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# ACTIVATION OF SUCCINATE DEHYDROGENASE BY 2,4-DINITROPHENOL—A COMPETITIVE INHIBITOR

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SUMMARY

1. The reported inhibition of the succinate oxidase system at high concentrations of dinitrophenol, considered to be at the primary dehydrogenase level, is now confirmed by measuring the activity of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) in the presence of dinitrophenol, using the dye reduction method.

2. The results indicate that the inhibition of substrate-activated succinate dehydrogenase by dinitrophenol is competitive.

3. Low concentrations of dinitrophenol inhibited the basal activity, while at higher concentrations the kinetics were complicated by an apparent activation.

4. Preincubation of mitochondria with dinitrophenol stimulated the enzyme activity, a phenomenon shown by succinate and competitive inhibitors. This activation was very rapid at 37°, compared to that by succinate; activation by dinitrophenol was observed even at 25°, under conditions where succinate had no effect.

5. Repeated washing of the activated mitochondrial samples with the sucrose homogenizing medium reduced the succinate-stimulated activity to the basal level, but only partially reversed the dinitrophenol activation.

6. The relevance of this activation phenomenon to the physiological modulation of this enzyme system is discussed.

## INTRODUCTION

Dinitrophenols were shown to have a specific inhibitory action on uncoupled respiration and ATPase activity in rat liver mitochondria[1]. Concentrations of 2,4-dinitrophenol greater than necessary for maximal uncoupling were required for inhibition of succinate oxidation[1–5]. This inhibition was shown to be competitive with respect to the substrate. At a given dinitrophenol concentration, less inhibition was obtained at higher substrate concentrations[2], leading to an increase in the appa-

Abbreviations: PMS, *N*-methyl phenazonium methyl sulfate (phenazine methosulfate); DCIP, 2,6-dichlorophenolindophenol.

rent $K_m$ for succinate without affecting the $v_{max}$ (ref. 4). This effect was also shown to occur in submitochondrial particles[6].

The competitive nature of the inhibition of succinate oxidation, the highly oxidized state of the respiratory chain carriers in the inhibited state, and the lack of effect on the cytochrome oxidase system indicated that the site of inhibition by dinitrophenol was between succinate and cytochrome *b*, presumably at the primary dehydrogenase[4]. It was therefore of interest to study the effect of dinitrophenol on the activity of succinate dehydrogenase (succinate:(acceptor) oxidoreductase, EC 1.3.99.1) assayed by the method of reduction of dyes. The results presented here with rat liver mitochondria show that dinitrophenol is indeed a competitive inhibitor of succinate dehydrogenase. By implication, a competitive inhibitor should also activate succinate dehydrogenase on preincubation, as observed with malonate, fumarate and other competitive inhibitors[7,8]. In this communication, a similar phenomenon is shown to occur in the case of dinitrophenol.

## EXPERIMENTAL

2,4-Dinitrophenol was purchased from E. Merck, Darmstadt (Germany) and phenazine methosulfate from Calbiochem, Inc., Los Angeles, Calif. (U.S.A.). All other chemicals used were of AnalaR grade.

### *Preparation of rat liver mitochondria*

Male albino rats weighing 150–170 g from the stock colony were used. The animals were killed by stunning and decapitation. After perfusion with 0.9% NaCl solution, the livers were homogenized in 10 vol. of cold 0.25 M sucrose medium (containing 0.01 M Tris, pH 7.4, and 5 mM EDTA) in a Potter–Elvehjem homogenizer. Mitochondrial fractions were obtained by differential centrifugation according to the procedure of SCHNEIDER AND HOGEBOOM[9], and were washed once with the homogenizing medium and suspended in the medium. Protein was determined by the biuret method[10]. The mitochondrial suspension generally contained 15–20 mg protein per ml.

