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# INHIBITION BY SURAMIN OF MITOCHONDRIAL ATP SYNTHESIS

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**Abstract**—Suramin, a drug intensively used in the chemotherapy of African trypanosomiasis and onchocerciasis, is currently being tested in clinical trials for AIDS treatment. Its effects on mitochondrial energy metabolism in mammals were studied. At low concentrations it inhibited ATP synthesis and ATPase activity in submitochondrial particles, as well as ADP-stimulated oxygen consumption and the uncoupler-stimulated ATPase activity in intact rat liver mitochondria. At higher concentrations it also inhibited uncoupled electron transport in both submitochondrial particles and intact mitochondria. From comparison of the kinetic patterns of those inhibitions, evidence suggesting that the adenine nucleotide translocase may be another target for the action of suramin was obtained. The relevance of these findings to the understanding of the biochemical basis of suramin toxicity is discussed.

Suramin [trisodium salt of 8,8′-(3″,3‴-ureylene-bis[3‴- benzamido- 4‴- methybenzamido])bis-1,3,5-naphtalenetrisulfonic acid], a drug used in the treatment of African trypanosomiasis [1] and onchocerciasis [2], has been described recently as a competitive inhibitor of the reverse transcriptase of a number of animal retroviruses [3]. Related to this observation, Mitsuya *et al.* [4] demonstrated that suramin blocks *in vitro* the infectivity and cytopathic effect of HTLV-III (a new retrovirus belonging to the HTLV family associated to AIDS) at doses that are clinically attainable in human beings [5, 6]. Several clinical trials [7–9] have been carried out to evaluate the potentiality of the drug for AIDS treatment. It is generally believed that suramin behaves as a virustatic and not as a virucidal drug [8, 10], hence suggesting that long-term treatments would be required in order to obtain favourable clinical responses.

Several toxic effects of suramin have been described [5, 6, 11, 12]. A number of enzymes and metabolic processes in mammals are affected by suramin, viz. ($Na^+$)-stimulated ATPase from guinea pig brain [13], ($Na^+$,$K^+$)-activated ATPase from erythrocytes [14], calcium transport in sarcoplasmic reticulum from rabbit muscle [15], succinate dehydrogenase from pig heart muscle [16], and DNA polymerases from mouse and calf thymus [17]. However, the biochemical basis of suramin toxicity is not yet well understood. Moreover, the known toxic effects have been described in connection with short-term treatments, and little or no information is available for long-term treatments such as those possibly needed for AIDS therapy [8].

Regarding its trypanocidal action, it has been reported by several researchers that suramin inhibits lactate dehydrogenase [18, 19], malate dehydrogenase [19], protein kinase I [20] and glycerol 3-phosphate oxidase [21] from protozoa. On those grounds it has been suggested that its main target in *Trypanosoma brucei* would be the energy metabolism of the parasite [22]. Moreover, it also has been demonstrated that suramin inhibits oxidative phosphorylation in mitochondrial preparations of *Crithidia fasciculata* [23], a trypanosomatid that, unlike *T. brucei*, has a well developed mitochondrial respiratory chain. This effect was related to the previously described [24] inhibition of a soluble $F_1$-like ATPase isolated from mitochondrial membranes from the same origin. However, the possibility of an effect of the drug on the adenine nucleotide translocase, similar to that described by Opperdoes *et al.* [25] in *C. luciliae*, was not discarded.

We describe in this paper the effects of suramin on the mitochondrial energy metabolism in mammals. We present kinetic evidence suggesting that the primary site of action of suramin in the energy metabolism of intact mitochondria is the adenine nucleotide translocase. We also discuss the possible relevance of those effects on the toxicity of the drug, in order to contribute to a better evaluation of its potentiality as a therapeutic agent.

## MATERIALS AND METHODS

Rat liver [26] and heavy bovine heart [27] mitochondria were prepared as described. Phosphorylating submitochondrial $Mg^{2+}$-ATP particles (SMP†) were prepared from heavy bovine heart mitochondria as described [28]. Beef-heart $F_1$ was prepared according to Knowles and Penefsky [29], stored at liquid nitrogen temperature, and processed before used as described [30].

