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Kinetic mechanism of mitochondrial adenosine triphosphatase

ADP-specific inhibition as revealed by the steady-state kinetics

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1. A substantial increase of the initial rate of ATP hydrolysis was observed after preincubation of bovine heart submitochondrial particles with phosphoenolpyruvate and pyruvate kinase. 2. The activation was accompanied by an increase of $V_{max.}$, without change of K_m for ATP. 3. The activated particles catalysed the biphasic hydrolysis of ATP in the presence of an ATP-regenerating system; the initial rapid phase was followed by a second, slower, phase in a time-dependent fashion. 4. The higher the ATP concentration used as a substrate, the higher is the rate of transition between these two phases. 5. The particles catalysed the hydrolysis of ITP with a lag phase; after preincubation with phosphoenolpyruvate and pyruvate kinase, ITP was hydrolysed at a constant rate. 6. Qualitatively the same phenomena were observed when soluble mitochondrial ATPase (F_1-ATPase) prepared by the conventional method in the presence of ATP was used as nucleotide triphosphatase. 7. A kinetic scheme is proposed, in which the intermediate active enzyme–product complex (E·ADP) formed during ATP hydrolysis is in slow equilibrium with the inactive E*·ADP complex forming as a result of dislocation of ADP from the active site of ATPase to the other site, which is not in rapid equilibrium with the surrounding medium.

It is well established that the coupling factor F_1-ATPase, having a high hydrolytic activity when separated from the mitochondrial membrane (Pullman *et al.*, 1960; Penefsky *et al.*, 1960), plays an essential role in the molecular mechanisms of ATP formation during oxidative phosphorylation. Lardy & Wellman (1953) were the first to formulate clearly the concept according to which the ATPase activity of mitochondrial preparations reflects the reversal of the sequence of events taking place during ATP synthesis. Most of the current models for the mechanism of ATP formation take into account the numerical data on the properties of the ATPase reaction catalysed by mitochondria, by submitochondrial particles, by isolated F_1-ATPase or by its individual subunits (Slater, 1974; Williams, 1975; Kozlov & Skulachev, 1977; Harris, 1978; Boyer, 1979; Mitchell, 1979), although the identities of the enzyme substrate-binding sites participating in the ATP hydrolysis and synthesis have been questioned (Penefsky, 1974; Pedersen, 1976).

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The steady-state kinetics of oxidative phosphorylation (Kayalar *et al.*, 1976; Schuster *et al.*, 1977), the ATP-dependent energy-linked reactions of submitochondrial particles (Hommes, 1963; Huang & Mitchell, 1972; Pedersen, 1976) and the ATPase reactions (Selwyn, 1967; Hammes & Hilborn, 1971; Akimenko *et al.*, 1972; Adolfson & Moudrianakis, 1973; Schuster *et al.*, 1975; Roveri *et al.*, 1980) have been widely investigated. Several kinetic schemes of ATP hydrolysis have been proposed (Harris *et al.*, 1978; Roveri *et al.*, 1980; Vasilyeva *et al.*, 1980); none of them has been conclusively established and the kinetic mechanisms of ATPase (or ATP synthesis) are far from being well understood. Moreover, there are some contradictory reports in the literature on the kinetics of mitochondrial ATPase. Hammes & Hilborn (1971) found simple Michaelis–Menten kinetics for soluble bovine heart F_1-ATPase and membrane-bound bovine heart ATPase with a simple competitive inhibition by ADP, whereas Schuster *et al.* (1975) and Pedersen (1976) observed a strong deviation from linearity in the double-reciprocal plots for rat liver and bovine heart ATPases. An even more complicated steady-state behaviour has been

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reported (Alexandre *et al.*, 1975). Obviously, some discrepancies in the literature may be due to an unjustified comparison of the results obtained under different conditions (pH values, ionic compositions of assay mixtures, ranges of nucleotide concentrations), by different methods (the presence or absence of the ATP-regenerating system, continuous or discontinuous registration of the 'initial' rates) and with different sources of the enzyme (soluble F_1-ATPases from bovine heart or rat liver, various types of submitochondrial particles of different degrees of resolution).

