# INHIBITION OF PHOTOPHOSPHORYLATION BY β-BROMO-β-NITROSTYRENE

P.C. BRANDON

*Philips Research Laboratories, N.V. Philips' Gloeilampenfabrieken, Eindhoven, The Netherlands*

Received 9 February 1971
Revised version received 21 March 1971

## 1. Introduction

Inhibition of energy transfer in photophosphorylation is revealed by inhibition of phosphorylation and the associated electron transport; electron transport in the absence of phosphate is not or only slightly inhibited.

The known energy-transfer inhibitors in photophosphorylation can be divided into two groups: (a) Compounds acting on terminal steps in the phosphorylation mechanism; this inhibition of coupled electron transport is relieved by addition of uncouplers. (b) Compounds acting on the initial steps of the phosphorylation mechanism, thus on a site close to the electron transport chain; inhibition is not abolished by addition of uncouplers. Examples of group (a) are phlorizin [1], Dio-9 [2], *N,N'*-dicyclohexylcarbodiimide [3], chlorotri-*n*-butyltin [4], and synthalin [5]; examples of group (b) are 4,5,6,7-tetrabromo-2-trifluoro-methylbenzimidazole [6], and 1-ethyl-3-thiocyanato-indole [7].

In the present communication a new energy-transfer inhibitor, β-bromo-β-nitrostyrene (BNS) is reported; its mode of action is intermediate between that of the two groups of known energy-transfer inhibitors. Binding by BNS of the non-phosphorylated high-energy intermediate in the energy-conversion mechanism is proposed.

## 2. Materials and methods

Spinach chloroplasts were isolated according to Izawa and Good [8]. Ferricyanide reduction and ATP formation were measured as described before [7]. Phosphorylation driven by acid-base transition was performed exactly as by Miles and Jagendorf [9]. Light-induced proton uptake was determined as described previously [7]. $Ca^{2+}$-dependent ATPase was activated in chloroplasts by treatment with trypsin [10]. Chloroplasts were, therefore, isolated in the absence of $Mg^{2+}$, they (50 μg chlorophyll) were pre-incubated for 5 min at 30° in the presence of 20 μmoles tris (pH 8.0), 5 μmoles $CaCl_2$ and 50 μg trypsin, 5 μmoles of ATP were then added and the incubation continued for 15 min. To trigger $Mg^{2+}$-dependent ATPase activity, chloroplasts (40 μg chlorophyll) were illuminated for 1 min at 20° with 20 mW/cm² of red light in the presence of 0.1 μmole PMS, 10 μmoles $MgCl_2$, 15 μmoles dithiothreitol, and 20 μmoles tris (pH 7.8). 10 μmoles ATP were added and the incubation continued for 10 min in light or dark. Inorganic phosphate was determined as described by Marsh [11]. BNS was obtained from N.V. Philips-Duphar (section phytosynthese), Weesp, The Netherlands. The compound was added as a methanolic solution; final concentration of methanol in the reaction mixtures was 1% (v/v).

## 3. Results and discussion

The effect of BNS on photoreduction of ferricyanide in the presence and absence of Pi is shown in fig. 1. Phosphorylation accompanying reduction of ferricyanide in the presence of Pi is almost completely abolished by BNS ($10^{-4}$ M). The electron transport rate is reduced to that in the absence of Pi (the basal rate), this basal rate being only weakly affected by BNS. Cyclic phosphorylation (PMS-catalyzed) is also

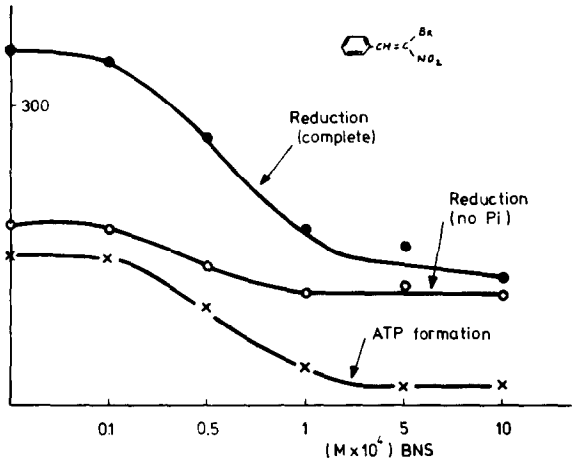


Fig. 1. The effect of BNS on photoreduction of ferricyanide. Reaction medium and conditions are outlined in [7]. Activities are given in μmoles/hr/mg chlorophyll.

