Phytochemistry, 1968, Vol. 7, pp. 2071 to 2081. Pergamon Press. Printed in England

NUCLEOTIDE STIMULATION OF THE UNCOUPLER-INITIATED INHIBITION OF MITOCHONDRIAL SUCCINATE OXIDATION

ROBERT E. DRURY,* JOHN P. MCCOLLUM, STEPHEN A. GARRISON† and DAVID B. DICKINSON

Department of Horticulture, University of Illinois, Urbana, Illinois

(*Received* 3 *April* 1968)

Abstract—Tomato fruit mitochondria depended upon adenine nucleotide to relieve the inhibition of succinate oxidation initiated by uncouplers of oxidative phosphorylation. Both ADP and ATP were effective. Magnesium and other divalent cations enhanced this effectiveness. Oligomycin, sufficient to repress state 3 respiration in coupled mitochondria, slightly diminished but did not eliminate the effect of ADP. Relief by ADP depended on orthophosphate, negating the possibility that ATP, synthesized by adenylate kinase, was the basis of relief. The stimulation by the nucleotides thus did not appear to require the energy of ATP or to function through a reversible reaction involving ATP and ADP plus Pi. Stimulation may, therefore, result from a binding by nucleotide which effects a favorable conformation for respiration and/or an unfavorable condition for binding by an inhibitor. Pyruvate prevented the inhibition, indicating that oxaloacetate was the immediate cause of the inhibition.

INTRODUCTION

PARDEE and Potter[1] first reported the stimulation by ATP of succinate oxidation inhibited by oxaloacetate (OAA) in 1948, but the role of ATP in this relief has not been resolved. Azzone and Ernster[2,3] noted the similar dependence of succinate oxidation on ATP in uncoupled mitochondria treated with arsenate. They suggested that the inhibition was due to a depletion of a high energy status of an electron transfer component. Oligomycin, however, did not block the ATP effect making it unlikely that ATP furnished energy to the electron transfer system.[4,5] Although no correlation existed between this inhibition and the concentration of OAA,[6] a source of OAA had to be present to obtain inhibition.[4,7]

The disappearance of OAA from a reaction medium will bring about the relief of inhibition,[8,9] but the concentration of OAA is not of itself an index of the inhibitory action of OAA on succinate oxidation.[5,10,11] This latter observation led Tyler,[10] in 1955, to postulate that ATP was effective, not by decomposing OAA, but by forming a non-inhibitory complex with

* Present Address: Dept. of Seed Investigations, N.Y.S. Experiment Station, Geneva, N.Y.
† Present Address: Dept. of Horticulture and Forestry, Rutgers, The State University, New Brunswick, N.J.

[1] A. B. PARDEE and V. R. POTTER, *J. Biol. Chem.* **176**, 1085 (1958).
[2] G. F. AZZONE and L. ERNSTER, *Nature* **187**, 65 (1960).
[3] G. F. AZZONE and L. ERNSTER, *J. Biol. Chem.* 236, 1518 (1961).
[4] J. B. CHAPPELL, in *IUB/IUBS Symposium on Biological Structure and Function* (edited by T. W. GOODWIN and O. LINDBERG), Vol. 2, p. 71, Academic Press, New York (1961).
[5] L. ERNSTER and C. P. LEE, *Ann. Rev. Biochem.* **33**, 729 (1964).
[6] P. SCHOLLMEYER and M. KLINGENBERG, *Biochem. Biophys. Res. Commun.* **4**, 43 (1961).
[7] G. F. AZZONE, in *IUB/IUBS Symposium on Biological Structure and Function* (edited by T. W. GOODWIN and O. LINDBERG), Vol. 2, p. 193, Academic Press, New York (1961).
[8] M. AVRON (ABRAMSKY) and J. B. BIALE, *J. Biol. Chem.* **225**, 699 (1957).
[9] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *J. Exp. Botany* **18**, 277 (1967).
[10] D. B. TYLER, *J. Biol. Chem.* **216**, 395 (1955).
[11] W. KUNZ, *Z. Physiol. Chem.* **334**, 128 (1963).

it. The dissociation of OAA from its site of inhibition by ATP, would explain the role of ATP irrespective of coupling, but though an admitted possibility, it is not a favored view.[12] Most workers envision mechanisms involving the removal of OAA. Current views hold that ATP furnishes energy to produce P-enolpyruvate from OAA in uncoupled mitochondria,[12, 13] while in coupled mitochondria ATP also reverses electron transfer through the first phosphorylation site preventing NADH oxidation and OAA formation from malate.[11, 14]

Although Wiskich and Bonner[12] observed that ADP stimulated O_2-uptake in uncoupled plant mitochondria, they surmise it was effective through conversion to ATP by adenylate kinase (E.C. 2.7.4.3). Malate oxidation was also dependent on the adenine nucleotide concentration in uncoupled mitochondria from cauliflower buds.[15]

This study characterizes the inhibition of succinate oxidation initiated by uncouplers and its relief by adenine nucleotides in tomato fruit mitochondria. The results support the view that the function of adenine nucleotide involves the dissociation of OAA from its site of inhibition.

