THE ACTION OF 2,4-DINITROPHENOL ON CORN ROOT MITOCHONDRIA

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Summary

A study has been made of the action of a wide range of concentrations of 2,4-dinitrophenol (DNP) on the partial reactions of oxidative phosphorylation. DNP at a concentration of 1×10^{-4}M caused classic uncoupling of oxidative phosphorylation. Higher concentrations inhibited phosphorylation and oxidation. The inhibition of oxidation was due to an inhibition of malate dehydrogenase. The P : O ratio was reduced by uncoupling and inhibition of the enzymes involved in ATP formation.

At a concentration of 1×10^{-3}M, DNP has a second peak of stimulation on ATPase activity; mitochondrial swelling was also stimulated but contraction was not affected. Increased swelling at 5×10^{-3}M DNP resulted in disrupted mitochondrial structure and concomitant inhibition of enzyme function, which is shown by an inability of the mitochondria to utilize an energy source to contract, and in an inhibition of ATPase activity.

The action of DNP on mitochondria is not a simple enzyme–substrate–inhibitor interaction but involves a number of sites in the overall mitochondrial function.

I. Introduction

In recent years it has been shown that the high-energy intermediates of oxidative phosphorylation can be studied by means of a number of so-called partial reactions, namely salt uptake (Mg^{2+}, Ca^{2+}, Sr^{2+}), mitochondrial contraction, and ATP phosphohydrolase (ATPase) activity (Lehninger 1964). Hanson and coworkers have studied these reactions extensively in plant mitochondria (Stoner, Hodges, and Hanson 1964; Hodges and Hanson 1965; Stoner 1965). Millard, Wiskich, and Robertson (1964) presented preliminary work on substrate-powered Mg^{2+} and P_i accumulation by beet mitochondria, and there are several reports on studies of plant mitochondrial ATPase (Forti 1957; Reid, Gentile, and Klein 1964).

Stoner, Hodges, and Hanson (1964), to account for the results in corn shoot mitochondria, have suggested the scheme in Figure 1, which is an extension of schematic relationships drawn up by Slater (1962), Charnock, Rosenthal, and Post (1963), Brierley, Murer, and Buchmann (1964), Connelly and Lardy (1964), and Green *et al.* (1964). The scheme may not apply in detail to animal mitochondria, for corn mitochondria readily exhibit spontaneous swelling and will contract rapidly on addition of Krebs cycle intermediates or NADH (Stoner, Hodges, and Hanson 1964).

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Ordinarily, swelling in animal mitochondria is achieved with a variety of swelling agents, and contraction is achieved with ATP or phosphorylating respiration (Lehninger 1964). Furthermore, corn root mitochondria do not require ATP for substrate-driven calcium and phosphate uptake, and the accumulation of phosphate requires calcium; magnesium will not substitute (Hodges and Hanson 1965).

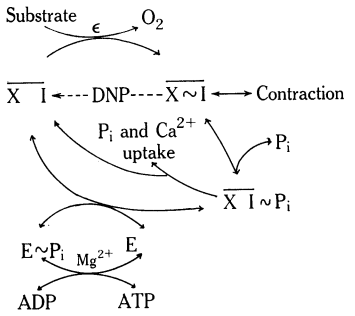


Fig. 1.—Reaction scheme showing alternative use of high-energy intermediates of oxidative phosphorylation (after Stoner, Hodges, and Hanson 1964).

The scheme (Fig. 1) is a modification of the widely used "type I" scheme of Slater (Lehninger and Wadkins 1962). The rationale of Stoner, Hodges, and Hanson (1964) is as follows: an unidentified intermediate $\overline{X\ I}$, essential to the passage of electrons though the coupling sites of the electron transport chain, forms a high energy bond ($\overline{X \sim I}$). This bond is associated in some fashion with contraction. The addition of phosphate yields a high-energy phosphorylated intermediate $\overline{X\ I} \sim P_i$, which in turn passes the phosphate to ADP, regenerating $\overline{X\ I}$. An alternative use for the phosphorylated intermediate $\overline{X\ I} \sim P_i$ is thought to be in phosphate (and calcium) accumulation. 2,4-dinitrophenol (DNP) is considered to promote the hydrolysis of the high energy bond of $\overline{X \sim I}$, whilst oligomycin is depicted as preventing the reversible transfer of P_i between ATP and $\overline{X\ I} \sim P_i$. The ATPase activity is due to the reversibility of these steps; the DNP stimulation of ATPase lies in the irreversibility brought about by the hydrolysis of $\overline{X \sim I}$.

