [9-13] and artificial lipid membranes [13] DNP and valinomycin were inferred to raise the membrane's permeability to protons and potassium ions, respectively. In similar studies [13-16] gramicidin and nigericin, which are very good uncouplers in chloroplasts [5,17], have been inferred to increase permeability to *both* protons and monovalent cations (such as K^+).

Assuming that permeability to both protons and a counter-transported ion (K^+ or Na^+) into the chloroplasts must be raised for uncoupling activity to be manifested, we considered the possibility that DNP and valinomycin may uncouple, only when added together. The experiments reported here demonstrate that DNP and valinomycin indeed uncoupled, while either compound alone was ineffective. Table 1 demonstrates that DNP and valinomycin together efficiently uncoupled the chloroplasts. ATP synthesis and the associated electron transport (+ Mg, ADP, P_i) were somewhat inhibited by either DNP or valinomycin alone, but the two agents together lead to stimulation of electron transport and complete inhibition of phosphorylation. Basal electron transport (−Mg, ADP, P_i) was stimulated two to four fold in different experiments.

Fig. 1 shows the effect of DNP and valinomycin on the extent of the light-induced proton uptake into chloroplasts [19,20]. Like other uncouplers, the combination DNP and valinomycin abolished the proton uptake, although, as seen from fig. 1, either agent alone at these concentrations had no effect.

In fig. 2 the extent of the proton uptake is plotted as a function of KCl concentration for a medium containing only KCl, and as a function of the KCl to NaCl ratio for a medium of constant ionic strength. As can be seen, the uncoupling by DNP and valinomycin with KCl alone was essentially complete down to 8 mM KCl. The well documented specificity of valinomycin to K^+ rather than to Na^+ [13,14] is demonstrated by the complete lack of uncoupling in a medium containing only Na^+.

In the medium with Na^+ and K^+, Na^+ somewhat inhibited the uncoupling by DNP + valinomycin. Thus, with 8 mM KCl, DNP and valinomycin were more inhibitory in the presence of KCl alone than in the presence of KCl and NaCl. This inhibition by Na^+ was not competitive with K^+ (not shown). No uncoupling of the proton uptake was observed when $MgCl_2$ replaced KCl. However, studies with KCl and $MgCl_2$ mixtures of the type recorded in fig. 2 indicated that $MgCl_2$, in contrast to NaCl, did not inhibit the uncoupling by DNP + valinomycin.

The combination of DNP and valinomycin at the appropriate concentrations, progressively reduced the extent of the photo-induced light scattering

Abbreviations: DNP, 2, 4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Table 1
Uncoupling of photophosphorylation by DNP + valinomycin.

Additions	Electron Transport (No Mg, ADP, P_i) μmoles electrons /mg Chl/hr	Electron Transport (With Mg, ADP, P_i) μmoles electrons /mg Chl/hr	ATP Synthesis (With Mg, ADP, P_i) μmoles ATP /Mg Chl/hr	P/2e⁻
None	104	296	150	1.02
+ DNP (33 μM)	113	278	152	1.09
+ DNP (100 μM)	113	235	119	1.01
+ Valinomycin (0.1 μM)	104	252	133	1.05
+ Valinomycin (0.33 μM)	100	209	83	0.80
+ DNP (33 μM) + Valinomycin (0.33 μM)	161	213	39	0.36
+ DNP (100 μM) + Valinomycin (0.33 μM)	252	291	6	0.04

The reaction mixture contained in 3.0 ml, in micromoles: tricine, pH 7.5, 45; KCl 60; potassium ferricyanide, 1.5; and chloroplasts prepared as previously described [5] containing 33 micrograms of chlorophyll. $MgCl_2$ 12; potassium phosphate, pH 7.5, 12 (containing 5×10^6 cpm $^{32}P_i$) and ADP, 2, were included where indicated. The reaction mixture was illuminated with 160,000 lux of white light for 2 minutes. ATP and ferrocyanide formed were assayed as previously described [5].

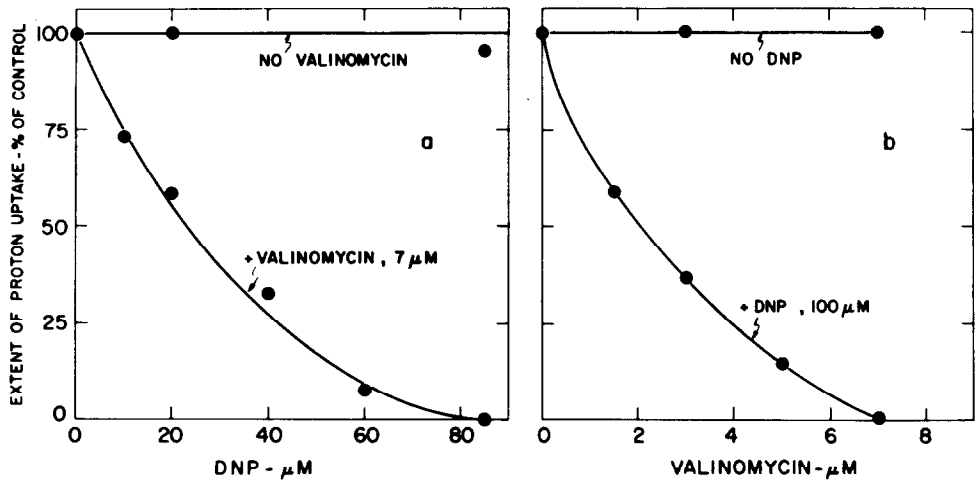

Fig.1. Effect of DNP and valinomycin on light-induced proton uptake into chloroplasts. The preparation of the chloroplasts and methods were as described before [18]. The reaction mixture contained, in a total volume of 2.5 ml, chloroplasts containing 50 μg chlorophyll/ml; KCl, 32 mM and pyocyanine, 15 μM. The initial pH was adjusted to 6.5. DNP and valinomycin were added at the concentrations indicated. Control activity was 0.43 μmoles H^+ taken up per mg chlorophyll.

