Biochimica et Biophysica Acta, 347 (1974) 87–101

BBA 46729

ADP AND Mg^{2+} REQUIREMENT FOR Ca^{2+} ACCUMULATION BY HOG HEART MITOCHONDRIA

CORRELATION WITH ENERGY COUPLING

PIERRE LEBLANC and HUBERT CLAUSER

Institut de Biochimie, Université de Paris-Sud, Centre d'Orsay, 91405-Orsay (France)

(Received September 19th, 1973)
(Revised manuscript received November 22nd, 1973)

SUMMARY

1. The action of ADP and Mg^{2+} on the respiration-driven accumulation of calcium phosphate by hog heart mitochondria, in the presence of oligomycin, has been studied. The accumulation is stringently ADP-specific and this requirement does not depend on the substrate used. The site of action of the nucleotide is located at the inner side of the inner mitochondrial membrane. It does not imply the involvement of adenylic translocase.

2. Maximal accumulation of calcium phosphate is observed, even in the absence of exogenous Mg^{2+}, when ADP is present in the medium from the onset of the incubations. In the absence of ADP, small accumulations of calcium phosphate are obtained, the levels of which do not depend on the addition of Mg^{2+}.

3. Conversely, once the calcium phosphate accumulation has stopped due to lack of ADP, both this nucleotide and Mg^{2+} are needed to reactivate the accumulation process. In this case, the extent of the reactivation is proportional to the Mg^{2+} concentration. The fact that it is possible to reverse the inhibition of calcium phosphate accumulation, indicates that this is not due to permanent damage of the mitochondrial membrane.

4. In the initial presence of ADP, calcium accumulation and oxygen uptake appear to be closely correlated. In its absence both parameters are independent: calcium phosphate accumulation stops, whereas oxygen uptake is maximally stimulated.

5. In the absence of ADP, ruthenium red and ethyleneglycol-bis-(aminoethyl)-tetraacetic acid (EGTA) have no effect on the maximally stimulated oxygen uptake. When the nucleotide is initially present, ruthenium red and EGTA promote respiratory control immediately. Once lost, respiratory control in response to ruthenium red is restored when ADP and Mg^{2+} are both present. However, with EGTA, Mg^{2+} alone is active in re-establishing respiratory control.

Abbreviations: Ado-PCP, α,β-methylene adenosine diphosphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; EGTA, ethyleneglycol-bis-(aminoethyl)tetraacetic acid.

6. Exchange between the calcium accumulated and the calcium of the incubation medium has been studied under experimental conditions, which caused any net calcium accumulation to stop. The exchange is inhibited by low concentrations of ruthenium red under conditions where no effect of this inhibitor on the stimulated oxygen uptake can be detected. This contradicts the hypothesis according to which this stimulation could be due to a cyclic movement of Ca^{2+}.

7. In order to explain the results observed, the lack of symmetry between the respective ADP and Mg^{2+} requirements is emphasized. It is postulated that magnesium is stringently required for energy coupling between calcium phosphate accumulation and oxygen uptake. The role of ADP is supposed to be a regulatory one and to consist in an increase of the affinity of the magnesium binding sites for this cation.

INTRODUCTION

Large amounts of Ca^{2+} (1–2 μatoms/mg protein) are accumulated by isolated mitochondria from mammalian tissues in the presence of phosphate ions, by a process requiring energy from either substrate oxidation or ATP hydrolysis (for extensive reviews of this aspect of mitochondrial metabolism, see refs 1–3). Phosphate is taken up in amounts stoichiometric to Ca^{2+} uptake and calcium phosphate precipitates in the mitochondrial matrix, forming small opaque spherical conglomerates, the so called "electron-dense granules" [4–7]. But even when the uptake is respiration driven, maximal calcium accumulation requires the presence of a nucleotide (ADP or ATP) in the incubation medium [8]. In this case the nucleotides do not act as energy sources, since oligomycin modifies neither the respiration-dependent Ca^{2+} accumulation [9–11], nor its nucleotide requirement [12]. In a previous work [13], it has been established that this requirement is specific for ADP with all mitochondria tested (heart, liver and kidney) and that, at least in the case of heart mitochondria, it is not directly linked to calcium phosphate precipitation [6, 12], since in heart mitochondria, as opposed to liver mitochondria [12], no significant amounts of adenylic nucleotides are accumulated with calcium phosphate.

The present work attempts to demonstrate, that in heart mitochondria, calcium accumulation requires not only ADP, but also Mg^{2+}, which seem to play an active part in the phenomenon studied, whereas ADP appears to regulate the affinity of the Mg^{2+}-requiring sites for this cation. A short preliminary account of these results has been published [14].

MATERIALS AND METHODS

Hog heart mitochondria were prepared as outlined in a previously published work [15]. The basic incubation medium used was as follows: sucrose 200 mM, L-histidine 35 mM [16], KH_2PO_4 12 mM, KCl 6 mM, bovine serum albumin (delipidated) type F 0.75 mg/ml, oligomycin 5 μg/ml; the pH was adjusted to 7.0. Various additions were made to the basic medium as indicated under Results.

Oxygen uptake was measured with a Gilson Oxygraph (Clark microelectrode) in a 1.6-ml cuvette. ^{45}Ca was used to follow accumulation and exchange kinetics.

The mitochondria were separated from the incubation medium by filtration

under negative pressure through a 0.6-μm Schleicher and Schull filter and subsequently washed with 10 ml of the following buffer: sucrose 100 mM, Tris–HCl 100 mM, pH 7.2. The filter was dried and the radioactivity counted in 5 ml of the usual scintillating mixture (toluene–PPO–POPOP) in a Packard scintillation counter No. 3375 on the C-channel.

Proteins were measured according to Jacobs et al. [17].

All the chemical reagents were of analytical grade. α,β-Methylene adenosine diphosphate (Ado-PCP) was purchased from Miles Laboratories Inc., Ruthenium Red from Fluka AG., bovine serum albumin from Sigma Chemical Company, Atractyloside from Calbiochem, bongkrekic acid was a generous gift from Dr Alain Gaudemer (Gif-sur-Yvette, France). $^{45}CaCl_2$ was purchased from CEA, Saclay, France.