The scheme is very simple, and undoubtedly will require modifications as the intermediates become known, but it does serve to show that uncoupling agents can affect several processes. This paper describes the uncoupling action of DNP in the context of its effects on the partial reactions of oxidative phosphorylation.

II. Methods

(*a*) *Isolation of Mitochondria*

Corn plants were grown as previously described (Bottrill and Hanson 1968). Approximately 200 g of roots from 3-day-old plants were washed twice with 400 ml of water at 0–4°C, allowed to drain, then tipped into an ice-cold mortar and ground in 200 ml of grinding medium (0·5M sucrose, 0·1M Tris, 0·05M maleic acid, and 0·005M EDTA adjusted to pH 7·5 with KOH). The pulp was strained through cheese cloth and the solution centrifuged at 1,000 *g* for 10 min to remove nuclei, plastids, and cell debris. The supernatant was recentrifuged at 12,000 *g* for 10 min to sediment the mitochondria. The mitochondrial pellets were suspended in 0·5M sucrose containing 0·005M EDTA adjusted to pH 7·5 with KOH, pooled, and made to 40 ml and centrifuged at 12,000 *g* for 15 min. The washed pellet was suspended in 0·5M sucrose.

(*b*) *Assay of Respiration and Oxidative Phosphorylation*

The standard method of Warburg manometry was used for oxidative phosphorylation studies (Umbreit, Burris, and Staufer 1957). The bath temperature was 30°C. Bovine serum albumin was added to reaction vessels to remove endogenous uncouplers, possibly fatty acids

(Dalgarno and Birt 1963). Flasks were shaken at 140 cycles/min. After an equilibration period of 15 min the stopcocks were closed and 1·0-ml samples were removed from replicate tubes, added to 3·0 ml of cold 5% trichloroacetic acid (TCA), and centrifuged at 1,000 *g* for 10 min. The supernatant was assayed for initial phosphate (Fiske and Subbarow 1925). Oxygen uptake was measured for a 30-min period, and duplicate 1·0-ml samples were treated as above for final phosphate levels. Mitochondrial nitrogen was determined by digestion and Nesslerization (Lanni, Dillion, and Beard 1950).

(*c*) *ATPase Assay*

Two types of experiments were carried out, a study to determine the reaction in a fixed time (30 min), and time-course studies, to determine the rate of reaction. ATPase activity was determined by measuring the liberation of P_i from ATP. Approximately 10% of the ATP was hydrolysed during the reaction.

Mitochondria were added to the reaction mixture (see Fig. 4) and 15 sec later 2·0 ml were removed and added to 1 ml of 15% TCA at 0°C, for the initial P_i assay. A second 2-ml aliquot was removed 30 min later for the final P_i assay. After centrifugation at 1,000 *g* for 10 min, 1·0 ml of the TCA supernatant was assayed for P_i (Fiske and Subbarow 1925).

In time-course studies the P_i level at zero time was determined by adding the mitochondria directly to the TCA solution containing the appropriate volume of the basic reaction mixture.

(*d*) *Studies on Mitochondrial Swelling and Contraction*

The basic techniques used for mitochondrial swelling and contraction were those described by Stoner (1965) for corn shoot mitochondria. Contraction was powered by substrate or ATP. In each case only those additives considered necessary for the utilization of the energy source were added to the reaction vessel, and no attempt was made to define further the requirements.

Swelling and contraction were followed by changes in optical density at 520 mμ, using a 1-cm cuvette in a Beckman model DB recording spectrophotometer at 27°C. Stoner (1965) has shown that changes in light scattering at 520 mμ rather accurately reflect the changes in water content of corn mitochondria. Swelling was studied in the presence and absence of substrate or ATP, and in the presence of the inhibitor. Contraction was studied by adding the energy source, in a small volume, to swollen mitochondria, and following the increase in optical density at 520 mμ with time. The addition of energy source was in a small volume (0·1–0·2 ml), but even this lowered the optical density by dilution. The reported curves have been adjusted for such dilution by shifting the curve as a whole by the dilution factor. This adjustment only applies to the swelling curve in the absence of the energy source.

(*e*) *Assay of Malate Dehydrogenase*

The method of Wolfe and Neilands (1956) was used for the assay.

