CHAPTER 8

Organotins as Mitochondrial Toxins

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Abstract: *In vitro* toxicity, molecular study of the toxicity mechanisms, which should account for the observed *in vivo* effects of toxic compounds, is a complicate issue. The study of organotin compounds is particularly complicate, since organotins, being chemically versatile, are able to bind covalently and non-covalently to many biomolecules; also organotins are soluble in lipophilic environments and can affect a wide variety of biological functions. Therefore, it is difficult to identify the biological mechanism and the molecular target inhibited by the lowest effective dose. In the interactions of organotin compounds with mitochondria, the problem is "simplified" since many organotins cause *in vivo* acute toxicity. Being mitochondria the energy source of the cell, mitochondrial impairment induces cell damage; therefore, in many cases, mitochondria are the molecular target responsible for the *in vivo* acute toxicity. On these bases, ATP synthesis mechanism in mitochondria should be carefully analysed, in order to individuate the step(s) inhibited by the toxic compounds. Many organotin effects on the mitochondrial functions are correlated with the ATP synthesis inhibition. In isolated mitochondria, the $(alkyl)_3$-Sn- compounds inhibit all the steps involved in the ATP synthesis mechanism, but experiments performed in isolated cells suggest that the mitochondrial ATP synthesis inhibition is probably related to the opening of the membrane permeability transition pore. In addition, also triphenyltin opens the permeability transition pore and triggers apoptosis. Other putative and proven mechanisms of organotin action in mitochondria are considered in detail.

Keywords: Organotin – mitochondria - membrane permeability transition pore (MPTP) - protonophores - ATP synthesis inhibition - mitochondrial respiratory chain (MRC).

THE MITOCHONDRIAL RESPIRATORY CHAIN (RC) AS TARGET OF TOXIC COMPOUNDS: THEORETICAL BASIS AND APPLICATIONS OF POLAROGRAPHIC STUDIES

The respiratory chain (RC) is common to all organisms, thus suggesting that toxicity mechanisms on RC could be the same (or very similar) in all living organisms. Moreover, it is well conserved, being the same or very similar in all living organisms. This situation, together with the observation that all biological membranes have a similar behaviour is of relevance in the understanding of the molecular mechanisms of organotin compounds and to explain why the toxicity is not species-specific, but it shares similar features and mechanisms with all living organisms, as demonstrated by the high sensitivity of all living organisms to rotenone and cyanide.

As well known [1-3], according to the chemiosmotic hypothesis the electron flow in the RC is coupled to a stoichiometric proton extrusion from the mitochondrial matrix to the external medium. Since the mitochondrial membrane is not permeable to protons, their extrusion induces a pH difference (ΔpH) and an electrical potential difference ($\Delta\Psi$). The sum of these two terms is called proton motive force (p.m.f, where p.m.f. = ΔpH + $\Delta\Psi$). The p.m.f. is the way by which the energy from the oxidation of the substrates is stored and subsequently given to ADP to synthesize ATP. This occurs through a high molecular weight protein, the ATPase which is positioned in the mitochondrial inner membrane and protrudes in the matrix. A selective proton channel in the ATPase allows the slow return of the protons from the external medium into the matrix. As a consequence of the p.m.f.-driven proton flow in the ATPase channel ATP is synthesized from ADP.

RC functioning can be investigated polarographically using an oxygen electrode (Clark oxygen selective electrode) which allows respiratory rate measurement. As the oxygen concentration varies greatly with temperature, Clark

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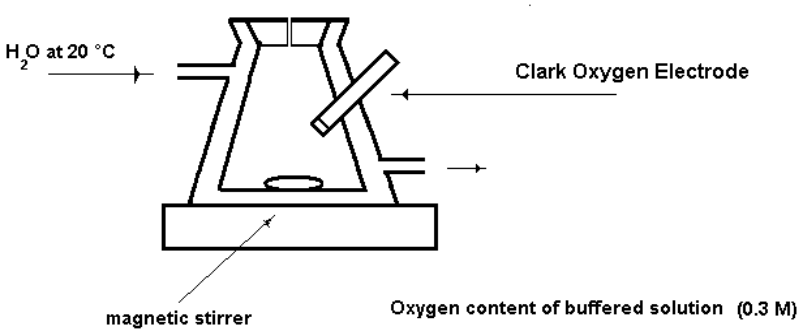
electrode is set in a thermostated vessel, usually at 20 °C. The vessel contains a stirred buffered solution to which mitochondria can be added. The solution chamber is closed by a Teflon-cap in which a thin hole allows insertion of a syringe needle to add substrates and inhibitors, but impedes air and thus oxygen entry. Therefore, only oxygen occurring in solution at the experiment start, according to the assay temperature, is consumed by mitochondrial respiratory activity. The apparatus is shown in Fig. (**1**).



Oxygen content of buffered solution (0.3 M)

Temperature (°C)	ng atom O/ml
15	575
20	510
25	474
30	445
35	410

Figure 1: The rate of oxygen consumption in mitochondria can be followed by means of a selective oxygen electrode (Clark electrode).