RESULTS

Specific ADP-requirement of respiration dependent Ca^{2+} accumulation in heart mitochondria

Table I shows the levels attained after an accumulation period of 10 min with ascorbic acid+*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as a substrate (rotenone added) [18], in the presence of oligomycin (5 μg/ml). Maximal accumulation is observed only in the presence of ADP, neither ATP nor AMP or various other nucleotides may substitute for ADP under these conditions. This also appears to be the case for the diphosphonic ADP analogue Ado-PCP; moreover, this analogue proves to be strongly inhibitory, when compared to the control, in the absence of any added nucleotide. A similar inhibition of Ca^{2+} accumulation takes place with a combination of ATP+phosphocreatine, which acts as an ADP trap, since heart mitochondria contain a powerful creatine kinase (EC 2.7.3.2) [19]. It is obvious, that both the penetrating nucleotide ATP and the penetrating nucleo-

TABLE I

RESPIRATION-DRIVEN CALCIUM PHOSPHATE ACCUMULATION BY HOG HEART MITOCHONDRIA, IN THE PRESENCE OF VARIOUS NUCLEOTIDES

Standard incubation medium: ascorbic acid 12 mM, TMPD 250 μM, rotenone 2 μg/ml, oligomycin 5 μg/ml; protein content 0.5 mg/ml, final volume 4.3 ml; pH 7.0, 22 °C; $CaCl_2$ 2.2 mM and $^{45}Ca^{2+}$ (2 μCi) are added to the medium 3 min after the mitochondria. 10 min later, 500 μl of the incubate are pipetted off with an Eppendorff micropipette and filtered under negative pressure (see Materials and Methods).

Nucleotide present	Ca^{2+} uptake (μatoms/mg protein in 10 min)
None	0.65
ADP 2.4 mM	1.2
ATP 1.2 mM	0.75
AMP 2.4 mM	0.54
ATP 1.2 mM+phosphocreatine 3.1 mM	0.37
Ado-PCP 2.4 mM	0.16
GDP 2.4 mM	0.58
CDP 2.4 mM	0.70

TABLE II

INFLUENCE OF PHOSPHOCREATINE AND OF ADENYLIC NUCLEOTIDE TRANSLOCATION INHIBITORS ON CALCIUM PHOSPHATE ACCUMULATION BY MITOCHONDRIA

Incubation medium: sucrose 60 mM, Tris–HCl 60 mM, succinate 12 mM; rotenone 2 μg/ml, KH_2PO_4 12 mM, KCl 6 mM, $MgCl_2$ 5 mM; oligomycin when present 5 μg/ml. Protein content 1 mg/ml, pH 7.3, 22 °C. $CaCl_2$ 2.2 mM and $^{45}Ca^{2+}$ 2 μCi are added to the medium 7 min after the mitochondria. Atractyloside (400 μM) is added 20 s before calcium, bongkrekic acid (5 μM) is added 3 min before calcium addition. ATP 1.2 mM; ADP 2.4 mM, phosphocreatine 3.1 mM.

Compounds or reagents present	Ca^{2+} uptake (μatoms/mg protein in 10 min)	
	+oligomycin	−oligomycin
ATP+ADP	1.53	–
ATP+phosphocreatine	0.75	1.7
ATP+ADP+bongkrekic acid	1.6	–
ATP+phosphocreatine+bongkrekic acid	1.5	–
ATP+ADP+atractyloside	1.4	–
ATP+phosphocreatine+atractyloside	1.25	–

tide analogue Ado-PCP [20] strongly inhibit Ca^{2+} accumulations with respect to the control (in the absence of any added nucleotide); this may be due to the displacement of endogenous mitochondrial ADP, which is subsequently trapped by phosphocreatine or diluted in the medium.

Table II shows that phosphocreatine is not an intrinsic inhibitor of calcium accumulation, as appears to be the case for phosphoenolpyruvate with liver and heart mitochondria [21]: when oligomycin is absent from the incubation medium, the ATPase activity induced by calcium uptake [1–3] gives rise to an intramitochondrial formation of ADP and no inhibition takes place under these conditions in the presence of phosphocreatine. These results confirm previous data [13] according to which, even in the presence of oligomycin, the addition of AMP+phosphocreatine, stimulates calcium phosphate accumulation, possibly through a series of creatine kinase and adenylate kinase (EC 2.7.4.3)-mediated reactions which continuously regenerate ADP.

Substrate specificity

The results outlined in Table I confirm and extend the data of a previous paper [13]; however, ascorbic acid plus TMPD [22] were the substrates added, instead of succinate which was used previously. Similar results (not shown in the present work) have been observed with pyruvate+malate as substrates. These results demonstrate that the specific requirement of sustained Ca^{2+} accumulation for ADP does not depend on the substrate used and hence on the "coupling site". However, when the substrates used are pyruvate+malate, progressive inhibition of the Ca^{2+}-stimulated oxygen uptake tends to complicate both the experiments and their interpretation: this phenomenon may imply a leak of the mitochondrial membrane for NAD^+ and NADH [23]. With ascorbic acid+TMPD, a progressive uncoupling of calcium versus oxygen uptake is observed, even in the presence of ADP (under experimental conditions similar to those used in Fig. 3) and prevents the accumulation

of Ca^{2+} to optimal levels; this may be due to an irreversible damage of the mitochondrial membrane [24, 25]. To obviate these experimental difficulties, succinate (in the presence of rotenone) has been systematically chosen as a substrate for all further experiments reported, and ATP has been added to all incubation media in order to prevent the inactivation of succinate dehydrogenase [26, 27].

The site of action of ADP

Table II shows that inhibitors of adenylic nucleotide translocation, like atractyloside [28–31] and bongkrekic acid [32–34] strongly counteract the inhibition observed with ATP and phosphocreatine. This may have been expected, if ATP plus phosphocreatine act by trapping endogenous ADP which has been displaced from its initial location in the mitochondrion.

All these results seem to exclude any competition phenomenon at the outer side of the inner mitochondrial membrane, indicating that ADP has an effect on Ca^{2+} accumulation at the inner side of this membrane. If this explanation holds, the lack of effect of the non-penetrating nucleoside diphosphates [29, 35], GDP, CDP (see Table I) is inconclusive. It may further be assumed that the site of action of ADP is distinct from the adenine nucleotide translocase, since numerous experimental

