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Interactions of hexachlorocyclohexanes with the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum

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Hexachlorocyclohexanes have been shown to inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase of muscle sarcoplasmic reticulum reconstituted into bilayers of dioleoylphosphatidylcholine. However, for the ATPase reconstituted into bilayers of dimyristoleoylphosphatidylcholine, a pattern of activation at low concentration followed by inhibition at higher concentration is seen for hexachlorocyclohexanes and alkanes such as decane and hexadecane. The ATPase in sarcoplasmic reticulum vesicles is also inhibited by the hexachlorocyclohexanes. The effects of hexachlorocyclohexanes on activity are largely independent of concentrations of Ca^{2+} and ATP. Inhibition is more marked at lower temperatures. The hexachlorocyclohexanes quench the tryptophan fluorescence of the ATPase, and the quenching can be used to obtain partition coefficients into the membrane system. As for simple lipid bilayers, partition exhibits a negative temperature coefficient. Binding is related to effects on ATPase activity.

Lindane, the γ-isomer of hexachlorocyclohexane is an insecticide whose site of action is believed to be in the membrane [1]. It has been shown to affect a variety of transport ATPases, including $(Ca^{2+} + Mg^{2+})$-ATPases [2], and it has been suggested that the effect of lindane on neurotransmitter release at the frog neuromuscular junction could follow from an increased level of internal calcium [3]. Effects of lindane on membrane proteins could follow indirectly from effects on the lipid component of the membrane or directly from binding to the proteins. Haydon and Urban [4] have suggested that the effects of a variety of small hydrophobic molecules such as cyclohexane and carbon tetrachloride on squid axon could follow from a thickening of the lipid component of the membrane which in turn would affect the functioning of sodium channels in the membrane. From changes in membrane impedance it was argued that these hydrophobic molecules bind in the centre of the lipid bilayer and can increase the thickness of the bilayer by 10 or more Angstrom units [4]. However, Franks and Lieb [5] measured bilayer thicknesses directly using X-ray techniques and could find no effect of cyclopropane on membrane thickness. McIntosh et al. [6] report that although short chain alkanes partition into the centre of lipid bilayers and cause an increase in thickness, alkanes with C_{12} chains and longer line up parallel to the lipid fatty acyl chains and have no effect on bilayer thickness. Further, for the hydrophobic pyrene molecule, fluorescence emission spectra are consistent with a location near the glycerol back-

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetracetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; NPTH, *N*-palmitoyl-L-tryptophan *n*-hexyl ester.

bone of the lipid rather than the bilayer centre (Ref. 7 and Jones, O.T. and Lee, A.G., unpublished data).

The possible importance of membrane thickness for the activity of membrane proteins has been tested with the $(Ca^{2+} + Mg^{2+})$-ATPase purified from sarcoplasmic reticulum. The ATPase can be reconstituted into bilayers of defined composition, and ATPase activity has been shown to be sensitive to the chemical structure of the surrounding phospholipids [8–10]. There is an optimal fatty acyl chain length for activity of about 18 or 20 carbons [8,9]. It has been shown that simple alkanes like decane will increase the activity of the ATPase reconstituted with a short chain lipid to above that seen with a phospholipid of optimal structure and it has been suggested that this could follow from partition of the alkane into the centre of the bilayer, increasing bilayer thickness towards the optimal value [9]. Addition of too high a concentration of alkane causes inhibition, and it has been suggested that this follows from overthickening of the bilayer [9]. Similar patterns of increasing followed by decreasing activity have, however, also been observed on addition of a variety of hydrophobic molecules that are unlikely to partition into the centre of the bilayer, including fatty acids, cholesterol and cholesterol hemisuccinate [11,12]. For such molecules an alternative model has been proposed. At high concentrations it is suggested that the hydrophobic molecules will bind at the lipid-protein interface (annular sites) of the ATPase, displacing phospholipid. Since the activity of the ATPase is known to be very sensitive to the chemical structure of the surrounding molecules, this will generally lead to a decrease in activity. It is proposed that the initial increases in activity observed at lower concentrations of additive follow from binding to other (non-annular) sites on the ATPase [11–13].

In the preceding paper [14] we showed how quenching of the fluorescence of a hydrophobic analogue of tryptophan could be used to obtain partition coefficients for hexachlorocyclohexanes into simple lipid bilayers. Here we study effects of hexachlorocyclohexanes on the fluorescence of tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum, and obtain partition coefficients which can be related to effects of hexachlorocyclohexanes on activity.

