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The comparative influence of substituted phenols (especially chlorophenols) on yeast cells assayed by electro-rotation and other methods

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The toxicity of 31 phenols was studied by electro-rotation of yeast cells. Control yeast cells show both anti-field and co-field rotation, depending upon the field frequency applied. After treatment with supra-threshold amounts of phenols the anti-field rotation is weakened or abolished and a stronger co-field rotation can be seen. The proportion of cells showing the co-field rotation was found to be a sensitive measure of toxicity. Doses of 2.2 μmol/l of pentachlorophenol, or of 0.3 μmol/l of pentabromophenol were detectable after 3 h incubation at pH 4.0. At a given pH, the toxicity of the chlorophenols correlated extremely well with their octanol:water partition coefficients (P_{ow}). The complete set of phenols showed fair overall correlation with P_{ow}, but less good correlation with their acidity constants (pK_a). In particular the toxicity of a given phenol was less than predicted from its pK_a if the incubation pH was higher than the pK_a. Biochemical assays on 23 of the phenols showed that the rotational sensitivity runs closely parallel to the sensitivities of cell growth rate and of the plasmamembrane ATPase, but less closely to the inhibition of purine incorporation. It appears that the electro-rotation method provides a useful and rapid test for the presence of organic ecotoxins. The test enables us to distinguish differences between single cells, and is comparable in sensitivity to biochemical tests that use vesicles or homogenates derived from a cell population.

Introduction

Phenol and its derivatives such as pentachlorophenol [1] are important environmental toxins, being present as components of waste water from the processing of oils, and from the manufacture and spillage of anti-oxidants, wood preservatives, disinfectants and other types of biocides. Methods for the assay of these compounds are therefore of interest, particularly when the assays use living organisms which may be sensitive to some aspect of the toxicity that is not easily detected by chemical or physical methods. On the grounds of ethics, rapidity and economics, tests carried out on a micro-organism such as yeast are preferable to toxicity evaluation in animals.

It is known that substituted phenols (particularly halophenols) affect various membrane functions, such as hypoxanthine transport [2], and both mitochondrial [3] and photosynthetic [4] elec-

Abbreviations: P_{ow}, partition coefficient between octanol and water; Mes, 4-morpholineethanesulphonic acid.

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tron transport. It can therefore be expected that the effects of these compounds should be detectable using electric-field-induced cell rotation [5,6], because the characteristics of the membrane have been shown to strongly affect electro-rotation [7–10]. Cell rotation has the useful property that differences in the degree of damage between single cells can be observed [9,10].

In this work we show that sufficient concentrations of the phenols produce a change in the rotation spectrum of most of the yeast cells that is very similar to that produced by treatment with heavy metals [8,9] or tetraphenylborates [10]. However, the kinetics, the dependence on cell density in the incubation medium and the pH sensitivities are different.

The bio-availability and therefore the toxic potential of the majority of organic toxins are expected to depend on the rate at which they can cross the plasmamembrane. In the absence of specific transport systems, we therefore expect a correlation of toxicity with 'lipophilicity' [11–13]. The best index of lipophilicity is usually taken to be the partition coefficient (P_{ow}) between octanol and water, because this coefficient has been found to have a better correlation than other partition coefficients to a wide range of toxicity data [11]. P_{ow} values for the majority of the phenols measured here have recently been measured [14,15].

A further index that has been correlated with toxicity is the pK_a, or acidity constant. In the case of the phenols generally, the more acidic the compound, the more toxic it is. This raises an apparent contradiction, in that more acidic materials should ionize more readily, and therefore be less toxic. This is because ionic forms require the Born energy to bring them from a high dielectric constant medium into the low dielectric constant membrane interior [16–18]. However, ions can exhibit a high toxicity if they are sufficiently large and hydrophobic [10]. The difference in dielectric properties between aqueous solutions and membranes may also cause alterations in the physicochemical properties of materials when they transfer between these two phases. For example, the pK_a of membrane bound PCP is significantly lower than in solution [19].

To elucidate the above points we tested a series of substituted phenols and attempted to compare their effects on cell rotation at several pH values with their partition coefficients and pK_a values. We present correlations of the rotation data with other assays using the same strain of yeast. These other assays are based on the inhibition by these phenols of the plasmamembrane ATPase, of purine incorporation [2,14,15] and of growth rate [20] of R XII yeast.

Material and Methods

(a) Chemicals. All water used was double-distilled of conductivity 1.5 μS/cm or less. The phenols used are listed in Table I where each is given a reference letter to enable uncluttered labelling of figures. The pK_a values presented there are derived from standard works [13,21], or estimated with reference to phenol by the use of 'sigma' values [13]. The phenols were from the sources shown in Table I; analytical grade was obtained where available. In cases where two suppliers are shown, either an independently prepared batch of toxin was used to check a particularly interesting result or else a purer preparation became available. All other chemicals were of purest grade available from Merck (D6100 Darmstadt, F.R.G.).

Stock solutions of the phenols were made (by direct weighing and dilution, where necessary) at concentrations approximately 100 × those to be used in the assays, so that volumes of 10–100 μl had to be added to the assays (5 ml). The low aqueous solubility of many phenols made it necessary to use ethanol as the solvent for the stock solutions: in these cases ethanol was added to all assays (including controls) so that they all contained the same amount (maximum 100 μl per 5 ml). The solubility limit of the least-soluble phenols was determined by observing the conductance of 50 ml aqueous solution while 50-μl aliquots of ethanolic solution were added.

In order to ensure that the buffer capacity of the assay was not overloaded by the addition of relatively acidic toxins, such as picric acid, 2-methyl-4,6-dinitrophenol or pentafluorophenol, these toxins were neutralised with KOH before use. In all other cases the concentration of phenol assayed was so low, or the pK_a so high, that the buffer capacity was not in doubt.