*Electron transport.* Electron transport in SMP (0.3 mg protein) was determined by following oxygen

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† Abbreviations: DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $P_i$, inorganic ortophosphate; RLM, rat liver mitochondria; SMP, submitochondrial particles; and TCA, trichloroacetic acid.

consumption with a Clark electrode connected to a Gilson oxygraph in a reaction medium (1.65 ml) containing 250 mM sucrose and 50 mM Tris–HCl (pH 7.5), using either 10 mM succinate and 3 $\mu$M rotenone or an NADH-regenerating system composed of 50 mM ethanol, 0.2 mM NADH and 125 I.U./ml of yeast alcohol dehydrogenase (EC 1.1.1.21). In rat liver mitochondria (RLM), electron transport was measured as above in a medium containing 250 mM sucrose, 30 mM KCl, 1 mM EDTA, 25 mM Tris–HCl (pH 7.4), 1.5 mM $MgCl_2$, 10 mM $KH_2PO_4$ and succinate or malate plus glutamate as indicated. Rate values reported correspond to the slopes of linear traces drawn by the oxygraph.

*ATP synthesis.* The reaction was measured in a medium containing (final concn) 180 mM sucrose, 1 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 50 mM glucose, 15 I.U. of yeast hexokinase (EC 2.7.1.1), 125 I.U. of yeast alcohol dehydrogenase (EC 1.1.1.21), 50 mM ethanol and ADP as indicated. SMP (0.4 mg protein) were added to this medium and incubated for 5 min. ATP synthesis was started by adding inorganic phosphate (as indicated), $2 \times 10^6$ cpm carrier free $^{32}P_i$ and 3 $\mu$mol $MgCl_2$. The final volume was 1 ml. The preincubation and the reaction were carried out under aerobiosis obtained with a gyratory waterbath shaker. After 5 min the reaction was stopped and inorganic phosphate was quantitatively precipitated by a modification [23] of the procedure of Sugino and Miyoshi [31]. After separating the ppt by centrifugation (10 min, 3000 rpm), aliquots were withdrawn and analyzed for [$^{32}P_i$]glucose-6-phosphate by Cerenkov counting in a Beckman 8100 liquid scintillation counter.

*ATPase activity of $F_1$ and SMP.* ATPase activity was determined spectrophotometrically essentially as described by Pullman *et al.* [32]. The ATPase activity of $F_1$ (3 $\mu$g protein) was determined in a reaction medium (2 ml) containing 100 mM sucrose, 1 mM $MgCl_2$, 10 mM sodium bicarbonate, 4 mM phosphoenolpyruvate, 0.350 mM NADH, 60 I.U. of pyruvate kinase (EC 2.7.1.40), 50 I.U. lactate dehydrogenase (EC 1.1.1.27), 40 mM Tris–HCl (pH 8) and $Mg^{2+}$-ATP. The ATPase activity of SMP (0.1 mg protein) was determined in a similar manner except that sucrose was 180 mM and 3 $\mu$M rotenone was also added in order to inhibit the NADH dehydrogenase. The oxidation of NADH was followed at 360 nm ($\varepsilon^{360}_{mM} = 3.92\ cm^{-1} \cdot mM^{-1}$) in order to avoid the interference due to suramin absorption at 340 nm.

*$P_i$-ATP exchange reaction.* SMP (1 mg protein) were added to 1 ml of a reaction medium containing 180 mM sucrose, 10 mM ATP, 10 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 10 mM $P_i$ and $2 \times 10^6$ cpm of carrier free $^{32}P_i$. After 5 min, the reaction was stopped by adding modified [23] Sugino and Miyoshi's reagent [31] and, after centrifuging at 3000 rpm for 10 min, the supernatant fraction was analyzed for [$\gamma$-$^{32}$P]ATP by Cerenkov counting.

*DNP-stimulated ATPase activity in RLM.* RLM (1 mg protein) were added to a medium containing 100 mM KCl, 50 mM Tris–HCl (pH 7.5) and 100 $\mu$M 2,4-dinitrophenol (DNP). After a 5-min preincubation the reaction was started by adding ATP (as indicated) and stopped 3 min later by the addition of 0.05 ml of 100% trichloroacetic acid (TCA). After centrifugation (10 min, 3000 rpm), aliquots were withdrawn from the supernatant fraction and analyzed for inorganic phosphate according to Sumner [33].

*Phosphorylation of endogenous ADP.* The phosphorylation of endogenous ADP was carried out in intact RLM essentially as described by Kemp *et al.* [34] at 8°.