Mitochondria were prepared by the above procedure but were suspended in 0·01M potassium phosphate buffer, pH 7·4. The mitochondria were then disintegrated ultrasonically at 0°C with a Bronwill Biosonic (model BP 1 Bronwill Scientific, Rochester, N.Y., manufactured by Blackstone Ultrasonics) for three 30-sec and one 50-sec treatments at full power, with a 30-sec cooling interval, during which the ultrasonic probe was plunged into an ice bath. The sample was then diluted so that a constant rate of reaction was obtained in the reaction vessel. NADH formation was dependent on the presence of malate and independent of the presence or absence of 1 mM amytal.

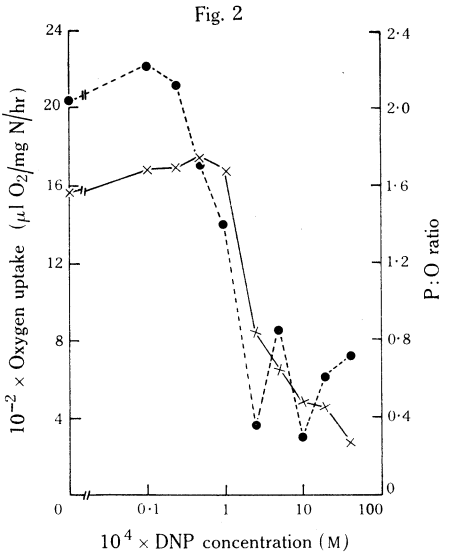
III. Results

(*a*) *Studies on Oxidative Phosphorylation*

At concentrations in excess of 2×10^{-5}M, DNP caused a reduction in the P : O ratio* (Fig. 2). The results at high DNP concentrations are variable since the rates of

* Defined as μmoles of orthophosphate esterified per μg-atom of oxygen consumed.

respiration and phosphate fixation were extremely low at this concentration. DNP at a concentration of 1×10^{-4}M caused little change in respiration, although the P : O ratio was markedly reduced, indicating uncoupling of oxidation and phosphorylation. Higher concentrations inhibited the rate of oxygen uptake. Malate dehydrogenase was strongly inhibited by these concentrations (Fig. 3).



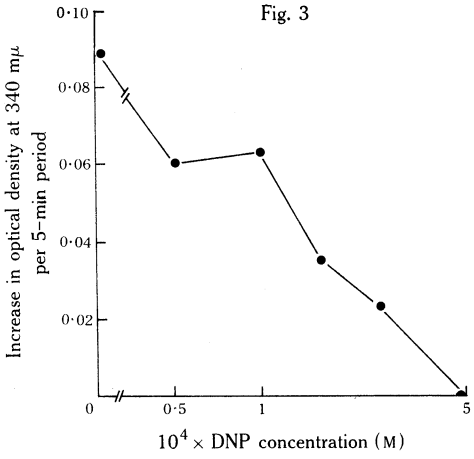


Fig. 2.—Effect of DNP on oxidative phosphorylation. The reaction mixture contained 0·02M potassium phosphate buffer (pH 7·5), 0·016M potassium malate, 0·016M potassium pyruvate, 40 mM glucose, 4 mM magnesium sulphate, $9{\cdot}6 \times 10^{-4}$M ATP, $5{\cdot}2 \times 10^{-2}$M coenzyme A, $2{\cdot}18 \times 10^{-4}$M NAD^+, $1{\cdot}67 \times 10^{-4}$M thiamine pyrophosphate, 2 mg/ml bovine serum albumin, 25 KM units of hexokinase (Sigma, type II), 0·05M sucrose, approximately 0·1 mg of mitochondrial nitrogen, and a range of DNP concentrations in a final volume of 2·5 ml. The centre well contained 0·2 ml of 4N potassium hydroxide. Respiration (×) and oxidative phosphorylation (●) were measured.

Fig. 3.—Effect of DNP on malate dehydrogenase activity. The reaction mixture contained 0·01M glycine–NaOH buffer (pH 10·0), 0·0962M potassium malate, 0·1 mM NAD^+, and 0·625 μg of mitochondrial nitrogen, in a final volume of 2·5 ml. The reaction was carried out at 27°C in a Beckman model DB spectrophotometer, and the rate of formation of NADH followed at 340 mμ.

Little stimulation of oxidation was caused by concentrations of DNP causing uncoupling since a high-energy trapping system was used and ADP was not rate limiting.