TABLE I

IDENTIFICATION OF THE 31 PHENOLS AND THEIR SOURCES, THEIR ACIDITY CONSTANTS, AND THE CONCENTRATIONS OF THEM THAT GAVE 20%, 50%, AND 90% CO-FIELD ROTATION

Each phenol has been given code letter(s) to allow identification of the data points in the figures. The suppliers are also identified by code letters as follows: Al, Aldrich-Chemie, D-7924 Steinheim; Bl, prepared by the Institute for Experimental Biology and Medicine of the Forschungsinstitut Borstel (D2061 Borstel, F.R.G.); Eg, Ega Chemie, D-7924 Steinheim-Albuch; Fl, Fluka, D-7910 Neu-Ulm; Mk, Merck, D-6100 Darmstadt. m, value confirmed by direct titration. S: value probably affected by poor solubility of the phenol. n.s., not sufficiently soluble to allow a determination.

Abbreviations: $pCFR_x = -\log_{10}$ (concentration that gave rise to a CFR of x%). Except where otherwise shown, the pK_a values were obtained by use of Hammett sigma values following the recommendations and substituent constants given by Hansch and Leo [13]. A pK_a of 10.0 [21] was taken for phenol itself.

Reference letter	Substance	Source	pK_a	$pCFR_{20}$		$pCFR_{50}$	$pCFR_{90}$
				pH 4	pH 6	pH 6	pH 6
A	Phenol	Al, Mk	10.0	1.57	1.20	1.17	1.11
B	4-Chlorophenol	Mk	9.4	2.66	2.33	2.07	2.00
C	2,3-Dichlorophenol	Al	7.7	3.22	2.92	2.68	2.52
D	2,4-Dichlorophenol	Eg, Mk	7.9	3.22	3.00	2.77	2.57
D′	3,4-Dichlorophenol	Mk	8.5	3.22	–	–	–
E	2,6-Dichlorophenol	Al, Mk	7.1	2.80	2.75	2.52	2.34
F	2,4,5-Trichlorophenol	Mk	7.1	4.05	3.51	3.26	3.17
F′	3,4,5-Trichlorophenol	Fl	7.7	3.80	3.36	3.30	3.14
G	2,4,6-Trichlorophenol	Mk	6.5	–	3.64	3.28	n.s.
G′	2,3,5-Trichlorophenol	Al	6.15	3.52	–	–	–
H	2,3,4,5-Tetrachlorophenol	Eg, Fl	6.1	4.80	4.14	3.92	3.82
J	Pentachlorophenol	Fl, Mk	4.8	5.66	4.48	3.96	3.82
K	2-Bromophenol	Eg	7.7	–	2.51	2.08	1.90
K′	4-Bromophenol	Fl	9.4	2.87	2.52	2.40	2.28
L	4-Nitrophenol	Fl	7.1	–	3.40	2.85	2.59
N	2,4,6-Trinitrophenol	Bl, Mk	1.0 [a]	–	2.05	1.74	1.57
P	3,5-Dimethoxyphenol	Mk	9.4	–	1.77	1.54	1.39
Q	4-Hydroxyphenol	Bl	10.3	–	0.77	n.s.	n.s.
R	4-Hydroxybenzoic acid	Mk	5.2	–	1.96	1.75	n.s.
S	4-Chloro-3,5-dimethylphenol	Eg	9.5	–	3.17	2.96	2.82
T	2-Amino-4-methylphenol	Eg	5.6 [b]	–	1.48	1.07	n.s.
U	4-Methyl-2-nitrophenol	Al	6.9	3.00	2.62	2.10	n.s.
V	2-Methyl-4,6-dinitrophenol	Al	4.0	–	3.26	2.62	n.s.
W	3-Hydroxyphenylurea	Al	10.1	–	1.40	n.s.	n.s.
X	3-(Trifluoromethyl)phenol	Al	8.9	–	2.64	2.31	2.14
Y	3,5-Di(*t*-butyl)-phenol	Al	10.7	–	4.22	3.96	3.77
Z	2,4,6-Triiodophenol	Al	6.4	n.s.	4.96	4.30	n.s.
BI	4-Iodophenol	Fl	9.3	3.15	2.59	2.40	–
GB	2,4,6-Tribromophenol	Fl	5.5	4.47	n.s.	n.s.	n.s.
JB	Pentabromophenol	Al, Eg	3.7	6.52	6.20	5.7 (S)	n.s.
JF	Pentafluorophenol	Fl	5.7 (m)	2.92	2.34	2.05	n.s.

[a] Literature value from Ref. 21.

[b] This pK is that of the amino group. The pK of the hydroxyl group lies beyond the pH of interest here.

(b) Yeast. Strain R XII of *Saccharomyces cerevisiae* was donated by Dr. A. Kotyk, Czechoslovak Academy of Sciences, Prague. This strain was used in place of the brewery yeast used earlier [8,9] because of the amount of biochemical [22,23] and toxicological data [14,15,24,25] available on it. Culture media were from Difco.

(c) Biochemical and growth assays. Assays of plasmamembrane ATPase, of purine transport, of growth rate, and of stationary phase cell density

are described in detail elsewhere [2,14,15,20]; therefore these will only be outlined here.

Purine transport was assessed by measurement of the uptake of [8-^{14}C]hypoxanthine. The purine carrier is an energy-dependent H^+-symport, K^+-antiport system [22,23], therefore the R XII cells were pre-incubated in 2% glucose in 50 mM citrate buffer (pH 5.5) [23]. Activity of the Mg^{2+}-dependent plasmamembrane ATPase was assessed by measurement of the inorganic phosphate released from inside-out plasmamembrane vesicles prepared [26] from protoplasts of R XII.

Growth rate was assessed during the exponential growth phase of R XII cultures in YEPG (1% yeast extract, 5% peptone, 2% glucose) medium.

(d) Cell culture for rotation assay. Cultures of R XII were maintained and grown up as already described for the brewery yeast in [8]. The final culture was in YNBGC medium (0.67% yeast nitrogen base, 0.5% glucose, 50 mM citric acid adjusted to pH 4.5 with KOH). A synthetic medium was used here to reduce the possibility of the transfer of variable amounts of complex nutrients.

Cell density at harvesting was $(7–15) \cdot 10^6$ cells/ml. After washing the cells three times (centrifugation through water at $2700 \times g$ for 10 min) they were resuspended and recounted.

