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Studies on the Role of Mg^{2+} and the Mg^{2+}-Stimulated Adenosine Triphosphatase in Oxidative Phosphorylation†

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ABSTRACT: When heart mitochondria were incubated with NAD-linked substrates at pH 7.4, added $MgCl_2$ markedly reduced the respiratory control and P:O ratios. The magnitude of the effect of Mg^{2+} on the P:O ratio was a direct function of the ratio of ATP:ADP present in the suspending medium. Added Mg^{2+} stimulated "state 4" respiration in a transcient and biphasic manner. This response was completely blocked by oligomycin or atractyloside, and markedly reduced by aurovertin, an ADP trap (phospho*enol*pyruvate *plus* pyruvic kinase), or by the absence of inorganic phosphate. The amount of oxygen consumed during the initial rapid phase of respiration was proportional to the amount of adenine nucleotide present, but was independent of the concentrations of added Mg^{2+} or mitochondrial protein. The rapid phase of Mg^{2+}-stimulated respiration was accompanied by an immediate and marked dephosphorylation of ATP to ADP, followed by a much more gradual increase in the ratio of ATP to ADP in the suspending medium. In contrast, the intramitochondrial ATP:ADP ratio was unaffected by Mg^{2+}. The Mg^{2+}-ATPase was blocked by oligomycin but was only partially arrested by aurovertin and was insensitive to atractyloside. It is concluded that all of the effects of Mg^{2+} reported herein are mainly, if not wholly, accounted for by the Mg^{2+}-stimulated ATPase activity which ensues simultaneously with oxidative phosphorylation. This activity appears to be regulated, in turn, by the ATP:ADP ratio. This ATPase, which is in all respects inert when no Mg^{2+} is added, and which functions as an "external" ATPase in the presence of Mg^{2+}, is probably identical with the ATPase-coupling factor of oxidative phosphorylation which is present as a contaminant of fully intact mitochondria. The failure of uncouplers to reverse the inhibition of the oxidation of α-oxoglutarate or glutamate by mitochondria incubated in the absence of Mg^{2+} is interpreted to be due not to inhibition of nucleoside diphosphate kinase or of succinyl-CoA synthetase, since the activities of the isolated enzymes were not affected by oligomycin. Rather, it is suggested that depletion of extramatrix Mg^{2+} is responsible for failure of nucleoside diphosphate kinase to furnish GDP required for respiration under these conditions.

Early reports of studies on isolated heart sarcosomes (see *e.g.*, Cleland and Slater, 1953; Maley and Plaut, 1953; Chance and Baltschefsky, 1958; Harman and Fiegelson, 1952) described them as "loosely coupled" due to the presence of adenosine triphosphatase (ATPase) activities. These preparations differed from "tightly coupled" mitochondria in that rapid respiration was not dependent on the presence of added ADP. Packer (1957, 1958) later reported that heart sarcosomes in sucrose containing EDTA were tightly coupled, if they were incubated in a medium without added magnesium ion. He also reported that the measurable endogenous ATPase of these sarcosomes was very low, and that it was markedly stimulated by Mg^{2+} or Ca^{2+} (Packer, 1958). The phosphorylation efficiency (ADP:O ratio) was decreased when Mg^{2+} was added.

One of us (Davis, 1965a) reported that acetate is oxidized rapidly by tightly coupled heart sarcosomes prepared in EDTA, but that if Mg^{2+} and 2,4-dinitrophenol were included in the incubation mixture, acetate was not oxidized. These results were taken as indicative that the Mg^{2+}-activated mitochondrial ATPase prevented acetate activation. There is convincing evidence that bound Mg^{2+} is absolutely required to sustain oxidative phosphorylation (see, *e.g.*, Kielley and Bronk, 1957; Pullman *et al.*, 1960; Lee and Ernster, 1966). However, the energy-coupling mechanism is very efficient in intact mitochondria which are prepared in the presence of a chelating agent and are incubated in the absence of added divalent metal ions. The demonstrable endogenous ATPase is quite low in these mitochondrial preparations and is markedly stimulated by added Mg^{2+}.

Davis (1965b) reported that uncouplers of oxidative phosphorylation failed to reverse the inhibition by oligomycin of α-oxoglutarate or glutamate oxidation by heart mitochondria prepared in sucrose containing EDTA, and incubated in the absence of added Mg^{2+}. The latter prevented, but did not reverse this unexpected effect. No satisfactory explanation has been given for this observation.

The oligomycin-sensitive portion of the ADP–ATP ex-

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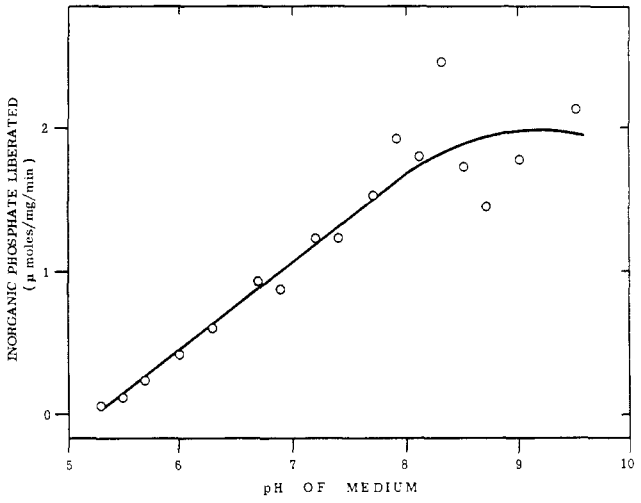

FIGURE 1: The pH profile of the Mg^{2+}-stimulated ATPase of guinea pig heart mitochondria. The P_i liberated was determined after incubation at 30° for 60 sec. The concentration of Mg^{2+} was 5 mM.

change, which is considered by many workers to represent the terminal reactions of oxidative phosphorylation, is increased in inner-membrane matrix preparations from liver mitochondria (Schnaitman and Pedersen, 1968; Pederson and Schnaitman, 1969). The greater specificity of the exchange for adenine nucleotides, and the more pronounced inhibition by oligomycin has been correlated with removal of ancillary reactions (nucleoside diphosphate kinase and adenylate kinase) from their mitochondrial preparation. Sensitivity of the exchange to oligomycin was abolished by added Mg^{2+}.

These various observations seemed to point to a dual role of Mg^{2+} in the coupling of respiratory energy to phosphorylation: (1) an absolute requirement, at low concentrations, for the coupling mechanism, and (2) at higher concentrations, a modification by Mg^{2+} of the sensitivity of various partial reactions involved in the energy coupling system to inhibitors of oxidative phosphorylation.

In the present communication, we report the results of experiments carried out with guinea pig and bovine heart mitochondria prepared in the presence of excess chelating agent, in order to remove as much "free" metal ions as possible. The effects of added Mg^{2+} on various aspects of mitochondrial metabolism are reported.

