EQUILIBRATION OF CHLORIDE AND PYRUVATE DISTRIBUTIONS BETWEEN LIVER MITOCHONDRIA AND MEDIUM MEDIATED BY ORGANO-TIN SALTS

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1. Introduction

Rat liver mitochondria are normally impermeable to chloride [1] so when separated from suspensions they carry about the same volume of Cl^--accessible water as sucrose-accessible water [2]. In contrast to Cl^-, most organic anions are internally accumulated to concentrations exceeding those outside, and such penetrant anions intercompete for occupation of the interior [3, 4].

Manger [5] observed that triethyl tin sulphate, added to a suspension in a 150 mM KCl based medium lessened the contents of organic anions. Studies of the swelling of liposomes, erythrocytes and mitochondria in response to organo-tin compounds led Selwyn et al. [6] to conclude that a Cl^--for-OH^- exchange was mediated by the compounds. Taken together the two findings point to the organo-tin mediated Cl^-entry providing another permeant anion to compete with the organic anions accumulated in the mitochondrial interior. This displacement of substrate anions is one possible explanation of the inhibition of respiration obtained by additions of organo-tin salts in Cl^--containing media [7]. The object of this work was to obtain direct evidence for the Cl^- entry and to compare its extent with that of a penetrant substrate (pyruvate). It has been observed [8] that the organo-tins inhibit phosphorylation in Cl^--free media; another action unrelated to Cl^- movement was sought, and this is also described. A model system was employed to confirm that the Cl^--for-OH^- exchange will take place across an oil layer.

2. Methods

Rat liver mitochondria were prepared as described before [9], and finally suspended in 0.25 M sucrose. The anion distribution studies were made at 20° with 3–5 mg protein/ml suspended in 150 mM potassium methane sulphonate, 20 mM Tris methane sulphonate and 50 mM sucrose at pH 6.8. Trace amounts of tritiated water were added, along with about 0.5 mM ^{36}Cl and 0.5 mM pyruvate as Tris salts. In a few experiments, [^{14}C] dextran, [^{14}C]sucrose, or [^{14}C]-ADP were substituted for the ^{36}Cl. The distribution of solutes between the medium and the mitochondrial pellet was found after separation of the suspension by centrifugation in Coleman Microfuges in tubes preloaded with 1.5 M perchloric acid beneath a layer of silicone oil (General Electric Versilube F.50) as described before [5]. Pyruvate was measured by enzymatic assay, phosphate by extraction of phosphomolybdate in isopropyl acetate [10]. ADP entry was measured by incubating the suspension with [^{14}C]ADP in presence of hexokinase (2 units), $MgCl_2$ (0.5 mM) and glucose (5 mM) in the medium. The movement was stopped by adding atractylate to 10 μM [11]. The mitochondria were separated as described to find their content of ^{14}C-labelled nucleotide.

Model experiments to investigate the counter movements of Cl^- and OH^- were made in the apparatus sketched in fig. 1. The aqueous media in the two U-tubes were in communication only via the oil layer at their common top. The contents of the U's and the floating oil layer were stirred. One U was charged with 3 M KCl buffered with 0.1 M Tris chloride to pH 7.4.

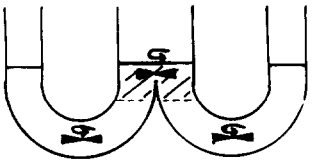

Fig. 1. Sketch of model system used to show the counter transfer of Cl^- in one sense and OH^- in the other through an interposed 'oil' layer between two aqueous media. One aqueous solution was buffered 3 M KCl, the other was 10 mM Cl-free buffer into which a Radiometer 'pH-Stat' fed Tris base to hold the pH at 7.4. The tin compound was added to the oil layer.

The other U was charged with 10 mM Tris methane sulphonate at pH 7.4; a Radiometer pH stat was set to hold the pH of this latter side constant by addition of 0.1 M Tris base. With an oil made up with 2 parts toluene and 1 part n-butanol, a slow tritration rate was observed. This rate and the effects on it of additions of triorgano-tin salts to the oil layer are given in sect. 3.

3. Results

3.1. Solute volumes associated with the mitochondrial pellets

Measurements of the amounts of alternative solutes carried down with the pellets into the acid beneath the silicone oil are shown in table 1. When tributyl tin acetate was present at 4 nmole/mg protein and Cl^- at 0.35 mM, there was no change of tritiated water, sucrose or dextran spaces, but the calculated Cl 'space' increased to double or more. With more tributyl-tin salt in presence of 0.77 mM Cl the tritiated water space increased slightly (by less than 10%) while the Cl 'space' doubled. The movement of Cl^- in these conditions is not accompanied by gross movement of water so it must be inferred that the Cl^- is being accumulated in the matrix. It was noted in these experiments that if tributyl-tin salt in excess of 10 nmole/mg were added to suspensions, the particles swelled, even in Cl-free media. This effect was observed both by optical density and packed volume measurements.

