Energy-Dependent Uptake of Arsenite by Rat Liver Mitochondria

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Uptake of arsenite by rat liver mitochondria is energy-dependent, as shown by comparing values without and with either uncoupling agent or respiratory inhibitor present. The uptake is inhibited by mersalyl and *N*-ethylmaleimide, which can be used as 'stopping' agents to obtain uptake kinetics. At 20°C the process is nearly complete in 1 min. The relation between the quantity in the energized mitochondria and the applied concentration corresponds to at least two different modes of binding of the arsenite. Competition occurs between arsenite and other anions (for example, phosphate) for intramitochondrial accumulation.

Conventionally, arsenite at 0.5–1 mM is used to inhibit the mitochondrial oxidation of pyruvate and 2-oxoglutarate. This action is attributed to a combination of arsenite with the reduced form of lipoic acid so that the lipoic acid can no longer pass reducing equivalents to the nicotinamide nucleotides. The inhibition is reversed by excess of dithiol compounds such as dithioglycerol and dithioerythritol (one of Cleland's reagents). When present in equivalent concentration to the arsenite, dithioglycerol has an uncoupling action and causes swelling (Fluharty & Sanadi, 1961, 1962; Hassinen & Hallman, 1967). There is little information about the mitochondrial uptake of arsenite, although in some conditions preincubation with it was required to stop respiration in the presence of 2-oxoglutarate as added substrate (Csillag, 1971).

The present investigation studied the time course and extent of arsenite uptake, and the effects of the energized state and certain thiol reagents on arsenite translocation.

Methods

Rat liver mitochondria were prepared as described before (Harris *et al.*, 1971). Incubations of about 4 mg of protein/ml were made at 20–21°C in a medium containing 125 mM-KCl, 20 mM-Tris/Hepes, pH 7.2, 3% Dextran C and the substrate(s) noted in the text and Tables. Arsenite was added as required from a stock solution containing a trace amount of ⁷³As- and ⁷⁴As-labelled material added to 100 mM-sodium arsenite. The radio-labelled arsenic is supplied as sodium arsenate, but the As rapidly exchanges with that of added arsenite

(material and information from The Radiochemical Centre, Amersham, Bucks., U.K.) and the stock solution was stored over a few milligrams of previously arsenite-equilibrated Dowex 1 anion-exchange resin. This would preferentially remove the more highly charged arsenate ions from the solution.

Fig. 1. Inhibition of stimulated respiration by arsenite when oxoglutarate is the added substrate

The change takes place within 1 min; the slow continued respiration is presumably due to endogenous substrate. The medium contained 120 mM-KCl, 20 mM-Tris/Hepes, pH 7.2, and 3 mM-oxoglutarate. The following additions were made (final concentrations): (a) 2 μM-dinitrophenol (DNP), 40 μM-arsenite (As), 0.4 mM-dithioerythritol (DTE); (b) 3 mM-phosphate initially, then 200 μM-ADP (ADP), 120 μM-arsenite (As). The temperature was 20°C.

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§ Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Fig. 2. Mitochondrial content of arsenite as function of the applied concentration

(a) The two top curves (● and ○) were obtained from two different preparations. The lower curves are contents of the pellets obtained in the presence of either 133 μM-mersalyl (■) or 266 μM-*N*-ethylmaleimide (□). These values are in the range expected for the sucrose-accessible volume of the pellet (2.5–3.0 μl/mg of protein). (b) Scatchard plot of the net (● minus ■) contents, showing that more than one class of site is involved. The conditions are as in Table 1.

The distributions of arsenite between the mitochondria and the medium were measured by centrifuging portions of the stirred suspension in a bench centrifuge in small plastic cups. The medium was then removed from above the pellet and two washes without resuspension were given; the inside of the tube was wiped and the pellet was dissolved

Fig. 3. Time courses of arsenite uptakes

The curves are for 21 (●) and 1°C (○); uptakes were terminated with *N*-ethylmaleimide added to 0.4 mM. In special experiments the incubation was either with antimycin (■) at 10 μg/ml for 1 min, or with tetrachlorotrifluoromethylbenzimidazole (□) at 3 μM for 1 min, or after a normal 1 min incubation the antimycin or uncoupler was added, and incubation was continued for 1 min more (both agents gave the same result, ▲). Conditions are as given in Table 1.

in 30% formic acid before transfer to a vial for scintillation counting. When it was found that *N*-ethylmaleimide and mersalyl were applicable as stopping agents for the uptake process, some kinetic measurements were made by successively adding the arsenite and, after a short delay, the *N*-ethylmaleimide to small portions of a stirred suspension. The 'stopped' mixture was centrifuged and the pellet washed and treated as before.