Materials and Methods

Guinea pig and bovine heart mitochondria were prepared in 250 mM sucrose–10 mM EDTA (pH 7.4) as described previously (Davis, 1967). Oxygen consumption was measured at 25° with a Gilson Medical Electronics "oxygraph". The basic reaction media contained 250 mM sucrose, 10 mM KCl, 10 mM Tris-HCl (pH 7.4), and other additions as indicated in the tables and figures. Adenine nucleotides were determined either enzymatically (Adam, 1965) or by column elution when radioactive nucleotides were used. For column elution the procedure of Groot and van den Berg (1968) was followed except that AMP was first quantitatively eluted with 0.5 M formic acid after thorough washing of the column with water. Separation of mitochondria from the suspending medium was accomplished by rapid centrifugation through a layer of silicone (Werkheiser and Bartley, 1957). Incubations for ATPase were carried out in the basic incubation medium containing 10 mM ATP. Inorganic phosphate was determined colorimetrically as described by Lindberg and Ernster (1954). Protein was determined by the Biuret method after dispersion with deoxycholate. Succinyl-CoA synthetase (succinate:CoA ligase (GDP), E.C. 6.2.1.4), and nucleoside diphosphate kinase (ATP:nucleoside diphosphate phosphotransferase, E.C. 2.7.4.6) were prepared following Cha *et al.* (1967a,b), except that bovine heart was used instead of pig heart. These two enzymes, which were purified through the first stage described by these authors, had about the same specific activities as they reported (20 and 87 μM units, respectively). Enzymes and unlabeled coenzymes were obtained from Sigma Co., St. Louis, Mo.; labeled coenzyme from Schwarz BioResearch, Orangeburg, N. Y.; and "Enzyme grade" sucrose from Mann Research Laboratories.

The terminology of Chance and Williams (1956) for the various mitochondrial states and for respiratory control ratios is used.

TABLE I: Effect of Mg^{2+} on ATPase Activity of Guinea Pig Heart Mitochondria; Inhibition by Oligomycin, Aurovertin, and Atractyloside.[a]

Expt I $[MgCl_2]$ (mM)	nmoles of P_i Released/min per mg of Protein		% Inhibition by Oligomycin
	−Oligomycin	+Oligomycin	
0	78		
1.0	671	17	97
5.0	716	44	94

Expt II $[MgCl_2]$ (mM)	[Aurovertin] μg/mg of Protein	nmoles of P_i Released/min per mg of Protein	% Inhibition by Aurovertin
2.5	0	815	
2.5	0.10	825	0
2.5	0.25	660	19
2.5	1.25	530	33

Expt III $[MgCl_2]$ (mM)	[Atractyloside] μg/mg of Protein	nmoles of P_i Released/min per mg of Protein	% Inhibition by Atractyloside
2.5	0	750	0
2.5	5	810	−8
2.5	25	760	0
2.5	50	830	−10

[a] Incubations contained 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM KCl, and 10 mM ATP in a volume of 3.0 ml. Reactions were started with mitochondria (1.6 mg of protein). Incubation time, 1.0 min at 30°. When added, oligomycin was present at a concentration of 1.0 μg/mg of protein. Final pH, 7.4.

Results

Magnesium-Stimulated ATPase. Table I and Figure 1 show the results of typical experiments carried out on the stimulation by Mg^{2+} of ATPase by guinea pig heart mito-

TABLE II: Effect of Monovalent Cations and Bovine Serum Albumin on the ADP:O Ratio and Respiratory Control Ratio of Guinea Pig Heart Mitochondria Oxidizing Glutamate.[a]

Incubation Medium	Mg^{2+} Added (2.5 mM)	Bovine Serum Albumin[b]	ADP:O	Respiratory Control Ratio
Standard sucrose medium	−	−	3.0	15.0
	+	−	2.1	3.2
K^+-free medium	−	−	2.9	14.0
	+	−	1.8	1.3
Na^+ medium	−	−	2.9	13.5
	+	−	1.7	2.3
Standard sucrose medium	−	−	2.9	14.0
	+	−	1.8	2.3
	+	+	2.0	2.3

[a] Mitochondria (0.80 mg of protein) were incubated in either standard sucrose medium, K^+-free sucrose medium, or Na^+-(10 mM) supplemented sucrose medium containing 10 mM glutamate. The state-3 respiration was initiated by the addition of 600 nmoles of ADP (Tris). Polarographic experiments, at 25°. [b] Bovine serum albumin (fatty acid poor) 5 mg added if present. Obtained from Gallard Schlesingerchen Manufacturing Company, New York, N. Y.

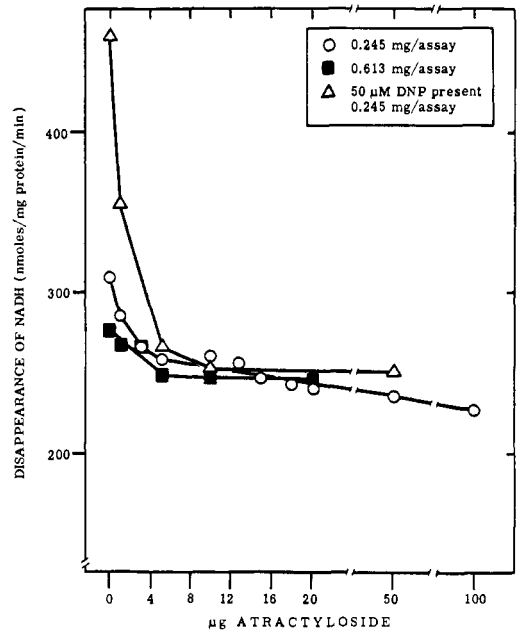

FIGURE 2: The effect of atractyloside on the initial rate of Mg^{2+}-stimulated ATPase at a constant concentration of ATP. Guinea pig heart mitochondria were incubated in the coupled ATPase assay medium containing 250 mM sucrose, 10 mM KCl, 10 mM Tris-HCl (pH 7.4), 2 mM phosphoenolpyruvate, 0.2 mM NADH, 0.5 mM ATP, 1 mM sodium arsenite, 2 μg of rotenone/mg of protein, 20 μg of pyruvate kinase, and 10 μg of lactate dehydrogenase. Reactions were started by the addition of ATP at room temperature. Linear initial velocity lasted for several minutes. The initial velocity was plotted against micrograms of atractyloside added.

chondria. ATPase was stimulated in a number of experiments by Mg^{2+} 8- to 12-fold. 2,4-Dinitrophenol (250 μM) stimulated the ATPase activity an additional 80–100% (not shown). In agreement with earlier reports (Lardy *et al.*, 1958; Huijing and Slater, 1961; Lardy *et al.*, 1964), oligomycin blocked the ATPase, but aurovertin was much less effective—maximum inhibition with the latter compound being only about 30%. Atractyloside, on the other hand, was completley without effect (Table I). Mg^{2+}-stimulated ATPase was markedly pH dependent, reaching a maximum at about pH 8.5–9.0 (Figure 1). This pH–activity curve is quite similar to that reported by Holton *et al.* (1957).

In view of the known inhibition by atractyloside of the translocation of adenine nucleotides (Klingenberg and Pfaff, 1966), the lack of effect on the Mg^{2+}-ATPase was unexpected. However, the inhibition of the translocation of ADP and ATP by atractyloside is competitive, so that we considered it possible that the high concentration of ATP present in the usual ATPase assay could perhaps effectively mask inhibition by atractyloside. Figure 2 shows the results of an experiment designed to show the effects of atractyloside on the Mg^{2+}-ATPase when the level of ATP was held relatively low (0.5 mM) and constant. As seen from these data, atractyloside had only a small effect on the ATPase stimulated by Mg^{2+}, but blocked the additional stimulation by dinitrophenol.