An important factor that strongly modulates the ATPase activity of submitochondrial particles and soluble F_1-ATPase in a fashion clearly distinct from the usual product inhibition is ADP (Fitin *et al.*, 1979; Minkov *et al.*, 1979; Vasilyeva *et al.*, 1980). It has been shown that the occupation of the site with a dissociation constant for ADP of about 10^{-8} M causes complete inhibition of the ATPase, and its activation in the presence of ATP occurs at a rate that is much lower than the enzyme turnover (Vasilyeva *et al.*, 1980). A kinetic scheme for interaction of ADP and ATP with mitochondrial ATPase has been proposed. It follows, from the kinetic constants measured, that the ADP-dependent slowly reversible inhibition might interfere with the steady-state characteristics of ATP hydrolysis. Indeed, in the present paper we provide evidence for the existence of two slowly interconvertible F_1-ATPase·ADP complexes during ATP hydrolysis, one of which is inactive in catalysis. In the accompanying paper (Vasilyeva *et al.*, 1982) we show that the effects of some inhibitors and activators (azide and sulphite respectively) are realized through this slow equilibrium primarily controlled by ADP.

Experimental

Bovine heart submitochondrial particles free of protein ATPase inhibitor (AS-particles) and F_1-ATPase precipitated from the solution containing ATP were prepared, stored and assayed as described in our previous papers (Minkov *et al.*, 1979; Vasilyeva *et al.*, 1980). The ATPase activity was measured spectrophotometrically at 340nm in the presence of the ATP-regenerating system as described previously (Vasilyeva *et al.*, 1980); the experimental details are indicated in the legends to the Figures. Special care was taken to ensure that the 'registering' enzyme system (pyruvate kinase and lactate dehydrogenase) was never limiting, so that the response time after addition of small calibrated amount of ADP or IDP was never more than 3s.

Perhaps the most important result of the present study is that the rate of ATP hydrolysis catalysed by submitochondrial particles or F_1-ATPase is almost never constant during assay in the presence of the ATP-regenerating system. Nevertheless the time scale for the activities change is much greater than that for the enzyme turnover, so at any given time the activities were treated assuming the steady-state approximation.

The nucleotides (potassium salts), pyruvate kinase and lactate dehydrogenase were obtained from Reanal (Budapest, Hungary). The enzymes were extensively dialysed before use. Other chemicals were of the purest grade commercially available.

Results

During our studies on the MgADP-induced inhibition of ATPase (Vasilyeva *et al.*, 1980) we noted that, when phosphoenolpyruvate and pyruvate kinase were added to re-activate the ADP-inhibited ATPase of AS-particles, the final activity reached in the system was variable. A closer examination of this variability revealed that the final activity depended on the total amount of ADP in the preincubation medium containing AS-particles, ATPase and pyruvate kinase. When the amount of ADP added to inhibit ATPase was just enough to inhibit ATPase completely, the final activity recovered after treatment with pyruvate kinase was always substantially higher than the original one (i.e. before inhibition by ADP). This is illustrated by Fig. 1, where the activation of AS-particles (as prepared) by pyruvate kinase is also shown.

The time courses of ATP hydrolysis by 'activated' and 'non-activated' particles were found to be qualitatively different. As shown in Fig. 2, the initial burst of hydrolysis followed by the lower, practically constant, rate was observed when the reaction was started by 'activated' particles. No such burst was seen when the reaction was initiated by 'non-activated' ATPase. The following treatments were found to have no effect on the appearance of a burst after activation: 10-fold dilution of the particles in the assay medium; 10-fold increase of pyruvate kinase and lactate dehydrogenase in the assay medium; the presence of uncouplers; pH change of the assay buffer from 7 to 9. When the initial rates of the reaction catalysed by an activated enzyme were plotted as a function of ATP concentration and compared with results obtained with the 'non-activated' enzyme, the non-competitive type of activation was evident (Fig. 3).

