Effects of ethidium bromide, tetramethylethidium bromide and betaine B on the ultrastructure of HeLa cell mitochondria in situ
A comparative binding study

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Summary. Several investigators have described the ultrastructural changes that occur in the mitochondria of cells in tissue cultures after treatment with the drug ethidium bromide (E). The mitochondria swell and the cristae become greatly altered and finally disappear; in the cristae-free region of the matrix electron-dense granules can be observed. It has been assumed that intercalation of E between the base pairs of the mitochondrial DNA induces the formation of the granular inclusions. To investigate whether intercalation is really the initial step in the generation of dense granules inside the matrix, we performed a comparative incubation study of HeLa-cell mitochondria in situ using three closely related dyes (D), i.e., E, tetramethylethidium bromide (TME) and betaine B (B). They strongly differ with regard to their affinity for DNA and their ability to cross membranes. E was used as a reference dye. TME does not intercalate, but is externally bound to DNA only weakly. The neutral B is not bound at all, but can cross membranes more easily than the cation E. Moreover, in aqueous solutions at pH ≈ 7.0, B is in equilibrium with its protonated cation BH. BH and E have almost equal affinities for DNA. Therefore, B may quickly pass the inner mitochondrial membranes and the cristae, and should then be bound inside the matrix, thus forming a BH-DNA complex. On the assumption that intercalation is necessary for the generation of intramitochondrial electron-dense bodies, we predicted that BH/B should be more efficient than E, while TME should be relatively ineffective. In experiments using HeLa cells, these predictions were found to be inaccurate. E, TME and BH/B produced almost the same mitochondrial alterations, but at different concentrations and after different incubation periods. In contrast to our expectations TME was much more effective than E and BH/B, with the last two behaving rather similarly.

Therefore, it seems unlikely that the drugs penetrate the inner mitochondrial membrane system by simple physical diffusion or that intercalation is the preliminary step for the generation of dense granules inside the matrix. Instead, we assume that hydrophobic interaction between the dye cations E, BH and TME and the cristae is the main cause of the mitochondrial changes. The favoured binding partner of the dye cations may be the divalent anion, cardiolipin; this phospholipid is an essential part of the inner membrane system but is absent in other membranes of cells. By distributing the dyes between a lipophilic phase and water, it was shown that TME is more lipophilic than E and BH; this may explain the greater effectiveness of TME. The bound dye cations disturb the organization of the cristae, which become altered and finally disappear. We assume that the electron-dense granules in the matrix are mainly composed of the dyes and former membrane materials such as phospholipids and proteins, as well as perhaps some other hydrophobic matrix materials. This would also explain why it was impossible to digest the dense granules by DNase treatment. The drugs enter the mitochondrial matrix by disordering and finally destroying the cristae.

Introduction

Some years ago, several investigators described ultrastructural changes that occur in the mitochondria of cells in tissue cultures treated with ethidium bromide (E; formula scheme; Soslau and Nass 1971; McGill et al. 1973, 1976; Heinen et al. 1974). They all observed effects that were more or less the same in the various cells studied. With increasing drug concentrations and incubating periods, the mitochondria swell, and the cristae exhibit alterations before almost completely disappearing. In the cristae-free region of the mitochondrial matrix, relatively large, electron-dense structures can be detected by electron microscopy. These dense granules are assumed to be E-mtDNA complexes of ethidium intercalated between the base pairs of the mitochondrial nucleic acid. It is possible that proteins are also present in these granules (McGill et al. 1976). From binding studies, it is known that DNA-bound E produces strong fluorescence (LePecq and Paoletti 1967). Indeed, it is also possible to visualize these mitochondrial granular inclusions using fluorescence microscopy (Heinen et al. 1974).

Several questions remain to be answered. The inner mitochondrial membrane system is unusually impermeable to cations, unless they are transported by a specific mechanism. It is highly improbable that cells should have such a transport system for ethidium cations. Therefore, the question arises as to by what pathway ethidium cations cross the membranes into the mitochondrial matrix. Moreover, it may be asked whether the intercalation of E into DNA is indeed the initial step of the generation of mitochondrial dense bodies.
In an attempt to answer these questions, we performed a comparative study using substances which differ considerably with regard to their ability to bind to DNA and to cross membranes. The following substances were used in our staining experiments (formula scheme): E, tetramethyl-ethidium bromide (TME; LeBret and Chalvet 1977) and betaine B (B; Finkentey and Zimmermann 1981). The last is an electrically neutral, dipolar molecule. In aqueous solutions, it undergoes protonation, thus forming BH cations (pK_A = 7.15). Therefore, at pH 7.0, an equilibrium consisting of nearly equal parts of the neutral species B and the cations BH is obtained. The four drugs D (E, TME, B and BH) are fluorescent dyes, that are closely related both chemically and sterically. Tissue cultures of HeLa cells were used in our incubation experiments.