Several possibilities for explaining the Mg^{2+} "uncoupling" and ATPase were considered: (1) K^+ translocation could be coupled to the Mg^{2+} effects. To test this possibility, mitochondria were prepared in the usual way except that the isolation medium contained 10 mM Na-EDTA instead of K-EDTA. Incubation media as well as substrates were prepared in Na^+ or Tris form to substitute for K^+. (2) The possibility was considered that Mg^{2+} may stimulate the release of free fatty acids which, in turn, may elicit an uncoupling effect. As shown in Table II, the ADP:O ratio and respiratory control were essentially unaffected by the presence or absence of K^+, Na^+, or bovine serum albumin in the incubation medium. Furthermore, when added at state 4, Mg^{2+} stimulated respiration under all incubations tested.

Effect of $[Mg^{2+}]$ *on the ADP:O and Respiratory Control Ratios.* Figure 3 shows the effect of increasing concentrations

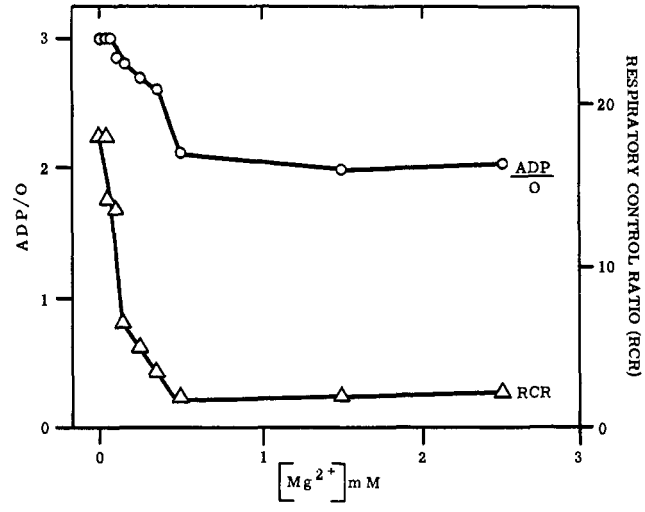

FIGURE 3: The effect of Mg^{2+} concentration on the ADP:O and respiratory control ratios of guinea pig heart mitochondria oxidizing glutamate. Polarographic experiment carried out in the basic medium (pH 7.4) containing 10 mM potassium glutamate and 10 mM potassium phosphate. State-3 respiration was initiated by addition of 600 nmoles of ADP. As indicated, various amounts of Mg^{2+} were added before ADP.

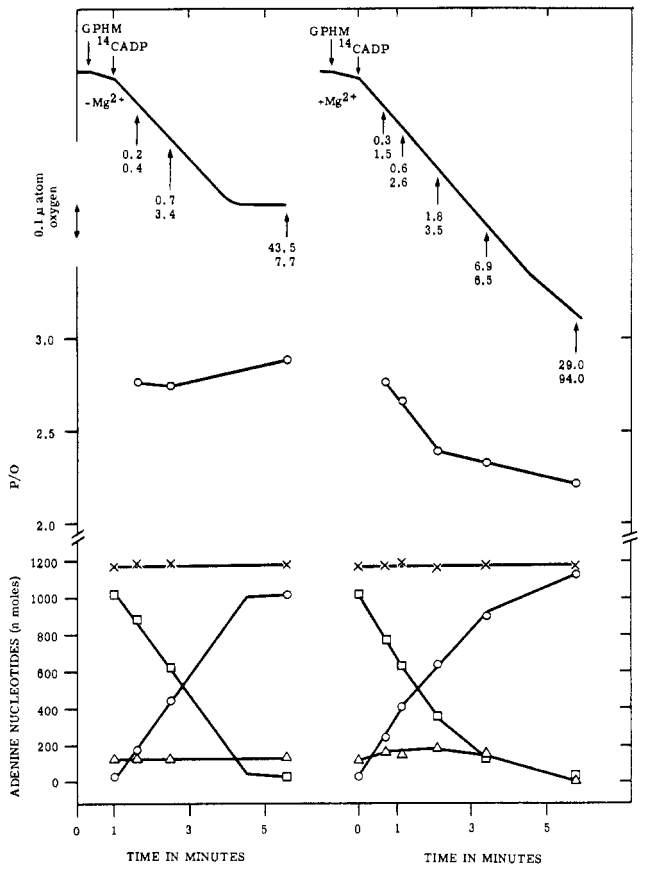

FIGURE 4: The effect of added Mg^{2+} on the P:O ratio of guinea pig heart mitochondria oxidizing glutamate. Correlation with the ATP:ADP ratio. Mitochondria (0.79 mg of protein) were incubated in the standard sucrose medium containing 10 mM potassium glutamate and 10 mM potassium phosphate (pH 7.4). Respiration was initiated by addition of 1.17 μmoles of [^{14}C]ADP. Reactions were stopped at times indicated with $HClO_4$ and [^{14}C]adenine nucleotides were eluted and counted. Adenylate kinase activity was corrected for by subtraction of AMP formed. ATP:ADP and ATP:AMP ratios (upper and lower numbers at each point of sampling) were those found at each incubation period. P:O ratios were calculated for the entire period of incubation. The [^{14}C]ADP originally contained 1.5 and 7.9% of [^{14}C]ATP and [^{14}C]AMP, respectively. Changes in the amounts of ATP (○–○), ADP (□–□), AMP (△–△), and total adenine nucleotides (×–×) are also shown.

TABLE III: Effect of $[Mg^{2+}]$ on Stimulation of State 4 Respiration.[a]

$[MgCl_2]$ Added at State 4 (mM)	Initial Rate of Respiration after Addition of $MgCl_2$ (natoms of Oxygen Consumed/mg of Protein per min)
0	30
0.01	27
0.05	31
0.10	31
0.20	41
0.30	54
0.35	77
0.40	103
0.50	132
1.0	168
2.5	162

[a] Mitochondria (1.0 mg of protein) were incubated in the basic medium including 10 mM potassium phosphate (pH 7.4) and 10 mM glutamate. Respiration was initiated with 0.5 μmole of ADP. After all of the ADP was phosphorylated, increasing amounts of $MgCl_2$ were added to stimulate respiration.

of $MgCl_2$ on the "tightness" of coupling of mitochondria oxidizing glutamate. Even at quite low concentrations (0.10–0.25 mM) Mg^{2+} had noticeable effects on both ADP:O and respiratory control ratios. It is likely that an effect by Mg^{2+} would have been observed at much lower concentrations if there were not an excess of EDTA in the mitochondrial suspension. Exactly similar effects by Mg^{2+} were observed when pyruvate plus malate, or α-oxoglutarate was substrate. These data are in qualitative agreement with those reported several years ago by Packer (1957, 1958).