Assuming that the only conceivable event taking place during preincubation of the particles with pyruvate kinase and phosphoenolpyruvate is the interconversion of ADP into ATP, and taking into account an extremely high affinity of AS-particle ATPase for ADP (Fitin *et al.*, 1979), the process of activation can be visualized as the slow removal of ADP (endogenously present in AS-particles or

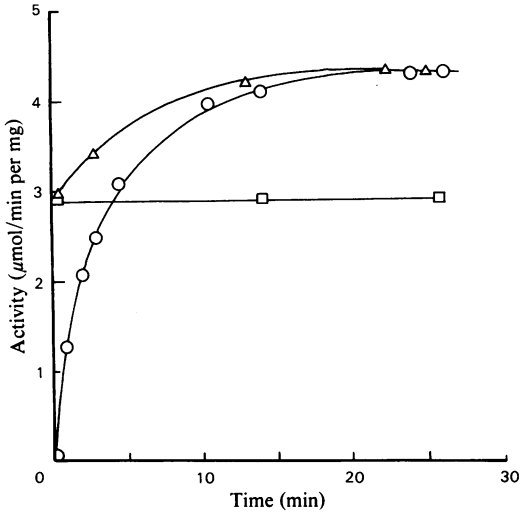


Fig. 1. *Activation of ATPase by the treatment with phosphoenolpyruvate and pyruvate kinase*

○, AS-particles (800 μg) were incubated for 3 min at 25°C in 0.4 ml of a mixture containing (final concentrations) 0.1 M-KCl, 2 mM-$MgCl_2$, 50 μM-EDTA, 10 mM-Tris/HCl buffer, pH 8.0, and 3 μM-ADP; 2 mM-phosphoenolpyruvate and 25 units of pyruvate kinase/ml were added at zero time and incubation was continued. △, AS-particles were treated as described above except that ADP was omitted. The activities were measured as the initial rates of NADH absorption decrease in the cuvette containing (final concentrations) 0.1 M-KCl, 10 mM-Tris/HCl buffer, pH 8.0, 2 mM-$MgCl_2$, 2 mM-KCN, 2 mM-potassium phosphoenolpyruvate, 0.3 mM-NADH, 3.3 units of pyruvate kinase/ml, 3.3 units of lactate dehydrogenase/ml, 0.2 mM-ATP and 9 μg of AS-particles/ml. The horizontal line (□) indicates the activity in the samples that were not treated with ADP, phosphoenolpyruvate and pyruvate kinase.

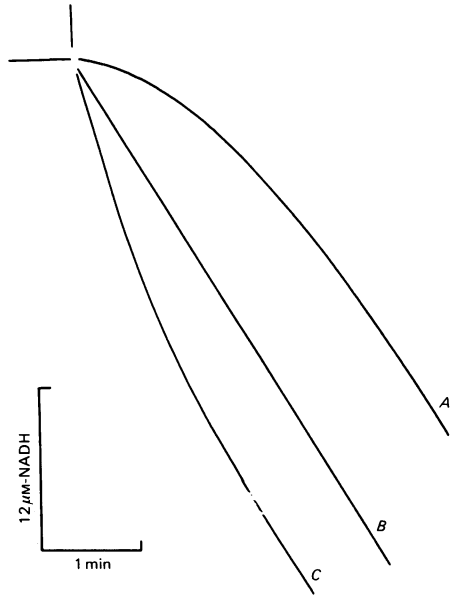


Fig. 2. *Time course of the ATP hydrolysis by AS-particles*

Particles were pretreated and assayed in the presence of 1 mM-ATP; curve *A*, as ○, in Fig. 1 at zero time; curve *B*, as □ in Fig. 1; curve *C*, as △ in Fig. 1 after 30 min activation.

added). Conversely, the decrease in the rate of ATP hydrolysis after the initial burst might be explained as slow equilibration between E·ADP complex, which is an intermediate in the overall process of ATP hydrolysis, and another complex E*·ADP, which is that described previously (Fitin *et al.*, 1979). To test this hypothesis, the advantage of a high specificity of a high-affinity inhibitory site for ADP (Vasilyeva *et al.*, 1980) was taken. As shown in Fig. 4, AS-particles catalyse the hydrolysis of ITP with a considerable lag. After activation the lag period disappears and the hydrolysis of ITP occurs at a constant rate. Since ADP inhibits the hydrolysis of other nucleoside triphosphates and IDP does not affect the ATPase activity (Vasilyeva *et al.*, 1980), this result supports the idea that ADP is the cause of the decrease in ATPase activity during the assay.