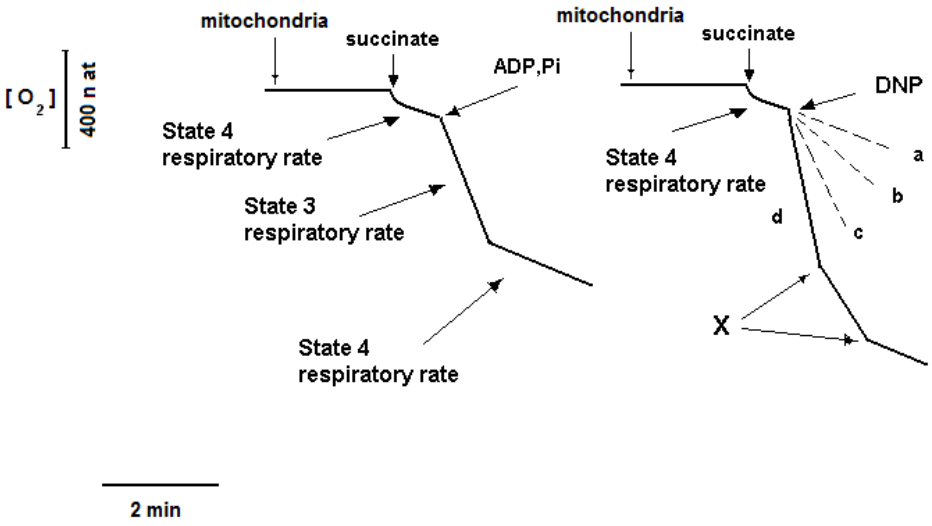


Figure 2: Polarographic measurement of mitochondrial respiratory activities. The addition of a substrate (succinate) to the medium containing mitochondria causes an oxygen consumption which is fast initially, then it decreases up to a stationary value called State 4. The addition in state 4 of ADP and phosphate (Pi), induces an increase of the respiratory rate called State 3. After ADP consumption (and ATP production), the systems returns to the State 3 respiratory rate. The addition in State 4 of a protonophore such as DNP, induces a similar increase of the respiratory rate to that detected in State 4. The increase is proportional to the DNP concentration (dotted lines) up to a maximum value which in the case of DNP is obtained by a DNP concentration of 80 μM. In a, [DNP]=0, in b, [DNP]=20 μM, in c, [DNP]=80μM. The addition in c of a generic RC inhibitor (X), induces an inhibition of the maximal respiratory rate.

In a typical respiratory activity measurement, at the experiment start the vessel contains a 0.25 M sucrose, 10 nM Tris-MOPS buffered solution, pH 7.4. First, mitochondria of known protein concentration are added to the solution, and 2 min later the addition of a reducing substrate (as succinate), induces oxygen consumption. At first, oxygen consumption is rapid, then it slows down and the respiratory rate (detected as the slope of the straight line obtained when oxygen concentration is plotted against time) remains low (about 10 n moles oxygen•mg protein^{-1}min^{-1}). This situation is called *State 4* respiratory rate as illustrated in Fig. (**2**).

According to the chemiosmotic hypothesis, the reducing substrate induces a proton extrusion with consequent ΔpH and ΔΨ formation. As ΔpH and ΔΨ increase, H^+ extrusion becomes more difficult, since ΔpH (as pH in the matrix side is alkaline) and ΔΨ (as Ψ is negative inside) are both forces which oppose to the proton extrusion. This explains why the respiratory rate is rapid initially, then slows down. The (low) respiratory rate in State 4 is a measure of the rate of H^+ passage in the ATPase proton channel.

The addition of ADP and inorganic phosphate (Pi) to the solution containing respiring mitochondria in State 4 induce an enhancement of the respiratory rate, called *State 3*, in which ATP is formed. The respiratory rate enhancement is due to the ATP synthesis that utilises the p.m.f.; therefore, in State 3, both ΔpH and ΔΨ are low, allowing a rapid proton extrusion by the RC. After all ADP and Pi are consumed by ATP synthesis, the system returns in State 4.

The respiratory rate enhancement from State 4 can also be obtained by addition of chemical compounds called uncouplers (or protonophores). As Fig. (**2**) shows, the addition of DNP (2,4-dinitrophenol) the first ever studied uncoupler, induces an increase in the respiratory rate which is proportional to the protonophore concentration up to a maximum value. The chemiosmotic hypothesis elegantly explains the respiratory rate increase and the correlated ATP synthesis inhibition. The uncouplers are weak acids (or weak bases): pK of DNP is 4.7. Therefore in solution the phenate anion is at equilibrium with its non-dissociated form, Fig. (**3**).

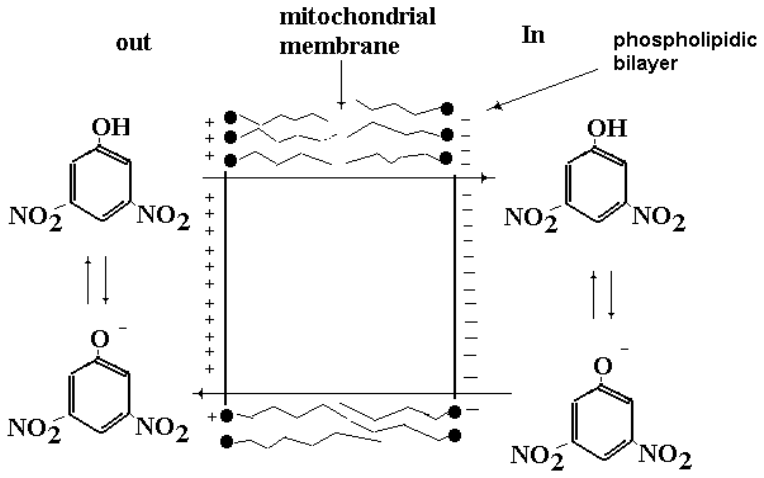


Figure 3: The DNP cycle. The protonophore DNP enters the mitochondrial matrix as non-dissociated electroneutral compound, since the pH is alkaline-inside. Once inside, it is extruded as phenate by the negative-inside potential. Any cycle, involves the passage of a proton.