(e) Incubation for rotation assay. The anti-field measurements used cells that had been incubated with the phenol under test in YEPG medium at pH 4.5 for 2 h at 30 °C in a shaking water bath. Cell suspension density was $1 \cdot 10^7$/ml.

Two sets of co-field measurements were carried out: one set at pH 4.0 as a direct comparison with the anti-field measurements, the other at pH 6.0 to allow comparison with biochemical assays which had been carried out at that pH. In both cases, the incubation medium contained only the phenol under test and a buffer. At pH 4.0 (10 mM citric acid/KOH buffer) a suspension density of $2 \cdot 10^5$/ml was used with incubation at 30 °C for 3 h. At pH 6.0 (25 mM Mes/KOH buffer) the same conditions were used except that the incubation time was 1–2 h.

(f) Rotation assays. Two assay methods were used, both of which depend on the observation that the rotational spectrum of yeast cells changes dramatically after treatment with sufficient quantities of the phenols. Fig. 1 illustrates this change after treatment with 10 μmol/l PCP. Untreated cells (single or budded) show both an anti-field rotation (for field frequencies between 1 kHz and 300 kHz) and a co-field rotation (when the field frequency is about 300 kHz). The majority of cells that had been treated with sufficient phenol exhibited only a co-field rotation, which has a definite maximum just below 1 MHz (Fig. 1).

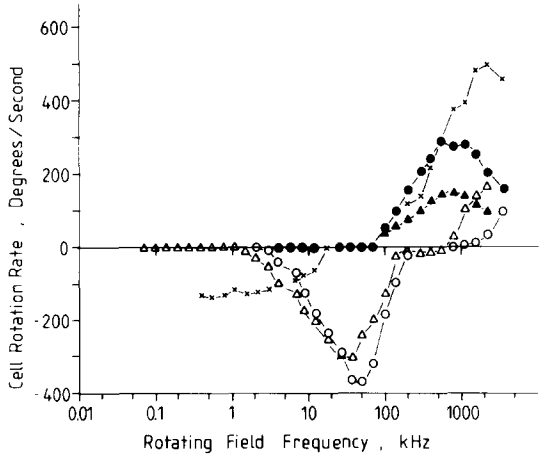

Fig. 1. Rotation spectra of R XII cells obtained by scanning the frequency of field rotation over the range from 3.28 MHz to 70 Hz. Control cells (hollow symbols) show an anti-field rotation with a maximum near to 40 kHz; after treatment with 100 μmol/l PCP (solid symbols) most cells show a spectrum with a single co-field rotation peak centered on 800 kHz. A few treated cells (×) exhibit an intermediate spectrum with not only a pronounced co-field peak but also a weak anti-field rotation at lower field frequencies. The spectrum denoted by ●, is the mean of the spectra from six cells having very small or no buds (there is no room to show standard deviations, which were approximately ±25% of the means). The other spectra are from single cells as follows: ▲, double cell; ○, unbudded cell; △, budded cell; ×, unbudded cell. The medium conductivity was 10 μS/cm, and the rotating field was of 89 V/cm field strength.

As discussed in earlier publications [8,9], the disappearance of the anti-field rotation corresponds to the loss of the insulating plasmamembrane, and the 800 kHz peak exists because the cell wall is rather more conductive than the medium. Although the R XII cells used here are smaller [10] than the brewery yeast used in the earlier work, the changes in spectra produced in most cells by treatment with organic toxins are closely similar to those seen in response to heavy

metals. A few treated cells (as shown by points × in Fig. 1) still exhibit a rather weak anti-field rotation (with no peak but rather an extension to much lower frequencies). These cells also have a co-field rotation that is faster than that of the majority, and the maximum is at a frequency higher than 1 MHz. This behaviour is not easily explicable, although this modified co-field rotation would be expected of cells which are only partially permeabilised, and which possessed an internal conductivity that was still a little in excess of the medium.

The difference between these spectra suggests two ways of quantifying what proportion of the cells have been affected:

(1) By counting the cells which still show an anti-field rotation. This was done using the frequency (42 kHz) that gave the fastest rotation of control cells in 10 μS/cm media. In response to increasing doses of phenol in the incubation, decreasing numbers of cells exhibit anti-field rotation. However, cells that still showed a weak anti-field rotation might be counted as unaffected, even though examination of the full spectrum would show otherwise.

(2) By counting the cells which show the increased co-field rotation. In a media of 10 μS/cm conductivity this change is easiest to observe if a frequency of 300 kHz is used, because control cells show (Fig. 1) no rotation there. This is the basis of the co-field rotation method [8,9]. Even the few cells with the intermediate rotation spectrum (points × in Fig. 1) give a strong co-field rotation at 300 kHz.

The apparatus used for rotating-field generation has been described before [5,8,27]; the procedure for measuring the co-field-rotation of yeast cells is detailed in Refs. 8 and 9.

Results and Discussion

(a) Comparison of anti-field and co-field measurements

Cells taken from incubations to which various concentrations of chlorophenols had been added were assessed for their rotation using field frequencies of 42 kHz or 300 kHz, which excite either anti-field or co-field rotation, respectively (see Methods).

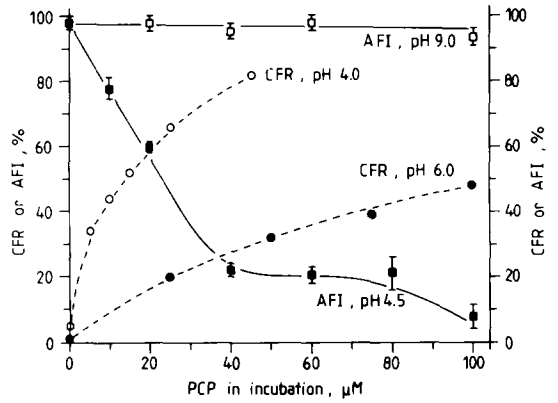

Fig. 2. Effect of increasing the pentachlorophenol (PCP) concentration in the incubation medium on the yeast cell rotation measured after washing the cells. Two types of rotational measurement were made, each after incubation in two different media. One measurement was of the decreasing percentage of cells that still showed anti-field rotation (AFI) at 42 kHz field frequency (after incubation at pH 9.0 (□); or else at pH 4.5 (■). The other was of the increasing percentage of cells that showed co-field rotation (CFR) at 300 kHz (after incubation at pH 4.0 (○); or else at pH 6.0 (●).