The effect on respiration with 2-oxoglutarate in the KCl/Tris/Hepes medium as substrate was followed with a Clark-type polarograph. It was confirmed that phosphorylation took place normally when succinate was used as substrate even in the presence of 0.5 mM-arsenite.

Results and Discussion

Respiratory inhibition

Fig. 1 shows that respiration stimulated either with dinitrophenol or with ADP+P_i is inhibited within 1 min by addition of arsenite. The longer delay noted by Csillag (1971) may be attributed to reserves of endogenous arsenite-insensitive substrate. Fig. 1 also shows the relief of inhibition given by excess of dithioerythritol.

Arsenite uptakes

Arsenite contents of mitochondria measured over a range of applied concentrations are shown in

Fig. 2 for two different preparations. The results of similar measurements made with suspensions to which either *N*-ethylmaleimide or mersalyl had previously been added are also indicated. With either agent present, the content of the pellet approximated to that in the sucrose space measured in other experiments. When the net amounts obtained by subtraction of the *N*-ethylmaleimide-inhibited (Fig. 2*a*, □) from the normal uptakes (Fig. 2*a*, ● or ○) were plotted by the Scatchard *et al.* (1957) procedure, the existence of at least two modes of binding can be inferred (Fig. 2*b*). One of these might be an attachment to thiol groups of the membrane and the other an accumulation of free or bound arsenite in the matrix space.

In view of the inhibition of uptake seen with *N*-ethylmaleimide and mersalyl, either agent appeared potentially useful as a 'stopping' agent to interrupt uptake and permit centrifugal separation. Adding *N*-ethylmaleimide to a concentration of 0.4 mM 10 s after arsenite, followed by quick separation, gave a content of arsenite of 3.64 nmol/mg, and if 2 min delay was interposed before separation the content was 3.55 nmol/mg, so evidently there was no rapid leak or displacement of arsenite by the *N*-ethylmaleimide. In Fig. 3 the time courses of arsenite uptake with *N*-ethylmaleimide as stopping agent are shown for temperatures of 21 and 1°C. The slow part of the rise of content at 1°C could be on account of an energy-dependence, and this factor was brought out in some special incubations whose results are also displayed in Fig. 3. Single points show the contents after 1 min at 21°C in the presence of antimycin to inhibit respiration (■), or uncoupler to dissipate energy (□), and the content measured after 1 min normal incubation plus 1 min further incubation with either uncoupler or antimycin (▲).

Table 1. *Expulsion of arsenite by other anions*

The incubation medium (see the Methods section) contained (apart from experiments with succinate) 1.5 mM-succinate, 1 µg of rotenone/ml, 80 µM-arsenite and 3.1 mg of protein/ml. For experiments with succinate the incubation medium contained 3 mM-ascorbate, 30 µM-tetramethylphenylenediamine and 2.1 mg of protein/ml. Incubations with the additions shown were for 1 min at 21°C.

Addition	Concn. (mM)	Mitochondrial arsenite content (% of control)
Phosphate	6	27
	12	24
Pyruvate	12	83
	24	53
Oxoglutarate	12	89
	24	43
Succinate	6	58
	12	41

Table 2. *Expulsion of pyruvate by arsenite*

The incubation medium (see the Methods section) contained 3 mM-ascorbate, 50 µM-tetramethylphenylenediamine, 1 µg of antimycin/ml, 1.66 mg of protein and 0.22 mM-pyruvate. Incubations with added arsenite were for 1 min at 21°C.

Addition	Concn. (mM)	Pyruvate content (% of control)
Arsenite	0.5	70
	1.0	40

Evidently arsenite accumulation is strongly energy-dependent. For this reason, incubation with arsenite at 0°C may be less effective at causing inhibition than when it is applied at a higher temperature. The kinetic results here accord with inhibition taking less than 1 min, even at 21°C.

It was previously shown (Harris & Manger, 1968) that anions intercompeted for accumulation within the mitochondrial interior, and arsenite shares this property; the values in Table 1 show that succinate, pyruvate, oxoglutarate and phosphate additions tend to lessen the arsenite content. The expulsion of arsenite by pyruvate is mirrored by an expulsion of pyruvate by added arsenite (Table 2). It follows that when arsenite is used in pyruvate accumulation studies the competition will lead to lower values for internal pyruvate than would hold in the absence of inhibitors.

The effect of the presence of dithioglycerol in equivalent amount to the arsenite is complicated by the uncoupling effect of the mixture. Fluharty & Sanadi (1961) suggested that this compound mediated an additional uptake of the arsenite, but measurements (not shown here) showed that this was not the case up to 100 µM-arsenite; higher concentrations of the mixture led to lower arsenite uptakes than found in controls without dithioglycerol.

The energy-dependence of arsenite accumulation recalls that described for azide (Palmieri & Klingenberg, 1967) and glutamate (Harris *et al.*, 1973).

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