*General.* Protein determinations were carried out according to Lowry *et al.* [35] (for soluble proteins) or by a modified biuret procedure [36] (for SMP and RLM).

Proper controls were carried out in order to test that the coupled systems used (alcohol dehydrogenase, hexokinase and pyruvate kinase plus lactate dehydrogenase) were not rate-limiting factors in the assays, even at the highest suramin concentration.

Rates reported are average of determinations in duplicate which agreed within 10%. Kinetic data were analyzed by linear and non-linear regression analysis using a computer program provided by Rossi and Garrahan.*

Measurements were carried out at 30°.

*Chemicals.* The nucleotides, oligomycin, atractyloside, hexokinase (Type IV, from baker's yeast), pyruvate kinase (Type III, from rabbit muscle), lactate dehydrogenase (Type IX, from rabbit muscle) and alcohol dehydrogenase (from baker's yeast) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). FCCP was obtained from Dr. P. G. Heytler, E. I. Du Pont de Nemours (Wilmington, DE, U.S.A.) and efrapeptin (A 23871) from Dr. R. Hamill, Eli Lilly Co. (Indianapolis, IN, U.S.A.). Suramin (Sterling-Winthrop Research Institute, Rensselaer, NY, U.S.A) was obtained from Dr. G. C. Hill, Colorado State University (Fort Collins, CO, U.S.A.).

Efrapeptin, oligomycin and FCCP were dissolved in ethanol and suramin and atractyloside in water. In all experiments, controls with the same amount of solvent were run simultaneously.

The concentration of adenine nucleotides was determined spectrophotometrically at 260 nm.

## RESULTS

*Effect of suramin on energy-linked reactions in submitochondrial particles.* Suramin inhibited electron transport from succinate to oxygen in SMP (50% inhibition was obtained with 60 $\mu$M suramin). On the other hand, NADH oxidation was not affected at all by 100 $\mu$M suramin and was only inhibited by higher suramin concentrations (Fig. 1).

ATP synthesis coupled to NADH oxidation was strongly inhibited by suramin concentrations lower than 100 $\mu$M. The inhibition was competitive towards $P_i$ (Fig. 2A) with $K_i = 3.1$ $\mu$M and noncompetitive towards ADP ($K_i = 7.7$ $\mu$M, see Fig. 2B).

Suramin also inhibited the ATPase activity of SMP and of the isolated coupling factor $F_1$, behaving as a mixed inhibitor with $K_i = 5.9$ and $K'_i = 11$ (Fig. 3A) and as a noncompetitive inhibitor with $K_i = 9$ $\mu$M (Fig. 3B) respectively.

* R. C. Rossi and P. J. Garrahan, *Fourth Panamerican Biochemistry Congress*, Abstr. 470 (1984).

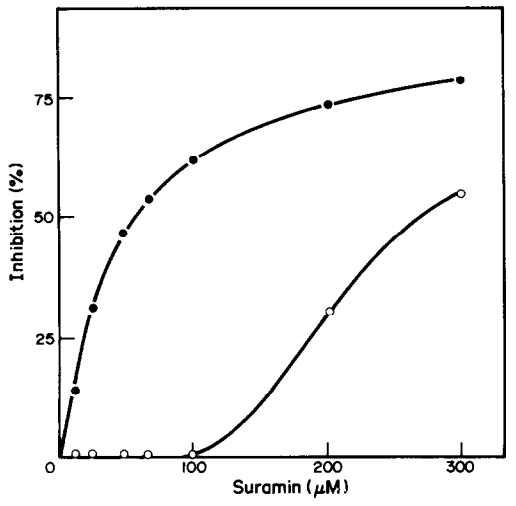



Fig. 1. Inhibition by suramin of electron transport in SMP. Electron transport was measured as indicated under Materials and Methods using either succinate (●) or an NADH-regnerating system (○) as substrate. Control activities were: 292 and 450 nat oxygen/min/mg protein for succinate and NADH respectively.

