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ROLE OF SUBSTRATES IN Sr^{2+}-INDUCED OSCILLATIONS OF IONIC FLUXES IN RAT LIVER MITOCHONDRIA

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(1) The reason for substrate specificity of Sr^{2+}-induced oscillating cation fluxes in isolated rat liver mitochondria was investigated. (2) With succinate as substrate, rotenone prevented oscillation. In this case the mitochondria were only partially able to take up added Sr^{2+} and did not take up any of the released K^+. Addition of substances decreasing the mitochondrial NADH/NAD^+ ratio (oxaloacetate or acetoacetate) restored the ability for reuptake of K^+ and for complete uptake of Sr^{2+} and, therefore, oscillation. (3) Inhibition of substrate-level phosphorylation by arsenite or uncoupling of substrate-level phosphorylation by arsenate in the presence of oligomycin also suppressed the reuptake of cations. This effect of inhibition of substrate-level phosphorylation on oscillation could be circumvented by addition of ATP in the presence of oligomycin. (4) Prevention of the intramitochondrial regeneration of 2-oxoglutarate from acetyl-CoA and oxaloacetate by fluorocitrate or from endogenous glutamate by aminoxyacetate shortened the time during which oscillation with succinate as substrate could be observed. (5) From the key role of substrate level phosphorylation it is concluded that for the reuptake of K^+ and Sr^{2+} during oscillation, sufficient GTP generation by the succinyl thiokinase (EC 6.2.1.4) reaction is essential. Therefore substrate level phosphorylation seems to be a necessary energy source additional to the respiratory chain. Since the latter process drives the active cation movements, the former may be required for the restoration of a sufficiently low proton conductance of the mitochondrial inner membrane. Oscillation in the absence of exogenous ATP therefore demands 2-oxoglutarate as substrate or the intramitochondrial generation of 2-oxoglutarate for the maintenance of a sufficient GTP production for a longer time.

Introduction

The main source of mitochondrial energy transformation is the oxidation of substrate hydrogen by the multienzyme complex of the respiratory chain. Generally, most of this energy is transferred into ATP. The two processes, substrate oxidation and formation of ATP, are linked one to the other by the proton electrochemical gradient which is an intermediate energy store [1–4]. In this form, energy may be utilized directly as a driving force for ion fluxes through the mitochondrial inner membrane. Under particular conditions it is possible to induce these ion fluxes in a reversible, oscillating manner [5–9]. Such oscillating systems require a specific control mechanism and are therefore suitable models for investigations of control phenomena in energy transformation.

As previously described, Sr^{2+}-induced oscillation in hypotonic phosphate-free medium reveals a dependence on the kind of the substrate oxidized by the mitochondria [10,11]. Particularly, oscillation could not be observed with succinate in the presence of

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rotenone, whereas it was possible in the absence of rotenone. Since regulatory properties of the substrate-oxidizing dehydrogenases may be involved in the mechanism of oscillation, this substrate dependence was investigated in more detail. From the actions of different substances, which prevented or reactivated the oscillation, it was concluded that an operational substrate level phosphorylation or other energy sources besides the energy transformation in the respiratory chain is a strict prerequisite for oscillating ion fluxes. These alternative energy sources are probably required for the restoration of the permeability properties of the mitochondrial inner membrane rather than for driving the active movements of ions.

Materials and Methods

Preparation of mitochondria

Mitochondria were prepared from rat liver by a standard procedure [12], washed and finally resuspended in 250 mM sucrose adjusted to pH 7.4 with 1 mM Tris-HCl (40 mg mitochondrial protein per ml).

For determination of the respiratory control index mitochondrial respiration was measured using a Clark-type electrode in a closed chamber at 25°C. The medium comprised 110 mM sucrose/60 mM KCl/60 mM Tris-HCl/10 mM potassium phosphate/5 mM $MgCl_2$/0.5 mM EDTA/1 μM rotenone/10 mM succinate (pH 7.4). Only mitochondria with a respiratory control index better than 4 were used for oscillation experiments. Mitochondrial protein was determined by the biuret method [13].