RESULTS AND INTERPRETATION

Dependence of DNP-Stimulation on ATP

Figure 1 shows the pattern of respiratory dependence upon ADP, the inhibition of state

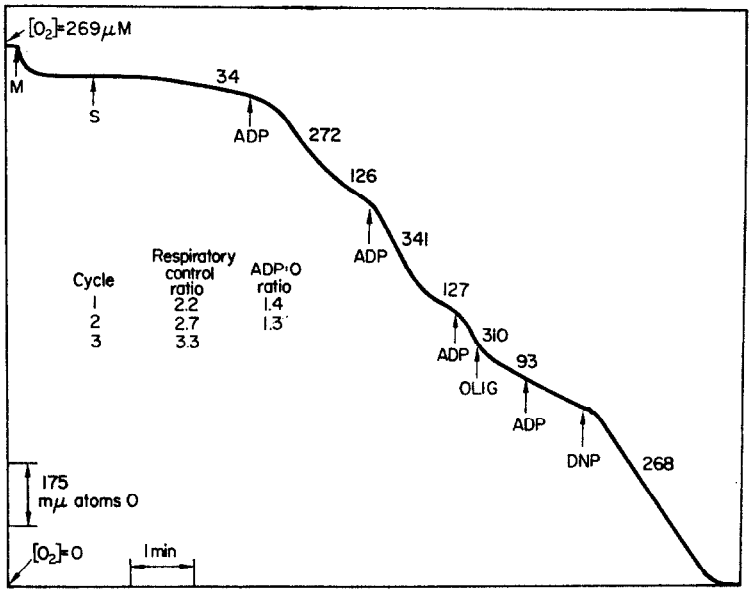

FIG. 1. INHIBITION OF SUCCINATE OXIDATION BY OLIGOMYCIN IN TIGHTLY COUPLED MITOCHONDRIA AND THE SUBSEQUENT STIMULATION BY DNP IN THE PRESENCE OF ADENINE NUCLEOTIDE.

The numbers along the trace are rates in mμatoms/min. M, mitochondria; S, succinate; ADP, 0·1 mM each addition; OLIG, 2·5 gamma total oligomycin; DNP, 0·1 mM. The respiratory control ratio is the ratio of state 3 to state 4.[16]

[12] J. T. WISKICH and W. D. BONNER, JR., *Plant Physiol.* **38**, 594 (1963).
[13] S. PAPA, N. E. LOFRUMENTO and E. QUAGLIARIELLO, *Biochim. Biophys. Acta* **110**, 442 (1965).
[14] B. CHANCE and B. HAGIHARA, *J. Biol. Chem.* **237**, 3540 (1962).
[15] G. G. LATIES, *Physiol. Plantarum* **6**, 199 (1953).

3[16]-oxygen uptake due to the inhibition of phosphorylation by oligomycin, the failure of additional ADP to stimulate in the presence of oligomycin, and the restimulation of the oligomycin-inhibited rate through uncoupling by dinitrophenol (DNP). The DNP-stimulated rate was 87 per cent of the average state 3 rate and almost eight times the substrate-state 4 rate (no exogenous nucleotide present) of 34 mμatoms/min. In contrast, when DNP was added during substrate-state 4, the stimulation of this rate was only 35 per cent (Fig. 2). Only

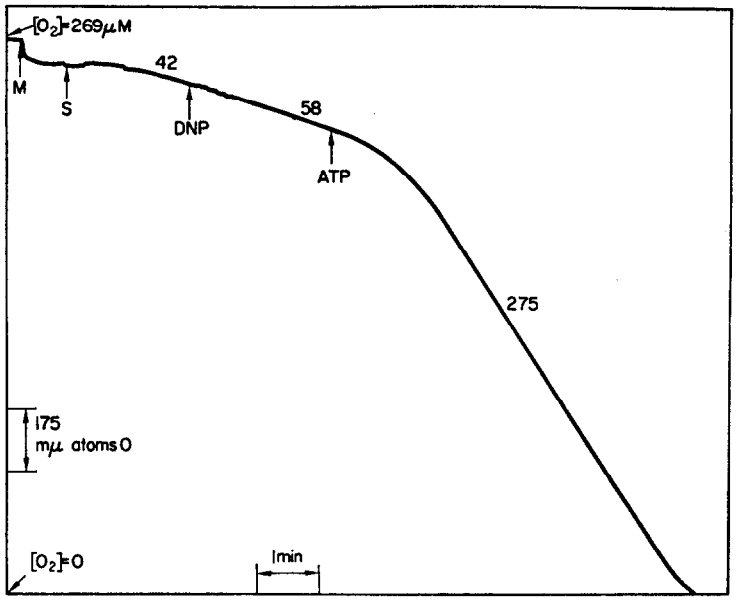

FIG. 2. DEPENDENCE OF DNP ON ATP FOR STIMULATION OF SUBSTRATE-STATE 4.
Mitochondria were from the same isolation as those of Fig. 1. Numbers along the trace are rates in mμatoms/min. M, mitochondria; S, succinate; DNP, 0·1 mM; ATP, 0·2 mM.