The biological membranes (in absence of membrane carriers) are generally not permeable to charged ions (such as protons, as above discussed). In the case of DNP, the neutral chemical form can pass across the membrane and will tend to accumulate in the matrix, with the alkaline-inside pH being the driving force. Once inside, the phenate anion is extruded. In this case, the driving force for the extrusion is the negative-inside potential. Once outside, will DNP return inside again, thus giving rise to a cyclic mechanism, Fig. (**3**). At any cycle, the entry of un-dissociated DNP and the extrusion of the phenate anion, corresponds to the entry of a proton. For this reason, the uncouplers are also called protonophores. This mechanism in particular explains:

- The low doses of DNP required, since the mechanism is cyclic (and this is true for all protonophores);
- The respiratory rate enhancement, since the entry of protons induces ΔpH and ΔΨ collapse;
- The ATP synthesis inhibition, since ΔpH and ΔΨ are the energy source for ATP synthesis.

All these aspects, are explained thanks to the chemiosmotic hypothesis.

Other experimental results obtained by the use of uncouplers can be explained by means of the chemiosmotic hypothesis, but this aspect will be treated later, since all protonophores are toxic compounds.

One of the proposals regarding the toxicity mechanism of organotin compounds is that they are protonophores. Then, before studying the *in vitro* interactions of these chemicals with mitochondria, it is indispensable to know, or to propose, a transport mechanism to justify their presence in the cytoplasm and their possible transport into the mitochondria.

POTENTIAL MITOCHONDRIAL TARGETS: GENERAL ORGANOTIN BEHAVIOUR

In the previous sections, the description of physiological behaviour of mitochondria and, especially of ATP synthesis mechanism, aimed at drawing a propaedeutic scenario to depict how toxic compounds can act on such mechanisms. Once established that a compound inhibits the ATP synthesis in mitochondria (also identifying the lowest effective dose!), it is necessary to individuate which step in the ATP synthesis is inhibited by the organotin compound under study.

On considering the ATP synthesis mechanism, the toxicity of a compound can be due to the concomitance of different affected targets:

a. The RC;

b. The ATPase;

c. The membrane permeability.

In turn, the membrane permeability enhancement can be due to:

c1. A protonophore effect;

c2. Membrane pore opening.

Schematically, *i.e.*:

ATP synthesis inhibition { a. RC inhibition; b. ATPase inhibition; c. membrane permeability increase { c1. protonophore effect; c2. pore opening }

Therefore, to assess the molecular target of a toxic compound which produces inhibition of ATP synthesis, the procedure must be the following:

Determination of the extent of ATP synthesis inhibition in the presence of the toxic compound to obtain quantitative information on the dose/response relationship, Fig. (**8**).

Analysis of each step (a, b and c) involved in ATP synthesis. The step responsible for the ATP synthesis inhibition requires the lowest dose of toxic compound and must give a quantitatively similar dose/response relationship to that obtained for ATP synthesis inhibition, Fig. (**4**).

Therefore, it is essential to measure the ATP synthesis in mitochondria, and many enzymatic procedures can be used to measure it. However, some enzymatic assays cannot be carried out in the presence of compounds which inhibit the ATP synthesis. In fact, there is a high probability that if a compound inhibits the ATP synthesis, at the same time will inhibit the enzymes utilized for ATP measurement, thus giving spurious results.

The alternative is to use a procedure which utilizes the correlation of the reactions a) and c) shown in the first Section, with pH changes [4,5]. Therefore, in the presence of an ATP synthesis inhibitor, it is possible to follow the ATP synthesis through the alkalinisation which accompanies the reaction a or b in low buffered medium.

Another procedure exploits the RC properties illustrated in the first Section. The ratio R between the mitochondrial respiratory rate in state 3 and state 4 is a measure of the ATP synthesis efficiency [6]. In well coupled mitochondria, R is about 5. In mitochondria unable to synthesize ATP, R is 1. This changed ratio stems from different effects on the mechanisms involved in ATP synthesis: a pure uncoupler stimulates the State 4 respiration rate. An inhibitor of the RC or of the ATPase inhibits the State 3 respiration rate. Irrespective of the method, it is possible to quantify the percentage of ATP synthesis inhibition in relation to the concentration of the tested compound X. The results can be graphically plotted as shown in Fig. (**4**).

