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The influence of tetraphenylborates (hydrophobic anions) on yeast cell electro-rotation

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The action of a series of tetraphenylborate ion (TPB) derivatives on yeast cells was studied by electro-rotation of the pre-treated cells. TPB derivatives in which all four phenyl groups were substituted with fluorine, chlorine or trifluoromethyl were much more toxic than the unsubstituted compound, the effect increasing dramatically with increasing size of substituents. These observations suggest that the toxicity of these hydrophobic ions is determined mainly by their size and possibly also by the chemical inductivity of their substituent groups. The order of the toxicities of these ions was in fair agreement with literature values for their translocation rates across artificial bilayers. Incubation times of 3 h were used as standard, longer incubations (up to 48 h) showed that the number of cells affected by low doses of TPB increased with the logarithm of time after the first hour of incubation. Although measurements of the percentage of cells showing co-field rotation showed that controls were not adversely affected by incubations as long as 9 h, rotation spectra showed that some cells suffer loss of internal conductivity during extended incubations. Decrease of the pH of the incubation medium, or inclusion of high concentrations of NaCl or KCl, potentiated the effects of these hydrophobic ions. The toxicity developed slowly, and the sensitivity of the assay was only very weakly dependent on the cell suspension density.

Introduction

In a parallel study using a wide range of phenols [1], the toxicity of halophenols was found to increase regularly with the degree of substitution. Substitution with halogens is expected to increase the hydrophobicity of these phenols [2], and indeed the octanol: water partition coefficient was found to correlate very well with the toxicity in most cases.

However, precise interpretation of these effects was difficult because the pK_a of these chemicals is also a function of substitution. When the pK_a lies below the pH of the plasmembrane surface, then the toxicity was reduced, presumably because the ionized forms of the phenols have a very low membrane solubility. It is known that increasing the size of an ion decreases the energy required to bring it into a membrane, and indeed it is known that some larger organic ions adsorb well to artificial membranes. It was therefore of interest

Abbreviations: TPB, tetraphenylborate; TFPB, tetrakis(4-fluorophenyl)borate; TCPB, tetrakis(4-chlorophenyl)borate; TTFPB, tetrakis(3-trifluoromethylphenyl)borate; CNTPB, cyanotriphenylborate; PCP, pentachlorophenol.

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whether these hydrophobic ions could exert toxic effects at concentrations comparable to those at which the phenols are toxic.

For this purpose, tetraphenylborate (TPB) and its halogen-substituted derivatives form a useful series of hydrophobic ions because their binding to and translocation across artificial bilayers [3–8] and some natural membranes [9] have been measured. It is interesting to note that some of these ions (notably TCPB) are widely used in selective electrodes as a means of increasing the permeability of thicker artificial membranes to (polyvalent) cations [10]. This effect may possibly be interesting in connection with synergy between organic ecotoxins and heavy metals.

The study of the toxicity of these ionic compounds was carried out using cell rotation. This has proved to be a flexible method capable of high sensitivity and of observing effects in single cells, but also able to gather statistically significant data on trends within a cell population [11,12].

Materials and Methods

(a) Chemicals. NaTPB and KTCPB were obtained from Fluka; NaCNTPB from Schuchardt, München; TTFPB was a kind gift from Dr. Ross W. Flewelling, University of Los Angeles. KTFPB and initial supplies of NaTCPB (kindly donated by Dr. Shigura Itoh, National Institute, Okazaki) were manufactured by Dojindo Laboratories, Kumamoto, Japan.

Components of culture media were from Difco. All other chemicals were from Merck, and were of the highest purity available.

(b) Yeast culture and incubation. Strain R XII of *Saccharomyces cerevisiae* was donated by Dr. Kotyk, Prague. Cultures were maintained and grown up as already described for the brewery yeast in [11,12], except that a second foreculture was made. Both this second foreculture and the final culture were in YNBGC medium (0.67% yeast nitrogen base, 0.5% glucose, 50 mM citric acid adjusted to pH 4.5 with KOH). The use of the second foreculture was found to give more reproduceable assays than inoculation from yeast extract/peptone medium, presumably because it eliminated the possibility of the transfer of variable amounts of complex nutrients.

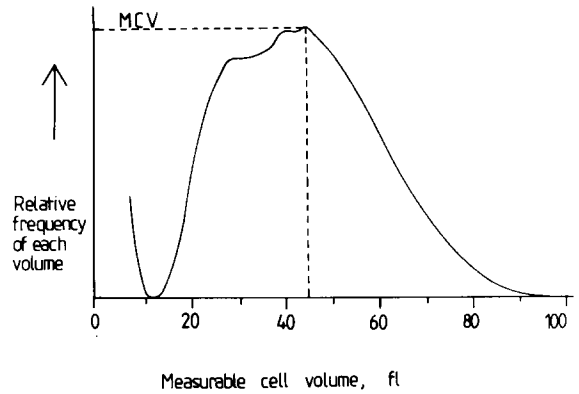

Fig. 1. The volume distribution of R XII yeast cells taken from the exponential growth phase in YNBGC medium. The rise of the curve below 12 fl is due to instrumental noise and can be neglected. The most common volume (MCV) is 43 fl, and integration of the complete curve gives a mean cytoplasmic volume of 44 fl.