### *Enzyme assay*

Succinate dehydrogenase activity was determined spectrophotometrically with phenazine methosulphate–2,6-dichlorophenolindophenol (PMS–DCIP) as the electron acceptor system as described by ARRIGONI AND SINGER[11]. The reaction mixture contained 100 $\mu$moles of potassium phosphate buffer (pH 7.6), 1.2 $\mu$moles of KCN (freshly neutralized), 0.75 $\mu$mole of $CaCl_2$ and 1–2 mg of mitochondrial protein in a volume of 2.7 ml. The reaction was started by successive additions of 50 $\mu$moles of succinate (in 0.1 ml); 0.04 $\mu$mole of DCIP (in 0.1 ml) and 0.1 ml of 1% PMS solution and the decrease in $A_{600\ nm}$ was determined in a Beckmann model DB recording spectrophotometer. The enzyme activity is expressed as nmoles of dye reduced per min per mg protein. Unless otherwise mentioned all enzyme assays were carried out at 25°. In the PMS assay of succinate dehydrogenase activity, it is recommended that the activity is determined at varying dye concentrations and extrapolated to $v_{max}$. It was found that a concentration of 1 mg of PMS in a total assay volume

of 3 ml was sufficient to give almost maximal activity. The enzyme activities were therefore measured at this fixed concentration of PMS and the values compared.

*Preincubation of mitochondria for activation*

Isolated mitochondria were activated by preincubation at 37°, or at 25° where specified, for 7 min essentially as described by KIMURA *et al.*[8]. The mixture usually contained about 10 mg of mitochondrial protein, 100 μmoles of potassium phosphate buffer (pH 7.6) and 50 μmoles of succinate, or dinitrophenol at different concentrations where indicated, in a total volume of 1 ml of which an aliquot was used for determining enzyme activity after preincubation. These preparations were referred to as "succinate-activated". Similar conditions were used for activation by dinitrophenol.

*Washing the activated mitochondrial preparations*

The activated mitochondrial preparations were diluted with 5 vol. of the sucrose medium and sedimented at 8300 × *g* for 10 min in a refrigerated Sorvall RC-2B centrifuge. The sediment obtained was resuspended in the same volume of medium as above and recentrifuged. The pellet obtained was resuspended in the sucrose medium and used for the enzyme assay. In these experiments generally 25 mg of the mitochondrial protein were used.

## RESULTS

*Inhibition of succinate dehydrogenase by dinitrophenol*

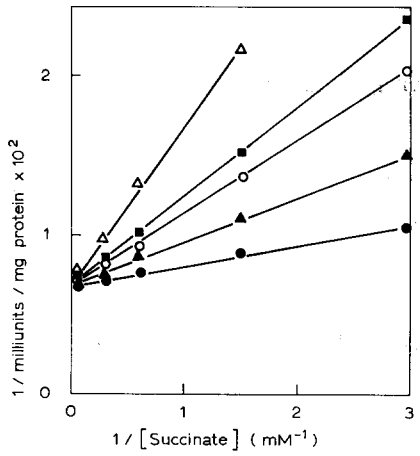
In the first set of experiments, the effect of adding dinitrophenol to the reaction mixture after the preincubation procedure on the activity of succinate dehydrogenase assayed by the reduction of the dye system (PMS and DCIP) was studied. Increasing concentrations of dinitrophenol led to increasing inhibition (Table I) A maximum inhibition of 20% was reached at 3.2 mM dinitrophenol and increasing dinitrophenol above this concentration did not further decrease the activity. Since the concentration of succinate was far in excess of that required for saturation, inhibition of the activity at different concentrations of both succinate and dinitrophenol was studied next. The data plotted according to LINEWEAVER AND

TABLE I

INHIBITION OF SUCCINATE DEHYDROGENASE BY DINITROPHENOL

The enzyme activities were assayed at a saturating succinate concentration of 50 μmoles per 3 ml of the assay medium. The values indicated for the percentage inhibition are the mean of six independent determinations carried out with different samples of mitochondria activated by preincubation with succinate. The variation between individual values was less than 5%.

| *Concentration of dinitrophenol in the assay (mM)* | *Inhibition (%)* |
|---|---|
| 0.8 | 12.0 |
| 1.6 | 14.7 |
| 3.2 | 20.4 |
| 6.4 | 20.4 |