(*b*) *Studies on ATPase*

The effect of a range of DNP concentrations on P_i liberated after a 30-min incubation is shown in Figure 4. Concentrations causing classic uncoupling of oxidative phosphorylation caused a stimulation of P_i liberation, which was reduced at higher concentrations, followed by another peak of stimulation and inhibition at concentrations where respiration was inhibited.

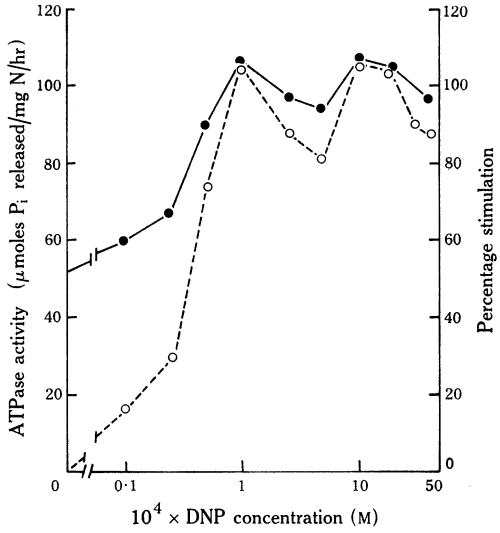


Fig. 4.—Effect of DNP on ATPase activity. The reaction mixture contained 0·2M KCl, 0·02M Tris–0·01M maleic acid buffer (pH 7·5), 0·01M sucrose, 5 mM ATP [the disodium salt of ATP (Sigma) was adjusted to pH 7·5 with potassium hydroxide], 4 mM magnesium sulphate, 2 mg/ml bovine serum albumin, a range of DNP concentrations, and approximately 0·1 mg of mitochondrial nitrogen in a total volume of 5·0 ml. P_i liberated in 30 min (●) was measured as described in Section II and the percentage stimulation by DNP calculated (○).

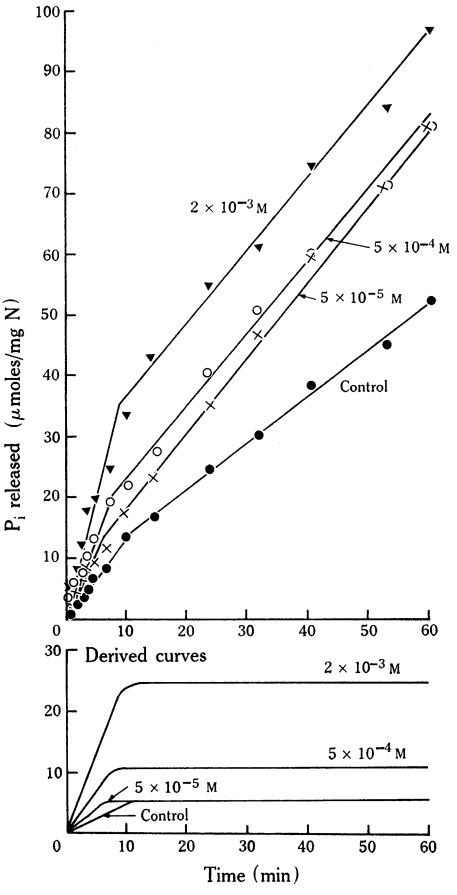


Fig. 5.—Time-course study of the effect of various DNP concentrations on ATPase activity. The reaction mixture was as for Figure 4 except that 0·6 mg of mitochondrial nitrogen was added in a total volume of 30 ml. Samples were removed at intervals as indicated. The derived curves were obtained by subtracting the final reaction rates from the overall rates.

The ATPase reaction was dependent on the presence of magnesium and was inhibited by oligomycin at concentrations in excess of 0·1 μg/ml. This concentration inhibited the DNP stimulation of ATPase.

The rate of P_i liberation was not constant over the 30-min incubation period (Fig. 5). Two rates of reaction were observed. Uncoupling concentrations of DNP (5×10^{-5}M) were found to increase both rates. Increasing concentrations increased the first rate but reduced the second (Fig. 5).

(c) *Studies on Swelling and Contraction*

DNP caused increased swelling at concentrations in excess of 1×10^{-4}M in the presence or absence of ATP [Fig. 6(*a*)]. The presence of ATP caused little change in the shape of the response curve but caused a reduction in the amount of swelling at all concentrations.

