2-*p*-TOLUIDINONAPHTHALENE-6-SULPHONATE (TNS)

An energy-transfer inhibitor in chloroplasts

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1. Introduction

The amphipathic fluorescence probe TNS has been used to investigate the surface electrical properties of chloroplast thylakoid membranes [1]. When TNS interacts with chloroplast membranes its fluorescence yield increases and its emission maximum shifts from 550 nm to ~440 nm. Such an increase in fluorescence (although to a smaller extent) was observed also with ANS.

Illumination of bacterial chromatophores causes an increase in fluorescence of ANS; this fluorescence change is closely related to the energisation of the membrane [2]. No such changes in fluorescence of ANS or TNS have been observed by us (unpublished results), or reported by others, in chloroplast preparations. It is possible that this difference reflects the absence of a significant membrane potential across chloroplast thylakoids under steady state illumination [3]; however, it also prompted us to investigate whether TNS has any direct effect on chloroplast reactions. In the course of these studies we observed that in chloroplasts TNS acts as an energy transfer inhibitor and with progressive illumination also as an uncoupler.

Abbreviations: TNS, 2-*p*-toluidinonaphthalene-6-sulphonate; ANS, 1-anilinonaphthalene-8-sulphonate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MV, methylviologen; DPIP, 2,6-dichlorophenolindophenol; Tricine, *N*-Tris (hydroxymethyl) methylglycine

2. Materials and methods

Chloroplasts were prepared from lettuce leaves as in [4]. Chlorophyll was determined following the method in [5]. Electron flow was measured by monitoring the changes in oxygen concentration with a YSI Clark-type oxygen electrode. Actinic illumination was provided with a 500 W projector lamp. All experiments were performed at a constant temperature of 25°C. Photophosphorylation was measured by the incorporation of ^{32}P into ATP according to [6]. Ca-ATPase was assessed either in trypsin-treated chloroplasts, or in an isolated and partially purified coupling factor [7]. The reaction was measured by following the release of P_i according to [8].

2-*p*-toluidinonapthalene-6-sulphonate, potassium salt was purchased from Sigma Chemicals Co.; it was dissolved in ethanol : water (1 :1, v/v) and stored as a 1% solution at 0°C in the dark.

3. Results and discussion

Light-dependent ATP synthesis in chloroplasts can be inhibited in three different ways:

(i) By blocking electron transport;
(ii) By uncoupling ATP synthesis from electron transport;
(iii) By interfering with the process of ATP synthesis proper.

Compounds which belong to the third category are designated, energy transfer inhibitors [9].

According to several criteria (described below)