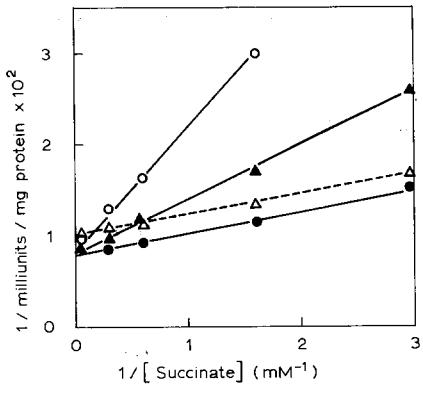




Fig. 1. Lineweaver–Burk plot for the inhibition by dinitrophenol of succinate dehydrogenase activated by preincubation with succinate. The concentration of dinitrophenol added in the assay medium: ●, nil; ▲, 0.8 mM; ○, 1.6 mM; ■, 3.2 mM; △, 6.4 mM.

Fig. 2. Lineweaver–Burk plot for the inhibition of non-activated succinate dehydrogenase by dinitrophenol. The concentration of dinitrophenol added in the assay medium: ●, nil; ▲, 0.8 mM; ○, 1.6 mM; △, 3.2 mM.

BURK[12] (Fig. 1) showed linear plots with a common intercept on the ordinate. This indicated that inhibition by dinitrophenol was competitive with respect to the substrate. The same $v_{max}$ was obtained with increasing apparent $K_m$ for succinate at increasing dinitrophenol concentrations.

*Effect of dinitrophenol on the basal activity of succinate dehydrogenase*

In the second set of experiments, the effect of dinitrophenol on the basal activity of succinate dehydrogenase was tested in freshly prepared mitochondria which had not been preincubated. The Lineweaver–Burk plots at low dinitrophenol concentrations were essentially similar to those obtained with "succinate-activated" preparations (Fig. 2). However, the effect of dinitrophenol at a concentration of 3.2 mM was more complex than at 1.6 mM, indicating an apparent activation (see Fig. 2).

*Kinetic constants*

The kinetic constants for the inhibition of succinate dehydrogenase by dinitrophenol are shown in Table II. The value of the $K_m$ for succinate for the "succinate-activated" enzyme activity was $1.7 \cdot 10^{-4}$ M and for the unactivated enzyme was $3 \cdot 10^{-4}$ M, which is in agreement with values reported previously[13,14]. The inhibitor constant ($K_i$) for dinitrophenol, calculated from the above data, was found to be fairly constant in the range $5 \cdot 10^{-4}$–$8.5 \cdot 10^{-4}$ M, for the succinate-activated succinate dehydrogenase at varying dinitrophenol concentrations. It is interesting to note that, at a dinitrophenol concentration of 3.2 mM which gave maximum inhibition, the $K_m$ value for succinate and the $K_i$ value for dinitrophenol were nearly the same for the succinate-activated enzyme.

TABLE II

KINETIC CONSTANTS FOR THE INHIBITION OF SUCCINATE DEHYDROGENASE BY DINITROPHENOL

Enzyme activities were determined with and without preincubation with succinate as described in the text.

| *Concentration of dinitrophenol in the assay (mM)* | *Apparent $K_m$ for succinate (M)* | *$K_i$ for dinitrophenol (M)* |
|---|---|---|
| *"Succinate-activated" succinate dehydrogenase activity* | | |
| None | $1.7 \cdot 10^{-4}$ | — |
| 0.8 | $4.2 \cdot 10^{-4}$ | $5.6 \cdot 10^{-4}$ |
| 1.6 | $6.7 \cdot 10^{-4}$ | $5.6 \cdot 10^{-4}$ |
| 3.2 | $8.2 \cdot 10^{-4}$ | $8.4 \cdot 10^{-4}$ |
| 6.4 | $15.0 \cdot 10^{-4}$ | $8.5 \cdot 10^{-4}$ |
| *Non-activated or basal succinate dehydrogenase activity* | | |
| None | $3 \cdot 10^{-4}$ | — |
| 0.8 | $7.4 \cdot 10^{-4}$ | $5.5 \cdot 10^{-4}$ |
| 1.6 | $16.7 \cdot 10^{-4}$ | $3.5 \cdot 10^{-4}$ |