Simultaneous registration of respiration and of ionic fluxes

The investigation of ionic fluxes and of oxygen consumption was carried out in an open temperature-controlled cell with constant stirring at 30°C. The total volume was 2.2 ml. The incubation medium for the registration of mitochondrial oscillation comprised 20 mM sucrose/1 mM KCl/12 mM Tris/5 mM succinic acid (pH 7.4). In some experiments succinic acid was replaced by 5 mM 2-oxoglutaric acid or 2.5 mM malic acid plus 2.5 mM glutamic acid. The activity of K^+ and Sr^{2+} in the incubation medium and the oxygen consumption were continuously registered. The determination was performed with ion-selective membrane electrodes (Radiometer, Copenhagen Type F2312 K and F2112 Ca and an oxygen electrode (Clark-Type). The sensitivity of the F2112 Ca electrode for Ca^{2+} was 3-times higher than for Sr^{2+}. As reference for K^+- and Ca^{2+}-electrodes an $Hg|Hg_2Cl_2$-electrode (Radiometer, Copenhagen, Type K 4112) with a second K^+-free salt bridge was used. The electrode potentials were amplified by vibrating capacitor electrometers (Vacutronic, Dresden, Type VA-J-51). The current of the Clark-Type oxygen electrode was amplified by an operational amplifier. After amplification, the signals from the electrodes were recorded with a four-channel pen recorder (Kutesz, Budapest, Type 175).

For estimation of changes in membrane permeability for cations caused by inhibitors of oscillation, the linear parts of the K^+-flux curves during the influx or efflux phases of the second cycle (Fig. 1) $\log[K^+]/\Delta t = V_{inh}$ were compared with the K^+-flux rate $\log[K^+]/\Delta t = V$ in the absence of inhibitors $(V_{inh}/V) \cdot 100$ (%).

Materials

ADP, ATP, 2-oxoglutaric acid and oxaloacetate were purchased from Boehringer-Mannheim (F.R.G.). Valinomycin, oligomycin and α-aminoxyacetic acid were obtained from Calbiochem, Lucerne (Switzerland). Rotenone and acetoacetate were products from Sigma (USA), succinic acid from Merck, Darmstadt (F.R.G.), L-malic acid from Ferak (West-Berlin), glutamic acid from Berlin-Chemie (G.D.R.) and arsenite from Riedel-De-Haen, Hannover (F.R.G.), respectively. Barium fluorocitrate was a product of K & K Laboratories, Plainview NY (U.S.A.). The barium fluorocitrate (10 mM) was changed into potassium fluorocitrate using 10 mM K_2SO_4 in a solution of 0.1 M HCl. After centrifugation the supernate was neutralized with KOH. Sucrose was purified by ion-exchange chromatography on columns of Dowex 50 (Serva, F.R.G.). All other chemicals were of analytical grade.

3. Results

The necessity for substrate level phosphorylation

A typical example of Sr^{2+}-induced oscillating ion fluxes in the presence of valinomycin is presented in

Fig. 1. As demonstrated by the full lines, there is, besides a small phase shift, a synchronized efflux of K^+ and Sr^{2+} (together with endogenous Ca^{2+}) followed by the reuptake during an influx phase. The changes in the direction of ion fluxes occur concomitantly with activation and deactivation of the oxygen consumption. It has been shown elsewhere that the redox state of the pyridine nucleotides [11], the membrane potential [15] and the direction of H^+-fluxes through the membrane [10,14] also oscillate.

The dotted lines in Fig. 1 demonstrate what happens in the presence of rotenone. This inhibitor prevented the reuptake of K^+ after the initial efflux as well as the complete accumulation of added Sr^{2+}, and the oxygen consumption reached a very high stationary state. Rotenone also affects succinate oxidation under non-oscillating conditions. As in the case of amytal, it prevents the intramitochondrial formation of oxaloacetate from succinate [16,17]. Since oxaloacetate is a potent inhibitor of succinate dehydrogenase [18–20], the possible participation of cyclic activation and deactivation by such mechanism during oscillation should be considered. For this reason, the effect of added oxaloacetate on the rotenone-inhibited system was tested. Fig. 2A demonstrates for the example of K^+ movement, that oxaloacetate restores the influx and the subsequent oscillation.