The size distribution of the cells (Fig. 1) was determined by measurement with a hydrodynamically-focussing Coulter counter. The broad peak is a consequence of the population of cells being a mixture of budded cells and single cells. It is usually assumed that the cell wall is highly porous, so that it will be electrically invisible in high conductivity solutions such as used in the Coulter counter. Therefore the measured cellular mean volume of 44 fl represents only the volume enclosed by the plasmamembrane.

Cell density at harvesting was $(7–5) \cdot 10^6$ cells/ml. After washing the cells three times (centrifugation through water at $2700 \times g$ for 10 minutes) they were resuspended at approximately $3 \cdot 10^6$ cells/ml and counted so that the incubation density of $2 \cdot 10^5$ could be attained by appropriate dilution. All solutions of tetraphenyl borates were prepared in ethanol. The appropriate small quantities were added to the assay tubes which contained 5 ml medium (usually 2 mM citric acid/NaOH buffer pH 4.0 in 160 mM NaCl). Incubation was in a shaking water bath (1 Hz, stroke 4 cm) at 30 °C.

(c) Rotation. The co-field rotation assay is described in earlier [11,12] and in the accompanying paper [1], and references to suitable equipment can also be found there. The principle of this method is that fresh cells or those from control incubations do not rotate when exposed to a 300

kHz rotating field, whereas pre-treatment with supra-threshold quantities of many types of toxins gives co-field rotation.

The most critical point experimentally is the attainment of a conductivity of 10 μS/cm within the chamber. In this work, this was especially critical because the cells had usually been incubated in high concentrations of NaCl. The washing procedure consisted of centrifugation ($3000 \times g$, 8 min) through distilled water, which was repeated until the pellet, when resuspended in 5 ml, gave a suspension with a conductivity of 5 μS/cm or less. Three washes were usually sufficient. The conductivity was increased to 10.0 ± 0.05 μS/cm by the careful addition of 2.5 mM KCl. The rotation chamber (10 μl volume) was washed repeatedly with distilled water and with the cell suspension before measurements were attempted. Each measurement was of 150–200 cells.

Rotation spectra were taken on single cells using a frequency generator that was programmed to step from 3.28 MHz to 100 Hz in conjunction with a video recorder having single-frame playback.

Results

(a) Relative toxicities and dose dependence

Pre-incubation of yeast cells with phenylborate concentrations above a certain threshold caused a dose-dependent fraction of the cells to show co-field rotation in the assay. Fig. 2 shows dose-response curves for the four most toxic of the hydrophobic ions tested here. Insufficient data was obtained on CNTPB for a dose-response curve, but the results of five simultaneous incubations each using one of the five borates at a single concentration (1 μM) are shown in the bar graph in Fig. 3a. In addition, Fig. 3a shows the means and standard deviations of several determinations made on different occasions. Comparison of the dose-response curves and of the mean results at 1 μM concentration allows us to state that the toxicities of the various phenyl borates were in the order:

TTFPB ≈ TCPB > TFPB > CNTPB ≈ TPB

In the cases of TTFPB and TCPB, the percentage co-field rotation increased from control

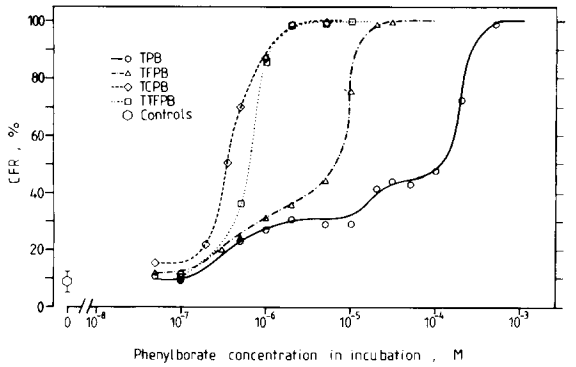

Fig. 2. The percentages of cells showing co-field rotation (CFR) after treatment with four different tetraphenylborate ions. Each point is the mean of typically three determinations (five in the case of TPB), each of which used 300 cells. The incubation was for 3 h, and used $2 \cdot 10^5$ cells/ml, in 160 mM NaCl, buffer 2 mM citric acid/NaOH (pH 4.0).