upon the addition of adenine nucleotide, in this case ATP, was this rate of oxygen uptake greatly stimulated, namely, to 275 mμatoms/min, or six and one-half times the substrate-state 4 rate. This is comparable to the behaviour of uncoupled animal mitochondria treated with arsenate.[2, 3, 13]

In a few preparations, DNP stimulated the substrate-state 4 rate as much as 100 per cent, but this rate rapidly diminished to a severely inhibited rate. However, such a pattern of uncoupler-initiated inhibition always occurred when $MgCl_2$ was omitted from the reaction medium (Fig. 3A). DNP caused less initial stimulation when mitochondria were assayed several hours after isolation. The ATP effect depended upon Mg^{2+} in that the final rate of oxygen uptake was greater and was achieved more rapidly in the presence of 5 mM exogenous Mg^{2+} than in its absence (Fig. 3A and B).

Effectiveness of ADP and its Dependence on Divalent Cations

Weinbach and Garbus found that ATP stimulated the rate of succinate oxidation by rat liver mitochondria in the presence of the uncoupler, pentachlorophenol, but observed no

[16] In the terminology of B. CHANCE and G. R. WILLIAMS, *Advan. Enzymol.* **17**, 65 (1956), state 3 is the condition promoting rapid respiration in the presence of ADP, and state 4 the condition in its absence, both with high oxygen and substrate concentrations.

effect by ADP.[17] However, preincubation with ADP in the presence of DNP stimulated succinate oxidation by sweet potato mitochondria.[12] Table 1 shows that ADP reversed the DNP-initiated inhibition of succinate oxidation in tomato fruit mitochondria upon its addition to the reaction medium.

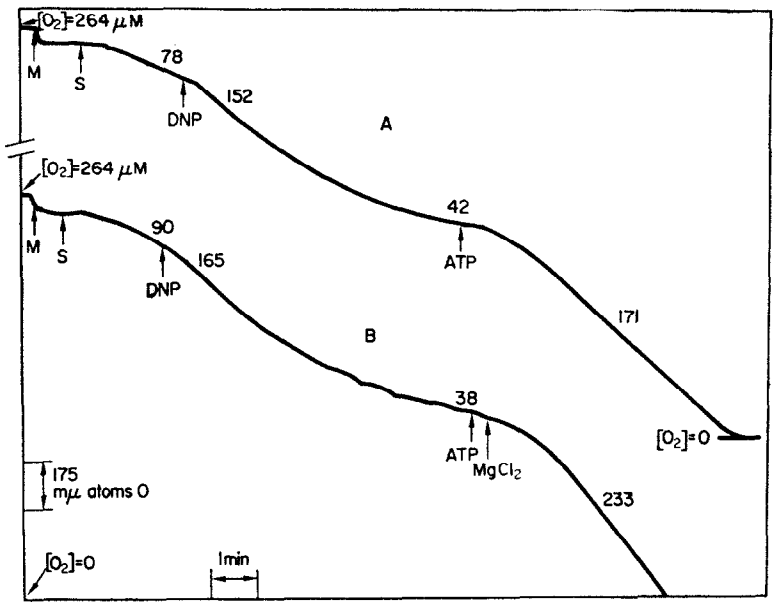

FIG. 3. EFFECT OF Mg^{2+} ON THE STIMULATION OF THE DNP-INHIBITED RATE BY ATP.

$MgCl_2$ was omitted from the reaction medium. Numbers along the trace are rates in mμatoms/min. M, mitochondria; S, succinate; DNP, 0·1 mM; ATP, 0·2 mM; $MgCl_2$, 5 mM. (A) No Mg^{2+} throughout. (B) $MgCl_2$ added shortly after ATP.

TABLE 1. ADP-STIMULATION OF THE DNP-RATE

	Rates (mμatoms/min)*	
	Expt. 1	Expt. 2
Substrate-state 4 rate	79	73
DNP-rate, initially	91	101
DNP-rate prior to ADP-addition	61	17
Time, after DNP, of ADP-addition (min)	5·7	21·8
Maximum, steady ADP-rate	416	266†
Time to maximum rate	1·8	1·9†

* Experiments were performed as in Fig. 2. DNP, 0·1 mM; ADP, 0·2 mM.

† These values are probably underestimated responses due to termination of the experiment by exhaustion of oxygen in the reaction vessel.