Correlation of the Effect of Added Mg^{2+} on the Efficiency of Oxidative Phosphorylation with the ATP:ADP Ratio. In the experiment shown in Figure 4, guinea pig heart mitochondria were incubated with glutamate and [^{14}C]ADP, in the presence and absence of added MgCl. It should be noted that in this experiment, the mean P:O ratio was recorded for the entire period starting from the time of addition of [^{14}C]-ADP until termination of the incubation. Thus, in the presence of Mg^{2+} the ratios obtained for the later *increments* of state-3 respiration were considerably less than calculated for the entire state-3 interval. After separation and counting of the [^{14}C]adenine nucleotides, the amount of [^{14}C]AMP was subtracted from the total [^{14}C]ATP to correct for adenylate kinase. It is clear from this experiment that the decrease in P:O ratio observed in presence of Mg^{2+} varies as a direct function of the ATP:ADP ratio. This result is consistent with the interpretation that the Mg^{2+} effect is a direct result of ATPase which acts, not *in lieu* of, but simultaneously with, oxidative phosphorylation. In this framework, Mg^{2+}-ATPase is stimulated by increasing concentrations of ATP, and inhibited by ADP (or a high ADP:ATP ratio).

Stimulation of State 4 Respiration by Mg^{2+}. It was routinely observed that, with mitochondria oxidizing NAD-linked substrates in sucrose media in the absence of added Mg^{2+}, the P:O and respiratory control ratios were near 3.0 and 10–20, respectively. However, on subsequent addition of Mg^{2+}, respiration was markedly stimulated. The initial rate of respiration was usually almost as fast as state 3, followed by a somewhat slower rate (60–80% of the rapid phase). The following experiments were carried out in order to gain more insight into the nature of this effect.

Table III shows that there is a noticeable response to addition of Mg^{2+} at concentrations as low as 0.1 mM but that the maximum effect is not seen until the Mg^{2+} concentration reaches approximately 1 mM. The range of concentration required for this effect is similar to that required to decrease the P:O and respiratory control ratios (Figure 3).

If organic phosphate was not present in the incubation medium, the response on addition of Mg^{2+} was small. Half-maximal stimulation of respiration by Mg^{2+} was seen when the phosphate concentration was about 0.5 mM (Table IV). This value is consistent with the estimated Michaelis constant of inorganic phosphate for oxidative phosphorylation (Chance and Hagihara, 1963). The respiratory response to

TABLE IV: Concentration Dependence of Added Inorganic Phosphate for Stimulation by Mg^{2+} of Respiration of Mitochondria in the Presence of Added ATP.[a]

Phosphate Added (mM)	Initial Respiration Rate on Addition of ATP (natoms of Oxygen Consumed/mg of Protein per min)
0	38
0.2	59
0.6	74
1.2	100
1.6	126
2.0	133
4.0	119
6.0	121

[a] Polarographic experiment. Mitochondria were incubated in the standard sucrose medium (minus phosphate) with 10 mM glutamate and 2.5 mM Mg^{2+}. Potassium phosphate buffer was added as indicated. Rapid respiration was initiated by addition of 500 nmoles of ATP.

TABLE V: Effects of Inhibitors on Stimulation by Mg^{2+} of Respiration by Mitochondria under State 4 Conditions.[a]

Additions	natoms/mg of Protein per min	
	Mg^{2+}-Stimulated Respiration	State 4 Respiration without Mg^{2+}
None	159	12
Oligomycin	12	
None	174	10
Aurovertin	31	
None	183	12
Atractyloside	7	
None	177	15
Phosphoenolpyruvate plus pyruvate kinase	52	

[a] Guinea pig heart mitochondria (1.0 mg of protein) were incubated at 25° in the basic medium containing 10 mM potassium phosphate (pH 7.4) plus 10 mM potassium glutamate. ADP (0.5 μmole) was added to initiate state 3 respiration. One minute after the state-3–state-4 transition inhibitor was added, followed 2 min later by $MgCl_2$ (2.5 mM). Other additions when present were: oligomycin, 1 μg/mg of protein; atractyloside, 12.5 μM; aurovertin, 1 μg/mg of protein; phosphoenolpyruvate, 5 μM; pyruvate kinase, 2 enzyme units. Each study was carried out on a separate mitochondrial preparation.

added Mg^{2+} was also blocked by inhibitors of oxidative phosphorylation (oligomycin or aurovertin) and by conditions designed to decrease the availability of ADP (in the presence of atractyloside, or phosphoenolpyruvate plus pyruvate kinase) (Table V).

Effects of Added ADP and ATP, and of Mg^{2+} and Mitochondrial Protein Concentrations on the Length of the Rapid Phase of Respiration Observed on Addition of Mg^{2+} to Mitochondria Respiring in State 4. Although Mg^{2+} had little or no effect on respiration when no nucleotide was added, respiration was stimulated by Mg^{2+} if either ADP or ATP was added initially (Table VI). Within a given experiment the length of the rapid phase of respiration was proportional to the amount of nucleotide added. The ratio, extra oxygen consumed/ADP or ATP was about 0.30 and 0.17, respectively. The exact stoichiometry varied somewhat from experiment to experiment; however, the extra oxygen consumed during the rapid phase of respiration was always proportional to the amount of nucleotide added. In contrast, when the amount of added ADP was held constant, varying the amount of mitochondrial protein from 0.65 to 1.95 mg or the [Mg^{2+}] from 0.5 to 5.0 mM had no effect on the length of the rapid phase of respiration.

Effect of Added Mg^{2+} on Adenine Nucleotide Ratios When Added to Mitochondria Respiring under "State-4" Conditions. Figure 5 shows the results of an experiment carried out to determine the correlation of nucleotide ratios with Mg^{2+}-stimulated respiration. It should be noted that the ratios recorded here are those calculated for the total [^{14}C]nucleotides present, *i.e.*, the *sum* of the intra- and extramitochondrial nucleotides. It is clear from this figure that in state-4 conditions in the absence of added Mg^{2+}, more than 93% of the total nucleotide pool is in the form of ATP. In some other experiments, ATP accounted for as much as 97% of the total labeled nucleotide pool. However, on addition of $MgCl_2$, there was a very rapid dephosphorylation of ATP, which coincides with a rapid burst of respiration. It can be calculated that, in the first interval after addition of Mg^{2+} (0.4 min), approximately 140 nmoles of ATP are dephosphorylated. This value represents a minimum rate of ATP hydrolysis of 490 nmoles/min per mg of protein. It appears likely that this value represents the *sum* of two competing reactions—oxidative phosphorylation, and a Mg^{2+}-dependent ATPase. However, the relative rates of these two reactions cannot be inferred from the data presented. However, there is net rephosphorylation of ADP during subsequent intervals of incubation, until new steady-state ratios of ATP:ADP, and of ATP:AMP are apparently reached. This new steady-state roughly coincides with the termination of the rapid phase of Mg^{2+}-stimulated respiration.

Effect of Added Mg^{2+} on the Intramitochondrial Adenine Nucleotide Ratios. Since addition of Mg^{2+} had a marked, but transcient effect on the ATP:ADP ratio, we considered the possibility that this ATPase could be accounted for by a rapid redistribution of the adenine nucleotides themselves. Experiments were therefore carried out with the aim of estimating the effect of added Mg^{2+} on the intra- as well as extramitochondrial ATP:ADP ratios. Mitochondria were incubated for various periods either in the presence or absence of added Mg^{2+}, and in the presence of [^{14}C]ADP. Reactions were terminated by rapid centrifugation of the mitochondria through a silicone layer into acid. The adenine nucleotides in the suspending medium over the silcone layer, and in the mitochondrial pellet were separated and counted. No correction was attempted for nucleotides present in the sucrose-permeable space of the mitochondria. As seen in Figure 6, added Mg^{2+} had a pronounced effect on the *extramitochondrial* adenine nucleotide ratios, but was without effect on the ratio of ATP to ADP present in the mitochondrial pellet.