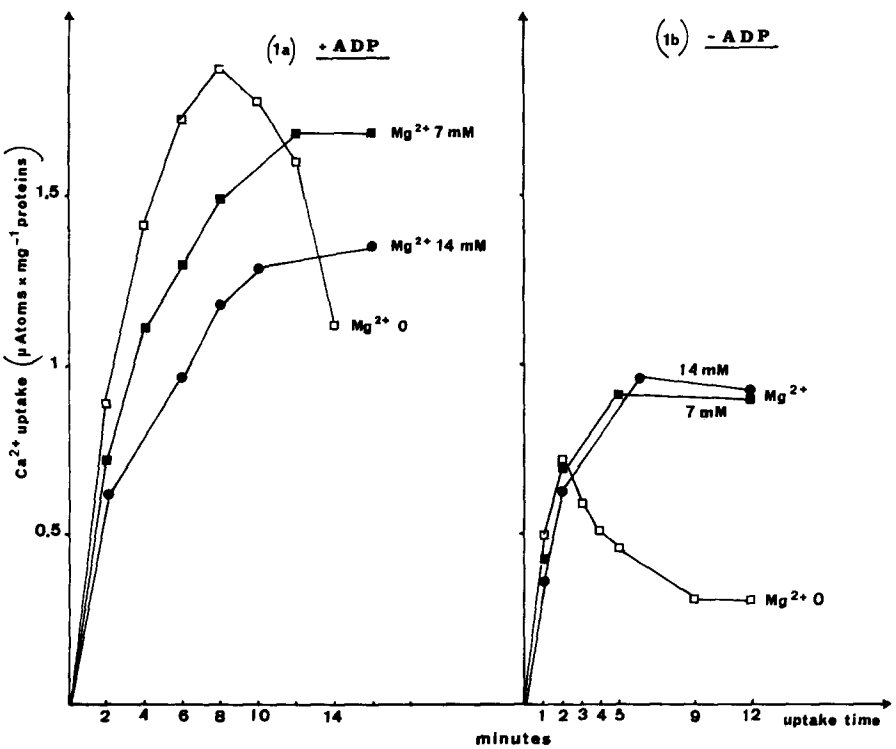

Fig. 1. Influence of Mg^{2+} on calcium phosphate accumulation by heart mitochondria, in the presence and in the absence of ADP. Standard incubation medium; succinate 13 mM, rotenone 2 µg/ml, oligomycin 5 µg/ml, ATP 1.2 mM + ADP 2.4 mM (Fig. 1a); ATP 1.2 mM + phosphocreatine 3.1 mM (Fig. 1b). Protein content 1 mg/ml, pH 7, 22 °C; for other conditions, see Table I (legend).

results show [36, 37] that the binding of ADP, ATP and Ado-PCP to this carrier have qualitatively identical effects on the conformational changes of mitochondrial membranes. It must also be remembered that atractyloside and bongkrekic acid have opposite effects on these latter parameters, since atractyloside prevents binding of the nucleotides to the translocase, whereas bongkrekic acid inhibits their dissociation [15, 38–41]. Both these compounds, however, impede translocation as such, which further substantiates the interpretation outlined above.

The action of Mg^{2+} on Ca^{2+} accumulation with or without ADP

Fig. 1 shows the accumulation kinetics of calcium phosphate with (Fig. 1a) or without (Fig. 1b) ADP, as a function of Mg^{2+} concentrations: when ADP is present from the onset of the incubation period (Fig. 1a), before the addition of calcium, maximum accumulation is observed in the absence of Mg^{2+}; however calcium phosphate is not retained by the mitochondria and appears to leak out after a certain delay. Under these conditions the effect of Mg^{2+} is 2-fold: Ca^{2+} accumulation rates are decreased, but no leaking out of accumulated calcium phosphate occurs. In the absence of ADP (Fig. 1b), accumulation proceeds to a maximal value (although a much lower one than with ADP), the rate depending on the Mg^{2+} concentration; Mg^{2+} does not seem to modify the maximum accumulation attained, but definitely

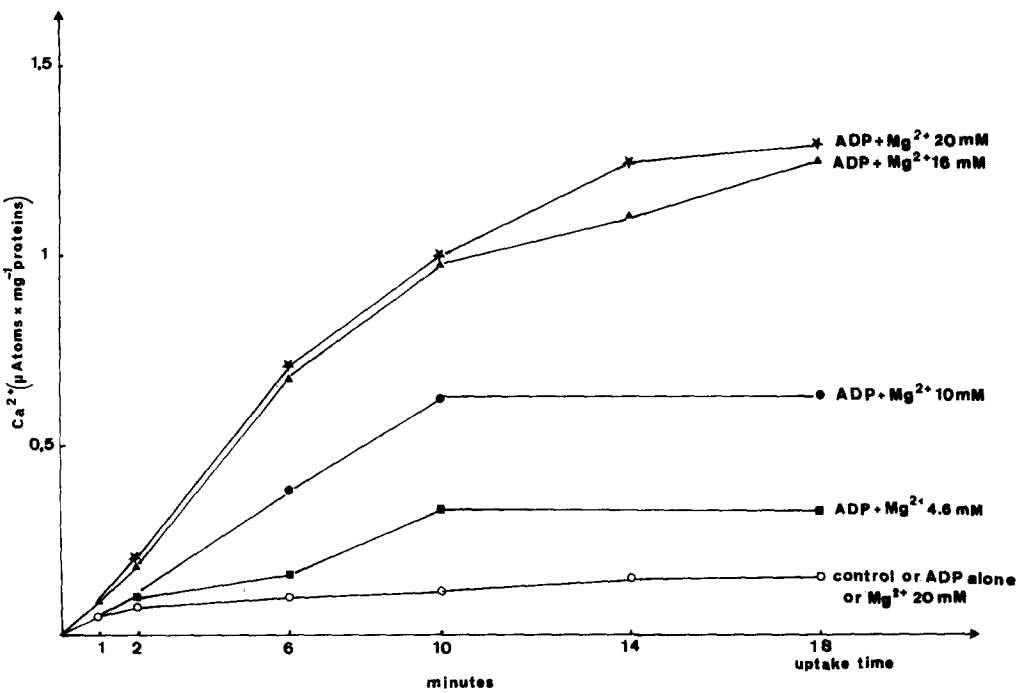

Fig. 2. Effect of Mg^{2+} on the reactivation of calcium phosphate accumulation, inhibited through lack of ADP. Standard incubation medium, without Mg^{2+}; succinate 13 mM, rotenone 2 μg/ml, ATP 1.2 mM, phosphocreatine 3.1 mM. $CaCl_2$ 2.2 mM is added 3 min after the mitochondria and $^{45}Ca^{2+}$ (2 μCi), 4 min after the cold $CaCl_2$ with or without ADP 2.4 mM and $MgCl_2$ at the concentrations stated. 500 μl of the incubate are pipetted off at the times indicated (and processed as in Table I) (legend). Protein content 1 mg/ml, pH 7.0, 22 °C.

prevents leaking out of calcium phosphate, as already observed in the presence of ADP.

Effect of Mg^{2+} on the restoration of calcium phosphate accumulation, after its prevention through lack of ADP

Fig. 2 shows the effect of Mg^{2+}, ADP or Mg^{2+}+ADP on the Ca^{2+} accumulation in mitochondria, after they have been previously allowed to accumulate the limited amount of calcium phosphate which may be taken up in the absence of exogenous Mg^{2+} and ADP (Fig. 1b). The addition of both ADP and Mg^{2+} is required to reactivate a calcium phosphate accumulation, the level of which appears to be proportional to the Mg^{2+} concentration. ADP alone, or Mg^{2+} alone, have no effect. The possibility to reverse the inhibition of calcium phosphate accumulation indicates that it is not due to permanent damage to the mitochondrial membrane.