[17] E. C. WEINBACH and J. GARBUS, *J. Biol. Chem.* **241**, 3708 (1966).

TABLE 2. EFFECT OF Mg^{2+} ON THE SUBSTRATE-STATE 4 AND DNP RATES

	Rate (mμatoms/min)	
	$+Mg^{2+}$	$-Mg^{2+}$*
Substrate-state 4	66± 7	91±13
DNP	96±13	151±25

Experiments were performed as in Fig. 2. DNP, 0·1 mM. Values are means ± std. error for seven isolations.

* $MgCl_2$ was omitted from the reaction medium.

Mg^{2+} of itself did not stimulate since it was present (5 mM) throughout the experiments of Table 1. In fact Mg^{2+} depressed the substrate-state 4 and DNP rates (Table 2). But Mg^{2+} also enhanced the relief by ADP which was more dependent on Mg^{2+} than was relief by ATP (Figs. 3 and 4). This enhancement was not specific to Mg^{2+}. The addition of 14·5 μmoles of $CaCl_2$, $CoCl_2$ or $MnCl_2$ to the reaction medium in place of $MgCl_2$ was also effective. This is

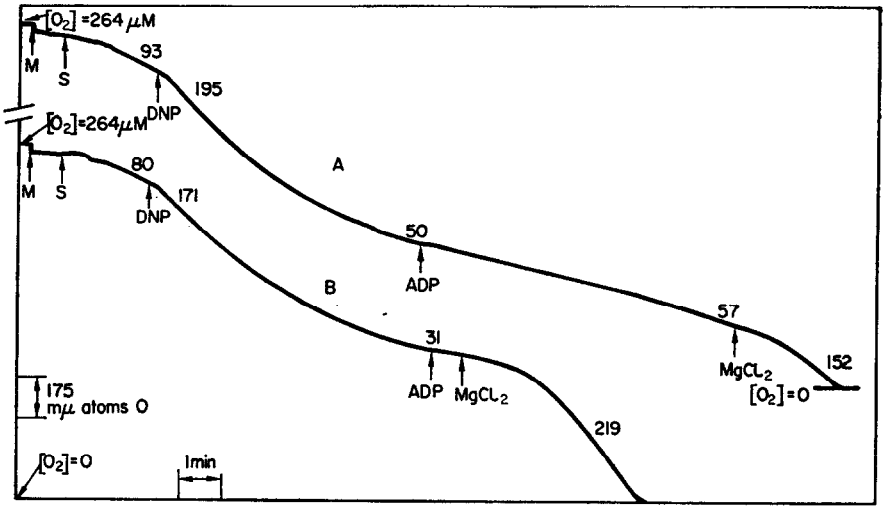

FIG. 4. EFFECT OF Mg^{2+} ON THE STIMULATION OF THE DNP-INHIBITED RATE BY ADP.

$MgCl_2$ was omitted from the reaction medium. Mitochondria were from the same isolation as those of Fig. 3. Numbers along the trace are rates in mμatoms/min. M, mitochondria; S, succinate; DNP, 0·1 mM; ADP, 0·2 mM; $MgCl_2$, 5 mM. (A) $MgCl_2$ added *ca.* 7 min after ADP. (B) $MgCl_2$ added shortly after ADP.

in contrast to the work of Tyler[10] who showed that Ca^{2+} reversed the relief afforded by Mg^{2+} in kidney homogenates treated with OAA. The tomato mitochondria still showed respiratory control by ADP in the presence of these cations in the absence of uncouplers. The Cl^{1-} anion was not an effective ion, nor could K^+ replace Mg^{2+} (Table 3a). ADP gave less stimulation in the presence of endogenous cations than when these were supplemented by exogenous

Mg^{2+} and gave even less stimulation when endogenous cations were chelated by EDTA (Table 3b). Table 3b also shows that the effectiveness of the nucleotide-magnesium relief was not mediated through a gross alteration in the pH of the reaction medium which could cause

TABLE 3. EFFECT OF IONS AND pH ON STIMULATION OF THE DNP-RATE BY ADP

Reaction medium	DNP-rate prior to ADP addition (mμatoms/min)	Additions after ADP	Time from ADP to maximum rate (min)	Maximum rate (mμatoms/min)	pH at end
(a) Complete	61	None	1·8	416	—
-$MgCl_2$, +10 mM KCl	93	None	0·0	93	—
(b) Complete (exogenous Mg^{2+})	45	None	2·4	268	7·50
-$MgCl_2$, -EDTA (endogenous cations)	42	None	3·9	232	7·50
-$MgCl_2$ (cations chelated)	47	None	8·5	129	7·45
-$MgCl_2$ (cations chelated)	43	1·45 μmoles HCl after 4 min	10·6	115	6·80

Experiments were performed as in Fig. 4. DNP, 0·1 mM; ADP, 0·2 mM. The complete reaction medium is described in the Experimental section.

the non-enzymatic decarboxylation of OAA. Mg^{2+} is a known stimulant to the nonenzymatic decarboxylation of OAA[9] but the dual dependence on Mg^{2+} and nucleotide indicates that the stimulation under study here is mainly enzymatic.

