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The Effect of Nucleotides and Inhibitors on Respiration in Isolated Wheat Mitochondria[1]

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ABSTRACT

The effect of mono-, di-, and trinucleoside phosphates and respiratory inhibitors on respiration in winter wheat (*Triticum aestivum* L. cv. Rideau) mitochondria has been examined. When added during state 4 respiration, subsequent to addition of ADP, all of the dinucleotides stimulated oxidation and induced respiratory control with all substrates examined. Similar results were obtained with AMP, but other mononucleotides and all trinucleotides did not affect the rate of oxidation. Nucleoside diphosphates did not stimulate respiration when added prior to the addition of ADP, but subsequent addition of AMP, ADP, or ATP re-established coupled respiration in the presence of the dinucleotides.

The duration of 2,4-dinitrophenol stimulated respiration during oxidation of α-ketoglutarate was found to be dependent on the amount of AMP, ADP, or ATP added, either prior, or subsequent to, addition of the uncoupler. The addition of oligomycin during 2,4-dinitrophenol stimulated respiration re-established coupled respiration with low ADP/O ratios, when added after addition of ATP or conditions which allow formation of ATP from added ADP. The nucleoside diphosphates, other than ADP, did not stimulate oxidation of α-ketoglutarate in the presence of 2,4-dinitrophenol until a small amount of adenine nucleotide was added to the system. The results suggest that dinucleotides other than ADP, are able to participate in the energy conversion processs of the mitochondria, probably via transphosphorylation reactions.

Studies on the respiratory properties of mitochondria isolated from different plant sources and by different isolation procedures have indicated that considerable variation may exist in their response to various substrates in the presence of uncouplers and respiratory inhibitors (3, 16, 27, 32). Therefore, it is often difficult to extrapolate results obtained from one system to another. The question of which nucleotides are able to act as phosphate acceptors has been examined in several systems from both plant and animal sources (14, 21, 24). Earlier reports had suggested that dinucleotides other than ADP could act as phosphate acceptors in submitochondrial particles, but not in intact mitochondria (18) and that AMP was an earlier acceptor of phosphate than ADP during oxidative phosphorylation in animal mitochondria (21). Recently (12), it has been demonstrated that ADP is the prime phosphoryl acceptor in rat liver mitochondria but other dinucleotides can participate in the energy conversion process via transphosphorylation reactions. Evidence has been presented that ADP is the prime phosphate acceptor in photophosphorylation of chloroplasts (14), but the role of other nucleotides in phosphorylation reactions in plants is not clear.

The present study was undertaken in an attempt to characterize the effect of nucleotides and respiratory inhibitors on respiration in the winter wheat mitochondrial system currently being used in this laboratory to examine structural and functional changes in mitochondrial membranes in relation to growth at low temperature (20).

MATERIALS AND METHODS

Isolation of Mitochondria. Seedlings of winter wheat (*Triticum aestivum* L. cv. Rideau) were germinated and grown in the dark at 24 C on moist filter paper for 2 days. Mitochondria were isolated from the shoots by differential centrifugation (22) and where indicated, further purified by sucrose density gradient centrifugation. Sucrose gradients were prepared by layering 3.7 ml each of 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2 M sucrose solutions containing 10 mM KCl, 10 mM tris-HCl 10 mM KH_2PO_4, and 0.75% (w/v) BSA at pH 7.2 in 30-ml centrifuge tubes and stored 24 hr at 2 C to produce a linear gradient. A 2.2-ml aliquot of the mitochondrial preparation was layered on top of the gradient and centrifuged for 1 hr at 23,000*g* in a Spinco SW 25-1 rotor. A clear zone visible at a position in the tube corresponding to 1.4 to 1.6 M sucrose was removed using a hypodermic syringe, diluted dropwise five times with the same buffer (minus sucrose) used in the gradient, centrifuged at 20,000*g* for 15 min, and the pellet was resuspended in a suitable medium.

Respiratory Measurements. Oxygen uptake was measured polarographically at 24 C with a conventional Clark electrode using media and methods previously described (22). The efficiency of phosphorylation in the presence of nucleotides other than ADP was calculated from O_2 uptake upon addition of a given amount of nucleotide and expressed as apparent P/O ratios. All organic chemicals were obtained from Schwartz/Mann Ltd. or Sigma Chemical Company. All inorganic reagents were of analytical reagent grade and solutions prepared in double distilled water were dissolved in small values of ethanol and added to a final concentration not exceeding 2% in the reaction cuvette. Mitochondrial protein was determined in BSA-free suspensions by the method of Lowry *et al.* (19).

Determination of APTase and Adenylate Kinase Activity. ATPase and adenylate kinase activities were determined by a modification of the methods of Sarkissian and Srivastava (26)