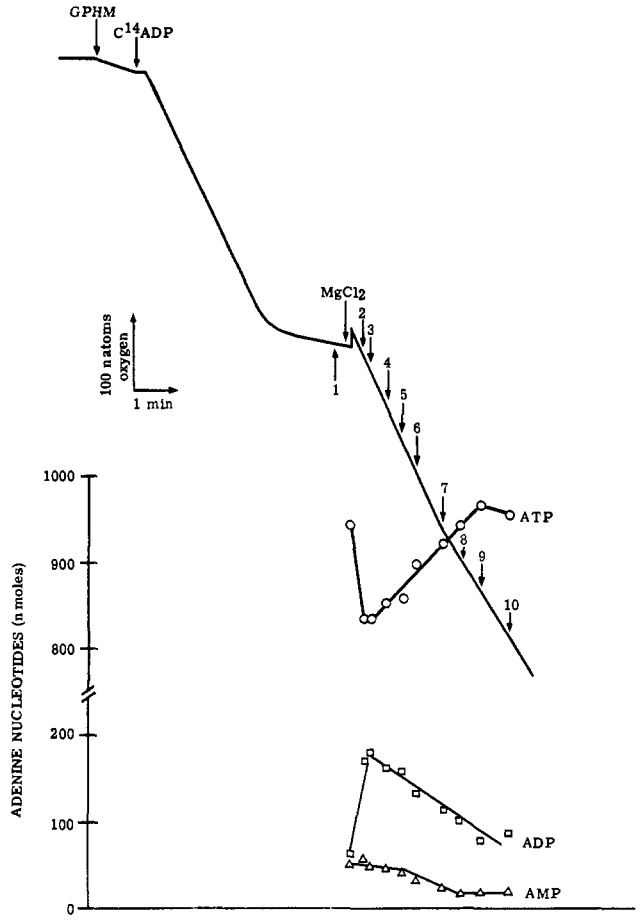

FIGURE 5: The influence of added Mg^{2+} on the ratios of adenine nucleotides when added under state 4 conditions. Mitochondria (0.92 mg of protein) were incubated in the standard sucrose medium (pH 7.4) containing 10 mM potassium glutamate and 10 mM potassium phosphate; 1056 nmoles of [^{14}C]ADP was added and respiration was allowed to proceed until the state-3–state-4 transition. Incubations were stopped with $HClO_4$ at state-4 and at the various times indicated after addition of 5 μmoles of $MgCl_2$. Nucleotides were separated and counted. The [^{14}C]ADP initially contained 2.0 and 5.4% of [^{14}C]ATP and [^{14}C]AMP, respectively.

TABLE VI: Duration of the Mg^{2+}-Stimulated Rapid Phase of Respiration as Affected by the Amount of ADP or ATP Added Initially.

Expt I[a] (nmoles of ADP Added)	Length of Rapid Phase of Mg^{2+}-Stimulated Respiration (natoms of Oxygen)
0	0
105	35
210	72
315	92
420	120
525	160
630	183
845	240
1055	275
Expt II[b] (nmoles of ATP Added)	
500	94
1000	158
2000	321

[a] Expt I: Guinea pig heart mitochondria (0.65 mg of protein) were incubated in the standard medium containing 10 mM phosphate buffer (pH 7.4) and 10 mM glutamate. Various amounts of ADP were added to stimulate respiration. One minute after the state-3–state-4 transition, Mg^{2+} was added to a final concentration of 2.5 mM. [b] Expt II: Mitochondria (1.0 mg of protein) were incubated in the same medium, plus various amounts of ATP. Respiration was again stimulated with 2.5 mM $MgCl_2$.

Indeed, this latter ratio was remarkably constant, regardless of the relative concentrations of nucleotides present outside the mitochondria. In addition, the total pool of radioactive nucleotides (ATP *plus* ADP *plus* AMP) in the mitochondrial pellet was essentially invariant under all conditions. It therefore appears that the mitochondrial adenine nucleotide translocase activity is not rate limiting for maintaining a relatively constant intramitochondrial supply of ADP, and that the intramitochondrial pool of nucleotides, once established, is constant. These results are perhaps expected in view of the detailed data obtained by Klingenberg and coworkers (see *e.g.*, Klingenberg, 1970) on the adenine nucleotide translocase system of liver mitochondria.

Hexokinase in Mitochondrial Preparations. In our early studies, ATP formed in short-term incubations was measured enzymatically, using hexokinase, glucose-6-phosphate dehydrogenase, and $NADP^+$ (Lamprecht and Trautschold, 1965). It was observed that when mitochondria were incubated with ADP in the absence of added Mg^{2+}, nucleotide was quantitatively recovered as ATP. However, when Mg^{2+} was also present, more NADPH was formed in the enzymatic assay for ATP than the amount of ADP added. On the other hand, there was no such discrepancy when [^{14}C]-ADP was added, and the radioactive nucleotides were subsequently separated and counted. The discrepancy in the enzymic assay was found to be due to the formation of small amounts of glucose 6-phosphate during incubation of the mitochondria under state-3 conditions in the presence of added Mg^{2+}. For this to occur, the mitochondria as isolated must contain hexokinase, and there must be a contamination in the medium of glucose. Indeed, during the period when this anomaly was first observed, incubation media were prepared and stored with the desired substrate (in this case, glutamic acid). The medium was then neutralized immediately before use. This method of storage caused substantial acid hydrolysis of sucrose. If both enzyme and substrate (glucose) were present in adequate quantities, the lowered P:O ratio due to Mg^{2+}, as well as the Mg^{2+}-induced stimulation of state 4 respiration could be accounted for by this "ATPase" system. The latter effect would also be oligomycin sensitive. It was therefore necessary to evaluate the importance of glucose *plus* hexokinase on these observations.

Sonicated guinea pig heart mitochondria were capable of phosphorylation of glucose to glucose 6-phosphate at a rate of 0.2 μmole/mg of protein per min at 25°. Essentially the same rate was observed with intact guinea pig or beef heart mitochondria, both in the presence and absence of 12.5 μM atractyloside.