*Effects of suramin on energy-linked reactions in intact rat liver mitochondria:* Suramin also inhibited the oxidation of malate/glutamate by RLM. However, the inhibition strongly depended on the metabolic state of the mitochondria: the inhibition in state 3 was stronger than in the presence of uncoupler (Fig. 4). Moreover, the inhibition of the uncoupled state diminished when the concentration of malate and glutamate increased (data not shown), whereas the inhibition of the ADP-stimulated respiration was strictly competitive ($K_i = 1.7\ \mu M$) with respect to ADP (Fig. 5A). Similar results (data not shown) were obtained when malate and glutamate were replaced by succinate.

The uncoupler-stimulated ATPase activity in RLM was also inhibited by suramin. In this case the inhibition was competitive towards ATP ($K_i = 69\ \mu M$, see Fig. 6A).

When mixtures of suramin and atractyloside (a known inhibitor of the adenine nucleotide translocase [38] were tested on the ADP-stimulated respiration, parallel lines were obtained when 1/v was plotted against suramin concentration at several fixed atractyloside concentrations (Fig. 5B). Hence, suramin and atractyloside behaved as mutually exclusive inhibitors [39]. Similar behaviour was obtained when the same procedure was followed for the uncoupler-stimulated ATPase activity (Fig. 6B). On the other hand, when suramin was used in combination with either efrapeptin or oligomycin (inhibitors that act on the ATPase complex, at the level of the $F_1$ and $F_0$, respectively), the behaviour corresponded to mutually non-exclusive inhibitors (data not shown).

## DISCUSSION

Suramin inhibited succinate oxidation and ATP synthesis coupled to electron transport from NADH

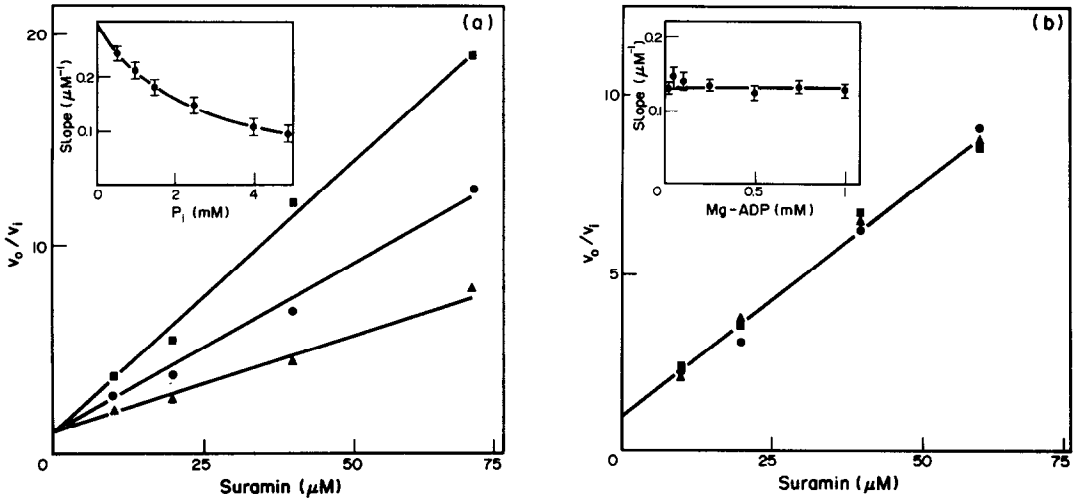



Fig. 2. Inhibition of ATP synthesis by suramin. ATP synthesis was measured as described under Materials and Methods. In the figure, $v_o/v_i$ values are plotted against suramin concentration, with $v_o$ and $v_i$ equal to the velocity measured in the absence and in the presence of inhibitor respectively. Slope values, estimated from the primary plots by linear regression analysis, are plotted in the inset as a function of ADP or $P_i$ concentration. The bars indicate the corresponding standard deviations. (a) Measurements were carried out at fixed ADP concentration (2 mM) and at variable $P_i$ concentrations: 0.5 (■), 2.5 (●) and 5 (▲) mM. The line in the inset is the best fit obtained by non-linear regression analysis [R. C. Rossi and P. J. Garrahan, *Fourth Panamerican Biochemistry Congress*, Abstr. 470 (1984)] using the following equation: slope = $K_s/(K_i \cdot (K_s + [S]))$, that corresponds to a linear competitive inhibitor [37] ($K_i = 3.1 \pm 0.1\ \mu M$ and $K_s = 2.3 \pm 0.3$ mM). (b) Measurements were carried out at fixed $P_i$ concentration (5 mM) and at variable ADP concentrations: 0.02 (●), 0.1 (■) and 0.25 (▲) mM. The plot shown in the inset identifies [37] a linear noncompetitive inhibitor with $K_i = 7.7 \pm 0.9\ \mu M$ (slope = $1/K_i$).