(5–12%) to near-saturation (90%) values over a 1 : 10 range of borate concentration. Therefore dose-response curves elicited by these ions are parallel when presented as semi-logarithmic plots (Fig. 2). TFPB exhibited a less steep dose-response curve, and TPB itself was unlike the substituted

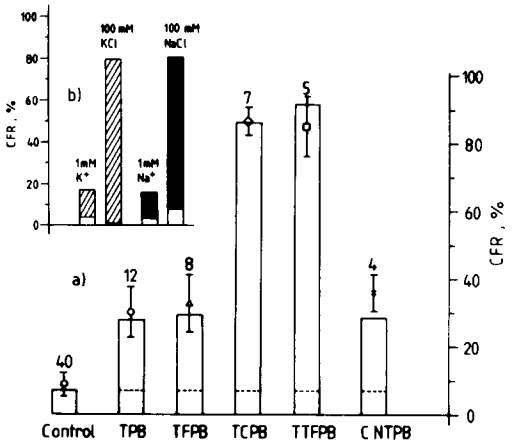

Fig. 3. (a) The relative toxicities of the five organoborates (all at 1 μmol/l) tested in a single day are shown as the bar-graph. Mean and standard deviations from larger numbers (as shown) of determinations on various occasions are also shown. Incubation conditions as for Fig. 2. (b) The increase in toxicity of 6 μmol/l TTFPB brought about by use of high-salt incubation media. The unshaded areas represent the control values. Incubation conditions as for Fig. 2, except that only 1 mM buffer and the indicated amounts of salt were used. CFR, co-field rotation.

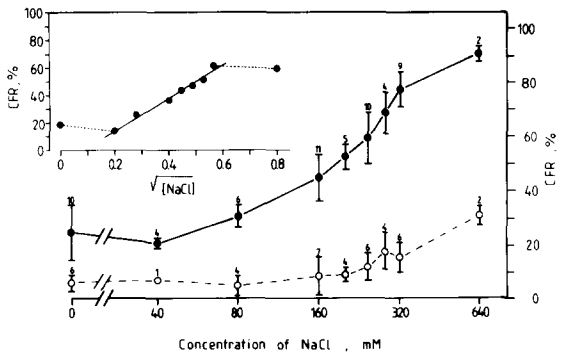

Fig. 4. The change in co-field rotation (CFR) brought about by increase of the NaCl content of the medium. The open symbols represent controls, the others are the results of treatment with 30 μmol/l TPB, incubation as for Fig. 2. The horizontal axis is logarithmic. The means, standard deviations and number of determinations are shown. (Inset) The same mean values, here expressed net of the controls, plotted against the square root of the salt concentration (units: $M^{0.5}$). The data points joined by the solid line exhibit a linear regression coefficient $r = 0.983$.

compounds in that the co-field rotation value rose in at least two phases, so that the co-field rotation was within the range 20% to 90% over at least three decades of concentration. This may be explained by saturation of transport or binding, or perhaps by the formation of an insoluble layer of KTPB.

As will be detailed below the toxicity of these ions was increased if the incubation medium contained high concentrations of salts, or if the pH was lowered. However, the relative toxicities of the series of ions remained the same.

(b) Effect of salt concentration in the medium

Figs. 3b and 4 show that use of high concentrations of salt in the incubation medium considerably increased the toxicity of near-threshold concentrations of the phenylborates. The TPB concentration used in Fig. 4 (30 μM) is sufficient to give a co-field rotation of 25% after incubation in low-salt medium. Increase of the salt concentration to 40 mM caused at most a slight decrease in co-field rotation, but above this concentration the co-field rotation value increased: 90% was reached after incubation in 640 mM salt. Control incubations were not affected by the increased salt concentrations until these reached values of 160 mM or higher.

The quantitatively equivalent effects of KCl and of NaCl on the toxicity of 6 μM TTFPB (Fig. 3b) indicated that this effect was not ion-specific. (TPB could not be used in such a comparison, because its potassium salt is almost insoluble.) It is therefore a reasonable working hypothesis to consider this as an ionic strength-dependent effect. There are two properties of the phenylborates that may give rise to an ionic-strength dependence of their toxicity: these are their hydrophobicity and their charge.

Hydrophobic toxins in aqueous solution should be made more toxic by increase in salt concentration because their activity coefficients (γ) are increased by the presence of most ions. This is the same effect as that giving rise to the salting-out of proteins and other molecules: the strength of this 'lyotropic' effect depends on the species of ions present, as described by the Hofmeister series [13–16]. According to Von Hippel and Schleich [14], the water structure that is induced around most ions (especially anions of high charge density) is incompatible with the water structure necessary for the solvation of hydrophobic substances such as benzene or (partly hydrophobic) protein molecules. In view of their charge, it may be doubted whether a similar salting-out effect is expected for the phenyborate ions.

It has recently been shown [7] that γ_{TPB} indeed increases with ionic strength between 100 mM and 1 M, and that this causes increased binding of hydrophobic ions by uncharged artificial membranes. At low ionic strength, γ at first decreases until a minimum is reached at 40–100 mM [7]. These changes are qualitatively consistent with the toxicity data given in Fig. 4, and are also highly suggestive of the phenomena of salting-in and of salting-out. However, quantitative estimation reveals that this effect due to hydrophobicity can only explain part of the salt-dependent increase in toxicity. (We shall not consider the salting-in effect, because the decrease in toxicity to which this should give rise was not visible at a statistically significant level in the yeast cell measurements.)