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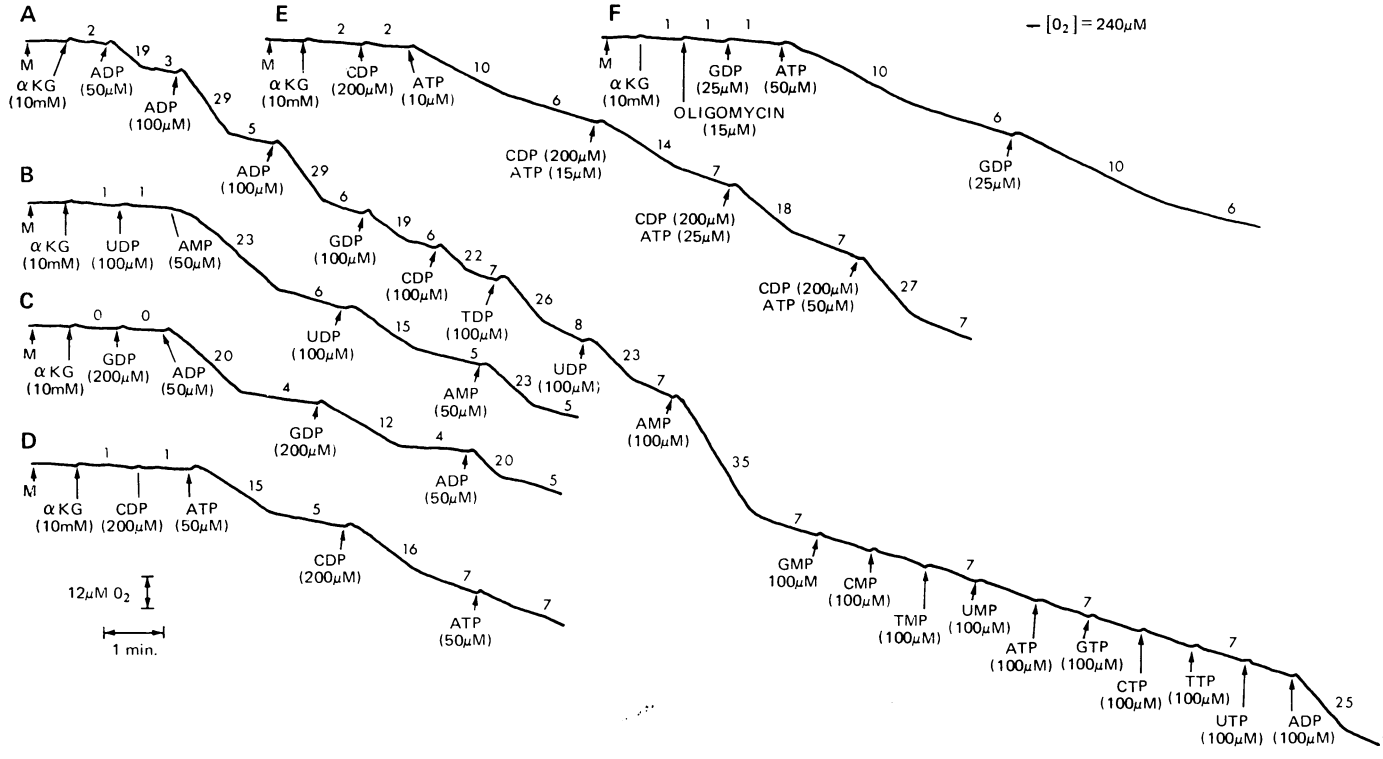

FIG. 1. Polarograph traces showing the effect of nucleotides on α-ketoglutarate oxidation. A: Effect of mono-, di-, and trinucleotides added after addition of ADP; B, C, D: effect of dinucleotides added prior to addition of adenine nucleotides, and subsequent addition of AMP, ADP and ATP, respectively; E: effect of ATP concentration on respiration in the presence of CDP; F: effect of oligomycin on respiration in the presence of CDP; F: effect of oligomycin on respiration in the presence of CDP and ATP. Numbers along the traces are rates of O_2 uptake in nmoles/min·1.5 ml. M indicates addition of mitochondria containing approximately 0.50 mg of mitochondrial protein.

and Blackmon and Moreland (4). Mitochondria were isolated as previously described using a grinding medium consisting of 0.25 M sucrose, 3 mM EDTA and 25 mM tris-HCl at pH 7.2. The pellet was suspended in a medium containing 0.3 M sucrose, 1 mM $MgSO_4$, and 1 mM tris-HCl at pH 7.2 and either assayed directly, or further purified on a sucrose density gradient containing 1 mM $MgSO_4$ and 1 mM tris-HCl. Mitochondrial ATPase and adenylate kinase activities were measured, as previously described (26), using a reaction mixture containing 0.1 M sucrose, 0.1 M tris-HCl, 3 mM $MgCl_2$, and 15 mM KCl at pH 7.3.

Electron Microscopy. The mitochondrial pellets were fixed, processed, stained, and examined in a Siemens Elmiskop I (2).

RESULTS

Effect of Nucleotides. The effect of adding mono-, di-, and trinucleotides to isolated wheat mitochondria respiring with relatively good respiratory control and ADP/O ratios is shown in Figure 1A and Table I. All four substrates exhibited a relatively high degree of coupled respiration in the presence of GDP, CDP, UDP, TDP, and AMP, but the remaining mononucleotides and all trinucleotides used did not stimulate state 4 oxidation rates of the four substrates. Respiratory control values in the presence of nucleotides other than ADP varied somewhat, but were generally similar to those obtained with ADP. The efficiency of phosphorylation, as indicated by P/O ratios, varied depending upon which nucleotide was being provided as a source of phosphate acceptor (Table I). With ADP as acceptor ADP/O ratios for all substrates were slightly lower than the generally accepted theoretical maxima of 4, 3, 2, and 2, respectively, for α-ketoglutarate, malate, succinate, and exogenous NADH, while considerable variation was observed in apparent P/O ratios in the presence of other nucleotides.