Increase in oxygen uptake linked to calcium phosphate accumulation

The action of ADP and Mg^{2+}. In Fig. 3, the kinetics of oxygen uptake and calcium phosphate accumulation are compared in the presence (Fig. 3a) and in the absence (Fig. 3b) of ADP. It appears that both parameters correlate satisfactorily

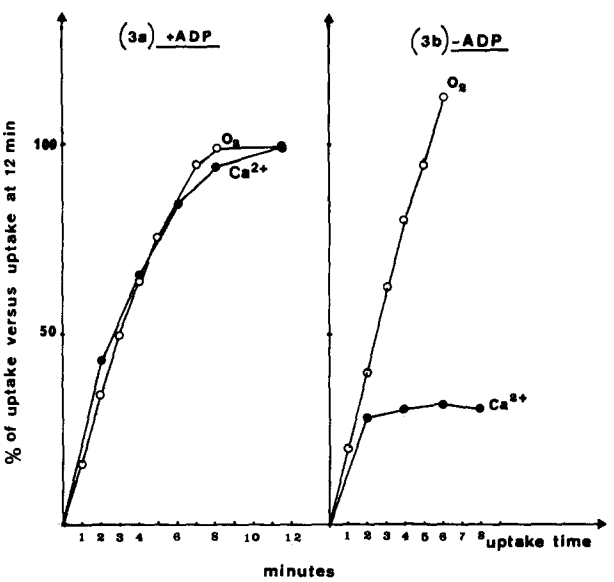

Fig. 3. Comparison between calcium phosphate accumulations and stimulation of oxygen uptake linked to this accumulation in the presence and in the absence of ADP. Standard incubation medium; succinate 13 mM, rotenone 2 μg/ml Mg^{2+} 7 mM. ATP 1.2 mM+ADP 2.4 mM (Fig. 3a), ATP 1.2 mM+phosphocreatine 3.1 mM (Fig. 3b); protein content 0.5 mg/ml, pH 7.0, 22 °C. Oxygen uptake and calcium phosphate accumulation are measured separately, in parallel experimental set-ups under identical conditions. Stimulation of oxygen uptake is expressed as the increments obtained in the presence of calcium after subtraction of control uptakes in the absence of this cation. For the sake of comparing the kinetics of oxygen uptake and calcium phosphate accumulation, both these parameters are plotted as percentages of maximal stimulation or maximal accumulation estimated in the presence of ADP for 12 min.

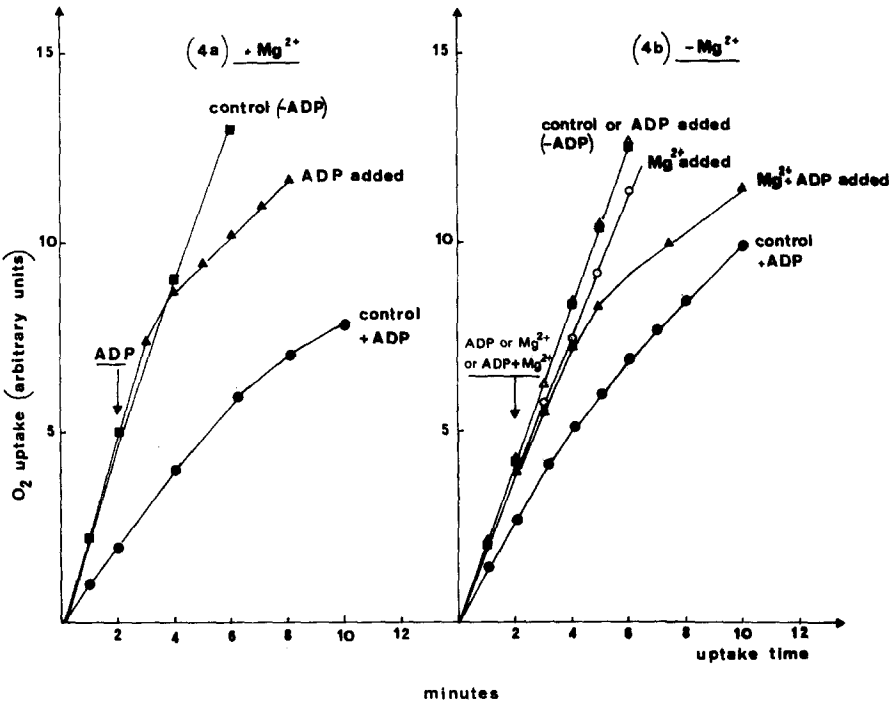

Fig. 4. Simultaneous requirement for ADP and Mg^{2+} to restore respiratory control in heart mitochondria after accumulation of limited amounts of calcium phosphate in the absence of ADP. Standard incubation medium; succinate 13 mM, rotenone 2 μg/ml, ATP 1.2 mM, phosphocreatine 3.1 mM, Mg^{2+} (Fig. 4a); ATP 1.2 mM, phosphocreatine 3.1 mM, no Mg^{2+} (Fig. 4b). Protein content 0.5 mg/ml, pH 7.0, 22 °C. Stimulation of oxygen uptake is expressed as the increments obtained in the presence of calcium after subtraction of control uptakes in the absence of this cation. Reagents are added to the medium at the time indicated by the arrow. (a) Mg^{2+} 7 mM initially present. (b) Mg^{2+} absent.

in the presence of ADP; in its absence no correlation is observed, which agrees with previous results of Rossi and Lehninger [8]: oxygen uptake undergoes permanent stimulation, whereas calcium phosphate accumulation stops. When Mg^{2+} is initially present, the addition of ADP (Fig. 4a) re-establishes respiratory control; in the initial absence of Mg^{2+}, the addition of both this cation and ADP is required to reinstate respiratory control (Fig. 4b).

The action of EGTA on the stimulation of oxygen uptake. Ethyleneglycol-bis-(aminoethyl)tetraacetic acid (EGTA) (4 mM) complexes the Ca^{2+} of the incubation medium and decreases its concentration below the threshold which activates oxygen uptake [42]. When Mg^{2+} is absent and ADP present (Fig. 5a), the stimulation of oxygen uptake is prevented by EGTA, but if both ADP and Mg^{2+} are absent (Fig. 5b) no inhibition of the highly stimulated oxygen uptake is observed. Once again the addition of ADP plus Mg^{2+}, 2 min after EGTA, restores the respiratory control immediately. It is remarkable that in this case Mg^{2+} alone re-establishes respiratory control, although more slowly than in the simultaneous presence of ADP.

