BLOCKING OF VALINOMYCIN-MEDIATED BILAYER MEMBRANE CONDUCTANCE BY SUBSTITUTED BENZIMIDAZOLES

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ABSTRACT Valinomycin selectively transports alkali cations, e.g. potassium ions, across lipid bilayer membranes. The blocking of this carrier-mediated transport by four substituted benzimidazoles has been investigated. The compounds are 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole, (TTFB); 4,5,6,7-tetrachloro-2-methylbenzimidazole, (TMB); 2-trifluoromethylbenzimidazole, (TFB); and 2-methylbenzimidazole, (MBM). Because of its low acidic dissociation constant (pK_a = 5.04), the blocking efficiency of TTFB in both neutral and anionic forms in the aqueous phase could be studied. The compounds exhibit the blocking efficiency sequence, $TTFB^- > TTFB^0 > TMB^0 > TFB^0 > MBM^0$. The corresponding scale of decreasing lipophilicity, as determined by octanol/water partitioning, is $TTFB^0 > TMB^0 > TTFB^- > TFB^0 > MBM^0$. Comparison of neutral species establishes a positive correlation of blocking efficiency with lipophilicity, with the latter being conferred primarily by chlorination of the benzenoid nucleus. Anionic TTFB, on the other hand, is the most effective blocking agent studied in spite of the fact that its dissociation in the aqueous phase markedly impedes its entry (presumably as a neutral species) into a bulk hydrocarbon phase. This observation suggests that the blocking of valinomycin-mediated bilayer membrane conductance takes place at the membrane/solution interface.

INTRODUCTION

In a previous publication (1) we reported that valinomycin-mediated bilayer membrane conductance could be blocked in the presence of the compound 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole, (TTFB). In this paper we present the results of additional studies using this and three additional substituted benzimidazoles. The structures of all four compounds are illustrated in Fig. 1. Abbreviations identifying each compound are also shown.

To develop an appropriate background for the presentation and interpretation of this work we first review briefly the operation of carrier transport mechanisms, as typified by the valinomycin-mediated transport of potassium (K^+) ion across synthetic lipid bilayer membranes. Then the present state of knowledge regarding structure-herbicidal activity relationships of substituted benzimidazoles is outlined. A correla-

tion between lipophilicity and herbicidal activity found for these compounds raises the possibility of a similar correlation with their ability to block carrier-mediated ion transport across bilayer membranes.

Spectroscopic studies of complexation in organic solvents (2), as well as X-ray analysis (3), indicate that valinomycin can form 1:1 complexes with K^+ ions and suggests that the neutral carrier enhances bilayer membrane conductance by forming such charged lipid-soluble complexes. A theoretical description of such an ion transport mechanism, based upon appropriate chemical equilibria between aqueous and membrane phases, has been given (4). An alternative approach (5) considers the kinetics of formation and dissociation of complexes at the membrane/solution interface, and of translocation of the complexes across the membrane. The kinetic description is most appropriate under conditions where a field-independent rate-limiting step restricts current flow at high applied electric field. The two approaches are, however, equivalent in the ohmic or low field limit (6). In both cases the ohmic conductance is proportional to the product of two factors, namely, (*a*) a partition coefficient or equivalent set of rate constants which establishes the concentration of the complex in the membrane interface, and (*b*) a mobility term or equivalent rate constant governing movement of the complex (or the uncomplexed carrier in certain cases) across the membrane. The blocking of carrier-mediated conductance could be interpreted as a modification of either or possibly both of these transport steps.

Substituted benzimidazoles have been reported to be effective herbicides (7, 8) as well as efficient uncouplers of mitochondrial oxidative phosphorylation (9–11). An extensive study by Büchel et al. (12) indicates that the herbicidal activity of these compounds may be attributed to inhibition of photosynthesis. The second light reaction, involving photolysis of water and evolution of oxygen, is inhibited, as revealed by in vitro studies of suppression of the Hill reaction (13) in broken chloroplast preparations. Cyclic photophosphorylation is also suppressed at higher concentrations of inhibitor (12).