Uncoupling by Oleic Acid

Inhibition of succinate oxidation is not peculiar to phenolic uncouplers. Oleic acid gave results comparable to DNP. The stimulation by ATP during uncoupling by oleate was more dependent on Mg^{2+} than it was during uncoupling by DNP.

Relative Effectiveness of ATP and ADP

Table 4 shows that although the maximum rate of oxygen uptake achieved by 0·2 mM ATP or ADP was approximately the same, when ADP was the stimulant it took longer to achieve this rate and more oxygen was consumed in doing so. The stimulating effectiveness of 0·1 mM ATP was intermediate to that of 0·2 mM ADP and 0·1 mM ADP. Decreasing the concentration of ATP from 0·2 mM to 0·1 mM, did not decrease the maximum rate achieved, but decreasing the ADP concentration in like manner did decrease the maximum rate.

Mitochondria from the isolations used in the experiments of Table 4 had an ADP:O ratio of $1·21 \pm ·06$[18] with succinate in the absence of DNP. This corresponded to 242 ± 12 mμatoms of oxygen consumed in the conversion of 0·29 μmoles of ADP (0·1 mM) to ATP. Thus, the oxygen consumed in reaching the maximum rate of oxygen uptake in DNP-inhibited mitochondria relieved by 0·2 mM ADP is 60·6 per cent of the oxygen required under the most favorable conditions to oxidatively phosphorylate the ADP present. This would

[18] ± standard error.

TABLE 4. EFFECT OF CONCENTRATION AND TYPE OF ADENINE NUCLEOTIDE ON THE STIMULATION OF THE DNP-RATE

Addition	Concentration (mM)	Time to maximum rate (min)	Oxygen consumed to maximum rate (mμatoms)	Maximum rate (mμatoms/min)
ATP	0·2	1·87±0·11	253±17	275±16
ADP	0·2	2·20±0·02	294±15*	286± 8
ATP	0·1	2·52±0·13	334±12	274±14
ADP	0·1	3·28±0·31	431±69	258±16

Experiments were performed as in Fig. 2. Each value is the mean ± std. error from three and four isolations, for concentrations of 0·1 mM and 0·2 mM, respectively. The time and oxygen consumed to the maximum rate were measured from the addition of the nucleotide.

* This is in contrast to 242±12 mμatoms required by these mitochondria to phosphorylate 0·1 mM ADP in the absence of uncouplers.

result in 0·12 mM ATP when stimulation was complete, if the mitochondria were not uncoupled. Since 0·1 mM ATP is notably less effective than 0·2 mM ADP, it must be concluded that the effectiveness of ADP as a stimulant is not through its conversion to ATP through oxidative phosphorylation. The conversion of ADP to ATP by any other mechanism than oxidative phosphorylation would increase the ADP:O ratio to a value greater than what it would be if oxidative phosphorylation were the only mode of ATP formation. The ADP:O ratio of 1·2 and the tight coupling, observed in the absence of DNP, give no indication that another mode of ATP formation was functioning.

One way of explaining these data while maintaining that ADP is not a stimulant, *per se*, is based on the possibility of compartmentation. The effective site of ATP action could be within a compartment during permeation of which ADP was converted to ATP. The rate of permeation could be equally slow for both ADP and ATP, and a function of the external nucleotide concentrations. Stimulation could then depend upon large amounts of exogenous nucleotide while in fact being effected by the small amount of ATP within the compartment, independently of oxygen consumption.

Failure of Oligomycin to Nullify the ADP Effect

Oligomycin, sufficient to inhibit state 3 respiration, did not eliminate the ADP relief of DNP-initiated inhibition of succinate oxidation, although the relief was somewhat diminished by it (Fig. 5). This also indicates that the ADP relief was not the effect of ATP formed through oxidative phosphorylation.

Involvement of Pi in the ADP Effect

Wiskich and Bonner[12] proposed that the formation of ATP from ADP by adenylate kinase was the reason for the effectiveness of relief by ADP of the DNP-initiated inhibition of succinate oxidation in sweet potato mitochondria. There are two reasons to believe that this is not the case with tomato fruit mitochondria. These are: (1) adenylate kinase activity would cause an apparent high ADP:O ratio whereas that obtained with coupled tomato fruit mitochondria is less than the theoretical maximum of two, in spite of the fact that relatively large amounts of ATP would be required for relief as noted above in the contrasted effectiveness of ATP and ADP; and (2) the effectiveness of ADP as an agent of relief is dependent on orthophosphate (Fig. 6).