The addition of dinucleotides, in the absence of exogenous ADP did not stimulate O_2 consumption (Fig. 1, B, C, and D). However, the subsequent addition of any of the three adenine nucleotides stimulated O_2 uptake, and further additions of the dinucleotides produced controlled respiration with respiratory control values and P/O ratios similar to those observed when the dinucleotides were added after initial addition of ADP (Table I). The duration of state 3 respiration was proportional only to the amount of dinucleotide (other than ADP) added when ATP was used to initiate state 3, but was proportional to the amount of dinucleotide plus ADP or AMP when the latter was added to initiate state 3 respiration (Fig. 1, B and C). The rate of O_2 consumption in the presence of the dinucleotides also was dependent on the concentration of adenine nucleotide added to initiate state 3 respiration (Fig. 1E). The rates of both state 3 and state 4 respiration attained maxima when the concentration of ATP or ADP reached approximately 50 μM. This observation is in accord with an earlier report by Garber and Ballard (15) who showed that transphosphorylation reactions in guinea pig mitochondria do not occur at low ATP concentration. Respiratory control and a low level of phosphorylation were retained with the dinucleotides during oxidation of α-ketoglutarate in the presence of oligomycin (Fig. 1F), whereas respiratory control was abolished during oxidation of malate.

Effect of Density Gradient Purification. The properties of mitochondrial preparations purified by differential centrifugation and after further purification by sucrose density gradient centrifugation are compared in Table II. Mitochondrial yield,

as indicated by protein content, was reduced approximately two-thirds by density gradient purification but no appreciable differences were observed in the rates of oxidation of α-ketoglutarate, when expressed on a protein basis, and respiratory control values and ADP/O ratios were similar for the two methods. However, electron micrographs of mitochondrial pellets obtained by the two procedures (Fig. 2) revealed that gradient purification yields preparations which contain a much higher proportion of uniform intact mitochondria.

The effect of various nucleotides on the oxidation of α-ketoglutarate by mitochondria isolated by the two procedures is compared in Table I. Irrespective of the method used, the rate of O_2 consumption by mitochondria in state 3 and state 4 was similar. Respiratory control values and apparent P/O ratios were also similar, indicating that the respiratory properties of wheat mitochondria are not altered appreciably by further purification through density gradient centrifugation. Similar results were obtained with malate, succinate, and NADH as substrates.

The levels of ATPase and adenylate kinase activities of mitochondrial preparations obtained by the two procedures were not significantly different. However, the activities of both enzymes were considerably lower than those previously reported for wheat mitochondria (26).

Table I. *Effect of Nucleotides on Oxidative Phosphorylation by Isolated Wheat Mitochondria*

Nucleotides were added during state 4 respiration, subsequent to addition of ADP, using the reaction medium described in Table II. "Other" nucleotides include GMP, CMP, UMP, TMP, GTP, CTP, UTP, and TTP. Values in parentheses are for mitochondria purified by sucrose density gradient. Data are averages from at least three experiments.

Substrate	Nucleotide	Oxygen Uptake		Respiratory control	Apparent P/O
		State 3	State 4		
		nmoles/min·mg protein		*ratio*	
α-Ketoglutarate	ADP	117.8	26.4	4.5 (4.3)	3.3 (3.4)
	GDP	78.8	22.7	3.5 (3.1)	7.1 (5.6)
	CDP	90.9	24.8	3.6 (2.3)	7.4 (8.1)
	UDP	87.0	25.2	3.4 (3.2)	4.3 (4.4)
	TDP	93.6	25.8	3.6 (3.2)	3.4 (2.4)
	AMP	122.1	24.3	5.0 (4.9)	1.6 (1.6)
	Others	26.4	26.4	...	...
L-Malate	ADP	118.5	25.8	4.6	2.5
	GDP	83.3	26.3	3.2	6.7
	CDP	96.9	28.4	3.4	7.0
	UDP	84.3	21.8	3.8	4.0
	TDP	85.8	21.5	4.0	3.3
	AMP	124.8	37.2	3.4	1.2
	Others	25.8	25.8	...	...
Succinate	ADP	186.6	86.3	2.2	1.7
	GDP	179.3	93.3	1.9	2.7
	CDP	163.4	87.0	1.9	3.3
	UDP	186.2	92.1	2.0	1.6
	TDP	178.2	72.9	2.5	1.4
	AMP	202.8	81.2	2.5	0.8
	Others	86.3	86.3	...	...
NADH	ADP	147.8	59.1	2.6	1.9
	GDP	107.7	58.8	1.8	2.1
	CDP	102.6	64.1	1.6	3.1
	UDP	133.4	63.5	2.1	1.6
	TDP	157.2	56.1	2.6	1.3
	AMP	157.8	60.6	2.6	0.6
	Others	59.1	59.1	...	...

Table II. *Comparison of Properties of Mitochondria Isolated by Differential Centrifugation and after Further Purification by Sucrose Density Gradient Centrifugation*

Respiratory measurements were determined as previously described (22) in a medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4,10 mM tris-HCl buffer, 0.75% (w/v) BSA, and 10 mM α-ketoglutarate. Data are averages from at least five experiments.