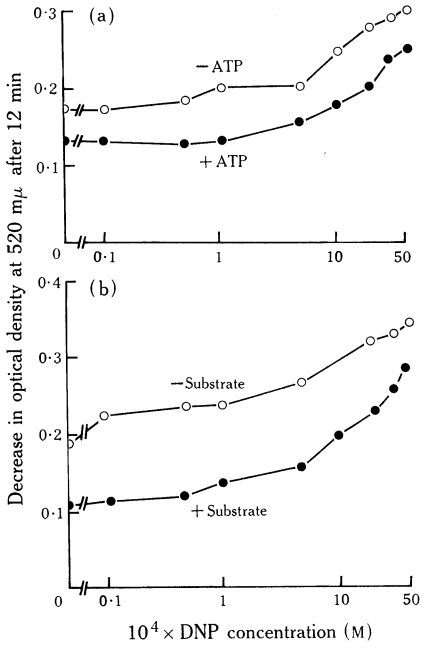


Fig. 6.—Effect of DNP on mitochondrial swelling (measured as decrease in optical density at 520 mμ) in the presence or absence of ATP or substrate. The basic reaction mixture contained 2 mg/ml bovine serum albumin, 0·2M KCl, 0·02M Tris–0·01M maleic acid buffer (pH 7·5), and approximately 0·1 mg of mitochondrial nitrogen in a final volume of 2·5 ml. 4 mM magnesium sulphate was added when 5 mM ATP was the energy source, and $5\cdot2\times10^{-5}$M coenzyme A, $2\cdot2\times10^{-4}$M NAD^+, and $1\cdot7\times10^{-4}$M thiamine pyrophosphate when substrate (8 mM potassium malate–8 mM potassium pyruvate) was the energy source.

Low concentrations of DNP (1×10^{-5}M) caused an increased swelling of the mitochondria in the absence of substrate but in the presence of substrate no response was observed at concentrations of DNP less than 1×10^{-4}M [Fig. 6(*b*)]. Unlike the response to ATP, substrate did not cause a proportional reduction in swelling over the concentration range but had reduced effect at high DNP concentrations.

Addition of ATP or substrate to mitochondria swollen in the presence of various DNP concentrations resulted in contraction except at high concentrations (Fig. 7). Where substrate was used there was a gradual reduction in contraction prior to the marked inhibition, possibly due to an inhibition of substrate utilization. Where DNP caused an increased swelling of the mitochondria, ATP or substrate caused a compensating increased contraction.

IV. Discussion

The classic uncoupling action of DNP, where the reactions involved in high-energy fixation are separated from, and hence fail to regulate, the reactions involved in oxidation was also observed here (Fig. 2). Higher concentrations of DNP also inhibit oxygen uptake. At these concentrations DNP inhibited malate dehydrogenase (Fig. 3) and it is possible that the inhibition of oxidation (Fig. 2) is at the substrate level. Wilson and Merz (1967) also observed that the inhibition of respiration by nitrophenols was by an inhibition at the substrate level and not by a reaction common with the uncoupling mechanism as suggested by Hemker (1964).

The action of DNP on ATPase has been extensively studied by Slater (1962), who observed that ATPase activity induced by DNP was inhibited by increasing DNP concentrations. However, a second peak of stimulation can be observed on using higher concentrations of DNP, again followed by an inhibition (Fig. 4). Both these peaks of stimulation were inhibited by oligomycin, and were considered to involve the reactions of the high-energy fixation sequence.

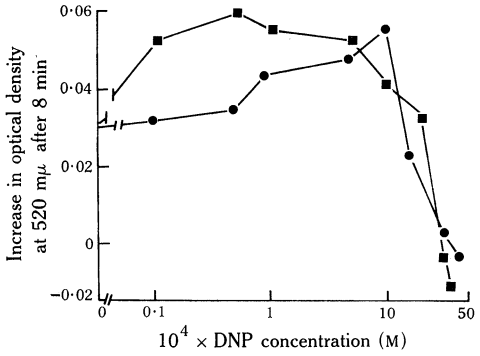


Fig. 7.—Effects of ATP and substrate in reversing DNP-induced swelling. Conditions were as for Figure 6. Mitochondria were allowed to swell for 12 min in the absence of energy source as described and contraction (increase in optical density at 520 mμ) was measured after addition of ATP (●) or substrate (■).