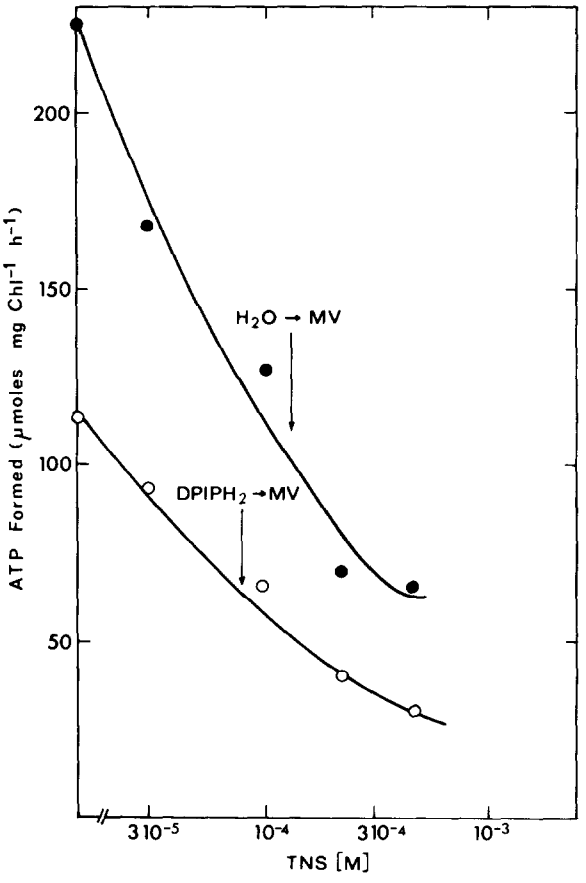

Fig 1 Effect of TNS on ATP formation The reaction mixture contained in 2 0 ml 20 mM NaCl, 30 mM Tricine (pH 7 5), 1 mM $MgCl_2$, 1 mM NaN_3, 3 mM P_i labelled with ^{32}P, 1 mM ADP, 0 2 mM MV, chloroplasts equivalent to 60 μg chlorophyll The reaction mixture for testing PSI phosphorylation contained in addition 2 mM ascorbate, 0 01 mM DCMU, 0 2 mM DPIP Illumination was performed for 60 s with white light filtered through a water bath The light intensity at the surface of the vessel was 6 × 10^5 ergs cm^{-2} s^{-1}

TNS acts as an energy transfer inhibitor In fig 1, it is shown that TNS inhibits the rate of ATP formation Approximately the same inhibition is obtained whether ATP formation is supported by electron flow through both photosystems ($H_2O \rightarrow MV$) or by photosystem 1 alone ($DPIPH_2 \rightarrow MV$, in the presence of DCMU), 0 1 mM TNS causes ~50% inhibition These (and subsequent) reactions were performed at pH 7 5 (although it is suboptimal for electron transport and ATP formation in lettuce chloroplasts), since the inhibition by TNS is more pronounced at pH 7.5 than at higher pH values

'Coupled' electron transport (i e , electron transport in the presence of phosphorylating reagents) is strongly inhibited by 80 μM TNS and the inhibition is released by adding 2 mM NH_4Cl (fig 2A) In comparison, 'basal' electron flow (in the absence of ADP and P_i) is inhibited by the same concentration of TNS to a much smaller extent (fig 2B) Finally, TNS does not inhibit 'uncoupled' electron flow, i e , electron flow which is stimulated by the addition of 2 mM NH_4Cl (fig 2C)

For comparison, the effect of TNS was studied also in pea chloroplasts and a similar pattern was observed. In pea chloroplasts also ANS was found to inhibit 'coupled' electron flow, and the inhibition was released by NH_4Cl

Various treatments are known which cause an induction of ATPase activity in chloroplasts or in the isolated coupling factor, CF_1 [10] Hydrolysis of ATP under these conditions is catalyzed by the same enzyme which 'normally' acts as an ATP synthase during phosphorylation [10] We tested the effect of TNS on Ca-ATPase which was induced by trypsin in chloroplasts or by dithiothreitol and a heat treat-

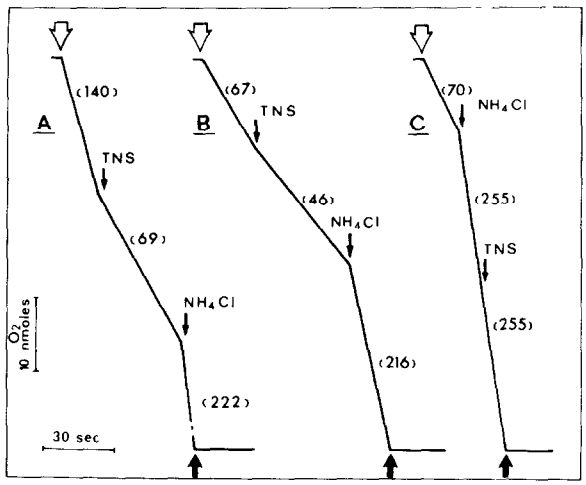

Fig 2 Effect of TNS on electron-transport Reaction mixture of experiment 'A' as in fig 1, in experiment 'B' and 'C' P_i and ADP were omitted Where indicated, NH_4Cl at 2 mM and TNS at 80 μM were added The numbers in parentheses denote the rate of electron flow in μequivalents mg chlorophyll^{-1} h^{-1}