inhibited; 60% inhibition was obtained with 0.1 mM BNS. These observations suggest that BNS acts as an energy-transfer inhibitor.

Inhibition of the energy-conversion mechanism may imply an inhibition of the initial reactions (production of a non-phosphorylated high-energy intermediate), or inhibition of the terminal reactions (utilizing this intermediate for ATP synthesis). In the latter case, a rapid decomposition of the non-phosphorylated high-energy intermediate by addition of uncouplers eliminates the terminal reactions. As shown in table 1A addition of the uncouplers $NH_4Cl$, methylamine, atebrin, or triton X-100, relieves inhibition by BNS, therefore localizing its site of action to terminal reactions in the energy-conversion mechanism. Uncoupling by arsenate is thought to originate by formation of an unstable ADP $\sim$ $AsO_4$ complex [12] at the site of phosphate entry in the terminal reactions. In accordance with Avron and Jagendorf [12], stimulation of the basal rate of electron transport was less with arsenate than with phosphate (table 1B). This arsenate-stimulated electron transport rate was decreased to the basal rate by addition of BNS. Thus the site of action of BNS is prior to that of phosphate entry.

ATP synthesis, not involving electron transport but driven by a pH gradient, [9] was also inhibited by BNS (table 2). Inhibition (only 40%) of this kind of ATP synthesis was less than the electron transport

Table 1
The effect of uncouplers on inhibition of electron transport by BNS.

| Additions | Ferricyanide reduction (μmoles/hr/mg chlorophyll) |
|---|---|
| *Experiment A* | |
| – | 100 |
| 0.1 mM BNS | 60 |
| 5 mM $NH_4Cl$ | 458 |
| 0.1 mM BNS + 5 mM $NH_4Cl$ | 394 |
| 10 mM methylamine | 470 |
| 0.1 mM BNS + 10 mM methylamine | 460 |
| 0.1 mM atebrin | 280 |
| 0.1 mM BNS + 0.1 mM atebrin | 184 |
| 0.007% Triton X-100 | 234 |
| 0.1 mM BNS + 0.007% Triton X-100 | 194 |
| *Experiment B* | |
| – | 388 |
| minus Pi | 196 |
| minus Pi + 0.1 mM BNS | 158 |
| arsenate substituted for Pi | 294 |
| arsenate + 0.1 mM BNS | 184 |

Reaction medium and conditions are described in [7]. Experiment A was performed at pH 7.8, because of the uncoupler-induced 'acid shift' [13] in pH optimum for electron transport. Under our conditions this pH optimum was around 7.8. Experiment B was performed at pH 8.4; the P/2e ratio of the control was 0.94; in the absence of Pi or with arsenate no phosphorylation was observable.

driven phosphorylation. However, a far greater amount of chloroplasts (250 μg of chlorophyll compared to 40 μg in the electron transport driven phosphorylation) was used in the acid–base transition, while the BNS concentration could not be increased because of its limited solubility in water. Using 250 μg chlorophyll in the experiments with ferricyanide, 34% inhibition was obtained with the same BNS concentration. Because electron-transport activity in phosphorylation driven by acid–base transition is highly improbable [9], the action of BNS as an energy-transfer inhibitor can be safely assumed.

Table 2
The effects of BNS on phosphorylation by acid–base transition.

| | (ATP nmoles per mg chlorophyll) |
|---|---|
| Control | 126 |
| 0.5 mM BNS in acid phase[a] | 72 |
| 0.5 mM BNS in base phase[a] | 76 |

[a] The final concentration of BNS after mixing the acid and base phase was 0.25 mM.