If the biphasic hydrolysis of ATP is the result of isomerization of the active intermediate enzyme·ADP complex into an inactive one, it might be expected that the rate of interconversion must depend on the concentration of ATP. As shown in Fig. 5, this is indeed the case: when a low concentration of ATP is used ($\ll K_m$) no deviation from linearity during the time of experiment was observed; with higher concentrations of ATP the decrease in the hydrolysis rate became evident.

It has been shown that ADP-induced inhibition is the common feature of both membrane-bound and soluble F_1-ATPase (Minkov *et al.*, 1979). Therefore it was decided to find out whether the biphasic kinetics of ATP hydrolysis are also typical for F_1-ATPase. As shown in Fig. 6, the reaction catalysed by F_1-ATPase prepared by precipitation from the solution containing ATP and EDTA (Senior & Brooks, 1970) is biphasic. The first rapid phase can be abolished by preincubation of F_1-ATPase with a very low concentration of ADP in the presence of Mg^{2+}, and by this treatment the time course of ATP hydrolysis can gradually be changed from a biphasic one (with a rapid initial burst) into a curve with a lag (results not shown).

The following properties were shown to be the

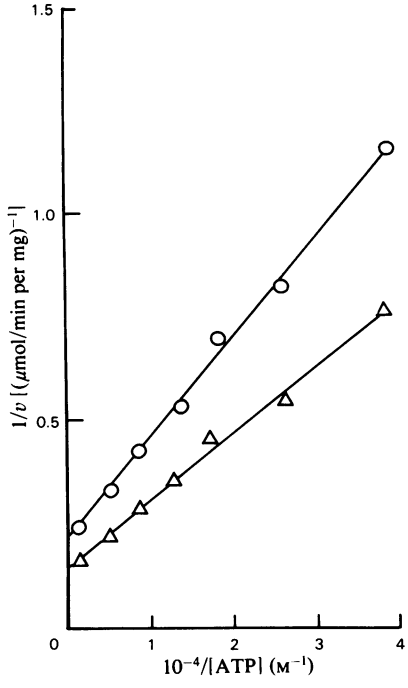


Fig. 3. *Non-competitive activation of ATPase by phosphoenolpyruvate and pyruvate kinase*
The conditions of the experiment were as given in Fig. 1. The time of activation was 45 min. ATPase was assayed as given in Fig. 1 in the presence of the indicated concentrations of ATP. The initial rates of the control (○) and activated (△) ATPases were measured.

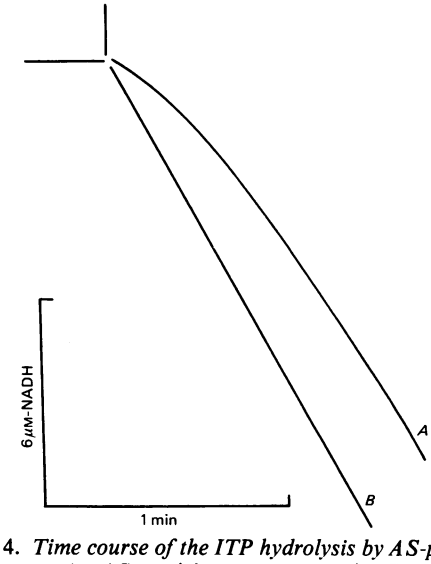


Fig. 4. *Time course of the ITP hydrolysis by AS-particles*
Curve *A*, AS-particles as prepared. Curve *B*, AS-particles were activated as indicated in Fig. 1 for 45 min. The activities were determined as given in Fig. 1 in the presence of 0.5 mM-ITP, except for the amounts of pyruvate kinase and AS-particles, which were increased 6- and 4-fold respectively.