Table 1

Solute-accessible volumes carried by the mitochondrial pellet into the acid beneath the oil calculated assuming that there is no change of concentration.

Expt.1 (KCl present at 0.35 mM)

	Volumes occupied by solutes at their external conc. in the pellet (ml/g protein).			
	T_2O	Cl^-	Dextran	Sucrose
Before tin salt	3.72	3.63	2.76	3.22
After tributyl tin at 4 μmole/g	3.71	7 to 10 over 5 min	2.80	3.22

Expt. 2 (KCl present at 0.77 mM)

Before tin salt	3.09	3.14	Not meas.	2.68
After tributyl tin at 5.7 μmole/g	3.29	6.3 at 6 min	Not meas.	2.62

Medium: As in Methods with KCl additions noted.
Values are means of 3 individual measurements which agree to within ± 0.15 (S.D.).

3.2. Induced Cl^- uptakes and their relation to pyruvate accumulation

When the triorgano-tin salts were added at 3–7 μmole/g protein, the Cl content rose over some minutes and the pyruvate content fell (fig. 2). With tributyl tin, which was most used, the uptake was complete within 5 min, and the contents of both Cl^- and pyruvate would then remain static or diminish slightly over 10–15 min. The result was obtained whether or not metabolism was inhibited (fig. 2). With triphenyl tin as additive the Cl^- uptake more obviously passed through a maximum and then fell, along with the pyruvate. In the results in table 2, a note is made of the times of measurement, and a remark about the constancy or otherwise of the pyruvate and Cl^- contents. Table 2 is arranged to show that after the tin salt addition the ratios internal pyruvate/external pyruvate and internal chloride/external chloride become equal. The result is obtained with each of the 6 different tin compounds tested, although the ratios applying to each anion were high in some conditions and low in others.

When cloride ions enter the mitochondria there is not an exactly equivalent displacement of pyruvate, since other anions will also redistribute. When pyruvate is being metabolised the citrate formed tends to accu-

Table 2
Changes in Cl and pyruvate contents of mitochondria in response to additions of triorgano-tin salts to suspensions.

Ref.	Extl. Cl (mM)	Contents (μmole/g) before			Addition and time after taking of samples	Extl. Pyr. (mM)	Contents (μmole/g) after				Ratios	
		Totals		Net			Totals		Net			
		Pyr.	Cl	Pyr.			Pyr.	Cl	Pyr.	Cl	Pyr.	Cl
1	0.35	10.3	0.99	7.06	Trimethyl tin chloride 9 μmole/g at 10 and 15 min	1.03	9.1	3.20	6.2	2.21	6.0	6.3
2	0.095	10.0	0.31	6.9	Triethyl tin sulphate 2.5 μmole/g at 3.3 and 4.3 min	0.87	8.6	0.94	5.7	0.63	6.5	6.6
3	0.245	13.9	0.68	11.8	Tripropyl tin acetate 7 μmole/g at 5 and 6 min	0.68	8.0	2.39	6.1	1.71	9.0	7.0
4	0.295	13.9	0.68	11.8	Tributyl tin acetate 7 μmole/g at 6 min	0.72	12.2	3.90	10.2	3.22	14.2	13.1
5	1.34	9.52	5.21	7.17	Tributyl tin acetate 5 μmole/g at 6 min	0.51	8.1	20.7	5.87	14.7	11.3	10.9
6	0.245	13.9	0.68	11.8	Triphenyl tin acetate 7 μmole/g at 3.6 min (falls later)	0.72	6.7	1.99	4.7	1.31	6.5	5.3
7	0.245	13.9	0.68	11.8	Tricyclohexyl tin methane sulphonate 7 μmole/g at 4.5 and 6 min	0.72	9.5	3.40	7.52	2.72	10.7	11.1

Medium: Cl^--free as Methods, plus Tris-pyruvate as noted, Tris-malate about 0.5 mM, Tris-chloride as noted.
Net internal contents in the matrix have been calculated assuming that the initial Cl^- content is all carried outside the matrix. Contents are referred to biuret protein. After the tin salts have induced Cl^- uptake, the ratios between the net contents and the respective concentrations of Cl^- and pyruvate become similar (last two columns).

mulate internally and compete out both pyruvate and chloride. As an example the internal citrate rose from 0.6 to 7.0 μmole/g protein in a 5 min incubation. The competitive effect of citrate was shown directly by adding it to the incubation medium and measuring the citrate, pyruvate and chloride contents (table 3). Again these anion ratios tended to the same value. Other experiments (not detailed) showed that Cl^- and pyruvate accumulation ratios could be diminished by adding more malate, pyruvate or chloride. These data indicate that the accumulated chloride penetrates the same mitochondrial compartments as pyruvate, malate and citrate [see 12, 13].