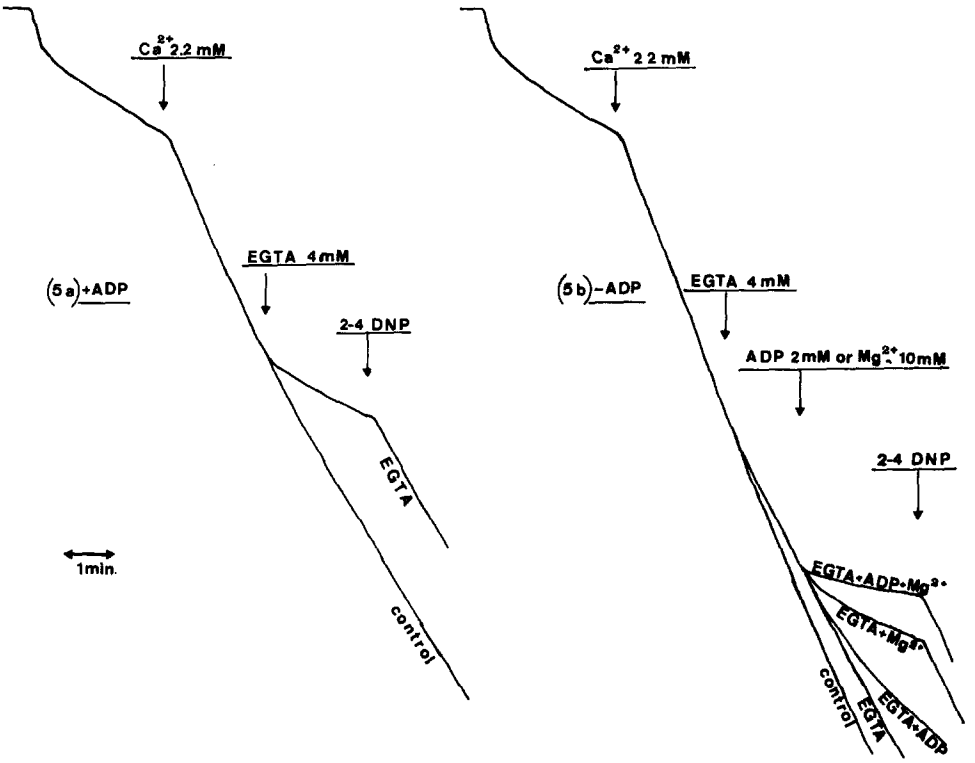

Fig. 5. The action of EGTA on the calcium-dependent stimulation of oxygen uptake in heart mitochondria. Standard incubation medium: succinate 13 mM, rotenone 2 μg/ml. ATP 0.7 mM, phosphocreatine 0.9 mM, no Mg^{2+}. Protein 1 mg/ml, pH 7.0, 22 °C. (a) ADP 2 mM initially present. (b) no ADP.

The action of ruthenium red on the stimulation of oxygen uptake. Ruthenium red inhibits calcium penetration in mammalian mitochondria, without interfering at low concentrations with the oxidophosphorylating system itself [43, 44].

Figs 6a and 6b show that provided ADP is initially present in the incubation medium, ruthenium red indeed inhibits the oxygen uptake linked to Ca^{2+} accumulation, both in the presence and in the absence of Mg^{2+}. This inhibition undoubtedly reflects the discontinuation of calcium uptake. In the absence of ADP (Figs 6c and 6d), ruthenium red has no effect on the greatly stimulated oxygen uptake, unless ADP plus Mg^{2+} (Fig. 6c), or ADP alone (in the initial presence of Mg^{2+}, Fig. 6d) are added to the medium simultaneously with ruthenium red: in this case only is the inhibitory action of ruthenium red completely recovered, which suggests that the strong stimulation of oxygen uptake in the absence of ADP and Mg^{2+} does not depend on calcium flow: this flow remains sensitive to ruthenium red, as demonstrated through the calcium-exchange experiments outlined below.

Studies on Ca/^{45}Ca exchange

Fig. 7a shows that in the absence of ADP (Mg^{2+} 7 mM), calcium phosphate accumulation stops completely, 2 min after the addition of Ca^{2+}. If labelled $^{45}Ca^{2+}$

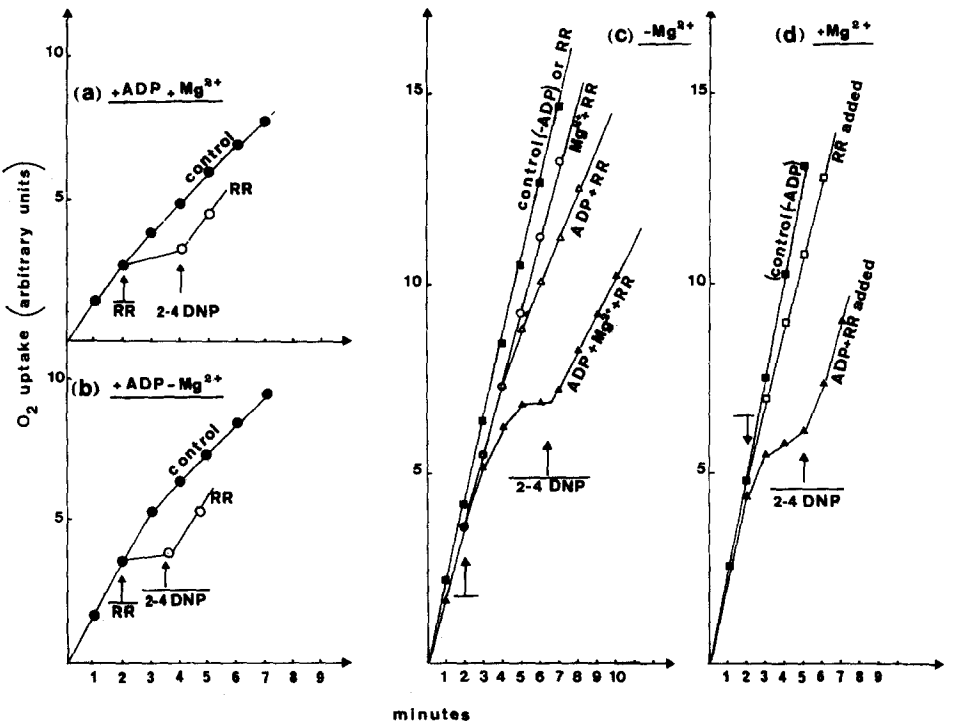

Fig. 6. The action of ruthenium red (RR) on the calcium-dependent stimulation of oxygen uptake in heart mitochondria. For experimental conditions and plots see Fig. 4 (legend). (a) ATP 1.2 mM, ADP 2.4 mM, Mg^{2+} 7 mM initially present. (b) ATP 1.2 mM, ADP 2.4 mM initially present; no Mg^{2+}. (c) ATP 1.2 mM, phosphocreatine 3.1 mM; no ADP, no Mg^{2+}. (d) ATP 1.2 mM, phosphocreatine 3.1 mM, Mg^{2+} 7 mM initially present, no ADP. Additions at the first arrow: ruthenium red plus (when indicated) ADP and Mg^{2+}.