The standard incubation medium was assayed for contamination with glucose, using the coupled enzymic assay sys-

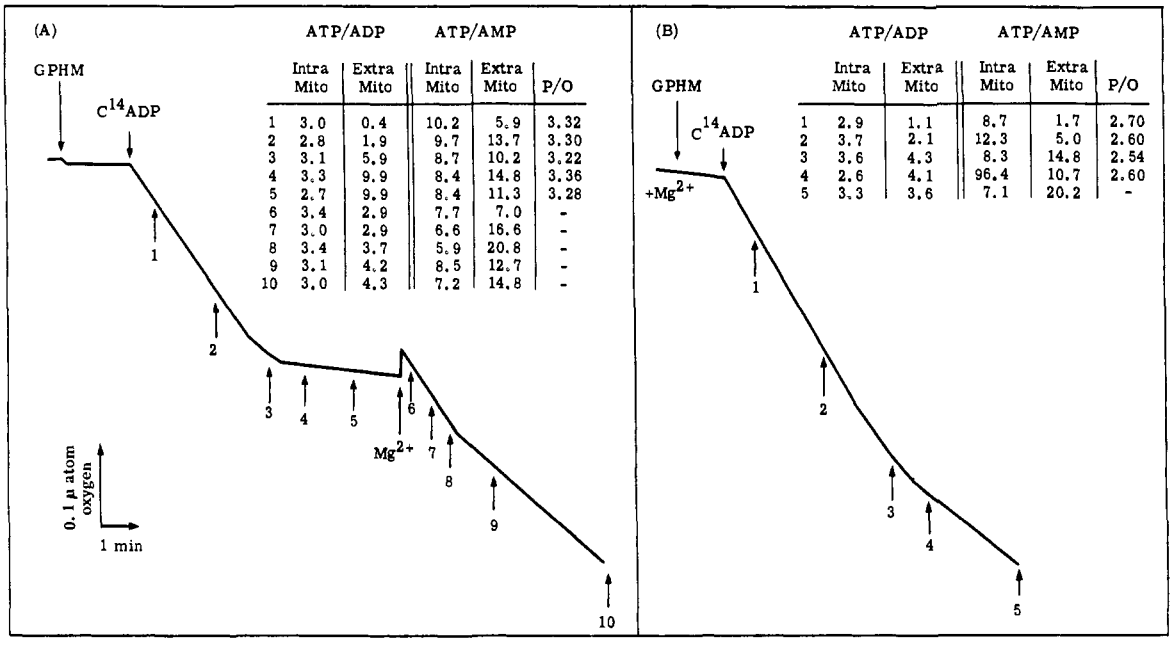

FIGURE 6: The effect of added Mg^{2+} on the P:O ratio and respiration at state-4. Correlation with the intra- and extramitochondrial ATP:ADP ratios. Guinea pig heart mitochondria (0.7 mg of protein) were incubated in standard sucrose medium (pH 7.4) containing 10 mM potassium glutamate and 10 mM potassium phosphate with (trace B) or without (trace A) 2.5 mM $MgCl_2$. Respiration was initiated by addition of 1.32 μmoles of [^{14}C]ADP. P:O ratios were calculated for the entire period of incubation. Correction was made for adenylate kinase activity.

tem described above, but with excess crystalline hexokinase. No glucose was detected in any of the additions except sucrose which, when freshly prepared, contained approximately 1 part of assayable glucose/10,000 parts of sucrose. This contamination did not increase on storage of sucrose solutions at neutral pH at 4° for at least 1 month.

Effect of Added Glucose on the Rapid Phase of Respiration Induced by Mg^{2+} in State 4 Conditions. Since we had established that the mitochondrial preparations used had adequate hexokinase present to function as an ATPase if glucose and Mg^{2+} were present, the effect of added glucose on the length of the rapid phase of respiration of mitochondria incubated in the presence of added ADP was measured. On addition of 5 mM $MgCl_2$ to mitochondria respiring under state-4 conditions, the presence of added glucose in the medium (20–200 nmoles) increased the length of the rapid phase of respiration to the extent of about 2.0 atoms of extra oxygen consumed per mole of glucose added. Control assays confirmed that all of the glucose had been phosphorylated during the incubation. These data show that if enough glucose is present, the glucose–hexokinase system is adequate for lowering the apparent efficiency of oxidative phosphorylation and the respiratory control of mitochondria incubated in presence of Mg^{2+}.

In order to demonstrate definitively whether or not the glucose–hexokinase system could account for the concentration-dependent, ATP-induced increase in the length of the rapid phase of Mg^{2+}-stimulated respiration, various amounts of ATP were added initially to mitochondria respiring in the standard sucrose medium, and glucose 6-phosphate formed was correlated with the length of rapid respiration on addition of Mg^{2+}. As shown in Table VII, the duration of the rapid phase of respiration is positively correlated with the amount of nucleotide added, but not to the amount of glucose 6-phosphate formed. Clearly, all of the glucose present in the incubation medium was phosphorylated at all levels of ATP added. These data show that, although hexokinase is apparently present in adequate quantity in our mitochondrial preparation to account for a substantial "ATPase" activity, the glucose present as a contaminant was inadequate to account for more than a very small portion of the rapid phase of respiration.

The results of an experiment designed to show the correlation of glucose 6-phosphate production and the effects of added Mg^{2+} on the P:O ratio and on Mg^{2+}-stimulated state-4 respiration are shown in Figure 7. No glucose 6-phosphate was formed unless Mg^{2+} was added. In the presence of added Mg^{2+}, a small amount of glucose 6-phosphate was formed. However, only a maximum of about 10% of the decrease in P:O ratio is accounted for by the formation of glucose 6-phosphate. Phosphorylation of glucose is probably insignificant in the stimulation of Mg^{2+} of state-4 respiration.

Effect of Oligomycin on the Activities of Partially Purified Succinyl-CoA Synthetase and Nucleoside Diphosphate Kinase.

TABLE VII: Correlation of ATP Concentration and Glucose 6-Phosphate Formation with the Length of the Rapid Phase Respiration Induced by Mg^{2+}.[a]

ATP Added (μmoles)	Length of Rapid Phase of Respiration (μatom of oxygen)	Glucose 6-Phosphate Formed (μmole)
0.5	0.09	0.033
1.0	0.16	0.029
2.0	0.32	0.032

[a] Guinea pig heart mitochondria (1 mg of protein) were incubated in the basic medium containing 10 mM potassium glutamate and 10 mM potassium phosphate at a final pH of 7.4. Various amounts of ATP were then added, followed by 5 μmoles of $MgCl_2$ to initiate rapid respiration.