3.3. Expulsion of DNP by Cl^- when tributyl tin is present

It has been observed [7] that triorgano tin salts will inhibit 2, 4-DNP-stimulated respiration in a chloride-containing medium. One explanation could be that the internal substrate anions are displaced. A second could be that the internal 2, 4-DNP level has been reduced (table 4). Most of the 2, 4-DNP is likely to be undissociated or in another compartment (membrane lipids, for example) which explains the high accumulation ratios. Only the dissociated acid ratio should be compared with other anion ratios.

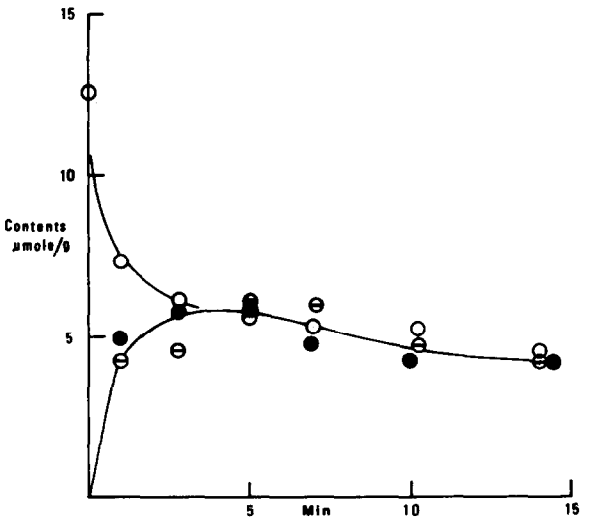

Fig. 2. Changes of mitochondrial pyruvate (○) and chloride (⊖ and ●) contents following an addition of tributyl tin acetate to the suspension at $t = 0$. The contents are corrected for the amounts carried in the non-specific volume associated with the pellet. The Cl points are from two parallel experiments, one with energy supplied by addition of hydroxy-butyrate and the other with energy supply inhibited. Media: as in Methods, with 0.5 mM pyruvate, 0.5 mM Cl and: (points ○ and ⊖) 0.5 mM Na arsenite, 5 mM Tris-hydroxybutyrate; (points ●) rotenone 1 μg/ml, oligomycin 5 μg/ml. Protein 4.5 mg/ml, pH 6.8, 20°.

Table 3
Equilibration between Cl^-, pyruvate and added citrate with trimethyl tin acetate present in the system at 8.7 nmole/mg.

Pyruvate			Chloride			Citrate		
External (mM)	Content (μmole/g)	Ratio	External (mM)	Content (μmole/g)	Ratio	External (mM)	Content	$(\text{Ratio})^{1/3}$
1.03	6.18	6.3	0.35	2.21	6.5	None added		
1.00	2.9	2.9	0.35	1.34	2.85	0.41	12.4	3.1
0.97	2.6	2.7	0.35	0.91	2.5	1.30	12.9	2.2

Medium: As in Methods, pH 6.5 plus 0.1 mM Tris-malate, Tris-pyruvate, citrate and chloride as noted.
Mitochondrial contents have been corrected for the amounts carried down in a volume equal to that occupied by the Cl^- before the tin addition. The cube root of the citrate ratio is noted because it would equal the ratio for the singly charged anions when they are equilibrated, provided the solvent volume is 1 ml per g protein.

Table 4
Changes of mitochondrial DNP content in response to addition of tributyl tin acetate to a suspension in a chloride-containing medium.

Experiment	DNP conc. (mM)	Ratio : content (μmole/g)/conc. (mM) Before; after; tin salt at:		(nmole/mg)
1	0.10	40.3	25.0	: 9
2	0.025	34.3	23.5	: 4.3
3	0.008	53.7	23.0	: 9.7

Medium: 150 mM KCl, Tris-chloride 20 mM, 30 mM sucrose, pH 7.2
The ratio between the content of DNP and the external concentration is noted.

