DINITROPHENOL AS AN UNCOUPLER OF PHOTOSYNTHETIC PHOSPHORYLATION.[1]

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DNP[2] is one of the best known uncouplers of oxidative phosphorylation. By contrast, it was claimed (Krogmann et al., 1959) that DNP was not an uncoupler, but only an inhibitor of electron flow for spinach chloroplasts. DNP was even found to replace other dyes as a catalyst for PSP (Wessels, 1959 and 1960).

In the experiments of Krogmann et al. (1959), run at pH 7.8, DNP inhibited FeCN reduction (Hill reaction) and its associated phosphorylation to about the same extent. Thus there was only a minor drop in the ATP/2e ratio due to DNP. Very recently we have found that the nature of the effect of DNP depends on the pH of the reaction. At pH 7.0 phosphorylation is selectively inhibited, with for instance a drop in the P/2e ratio from 0.84 down to 0.09 (Table I). At the lower pH, therefore, DNP meets the most crucial test of the definition of an uncoupling reagent.

An examination of the effect of DNP on the FeCN Hill reaction at different pH's is shown in figure 1. Using

1. Contribution number 408 from the McCollum-Pratt Institute.
2. Abbreviations: Tris for tris-(hydroxymethyl) aminoethane; ADP and ATP for adenosine di- and triphosphates; EDTA for ethylenediamine tetraacetic acid; TCA for trichloroacetic acid; DNP for 2,4-dinitrophenol; PSP for photosynthetic phosphorylation, FeCN for potassium ferricyanide.

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chloroplasts already uncoupled by EDTA treatment followed by water extraction (curves with triangles; see Jagendorf and Smith, 1962), DNP is found to inhibit at all pH's. The inhibition is somewhat more severe at higher pH's, however (90% at pH 8.5, down to 50% at pH 6.0). With normal chloroplasts, on the other hand, DNP inhibits electron flow above pH 7.0 but actually stimulates it below pH 7.0 (curves with circles). Thus in the lower pH range DNP meets the

Table I

The effect of DNP on ferricyanide reduction and ATP formation at pH 7.0

DNP molarity	Ferricyanide Reduced S.A.	ATP formed S.A.	ATP/2e
0	554	226	0.84
10^{-4}	539	200	0.75
3.3×10^{-4}	466	125	0.54
6.6×10^{-4}	365	56	0.31
10^{-3}	318	15	0.09

Each tube contained 150 umoles of Tris buffer at pH 7.0, 11 umoles of sodium phosphate at pH 7.0, and 500,000 c.p.m. of carrier free P^{32}. ATP was assayed according to Jagendorf and Smith (1962). Otherwise experimental conditions as in Fig. 1. S.A. = umoles per mg chlorophyll per hour.

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second criterion for an uncoupler, and can indeed stimulate the rate of electron flow in the absence of phosphorylation. The optimum concentration for stimulating electron flow at pH 6.0 is 3.3×10^{-4}M (Table II) which inhibits 20% at pH 8.0. Similar results are found in the presence or absence of ADP. Maximal stimulations are up to 140% of the control rates.

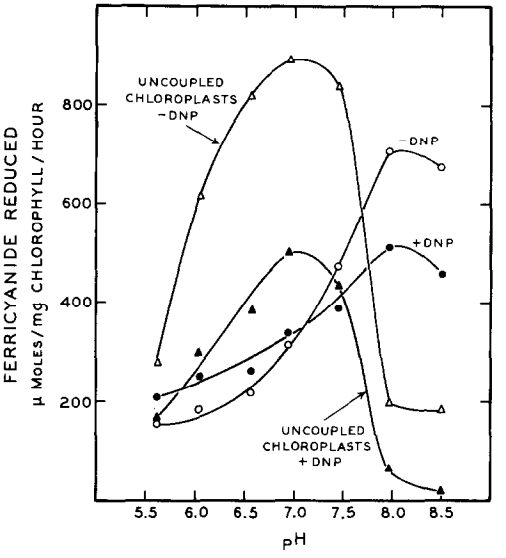

Figure 1. pH curves for the Hill reaction in normal and uncoupled chloroplasts, in the presence and absence of DNP.

Reaction mixtures of 3.0 ml final volume contained 0.045 mg of chlorophyll and in umoles: phosphate 150; Tris 150; NaCl 70; $MgCl_2$ 10, potassium ferricyanide 1.8, ADP 8 and DNP at a final concentration of 3.3×10^{-4}M, where indicated. Reactions were run in test rubes in triplicates exposed to 5000 ft-c of light from an incandescent lamp shielded by a water bath at room temperature, for 2 minutes. Readings of the deproteinized supernatants at 420 mu were always corrected for dark controls with or without DNP.