*Activation of succinate dehydrogenase by preincubating mitochondria with dinitrophenol*

Besides succinate, preincubation of mitochondria with low concentrations of competitive inhibitors such as phosphate[15], malonate and other compounds capable of combining at the active site, stimulated succinate dehydrogenase activity[7,8]. Since the addition of higher concentrations of dinitrophenol to the assay stimulated the basal enzyme activity at low succinate concentrations, it seemed possible that dinitrophenol which is a competitive inhibitor, could also activate the enzyme during the assay, despite its short period. As expected, preincubation of mitochondria with low concentrations of dinitrophenol in potassium phosphate buffer at 37° stimulated the succinate dehydrogenase activity. 10 mM dinitrophenol caused maximal stimulation of 85% over the control (Fig. 3), compared to 94% obtained with 50 mM succinate under similar conditions. The percentage stimula-

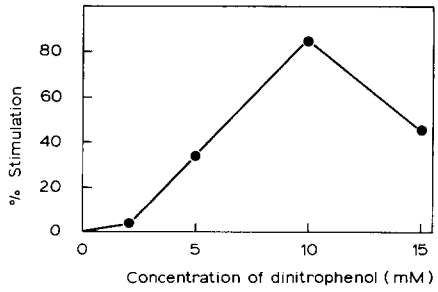
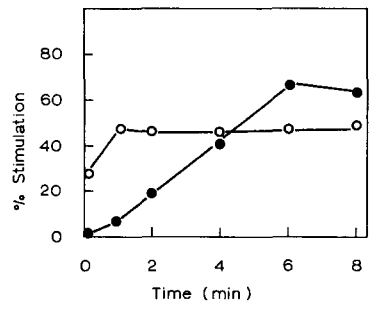



Fig. 3. Stimulatory effect of dinitrophenol on succinate dehydrogenase activity on preincubation with mitochondria. The preincubation mixture consisted of about 8 mg of mitochondrial protein, 100 μmoles of potassium phosphate buffer (pH 7.6) and dinitrophenol concentrations as shown in the figure, in a total volume of 1 ml. After preincubation at 37° for 7 min, suitable aliquots were taken for the enzyme assay. A similar experiment carried out using 100 μmoles of potassium phosphate buffer alone for preincubation served as the control. Preincubation of mitochondria with 50 μmoles of succinate and 100 μmoles of potassium phosphate buffer (pH 7.6) under similar conditions gave a stimulation of 94% over the control value.



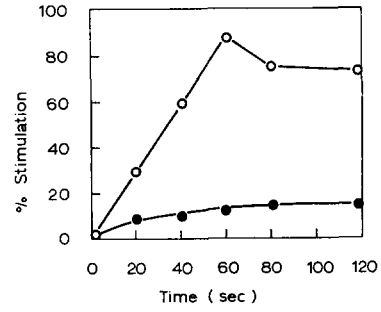




Fig. 4. Time course of the stimulation of succinate dehydrogenase by dinitrophenol and succinate. About 8 mg of mitochondrial protein were preincubated at 37° in 100 μmoles of potassium phosphate buffer (pH 7.6), 50 μmoles of succinate (●) or 10 μmoles of dinitrophenol (○) for various time intervals as shown in the figure. At the end of the incubation period, the tubes were cooled to 0° to prevent further activation, suitable aliquots were taken and the enzyme activities were determined at 25°. A similar experiment carried out by preincubation with potassium phosphate buffer alone served as the control.

Fig. 5. Time course of the stimulation of succinate dehydrogenase by succinate and dinitrophenol on preincubation at 25°. The method is the same as mentioned in the legend for Fig. 4. Preincubations with succinate (●) or dinitrophenol (○) were done at 25° for the time intervals shown and the enzyme activities assayed at 15° to prevent further activation during the assay.

tion obtained at a dinitrophenol concentration of 15 mM was less because of the simultaneous inhibition by dinitrophenol carried over from the preincubation to the assay.