Table 3
The effect of BNS on light-induced proton uptake.

| | Total pH rise (pH units) | pH rise/sec[a] | pH decay/sec[a] |
|---|---|---|---|
| Control | 0.64 | 0.068 | 0.052 |
| Control[b] | 0.60 | 0.070 | 0.056[b] |
| + 0.5 mM BNS | 0.21 | 0.020 | 0.019 |

[a] Rise or decay in pH units per sec, taken from the linear change in pH during the first 5 sec immediately after the start or the end of illumination.

[b] 0.5 mM BNS was added at the end of the illumination period.

Table 4
The effect of BNS on $Ca^{2+}$- and $Mg^{2+}$-dependent ATPase activities in chloroplasts.

| ATPase dependent type | Additions | μmoles Pi/hr/mg chlorophyll |
|---|---|---|
| | *Experiment A* | |
| $Ca^{2+}$ | – | 54.5 |
| | BNS[a] | 65.5 |
| | *Experiment B* | |
| $Mg^{2+}$ | – | 50.5 |
| | BNS[a] | 58.1 |

[a] 0.1 mM BNS.

The effect of BNS on the light-induced pH rise [14] is shown in table 3; proton uptake was inhibited, this inhibition not being caused by an acceleration of proton efflux.

The effects of BNS on chloroplast ATPase activity – activity representing the terminal reactions of the energy-conversion mechanism – are listed in table 4. BNS caused a slight stimulation of both types of ATPase. The effects of BNS differ from those of energy-transfer inhibitors acting on terminal steps in the energy-conversion mechanism (group (a) in the Introduction). These inhibitors have no effect on proton uptake but inhibit ATPase activity; with BNS the reverse is observed. There is also no agreement with energy-transfer inhibitors acting on the initial steps of the energy-conversion mechanism (group (b) in the Introduction). The actions of these inhibitors are not relieved by uncouplers, whereas the effect of BNS is abolished by addition of uncouplers.

The only explanation for the effect of BNS seems to be binding of the non-phosphorylated high-energy intermediate by BNS. With the concentrations used, the affinity of BNS for this intermediate must be higher than that of ADP, but lower than that of uncouplers.

## Acknowledgements

The author is indebted to Miss J.Claassen and Mrs. L.Salters for skilful technical assistance.

## References

[1] S.Izawa, D.Winget and N.E.Good, Biochem. Biophys. Res. Commun. 22 (1966) 223.
[2] R.E.McCarty, R.J.Guillory and E.Racker, J. Biol. Chem. 240 (1965) PC 4822.
[3] R.E.McCarty and E.Racker, J. Biol. Chem. 242 (1967) 3435.
[4] J.S.Kahn, Biochim. Biophys. Acta 153 (1968) 203.
[5] E.Gross, N.Shavit and A.San Pietro, Arch. Biochem. Biophys. 127 (1968) 224.
[6] Z.Gromet-Elhanan, Arch. Biochem. Biophys. 123 (1968) 447.
[7] P.C.Brandon, Arch. Biochem. Biophys. 138 (1970) 566.
[8] S.Izawa and N.E.Good, Biochim. Biophys. Acta 162 (1968) 380.
[9] C.D.Miles and A.T.Jagendorf, Biochemistry 9 (1970) 429.
[10] V.K.Vambutas and E.Racker, J. Biol. Chem. 240 (1965) 2660.
[11] B.B.Marsh, Biochim. Biophys. Acta 32 (1959) 357.
[12] M.Avron and A.T.Jagendorf, J. Biol. Chem. 234 (1959) 967.

[13] H.Y.Tsujimoto, B.D.McSwain, R.K.Chain and D.I. Arnon, in: Progress in Photosynthesis Research, Vol. 3, ed. H.Metzner (Tübingen, Germany, 1969) p. 1241.

[14] A.T.Jagendorf and J.Neumann, J. Biol. Chem. 240 (1965) 3210.