3.4. Inhibition of phosphorylation and adenine nucleotide exchange by triethyl and tributyl tin salts

The triorgano-tin salts inhibit phosphorylation even in the absence of chloride [8]. It was confirmed that in a chloride-free medium, addition of tributyl or triethyl-tin salts did not cause a loss of internal substrate; indeed, there was sometimes an increase of 2 to 4 μmoles of substrate anions and phosphate per g protein. Thus it was possible that access of the adenine nucleotide to the enzymic site or the site itself was being restricted. It has been suggested that the organo-tin salts have an effect similar to oligomycin; however, it seemed possible there was an alternative effect upon the adenine nucleotide translocator. The rate of ^{14}C-labelled ADP entry was measured in a Cl^--free medium using a high concentration of ADP maintained with hexokinase and glucose. The results for the ratio of internal to external ^{14}C-labelled nucleotide as a function of time show (fig. 3) that at 6 μmole of tributyl or triethyl tin added per g of protein some minutes before the ADP, the organo-tin salts strongly inhibit the uptake. There was little or no effect if the organotins were added at 2 μmole/g before the ADP or at 6 μmole/g at the same time as the ADP. Thus it is likely that these triorgano-tin salts react with the nucleotide exchanger groups.

3.5. Model system experiments

The model system was used to find the rate at which alkali had to be added to the weak buffer solution to maintain its pH when 3 M KCl was in the other

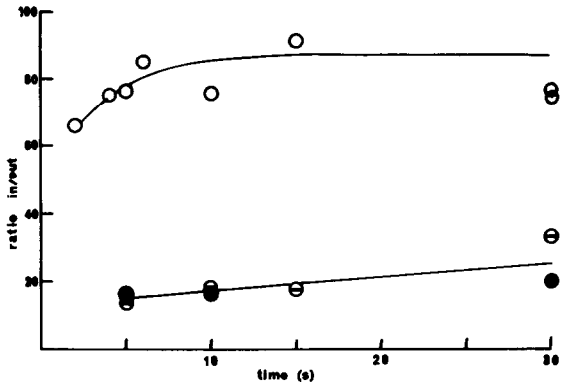

Fig. 3. The effect of triethyl and tributyl tin salts on the labelling of the internal adenine nucleotide pool from ^{14}C-labelled ADP at 0.2 μM. The exchange was stopped by atractylate (10 μM) at the different times. Pre-treatment for 10 min at 0° with the tin compounds at 6 nmole/mg protein give the results marked ⊖ (triethyl) and • (tributyl). Untreated controls are marked ○. Medium: Cl-free mixture as in Methods, pH 6.8, 20°.

Table 5
Titration rates measuring OH^- disappearance from the low Cl^- side of the model apparatus (fig. 1) when Cl^- moves into it across the oil layer.

Compound	Amount added in μatoms Sn to 5 ml oil	Rate of titration (μmole/min)
None		0.10
$((CH_3)_3SnCl$	8.5	0.41
	58.5	1.00
$((C_2H_5)_3Sn)_2SO_4$	9.0	0.23
	40.0	0.43
$(C_3H_7)_3SnAc$	10.0	0.62
$(C_4H_9)_3SnAc$	9.0	0.60
	45.0	0.90
$(C_6H_5)_3SnAc$	7.1	0.19
	28.4	0.75
$(C_6H_{11})_3SnAc$	10.0	0.29
	41.0	1.07

arm (table 5). Without any additions to the butanol–toluene there was a slow titration rate. Adding any of the tin compounds listed in table 2 caused an opaque layer to appear at the oil water interface. No absolute significance is attached to the observed rates because inadequate stirring and the plating phenomenon will have affected them. The point is that the compounds have comparable abilities to mediate the Cl^--for-OH^- exchange through a non-aqueous solvent.

4. Discussion

Our results show that triorgano tin salts tested in table 2 have more than one action on anion permeability. The mediation of Cl^- permeation, whatever its mechanism, allows the Cl^- to equilibrate with the pyruvate. Whether or not the two ions are at electrochemical equilibrium cannot be decided but they are equilibrated between themselves. Earlier results [10, 12, 13] suggest that pyruvate, malate and citrate are similarly equilibrated. If we accept that a free exchange of OH^--for-Cl^- is provided by the tin compounds when they dissolve in the membrane, then it would follow that the equilibrium holds between the anions and OH^- and implicitly H^+ also. As the accumulation occurs after respiration is inhibited (fig. 2), it is necessary to regard the inside as having been 'charged up' during previous metabolic activity [13, 14]. Our finding that the adenine nucleotide exchanger is inhibited extends the known toxic effects of the triorgano tin compounds. It does not preclude an additional oligomycin-like effect [15, 16]. It may be useful in providing an additional agent to atractylate for titrating the adenine exchanger groups.

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