Examples of the dose-dependence of the two sorts of measurement are shown in Fig. 2. At 42 kHz (the optimum for the anti-field rotation of control cells) the number of rotating cells decreased as the dose of PCP (preincubation at pH 4.5) was increased. At pH 9.0, no effect was seen. The saturation evident at pH 4.5 and concentrations above 40 μM may be due to the fact that the solubility of PCP in water is only 300 μM [28], and presumably less if the pH is held below the pK_a of 4.8. Measurements at 300 kHz (where control cells show no rotation) show an increase in the number of cells showing co-field rotation with pentachlorophenol dose. It can be seen that the method is much more sensitive at pH 4.0 than at pH 6.0: the pH dependence is discussed in section (b) below.

The doses required to give inhibition of the anti-field rotation of 20% of the cells (the AFI_{20}, measured at 42 kHz) and also the doses required to give an increase of 20% in the number of cells showing co-field rotation (the CFR_{20}, measured at 300 kHz) were determined for several other chlorophenols. To enable easy comparison of data taken on substances with very widely varying toxicity, we took the logarithm of these concentra-

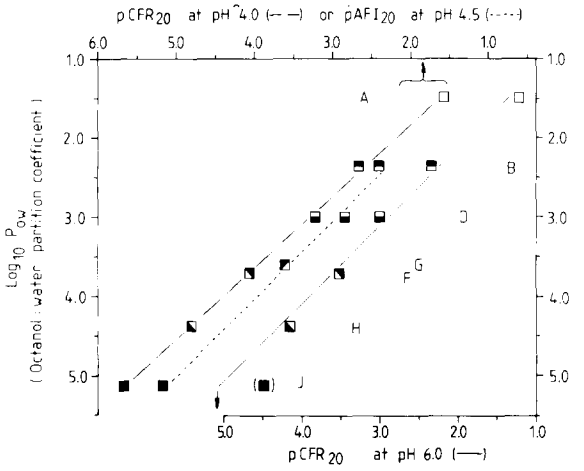

Fig. 3. Comparison between the sensitivities of the three types of measurement to a range of chlorophenols. The comparison is made easier by the linear correlation found between P_{ow} and all three rotation indices. The upper two correlation lines show that the co-field measurement after incubation at pH 4.0 (- - - - -) was much more sensitive than the anti-field measurement after pH 4.5 incubation (·····). The pH 6.0 co-field measurement exhibited a sensitivity that was so close to that of the pH 4.5 anti-field measurement that it has to be shown on an axis displaced by 0.6 unit. $pCFR_{20}$, $-\log_{10}$ (dose for 20% co-field rotation); $pAFI_{20}$, $-\log_{10}$ (dose for 20% decrease in anti-field rotation).

tions that gave 20% effect and defined (by analogy with pH):

$pCFR_{20} = -\log_{10}$ (dose to give 20% co-field rotation at 300 kHz)

and

$pAFI_{20} = -\log_{10}$ (dose to give 20% inhibition of anti-field rotation at 42 kHz)

In Fig. 3, these sensitivity indices are plotted against the logarithm of the octanol-water partition coefficients (P_{ow}) of these substances. This homologous series of phenols shows an excellent correlation between P_{ow} and all three rotation indices (except for pentachlorophenol at pH 6.0, as discussed below). The sensitivity of the anti-field method at pH 4.5 is close to that of the co-field method at pH 6.0 (so close that the axis for the co-field rotation results at pH 6.0 had be shifted to properly display the results). The co-field method at pH 4.0 has the highest sensitivity, and reference to Fig. 2 shows that the difference in sensitivity is particularly marked at the lowest detectable concentrations of toxin.

The higher sensitivity of the co-field rotation method relative to the anti-field method, especially in the threshold region, may be explained by assuming that in this region many affected cells display the intermediate type of rotation spectra (the points × in Fig. 1). As noted in the discussion of Fig. 1 in the Methods, cells with this type of spectrum may be counted as unaffected when judged by their anti-field rotation at 42 kHz, even though their co-field rotation at 300 kHz shows that they are at least somewhat damaged. The suggestion put forward in the Methods, that these cells are only partially permeabilised, is consistent with their formation in the presence of threshold concentrations of toxin.

In addition to the fact that the co-field method appeared to be more sensitive than the anti-field, it was also found easier to observe the threshold of toxicity with the co-field rotation method. This was because even a very small number of rotating cells made their presence very obvious against a background of stationary (unaffected) cells. Many measurements used incubations at pH 6.0 in order to allow comparison with other assay methods. The excellent correlations between P_{ow} and the chlorophenol rotation indices suggested that the rotation method might be useful for characterising the toxicity of a wider range of phenols. For further measurements we used only the co-field rotation method because of its higher sensitivity.

The correlations between the logarithms of the rotation indices and the $\log_{10}(P_{ow})$ values for the chlorophenols suggest interpretations of the mechanism of toxicity based on the effective solubility of these materials within the membrane [12] (see also section (e) below). However, it is apparent that pentachlorophenol (phenol J) does not correlate so well with the other chlorophenols at pH 6.0. The pentachlorophenol data point at pH 6.0 is the only one in Fig. 3 where the pK_a was much lower than the incubation pH (pentachlorophenol has $pK_a = 4.8$). This will have caused a significant degree of ionization of this compound, and the reduced toxicity leads to the conclusion that it is

the unionized form that is toxic. If this is the case, then pentachlorophenol should not deviate from the trend of the other phenols when the incubations are carried out below the pK_a. The results using the anti-field method at pH 4.5 and the co-field method at pH 4.0 confirm this (Fig. 3). However, it is apparent that the deviation of the pentochlorophenol data from the pH 6.0 least-squares regression line is approximately 0.5 pH unit, which would correspond to a value for the pK_a of pentachlorophenol of 5.7, if it is assumed that the ionized form of pentachlorophenol has negligible toxicity. This is almost one unit higher than the pK_a (4.8) of pentachlorophenol measured in aqueous solution.