Materials and Methods

Dioleoylphosphatidylcholine was obtained from Lipid Products and myristoleic acid from Sigma. Dimyristoleoylphosphatidylcholine was prepared by acylation of the cadmium chloride adduct of L-α-glycerophosphorylcholine with the anhydride of myristoleic acid according to the method of Selinger and Lapidot [16]. The lipid was purified either using silicic acid chromatography as outlined by Hanahan et al. [17] or using high pressure liquid chromatography [18], and gave a single spot on thin-layer chromatography (Silica Gel G; chloroform/methanol/acetic acid/water, 25 : 15 : 4 : 2 by volume). Potassium cholate (Sigma) was recrystallised from ethanol. The isomers of hexachlorocyclohexane were a gift from ICI.

Sarcoplasmic reticulum was prepared from rabbit skeletal muscle in the presence of dithiothreitol, largely as described in Ref. 19. $(Ca^{2+} + Mg^{2+})$-ATPase was purified from sarcoplasmic reticulum by treatment with cholate and centrifugation into a sucrose gradient as described by East and Lee [10]. Protein was estimated using the extinction coefficient of Hardwicke and Green [20] and lipid was estimated using the phosphate method of Bartlett [21]. The molar ratio of lipid to protein in the purified ATPase was 30 : 1, based on a molecular weight of 115 000 for the protein. ATPase activity was measured using the double-enzyme coupled assay [22,23]. Free calcium levels in the presence of EGTA and ATP, and MgATP concentrations were calculated using the binding constants given in [23].

Lipid titrations of the purified ATPase to give defined lipid-ATPase complexes were performed essentially as described previously [10]. Briefly a large excess of lipid (4 mg) was dispersed in buffer (180 μl; 250 mM sucrose/1 M KCl, 50 mM K_2HPO_4/KH_2PO_4, 5 mM MgATP, pH 8.0) containing potassium cholate (2.25 mg). The dispersion was then sonicated in a bath sonicator to clarity. $(Ca^{2+} + Mg^{2+})$-ATPase (0.5–1 mg) was added and the mixture left to stands for 20 min at room temperature, followed by 30 min on ice. The mixture was then diluted ten-fold with buffer as above, and small aliquots (10–20 μl) were diluted into assay medium (2.5 ml) for determination of activity. Effects of additive were tested either by

cosonication with lipid and cholate prior to addition of the ATPase or by direct addition and incubation in the assay medium. The two methods of addition gave identical results.

Fluorescence measurements were made using a Perkin-Elmer MPF44A fluorimeter, exciting fluorescence at 280 nm and measuring intensity at 340 nm. The buffer was 40 mM Hepes/100 mM NaCl/1 mM EGTA (pH 7.2). Hexachlorocyclohexanes were added from a stock solution in methanol, the final volume of methanol never exceeding 2% of the total volume. Measurements of light scattering were made either by recording absorbance at 590 nm or by measuring the intensity of scattered light at 90° to the incident light in a fluorimeter.

Results

The reconstitution procedure involves incubation of the ATPase system with a large excess of test lipid, followed by a 1000-fold dilution into an assay medium. On reconstitution with dimyristoleoylphosphatidylcholine, the original ATPase activity of approx. 12 I.U./mg at saturating Ca^{2+} and ATP at 37°C falls to 5 I.U./mg whereas on reconstitution with dioleoylphosphatidylcholine the activity increases to 16 I.U./mg (Fig. 1), in agreement with previous results [9,10]. As shown in Fig. 1, addition of decane causes an increase in activity for the ATPase reconstituted with dimyristoleoylphosphatidylcholine of up to 430%, maximum activity being observed for a molar ratio of alkane to lipid of 1 : 1 (0.25 : 1 on a weight basis). Further addition of decane causes a decrease in activity. A similar profile of stimulation and inhibition is seen on adding decane to the ATPase reconstituted with dioleoylphosphatidylcholine, although here the maximum stimulation was greatly reduced compared to the ATPase reconstituted with dimyristoleoylphosphatidylcholine. The effects of decane could be reversed by addition of excess bovine serum albumin (final concentration 10 mg/ml). These results agree with those published previously by Johannsson et al. [9]. As shown in Fig. 2, similar effects are seen with other alkanes, although maximum stimulation, and the concentration at which it occurred, depended on alkane structure. Stimulation by *n*-nonane and its positional isomer 2,2,4-trimethylhexane are similar, but with hexadecane the maximum stimulation was markedly less and occurred at a higher concentration (molar ratio of hexadecane/lipid 3 : 1, or 1 : 1 on a weight basis).