A kinetic study of ATPase activity indicated two rates of reaction, as shown by the change in slope of the curves in Figure 5. Using the nomenclature of Stoner, Hodges, and Hanson (1964), these two rates were explained by the following: on addition of ATP to the mitochondrial system $\overline{X\,I}$ reacted with ATP to form $\overline{X\,I} \sim P_i$, which then liberated P_i with the formation of $\overline{X \sim I}$. Initially there was a high concentration of $\overline{X\,I}$ and the rate of inorganic phosphate liberation was controlled by the rate of conversion of $\overline{X\,I}$ to $\overline{X \sim I}$. However, as soon as $\overline{X \sim I}$ was present the irreversible reaction to $\overline{X\,I}$ was brought about by uncoupling reagents. If the uncoupling reaction is slower than the rate of conversion of $\overline{X\,I}$ to $\overline{X \sim I}$ then this will become rate limiting. Hence, the first rate is due to a combination of the rate of the uncoupling reaction and the rate of formation of $\overline{X \sim I}$, while the second is simply due to the uncoupling reaction. The mitochondria were not tightly coupled in these experiments, with a maximum P : O ratio of 2·4, hence the relatively high rates of the uncoupling reaction. In support of this hypothesis a concentration of DNP found to cause classic uncoupling increased the rate of the uncoupling reaction while not affecting the equilibrium concentration of $\overline{X \sim I}$ as shown by the derived curve (Fig. 5). The derived curve was obtained by subtracting the rate of the uncoupling reaction from the overall rate. This assumes that the uncoupling rate is constant over

the whole of the reaction time, which is not strictly true since this rate is determined by the concentration of $\overline{X \sim I}$ which initially increases rapidly.

Higher concentrations of DNP do not increase the rate of the uncoupling reaction but rather cause it to decrease (Fig. 5). The initial rate, on the contrary, is increased. The derived curves indicate that the rate of formation of $\overline{X \sim I}$ and the equilibrium concentration are altered in this case. The double peaks obtained in Figure 4 are therefore due to two separate effects, the first peak being due to a simple uncoupling effect or conversion of $\overline{X \sim I}$ to $\overline{X\ I}$, while the apparent inhibition and subsequent stimulation is due to a separate effect on the level of $\overline{X\ I}$. It was found that other compounds could stimulate the rate of formation of $\overline{X \sim I}$ without affecting the uncoupling reaction or the effective level of $\overline{X\ I}$.

A study of the partial reactions involving swelling and contraction shed further light on the uncoupling action of DNP. The intermediate $\overline{X \sim I}$ is thought to be implicated in these reactions (Fig. 1). In the presence or absence of substrate or ATP mitochondrial swelling is increased by concentrations of DNP in excess of 1×10^{-4}M (Fig. 6), the concentration causing maximum classic uncoupling (Fig. 4). At concentrations in excess of 5×10^{-4}M the swelling is pronounced. On addition of ATP to mitochondria swollen in the absence of ATP contraction is enhanced (the effective level of $\overline{X \sim I}$ is increased) with increasing concentrations of DNP, until a concentration (1×10^{-3}M) is reached after which there is a failure to contract (Fig. 7). It is known from ATPase studies that such pretreatment has no effect on the initial and final rates of P_i liberation, hence these results may be regarded in the light of the ATPase results. 1×10^{-3}M DNP initiated the second inhibition of ATPase (Fig. 4) and also initiated a sudden failure of the mitochondria to contract; therefore, the second inhibition of ATPase can be explained by an uncontrolled swelling and disruption of the mitochondrial structure, with the concomitant inability of the mitochondria to utilize ATP.

The failure of substrate to cause a proportional reduction in swelling over the DNP concentration range [Fig. 6(*b*)] is possibly due to the inhibition by DNP of substrate utilization at the level of malate dehydrogenase. Due to this inhibition the increased contraction in response to increased swelling is not as pronounced at high DNP concentrations (Fig. 7), but otherwise substrate produces similar results to those obtained with ATP.

DNP can therefore be seen to uncouple mitochondria at low concentrations, inhibit malate dehydrogenase, and increase mitochondrial swelling, and thereby the effective concentration of $\overline{X \sim I}$, at higher concentrations, until a concentration is reached where mitochondrial structure is disrupted due to swelling, together with loss of enzyme function. The action of DNP on mitochondria cannot be interpreted in simple terms of a single enzyme–substrate–inhibitor interaction as Slater (1962) and Hemker (1964) suggest, but there is rather a complex of such interactions between DNP and possibly closely associated enzymes in the sequence of reactions involved in oxidative phosphorylation. The DNP response curves for each of these reactions overlap and make it difficult to use simple models to explain the DNP-induced uncoupling.

V. References

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