It is found that the degree of the dependence of activity coefficient on salt concentration is described by a coefficient k_s, which is a constant for any given hydrophobic substance and salt respec-

tively, as given by the Setschenow equation [13,14]:

$$\log \gamma \equiv \log (S_0/S_c) = k_s \cdot C \quad (1)$$

where S_0 and S_c are the solubilities of the hydrophobic solute at salt concentrations of zero (where $\gamma \equiv 1.0$) and of C (molar), respectively. Inspection of k_s values for a given salt type [14] shows that they increase with hydrophobicity and with molecular weight, in agreement with the above explanation of salting-out. The mean value of γ for KTPB increases to 1.45 at an ionic strength of 1 M [7], whereas that of KCl is reduced to 0.6 [7]. Assuming that the activity coefficients of K^+ and of Cl^- are equal at $\sqrt{0.6} = 0.77$, we obtain $\gamma_{TPB} = 1.88$. Use of Eqn. 1 gives a value for k_s of 0.27. This is in accord with TPB being salted-out as a hydrophobic particle; the smaller model hydrophobic molecule benzene has $k_s = 0.19$.

However, the increase in toxicity of the phenylborates caused by high salt corresponds to much higher values of k_s, if it is assumed that hydrophobicity is the only effect operating. It is apparent from Fig. 3b that 100 mM KCl or NaCl were sufficient to increase the number of cells affected from below 20% to 80%, equivalent to raising the TTFPB dose by a factor of approximately 3 (as estimated from Fig. 2). The appropriate molar value of k_s is 4.7, which is much higher than that seen in the artificial membrane work [7] and greater than that of even large proteins in NaCl [14]. It is necessary to conclude that the increase in activity coefficient can only account for part of the effect of NaCl seen when phenylborates are applied to yeast cells. This is confirmed by experiments (not shown) in which the NaCl was replaced by salts such as sodium citrate or phosphate. These are expected from their position in the Hofmeister series to give a much greater increase in activity coefficient: however, they gave slightly weaker effects than NaCl at the same osmolarity.

An alternative interpretation of the 'salt effect' is based upon the Coulombic interaction between the negatively charged phenylborate ions and the membrane, which is also negatively charged. The concentration of negative ions at the membrane surface will be lower than that in the bulk by a factor that depends on the surface potential ψ_0, which is determined by the charge density on the outside surface of the membrane, σ_{os}:

$$\psi_0 = \frac{\sigma_{os}}{z \cdot e} \left(\frac{kT}{2 \cdot N \cdot c_1 \cdot \epsilon_1} \right)^{1/2} \quad (2)$$

where kT is the product of Boltzmann constant and absolute temperature, z is the valency of the ions (symmetrical electrolyte assumed), e is the magnitude of the electronic charge, N is the Avogadro number, and C_1 and ϵ_1 are the electrolyte concentration and the absolute permittivity of the medium. In order to arrive at an analytical expression for ψ_0, the derivation of Eqn. 2 uses a simplification based on a series expansion of $\sinh(z \cdot e \cdot \psi_0/2kT)$ and so is only strictly valid for $z \cdot e \cdot \psi_0 \ll 2kT$ [17]. The restriction to symmetrical (1 : 1) electrolytes is also necesssary to obtain an analytical expression. The magnitude of ψ_0 was discussed in the accompanying paper [1].

The ratio between the anion concentrations at the surface (C_{os}) and in the bulk (C_1) will then be given by:

$$\log (C_{os}/C_1) = -\psi_0/(0.059 \cdot z \cdot e) \quad (3)$$

ψ_0 is invariably negative, so that $C_{os} < C_1$ for $z = -1$ (anion).

Eqns. 2 and 3 shows that the logarithm of the toxicity should increase with the square root of the ionic strength. The same rotation data (net of control values) was therefore plotted against the square root of the salt concentration (Fig. 4, inset). The linearity of the plot is very good, which may be considered surprising because the rotation index is usually linearly proportional to the toxin concentration, and not to its logarithm as demanded by Eqn. 3. However, in the case of TPB, the co-field rotation increases in several steps over a wide range of concentrations (Fig. 2) which may explain why the linear dependence shown in the inset to Fig. 4 was obtained.

The above hypothesis that the salt-dependent increase in toxicity of tetraphenylborates is mainly due to a decrease in ψ_0 is supported by preliminary results that show that salts of polyvalent cations are more effective in this respect than NaCl or KCl. Both this explanation of the salt effect and that based on the correlation with the

data on γ_{TPB} are consistent with the hypothesis that the toxicity is proportional to the quantity of ion bound, or transport-rate-limited by, a membrane (presumably the plasma membrane).