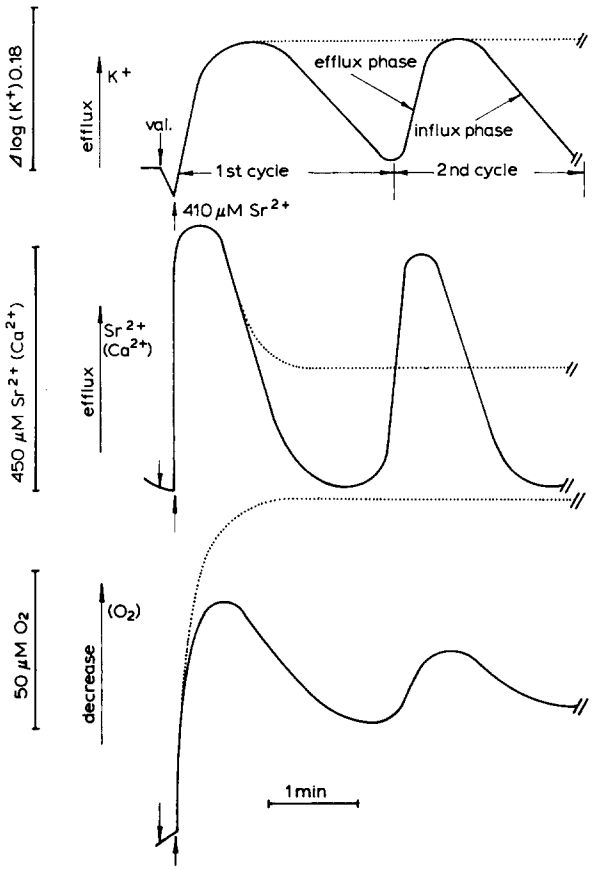


Fig. 1. Cyclic ion fluxes during succinate oxidation, and the effect of rotenone. Simultaneous recording of cyclic K^+- and Sr^{2+}-fluxes and the respiration as well as the effect of rotenone on ion fluxes and respiration. Basic medium: 20 mM sucrose/12 mM Tris/1 mM KCl/5 mM succinic acid (pH 7.4). Additions: 10.8 mg mitochondrial protein, 6 ng valinomycin (val.)/mg protein (full lines). Dotted lines: identical additions, but with an additional 425 ng rotenone/mg protein. Total volume 2.2 ml, t = 30°C. The measurements were carried out in an open system. The bottom trace y-axis is 'oxygen concentration': decreasing to the top indicates increased respiration in a qualitative manner.

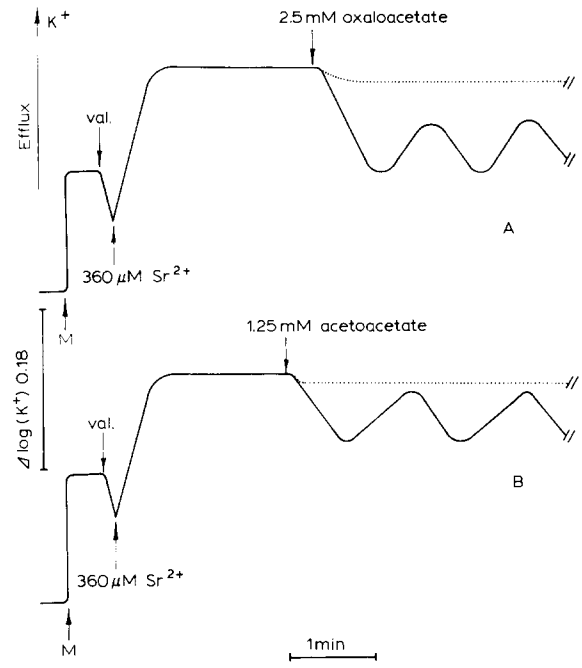


Fig. 2. Reactivation of rotenone-inhibited ion fluxes by oxaloacetate (A) or by acetoacetate (B) and the effect of arsenite. Additions: 9.7 mg mitochondrial protein and: 6.5 ng valinomycin/mg protein, 406 ng rotenone/mg protein (full lines). Dotted lines: identical additions, but with an additional 0.9 mM arsenite. Basic medium and conditions as in Fig. 1. M, mitochondria.

Cyclic changes in oxaloacetate concentrations as the reason for oscillation in samples without rotenone are not very likely because a relatively high added concentration restored the ability for cyclic ion fluxes. Furthermore, the circumvention of the rotenone effect is not specific for oxaloacetate, as is shown in Fig. 2B by the effect of acetoacetate. Therefore, it is more probable that the action of rotenone is mediated more directly by the influence of the redox state of the pyridine nucleotides, because both oxaloacetate and acetoacetate are able to oxidize NADH. Both oxaloacetate and acetoacetate could act as hydrogen acceptors and enable oxidation of endogenous intermediates of citrate cycle under these conditions.