ment in CF_1 [7]. As shown in fig.3, both preparations are inhibited to the same extent by TNS; 0.2 mM TNS caused 50% inhibition of Ca-ATPase activity. From these results we conclude that the primary effect of TNS in chloroplasts is an interference with ATP synthase. The interference with ATP synthase activity can account for the inhibition of ATP synthesis and the inhibition of 'coupled' electron flow. In these respects TNS resembles the energy transfer inhibitors Dio-9 [11], phlorizin [12] and efrapeptin [13]; but it differs from other energy transfer inhibitors like DCCD [12] and triphenyltin chloride [14] which do not inhibit Ca-ATPase and seem to inhibit ATP synthesis by affecting another site in the thylakoid membrane closely related to the energy conservation step [12,14].

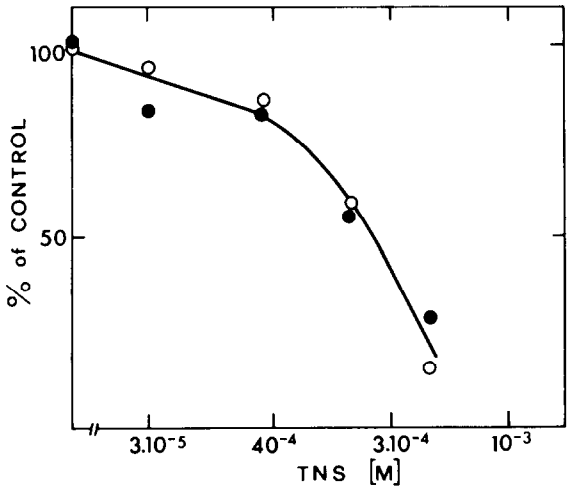

Fig.3. Effect of TNS on Ca-ATPase. The reaction mixture for the 'activation step' contained in 1.0 ml: Tris buffer 40 mM (pH 8.0); EDTA 2 mM; ATP 1 mM; chloroplasts equivalent to 200 μg chlorophyll; the activation was started with the addition of 800 μg trypsin and it was carried on for 10 min at 25°C; the activation process was terminated by the addition of 1.6 mg trypsin inhibitor. The isolation and purification of the coupling factor, up to and including the ammonium sulphate gradient step, was performed according to [7]. Activation was carried on for 4 min at 65°C in the presence of dithioerythrol [7]. Ca-ATPase activity was assessed for 20 min at 37°C in a reaction mixture which contained ATP 5 mM, $CaCl_2$ 5 mM, tricine 30 mM (pH 7.5) and either chloroplasts equivalent to 20 μg chlorophyll or CF_1 equivalent to 12 μg protein. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 3%. Inorganic phosphate was assayed according to [8]. Control rates: Ca-ATPase of chloroplasts was 36 μmol P_i hydrolyzed.mg chlorophyll^{-1}.h^{-1}; Ca-ATPase of CF_1 was 169 μmol P_i hydrolyzed.mg protein^{-1}.h^{-1}.

We have studied also the effect of TNS on light-induced proton uptake in the $H_2O \rightarrow MV$ system. When the extent of light-induced proton uptake was measured, an increase of ~50% was observed between pH 6.6, and pH 7.8. These results are similar to those obtained with Dio-9 [15,16]. When TNS was added to the reaction mixture after turning the light off, it did not affect the rate of proton efflux. However, when TNS was present during illumination (i.e., when added before the onset of the reaction), it did cause a subsequent stimulation of proton efflux in the dark; (the stimulation at pH 6.6 was 66%). This stimulation is presumably due to the fact that TNS has, in addition to its 'energy transfer' inhibitory activity, some uncoupling activity which increases with time of illumination of chloroplasts. Consequently, we studied the effect of TNS on electron transport and ATP formation as a function of length of illumination. In the results (fig.4) it can be seen that with increasing time of illumination there is (i) a decrease in P/2e and (ii) a decrease of extent of inhibition of electron transport. Both facts are indicative of uncoupling. In additional experiments we have observed that illumination of TNS alone does not