The effects of lindane and the α- and δ-isomers of hexachlorocyclohexane on the activity of the

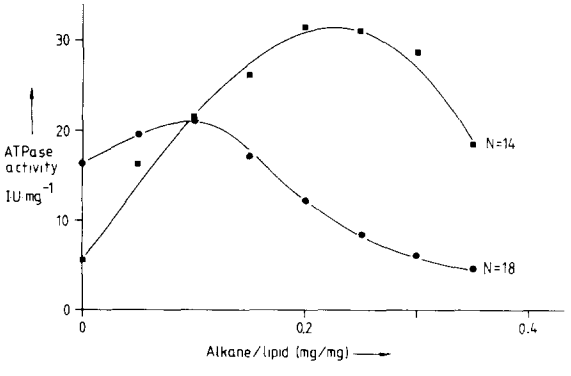

Fig. 1. The effect of decane on the activity of the ATPase reconstituted with either dimyristoleoylphosphatidylcholine (■) or dioleoylphosphatidylcholine (●), assayed as described in the text at 37°C. Concentrations of decane expressed as mg decane/mg lipid.

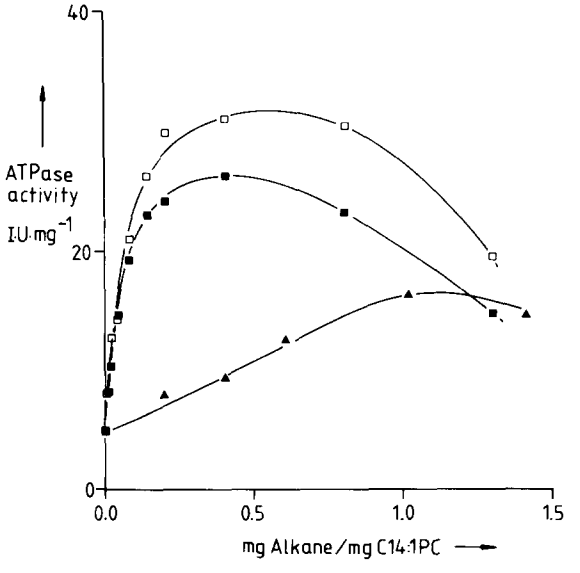

Fig. 2. The effects of nonane (■), 2,2,4-trimethylhexane (□) and hexadecane (▲) on the activity of the ATPase reconstituted with dimyristoleoylphosphatidylcholine, assayed as described in the text at 37°C.

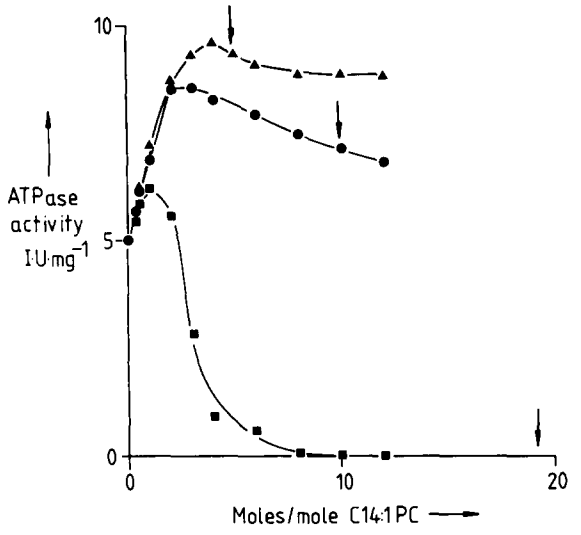

Fig. 3. The effects of lindane (●) and the α- (▲) and δ- (■) isomers of hexachlorocyclohexane on the activity of the ATPase reconstituted with dimyristoleoylphosphatidylcholine at 37°C. Protein concentration 0.018 μM; lipid concentration 10.7 μM. Arrows indicate the calculated molar ratio of hexachlorocyclohexane to lipid at which the aqueous solubilities will be exceeded.