Table II

The effect of various concentrations of

DNP on ferricyanide reduction

DNP M	Specific Activity pH 6.0	pH 8.0
0	93	588
10^{-4}	142	510
3.3×10^{-4}	214	473
10^{-3}	146	169

150 umoles of phosphate-tris (for each) at the specified pH were added to the reaction mix. No ADP was added. Otherwise the experimental conditions were the same as those of Fig. 1. Specific activity = umoles of ferricyanide reduced per mg chlorophyll per hour.

It is apparent from these data, especially from the curves of figure 1, that DNP has two effects. It is both an inhibitor of electron flow, and an uncoupler with consequent ability to stimulate electron flow in chloroplasts. The concentrations required for these two effects are essentially overlapping; however the uncoupling effect is stronger at the low pH, and the inhibitory effect is stronger at a higher pH. This is really not very different from the case for other uncouplers (see for instance Krogmann et al. 1959, Jagendorf and Smith 1962); however with most other reagents there is a greater separation between the concentration needed for uncoupling and that needed for inhibition. A stronger effect of substituted nitrophenols at lower pH's in stimulating mitochondrial ATPase (Hemker 1962) was interpreted as due to greater solubility of the undissociated molecule in the mitochondrial membranes. Chloroplasts still differ from mitochondria in requiring a higher concentration of DNP (3.3×10^{-4}M) for effective uncoupling and/or inhibition.

These concepts help to resolve several observations. The parallel inhibition of electron flow (FeCN reduction) and of phosphorylation noted by Krogmann et al (1959) occurred at pH 7.8 where the inhibitory effect of DNP is stronger than its uncoupling action. Earlier, Arnon et al. (1956) had found that anaerobic PSP (catalyzed by FMN + menadione) was inhibited more strongly by a given concentration of DNP than was the Hill reaction with benzoquinone as acceptor. Their reactions were performed at pH 7.2 where the uncoupling effect of DNP can stand out more clearly. The recent report that anaerobic cyclic phosphorylation with ferredoxin is inhibited by DNP at 3×10^{-5}M (Tagawa et al., 1963) is based on experiments

at pH 8.3, and thus may involve in part the inhibitory action. Interpretation of the latter experiments is somewhat complicated by the possibility that DNP itself was photo-reduced anaerobically (Wessels, 1960).

Correlative evidence for the uncoupling action of DNP was the finding that it inhibits a light-induced pH rise (Neumann and Jagendorf, 1964) without inhibiting electron flow. This rise in pH seems to be associated with the phosphorylation mechanism, and is easily measurable at pH 6.0.

We believe that the present results do not contradict the function of DNP as a catalyst of PSP (Wessels, 1959, 1960). Wessels experiments were anaerobic; DNP was first photo-reduced to 2-amino-4-nitrophenol, and the latter compound was the effective catalyst of PSP. With our aerobic, ferricyanide containing reaction mixtures DNP would not have been photo-reduced, and so the inhibitory and uncoupling actions of DNP itself could find expression.

Finally we would like to suggest the possibility that isolated chloroplasts might be, normally, partially uncoupled at pH 8.0 but not at pH 6.0. If true, this could help to account for both the failure of DNP to stimulate electron flow at pH 8.0, and the high pH optimum for the "basal" Hill reaction of fresh chloroplasts. The concept is supported by the fact that both the non-phosphorylated energy reserve (Hind and Jagendorf, 1963) and the light-induced pH rise (Neumann and Jagendorf, 1964) are much more unstable at the more alkaline pH. It seems somewhat paradoxical to postulate that chloroplasts are partially uncoupled at the optimum pH for the overall phosphorylation reaction. However this pH optimum is imposed by the final reactions of addition of

phosphate and ADP; these would be rate-limiting for electron flow at the lower pH's.

References

Arnon, D. I., Allen, M. B. and Whatley, F. R. Biochem. Biophys. Acta (1956) 20, 449.
Hemker, H. C. Biochem. Biophys. Acta (1962) 63, 46.
Hind, G. and Jagendorf, A. T. (1963). Proc. Natl. Acad. Sci. (U.S.) 49, 715.
Jagendorf, A. T. and Smith, M. (1962). Plant Physiol., 37, 135.
Krogmann, D. W., Jagendorf, A. T. and Avron, M. (1959). Plant Physiol., 34, 272.
Neumann, J. and Jagendorf, A. T. Arch. Biochem. Biophys. In press.
Tagawa, K., Tsujimoto, K. K. and Arnon, D. I. (1963). Proc. Natl. Acad. Sci. (U.S.) 50, 544.
Wessels, J.S.C. (1959). Biochim. Biophys. Acta 36, 264.
Wessels, J.S.C. (1960). Biochim. Biophys. Acta 38, 195.