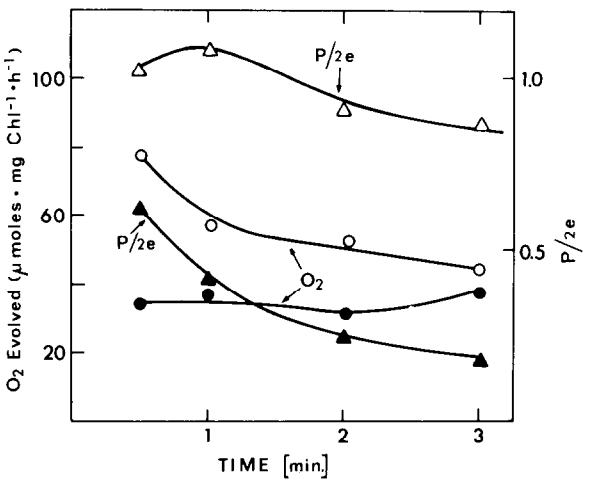

Fig.4. Effect of TNS on electron flow and P/2e as a function of time of illumination. Experimental conditions as in fig.1 in the $H_2O \rightarrow MV$ reaction: Open circles, control; Full circles, TNS 0.22 mM.

change its action on chloroplasts A time-dependent progressive uncoupling has also been observed with some trialkyltin compounds [14]

In [1] it was shown that the increase in fluorescence of TNS on addition of chloroplasts in the dark was further augmented by the presence of various cations The cations presumably shielded the negative charge on the thylakoid membrane and decreased the repulsion between the negatively charged anion TNS and the membranes, such a reduction in charge repulsion allowed a stronger hydrophobic interaction between TNS and the thylakoids On the other hand, addition of $MgCl_2$ (at the same concentration range) to the chloroplast system studied here did not change the extent of inhibition of phosphorylation by TNS (fig 5) This experiment was performed in the presence of a low salt concentration (~6 mM monovalent ions) in order to make it comparable to that in which TNS interaction with the membranes was studied in the dark [1] We therefore conclude that the specific inhibition of ATP-synthase by TNS studied here is in addition to the general hydrophobic interaction reported [1] In the former, the anionic part of TNS may play an important role since the binding sites for ADP-Mg and for P_i are obviously positively charged In support of the notion that the anionic part of TNS plays a role in inhibition is our finding that the inhibition of ATP formation by TNS is competitive with phosphate

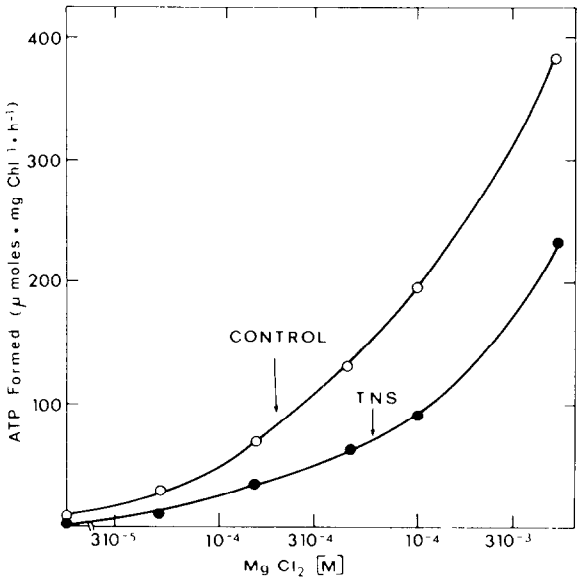

Fig.5 Effect of TNS on ATP formation at various $MgCl_2$ concentrations The reaction mixture contained in 2 0 ml tricine 20 mM (pH 7.5), ADP 0 5 mM, P_i 1 mM, NaN_3 1 mM, MV 0 2 mM, chloroplasts equivalent to 60 μg chlorophyll

4. Conclusions

TNS was shown to act as an energy transfer inhibitor in chloroplasts In addition it has uncoupling activity which increases progressively with time of illumination

The energy transfer inhibition activity of TNS seems to be due to the effect of TNS on CF_1 The binding of TNS, which has fluorescence properties, to CF_1 may be useful in further studies of the molecular structure and activity of CF_1

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