The time courses of the activation by succinate and dinitrophenol were next compared. At 37° with succinate, maximum activation was obtained after a 6-min preincubation (KIMURA *et al.*[8]) and no further increase was observed, whereas the activation by dinitrophenol was very rapid, maximal activation being attained in the first 2 min with no further change (Fig. 4). In view of the rapid activation by dinitrophenol, the preincubation was carried out at a lower temperature (25°) for short time intervals and was compared with succinate under similar conditions. The enzyme assay was carried out at 15° to prevent any further activation during assay. The stimulation by dinitrophenol at one minute was already maximal even at 25° while succinate did not show any appreciable stimulation for 2 min (Fig. 5).

*Effect of washing the mitochondria preincubated with succinate and dinitrophenol*

KIMURA *et al.*[8] demonstrated that the activation obtained by preincubating mitochondria with succinate was reversed on removal of the bound activating agent either by repeatedly washing the mitochondria or by exclusion on Sephadex gel in soluble preparations. On the contrary, competitive inhibitors like malonate were known to be firmly bound to the active site of the enzyme and were not easily removed by repeated washing.

The results given in Table III show that on preincubation of mitochondria with phosphate (100 μmoles/ml), succinate (25 μmoles/ml) and dinitrophenol (10 μmoles/ml) at 37° for 7 min, the basal activity of the enzyme could be raised by 14%, 76% and 66%, respectively. Repeated washing of these activated mitochondrial samples with the homogenizing medium could reduce the enzyme activities to the basal level in the case of the "phosphate- and succinate-activated" preparations.

TABLE III

EFFECT OF WASHING THE MITOCHONDRIA ON SUCCINATE DEHYDROGENASE ACTIVITY AFTER PREINCUBATION WITH PHOSPHATE, SUCCINATE OR DINITROPHENOL

The preincubation was carried out at 37° as described in the text. The figures in parentheses indicate the percentage increase over the basal activity.

| *Preincubation* | *Enzyme activity (nmoles of dye reduced/min per mg protein)* | | |
|---|---|---|---|
| | *Initial* | *Washed twice with the homogenizing medium* | *Preincubation repeated after washing* |
| None | 150 (100) | — | — |
| Phosphate | 171 (114) | 155 (104) | — |
| Succinate | 263 (176) | 157 (105) | 272 (182) |
| Dinitrophenol | 248 (166) | 206* (138) | 240 (160) |

* Preincubation with succinate gives 250 (167).

The enhanced activity of the enzyme obtained on preincubation with dinitrophenol could only be partially reserved to 38% above the basal level by the washing procedure. A second preincubation of this preparation with dinitrophenol (5 $\mu$moles/ml) at 37° for 7 min could reactivate the enzyme to the activated state. A second preincubation of the mitochondria obtained after washing the "succinate- and dinitrophenol-activated" preparations with succinate (50 $\mu$moles/ml) also activated the enzyme to the original activated state. The partial reversal of the activity obtained on washing the dinitrophenol-activated preparation shows that dinitrophenol binds more firmly to the enzyme.

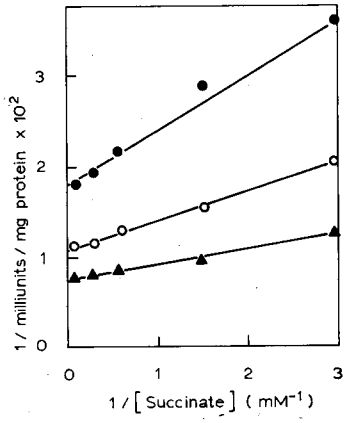



Fig. 6. Kinetic studies of the activation of succinate dehydrogenase by dinitrophenol and succinate. About 5 mg of mitochondrial protein were preincubated with 100 $\mu$moles of potassium phosphate buffer (pH 7.6) alone (●) or buffer and 50 $\mu$moles of succinate (▲) or buffer and 10 $\mu$moles of dinitrophenol (○) in a total volume of 1 ml, at 37° for 7 min. At the end of the incubation period, the tubes were cooled to 0° to stop further activation. Suitable aliquots of the mitochondrial protein were taken and the enzyme activities determined at varying succinate concentrations in the assay.