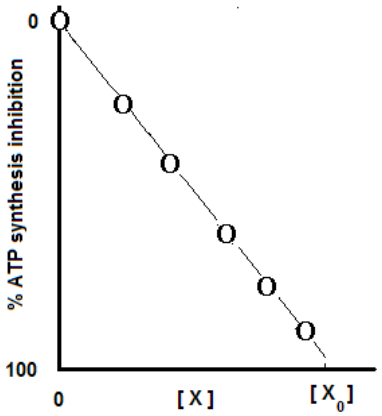


Figure 4: ATP synthesis inhibition by the generic X compound. Maximal (100%) inhibition is attained at $[X_0]$. Once established that the X compound inhibits the ATP synthesis, it is necessary to look into each step (a, b and c) involved in the ATP synthesis separately to identify the inhibited one.

a. The RC

If the X compound inhibits the RC (a), its effect can be quantitatively analysed using uncoupled mitochondria. In uncoupled mitochondria, the respiratory rate is maximal, as before explained, and unaffected by both uncouplers (obviously) and ATPase inhibitors, as shown by employing the classical ATPase inhibitor oligomycin. Under these conditions, the "pure" inhibitory effect on the RC can be analysed. A RC inhibitor reduces the respiratory rate (measured as the slope of the straight line of oxygen concentration plotted against time). The results can be plotted in a graph and compared with that of ATP synthesis inhibition. There are two possible outcomes:

1) The effective doses are similar. In this case, it is very likely that the RC is the step responsible for the ATP synthesis inhibition.

2) The doses needed to inhibit the RC are higher than those necessary to inhibit the ATP synthesis, Fig. (**4**). In this case it is possible to conclude that the RC, although inhibited, is not the step responsible for the ATP synthesis inhibition. In this case the other steps (b, c) must be analysed.

b. The ATPase

ATP hydrolysis is catalysed by the ATPase. Since the reaction induces an acidification of the medium, it can be easily followed through a pHmeter in a low buffered medium [4,5] and using uncoupled mitochondria. Fig. (**5**) shows the effects of an uncoupler (DNP) (A) and of the protonophore X added in the presence of 80 μM DNP (B).

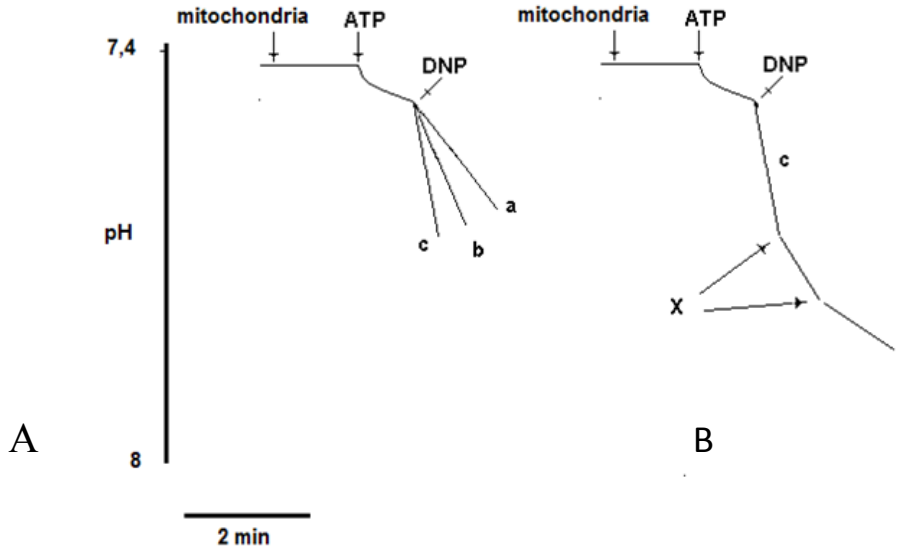


Figure 5: ATP hydrolysis induces acidification in the medium. A) The acidification rate is initially slow, since an equilibrium between p.m.f and ATP exists (ATP ⇆ ΔpH + ΔΨ). B) An increase in ATP hydrolysis rate in the presence of the uncoupler DNP is obtained by adding the protonophore X. The increase is proportional to the protonophore concentration. In a) [DNP]=10 μM, in b), [DNP]=20 μM, in c, [DNP]=80μM.

c. Membrane Permeability Enhancement

This effect can be due to a protonophore effect (c1) or to the opening of a membrane pore (c2).

c1. The protonophore effect mechanism (which, as we will see is one of the hypothesized mechanisms to explain the toxicity of the trialkyltin compounds) has been previously shown in the case of DNP. On these bases a selective procedure in order to highlight this effect was proposed [7,8] by using non respiring mitochondria (note that the RC is not utilized and nor the ATPase!). The mitochondrial matrix contained 0.1M K^+. If mitochondria were resuspended in a medium with a low (0.1 mM) potassium concentration, although in the presence of a concentration gradient, the potassium ion could not exit from the mitochondrial matrix, because biological membranes are not permeable to K^+. In the presence of valinomycin, a potassium ion carrier, only a negligible K^+ efflux was monitored by a selective potassium electrode. This result was due to the fact that K^+ is a charged species and K^+ efflux would generate a negative-inside potential which opposes to a complete K^+ efflux. Under these conditions, the addition of a protonophore, which allows H^+ entry, induced membrane potential collapse and a total K^+ efflux. K^+ efflux rate was proportional to the protonophore concentration, as shown in Fig. (**6**). The above detailed procedure allows us to individuate a protonophore compound, but it does not allow to obtain a dose/response relationship, Fig. (**6**).

However, other procedures can be exploited, at least in some cases, to individuate a protonophore compound and to obtain a dose/response relationship.