Correlation analysis of the Hansch type (14), carried out by Büchel and co-workers (12), indicates that substitutions which increase the lipophilicity of benzimidazole also enhance its effectiveness as an inhibitor of the Hill reaction. The situation is complicated by the fact that such substitutions generally increase the acidic dissociation constant (decrease the pK_a value) of the compound as well. The correlation analysis cited above leads to the conclusion that the undissociated form of the compound is the toxic agent. A survey of structure-activity relations and possible mechanisms of action of a variety of herbicides, including benzimidazoles, has been made by Büchel (15).

In view of this background we have sought a correlation between lipophilicity, as determined by measurement of *n*-octanol/water partition coefficients, and the ability of the substituted benzimidazoles studied to block valinomycin-mediated ion transport. The influence of the state of charge of the compound upon the blocking effect has also been investigated in the case of TTFB. The results and conclusions drawn are presented in this paper. A further objective of these studies, to be approached in a subsequent publication, is the description of the blocking mechanism in terms of modification of either the equilibrium or kinetic parameters which characterize valinomycin-mediated ion transport through bilayer membranes.

METHODS

Membrane Formation and Measurement Techniques

The membrane lipid used was bacterial phosphatidylethanolamine supplied by Supelco, Inc., Bellefonte, Pa. Reagent grade *n*-decane was employed as a solvent. Bilayer membranes were formed by the brush technique in a conductance cell of Teflon and Pyrex construction. Stationary state electrical measurements employed apparatus previously described (16), operated at voltage levels in the ohmic range of membrane conductance. Aqueous solutions were prepared using reagent grade salts and deionized distilled water. Ethanolic stock solutions of valinomycin (Calbiochem, San Diego, Calif.) and the four substituted benzimidazoles were prepared, with appropriate aliquots being added to the membrane-bathing solutions at the time of use. Resulting ethanol concentrations in the aqueous phases never exceeded $\frac{1}{2}\%$ by volume.

In a typical experiment involving both valinomycin and a substituted benzimidazole valinomycin was added first, but only after the membrane had become fully black. After a stable high conductance was reached, usually in about 15 min, stepwise additions of the benzimidazole were made, increasing its concentration from 10^{-8}–10^{-4} M. Sufficient time, usually at least 5 min, was allowed after each addition for the membrane conductance to stabilize. It was generally possible to cover the entire range of benzimidazole concentration with a single membrane.

Synthesis of Substituted Benzimidazoles

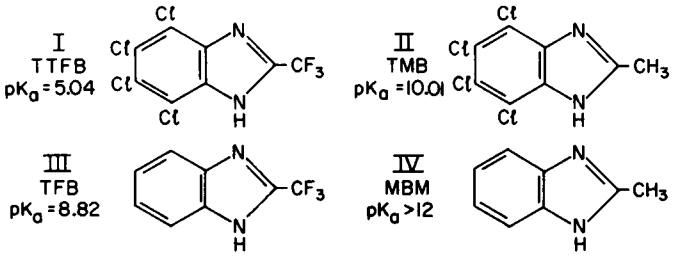
The first step in the synthesis of TTFB and of TMB was the treatment of benzotriazole with boiling aqua regia to yield 4,5,6,7-tetrachlorobenzotriazole (17). Reaction with zinc and hydrochloric acid then gave the intermediate compound 3,4,5,6-tetrachloro-*o*-phenylenediamine (18). Reaction with either trifluoroacetic acid or with acetic acid then yielded TTFB or TBM, respectively. TFB and MBM were obtained by reaction of *o*-phenylenediamine with the corresponding acids. Chlorination of TFB and MBM by direct substitution to yield TTFB and TMB has also been reported (9, 19).

Measurement of Octanol/Water Partition Coefficients

The *n*-octanol/water partition coefficients of all four substituted benzimidazoles have been measured spectrophotometrically using a Cary Model 14 recording spectrophotometer (Cary Instruments, Monrovia, Calif.). The ultraviolet absorption spectra of benzimidazole and MBM have been reported (20). In each experiment a known amount of the compound was added initially to the buffered aqueous phase, to insure more rapid equilibration with octanol. The aqueous phase was saturated with octanol, and the octanol saturated with water, prior to each experiment. All measurements were conducted at 25°C. Shaking on a mechanical agitator for $1\frac{1}{2}$ h was sufficient to ensure an equilibrium distribution of the compound between the two phases. After agitation complete phase separation was effected by centrifugation. The concentration of compound in each phase was determined by measurement of the extinction coefficient at a suitable wavelength (e.g., 220 nm for TTFB), and by comparison with calibration curves based upon measurements using solutions of known concentration. Estimated accuracy of the partition coefficients determined is $\pm 10\%$, except for TTFB at aqueous phase pH = 3.0. A lower limit was determined in this case. Results of partition measurements are presented in Table I of the following section.