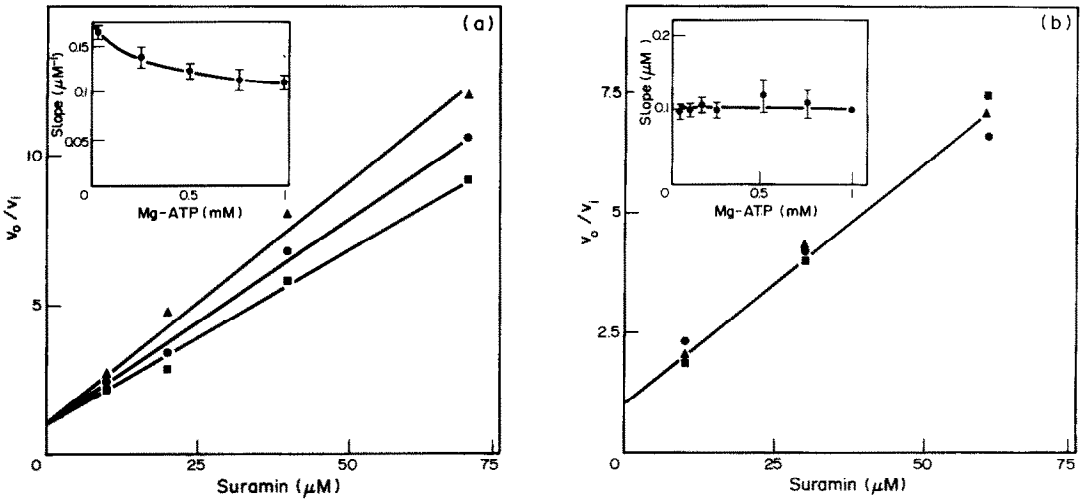



Fig. 3. Inhibition by suramin of the ATPase activity of SMP and of the soluble coupling factor $F_1$. Experimental conditions were as described under Material and Methods. (a) The ATPase activity of SMP was measured at 0.025 (▲), 0.25 (●) and 0.5 (■) mM ATP. The line in the inset was calculated as indicated in Fig. 2 using the equation (slope = $(K_s/K_i + [S]/K'_i)/(K_s + [S])$) that corresponds to a linear mixed-inhibitor [37]. The estimated values of $K_i$ and $K'_i$ were: 5.9 ± 0.2 and 11 ± 2 μM respectively. (b) The ATPase activity of $F_1$ was measured at 0.05 (▲), 0.175 (●) and 0.75 (■) mM ATP. The line in the inset corresponds to a linear noncompetitive inhibitor with $K_i = 9 \pm 1$ μM [37].

to oxygen in SMP (Figs. 1 and 2). The inhibition of succinate oxidation observed at concentrations lower than 100 μM can be attributed to interaction of suramin with a site in Complex II (succinate dehydrogenase) of the respiratory chain, as previously reported [16]. The occupation of this site by sumarin would not result (in agreement to the data shown in Fig. 1, open circles) in inhibition of NADH oxidation, since complex II is not in the path of electrons from NADH to oxygen. Therefore, the inhibition by suramin of ATP synthesis coupled to NADH oxidation cannot be explained by an effect on the electron transport chain, since the results were obtained at suramin concentrations (less than 100 μM) that did not affect NADH oxidation at all (see Figs. 1 and 2). Moreover, the ATPase activity of SMP (Fig. 3A) and the ATP-$P_i$ exchange reaction (data not shown) were also inhibited by suramin. Hence, the site of action of suramin on ATP synthesis in SMP can be tentatively located in the ATPase complex, more precisely at the level of the coupling factor $F_1$, since $F_1$-ATPase activity was also inhibited (Fig. 3B) by similar suramin concentrations.