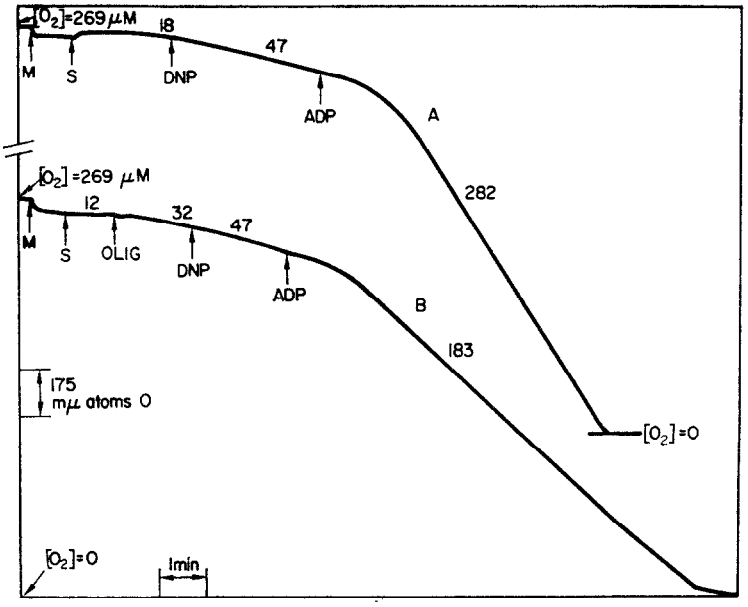

FIG. 5. EFFECT OF OLIGOMYCIN ON THE STIMULATION OF THE DNP-RATE BY ADP.

Numbers along the traces are rates in mμatoms/min. M, mitochondria; S, succinate; DNP, 0·1 mM; ADP, 0·2 mM; OLIG, 2·5 gamma total oligomycin. (A) Without oligomycin. (B) With oligomycin.

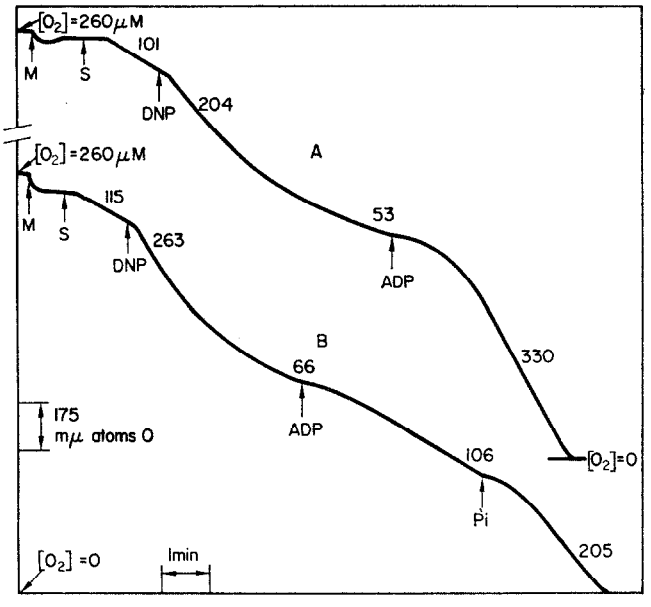

FIG. 6. EFFECT OF ORTHOPHOSPHATE ON THE STIMULATION OF THE DNP-RATE BY ADP.

Numbers along the traces are rates in mμatoms/min. M, mitochondria; S, succinate; DNP, 0·1 mM; ADP, 0·2 mM; Pi, 5 mM. (A) In the presence of Pi. (B) Pi omitted from the reaction medium and added *ca.* 3 min after ADP.

Specificity for Adenine Nucleotide

Guanosine triphosphate and inosine triphosphate were not effective in relieving the DNP-initiated inhibition either in themselves or through a transphosphorylation of ADP in the absence of orthophosphate.

Involvement of OAA in the Inhibition

When Mg^{2+} was omitted from the reaction medium, the substrate-state 4 rate remained fairly constant until oxygen was exhausted (Fig. 7A). If an uncoupler was added during this state, oxygen uptake was initially stimulated, but then severely inhibited (Fig. 7B). In