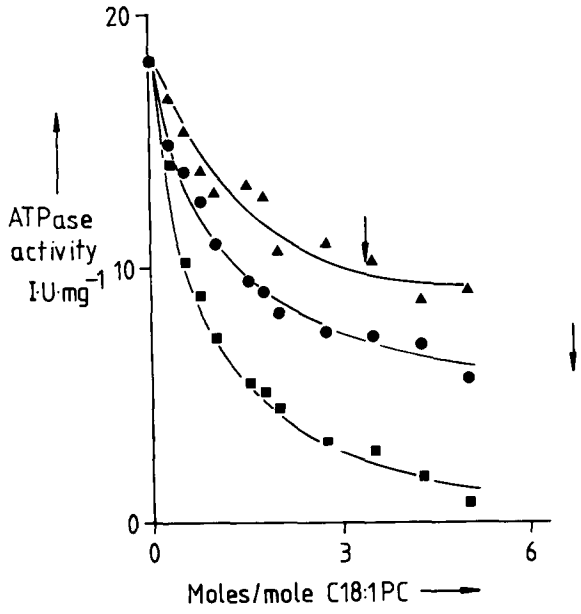

Fig. 4. The effects of lindane (●) and the α- (▲) and δ- (■) isomers of hexachlorocyclohexane on the activity of the ATPase reconstituted with dioleoylphosphatidylcholine at 37°C. Protein concentration, 0.036 μM; lipid concentration, 21.4 μM. Arrows indicate the calculated molar ratios of hexachlorocyclohexane to lipid at which the aqueous solubilities will be exceeded.

ATPase reconstituted with dimyristoleoylphosphatidylcholine are shown in Fig. 3. Stimulation is observed, but the effects are much less marked than for decane. For the δ-isomer the maximum stimulation was only 30% and complete inhibition was observed at a molar ratio of δ-hexachlorocyclohexane to lipid of about 10 : 1. Inhibition at this level was not reversible by addition of bovine serum albumin. Effects on the activity of the ATPase reconstituted with dioleoylphosphatidylcholine are shown in Fig. 4. Only inhibition was observed in these cases. Effects of the hexachlorocyclohexanes on the activity of the purified, untitrated ATPase are shown in Fig. 5, and are very similar to those for the ATPase reconstituted with dioleoylphosphatidylcholine except for the higher molar ratios of hexachlorocyclohexane to lipid used in the former experiments.

Possible effects of the hexachlorocyclohexanes on substrate affinities were investigated by studying activity as a function of Ca^{2+} (Fig. 6) or ATP (Figs. 7 and 8) concentration.

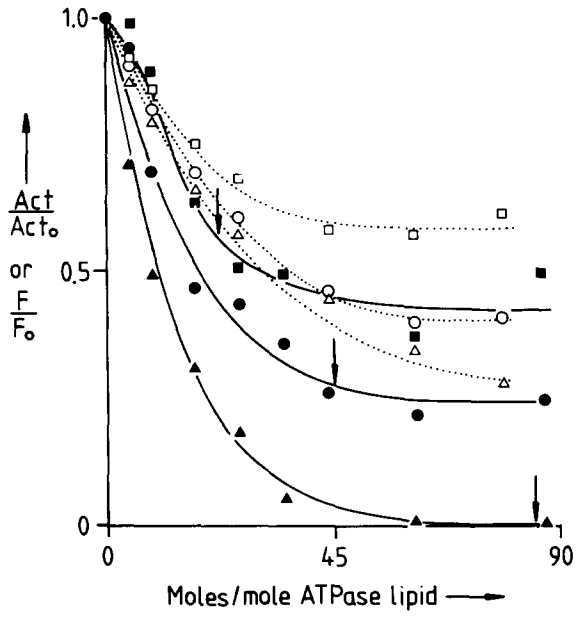

Fig. 5. Effects of lindane (●, ○) and the α- (■, □) and δ (▲, △)-isomers of hexachlorocyclohexane on the activity of the ATPase (solid lines, filled symbols) and on tryptophan fluorescence intensity (broken lines, open symbols) at 37°C. Protein concentration, 2.8 μM. Arrows indicate the calculated molar ratios of hexachlorocyclohexanes to lipid at which the aqueous solubilities will be exceeded.