The salt effect was used to increase the sensitivity of the method. The majority of the incubations in this study were carried out in 160 mM NaCl, the highest value that did not adversely affect control assays. (Rotation was in low-salt medium, as usual.) In studies of heavy metal toxicity [11,12], high salt concentrations were not advisable due to the possibility of complex formation. This cannot occur with these fully substituted borates; in fact high ionic strength is actually an advantage in suppressing the effects of the changes in buffer ion concentrations with pH.

(c) Incubation pH

The sensitivity exhibited by the yeast cells was markedly increased by decreasing the pH of the incubation medium, as shown in Fig. 5a. It can be seen that the effect of 30 μM TPB could be made to increase from almost zero up to almost maximal (90% co-field rotation) by decreasing the incubation pH from 8.0 to 3.0. In achieving the most sensitive assay conditions, the limiting factor was once again the rise in the co-field rotation of the control incubations. The lowest pH that gave

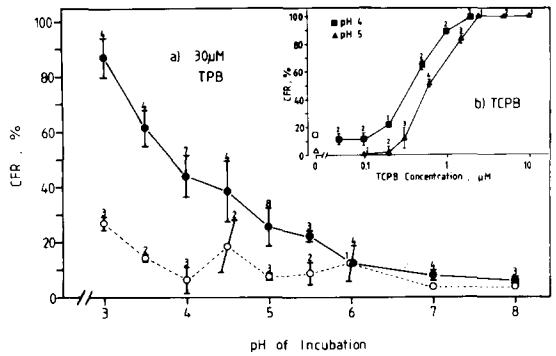

Fig. 5. The increase in the co-field rotation (CFR) caused by decrease of the medium pH. The solid symbols represent treatment with 30 μmol/l TPB. It can be seen that the controls (open symbols) are adversely affected if the pH is less than 4. Means, standard deviations and the number of determinations are shown for each point. (Other incubation parameters as for Fig. 2.) (Inset) The shift of the dose-response curve for TCPB brought about by decreasing the medium pH from 5.0 to 4.0.

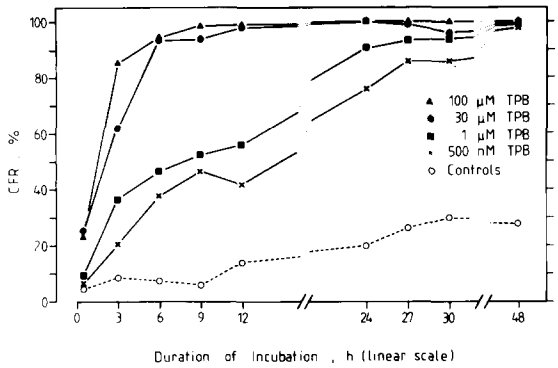

Fig. 6. The increase in the number of cells that were affected by TPB as the incubation time was increased. The progressive deterioration of the controls with incubation times longer than 9 h is also apparent. Other incubation conditions were as for Fig. 2. Values shown are means of two or more determinations. CFR, co-field rotation.

essentially unaffected controls was 4.0, therefore most subsequent work used this pH.

Measurements carried out after the incubation showed that there was no significant change in pH from the initial values. The buffer concentration of 1 or 2 mM appears to have been adequate, as expected at a cell concentration as low as $2 \cdot 10^5$ cells/ml. (The mean volume of the cells was 44 fl, giving a cytoplasmic volume of only 10 nl per ml suspension, or one part in 10^6. Even 1 mM buffer was equivalent to a concentration of 1000 M within the cell volume.)

Fig. 5b shows the increase in sensitivity to TCPB brought about by decreasing the incubation pH from 5.0 to 4.0. The increase in the control values is rather greater in this case.

(d) Incubation duration

The effect of TPB increased with incubation time, as may be seen from Fig. 6. The longest incubation made was 48 h, but even after this period the sensitivity was still increasing. Longer incubations were not carried out because the control co-field rotation started to increase after 9 h.

3 h incubations were used in the majority of this work to enable comparison with results obtained earlier. In that work, it was observed that the action of Ag^+ was complete after 20 min [11], whilst that of Hg(II) was slowing down after 3 h [12]. In these heavy metal systems, it appeared

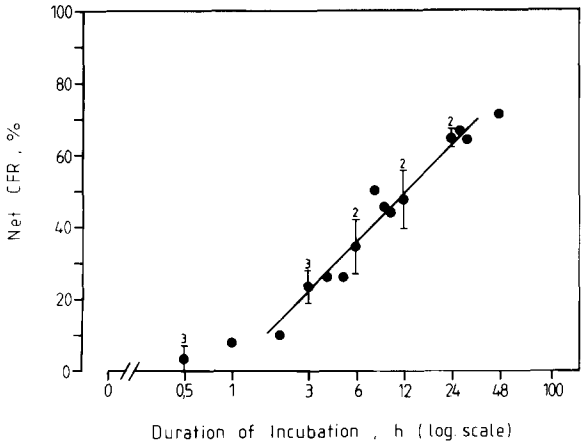

Fig. 7. The 1 μmol/l TPB data from Fig. 6 (but also including some values determined once only) plotted on a logarithmic time axis. The values are net of the controls, so that the rather high control values obtained after prolonged incubation do not contribute to the data. The line was obtained by the least-squares method applied to the data taken for incubation times between and 30 h, and the regression coefficient r is 0.957. CFR, co-field rotation.

reasonable to equate toxic effect with binding, which should also occur on a time scale of 3 h or less. We must conclude that the phenyl borate binding has no kinetic relationship to the toxic effect, because it is difficult to imagine why the binding should be so slow.