In the described procedure to test the ATPase sensitivity to toxic compounds by measuring the ATP hydrolysis rate as shown in Fig (**5**), uncoupled mitochondria are utilised; ATP hydrolysis rate is very slow since the ATP concentration and the p.m.f are in thermodynamic equilibrium. Therefore, ATP addition induces a ΔpH and a ΔΨ formation which oppose to ATP hydrolysis. Since an uncoupler (or protonophore) permeates the membrane to protons, the consequent ΔpH and ΔΨ collapse induce an increase in ATP hydrolysis rate. This effect is proportional to the protonophore concentration and by this procedure, it is possible to obtain a dose/response relationship, providing that the protonophore is not an ATPase inhibitor.

Analogously, as previously interpreted on the basis of the chemiosmotic hypothesis, the addition of a protonophore to mitochondria in the state 4 respiratory rate, induces an increase of the respiratory rate, providing, in this case, that the protonophore is not an inhibitor of the RC.

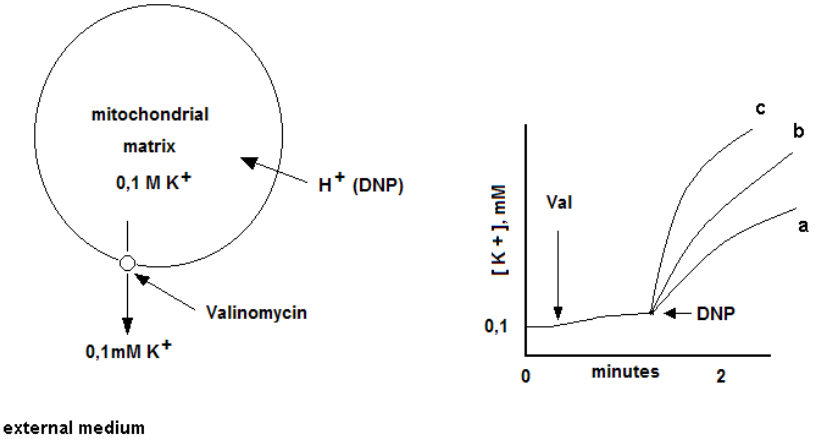


Figure 6: Mechanism of DNP toxicity. In non respiring mitochondria the rate of K^+ efflux (in the presence of valinomycin) is proportional to DNP concentration. A similar response is obtained with the $(alkyl)_3Sn$- compounds, thus demonstrating that they are protonophores. In a, [DNP]=10 μM, in b, [DNP]=20 μM, in c, [DNP]=30 μM.

Since $(alkyl)_3Sn$- compounds are inhibitors of both ATPase and RC (as we will see), both these last two procedures cannot be applied. Therefore, in the case of the trialkyl compounds it is possible to demonstrate that they are protonophores [7, 8], however without obtaining a dose/response relationship, and thus not providing evidence that the dissipation of ΔpH and ΔΨ is the step responsible for ATP synthesis inhibition.

c2. Membrane Transition Pore (MTP) Opening

In mitochondria, many chemicals, including metals and organometallic compounds, induce the opening of MTP pore. A conformational change related to pore opening, not completely clarified yet, allows the passage of solutes of molecular weight up to 1.5 KDa through the mitochondrial membrane. This passage promotes a mitochondrial swelling, caused by influx of water and solutes (sucrose) through the open pore driven by the osmotic gradient and due to the presence of not diffusible matrix proteins (*i.e.* a colloid-osmotic process). The process can be spectrophotometrically followed by an absorbance quenching at 540 nm [9, 10]. The pore opening induces, evidently, a corresponding ATP synthesis inhibition, since the p.m.f. collapses.

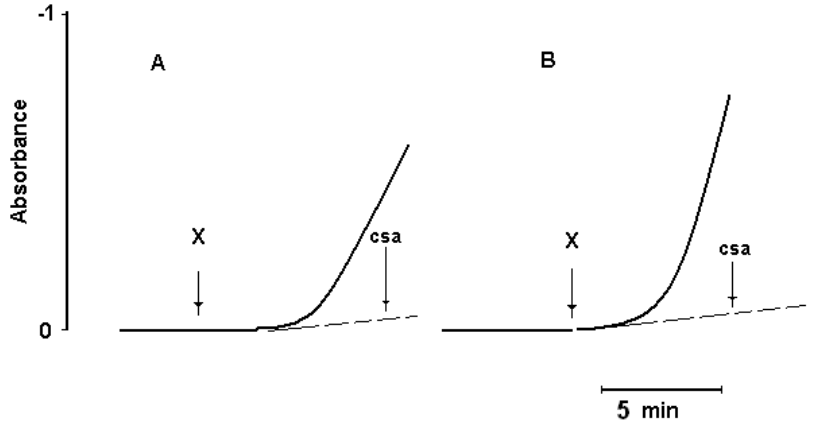


Figure 7: Spectrophotometrical measurement of mitochondrial swelling. The mitochondrial swelling is monitorable as absorbance decrease at 540 nm. The phenomenon is time-delayed and depends on the inducer concentration. The figure shows a typical experiment with two concentrations of the same inducer (X): $[X]_B > [X]_A$ are used. In the case of $(alkyl)_3Sn$- compounds, the response is cyclosporine-sensitive (csa) (dotted line).