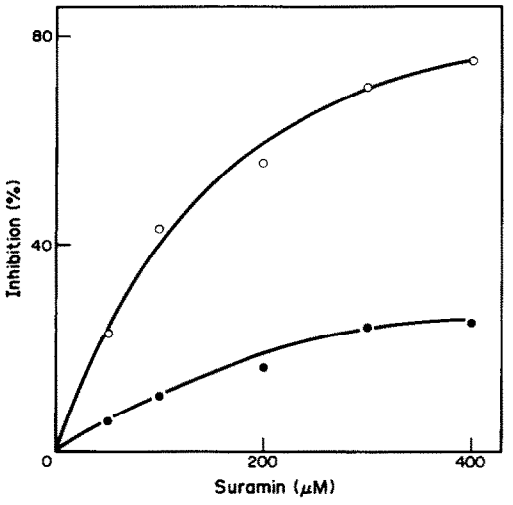



Fig. 4. Effect of suramin on electron transport in intact mitochondria. Experimental conditions were as described in Materials and Methods. Oxygen consumption was measured using 5 mM malate plus 5 mM glutamate as oxidizable substrate, in the presence of either 2 μM FCCP (●) or 1 mM ADP (○). In the latter, the percent inhibition reported corresponds to inhibition of the stimulus of oxygen consumption obtained by addition of ADP, calculated as the difference between the rates of oxygen consumption in state 3 and state 4 at the indicated suramin concentration. Activities of controls were: 6.5, 64 and 71 nat oxygen/min/mg protein for state 4, state 3 and uncoupled respiration respectively.

Two effects of suramin have been observed on intact RLM: (i) an inhibition of the uncoupled electron transport with either succinate or malate plus glutamate as substrate, and (ii) a more powerful inhibition of the ADP-stimulated respiration. Before discussing the possible location of the sites responsible for such effects, one must take into account that the ATPase complex and the substrate sites of the NADH and succinate dehydrogenases that were freely accessible to the media in SMP are in RLM facing the matrix of the mitochondrion. Therefore, suramin must cross the inner mitochondrial membrane in order to reach those enzyme systems or any intramitochondrial dehydrogenase. Suramin is an impermeant polyanion, unable to cross several membranes [40, 41]. Therefore, the inhibition of t uncoupled electron transport is more likely to exerted at the dicarboxylate carrier of the chondrial membrane rather than at the level o the matrix dehydrogenases or the mitochond