The simplest explanation of these data is that the pentachlorophenol anion is in fact only slightly less toxic than the unionized molecule. However, it should not be neglected that differences in properties between the cell membrane and the bulk may be operating here. There are two possibilities: either that the effective pK_a of the phenol was higher, or else that the effective pH was lower, than in the bulk.

The second possibility is highly plausible, because the pH at the cell surface is usually lower than that of the bulk. This is a consequence of the surface potential being negative under most conditions, leading to a concentration of positive ions. The pH difference can be expressed as (see Eqn. 3 of the following paper [10]:

$$pH_{membrane} - pH_{bulk} = \psi_0/0.059 \quad (1)$$

where ψ_0 is the surface potential (in V), which we at first assume to be the same over the entire cell surface.

It is usual to approximate the surface potential to the quantity that can be measured by cellular electrophoresis, the ζ-potential (the potential at the plane separating the bulk medium from the aqueous layer hydrodynamically bound to the cell). ζ-potential values for protoplasts of another yeast have been reported to be approximately 6 mV, 3 mV and 2 mV at pH 6.0, 4.5 and 4.0, respectively [29]. These correspond to the pH at the cell membrane being lower than that in the bulk by 0.1 unit or less in all cases, which is not sufficient to explain the above discrepancy.

The above interpretation of ζ-potentials as uniform surface potentials can be questioned in the case of cell membranes, the surface of which is not necessarily flat or homogeneous. Studies, in whole cells, of the transport of Rb^+ [30] and of the binding of the cationic dye 9-aminoacridine [31] indicate that at least some parts of the plasma-membrane (presumably transport proteins) are almost 10-times more negative than the ζ-potential. (Presumably the remainder of the surface must be close to zero potential, in order to give the slightly negative ζ-potentials measured above.) In this case the magnitude of the pH shift would certainly be sufficient to explain the above data, if the site attacked by the phenols is one of those areas showing this sort of discrete charge effect.

On the other hand, the possibility that the pK_a of pentachlorophenol is shifted significantly when it binds to a lipid membrane is supported by recent measurements on membrane-bound pentachlorophenol. Smejtek et al. [19] have shown that in uncharged artificial bilayers the effective pK_a of pentachlorophenol is increased by about one unit. It is not necessarily justifiable to use artificial membrane data for cellular membranes, nevertheless the value given by this approach ($pK_a = 5.8$) is in excellent agreement with the value ($pK_a = 5.7$) estimated from correlation of the data obtained at pH 6.0.

The above can be summarized as follows. Although it is clear that the anomalously low toxicity of pentachlorophenol at pH 6.0 (Fig. 3) seems to be caused by its low pK_a, it is not clear how much the overall toxicity is modified by the various surface effects.

(b) Effect of incubation pH

We have already indicated in connection with Figs. 2 and 3 that the sensitivity of the yeast cells to the chlorophenols increased as the incubation pH was decreased. Fig. 4 demonstrates the pH dependence of the toxicities of the three perhalophenols. In all cases the increase in sensitivity with decrease in pH is to be seen, although the control values also increased. Use of incubation pH values below 4.0 gave rise to such a large and variable increase in the control co-field rotation that we were not able to take repeatable measurements in this region.

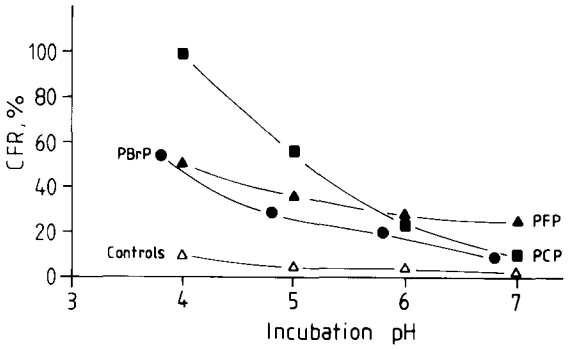

Fig. 4. The dependence of the co-field rotation (CFR) on the pH of medium in which they were incubated. The curves are results with the three perhalophenols. data points: ■, obtained after 3 h incubation with 40 μmol/l pentachlorophenol (PCP); ▲, obtained after 3 h incubation with 3 mmol/l pentafluorophenol (PFP); ●, obtained after 3 h incubation with 2.4 μmol/l pentabromophenol (PBrP); △, control, obtained after 3 h incubation without perhalophenols.

The pH dependence was not limited to the fully-substituted halophenols, but was observed for the complete range of phenols (see Table I). The trend is also evident from the wide range of chloro- and bromophenols shown in Fig. 5. The measurements were made not only at pH 4.0, where the highest useable sensitivity was obtainable, but also at pH 6.0 (to enable comparison with the biochemical assays [14,15,20] also run at this pH).

Examination of the semi-logarithmic plots of Fig. 5 shows that although the various halophenols have toxicities that cover a wide range, the shapes of plots are very similar. This observation is consistent with the hypothesis that the mechanism of toxicity is the same in all cases. In a few cases (e.g. pentachlorophenol at pH 6.0) the plots are unlike the others because relatively large amounts of toxin are required to reach 100% toxicity. At least in the case of pentachlorophenol, the explanation is that the solubility is so low (see section (a) above) that it becomes a limiting factor.

(c) Effects of incubation cell density

If toxin concentrations only slightly above the threshold are used, it can be shown that the percentage of cells affected by the toxin decreases as the cell density in the incubation medium is increased (Fig. 6). The dependency is rather weak, and does not resemble the curves seen in the case of Ag^+ [8] and Hg(II) [9] which led to the suggestion that the effect was due to binding. Although work with labelled pentachlorophenol [2] has shown that R XII yeast binds pentachlorophenol moderately well, this is apparently not enough to give a steep reduction in toxicity at higher cell concentrations.