Figure (**7**) shows an example of spectrophotometrical monitoring of mitochondrial swelling. It is necessary to remark that the absorbance quenching is time-delayed and progressive (not linear). This behaviour cannot be quantified as dose/response relationship and therefore, it is not possible to verify if MTP opening is responsible for ATP synthesis inhibition. However, a help can arise from the fact that in many cases the phenomenon is cyclosporine A sensitive, since the cyclosporine inhibits the pore opening and the consequent swelling. In this case it is possible to ascertain if the swelling is responsible for the ATP synthesis inhibition, by comparing the ATP synthesis obtained in the absence, as in the experiment illustrated in Fig. (**2**), and in the presence of cyclosporine. If a difference is observed, it allows undoubtedly to conclude that the MTP opening is the key step responsible for the ATP synthesis inhibition.

In conclusion, when more steps are inhibited, the investigation of the step responsible for the ATP synthesis inhibition, is complicate. This is the case of the organotin compounds, as we will see.

ORGANOTINS TARGET MITOCHONDRIA

In the last years, organotin compounds have been largely studied as a consequence of a chemical-theoretical scientific interest, and as a consequence of their practical applications.

Organotin compounds are classified as R_4Sn, R_3SnX, R_2SnX_2, and $RSnX_3$,where in industrially relevant compounds R is usually a butyl, octyl, or phenyl group, instead X is a chloride, fluoride, oxide, hydroxide, carboxylate, or thiloate function. Generally, mono-substituted organotin compounds ($RSnX_3$) have had a very limited application, but they are used as stabilizers in poly(vinyl chloride) films. Twice substituted organotin compounds (R_2SnX_2) are mainly used as stabilizers in polyvinylchloride, but also as catalysts in polyurethane foams production and *in silico*ne vulcanization processes performed at room-temperature. Tri-substituted organotin compounds (R_3SnX) have biocidal properties that are strongly influenced by the toxicological valence of R-groups. The most important of these compounds show tributyl-, triphenyl- and tricyclohexyltin as substituted groups: they are used as fungicides, bactericides, antihelminthics, miticides, herbicides, molluscicides, insecticides, nematocides, ovicides, rodent repellents, and antifoulants in boat paints. The tetrasubstituted organotin compounds (R_4Sn) are mainly used as intermediates in the preparation of other organotin compounds.

Among the wide range of organotin compounds which have been synthesized and investigated, the $(alkyl)_3Sn$- compounds (almost all organotin have a tetravalent Sn^{4+} structure) have been largely utilized as antifouling compounds. Despite their efficacy, they have been substituted by the $(phenyl)_3Sn$- compounds (TPhT) before to be definitively banned. Bans were due to the high toxicity of the $(alkyl)_3Sn$- and of the $(phenyl)_3Sn$- compounds and to their high environmental persistence. The $(phenyl)_3Sn$- compounds have been also utilized as agricultural fungicides before to be banned. Therefore, the toxicological problems of organotin compounds involve mainly the $(alkyl)_3Sn$- and the $(phenyl)_3Sn$- compounds.

Since the toxicity of organotin compounds, and especially of trisubstituted species, is an acute toxicity (which manifests its effects within 48 hours after exposure), there are many evidences that, as in many cases of acute toxicity, the mitochondria are the preferential target.

Low doses of $(alkyl)_3Sn$- compounds inhibit the ATP synthesis in isolated mitochondria. For TBT (tributyltin), TPT (tripropyltin) and TET (triethyltin), the concentration which 100% inhibits ATP synthesis is around 10^{-7} M. For $(methyl)_3Sn$- (TMT), the inhibition is attained at around 10^{-6} M [11-14].

As regards the mitochondrial function responsible for the ATP synthesis inhibition, Stockdale *et al.* found that the $(alkyl)_3Sn$- compounds inhibit phosphorylation more than oxidation [15]. This finding lead to exclude the RC inhibition as the step responsible for ATP synthesis inhibition. This conclusion was confirmed by the fact that the RC inhibition is a delayed effect, while the ATP synthesis inhibition is not a delayed effect [15-19].

It was also reported [15] that, in a sucrose medium, the $(alkyl)_3Sn$- compounds had an oligomycin-like effect. The doses necessary to inhibit the ATPase were about 2 μM and were close to those producing inhibition of the oxidative phosphorylation. This behaviour was subsequently confirmed by Bowman [20] who, accounting for the

protein concentration, found a similar inhibitory effect at around 3 μM TET. Taking into account the two experimental data, it can be supposed that the inhibitory effect of $(alkyl)_3Sn$- compounds is due to an inhibitory effect on the ATPase (oligomycin-like effect).

In a previous paper [21] Aldridge and Street observed that the ATP synthesis inhibition occurred with trialkyltin doses similar to those promoting mitochondrial swelling. In this case, a quantitative appraisal is not easy as illustrated in Section 2.2. In addition, those experiments were performed in 1964 [21] when the process of MPT opening and its cyclosporine-sensitivity were not known yet. Furthermore, the authors emphasized that the observed phenomena strongly depended on the resuspending medium. On considering the whole of data, it is possible to exclude an organotin effect on the RC; it remains the possibility of separate effects on the ATPase and on MPT opening as possible inducers of ATP synthesis inhibition.

Further investigations, however, allow to predict if the MTP opening is the step responsible for ATP synthesis inhibition. Trialkyltin compounds were found to induce apoptosis in various cell types by a cyclosporine-sensitive mechanism. Since mitochondria are involved in all kinds of apoptosis, and since mitochondrial swelling is cyclosporine-sensitive [22], the hypothesis that the MPT opening is the crucial step responsible for the trialkyltin toxicity is strongly supported. Although this process should be the most probable key event, a possible role of an uncoupling effect cannot be excluded.