Determination of pK_a Values

The aqueous phase pK_a values of the substituted benzimidazoles used were also determined spectrophotometrically at 25°C, with titration curves being based upon the pH-dependent amplitudes of absorption peaks in the vicinity of 290 nm (anion absorption) and of 270 nm (neutral molecule absorption). Aqueous phase pK_a values for TTFB and TFB have been reported previously (7), and are in good agreement with our measurements given in Fig. 1. The estimated maximum error of the values given is ± 0.02 pH units, except in the case of MBM for which only



I 4,5,6,7 - tetrachloro-2- trifluoromethylbenzimidazole
II 4,5,6,7 - tetrachloro-2 - methylbenzimidazole
III 2 - trifluoromethylbenzimidazole
IV 2 - methylbenzimidazole

FIGURE 1 Structures of the four substituted benzimidazoles used in this study are illustrated, and the aqueous phase pK_a values of each are given. Abbreviations used in the text are also listed.

a lower limit was determined. Potentiometric pK_a determinations for many substituted benzimidazoles in 50 vol % aqueous ethanol have also been reported (12, 21). Where comparisons can be made these values are elevated, relative to those measured in a pure aqueous solvent, by about 0.2 pH unit.

These compounds are amphoteric; pK_b values (proton uptake) of 5.53 for benzimidazole and of 6.19 for MBM have been reported (22). The presence of electronegative substituents on TTFB, TMB, and TFB should lead to significantly lower pK_b values for these compounds.

RESULTS

Typical results illustrating the blocking by TTFB of valinomycin-mediated K^+ conductance of bilayer membranes are shown in Fig. 2. It is seen that, at TTFB concentrations in excess of 10^{-6} M, membrane conductance in the presence of both valinomycin and TTFB does not differ significantly from that attributable to TTFB alone. At a pH of 8.0 the TTFB should be fully anionic, and the lipid-soluble anion alone produces a high level of both initial (23) and steady-state (24) bilayer membrane conductance.

The compound TMB can also block valinomycin-K^+ conductance, as illustrated by Fig. 3. In this case, however, the compound is neutral in aqueous solution at pH = 8.0, in view of its pK_a value of 10.01. As a consequence of this, as well as lower lipophilicity, the membrane conductance in the presence of TMB alone is low, and is in fact negligible in comparison with valinomycin-K^+ conductance at all TMB concentrations reached. The valinomycin-K^+ conductance is nevertheless decreased by about two orders of magnitude as the TMB concentration is increased from 0 to 10^{-4} M.

A comparison of the relative blocking efficiencies of TTFB and TMB is provided in Fig. 4. TTFB in the anionic form in the aqueous phase (pH = 8.0) is seen to be more effective as an antagonist of valinomycin-K^+ conductance than when it is in a predominantly neutral form (pH = 3.0). TTFB in the neutral form is, however, a more effective agent for the blocking of carrier-mediated conductance than is TMB.

Reference to Fig. 5 indicates that TFB produces only a slight blocking effect, while MBM does not block valinomycin-K^+ conductance at all. Elevation of the aqueous solution pH to 9.4, at which more than half of the TFB should be anionic, does not significantly enhance blocking (data not shown). Interpretation of this finding will be