is then added (at 4 min) an appreciable amount of the tracer is taken up (Figs 7a and 7b). Since accumulation of the cation has stopped, this result suggests the occurrence of an exchange either between free Ca^{2+} and calcium bound to the mitochondrial membranes, or between free Ca^{2+} and accumulated intramitochondrial calcium. If antimycin A, which inhibits the oxidation of the substrate (succinate) and thus prevents the energy-dependent accumulation of calcium phosphate, is added [1–3], before the addition of unlabelled calcium, and the exchange subsequently measured as outlined above (Fig. 7b), the amount of tracer taken up averages 12 natoms/mg protein (as compared to 150 natoms/mg protein without antimycin A). This small amount is believed to consist of calcium bound to the external and internal mitochondrial membranes, which is conspicuously short of the 60 natoms/mg of protein quoted in the literature [3]. However, the method used in the present work to recover the mitochondria from the incubation medium (filtration under negative pressure and washing with a large volume of the buffer) may explain this discrepancy. It is thus assumed that Fig. 7b actually shows the exchange between the calcium of the medium and calcium in the internal mitochondrial space.

Fig. 8 demonstrates that ruthenium red (1.2 μM) strongly inhibits this ex-

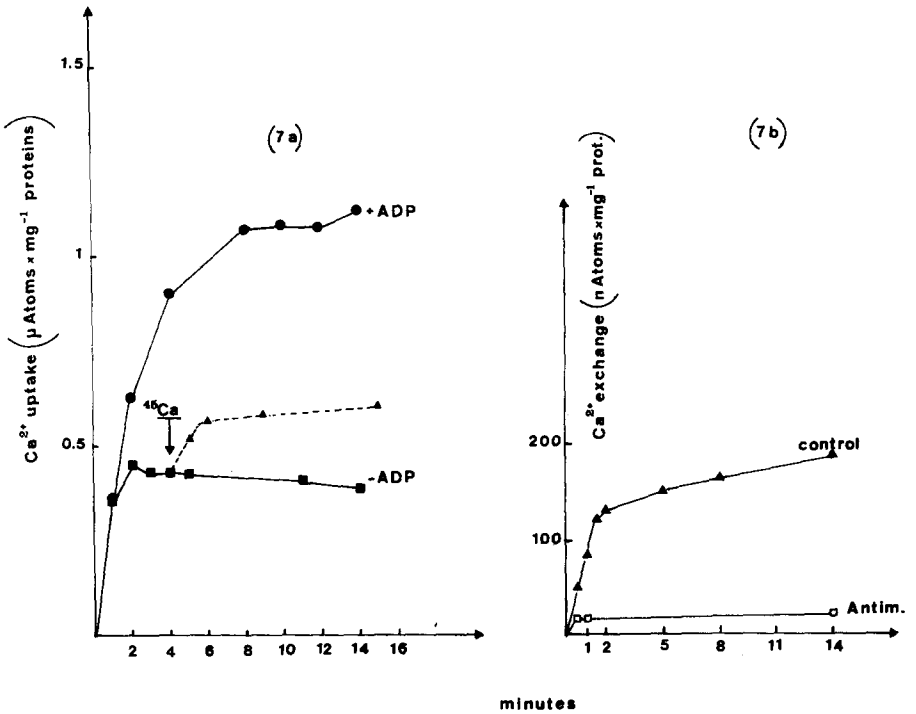

Fig. 7. Calcium-exchange studies. Experimental conditions as in Fig. 1 (legend). $MgCl_2$ 7 mM. (a) $CaCl_2$ 2.2 mM + $^{45}Ca^{2+}$ added 3 min after the sarcosomes. ●–●, Calcium accumulated with ATP 1.2 mM + ADP 2.4 mM initially present. ■–■, Calcium accumulated with ATP 1.2 mM + phosphocreatine 3.1 mM. When exchange is studied (▲- - -▲), unlabelled $CaCl_2$ is added 3 min after the sarcosomes and $^{45}Ca^{2+}$, 4 min after unlabelled $CaCl_2$. (b) Same conditions. ▲–▲, Same as in (a), expanded ordinate scale. □–□, Antimycin (0.15 μg/ml) added 3 min after the sarcosomes, $CaCl_2$ 2.2 mM, 1 min later and finally $^{45}Ca^{2+}$, 3 min later. In exchange experiments sarcosomes are separated from the incubation medium by filtration under negative pressure, similar to the procedure used for accumulation experiments.

change. The simultaneous addition of ADP with ruthenium red increases the inhibitory action of the latter compound; as expected, in its presence no restoration of calcium phosphate accumulation by ADP takes place.

It must be added that the initial rate of the exchange process (Fig. 8) is identical to the initial rate of Ca^{2+} accumulation and since both are inhibited by an identical concentration of ruthenium red, it may be postulated that both exchange and accumulation proceed through identical, ruthenium red-sensitive channels, presumably by means of the calcium carrier as described in the literature [45, 46].

Correlating these observations with the fact that ruthenium red has no effect whatsoever on the greatly stimulated oxygen uptake in the absence of ADP (Figs 6c and 6d), leads to eliminate one early hypothesis of Rossi and Lehninger [8] who postulated a cyclic Ca^{2+} movement through the mitochondrial membrane to explain the stimulation of oxygen uptake: according to this mechanism, Ca^{2+} uptake is believed to be energy requiring, but passive leaking out of the cation occurs from the mitochondria in the absence of ADP, since under these conditions calcium phosphate