An explanation based upon binding is also difficult to accept because very similar cell concentration dependencies are seen for phenols covering a wide range of toxicities. This is because it seems most unlikely that those chemicals which

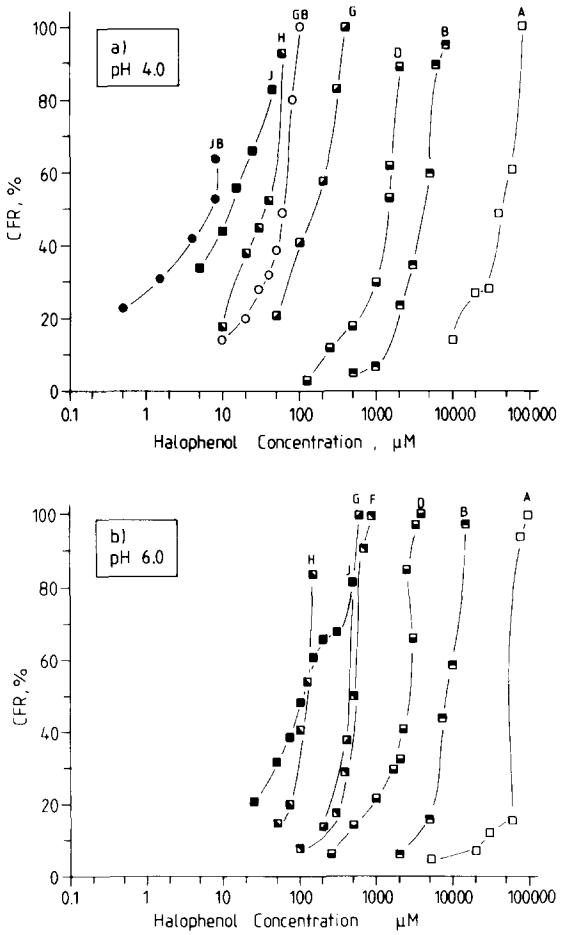

Fig. 5. The increases in co-field cell rotation (CFR) produced by incubation with a wide range of halophenols. A given symbol refers to the same phenol (which may be identified from Table I) in both parts of this and other figures. The sensitivities at pH 4.0 (upper part of the figure) are all at least a factor of two higher than the sensitivities at pH 6.0.

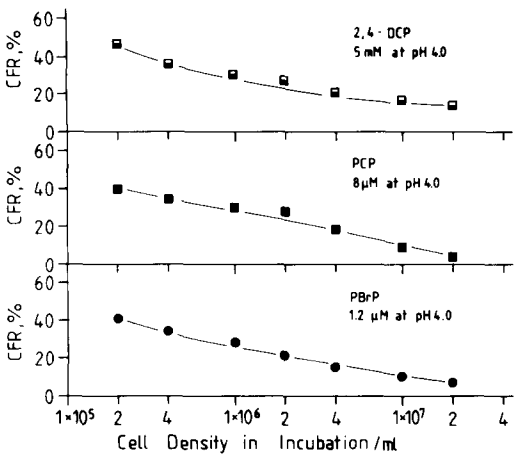

Fig. 6. The decrease in co-field rotation (CFR) upon increasing the cell density in the incubation. Three different toxins were used as indicated. 2,4-DCP, 2,4-dichlorophenol; PCP, pentachlorophenol; PBrP, pentabromophenol.

are the least toxic are bound in the greatest quantities. Indeed in the case of 2,4-dichlorophenol it can be estimated that the cellular concentration of rather more than 10 M would be necessary in order to give enough absorbtion, which is therefore not the mechanism.

(d) Kinetics

Fig. 7 shows that 3.5 mM 2,4-dichlorophenol caused a rapid increase in damaged cells which

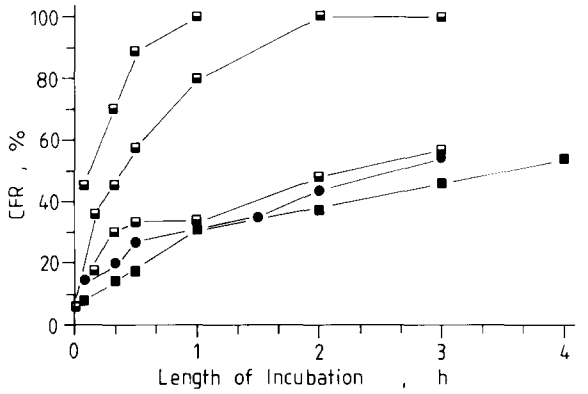

Fig. 7. The increase of co-field rotation (CFR) with incubation time for three concentrations of 2,4-dichlorophenol (□) and for near-threshold concentrations of pentabromophenol (2.5 μM at pH 4.0, ●) and of pentachlorophenol (150 μM at pH 6.0, ■). The 2,4-dichlorophenol incubations were at pH 6.0 and the concentrations were (from top downwards) 3.5, 3.0 and 1.0 mM, respectively.

reached 100% within 1 h. However, use of lower concentrations gave progressively slower kinetics, so that the percentage of damaged cells was still rising linearly with time after 3 h incubation. Other measurements on R XII yeast [10] show that the controls still show low percentages of damaged cells even after 8 h, so that the sensitivity could presumably be raised considerably if longer incubations are tolerable.

The above show that the toxic effect cannot be considered rapid, at least in the sense of the rapidity seen with Ag^+ (the effects of even threshold doses of Ag^+ were complete within 20 min [8]). Fig. 7 also shows that pentachlorophenol and pentabromophenol exhibit similarly slow kinetics to 2,4-dichlorophenol. This is interesting, because the much larger P_{ow} of these fully-substituted phenols should cause them to be much more rapidly transported into the cell [11,12]. It must be concluded that transport is not the cause of the slow kinetics, but that the sensitive site is only slowly attacked by these materials.

(e) Correlations with chemical data (P_{ow} and pK_a)

Fig. 8 shows attempts at correlation the doses of various phenols that are required to give 20% co-field rotation with their octanol: water partition coefficient P_{ow}. Data covering the complete range of phenols (Table I) are shown, not just the halophenols shown in Fig. 3). Except in the case of the pK_a (already logarithmic), all the parameters are expressed as their logarithms in order to cover the necessary range. In both cases correlations were estimated by the method of least squares. Despite the large number of phenols for which pK_a values were available, the correlation with pK_a was restricted to those phenols for which P_{ow} values were available in order to enable a fair comparison to be made between the two correlations.