The binding of the above-mentioned dyes to DNA was determined in aqueous solutions under comparable conditions in vitro using Scatchard isotherms (Pauluhn et al. 1978, 1979; Röding and Zimmermann 1982; references to earlier binding studies performed under different experimental conditions are given in these studies) as follows:

\[ r/c_{Df} = K_D(n - r), \]  

where \( c_{Df} \) and \( c_{Db} \) are the equilibrium concentrations of free (f) and bound (b) dye molecules (D), \( r = c_{Db}/c_{Df} \) is the number of bound molecules per mononucleotide unit (N), and \( n \) is the fraction of mononucleotides (\( \psi_N \)) capable of binding. At low concentrations of bound molecules \( c_{Df} \) the number of molecules that can bind is large compared with \( c_{Df} \). Therefore, the function \( G \) can either be neglected or replaced by its mean value \( \bar{G} \).

The binding constant \( K_D \) depends not only on the temperature (T) but also on the concentration \( c_N \) and type (S) of salts present in the solution, i.e. \( K_D = K_{D0}(T, c_N) \). \( K_{D0} \) rapidly decreases with increasing \( c_N \) values. Apparently, the molecules (D) are displaced by the action of the salts (S), i.e. a competitive effect is observed. However, in general, a residue of bound molecules (D) remains even at high salt concentrations. These findings can be explained by the assumption that D undergoes two types of binding to DNA (i.e. types 1 and 2), which can be distinguished by the competitive salt effect. Binding 1 is resistant to competitive salts and still occurs at high salt concentrations (non-competitive binding). It has been shown that non-competitively bound molecules intercalate (Ohmes et al. 1980). In contrast, in the case of binding 2, D is displaced by the action of competitors (S) like NaCl, KCl and MgCl_2 (competitive binding). This can be regarded as external or pre-intercalative binding, but it should not be confused with the 1:1 binding of D to DNA that occurs at high dye concentrations in the range of curved Scatchard plots.

At low dye concentrations \( c_{Df} \), the dependency of \( K_D \) on the competitor concentration \( c_S \) can easily be derived by means of equilibrium thermodynamics (for the derivation and discussion, see Zimmermann 1986):

\[ K_D = K_1 + K_2^* + K_3)/(1 + K_3 c_S). \]  

The three binding constants \( K_1 \), \( K_2^* \) and \( K_3 \) are related to the intercalation (1), the external binding of D (2) and the competitor (3), respectively. At low dye concentrations, the experimentally accessible constant, \( K_2^* = K_2(1 + G) \), differs only slightly from the real binding constant \( K_2 \) for externally bound D. Therefore, the function \( G \) can either be neglected or replaced by its mean value \( \bar{G} \).

The binding constants \( K_1 \), \( K_2 \) and \( K_3 \) as well as the parameter \( n \) of E, TME, B and BH were determined in the region of linear Scatchard plots, and are summarized in Table 1. Calf-thymus DNA was used as a model nucleic acid, and NaCl was used as the competitor (S).

The four molecules studied differ completely with regard to their binding behaviour. The cations E and BH both intercalate and are externally bound. The binding constant for the intercalation \( K_1 \), is more than one order of magnitude smaller than the binding constant for the external binding \( K_2 \). According to \( K_1 \lt K_2 \) the intercalated molecules are much more weakly bound to DNA than the externally bound cations. This finding is surprising and contradicts conventional opinions.

In contrast to E and BH, the cation TME is only externally bound but does not intercalate within the experimental range of error, \( K_1 \approx 0, K_2 \gt 0 \). For TME, the binding constant \( K_2 \) is significantly smaller than that found for its parent compound E.

Finally, the neutral molecule B neither intercalates nor undergoes external binding, i.e. \( K_1 \approx 0, K_2 \approx 0 \) (for details of the binding properties of E, TME, BH and B other molecules, see Zimmermann 1986).

Pure BH and B only occur in strongly acidic and alkaline solutions, respectively. However, our HeLa cells were cultured at pH 7.0, so that the medium contained both species in almost equal proportions. Therefore, when a binding experiment is started with B at pH 7.0, the sub-

<table>
<thead>
<tr>
<th>D</th>
<th>K_1</th>
<th>K_2</th>
<th>K_3</th>
<th>K_0</th>
<th>n</th>
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<tr>
<td>E</td>
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<td>1.4 x 10^5</td>
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<td>1.0 x 10^5</td>
<td>33</td>
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<td>0.21</td>
</tr>
<tr>
<td>TME</td>
<td>~0</td>
<td>1.6 x 10^5</td>
<td>30</td>
<td>2.9 x 10^5</td>
<td>0.21</td>
</tr>
<tr>
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<td>~0</td>
<td>~0</td>
<td>~0</td>
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<tr>
<td>BH/B</td>
<td>1.3 x 10^4</td>
<td>1.4 x 10^6</td>
<td>33</td>
<td>2.5 x 10^5</td>
<td>0.21</td>
</tr>
</tbody>
</table>

\( M = \text{moles per litre} \)

The \( K_D \) values were calculated with \( c_{NaCl} = 0.15 \text{ M} \), and the BH/B constants were calculated at pH 7.0.