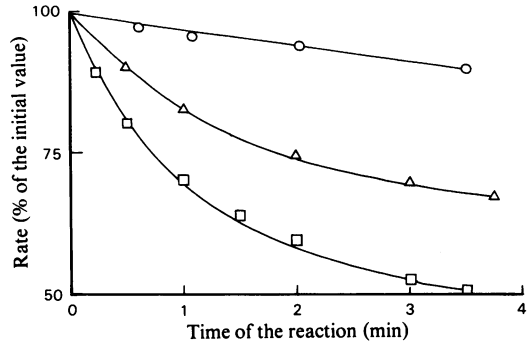


Fig. 5. *Effect of ATP concentration on the decrease of the ATPase activity during the time of assay*
AS-particles activated by phosphoenolpyruvate and pyruvate kinase as indicated in Fig. 1 for 45 min were assayed in the presence of 5 μM- (○), 40 μM- (△) and 1000 μM- (□) ATP. The 100% values of the initial rates correspond to 0.4, 2.8 and 8.1 μmol/min per mg of protein respectively for the concentrations of ATP given above.

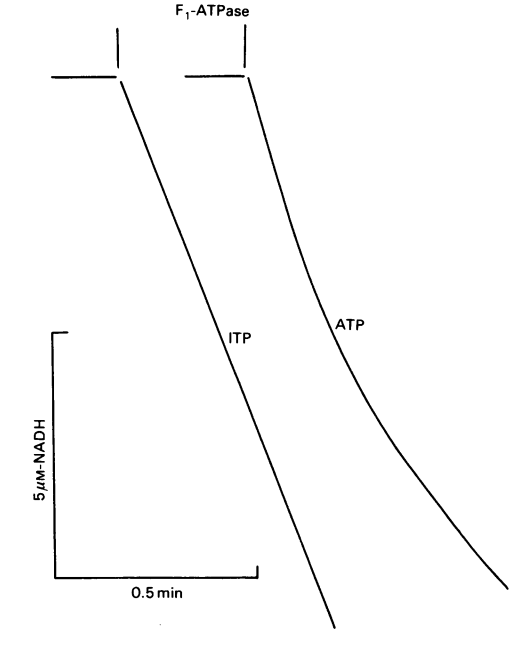
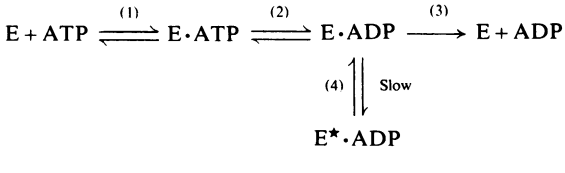


Fig. 6. *Time course of nucleoside triphosphates hydrolysis by F_1-ATPase*
The assay was performed in the medium containing the components indicated in Fig. 1, except for pH value, which was 7.5. The concentrations of ATP and ITP were 0.1 and 0.3 mM respectively. The reaction was started by the addition of 3.2 or 13 μg of F_1-ATPase to a 3 ml curvette for ATP or ITP hydrolysis respectively.

same for the nucleoside triphosphatase activity of AS-particles and F_1-ATPase: (i) the reaction is biphasic only if ATP is hydrolysed; (ii) the rate of interconversion between the first and the second phase depends on ATP concentration; (iii) only those preparations that are depleted of ADP show the biphasic kinetics. The differences found in the behaviour of soluble F_1-ATPase and membrane-bound ATPase of AS-particles are: (i) the absolute rate of interconversion from the first rapid phase into the second slow phase is higher for F_1-ATPase; (ii) when the results obtained for F_1-ATPase were plotted as in Fig. 3 the mixed type of activation was obtained. Thus it may be concluded that the phenomenon described in the present paper is a common feature of both soluble F_1-ATPase and membrane-bound ATPase.