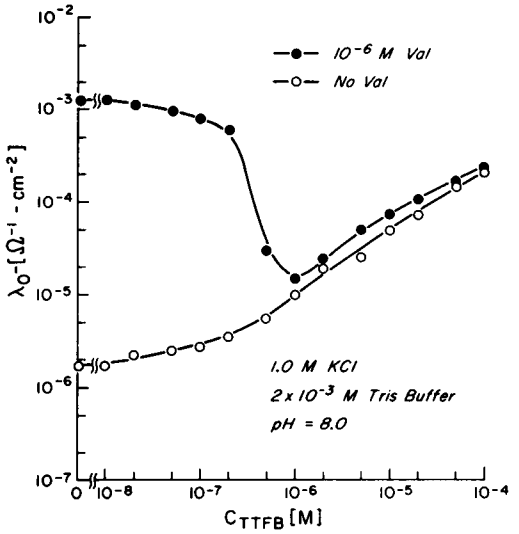


FIGURE 2

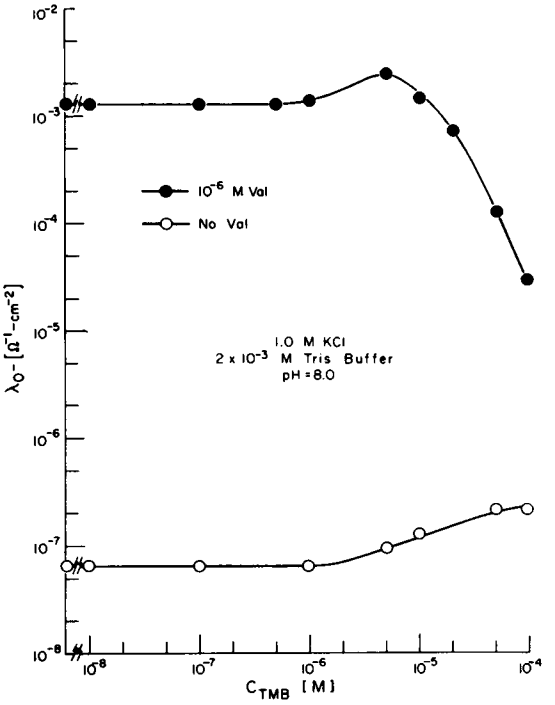


FIGURE 3

FIGURE 2 The blocking by TTFB of valinomycin-mediated K^+ ion conductance is illustrated. At TTFB concentrations in excess of 10^{-6} M the conductance in the presence of valinomycin does not differ significantly from that due to TTFB alone.

FIGURE 3 The blocking by TMB of valinomycin-mediated K^+ ion conductance is illustrated. The slight rise of conductance prior to the onset of blocking is a reproducible effect which will be interpreted in a subsequent publication. In this case the conductance due to TMB alone is negligible.

complicated by the fact that the membrane acquires a net negative charge at this higher pH, owing to deprotonation of the amine group on the phosphatidylethanolamine moiety of the lipid (25). A net negative membrane surface charge would be expected to impede entry of TFB^- into the membrane interface, while promoting entry of the valinomycin-K^+ complex.

Measured values for the *n*-octanol/water partition coefficients of the substituted benzimidazoles used in this work are listed in Table I. The higher lipophilicity of the chlorinated compounds TTFB and TMB is apparent. Comparison of results at pH = 8.0 and at pH = 3.0 for TTFB indicates that dissociation in the aqueous phase impedes its entry, doubtless as an associated and neutral species, into a bulk hydrocarbon phase. Such association would be required to maintain electroneutrality in the non-polar hydrocarbon phase.

DISCUSSION

Reference to the data of Figs. 4 and 5, together with the data of Table I, establishes that both lipophilicity and blocking efficiency of the compounds, when neutral in the