The hypothesis that organotins may act as uncouplers was firstly advanced by Selwin *et al.* [23] in order to explain phenomena occurring in concomitance with mitochondrial swelling, but the molecular details to support this hypothesis were not provided. The molecular mechanism and its demonstration were given by Bragadin *et al.* [7,8,24] who depicted a model based on the consideration that the $(alkyl)_3Sn$- compounds are weak acids [25]. Therefore, in solution the two forms are at equilibrium:

$$(alkyl)_3Sn\text{-}OH \leftrightarrows (alkyl)_3Sn^+ + OH^-$$

Being this the situation, in respiring mitochondria, $\Delta\Psi$ (which is negative-inside) drives the uptake of the $(alkyl)_3Sn^+$ cation. In the matrix, the electroneutral $(alkyl)_3Sn$-OH compound is extruded, leading to a cyclic mechanism, which gives rise to the transport of a proton in the matrix, at any cycle. The protonophore mechanism, which has been demonstrated following the same procedure utilized for DNP and other compounds as illustrated in Fig. (**7**), does not provides a threshold. Therefore, it can be always present, since the transport of the compounds $(alkyl)_3Sn$-OH and $(alkyl)_3Sn^+$ occurs, like all uncouplers, through the phospholipid bilayer and it is not possible to quantify the uncoupling role on ATP synthesis inhibition.

As regards other possibilities and other mechanisms, taking into account the effects found in the medium containing anions, a proposal regarding the role of $(alkyl)_3Sn$- compounds as Cl^-/OH^- electroneutral exchangers was advanced by Skilleter [26] who suggested that the trialkyl compound, in the form of $(alkyl)_3$-Sn-Cl is present in the cytoplasm and enters the mitochondria as electroneutral undissociated $(alkyl)_3$-Sn-Cl compound. Since an equilibrium between $(alkyl)_3$-Sn-Cl and $(alkyl)_3$-Sn-OH exists

$$(alkyl)_3\text{-}Sn\text{-}Cl \leftrightarrows (alkyl)_3\text{-}Sn\text{-}OH$$

the $(alkyl)_3$-Sn-OH form is extruded from the matrix. The balance of the consequent cyclic mechanism is an Cl^-/OH^- electroneutral exchange. Although the mechanism, which was firstly proposed to explain transport mechanisms in isolated phospholipids [27], has some weak points (the existence in solution of the undissociated trialkyl-Sn-Cl is not reported in the literature and therefore it can be only postulated), it is important because it suggests not only a mechanism which could contribute to explain the toxic effects, but also a transport mechanism. In general, this is one of the most important and preliminary topics in the *in vitro* study of the toxicity of all compounds. Indeed, before studying toxicant interactions with mitochondria, it is indispensable to know, or to postulate, a transport mechanism to justify the toxicant presence in the cytoplasm and its possible transport into mitochondria. Since non physiological compounds cannot have a specific carrier (!), in order to be transported inside the cell, these compounds must utilize a physiological carrier in the phospholipidic bilayer. Both the electroneutral and the protonophore models suggest a transport mechanism without the utilization of a physiological carrier.

The inner mitochondrial membrane possesses an anion uniport, known as the inner membrane anion channel (IMAC). Experiments performed by Powers and Beavis [28] evidenced that $(alkyl)_3Sn$-compounds inhibit the IMAC channel. The efficacy of the IMAC inhibition increases with the hydrophobicity in the following order:

trimethyltin<triethyltin<tripropyltin<triphenyltin<tributyltin

In the case of tributyltin (TBT), a complete inhibition is obtained at 0.9 nmoles/mg of protein. The above efficiency order suggests that the binding site of IMAC may be accessible from the phospholipid bilayer. The same investigation [28] confirmed that the ATPase was inhibited by 0.75 nmoles TBT/mg of protein. The comparison between the IMAC and ATPase inhibition data lead to exclude that the effects on mitochondria are due to the IMAC inhibition.

Toxicity of Bis(tributyltin) Oxide (TBTO)

The interactions of trialkyltin oxide compounds (used in wood preservation, marine antifouling, disinfection of circulating industrial cooling waters) with mitochondria were repeatedly studied [29,30]. All the trialkyl compounds inhibit the mitochondrial respiration [29]. The effects of TBTO and of the dibutyl diisooctyl thioglycolate are localized in the terminal step of the RC. Other alkyltins inhibit before the cytochrome c. The ATP synthesis inhibitory effect of TBTO was found to be similar to that exerted by oligomycin, a potent ATPase inhibitor. These data are in agreement with those obtained by Penninks *et al.* [31,32]; who suggested that the TBTO target is the mitochondrial ATPase which is inhibited by 0.5 μM TBTO.

Toxicity of Trialkyltin Derivatives

Although the trialkyltin compounds are persistent in the environment (and this is one of the reasons for their banning), they undergoes a slow decomposition to form, after some weeks, the di- and monoalkyl derivatives:

$$(alkyl)_3SnCl \rightarrow (alkyl)_2SnCl_2 \rightarrow (alkyl)SnCl_3$$

This situation offers the possibility that, like other toxic compounds, the decomposition products can be toxic and, in some cases, more toxic than the starting compounds.