Fig. 7 shows the co-field rotation (after subtraction of the control) plotted as a function of the logarithm of incubation time. The plot is linear for those incubation times longer than approximately 1 h. This is a remarkable dependence which no single mechanism can explain. A possible explanation assumes that the susceptibility of the cells varies over a very wide range, so that rise in co-field rotation of the population is effectively the sum of the contributions from a large number of sub-populations which become affected at widely different times.

(e) Rotational spectra after prolonged incubation

The use of prolonged incubation times such as discussed above raises the question of what changes take place in the cells over these long periods, and whether the rotational spectra can be assumed to be the same as those taken after the incubation period of 3 h used until now.

Fig. 8a shows typical examples of spectra of cells taken from control incubations after 24 h. It is clear that the anti-field rotation is still the dominating feature of the spectra. At 300 kHz, these cells show no rotation, just as do fresh controls. Accordingly, the co-field rotation values of controls were low up to at least 9 h (see Fig. 6). After 48 h incubation, cells with spectra (points ▲ in Fig. 8b) identical to those of toxin-treated cells began to more numerous, even in control incubations.

Other cells (points ○ in Fig. 8b) showed intermediate spectra, with a weakened anti-field rotation. In contrast to the intermediate type of spectrum of some pentachlorophenol-treated cells [1], this rotation possessed a definite peak. Modelling of spectra has shown (see Fig. 1 of Ref. 12) that this weakened co-field rotation peak can be attributed to cells with an electrically functioning membrane that have lost the majority of their inner conductivity. At the measurement frequency of 300 kHz, such cells showed a very slow co-field rotation, so that the rise in co-field rotation in the

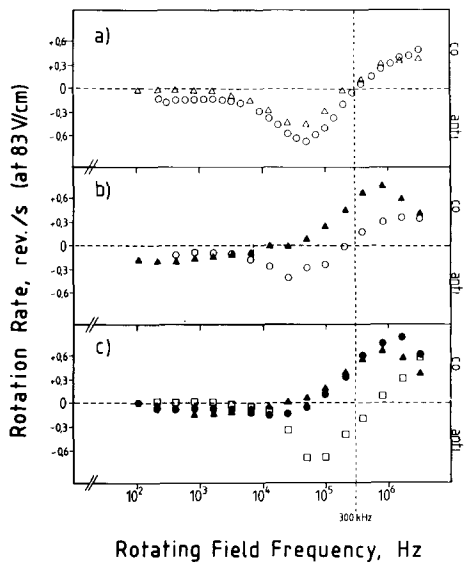

Fig. 8. Typical rotation spectra of single cells taken after prolonged (24 and 48 h) incubations. (Other conditions as for Fig. 2.) In all cases spectra typical of damaged cells (even in control incubations) are traced by filled symbols. Open symbols represent apparently undamaged or intermediate stages. (a) Control, 24 h. (b) Control, 48 h. (c) 1 μmol/l TPB, 48 h. For further description, see text.

control cell population (Fig. 6) was presumably due to the appearance of increasing numbers of cells of both types shown in Fig. 8b (fully-permeabilised, and ion-depleted).

The above show that incubation times longer than 9 h and more especially longer than 24 h constitute a stress to the cells. However, even after 48 h incubation in the presence of 1 μM TPB, there were still cells exhibiting spectra (points □ in Fig. 8c) identical with fresh controls. This was despite the fact that 87% of the cells from this incubation showed co-field rotation, with spectra (points ● and ▲ in Fig. 8c) attributable to fully-permeabilised cells such as seen after shorter but more acute toxin treatments. As in (d) above, it must be concluded that the cell population exhibits a very wide range of resistance.

(f) Cell concentration

Except where otherwise stated, all incubations were carried out at low suspension density. This was because work with Ag^+ and Hg(II) has shown that the cell concentration in the incubation can radically decrease the sensitivity to toxin.

However, preliminary results with the phenylborates show that increasing the cell density has only an extremely weak effect on the sensitivity. It is therefore not possible to estimate how much phenylborate is bound per cell, as was done in the case of Ag^+. In the case of Ag^+ and of Hg(II), it was possible to consider that each cell could bind (harmlessly) a certain amount of metal, thus explaining the pronounced decrease in toxicity as a function of cell concentration. The reason may be that (as suggested above) the binding of the phenylborates is very slow, perhaps due to transport limitation at the plasma membrane. If this were the case, then increasing the cell density would not decrease the concentration of phenylborate present after 3 h incubation, but only after much longer intervals.