Furthermore, the flux through the citrate cycle can be prevented by arsenite, which inhibits the lipoamide dehydrogenase in the 2-oxoglutarate dehydrogenase complex [21]. The dotted lines in Fig. 2 demonstrate that arsenite indeed inhibited the reactivating effect of both oxaloacetate and acetoacetate. The effect of arsenite in preventing oscillation could be realized by two different processes. Oscillation will be prevented either by inhibition of the flux of metabolites per se or by the prevention of the substrate level phosphorylation which takes place during oxidation of 2-oxoglutarate. By substitution of arsenate for arsenite it should be possible to discriminate between the two possibilities, since arsenate uncouples only the substrate-level phosphorylation without inhibition of the 2-oxoglutarate oxidation. Therefore, in the presence of arsenate the flux of citrate cycle intermediates is not interrupted. Fig. 3 shows the titration of the oscillating system with arsenate. These experiments were performed in the presence of oligomycin in order to prevent additional effects produced by the F_1-ATPase. They resulted in a linear correlation between the concentration of added arsenate and the inhibition of the K^+ uptake rate in the influx phase.

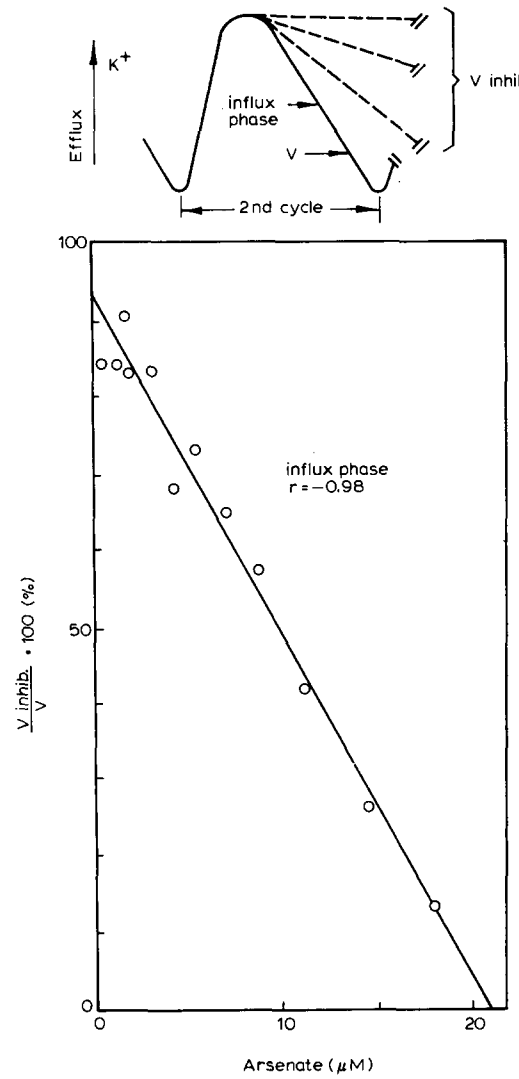


Fig. 3. Inhibition of K^+ influx rate with arsenate in the presence of oligomycin. Additions: 10.1 mg mitochondrial protein, 6 ng valinomycin/mg protein, 2 μg oligomycin/mg protein, concentrations of arsenate increasing from 0.45 μM to 18 μM. Starting with 360 μM Sr^{2+}. Basic medium and conditions as in Fig. 1. Each point represents one experiment.

The substitution of substrate level phosphorylation by exogenous ATP

From the foregoing results it follows that oscillation requires the operation of substrate-level phosphorylation in addition to oxidation of succinate by the respiratory chain. This suggests that substrate-level phosphorylation is necessary as an additional energy source. It should then be possible to exchange this additional source for another, e.g., by exogenous added ATP. In the experiment presented in Fig. 4A, which represents one typical experiment from five, the oscillation was inhibited by arsenite but could be restored by addition of ATP. Therefore the substrate level phosphorylation must be