The correlation between P_{ow} and $pCFR_{20}$ (Fig. 8a) is very good. This indicates that solubility in some membrane system (the plasmamembrane?) is very probably a principal factor in determining the toxicity. 4-Nitrophenol (L) was the only substance omitted from the P_{ow}/CFR_{20} regression analysis. This was done because its toxicity appears to be much greater than expected from the P_{ow} value, so that a specific effect appeared to

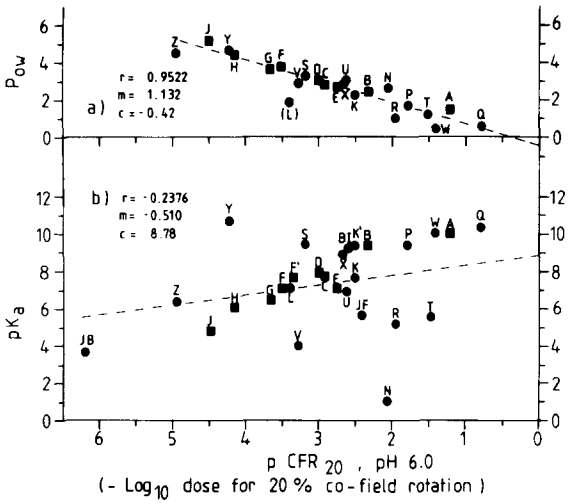

Fig. 8. The correlation between the cell toxicity measured by co-field rotation (CFR) and two chemical indices, P_{ow} and pK_a. The lines are least-squares fits to all the phenols indicated in the P_{ow} plot (except for L, see text). The additional phenols shown on the pK_a plot are not included in either regression analysis. The square symbols represent chlorophenols, and all phenols may be identified by reference to Table I.

be present. Indeed it is known that 4-nitrophenol is a much better uncoupler than expected from its hydrophobicity because the ionized form of the molecule can delocalize its charge by 'through-resonances' [32].

In contrast to the correlation between the P_{ow} values, the correlation between pK_a and $pCFR_{20}$ (Fig. 8b) is much poorer. However, it can be seen that if the analysis is restricted to a homologous series such as the chlorophenols (square symbols in Fig. 8b), then a much better correlation can be obtained. Therefore it seems that the use of pK_a as a toxicity index is restricted to compounds having similar substituents.

It is also apparent from Fig. 8b that phenols having very low pK_a values do not correlate very well with other compounds. Examples of this behaviour are phenols N and V (picric acid, pK_a = 1.0 and 2-methyl-4,6-dinitrophenol, $pK_a = 4.0$). It appears that if the phenol pK_a is so low that it is well beneath the pH of the incubation medium, then most of the toxin will be present as the anion, which is apparently less toxic than the unionized phenol. A less extreme example of the same effect was already discussed in connection with Fig. 3 (pentachlorophenol, $pK_a = 4.8$ is less toxic at pH 6.0 than is expected from its toxicity at 4.0).

The above data show that the P_{ow} seems to correlate much better with the toxicity measured in cells by rotation than does the pK_a. This is perhaps to be expected because P_{ow} measures the partitioning of the toxin into octanol, which appears to be the best obtainable bulk model for the stratified polar/non-polar/polar structure of the biological membrane [11].

It was noted above that the slight departure of pentachlorophenol from the $P_{ow}/pCFR_{20}$ correlation line (better visible in Fig. 3) was probably due to the pK_a of this substance being below the incubation pH of 6.0. Three other phenols in Fig. 8a have pK_a values below the incubation pH of 6.0: these were (Table I) N, V and R. It is not apparent that these all show the same trend as was apparent using the more closely correlated halophenols. It may be that the shift of the pK_a on binding these phenols is very different from that of pentachlorophenol.

(f) Correlation with growth and biochemical data

It was of interest to compare the rotation method with other biologically-based methods found useful for surveying the toxicity of phenols. As these other methods rely on completely different effects, it was hoped that this exercise might give a hint as to the mechanism of the toxicity.

Fig. 9 shows the correlation performed between the concentrations required for 20% co-field rotation and the inhibitory concentrations for 20% reduction in growth rate. All values are expressed as the negative logarithms (p values). Fig. 10 shows similar correlations using two biochemical indices, namely inhibition of purine transport and of the plasmamembrane ATPase activity. Specific effects of certain of the phenols (L, N and W) forced us to restrict the analyses to the same 20 phenols in all three cases in order to enable a comparison to be made between the various methods. Where data on the other phenols were available it is included in parentheses on the figures.

In the case of the inhibition of growth rate, good correlation is found with the co-field rotation data (coefficient $r = 0.919$ and it is also ap-

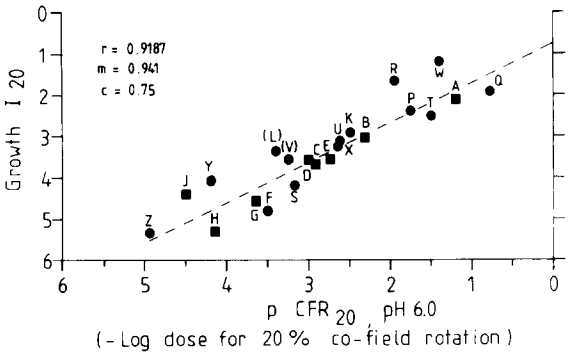

Fig. 9. Correlation between the dose of 20 phenols necessary for 20% inhibition of growth rate and the dose necessary to give 20% co-field rotation (CFR). In both cases the negative of the logarithm of the dose is used. The line is a least-squares fit to the logarithmic data (excluding the bracketed phenols), and exhibit correlation coefficient (r), gradient (m) and intercept on the vertical axis (c) as indicated. The square symbols are used to denote chlorophenols, as in Fig. 8. The phenols may be identified by reference to Table I.