Discussion

The results show a striking dependence of the toxicity on the size of the ions. A slight increase of the radius (about 50% [8]) of these ions results in an increase in toxicity (in a 3 h incubation) by almost 1000-times (Fig. 2). This is the reverse of the situation usually seen for size dependence of the membrane permeability of lipophilic molecules [2]. However, an interpretation of this trend in terms of permeability is nevertheless very attractive, because the toxicity series is remarkably similar to the series formed by comparing the transport kinetics of these ions through artificial bilayers [8]. The sensitivity can therefore be interpreted either as being due to a rate limitation of trans-membrane transport, or else in terms of the attainment of a critical concentration within the membrane. However, even in the second case, transport across the membrane may be a limiting factor because the interior of the cell will continue to deplete the membrane of ions until inside and outside are in equilibrium.

If it was indeed transport into the cell that was rate-limiting, this is equivalent to saying that the 3 h incubation was much shorter than the equilibration time. This is certainly the case for TPB as demonstrated in Figs. 6 and 7, from which it may be surmised that the TPB could actually be as toxic as its larger derivatives, if much longer incubation times were practical. (Preliminary data on TCPB indicates that it is indeed much faster than TPB, but more work is required to quantitate this).

The similarity with the data obtained on bilayers suggests that it should be possible to calculate the equilibration times for a cell-sized unilamellar vesicle having a PE- or PC-based bilayer membrane. The bilayer work gives values for, amongst other things, the partition coefficient β (units: M) of ions between bathing solutions and the membrane (both sides considered together) and the translocation rate k_i (units s^{-1}) of ions from one side to the other of the membrane.

The time constants measured on cells are of the order of minutes or hours, therefore we assume that aqueous diffusion resistance (unstirred layer effects etc.) and the kinetics of adsorption to and desorbtion from the membrane can be neglected, and that the internal membrane barrier is the only significant resistance to transport. This means that the time constant is given by:

$$\tau = C \cdot \text{Volume}/I_0 \qquad (4)$$

where C is the internal end-concentration of ion

(for a vesicle, assumed equal to the initial concentration in the medium), and I_0 is the initial rate of influx, given by multiplying the amount of ion bound to the outside of plasmamembrane by k_i:

$$I_0 = \text{Area} \cdot k_i \cdot C \cdot \beta / 2 \quad (5)$$

Approximating the cell to a sphere of radius a (m), we obtain:

$$\tau = a/(1.5 \cdot \beta \cdot k_i) \quad (6)$$

For a given cell size, the time constant for equilibration should be proportional to $1/(\beta \cdot k_i)$, and accordingly we have calculated this parameter from the data in [8] to allow comparison between the cell toxicity data and the trans-bilayer transport data (Table I). As may be seen, the differences in toxicity between the TPB derivatives parallel changes in $1/(\beta \cdot k_i)$ rather well, except in the case of CNTPB (which is not strictly a member of this homologous series and did not give reproducible effects in the cell toxicity tests). The parallel trend supports the hypothesis that it is the rate of transport across the hydrophobic membrane barrier, and therefore the size of the ion, that dominates the toxicity.

It is apparent that the bilayer data cannot be applied too directly to cells, because the time constants that can be calculated from Eqn. 6 are far shorter than those observed in cell toxicity experiments. Taking a value for a of 2.2 μm, (estimated from the mean cell volume of 44 fl shown in Fig. 1.), we calculate values of $5.6 \cdot 10^{-5}$ s and $4.5 \cdot 10^{-4}$ s for the time constant of TPB diffusion into similarly sized vesicles having PE or PC membranes. On the other hand, it can be seen from Fig. 7 that the observed time constant of TPB action on cells is of the order of 10^5 s (24 h). Therefore, if we wish to consider the biophysics of the bilayer and the cell membrane as similar, we must account for this observation that the transport across the cell membrane is a factor of roughly 10^9 slower than expected purely from bilayer work.

Roughly three orders of magnitude may be accounted for by the cell membrane potential (ψ_m). The influence of ψ_m on the translocation rate constant k_i amounts to one decade per 59 mV. According to Bakker et al. [18], the membrane potential of cells of the yeast *Endomyces* is -190 ± 13 mV at pH 4.5 and -275 ± 11 mV at pH 7.1, from which a value of ψ_m of -174 mV can be extrapolated for the pH used here (4.0). Therefore the membrane potential can be expected to have reduced the transport rate of the phenyl borates by $174/59 = 3.0$ orders of magnitude. Although this value of ψ_m is much larger than that found in the yeast *Pichia* by Höfer and Novacky [19] ($\psi_m = -48$ mV at pH 6.0), it is in agreement with the value reported for *Neurospora* [20]. Unfortunately, *S. cerevisiae* is too small to allow direct (microelectrode) measurements, and the cation-distribution method of measuring pH is reported to be inapplicable to yeasts [18].