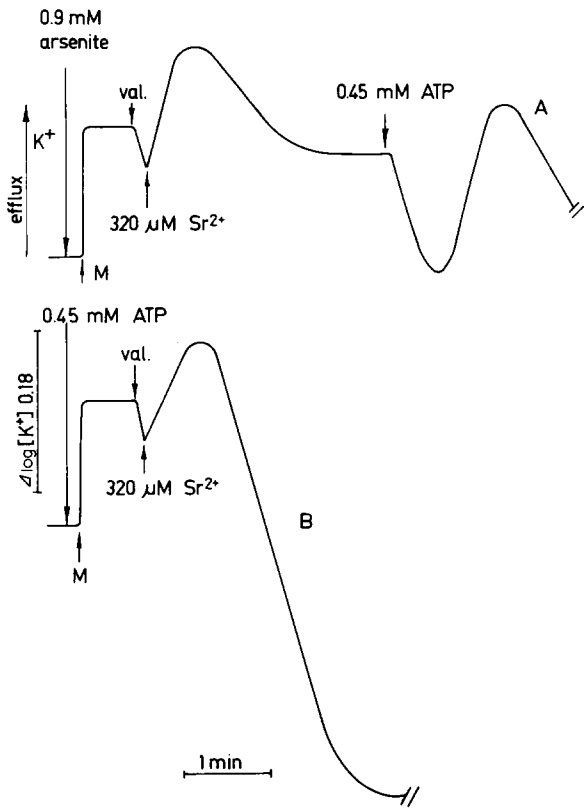


Fig. 4. Reactivation of arsenite prevented oscillations by ATP in the presence of oligomycin (A) and the effect of exogenous ATP on oscillations without arsenite (B). Additions: 8.0 mg mitochondrial protein and: 7 ng valinomycin/mg protein, 2 µg oligomycin/mg protein B. Basic medium and conditions as in Fig. 1. M, mitochondria.

operational for the delivery of a high-energy compound. Because these experiments were performed in the presence of oligomycin, the ATP could not be utilized for restoration of the proton electrical gradient by the proton pumping F_1-ATPase. Therefore it must be concluded that substrate-level phosphorylation is necessary for other energy-dependent processes which are obligatory for oscillation. It may be expected that these other processes are connected with the permeability properties of the membrane. In the absence of additional energy sources the mitochondria remained in the state which followed the efflux phase (Figs. 1–3). On the other hand, if the substrate-level phosphorylation was operating, addition of exogenous ATP produced a distinct influx of the cations (Fig. 4B). Following this influx, the mitochondria remained in the state which followed the influx phase. In other words, no further oscillation occurred.

The missing phase-dependence effect of substrate level phosphorylation

As shown above, operational substrate-level phosphorylation is necessary for the reuptake of K^+ (and alkali earth ions). The question arose as to whether the substrate-level phosphorylation also influences the efflux phase. This should be clarified by comparison of the effect of the influx and the efflux phases after partial inhibition of the 2-oxoglutarate dehydrogenase complex by arsenite. In Fig. 5 the results of corresponding titration experiments are summarized. Arsenite not only inhibited the rate of K^+ influx but also influenced the rate of K^+ release. It must be concluded, therefore, that the energy-dependent pro-

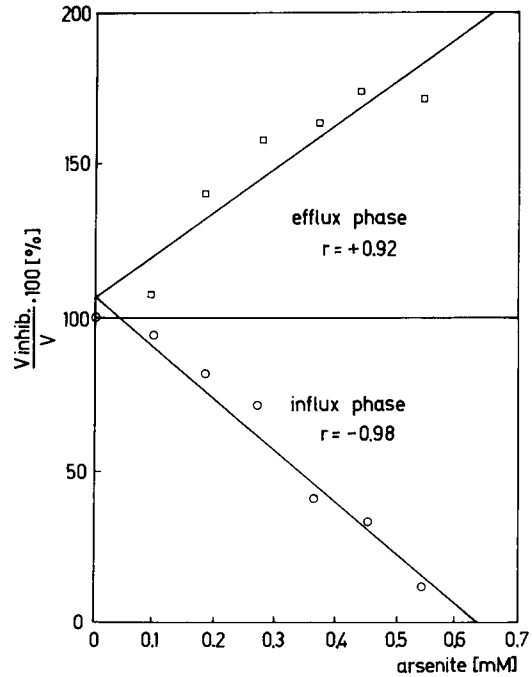


Fig. 5. Comparison of inhibition of K^+ influx rate and stimulation of K^+ efflux rate by arsenite. Additions: 9.3 mg mitochondrial protein, 7 ng valinomycin/mg protein, concentrations of arsenite increasing from 0.09 mM to 0.54 mM. Basic medium and conditions as in Fig. 1. Each corresponding pair of symbols represents one experiment.

cesses supported by substrate level phosphorylation operated during both phases. Functions of arsenite other than inhibition of substrate level phosphorylation [22] seem not to be responsible for the observed effects, because after inhibition with 0.9 mM arsenite added ATP reactivated normal oscillations (Fig. 4A).