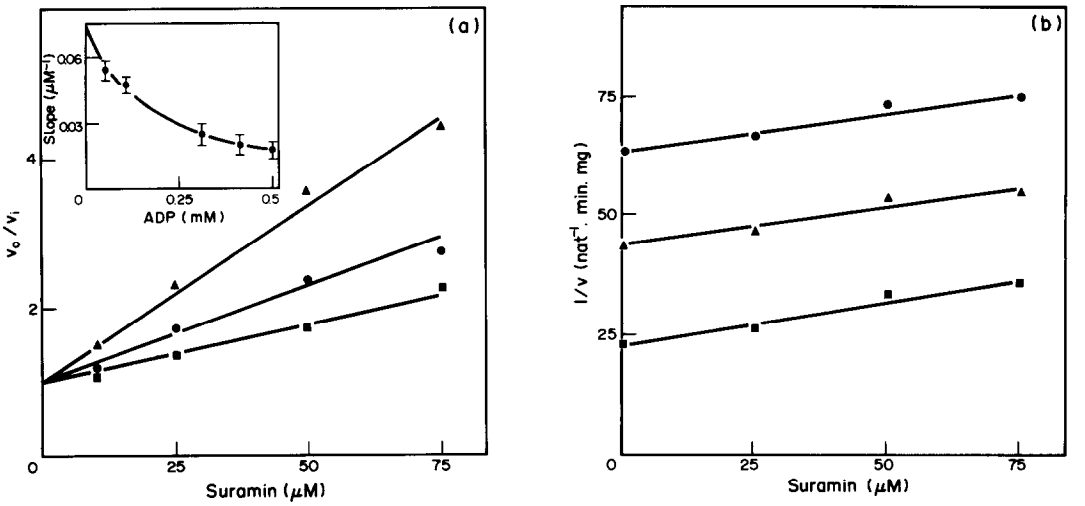
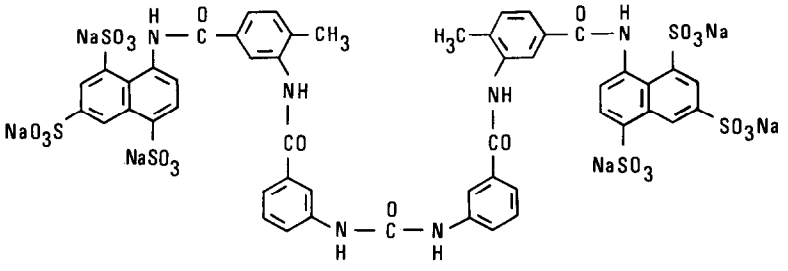
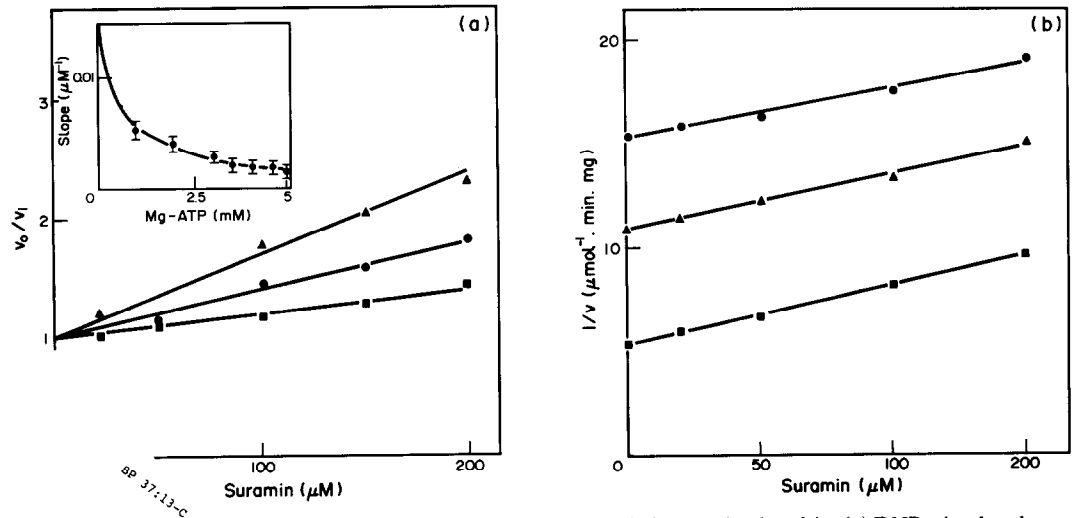



Fig. 5. Kinetic characterization of the inhibition of the ADP-stimulated respiration by suramin. Experimental conditions were as described in Materials and Methods. The oxidizable substrate was malate plus glutamate (10 mM each). (a) $v_0$ and $v_i$ were calculated as the increase in respiration obtained by adding 0.1 (▲), 0.3 (●) and 0.5 (■) mM ADP to RLM in state 4 in the presence of different concentrations of suramin. The line in the inset is the theoretical curve for a linear competitive inhibitor ($K_i = 13.4 \pm 1.7\ \mu M$ and $K_s = 0.139 \pm 0.04$ mM) obtained as indicated in the legend to Fig. 2A. (b) Oxygen consumption measurements were carried out with RLM in the presence of 0.2 mM ADP and 0 (■), 0.3 (▲) and 0.5 (●) μg/ml of atractyloside at different suramin concentrations.




SURAMIN






ɔn by suramin of the DNP-stimulated ATPase in intact mitochondria. (a) DNP-stimulated ɔf RLM was measured at 1 (▲), 2 (●) and 3 (■) mM ATP. Other conditions were as 2A for a competitive inhibitor ($K_i = 69 \pm 13\ \mu M$ and $K_s = 0.69 \pm 0.18$ mM). (b) carried out at 2 mM ATP in the presence of 0 (■), 1.5 (▲) and 3 (●) μg/ml of atractyloside at different suramin concentrations.

tron transport chain at the inner side of the mitochondrial membrane. For the same reason, it is difficult to explain the inhibition of the ADP-stimulated respiration in RLM by the above described action of suramin on the ATPase complex in SMP. Moreover, two kinetic studies support the hypothesis that suramin inhibited the ADP-stimulated respiration by acting on the transport of ADP into the mitochondria, at the level of the adenine nucleotide translocase: (i) the inhibition of the ADP-stimulated respiration in RLM exhibited a completely different kinetic pattern (competitive inhibition with respect to ADP, see Fig. 5A) than the inhibition of ATP synthesis in SMP (non-competitive inhibition, see Fig. 2B) and (ii) suramin behaved as a mutually exclusive inhibitor (Fig. 5B) with respect to atractyloside. This last result indicates that suramin and atractyloside cannot bind simultaneously, and strongly suggests that the more likely site of action of suramin in RLM is the adenine nucleotide translocase. Hence, the effect of suramin on the ADP-stimulated respiration would be secondary to an inhibition of the ADP transport into the mitochondrion. In agreement with such an hypothesis, the phosphorylation of endogenous ADP [34] was not affected by suramin (data not shown).