Isolation	Oxygen Uptake		Respiratory Control	ADP/O	Activity		Protein Content
	State 3	State 4			ATPase	Adenylate Kinase	
	nmoles/min·mg protein		*ratio*		*μmoles Pi/hr·mg protein*		*mg/g fresh wt*
Differential centrifugation	109.9	24.5	4.5	3.1	5.1	4.2	0.73
Density gradient	105.9	26.4	4.0	3.3	5.5	4.9	0.25

Effect of Respiratory Inhibitors and Uncouplers. The effect of respiratory inhibitors and uncouplers on the oxidation of α-ketoglutarate, malate, succinate, and NADH by mitochondria prepared by differential centrifugation is presented in Table III. The addition of the respiratory chain phosphorylation inhibitors, oligomycin (17) and atractyloside (12, 25), during state 3 respiration resulted in severe inhibition of ADP-stimulated respiration of all four substrates. Respiratory control was completely abolished with both inhibitors, except for a very low level retained during oxidation of α-ketoglutarate.

The extent of inhibition of respiration by amytal and rotenone varied considerably depending on the substrate being oxidized (Table III), but the results are generally in accord with those reported for other plant mitochondria (6, 8, 16, 32). However, in contrast with the findings of Carmeli and Biale (6) on sweet potato mitochondria, amytal severely inhibited state 3 oxidation of exogenous NADH. Also, the retention of a relatively high rate of α-ketoglutarate oxidation in the presence of amytal appears to be at variance with the postulation that all NAD-linked substrates are severely inhibited by amytal (13). The observation that ADP/O ratios declined by approximately one-fourth and one-third during oxidation of α-ketoglutarate and malate, respectively, in the presence of rotenone is consistent with a recent suggestion by Brunton and Palmer (5) that rotenone reduces NAD-linked substrate phosphorylation by the equivalent of one phosphorylation site, by by-pass of the first electron chain site.

The addition of the Cyt oxidase inhibitors cyanide and azide during state 3 oxidation of all four substrates resulted in severe inhibition of respiration (Table III). Respiratory control was abolished by both azide and cyanide during oxidation of the four substrates, except for α-ketoglutarate in the presence of cyanide. However, when the concentration of cyanide was increased from 0.01 mM to 0.1 mM, respiratory control was markedly decreased due to a decrease in state 4 respiration, whereas in the presence of 5 mM cyanide, both state 3 and state 4 respiration were reduced to 10% of normal state 3 and respiratory control was eliminated.

Numerous reports (3, 6, 28, 31) suggest the presence of cyanide and azide-insensitive alternate terminal oxidases in mitochondria from many plant sources to account for oxidation of substrates in the presence of Cyt oxidase inhibitors. The