Sources of the endogenous 2-oxoglutarate for substrate level phosphorylation

Arsenite and arsenate were found to prevent oscillation if succinate were the substrate. The action of both inhibitors is comprehensible only if mitochondria contain, or are able to form, some endogenous amount of 2-oxoglutarate. Oscillation could be observed for a time of 40–60 min [10]; therefore continuous regeneration of the endogenous 2-oxyglutarate pool must be expected. For the latter, two metabolic pathways can be taken into consideration: (i) from citrate formed from oxaloacetate as a product of succinate oxidation, plus endogenous acetyl-CoA generated from free fatty acids; or (ii) by utilization of endogenous glutamate via transamination.

Free fatty acids and glutamate may be produced by lytic degradation of mitochondrial phospholipids and proteins, respectively. In Fig. 6 two comparable

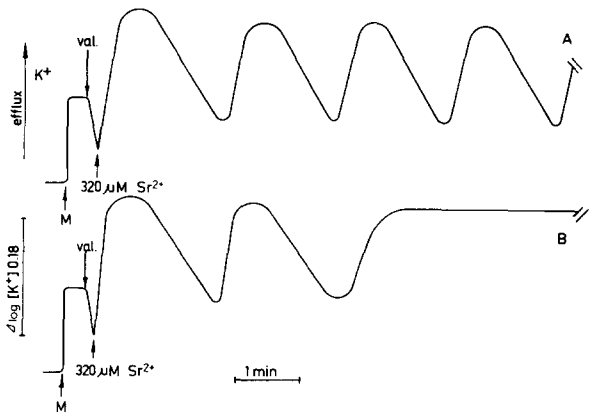


Fig. 6. The effect of fluorocitrate and aminoxyacetate on oscillations with succinate as substrate. Additions: 9.1 mg mitochondrial protein, 6 ng valinomycin/mg protein (A). (B) Same conditions as in (A) but mitochondria were preincubated for 1 min in fluorocitrate and aminoxyacetate (final concentrations 50 µM fluorocitrate, 2.5 mM aminoxyacetate). Basic medium and conditions as in Fig. 1. M, mitochondria.

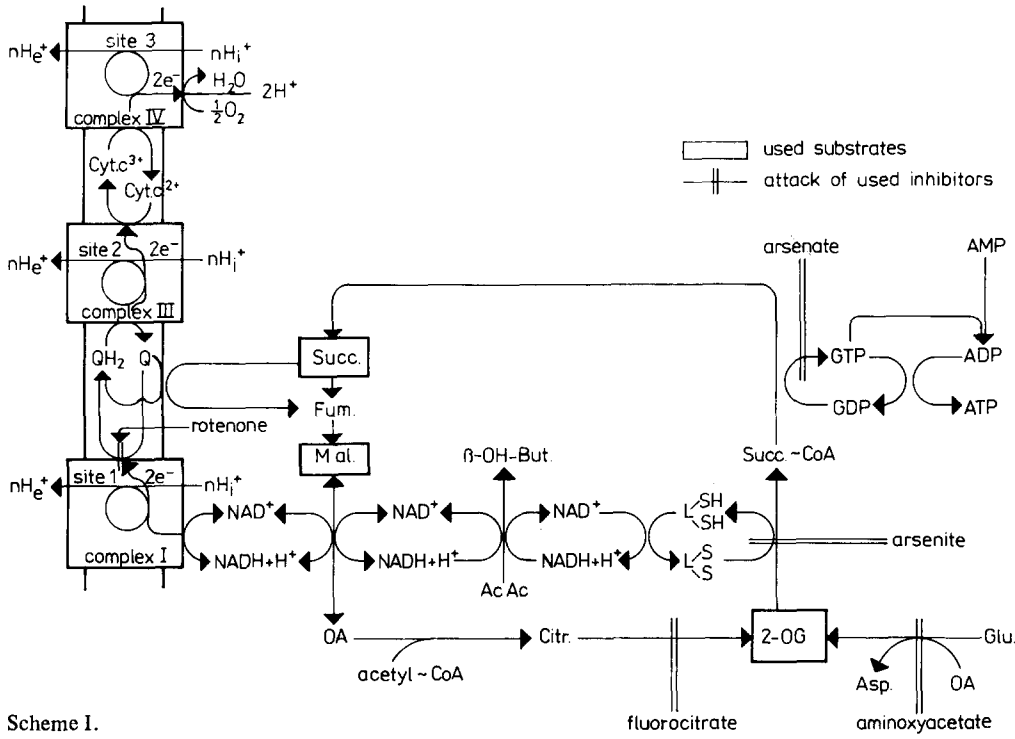
experiments are shown. In one of them (trace B) the regeneration of 2-oxoglutarate was inhibited. This was achieved by the combined addition of fluorocitrate and aminoxyacetate. The former inhibits the regeneration of 2-oxoglutarate from acetyl-CoA and oxaloacetate [23,24] and the latter from glutamate [25,26]. In the presence of both inhibitors, the course of oscillation was remarkably shortened. In further experiments, not demonstrated here, it was found that fluorocitrate alone, as well as aminoxyacetate alone, had an effect on the duration of oscillation.