*Effect of activation on $K_m$ values for succinate*

Succinate, fumarate and malonate are known to be effective activators of succinate dehydrogenase. In general, regular Michaelis–Menten type kinetics were obtained with each activated preparation (KEARNEY[7]). Fig. 6 compares the effectiveness of phosphate, succinate and dinitrophenol as activators. In this experiment the activation was performed by preincubating the mitochondria with the respective activator at 37° for 7 min, the period sufficient for maximal activation to be attained by all the activators. It is seen from Fig. 6, that the $v_{max}$ values were different for phosphate, dinitrophenol and succinate and increased in that order. A lower $v_{max}$ obtained in the case of dinitrophenol activation could be due to the simultaneous inhibition caused by dinitrophenol carried over from the preincubation to the activity measurements.

In a previous publication[13] from this laboratory, the activation obtained by preincubation with succinate was considered to be of the 'V-type' (ref. 16) without alteration of the $K_m$ value. In the present set of experiments the data obtained were treated by the method of least squares to obtain the lines shown in Fig. 6, with points showing the experimental values.

The $K_m$ values for succinate were calculated to be $3.4 \cdot 10^{-4}$ M, $3.2 \cdot 10^{-4}$ M and $2.2 \cdot 10^{-4}$ M for the enzyme preparations activated by preincubation with phosphate, dinitrophenol and succinate, respectively, indicating a small decrease for succinate-activated preparation.

## DISCUSSION

High concentrations of dinitrophenol inhibited the succinate oxidase system. Although this inhibition was postulated to be at the dehydrogenase level, this report is the first to substantiate this by measuring the activity of the flavoprotein using the dye reduction method, and the results confirmed the competitive nature of this inhibition. While testing the effect of dinitrophenol on the basal activity in freshly prepared mitochondria, it was found that, while inhibition was obtained at lower dinitrophenol concentrations, at higher concentrations the kinetics became complicated with an apparent activation. It was considered that this may reflect the activation observed with competitive inhibitors of succinate dehydrogenase such as malonate. This was indeed found to be true with dinitrophenol, whose activation was about 90% that of succinate. In contrast to succinate, the activation by dinitrophenol was very much more rapid and occurred at lower temperatures, explaining the activation obtained during the short period of the assay. In preparations fully activated by preincubation with succinate, where further activation was not possible, the inhibition became proportional to dinitrophenol concentrations.

There is one significant difference between the activation obtained with succinate and dinitrophenol. In the former case, this is reversible whereas the latter yields a partially stable activated preparation. This may be due to the firm binding of dinitrophenol known to occur at concentrations higher than necessary for uncoupling[16].

The above property seems to be relevant to physiological modulation of the activity of this enzyme system. Work from this laboratory (AITHAL AND RAMASARMA[13]) has shown that exposure of rats to low atmospheric pressure (0.5 Bar) resulted

in an activation of hepatic mitochondrial succinate dehydrogenase which was not reversed by washing the mitochondria, a phenomenon similar to that obtained with dinitrophenol. This appears to be an effect of lowered oxygen because it was also obtained when rats were exposed to a gas mixture containing 10% oxygen and 90% nitrogen (L. SUSHEELA, unpublished data) and therefore may be a compensatory mechanism to maintain the necessary oxygen uptake. The identification of the physiological agent causing this activation is under study.

The inhibitory effect of dinitrophenol, however, does not seem to be specific to succinate dehydrogenase alone. Other dehydrogenases may also be similarly affected as shown by the work of KATYARE *et al.*[5], and JURTSHUK *et al.*[17].

Finally, it should be pointed out that this activation obtained after pre-incubation is not related to the stimulation obtained by uncoupling because the concentration of dinitrophenol required for the former is far in excess of that required for the latter.

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