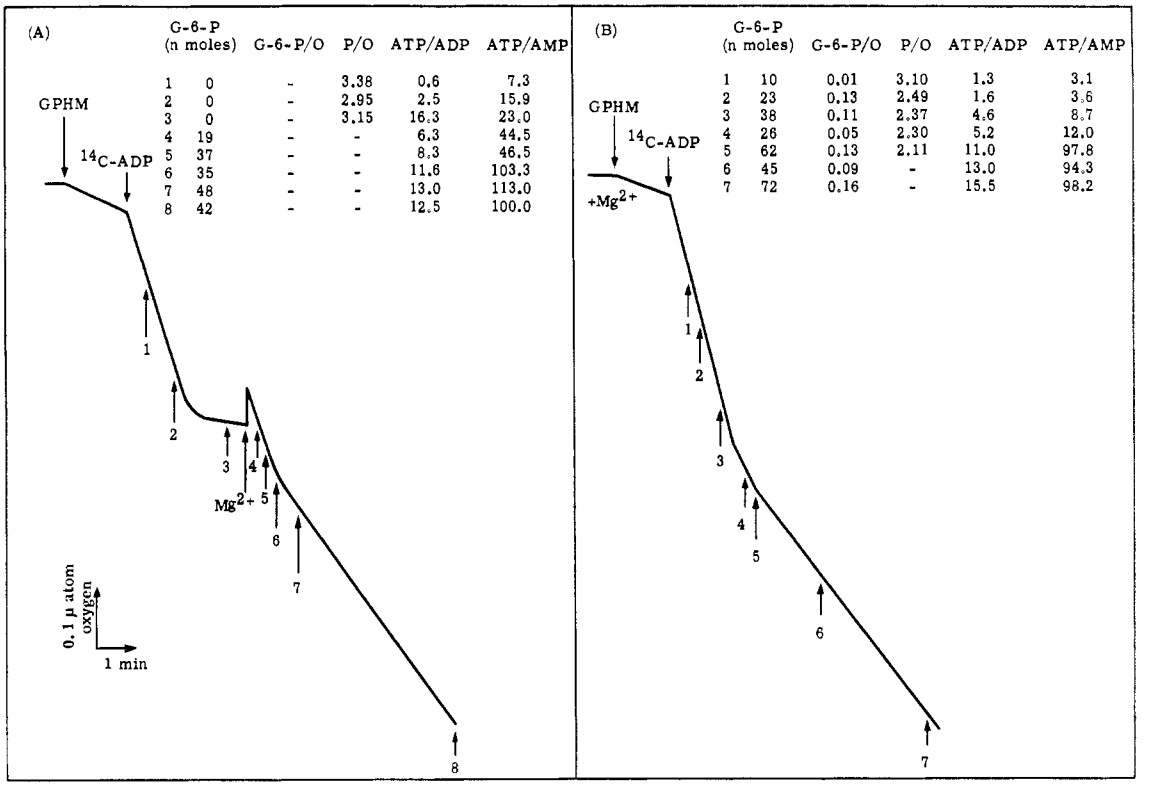

FIGURE 7: The formation of glucose 6-phosphate correlated with the Mg^{2+}-induced uncoupling and Mg^{2+}-stimulated respiration under state 4 conditions. Guinea pig heart mitochondria (1.0 mg of protein) were incubated with the standard sucrose medium (pH 7.4) with 10 mM potassium glutamate and 10 mM potassium phosphate. 1056 μmoles of [^{14}C]ADP was added to initiate state-3 respiration. Tracing A: no added Mg^{2+}; tracing B: 2.5 mM $MgCl_2$ was present.

The observation (Davis, 1965b) that uncouplers fail to relieve the inhibition of respiration of heart mitochondria oxidizing glutamate or α-oxoglutarate in the absence of added Mg^{2+} was confirmed, both with guinea pig and bovine heart mitochondria prepared in the presence of 10 mM EDTA (Figure 8). This phenomenon could most simply be explained by a direct inhibition by oligomycin of one of these enzymes, which might be looked upon as secondary to its effect on oxidative phosphorylation *per se*. We therefore incubated these enzymes (prepared as described in Methods) with various concentrations of oligomycin (0–5 μg/mg of protein) for periods up to 30 min, both in the presence and absence of 5 mM $MgCl_2$. In no case was there significant inhibition by oligomycin of either of these two activities.

Discussion

It was found in the present work, in agreement with Packer (1957, 1958), that Mg^{2+} caused a decrease in the P:O ratio obtained with several substrates, and that most of the phosphate acceptor control of respiration was lost. In addition, it was found that (a) if Mg^{2+} was added to heart mitochondria respiring under state-4 conditions, respiration was stimulated to approximately the state-3 rate, followed by reversion to a somewhat slower but constant rate of respiration. The slower (latter) phase of respiration in the presence of Mg^{2+} was much higher than the state-4 rate without Mg^{2+}, usually being of the order of 60% of the state-3 rate. (b) Mg^{2+} did not stimulate respiration when there was no *added* nucleotide. (c) The Mg^{2+}-stimulated respiration was accompanied by a rapid production of ADP, and the return of the ATP:ADP ratio to approximately the state-4 level coincided generally with the end of the rapid phase of Mg^{2+}-stimulated respiration. (d) The stimulation of state-4 respiration by Mg^{2+} was blocked by inhibitors of oxidative phosphorylation (oligomycin, aurovertin, and atractyloside), by the lack of inorganic phosphate, and by a system (phosphoenolpyruvate plus pyruvic kinase) which competes for available ADP. (e) When the efficiency of oxidative phosphorylation was followed throughout the period of phosphorylation of a limiting amount of added ADP, Mg^{2+} had minimal effect on the P:O ratio when the ADP:ATP ratio was high, and a maximal effect when this ratio was low. This accumulated evidence leads us to conclude that the stimulation of state-4 respiration, and the "uncoupling" effect of Mg^{2+} are accounted for solely by an ATPase which is turned on by added Mg^{2+} and that this ATPase is active at the same time as oxidative phosphorylation is taking place. That these effects are not mediated by the hydrolysis of a nonphosphorylated intermediate in the main pathway of oxidative phosphorylation is apparently excluded on the grounds that inhibitors of oxidative phosphorylation block the respiratory response.

It was found that the mitochondrial preparations contained an active hexokinase activity, and that the phosphorylation of added glucose could account for the rapid phase of Mg^{2+}-stimulated respiration. However, the possibility was finally rigorously excluded that the glucose–hexokinase system could account for Mg^{2+}-stimulated respiration and uncoupling under the usual incubation conditions.

In view of the accumulated evidence, two explanations seem possible for explaining the data presented here concerning the effects of Mg^{2+} on oxidative phosphorylation and those referred to in the literature.

(1) Heart mitochondria are damaged during preparation in such a manner that a significant portion have been broken up to a degree that there is no longer an atractyloside barrier to the entry of adenine nucleotides. In order for this explanation to be valid, these broken mitochondria would further be damaged to the extent that they were incapable of oxidation of NAD-linked substrates, since the stimulation by added Mg^{2+} of respiration by these mitochondria oxidizing glutamate, or pyruvate *plus* malate is completely blocked by atractyloside. The damaged mitochondria would be expected to have lost the sensitivity of coupled respiration to the action of this inhibitor. Such damage is not readily evident in electron micrographs of mitochondria incubated in the presence or absence of added Mg^{2+}. Essentially all of the mitochondria appeared intact, *i.e.*, having a distinguishable intact inner and outer membrane, gave little evidence of submitochondrial particles present in the preparations. It is possible, however, that damage incurred in the preparation of heart mitochondria is too subtile to be discernible by the usual tissue-fixing techniques used in electron microscopy.

The above explanation is very attractive in explaining a number of apparent differences between heart and liver mitochondria. (a) Preparations of heart mitochondria always exhibit a substantial ATPase activity in the presence of Mg^{2+}, whereas the ATPase of liver mitochondria is latent unless they are aged or otherwise damaged after preparation. (b) Most preparations of heart mitochondria oxidize NADH, the rate of which is variable from preparation to preparation, but is greatly increased by sonic disruption or treatment with detergents. On the other hand, liver mitochondria do not oxidize added NADH. (c) Heart mitochondria exert much poorer respiratory control with succinate as substrate than with NAD-linked substrates. With liver mitochondria, respiratory control with succinate is often comparable to that obtained with NAD-linked substrates. Hence, damaged particles in mitochondrial preparations from heart could account for the Mg^{2+}-ATPase, oxidation of added NADH, and the poor respiratory control observed when succinate is substrate. The last observation is explained by the presence of both intact and damaged mitochondria which contain an active succinic oxidase system. The observed respiratory control would be the average of the two populations.