Discussion

The framework of the reactions considered and their connections as well the targets of the inhibitors used are summarized in Scheme I. It should be pointed out that the oscillating system requires substrate oxidation to establish not only an electrochemical proton gradient, but also the formation of GTP by substrate-level phosphorylation. This explains the prevention of oscillation if the substrate level phosphorylation is uncoupled by arsenate or abolished by inhibition of 2-oxoglutarate oxidation with arsenite. Mitochondria contain a small pool of endogenous 2-oxoglutarate which is sufficient to support oscillation for some time. Furthermore, it was demonstrated that this pool can be regenerated by transamination of endogenous glutamate and via citrate available from endogenous sources of acetyl-CoA. This regeneration allows oscillation for a relatively long period if the intermediate reactions are not blocked by fluorocitrate and aminoxyacetate.

The necessity of substrate level phosphorylation also explains the inhibition of oscillation by rotenone if succinate is the substrate oxidized exclusively for oscillation. In this case, the substrate-level phosphorylation is prevented because of the high degree of reduction of the NAD-system [27]. Since oxaloacetate or acetoacetate delivers NAD^+ by oxidation of NADH, these substances can circumvent the inhibition.

The dependence of oscillation on GTP formation leads to two further questions. It was shown that addition of exogenous ATP may replace GTP formation. Therefore the question arises as to why the F_1-ATPase is unable to deliver a sufficient supply of en-



Scheme I.

dogenous ATP. It was found that under Sr^{2+}-induced oscillating ion fluxes the intramitochondrial ATP/ADP-ratio is very low (about 0.4) and that more than 65% of total mitochondrial adenine nucleotides are detectable in the form of AMP (Gellerich, F.N. et al., unpublished results). The other question concerns the process which is driven by GTP (or exogenous ATP). This process cannot be related to the proton-pumping F_1-ATPase, as is shown by its independence of added oligomycin. A rationale of both problems follows from investigations of phospholipid compositions during the influx and efflux phases.

It was found that the oscillations are concomitant by cyclic deacylation of phospholipids to lysocompounds and by reacylations which take place during both phases (Wiswedel, I. et al., unpublished results). Lysophospholipids and fatty acids enhance the conductivity of membranes [28,29] and their formation is considered to be responsible for the efflux phase (Refs. 10 and 11 and Wiswedel, I. et al., unpublished results). The reacylation requires the activation of fatty acids by ATP. Because ATP is transformed in this process into AMP it may explain the accumulation of this compound (Gellerich, F.N. et al., unpublished results) during oscillation. In the matrix, adenylate kinase is not present and intramitochondrially formed AMP can be rephosphorylated only by the GTP-AMP phosphotransferase [30].

In addition, it must be considered that during the efflux phase mitochondria are in a partially uncoupled state, because the conductivity of the membrane is high and the membrane potential is greatly decreased [15]. Therefore it can be expected that the electrochemical proton gradient may be too low for the formation of a sufficient intramitochondrial ATP level by the proton pumping F_1-ATPase. From this, it follows that ATP must be generated from ADP and GTP by the action of the nucleoside diphosphate kinase [30].

In conclusion, it is seen that the task of substrate-level phosphorylation is to guarantee the normal function of the mitochondrial inner membrane by a repair mechanism, at least under special conditions.

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