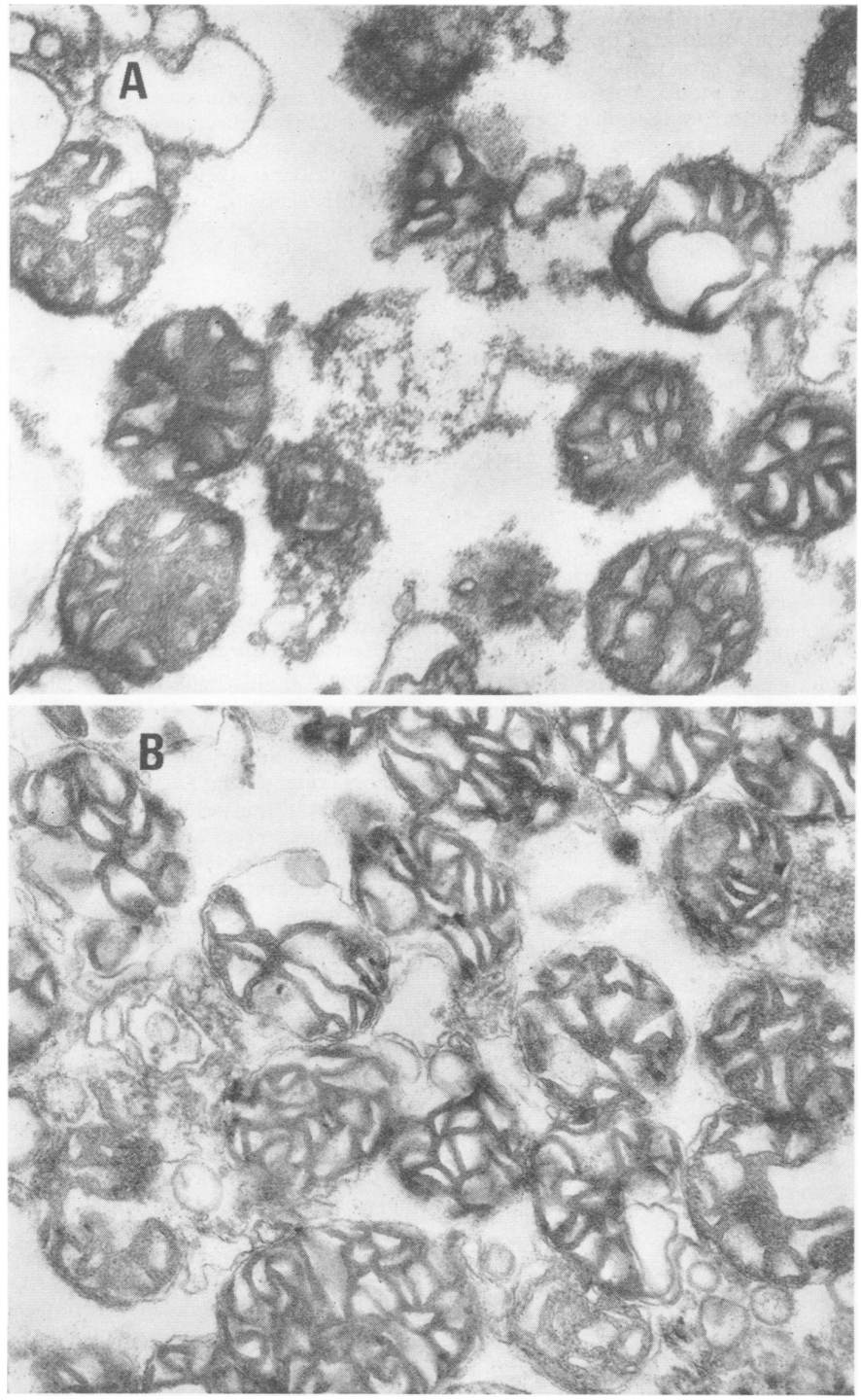

FIG. 2. Electron micrographs of mitochondria prepared by differential centrifugation (A), and after further purification by sucrose gradient centrifugation (B). × 37,500.

results obtained here indicate that such pathways exist in winter wheat mitochondria, since significant respiration was retained in the presence of both cyanide and azide. The retention of considerable respiratory control and relatively high ADP/O ratios during oxidation of α-ketoglutarate in the presence of cyanide suggest that α-ketoglutarate oxidation is less sensitive to cyanide inhibition than oxidation of other substrates. Other studies (3, 32) on mitochondria from different plant sources have demonstrated the existence of cyanide-insensitive respiration in the presence of varied cyanide concentration.

Antimycin A, a respiratory chain inhibitor which acts between Cyt *b* and c_1 (23, 29), severely inhibited O_2 consumption and abolished respiratory control during oxidation of all four substrates (Table III). This observation supports the view that an antimycin A-insensitive oxidase system is present in plant mitochondria (9, 29, 32).

The effect of several specific substrate inhibitors was ex-

Table III. *Effect of Antibiotics and Respiratory Inhibitors on Oxidative Phosphorylation*

Antibiotics and inhibitors were added during state 3 oxidation of mitochondria isolated only by differential centrifugation. Reaction medium as indicated in Table II. Data are averages of at least four experiments.

Antibiotic or Inhibitor	α-Ketoglutarate				L-Malate				Succinate				NADH			
	Oxygen uptake		R.C.[1]	ADP/O	Oxygen uptake		R.C.	ADP/O	Oxygen uptake		R.C.	ADP/O	Oxygen uptake		R.C.	ADP/O
	State 3	State 4			State 3	State 4			State 3	State 4			State 3	State 4		
	nmoles/min·mg protein		*ratio*		*nmoles/min·mg protein*		*ratio*		*nmoles/min·mg protein*		*ratio*		*nmoles/min·mg protein*		*ratio*	
None	120.0	24.8	4.8	3.2	122.7	24.9	4.9	2.7	201.3	92.4	2.2	1.7	164.4	63.8	2.6	1.8
Antimycin A, 4 μM	28.2	28.2	...	...	23.3	23.3	...	...	26.3	26.3	...	...	13.7	13.7	...	...
Amytal, 6 mM	74.9	35.7	2.1	1.4	22.2	22.2	...	...	151.5	111.9	1.4	1.2	86.0	86.0	...	...
Arsenite, 1 mM	15.0	15.0	...	...	120.3	24.0	4.9	2.7	198.9	87.9	2.2	1.6	151.7	55.5	2.7	1.7
Atractyloside, 15 μM	34.4	25.5	1.3	2.6	27.9	27.9	...	...	102.0	102.0	...	...	66.3	66.3	...	...
Azide, 10 mM	28.2	28.2	...	...	24.9	24.9	...	...	44.7	44.7	...	...	18.2	18.2	...	...
Cyanide, 0.1 mM	60.0	23.3	2.6	2.4	23.3	23.3	...	...	44.9	44.9	...	...	6.2	6.2	...	...
Dinitrophenol, 0.1 mM	102.8	35.4	2.8	0.8	104.3	104.3	...	...	171.0	171.0	...	...	160.2	160.2	...	...
Malonate, 5 mM	84.2	15.3	5.5	3.5	99.5	19.5	5.1	2.6	26.3	26.3	...	...	157.8	63.8	2.5	1.8
Oligomycin, 15 μM	48.0	30.0	1.6	0.6	16.1	16.1	...	...	93.6	93.6	...	...	47.9	47.9	...	...
Oxaloacetate, 1 mM	26.0	6.9	3.8	3.7	14.3	14.3	...	...	3.2	3.2	...	...	0	0	...	...
Rotenone, 8 μM	86.4	26.3	3.2	2.3	53.7	28.2	1.9	1.6	163.2	81.0	2.0	1.6	159.6	63.3	2.5	1.7

[1] Respiratory control.

amined also in this study. Arsenite, an inhibitor of dihydrolipoyl dehydrogenase (1), severely inhibited both state 3 and state 4 oxidation of α-ketoglutarate, but, as expected, did not affect the rate of O_2 consumption during oxidation of malate, succinate, or NADH. Malonate, an inhibitor of succinic dehydrogenase (16), markedly reduced both state 3 and state 4 respiration, and abolished respiratory control during oxidation of succinate, but with the other substrates only slight inhibition was observed. Oxaloacetate severely inhibited both state 3 and state 4 oxidation of all four substrates. The severe inhibition of α-ketoglutarate and malate oxidation, and almost complete inhibition of both succinate and NADH oxidation by oxaloacetate are consistent with its postulated roles as a competitive inhibitor of succinic dehydrogenase and an oxidant of NADH (10, 32).