(2) An alternative explanation which has been considered and which may account for the various Mg^{2+} effects is the Mg^{2+}-dependent formation of a high-energy intermediate which can be formed irreversibly either from ATP or electron transfer, and which can in turn be used to carry out various work functions. Its formation would not be on the main path of ATP *formation* from electron transfer. Such an intermediate would be identical with the work intermediate, W ~, proposed by Lardy *et al.* (1964), which is based largely upon the differential sensitivity of ATPase, swelling, contraction, and ion transport to the two inhibitors oligomycin and aurovertin. As visualized in Lardy's scheme, W ~ can be formed irreversibly from electron transport or from ATP. Its formation *via* electron transport would not be affected either by oligomycin or aurovertin, whereas its formation from ATP (ATPase) is inhibited by oligomycin, but not by aurovertin. Mg^{2+} is required for the formation and/or breakdown of W ~. Thus, Mg^{2+} "uncoupling" as well as the Mg^{2+} ATPase would be dependent on the formation of W ~ from ATP, and the physiological work equivalent *in vivo* of the breakdown of W ~ would be "turned on" by Mg^{2+} *in vitro*. We now reject this latter explanation of the Mg^{2+} effects reported in this communication due to our inability to observe any work parameter which is activated on the addition of Mg^{2+}, and since the data are in the main consistent with the first explanation.

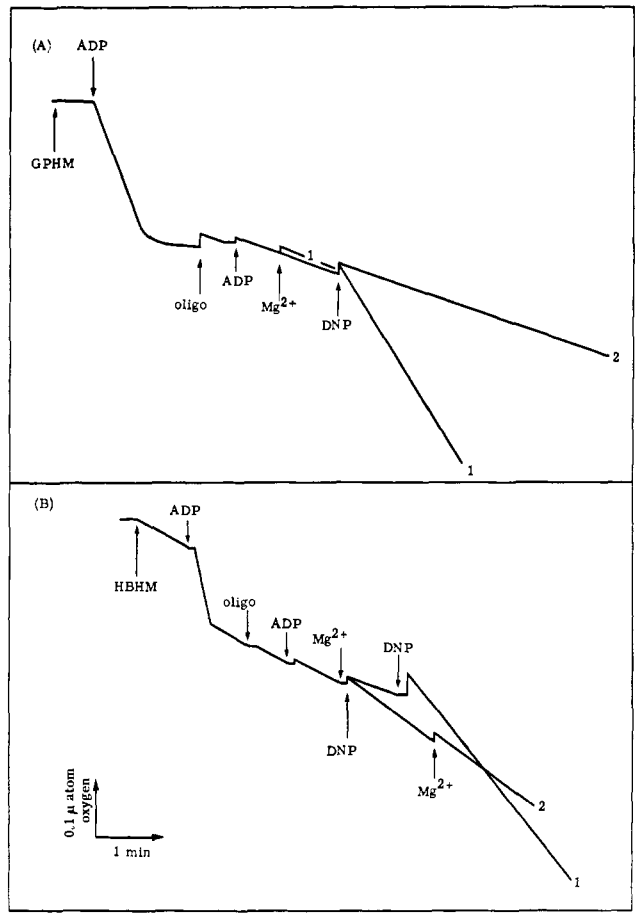

FIGURE 8: The effect of Mg^{2+} on the inhibition by oligomycin of glutamate oxidation by guinea pig (A) or bovine (B) heart mitochondria in the presence of 2,4-dinitrophenol. The basic incubation medium contained 10 mM potassium glutamate and 10 mM potassium phosphate. In curves 1, $MgCl_2$ (2.5 mM) was added before 2,4-dinitrophenol (DNP, 50 μM); in curves 2, no $MgCl_2$ was added before DNP. Incubations contained 1.8 mg (A) and 2.0 mg (B) of mitochondrial protein and, when present 2 μg of oligomycin and 2 μmoles of ADP (second addition). Reaction temperature, 25°.

The possibility that contamination of the mitochondrial preparations with the Mg^{2+}-stimulated ATPase localized in the endoplasmic reticulum could explain the various effects of Mg^{2+} is excluded, since this ATPase is completely insensitive to oligomycin at the concentrations used in the experiments reported here (van Groningen and Slater, 1963). Furthermore, the microsomal ATPase would not be active in the presence of the quite low K^+ and Na^+ concentrations used (Jöbsis and Vreman, 1963).

The observation by Davis (1965b) that uncouplers of oxidative phosphorylation (which do not directly affect substrate-linked phosphorylation) failed to relieve the block by oligomycin of the oxidation of α-oxoglutarate by mitochondria in the absence of Mg^{2+} could be most simply explained by a direct effect of oligomycin on either succinyl-CoA synthetase or nucleoside diphosphate kinase. Both of these enzymes are required for respiration of α-oxoglutarate.

Partially purified preparations of succinyl-CoA synthetase and nucleoside diphosphate kinase were prepared from beef hearts following the procedure of Cha *et al.* (1967a,b). Oli-

gomycin had no effect on these isolated enzymes. Therefore, the simplest and most obvious explanation for the observation of Davis was excluded.

A likely explanation of this observation can now be proposed on the basis of lines of evidence recently published from two separate laboratories. First, Kün and coworkers (Kün *et al.*, 1969, 1970) noted that there is a rapid loss of Mg^{2+} from rat liver mitochondria which are incubated in the presence of ADP and an uncoupler of oxidative phosphorylation (conditions identical with those of Davis' experiment). Furthermore, the oxidation of glutamate was blocked after this treatment. It therefore appears that the activities of nucleoside diphosphate kinase and/or succinyl-CoA synthetase are blocked due to a deficiency of Mg^{2+} at the site of their activity. Secondly, Schnaitman and Pedersen (1968), and Pedersen and Schnaitman (1969) studied the ADP–ATP exchange in whole liver mitochondria and in inner-membrane matrix preparations from these mitochondria. The latter preparation was essentially devoid of nucleoside diphosphate kinase and adenylate kinase, the sum of which, if present, could catalyze a nonspecific and oligomycin-insensitive ATP–ADP exchange. They found that in the absence of added Mg^{2+} the inner-membrane matrix fraction catalyzed an oligomycin-sensitive ADP–ATP exchange reaction which was specific for adenine nucleotides. These preparations were capable of acceptor control of respiration. Loss of oligomycin sensitivity of the ADP–ATP exchange, and loss of acceptor control of respiration was seen on addition of Mg^{2+}. In view of these findings, it is suggested that a rapid loss of Mg^{2+}, under the conditions described by Davis (1965) and by Kün *et al.* (1969) results in the failure of the uncoupler to reverse the inhibition by oligomycin of the oxidation of α-oxoglutarate or glutamate. In other words, depletion of mitochondrial Mg^{2+} would be functionally equivalent to removal of nucleoside diphosphate kinase or succinyl-CoA synthetase, since both reactions require Mg^{2+}.

The degree of respiratory control is often used as one of the most critical parameters for measuring "intactness" of a mitochondrial preparation. In view of the conclusions drawn from the present work, an added parameter may be useful determining the *uniformity* of a preparation, *i.e.*, to observe the effect of added Mg^{2+} on the respiratory control ratio. Presumably, if the mitochondria in a given preparation are uniformly intact, Mg^{2+} should have no effect on respiratory control observed in short-term incubations. In addition, these mitochondria would be incapable of oxidizing added NADH, would not have a demonstrable Mg^{2+}-dependent ATPase, and would exert respiratory control with succinate quite comparable to that with NAD-linked substrates.

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