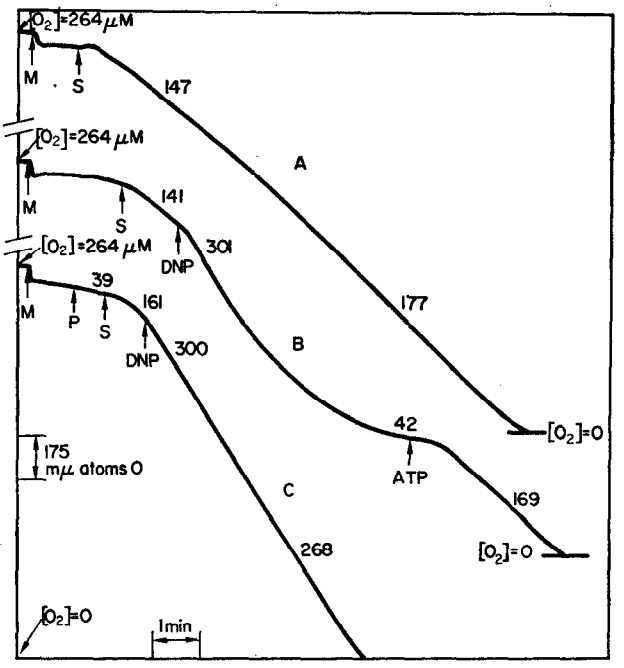

FIG. 7. EFFECT OF PYRUVATE ON STIMULATION OF SUBSTRATE-STATE 4 BY DNP.

$MgCl_2$ was omitted from the reaction medium. Numbers along the traces are rates in mμatoms/min. M, mitochondria; S, succinate; DNP, 0·1 mM; ATP, 0·2 mM; P, pyruvate, 20 mM (equimolar to succinate) plus 0·2 mg thiamine pyrophosphate, 0·4 mg NAD, and 0·1 mg coenzyme A. (A) Substrate 4 with succinate only. (B) Effect of DNP with succinate only. (C) Effect of DNP with succinate and pyruvate.

contrast, when pyruvate with succinate was used as substrate, the stimulation caused by the addition of the uncoupler continued to oxygen exhaustion (Fig. 7C). This indicates that DNP initiated the inhibition of succinate oxidation by permitting the formation of oxaloacetate. This conclusion is in agreement with those drawn from the work on mitochondria of other tissues.[5, 12, 13]

The rapid rate of succinate oxidation in the absence of the uncoupler and Mg^{2+} without inhibition occurring (Fig. 7A) probably reflects a slow rate of malate oxidation. The concentrations of exogenous succinate and endogenous ATP may have been adequate to maintain a reversed gradient through the first phosphorylation site. In this case, NAD would remain in the reduced state and prevent OAA formation from the malate produced by the oxidation of succinate. This condition would be comparable to that obtained with aged

pigeon heart mitochondria treated with ATP.[14] Upon the addition of the uncoupler such a reversed gradient would not be maintained and formation of and inhibition by OAA would ensue.

DISCUSSION

The relative effectiveness of ADP to ATP, the failure of oligomycin to render ADP ineffective, and the dependence of the ADP effect on Pi, preclude the possibility that ADP is converted to ATP in order to stimulate O_2 uptake in uncoupled tomato fruit mitochondria with succinate as substrate. Consequently, ADP + Pi must be effective *per se*. The effectiveness of both ATP and ADP + Pi eliminates, too, the possibility of the involvement of the nucleotides in reversible reactions which could remove OAA such as phosphopyruvate carboxylase (E.C. 4.1.1.32) as proposed for mitochondria for some tissues.[2, 12, 13] A plausible alternative to a chemical reaction involving ADP + Pi and ATP, is a non-energy-expending binding by the nucleotide which effects a favorable conformation for respiration and/or an unfavorable condition for binding by an inhibitor. Such a function is consistent with the observation by some investigators that relief by nucleotide does not require the removal of OAA from the reaction medium.[5] Two possible sites of such a binding are: (1) an allosteric site of succinate dehydrogenase (E.C. 1.3.99.1) which would control the relative affinities of succinate and OAA for the catalytic site; and (2) the phosphorylation sites since the relief is oligomycin sensitive to some extent. The role of magnesium in either case could be the differential enhancement of the binding of both the inhibitor and the nucleotide.

DNP may stimulate oxygen consumption by altering the structure of the mitochondrial membrane to permit the entry of water and hydrolysis of the high energy bonds resulting from substrate oxidation.[19] The binding of ADP and Pi could be required to effect this alteration of structure in the presence of oxaloacetate, However, DNP may operate by forming a readily hydrolyzed bond at the expense of a high energy intermediate, designated $I \sim X$, where X would be phosphorylated to $X \sim P$ during the normal course of phosphorylation.[20] The association of ADP with $X \sim P$ precedes ATP formation with the release of X. If oxaloacetate formed a stable complex with X and adenine nucleotide did not, DNP stimulation, which depends on the recycling of X, would cease until the addition of adenine nucleotide.