The above discussion in terms of the membrane potential would also account for increase in toxicity as the incubation pH was lowered (Fig. 5). The

TABLE I

COMPARISON OF THE TOXIC DOSES OF FIVE HYDROPHOBIC IONS WITH THEIR TRANSPORT PARAMETERS PREDICTED FROM BILAYER MEASUREMENTS [8]

The data enable comparison to be made between the dose of phenylborate required to give 90% co-field rotation and the rate of ion transport estimated from studies on PE (phosphatidylethanolamine) or on PC (phosphatidylcholine) bilayers (BLM). β is the partition coefficient between aqueous solution and artificial membranes of these lipids, and k_i is the translocation rate constant across the membrane [8]. For further details, see text and Eqns. 4–6.

Ion	$TTFPB^-$	$TCPB^-$	$TFPB^-$	TPB^-	$CNTPB^-$
Dose for 90% CFR (μmol/l)	1.2	1.1	14	350	≈ 100
$1/(\beta \cdot k_i)$ (m/s)					
PE BLM	$1.1 \cdot 10^{-1}$	$1.4 \cdot 10^{-1}$	$4.3 \cdot 10^{-1}$	$7.6 \cdot 10^{1}$	$1.2 \cdot 10^{4}$
PC BLM	$6.3 \cdot 10^{-1}$	1.4	4.0	$6.2 \cdot 10^{2}$	$6.2 \cdot 10^{4}$

membrane potential of yeast is measured to decrease as the pH is lowered [18]. However the large kinetic factor of roughly 10^6 remains unaccounted for.

It may be thought that sieving or binding by the cell wall could also contribute to the slow action of these ions in yeast cells. A sieving effect cannot be responsible for the observed data, because the toxicity of the substances increases with their size. In addition the yeast cell wall only restricts the diffusion of molecules of molecular weight $M_r > 760$ [21] whereas the series TPB to TTFPB have $M_r = 320$ to 592. Binding of negative ions is unlikely because the yeast cell wall has a negative charge [22].

A second interesting characteristic of the toxicity of these ions is that the form of the dose-response curves of TFPB and TPB is unlike that seen with heavy metals [11,12] or with any of the phenolic ecotoxins [1]. In the case of TPB a plateau is visible in the dose-response curve at concentrations rather above 1 μM. This is very interesting in view of the observation that saturation of TPB binding to artificial bilayers occurs at concentrations of 1 μM [3]. It is plausible to assume that the first plateau is due to a saturation in binding, but that considerably higher concentrations of tetraphenylborate are toxic via a different route.

In view of the above, and of the effect of salt concentration on toxicity (section (b) of Results), it seems possible that the toxic effects of these ions are due to their concentration within the plasma membrane. The related transport model is very attractive (see discussion of Table I), but the process in cells still seems much slower than predicted from the bilayer data. A possible explanation may be that the binding of amphipathic molecules to biomembranes is much lower than to artificial systems [23]. In addition, the dipole potential [17,24] of the yeast plasma membrane is unknown, but could also act to decrease the transport rate of lipophilic ions.

In support of the binding model of toxicity, it should be noted that one consequence of the incorporation of these ions in artificial membranes is a drastically increased permeability to cations [10]. If this were the case in the plasmamembrane also, then the cell would suffer a general metabolic collapse.

It is possible that the apparent insensitivity of the yeast cell toxicity when compared to the bilayer binding and translocation data could be due not to a lack of binding or translocation in the yeast cells, but to a very slow kinetics of action of the phenylborates once bound. In fact the long-term incubations (Fig. 6) do show that the sensitivity of this method may be increased by using longer incubations. Unfortunately, periods longer than 9 h give poor controls, and so this possibility must remain open for the present. The spectra of the cells that had been incubated for longer periods indicates that a gradual loss of cytoplasmic conductivity may be the cause of the increase in co-field rotation of the controls. In addition, the appearance of spectra intermediate (Fig. 8) between those typical of 'control' or 'damaged' from 3 h incubations may make use of the distinction between rotating and non-rotating cells rather difficult, and thereby rob the co-field rotation method of its simplicity of observation.

Conclusion

Interesting data on the kinetics of the toxicity of the phenylborates to yeast cells were obtained, so that striking correlation with artificial bilayer data could be made. The modification of this toxicity by medium pH and salt content were demonstrated. Although a parallel study [1] indicated that only the uncharged forms of phenols exhibited significant toxicity, it could be shown here that some of the hydrophobic ions are indeed toxic at very low doses (0.2 μmol/l). The toxicity of these ions increased dramatically with their size, probably because of a decrease in the energy required to bring them into the membrane adsorption plane, or to transport them across the membrane.

Use of the rotation technique allows the effects of long-term incubations to be observed in single cells, so that various types of damaged cells could be identified. Both single-cell and statistical observations indicate that the susceptibility of yeast cells to long term incubations varies over a wide range.

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