The acute toxicity of $(butyl)_2SnCl_2$ (DBTC) was *in vitro* studied. The pattern is complicate, since, as in the case of the precursor TBTC, DBTC inhibits many mitochondrial functions. An uncoupling effect was observed from a level of 8.3 nm DBTC/mg of protein [31], but the oxidation of the α-ketoglutarate was inhibited by 0.8 nm DBTC/mg of protein. This finding lead the authors to conclude that the uncoupling effect, although present, was not quantitatively crucial to inhibit ATP synthesis. Penninks and Seinen [32] found that all examined dialkyltin compounds, namely dimethyltin (DMT), diethyltin (DET), dibutyltin (DBT) and dioctyltin (DOT) inhibited the pyruvate dehydrogenase system in mitochondria. Analogously, Cain *et al.* [33] found, in addition to the α-ketoglutarate and pyruvate dehydrogenase complex inhibition which leads to the inhibition of the pyruvate and α-ketoglutarate oxidation, an inhibition of the oligomycin-sensitive complex at 2.8 n mol DBT/mg protein. The authors suggested that this was the effect responsible for the ATP synthesis inhibition. The inhibiting concentration value, if compared to that reported for the ATPase inhibition by TBT (about 3 μM), would suggest that DBT formed from TBT decomposition could be even more toxic than the starting compound.

The ATPase inhibition was confirmed by Cain *et al.* [34], while Aldridge and Cremer assessed that DBT inhibited oxidative phosphorylation as a consequence of the inhibitory effect of the dihydrolipoate cofactor in pyruvate and α-ketoglutarate dehydrogenase complexes [35]. It was observed that DBTC induces apoptosis [36]. Since mitochondria are involved in all kinds of apoptosis, this situation supports the possibility that the mitochondrial permeability pore (MTP pore) is the target of DBTC. This hypothesis is not supported by the results of Tomiyama *et al.* [37] who suggested that TBT induces apoptosis, while DBTC induces necrosis.

Toxicity of Triphenyltin (TPhT)

As regards the $(phenyl)_3Sn$- compounds (TPhT), as in the case of $(alkyl)_3Sn$- compounds, many effects were observed. The pattern is complicate and, surprisingly, in some cases, the effects (RC inhibition), even if slightly, depend on the species utilized for the mitochondrial preparation [38].

RC inhibition was first observed by Stockdale *et al.* [15], but the effect was time-dependent. Furthermore, since RC inhibition did not depend on the organotin compound, it was probably a consequence of the swelling. This lead to the conclusion that RC inhibition was a secondary effect. RC inhibition at the level of the cytochrome oxidase, was also observed by Barranco *et al.* [39]. Low doses of TPhT inhibit ATP synthesis [40] and induce apoptosis. As far as we are aware detailed data regarding specific interactions with the RC ase are not reported in the literature. Probably, as in the case of $(alkyl)_3Sn$- compounds, the ATP synthesis inhibition is correlated with the MPT pore opening [40].

Low doses of TPhT were found to inhibit the ATPase in submitochondrial particles which are obtained by ultrasound treatment of mitochondria [41]. Probably the swelling observed by Stockdale *et al.* [15] and by Zazueta *et al.* [42] in isolated mitochondria (although not cyclosporine-sensitive [43]), could be correlated with the MPT opening. The *in vitro* induced cell apoptosis by treatment with TPhT [40,43] was probably also due to the same phenomenon. The whole of data induce to suppose that the toxic effect of TPhT is due to the MPT opening in mitochondria and consequent induction of apoptosis.

CONCLUSIONS

Generally, toxicity of organotin compounds is due to an impairment to biological functions largely common to animal and plants, thus justifying the fact that many biological structures in different tissues and species exhibit the same sensitivity to the compounds. The fact that mitochondria are functionally and structurally similar in all eucaryots, as discussed in the first part, is an indispensable requisite in order to explain the widespread toxicity of organotin compounds.

A transport mechanism should be necessarily demonstrated or proposed to justify the presence of organotin compounds in cytoplasm and mitochondria. The utilization of physiological carriers has never been demonstrated, but, taking into account the properties of the biological membranes (even if the composition of the lipid bilayer can be different), organotin transport can be explained by means of the uncoupling mechanism or the Cl^-/OH^- exchange mechanism.

In isolated mitochondria, the $(alkyl)_3$-Sn- compounds inhibit all the steps involved in the ATP synthesis mechanism. The individuation of the step requiring the lowest effective dose is further complicated by the dependence of the responses in the presence of anions. A solution of the problem may come from experiments in isolated cells, where the induced apoptosis is cyclosporine-sensitive. Since an analogous cyclosporine-sensitivity was found in isolated mitochondria, the opening of the MPT pore is thought to represent the crucial step responsible for the ATP synthesis inhibition. This statement does not exclude the concomitance of other mechanisms, such as a protonophore effect and an Cl^-/OH^- electroneutral exchange, but at this stage it is impossible to quantitatively evaluate their effect.

Finally, the toxicity of the products formed by decomposition of the $(alkyl)_3Sn$- compounds, namely of partially dealkylated derivatives, has been undoubtedly assessed. Unfortunately contradictory results and controversial interpretations were provided and the mechanisms involved in their effects on mitochondrial functions are still a matter of debate.

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