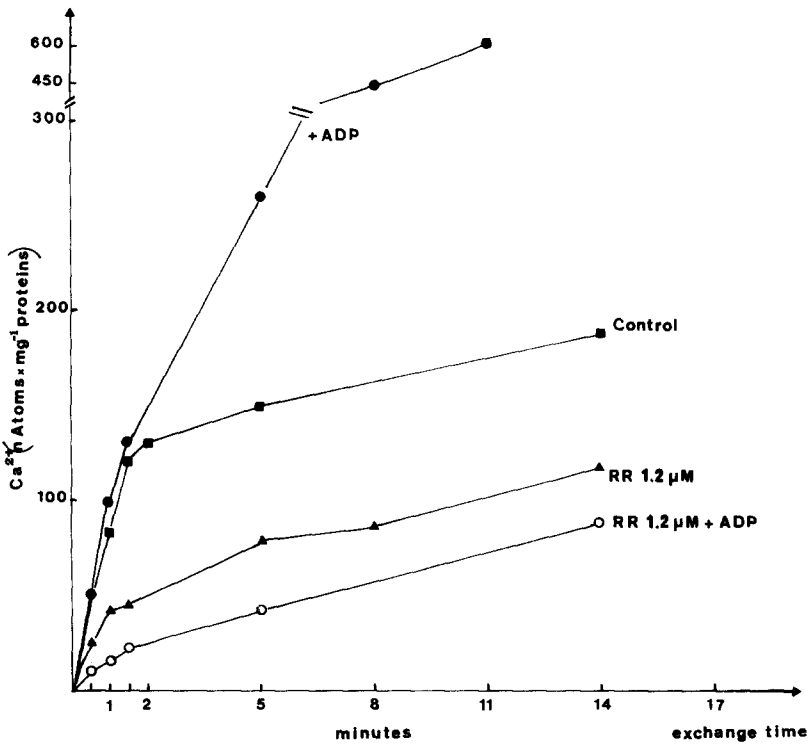

Fig. 8. The action of ruthenium red on calcium exchange in heart mitochondria. Conditions for exchange as in Fig. 7. In all experiments $^{45}Ca^{2+}$ is added 4 min after unlabelled $CaCl_2$ (2.2 mM). ■–■, Control, no addition; ●–●, ADP 2.2 mM added 1 min prior to $^{45}Ca^{2+}$; ▲–▲, ruthenium red 1.2 μM added 1 min prior to $^{45}Ca^{2+}$. ○–○, ruthenium red 1.2 μM + ADP 2.2 mM added 1 min prior to $^{45}Ca^{2+}$.

precipitation would not take place [12]: hence the sustained stimulation of the energy providing oxygen uptake. In this case, however, ruthenium red which inhibits Ca^{2+} uptake [43, 44], should eliminate active transport of this ion, and reimpose respiratory control in the absence of ADP just as in its presence, which clearly is not the case. Similarly, EGTA (Fig. 5) which complexes free Ca^{2+} [42] should suppress activation of oxygen uptake as it does in the presence of ADP. It may be concluded that, in the absence of this nucleotide, calcium flow, as demonstrated by exchange experiments, is independent of metabolic energy.

DISCUSSION

Comparison between the respective roles of ADP and Mg^{2+}

All observations reported in the present work indicate that ADP and Mg^{2+} do not play a symmetrical rôle with respect to the metabolic parameters studied. Concerning the respiration-driven calcium phosphate accumulation, Fig. 1a shows that provided ADP is present in the incubation medium prior to the addition of calcium, optimal accumulations are obtained without any added magnesium. The sub-

sequent loss of Ca^{2+} from the mitochondria under these experimental conditions appears to proceed from causes other than those outlined in the present paper (as visualized by the electron microscopic pattern of the calcium phosphate precipitate, unpublished observations). This will not be further discussed. On the contrary, Mg^{2+} alone does not support maximal Ca^{2+} accumulation (Fig. 1b). When small amounts of calcium phosphate are first accumulated in the absence of ADP under which conditions the uptake soon stops, ADP and Mg^{2+} are required to reinstate an accumulation which henceforth is proportional to Mg^{2+} concentrations (Fig. 2).

Analogous observations apply to the stimulation of oxygen uptake linked to calcium phosphate accumulation: respiratory control is demonstrated with ruthenium red only if ADP has been present in the incubation medium before the addition of calcium and indicates stringent coupling between both parameters under these conditions. Exogenous Mg^{2+} has no effect on the phenomenon (Fig. 6). When, however, respiratory control is lost by accumulation of small amounts of calcium phosphate in the absence of ADP, it needs both the presence of Mg^{2+} and ADP to restore this control (Figs 4 and 6).

A possible model for the mechanisms of action of ADP and Mg^{2+}

The results discussed in the preceding sections strongly suggest that the energy-driven accumulation of calcium phosphate uptake, coupled to the oxidation of a substrate depends on the integrity of a mitochondrial site which requires both Mg^{2+} and ADP to be present. Allowing for the well-established fact that the energy-transducing steps for calcium accumulation are identical to the mechanisms which result in the phosphorylation of ADP to ATP [1–3], this site may tentatively be localized within the multi-enzymatic system which couples oxidation to phosphorylation. At any rate, the site is clearly distinct from the calcium carrier itself, as evidenced by the fact that calcium exchange through the mitochondrial membrane is unaffected by the lack of ADP and magnesium.

The absence of symmetry between the requirements for ADP and Mg^{2+} under various experimental conditions points to a somewhat more particular model, which may be outlined as follows: ADP either regulates the affinity of a Mg^{2+}-binding site for this cation, or modifies its environment in such a way as to shield the magnesium bound to this site against displacing cations, such as calcium. Displacement of magnesium by calcium is supposed to induce structural changes of the mitochondrial membrane (resulting in a labilization of energy-rich intermediates or in an increased permeability of the membrane to protons) which in turn provoke the functional changes observed throughout the present work, such as the discontinuation of calcium phosphate accumulation and the loss of respiratory control.

This explanation, although not clearly demonstrated, is consistent with all the results reported above. If the presence of ADP modified the magnesium binding site in a way which prevents the displacement of Mg^{2+} by Ca^{2+}, exogenous Mg^{2+} should indeed not be required to maintain the metabolic functions studied, viz. sustained calcium phosphate accumulation, coupled to substrate oxidation; in the absence of ADP, calcium phosphate accumulation may ultimately result in the displacement of Mg^{2+} by Ca^{2+}, and the ineffectiveness of even high concentrations of Mg^{2+} without ADP to restore metabolism strongly suggests that on the unprotected binding site, competition between both cations is unfavourable to magnesium. The

small amount of endogenous ADP initially present, could account for the limited amount of calcium phosphate taken up at the start of the experiment prior to uncoupling. Once magnesium has been displaced by calcium, addition of ADP plus Mg^{2+} invariably reinstates the initial metabolic characters of the system: Mg^{2+} alone is ineffective, because it does not compete favourably with calcium, ADP alone has no effect because it is not the compound required primarily by the active site.

The observed effect of EGTA (Fig. 5) supports this interpretation: respiratory control is not re-established by this reagent in the absence of both ADP and magnesium ions, but with EGTA, magnesium alone (without ADP), promotes recoupling of the system. It may be postulated that under conditions where the concentration of free Ca^{2+} is strikingly decreased by EGTA, binding of Mg^{2+} to the active site becomes possible. It must be emphasized that in comparison, ADP+EGTA (without added Mg^{2+}) exhibits a slow effect on the coupling/uncoupling phenomenon, which might be due to some endogenous Mg^{2+}.

The existence of a magnesium-dependent energy transducing site has been previously postulated by Kun et al. [45–48], but some discrepancies remain between the results of this group and of our experiments; thus ADP in the presence of 2,4-dinitrophenol (substrate glutamate) appears to labilize bound magnesium ions, as shown by Kun et al. [45], whereas, according to the present results, ADP is thought to protect magnesium in the active site against displacement by Ca^{2+}. It seems premature to speculate on this apparent contradiction.