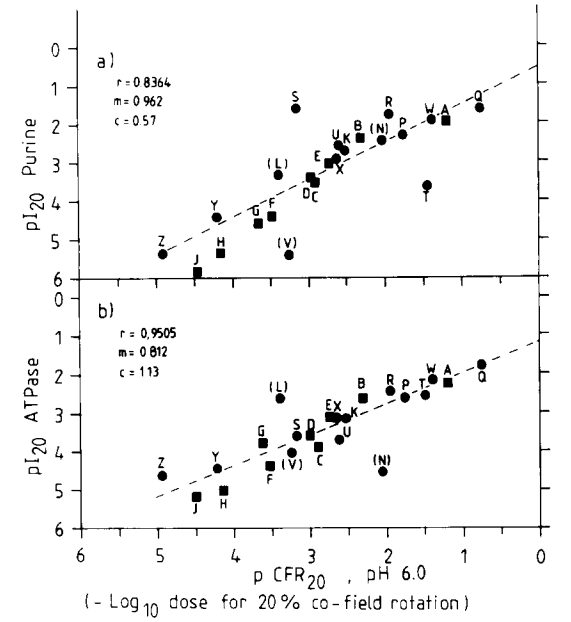

Fig. 10. Correlation between the dose of 20 phenols necessary for 20% inhibition of purine uptake by R XII yeast cells (a), for 20% inhibition of the activity of the plasma membrane ATPase (b) and the dose necessary to give 20% co-field rotation. In all cases the negative of the logarithm of the dose (p value) is used. The lines are least-squares fits to the logarithmic data (excluding the bracketed phenols), and exhibit correlation coefficients (r), gradients (m) and intercepts on the vertical axis (c) as indicated. The square symbols are used to denote chlorophenols, as in Fig. 8. The phenols may be identified by reference to Table I.

parent that this effect varies almost linearly with the co-field rotation index (gradient $m = 0.941$).

The biochemical data (Fig. 10) show that the correlation of purine transport inhibition with rotation is not so good as that of ATPase inhibition (coefficients r of 0.836 and 0.950, respectively). The correlation between CFR_{20} and I_{20} values for the plasmamembrane ATPase is interesting because the ATPase appears to be the site attacked by cadmium [25]. On the other hand the gradients (0.962 and 0.812, respectively) show that the dependence between the sensitivities measured by purine transport and rotation is linear, but that the toxicity to the ATPase varies nearly as the 0.8-th power of the cell-toxicity measured by rotation.

This observation is equivalent to saying that the ATPase sensitivity varies less strongly with the lipophilicity of the phenols than does the rotational sensitivity. This is reasonable in view of the position of the plasmamembrane ATPase: the vesicles used in the assay are inverted, so that the normally cytoplasmic side of the enzyme now faces outwards. It can be expected that it is this side of the enzyme that is the more sensitive, because the majority of its substrates come from the cytoplasm. This enzyme should therefore be more sensitive in inverted vesicles than in whole cells, and this increase in sensitivity will be greater for substances that cannot penetrate the membrane. This explains the relatively weak dependence on lipophilicity.

If we assume that the best-fit correlation line represents compounds with toxicities that are determined by their non-specific transport or partitioning constants, then the compounds that do not correlate well may have specific effects. In the case of the correlation of purine transport with rotation (Fig. 10a) the most notable example is phenol V (i.e. 2-methyl-4,6-dinitrophenol). As discussed above, the acidity ($pK_a = 4.0$) of this compound implies that, at the assay pH of 6.0, 99% is present as the anion. This may account for the relatively much weaker effect of this substance on the whole cell toxicity compared to its strong inhibition of purine transport. On the other hand, it must not be forgotten that 2,4-dinitrophenols are well-known as uncouplers, so that phenol V may remove the proton gradient on which the

purine pumping is energetically dependent. This latter may indeed be the case, because trinitrophenol (substance N) shows an even weaker effect on the purine transport than on rotation. In contrast to the dinitrophenols, this substance is so acidic that it is apparently excluded from the membrane and shows little uncoupling activity. A further substance that appears to have a disproportionately large effect on purine transport is phenol T (2-amino-4-methylphenol). Presumably the structural similarity to the purines causes this phenol to act as a competitive inhibitor.

Fig. 10b shows that the largest deviations from the correlations between ATPase inhibition and co-field rotation are substance L and N. The first of these is 4-nitrophenol which shows a considerably lower potency against the ATPase than against the whole cell. However, it was already noted to have more effect in the rotation assay than was expected from its pK_a or P_{ow}. As already noted, the through-resonance [32] possible in this compound makes it a good uncoupler, so that is also a good inhibitor of purine transport.

Substance N is trinitrophenol and appears to have a powerful inhibitory effect on the ATPase. In that no such effect is apparent in the other assays, this can be termed a specific effect. This is not necessarily due to a particularly strong affinity for the ATPase, but perhaps more to the fact that this strongly ionized phenol is the least likely of the entire set to be able to pass the plasmamembrane. However, it can still act on the transmembrane ATPase in inverted vesicles as discussed above.

Conclusion

We have demonstrated that the co-field rotation method can be used to obtain considerable data on the toxicity of phenols. Considerable differences from the heavy metal data obtained earlier [8,9] were found. These differences were in the rotation spectra, in the kinetics of development of damage, and in the dependence upon pH and cell density of the incubation suspension. From the kinetic data it could be deduced that partitioning into a membrane is the dominating factor in determining the toxicity of these compounds.

The importance of partitioning into the membrane was confirmed by the high correlation between the rotational toxicity data and the octanol-water partition coefficients. A much poorer correlation was found with the pK_a values, especially when these were so low as to cause ionization of the phenols at the pH of the incubation medium. Better correlations could be obtained with both P_{ow} and pK_a if the phenols used were restricted to the homologous series formed by the chlorophenols.

Comparison of the co-field rotation results with those from assays based on the inhibition of yeast growth rate and of plasmamembrane ATPase activity showed good correlations. In the case of purine incorporation, only fair correlation was evident. The sensitivity of the rotation method was not as high as some of these other methods, but it would appear to be possible to increase this by use of longer incubation times, and possibly also by increase of the ionic strength of the medium as used in Ref. 10.

Examination of single cells showed the existence of some cells with spectra intermediate between those of control cells or of the majority of poisoned cells. This type of behaviour confirms the observations made with Hg(II)-treated cells [9] that the rotation method is useful for detecting differences between members of the cell population. As in the case of Hg(II), the damage inflicted by the chlorophenols is only approximately an all-or-none effect.

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