Discussion

We were able to show that during the steady-state hydrolysis of ATP by mitochondrial ATPase in the presence of the ATP-regenerating system the enzyme is present as a slowly equilibrating mixture of active and inactive complexes. The data in the present paper, together with those previously reported (Fitin *et al.*, 1979; Minkov *et al.*, 1979; Vasilyeva *et al.*, 1980), leave little doubt that the inactive complex is the enzyme bound with ADP. Thus the kinetic scheme for ATP hydrolysis in the presence of the ATP-regenerating system can be represented as shown in Scheme 1 (where we leave out of consideration the participation of other substrates and products of the reaction, i.e. P_i, Mg^{2+}, water and protons). The following data reported in the represent paper are consistent with Scheme 1: non-competitive activation of ATPase by phosphoenolpyruvate and pyruvate kinase; the dependence of the interconversion rate on the substrate concentration [this would mean that the reactions (1) and (2) are in equilibrium]; the independence of the final activity on the amount of the ATP-regenerating system in the assay system. Some kinetic and thermodynamic parameters of formation and dissociation of the E*·ADP complex reported in the present paper and previously (Vasilyeva *et al.*, 1980) clearly indicate that the tight-binding site for ADP distinct from that participating in catalytic turnover of ATPase is involved in the formation of inactive enzyme.



Scheme 1.

Two questions merit special discussion. (1) How is the slow active–inactive interconversion of ATPase related to the other experimental observations and kinetic schemes for ATPase published in the current literature? (ii) What is the functional significance of the E*·ADP complex relevant to the mechanism of oxidative phosphorylation? The kinetics of mitochondrial ATPase have been recently re-investigated (Harris *et al.*, 1978; Roveri *et al.*, 1980). Only one E·ADP complex is included into the kinetic schemes suggested by both groups, although the latter authors have pointed out the possibility of what they call a 'slowly developing inhibitory side reaction'. The slowly developing ATP-reversed inhibition of ATPase by ADP described by Harris *et al.* (1978) is evidently a quite different phenomenon, since, in contrast with that described by us (Fitin *et al.*, 1979; Vasilyeva *et al.*, 1980; the present paper), it is seen only with soluble F_1-ATPase, does not need Mg^{2+} and has a much higher K_i value for ADP. We believe that the ADP-induced inhibitions described by Harris *et al.* (1978) for bovine heart F_1-ATPase and by Hockel *et al.* (1978) for *Micrococcus* F_1-ATPase are similar in nature, being due to the replacement of tightly bound nucleotides (Garrett & Penefsky, 1975; Harris *et al.*, 1977) by ADP.

Some recent reports in the literature (Minkov *et al.*, 1979; Hackney, 1979; Lowe *et al.*, 1980) have extended an earlier observation by Moyle & Mitchell (1975) on the slow active–inactive transition of mitochondrial ATPase. Non-linearity of the time course of ATP hydrolysis by rat liver F_1-ATPase in the presence of the ATP-regenerating system has also been claimed by Pedersen (1976). Although none of the authors cited above have considered ADP as the cause for inactivation, it seems very likely that the inactivation of ATPase they have observed and the phenomenon described in the present paper are due to the same process of E*·ADP complex formation being poised in equilibrium during steady-state ATP hydrolysis (the present paper) or shifted towards complete inactivation. No definite conclusion about the functional participation of the E*·ADP complex in the ATPase activity or oxidative phosphorylation can be drawn. The nucleotide-specificity of its formation is remarkably correlated with the specificity of nucleoside triphosphate-driven reversal of electron transfer, nucleoside triphosphate–$^{32}P_i$ exchange and oxidative phosphorylation reported by Harris *et al.* (1978).

An important clue for the functional role of ADP-inactivated ATPase is given in our report (Minkov *et al.*, 1980) that, though inactive as an ATPase, the complex E*·ADP is capable of ATP

synthesis. A hypothesis has been put forward that mitochondrial F_1-ATPase catalyses ATP synthesis and hydrolysis in two different slowly equilibrating conformational states and that this process is controlled by ADP. Should this hypothesis be true, the results presented in the present paper [and in the accompanying one (Vasilyeva *et al.*, 1982)] show that mitochondrial F_1-ATPase in the 'synthetase' conformation can be the subject of investigation under conditions of ATP hydrolysis.