The effect of DNP[2] on respiration varied widely, depending on which substrate was being oxidized. During oxidation of α-ketoglutarate, the addition of increasing concentrations of DNP during state 4 respiration (Table IV) resulted in enhanced rates of respiration, until a concentration of 100 μM DNP was reached, after which further addition of DNP was inhibitory. Both respiratory control and ADP/O ratios declined with increasing concentration of DNP. Therefore, 100 μM DNP was used in further experiments to study its effect on respiratory properties.

Dinitrophenol stimulated respiration of all substrates by more than 80% of the normal state 3 rates (Table III; Figs. 3 and 4) and completely abolished respiratory control during oxidation of malate, succinate, and NADH. However, DNP-stimulated respiration gradually declined after 1 to 2 min with malate and succinate as substrates and was unaffected by further additions of ADP or DNP. The addition of ATP, during this diminished rate of oxidation, stimulated O_2 consumption with succinate as substrate (11), while malate oxidation was unaffected by added ATP.

The addition of DNP during state 4 oxidation of α-ketoglutarate stimulated O_2 consumption slightly less than that observed during normal state 3 respiration (Table III, Fig. 4A).

[2] Abbreviation: DNP: 2,4-dinitrophenol.

Table IV. *Effect of 2,4-Dinitrophenol on Oxidative Phosphorylation by Wheat Mitochondria Utilizing α-Ketoglutarate as Substrate*

DNP was added during state 4 respiration. Data are averages from three to five experiments.

DNP	DNP-stimulated Rate		Respiratory Control	ADP/O
	of State 3	of State 4		
μM	%		*ratio*	
0	22	100	4.6	3.1
0.1	22	100	4.6	3.1
1.0	25	114	2.0	2.7
10	40	190	2.4	2.3
100	85	412	2.8	0.7
500	19	85	1.4	0.7
1000	14	66	...	...

The duration of this DNP-stimulated respiration was dependent on the amount of ADP added to the system, either prior to, or after addition of the uncoupler. Respiratory control was re-established during DNP-stimulated respiration by the addition of oligomycin (Fig. 4B). However, when DNP was added prior to the addition of ADP (Fig. 4D), the subsequent addition of oligomycin did not re-establish respiratory control. Similar results were obtained using AMP as a source of phosphate acceptor, although P/O ratios were lower than observed with ADP (Fig. 4A). The addition of ATP during state 4 oxidation of α-ketoglutarate did not stimulate O_2 consumption, but did extend the duration of subsequent DNP-stimulated respiration (Fig. 4C) in a manner similar to that observed with increased concentration of ADP (Fig. 4B). Other nucleoside diphosphates did not stimulate oxidation of α-ketoglutarate in the presence of DNP, prior to the addition of ADP (Fig. 4E).

All of the dinucleotides partially released oligomycin-induced inhibition of uncoupling by DNP during state 4 oxidation of α-ketoglutarate, although respiratory control values and apparent P/O ratios were lower than observed during respira-

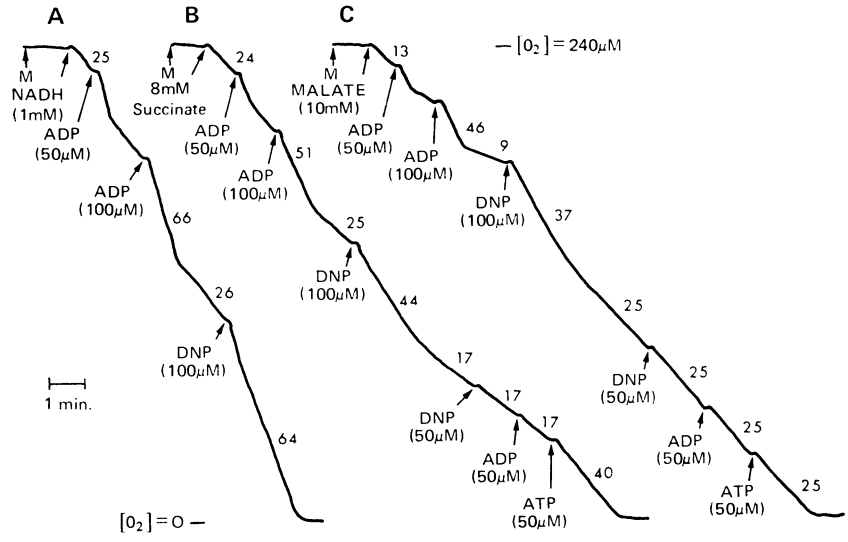

FIG. 3. Polarograph traces showing the effect of DNP on O_2 uptake during oxidation of NADH (A), succinate (B), and L-malate (C). Numbers along traces are rates of O_2 uptake in nanomoles/min·1.5 nl. M indicates addition of mitochondria containing approximately 0.50 mg of mitochondrial protein.