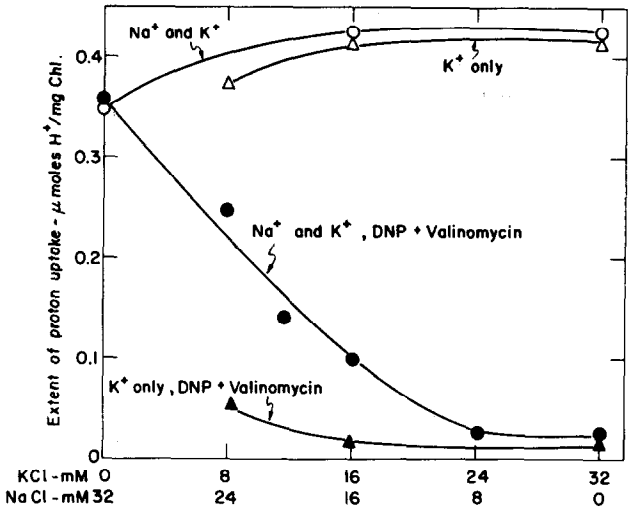

Fig.2. Effect of KCl and NaCl on the DNP and valinomycin uncoupling of light-induced proton uptake. The reaction mixture was as in fig. 1, except that KCl or KCl and NaCl concentrations were varied as indicated. DNP and valinomycin were added, where indicated, at a concentration of 100 μM and 7.0 μM, respectively.

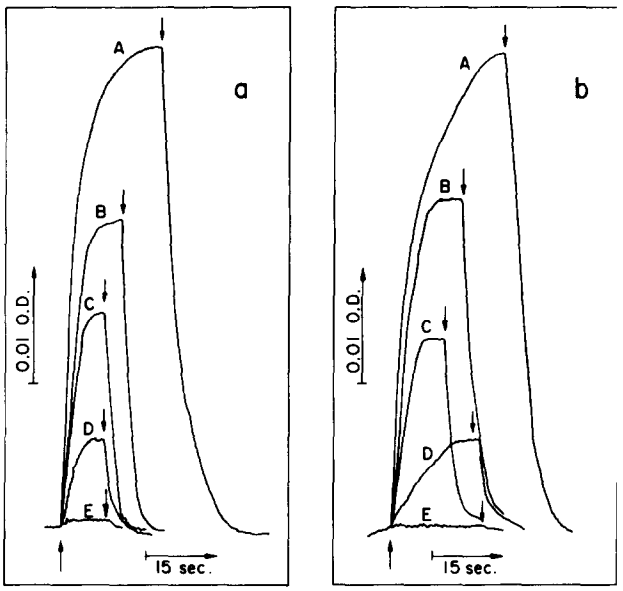

Fig.3. Effect of DNP and valinomycin on photoinduced light-scattering changes in chloroplasts. The reaction mixture was as in fig. 1 with the addition of tricine, 15 mM, pH 6.5. Light-induced scattering changes were measured at 538 mμ, in a Cary 14 spectrophotometer. Illumination was provided by light from a 500 watt projector through a 700 mμ (30 mμ half band width) interference filter. The phototube was protected from the actinic light by a 4-96 Corning filter. The concentrations of DNP and valinomycin added were as follows: (a) Valinomycin 7.0 μM; DNP: A, O; B; 10 μM: C, 25 μM; D, 50 μM; E, 100 μM. (b) DNP, 100 μM; valinomycin: A, O; B; 0.5 μM; C, 1.0 μM; D, 1.5 μM; E, 3.0 μM.

changes (fig. 3) while either compound alone had no effect. This uncoupling activity was again specific to K^+, rather than Na^+. Some other uncouplers, like FCCP and gramicidin have been observed to similarly affect light induced scattering changes, but apparently in a manner non specific towards ions [16,21].

We have recently observed [22] that concentrations of the uncoupler FCCP which alone are ineffective in uncoupling chloroplast reactions (around 10^{-8} M) became fully uncoupling on addition of valinomycin, FCCP has been deduced to increase membrane permeability to protons in various systems [13,14], and the couple FCCP and valinomycin thus seems similar to DNP and valinomycin. The observation that FCCP by itself uncouples chloroplasts around 10^{-6} M [5], may mean that at such a concentration it also permits the rapid penetration of Na^+ and/or K^+ across the chloroplast membrane.

The experiments reported here when considered in view of the available information are consistent with the hypothesis that uncoupling of chloroplasts by DNP or FCCP and valinomycin, gramicidin or nigericin, is caused by an increase in membrane permeability to protons and a cation (K^+ or Na^+ as the case may be). We have supposed, as a working hypothesis, that light-induced proton uptake implies the formation of a proton gradient driven by energy conserved as a high-energy intermediate ~ X [18]. In the presence of agents, raising permeability to H^+ and K^+, protons pumped into the relevant chloroplast space may exit by rapid exchange with an external K^+. This K^+ ion, now inside, may then itself exit by exchange with the next H^+ pumped in, and so on. Such consecutive exchange of K^+ into and out of the chloroplast, will prevent the establishment of any significant net proton gradient, while demanding a constant drain on the energy conserved as ~ X. Thus, the typical uncoupling effects will result.

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