If plant mitochondria are as permeable to adenine nucleotides as animal mitochondria, differential permeability would not explain the somewhat greater effectiveness of ATP than ADP in the stimulation of the inhibited rate of oxygen uptake. However, the specific exchange of the nucleotides as whole molecules in the presence of DNP could expain it.[21]

Papa *et al.*[13] have shown that in rabbit kidney mitochondria ATP relief of the DNP-initiated inhibition of succinate oxidation was not oligomycin sensitive, but that it was sensitive to a combination of oligomycin and atractyloside. They interpreted their results on the basis that atractyloside inhibited all intramitochondrial reactions, and that oligomycin did not. This effect of atractyloside is compatible with the suggestion that this phenomenon involves binding without the consumption of the energy of ATP, because atractyloside prevents the binding of exogenous nucleotides.[21]

[19] E. RACKER, *Advan. Enzymol.* **23**, 323 (1961).
[20] J. B. HANSON and T. K. HODGES, in *Current Topics in Bioenergetics* (edited by D. R. SANADI) in press, Academic Press, New York.
[21] E. PFAFF, M. KLINGENBERG and H. W. HELDT, *Biochim. Biophys. Acta* **104**, 312 (1965).

In *Mycobacterium avium*, 5 mM oxaloacetate did not inhibit succinate dehydrogenase, but a 1·6 mM concentration inhibited succinate oxidation.[22] Thus, the function of the adenine nucleotide in the relief of DNP-initiated inhibition is not necessarily at the dehydrogenase level. On the other hand, Kuramitsu[23] has reported the enhancement of malate dehydrogenase (E.C. 1.1.1.37) activity by adenine nucleotides.

Adenine nucleotides may have several structural or allosteric functions in mitochondrial metabolism distinguishable from their reactive roles in oxidative phosphorylation. Stuart and Williams,[24] e.g. have observed an oligomycin-insensitive role for ADP in the regulation of pyruvate metabolism. Also, Connelly and Hallstrom[25] have noted a nonphosphorylative role of adenine nucleotide in increasing the time onset of swelling induced by Pi. Oligomycin enhanced the effectiveness of ADP and diminished the effectiveness of ATP in this phenomenon.

EXPERIMENTAL

Tomato fruit (*Lycopersicon esculentum* Mill.) were picked in the greenhouse or field the day of use. Mature green fruit were used throughout. The entire mitochondrial isolation was done at 0° to 4°. A sample of outer pericarp, 62·5 g, was rinsed in distilled water and chilled in crushed ice, then finely sliced and ground in 125 ml of grinding medium in a Foley food mill. The grinding medium contained 0·5 M mannitol, 0·05 M Na-barbital (pH 7·8), 4 mM cysteine, 5 mM EDTA, and 1·5 mg/ml bovine serum albumin fraction V powder. During grinding the pH dropped to 7·4. Two samples were pressed through broadcloth, and mitochondria were collected as the precipitate between $1500 \times g$ and $15{,}000 \times g$. The mitochondria were washed in 40 ml, recentrifuged, and suspended in 2 ml of medium, which contained 0·5 M mannitol, 5 mM Na-barbital (pH 7·5), and 1·5 mg/ml albumin. Each centrifugation was 15 min. The final mitochondrial suspension was placed in an open, 50-ml centrifuge tube and kept in crushed ice until used.

O_2 uptake by mitochondria was determined by monitoring the O_2 concentration of the reaction medium at room temperature with a Clark oxygen electrode. The reaction medium contained 1·45 mmoles mannitol, 1·45 μmoles EDTA, 14·5 μmoles $MgCl_2$, 29 μmoles Tris (pH 7·5), and 29 μmoles K-phosphate (pH 7·5); 0·2 ml mitochondrial suspension and 58 μmoles of K-succinate were used in a total reaction volume of 2·9 ml. Further additions were made with a microsyringe. ADP:O ratios and respiratory control ratios (i.e. the ratio of state 3/state 4) were calculated by the method of Chance and Williams.[16]

Comparisons of mitochondrial metabolism were made within individual isolations of mitochondria. The 0·2 ml mitochondrial suspension used in each experiment represented 10 g fresh weight of fruit tissue. Measurement of coupled succinate oxidation on two out of every three of the isolations reported on here yielded the following means $\pm$ the standard errors: state 3, 376 $\pm$26 mμatoms O/min/10 g fresh weight and 1551 $\pm$128 μl O_2/hr/mg N; respiratory control ratio, 2·46 $\pm$0·09; ADP:O ratio, 1·25 $\pm$0·04. Mitochondrial nitrogen was determined by nesslerization[26] following acid digestion.

In other experiments using a differential respirometer and a hexokinase trap (unpublished data), the concentrations used here of oligomycin and DNP with mitochondria from 10 g of tissue individually inhibited phosphorylation.

[22] M. KUSUNOSE, S. NAGAI, E. KUSUNOSE and Y YAMAMURA, *J. Bacteriol* **72**, 754 (1956).
[23] H. K. KURAMITSU, *Biochim. Biophys. Res. Commun.* **23**, 329 (1966).
[24] S. C. STUART and G. R. WILLIAMS, *Biochemstry* **5**, 3912 (1966).
[25] J. L. CONNELLY and C. H. HALLSTROM, *Biochemistry* **6**, 1567 (1967).
[26] J. F. THOMPSON and G. R. MORRISON, *Anal. Chem.* **23**, 1153 (1951).