Another piece of evidence for that proposal comes from the analysis of the results obtained when the effect of suramin on the ATPase activity in SMP and in RLM was studied. Suramin inhibition of the ATPase activity in SMP can be explained by its action on the coupling factor $F_1$, since its ATPase activity was also blocked by the drug. The kinetic patterns of those inhibitions are slightly different, but such differences can be explained, taking into account that the conformation of the coupling factor $F_1$ depends on whether it is soluble or bound to $F_0$ in the inner mitochondrial membrane. More striking are the differences between the mixed-type inhibition observed for the ATPase activity in SMP (Fig. 3A) and the competitive inhibition observed for the uncoupler-stimulated ATPase in RLM (Fig. 6A). In addition, in this last case the inhibition was also mutually exclusive with respect to atractyloside, hence suggesting that the effect on the ATPase activity in RLM would be secondary to an inhibition of the adenine nucleotide translocase.

Therefore, the suggestion previously made [23] that the inhibition by suramin of oxidative phosphorylation in mitochondrial preparations of *C. fasciculata* could be explained by its action on the $F_1$-like ATPase must be revised. Moreover, the complex kinetic behaviour observed by these authors could easily be explained by the fact that their preparation was a mixture of right-oriented mitochondrial vesicles and inside-out submitochondrial particles, hence resulting in two populations carrying out ATP synthesis: (i) one inhibited by suramin acting on the adenine nucleotide translocase (the right-oriented vesicles), and (ii) another one inhibited by suramin at the level of the, in this case, freely accessible $F_1$-ATPase (the inside-out vesicles).

In summary, several enzymes and translocators related to the mitochondrial energy metabolism can be affected by suramin: (i) succinate dehydrogenase [16], (ii) ATPase complex, and (iii) adenine nucleotide translocase. The inhibition of any of these enzymes would result in an impairment of the cellular energy metabolism. When these results obtained *in vitro* are extrapolated to the *in vivo* situation in order to explain the toxic effects of suramin, we must consider the different permeability barriers that the drug must cross to reach its potential site of action. Since suramin is an impermeant anion [40, 41] that can enter into a cell by endocytosis associated with proteins [6, 21, 22], it is rather difficult to relate its toxicity to actions on the electron transport chain or on the ATPase complex. But provided it can be liberated into the cell sap from lysosomes, the inhibition of the adenine nuleotide translocase and, consequently, of the mitochondrial ATP synthesis must be considered as a possible target for its toxic action in the evaluation of its potentiality as therapeutical agent.

Wills and Wormall [42] have postulated two alternative general mechanisms for enzyme inhibition by suramin: (i) combination of two appropriately distant naphtylaminetrisulphonic acid groups with basic groups in the enzyme surface on opposite sides of the active site to form a bridge capable of blocking the entry of substrate, or (ii) a direct combination with the active centre itself, favoured by the spatial configuration and the presence of reactive group(s) in the molecule of suramin. The first mechanism would explain the effect of the drug on enzymes inhibited only at pH values lower than their isoelectric points (e.g. urease [42]), and the second one the effect on enzymes inhibited even at the alkaline side of their pI. Since the experiments reported herein have been carried out at pH over 7.5, the latter mechanism can be tentatively postulated for the different actions of suramin on the mitochondrial energy metabolism machinery. A description at a molecular level of suramin interaction with succinate dehydrogenase, ATPase and mitochondrial translocases remains to be established. However, we believe that attention must be paid to the presence of naphtylaminetrisulphonic acid groups and peptide-like linkages in the suramin molecule.

Finally, the possibility of interaction with cationic groups on the cell surface belonging either to anion transport systems or to cell receptors would be worthwhile exploring when trying to understand the biochemical basis of suramin toxicity.

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