References

Adolfson, R. & Moudrianakis, E. N. (1973) *Biochemistry* **12**, 2926–2933

Akimenko, V. K., Minkov, I. B., Bakeeva, L. E. & Vinogradov, A. D. (1972) *Biokhimiya* **37**, 348–356

Alexandre, A., Carignani, G. & Rossi, C. A. (1975) *FEBS Lett.* **52**, 111–115

Boyer, P. D. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G. & Ernster, L., eds.), pp. 461–479, Addison–Wesley, Reading

Fitin, A. F., Vasilyeva, E. A. & Vinogradov, A. D. (1979) *Biochem. Biophys. Res. Commun.* **86**, 434–439

Garrett, N. E. & Penefsky, H. S. (1975) *J. Biol. Chem.* **250**, 6640–6647

Hackney, D. D. (1979) *Biochem. Biophys. Res. Commun.* **91**, 233–238

Hammes, G. G. & Hilborn, D. A. (1971) *Biochim. Biophys. Acta* **233**, 580–590

Harris, D. A. (1978) *Biochim. Biophys. Acta* **463**, 245–273

Harris, D. A., Radda, G. K. & Slater, E. C. (1977) *Biochim. Biophys. Acta* **459**, 560–572

Harris, D. A., Gomez-Fernandez, J. C., Klungsøyr, L. & Radda, G. K. (1978) *Biochim. Biophys. Acta* **504**, 364–383

Hockel, M., Hulla, F. W., Risi, S. & Dose, K. (1978) *J. Biol. Chem.* **253**, 4294–4296

Hommes, F. A. (1963) *Biochim. Biophys. Acta* **77**, 173–182

Huang, C. H. & Mitchell, R. A. (1972) *Biochemistry* **11**, 2278–2283

Kayalar, C., Rosing, J. & Boyer, P. D. (1976) *Biochem. Biophys. Res. Commun.* **72**, 1153–1159

Kozlov, I. A. & Skulachev, V. P. (1977) *Biochim. Biophys. Acta* **463**, 29–89

Lardy, H. A. & Wellman, H. (1953) *J. Biol. Chem.* **201**, 357–364

Lowe, P. N., Linnett, P. E., Baum, H. & Beechey, R. B. (1980) *Abstr. Eur. Bioenerg. Conf. 1st* 215–216

Minkov, I. B., Fitin, A. F., Vasilyeva, E. A. & Vinogradov, A. D. (1979) *Biochem. Biophys. Res. Commun.* **89**, 1300–1306

Minkov, I. B., Vasilyeva, E. A., Fitin, A. F. & Vinogradov, A. D. (1980) *Biochem. Int.* **1**, 478–485

Mitchell, P. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G. & Ernster, L., eds.), pp. 361–372, Addison–Wesley, Reading

Moyle, J. & Mitchell, P. (1975) *FEBS Lett.* **56**, 55–61

Pedersen, P. L. (1976) *J. Biol. Chem.* **251**, 934–940

Penefsky, H. S. (1974) *J. Biol. Chem.* **249**, 3579–3585

Penefsky, H. S., Pullman, M. E., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* **235**, 3330–3336

Pullman, M. E., Penefsky, H. S., Datta, A. & Racker, E. (1960) *J. Biol. Chem.* **235**, 3322–3329

Roveri, O. A., Muller, L. M., Wilms, S. & Slater, E. C. (1980) *Biochim. Biophys. Acta* **589**, 241–255

Schuster, S. M., Ebel, R. E. & Lardy, H. A. (1975) *J. Biol. Chem.* **250**, 7848–7853

Schuster, S. M., Reinhart, C. D. & Lardy, H. A. (1977) *J. Biol. Chem.* **252**, 427–432

Selwyn, M. J. (1967) *Biochem. J.* **105**, 279–288

Senior, A. E. & Brooks, J. C. (1970) *Arch. Biochem. Biophys.* **140**, 257–266

Slater, E. C. (1974) in *Dynamics of Energy Transducing Membranes* (Ernster, L., Estabrook, R. W. & Slater, E. C., eds.), pp. 1–20, Elsevier, Amsterdam

Vasilyeva, E. A., Fitin, A. F., Minkov, I. B. & Vinogradov, A. D. (1980) *Biochem. J.* **188**, 807–815

Vasilyeva, E. A., Minkov, I. B., Fitin, A. F. & Vinogradov, A. D. (1982) *Biochem. J.* **202**, 15–23

Williams, R. J. P. (1975) *FEBS Lett.* **53**, 123–125