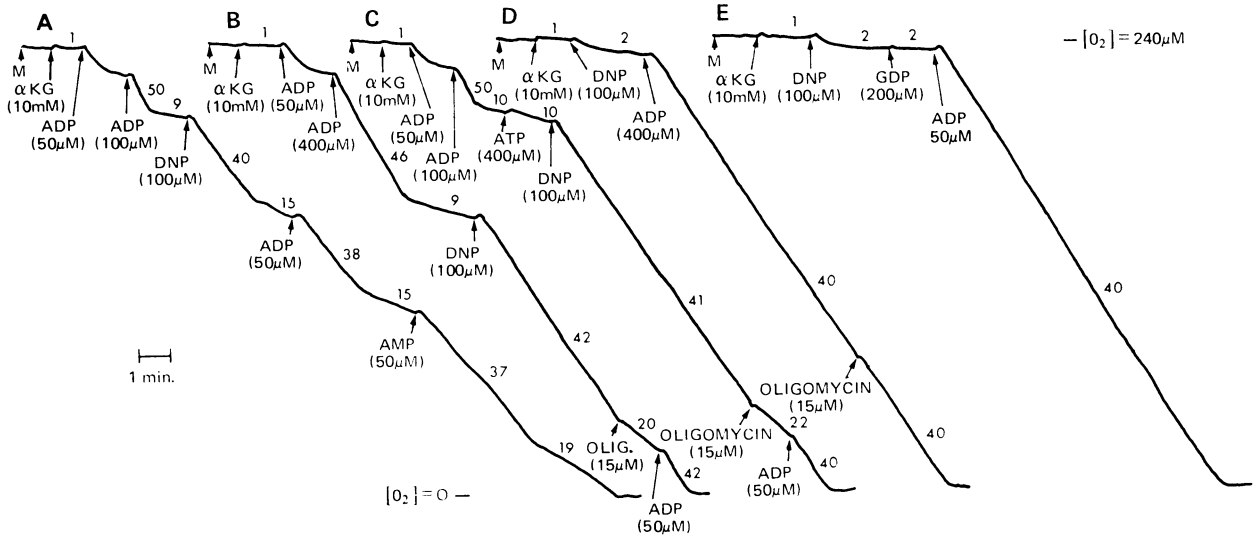

FIG. 4. Polarograph traces showing the relationship between added nucleotides and DNP-stimulated respiration during oxidtaion of α-Ketoglutarate. A, B: Effect of ADP concentration; C: effect of ATP; D: effect of ADP added after addition of DNP; E: effect of GDP. Numbers along traces are rates of O_2 uptake in nanomoles/min·1.5 ml. M indicates addition of mitochondria containing approximately 0.50 mg of mitochondrial protein.

tion in the absence of oligomycin and DNP (Table V). The trinucleotides, except ATP, also partially released the inhibition, but the rates of respiration were generally lower than those observed with the dinucleotide and apparent P/O ratios were much greater. Adenosine monophosphate also partially released the inhibition, and while respiratory control was similar to that observed with ADP, the P/O ratio was markedly lower.

DISCUSSION

An important problem in the field of oxidative phosphorylation is the question of which nucleotides can serve as phosphate acceptors in phosphorylation reactions. There is strong evidence from studies on both plant and animal mitochondria to suggest that both ADP and AMP can fulfill this role (12, 21, 24, 25), but considerable doubt still exists as to which of these two adenyl nucleotides is the first phosphoryl acceptor in oxidative phosphorylation. The results obtained in the current study clearly demonstrate that reasonably intact mitochondria oxidize Kreb's cycle intermediates with good respiratory control in the presence of AMP and several nucleoside diphosphates. Furthermore, the rate of O_2 consumption, respiratory control, and efficiency of phosphorylation are not altered by density gradient purification of the preparations suggesting that these observations cannot be attributed to the presence of submitochondrial particles as reported by Löw *et al.* (18) for beef heart.

All dinucleotides examined permit respiration to proceed with relatively good respiratory control. This poses the question whether all dinucleotides can themselves serve as primary phosphate acceptors in oxidative phosphorylation, or whether

Table V. *Effect of Nucleotides on Oligomycin-induced Inhibition of Uncoupling of Dinitrophenol during State 4 Oxidation of α-Ketoglutarate*

Oligomycin (15 μM) was added during state 4 respiration induced by ADP exhaustion. DNP (100 μM) was then added followed by addition of nucleotides. Data are averages of at least three experiments.

Nucleotide	Oxygen Uptake		Respiratory Control	Apparent P/O
	State 3	State 4		
	nmoles/min·mg protein		*ratio*	
None	...	29.7[1]	...	...
ADP	96.0	48.0	2.0	0.7
GDP	69.6	38.7	1.8	1.0
CDP	73.2	43.1	1.7	1.1
TDP	87.9	44.0	2.0	0.6
UDP	82.8	51.8	1.6	0.7
ATP	39.9	39.9	...	...
GTP	57.6	32.7	1.8	3.0
CTP	48.0	34.7	1.4	4.6
TTP	60.3	39.6	1.5	3.3
UTP	50.7	31.2	1.6	4.1
AMP	97.8	46.7	2.1	0.4
GMP	45.3	45.3	...	...
CMP	45.3	45.3	...	...
TMP	45.3	45.3	...	...
UMP	45.3	45.3	...	...

[1] Rate after addition of oligomycin and DNP, prior to addition of nucleotide.

they are involved in transphosphorylation reactions leading to the formation of ADP which in turn acts as phosphate acceptor. The latter possibility is supported by the fact that controlled oxidation of substrates occurs only in the presence of exogenous adenine nucleotides. However, the possibility that adenine nucleotides also may serve as an energy source for transport of the dinucleotides to the sites of oxidative phosphorylation cannot be excluded.