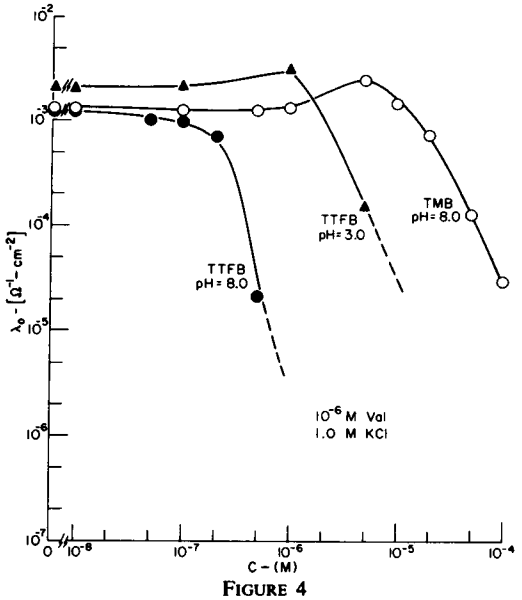


FIGURE 4

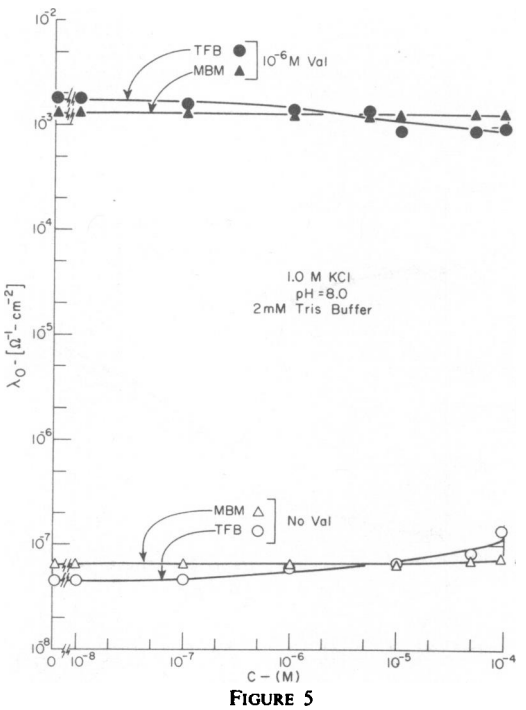


FIGURE 5

FIGURE 4 A comparison of blocking of valinomycin-K^+ ion conductance by anionic TTFB (pH = 8.0), predominantly undissociated TTFB (pH = 3.0), and predominantly undissociated TMB (pH = 8.0) is given. Background conductance of the compound alone has been subtracted in the case of TTFB.

FIGURE 5 The limited blocking of valinomycin-K^+ ion conductance by TFB and the complete absence of blocking by MBM are illustrated.

aqueous phase, are characterized by the sequence, TTFB > TMB > TFB > MBM. Since only three compounds exhibit measureable blocking, and of these only two exhibit large (order of magnitude) conductance changes, a regression analysis of the Hansch type (14) is clearly inappropriate. The results nevertheless establish an interesting parallel with the work of Büchel et al. (12) discussed in the introduction. The anion $TTFB^-$, however, though the most effective blocking agent, falls between TMB and TFB in the lipophilicity sequence. This will be considered further below.

The kinetic model of carrier-mediated transport developed by Läuger and Stark (5) provides a useful approach to a more detailed understanding of the mechanism of the blocking effect reported here. This is so because the model deals specifically with, (*a*) the surface reactions which establish the concentrations of neutral carrier and of ion-carrier complex in the membrane interfaces, and (*b*) translocation of both complexed and uncomplexed carriers across the interior of the membrane. The feasibility of determining separately the parameters characterizing these processes has been demonstrated for the valinomycin carrier system. The determination is based upon analysis of both stationary state (26) and transient (27) measurements of bilayer membrane conductance. Thus it should be possible to relate the blocking effect to a modification

TABLE I

n-OCTANOL/WATER PARTITION RATIO FOR SUBSTITUTED BENZIMIDAZOLES

Compound	Aqueous phase	Partition coefficient at 25°C
	pH	
TTFB	8.0	420
TTFB	3.0	>3,000
TMB	8.0	670
TFB	8.0	130
MBM	8.0	27

of one or more of the specific rate processes mentioned above. Experiments having this objective are in progress using TMB as the blocking agent; results will be presented in a forthcoming publication. TMB is a particularly suitable choice because the compound alone makes a negligible contribution to membrane conductance. Furthermore, because the compound is undissociated in the aqueous phase, complications due to surface potential effects (1) should be minimal.

The data of Fig. 4 show that anionic TTFB is a more effective blocking agent than the undissociated compound, in spite of the fact that the latter is more readily taken up by a bulk hydrocarbon phase (Table I). The hydrocarbon core of a bilayer membrane, on the other hand, appears to be a reasonable approximation to a bulk hydrocarbon phase. This view is supported both by image force calculations of the electrostatic potential of ions in membranes (28) and by experimental determination of activation enthalpies for translocation of charged complexes across bilayers (29, 30). While this evidence alone is not conclusive it nevertheless suggests that one or more of the interfacial steps in carrier-mediated ion transport, outlined in the preceding paragraph, are most likely to be modified upon introduction of a blocking agent.

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