ACKNOWLEDGEMENTS

The authors wish to thank Miss Maryvonne Bourdain for her excellent technical assistance. The present work has been performed thanks to two official grants of the C.N.R.S., Paris, France (ERA No. 33 and ATP No. 429.914), to a grant obtained from the D.G.R.S.T. (No. 72.7.0135), to a generous contribution of the Fondation pour la Recherche Médicale Française and to a participation of the CEA (Saclay, France) in the purchase of radioactive compounds.

REFERENCES

1 Chance, B. (1965) J. Biol. Chem. 240, 2729–2748
2 Lehninger, A. L., Carafoli, E. and Rossi, C. S. (1967) Adv. Enzymol. 29, 259–320
3 Carafoli, E. and Lehninger, A. L. (1971) Biochem. J. 122, 681–690
4 Greenawalt, J. W., Rossi, C. S. and Lehninger, A. L. (1964) J. Cell. Biol. 23, 21–38
5 Brierley, G. P. and Slautterback, D. B. (1964) Biochim. Biophys. Acta 82, 183–186
6 Weinbach, E. C. and Von Brand, T. (1965) Biochem. Biophys. Res. Commun. 19, 133–137
7 Greenawalt, J. W. and Carafoli, E. (1966) J. Cell. Biol. 29, 37–61
8 Rossi, C. S. and Lehninger, A. L. (1964) J. Biol. Chem. 239, 3971–3980
9 Vasington, F. D. and Murphy, J. V. (1962) J. Biol. Chem. 237, 2670–2677
10 Lehninger, A. L., Rossi, C. S. and Greenawalt, J. W. (1963) Biochem. Biophys. Res. Commun. 11, 441–446
11 Brierley, G. P., Murer, E. and Green, D. E. (1963) Science 140, 60–62
12 Carafoli, E., Rossi, C. S. and Lehninger, A. L. (1965) J. Biol. Chem. 240, 2254–2261
13 Leblanc, P., Bourdain, M. and Clauser, H. (1970) Biochem. Biophys. Res. Commun. 40, 754–761
14 Leblanc, P. and Clauser, H. (1973) 9th Int. Congress of Biochemistry 4fg, p. 50, Stockholm 1973

15 Leblanc, P. and Clauser, H. (1972) FEBS Lett. 23, 107–113
16 Connelly, J. L. and Myron, D. R. (1971) Biochemistry 10, 102–107
17 Jacobs, E. E., Jacobs, M., Sanadi, D. R. and Bradley, L. B. (1956) J. Biol. Chem. 223, 147–156
18 Packer, L. and Deuton, M. D. (1962) Fed. Proc. 21, 52
19 Jacobs, H., Heldt, H. W. and Klingenberg, M. (1964) Biochem. Biophys. Res. Commun. 16, 516–521
20 Duée, E. D. and Vignais, P. V. (1968) Biochem. Biophys. Res. Commun. 30, 420–427
21 Chudapongse, P. and Haugaard, N. (1973) Biochim. Biophys. Acta 307, 599–606
22 Tager, J. M., Howland, J. C. and Slater, E. C. (1962) Biochim. Biophys. Acta 58, 616–618
23 Vinogradov, A., Scarpa, A. and Chance, B. (1972) Arch. Biochem. Biophys. 152, 646–654
24 Hunter, F. E. Jr., Scott, A., Hoffsten, P. E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B.,, Fink, J., Ford, L. and Smith, E. (1964) J. Biol. Chem. 239, 604–613
25 Park, J. H., Heriwether, B. P., Park, C. R. and Spector, L. (1957) Fed. Proc. 16, 97
26 Gutman, M., Kearney, E. B. and Singer, T. P. (1971) Biochem. Biophys. Res. Commun. 44, 526–532
27 Rasmussen, U. F. (1972) FEBS Lett. 21, 173–178
28 Bruni, A., Luciani, S. and Contessa, A. R. (1964) Nature 201, 1219–1220
29 Pfaff, E., Klingenberg, M. and Heldt, H. W. (1965) Biochim. Biophys. Acta 104, 312–315
30 Duée, E. D. and Vignais, P. V. (1965) Biochim. Biophys. Acta 107, 184–188
31 Brierley, G. and O'Brien, R. L. (1965) J. Biol. Chem. 240, 4532–4539
32 Henderson, P. J. F. and Lardy, H. A. (1970) J. Biol. Chem. 245, 1319–1326
33 Klingenberg, M., Grebe, K. and Heldt, H. W. (1970) Biochem. Biophys. Res. Commun. 39, 344–351
34 Weideman, M. J., Erdelt, H. and Klingenberg, M. (1970) Biochem. Biophys. Res. Commun. 39, 363–370
35 Duée, E. D. and Vignais, P. V. (1969) J. Biol. Chem. 244, 3920–3931
36 Weber, N. E. and Blair, P. V. (1970) Biochem. Biophys. Res. Commun. 41, 821–829
37 Stoner, C. D. and Sirak, H. D. (1973) J. Cell Biol. 56, 51–64
38 Klingenberg, M., Grebe, K. and Scherer, D., (1971) FEBS Lett. 16, 253–256
39 Stoner, C. D. and Sirak, H. (1973) J. Cell Biol. 56, 65–73
40 Vignais, P. V. and Vignais, P. M. (1972) FEBS Lett. 26, 27–31
41 Vignais, P. V., Vignais, P. M. and Defaye, G. (1971) FEBS Lett. 17, 281–288
42 Crofts, A. R. and Chappell, J. B. (1965) Biochem. J. 95, 387–392
43 Moore, C. L. (1971) Biochem. Biophys. Res. Commun. 42, 298–305
44 Vasington, F. D., Gazzotti, P., Tiozzo, R. and Carafoli, E. (1972) Biochim. Biophys. Acta 256, 43–54
45 Reynafarje, B. and Lehninger, A. L. (1969) J. Biol. Chem. 244, 584–593
46 Lehninger, A. L. (1970) Biochem. J. 119, 129–138
47 Kun, E., Kearney, E. B., Lee, N. M. and Wiedemann, I. (1970) Biochem. Biophys. Res. Commun. 38, 1002–1008
48 Lin, D. C. and Nobel, P. S. (1971) Arch. Biochem. Biophys. 145, 622–632
49 Kun, E. (1972) in Biochemical Regulatory Mechanisms in Eukaryotic Cells (Kun, E. and Grisolia, S., eds), Chapter 10, p. 303, John Wiley Publ. N.Y.
50 Lin, D. C. and Kun, E. (1973) Biochem. Biophys. Res. Commun. 50, 820–825