Duée and Vignais (12) have suggested that transphosphorylation of AMP to ADP in rat liver mitochondria is catalyzed by a coupled GTP-AMP phosphotransferase and nucleoside diphosphokinase reaction resulting in the formation of 2 moles of ADP for each mole of AMP added to the reaction. This type of reaction could explain the results obtained with wheat mitochondria, where the addition of AMP yielded P/O ratios approximately one-half those observed with ADP (Table I). Ozawa (21) also reported that AMP/O ratios were approximately one-half the ADP/O ratios in respiring beef heart mitochondria, and suggested that AMP and not ADP is the prime external acceptor of phosphoryl groups.

Stimulation of O_2 uptake by dinucleotides, other than ADP, may also be mediated by a nucleoside diphosphokinase catalyzed transphosphorylation reaction with ATP, resulting in the formation of ADP. Subsequent oxidative phosphorylation of this ADP could then provide ATP for continuing the transphosphorylation reactions in a cyclic manner. This interpretation is consistent with the observation that all three adenine nucleotides initiated state 3 oxidation and subsequent respiratory control in the presence of other dinucleotides. Evidence for the existence of nucleoside diphosphokinases in several species of plants has been reported (30).

The generally lower respiratory control values obtained with the dinucleotides, other than ADP, are largely unexplained. The data show that reduced respiratory control is mainly the result of decrease in state 3 respiration. However, the dinucleotides themselves are not inhibitory, since no change in respiration occurred when GDP was added in the presence of ADP during state 3 oxidation of α-ketoglutarate. This suggests that state 3 oxidation in the presence of the dinucleotides was probably limited by the rates of transphosphorylation reactions leading to the formation of ADP. Since the presence of ADP is necessary for maximum O_2 consumption in tightly coupled mitochondria, the rate of ADP formation by transphosphorylation reactions would directly affect state 3 respiration.

The reason for the consistent differences observed in apparent P/O ratios among the dinucleotides is not clear. The results suggest that less ADP may be available for oxidative phosphorylation when GDP and CDP are added to the system since the duration of state 3 oxidations were much shorter than with either UDP or TDP. These observations could be accounted for either by dinucleotide specificity for transphosphorylation reactions, or by differential utilization of dinucleotides in other reactions before or during transphosphorylation.

The results obtained in the study of the relationship between uncouplers of phosphorylation and specific inhibitors of oxidative phosphorylation, during oxidation of malate, succinate, and NADH are in agreement with the well established pattern of complete inhibition of ADP-stimulated O_2 consumption by oligomycin (17) and marked stimulation by the uncoupler DNP, in the absence of ADP. However, with α-ketoglutarate as substrate, considerable respiratory control was retained, since substrate-level phosphorylation is insensitive to both oligomycin and DNP (7). Previous studies have shown that the addition of oligomycin during state 4 oxidation of α-ketoglutarate inhibits stimulation by the subsequent addition of DNP, and that this inhibition can be relieved by the addition of ADP or AMP (22, 33). Results reported in Table V confirm these observations, and further demonstrate that this inhibition is relieved by all dinucleotides, and by all trinucleotides, except ATP. The effect of the nucleoside diphosphates, other than ADP, is probably mediated via transphosphorylation reactions resulting in the formation of ADP, which can then serve as phosphate acceptor for substrate level phosphorylation. The addition of ATP does not release oligomycin-induced inhibition since the ATPase required for hydrolysis of ATP to provide ADP as a phosphate acceptor is inhibited by oligomycin (17, 33). The observation that nucleoside triphosphates, other than ATP, do relieve this inhibition suggests the presence of specific oligomycin-insensitive nucleoside triphosphatases capable of hydrolyzing trinucleotides to the corresponding dinucleotides from which ADP can be generated via transphosphorylation reactions.

The failure of DNP to uncouple oxidation of α-ketoglutarate in the presence of oligomycin has been attributed to inhibition of ATPase necessary to provide ADP for substrate-level phosphorylation (33). The results support this view, and further demonstrate that the duration of DNP-stimulated oxidation of α-ketoglutarate is dependent on the amount of any of the three adenine nucleotides in the reaction system. In the absence of oligomycin, ADP for substrate-level phosphorylation is readily synthesized from either ATP or AMP and hence, an increase in either of these nucleotides results in an increase in the duration of stimulated respiration. The addition of oligomycin during DNP-stimulated respiration in the presence of excess ATP results in immediate inhibition, since inhibition of ATPase prevents further formation of ADP. On the other hand, oligo-

mycin does not inhibit respiration in the presence of excess ADP or AMP since under these conditions, ATPase is not involved in the maintenance of respiratory control. These results clearly demonstrate that substrate-level phosphorylation is dependent on the action of ATPase under conditions of limiting ADP. However, the level of ATPase activity in normally respiring isolated winter wheat mitochondria must be low as shown by the relatively high degree of respiratory control exhibited by the preparations during oxidation of α-ketoglutarate and malate.

The observation that GDP does not stimulate the rate of oxidation of α-ketoglutarate via substrate-level phosphorylation, in the presence of DNP, until at least a small amount of adenine nucleotide is added, suggests that an exogenous source of energy may be required for transport of the dinucleotides into the mitochondria. This interpretation is in accord with the previously discussed observation that the nucleoside diphosphates (including GDP) also support controlled respiration, in the absence of uncoupler, only after addition of a small amount of adenine nucleotide. These results, however, do not preclude the possibility that transphosphorylation reactions occur after transport of the dinucleotides into the mitochondria, and indeed it seems likely that both systems are operable in isolated wheat mitochondria.

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