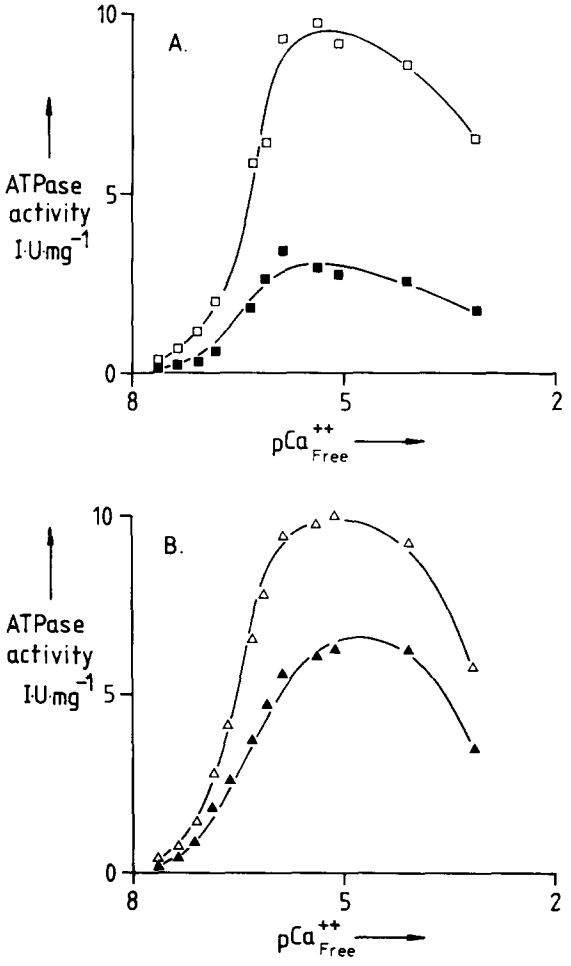

Fig. 6. The calcium dependence of ATPase activity in the absence (□, △) or presence of δ-hexachlorocyclohexane (A, ■) or lindane (B, ▲) at 37°C. Molar ratio of hexachlorocyclohexane to lipid was 17.5 : 1 and protein concentration, 0.018 μM.

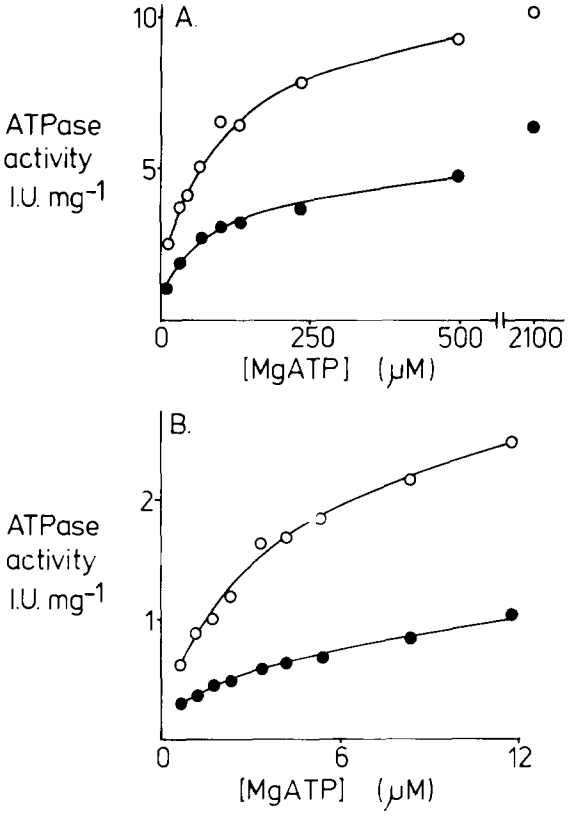

Fig. 7. The effects of high (A) and low (B) concentrations of MgATP on ATPase activity at 37°C, free Ca^{2+} = 1.2–1.4 μM in the absence (○) and presence (●) of 71.4 μM lindane, respectively. Protein concentration, 0.054 μM.

The effect of temperature on the inhibition by lindane of the ATPase reconstituted with dioleoylphosphatidylcholine is shown in Fig. 9, and clearly shows greater inhibition of ATPase activity at lower temperatures. A similar dependence on temperature was found for inhibition of the untitrated ATPase by the hexachlorocyclohexanes (Fig. 10).

Effects of lindane on the activity of vesicles of sarcoplasmic reticulum were also studied. Normally, in sealed sarcoplasmic reticulum, ATPase activity is limited by accumulation of calcium within the vesicle. In the presence of the ionophore A23187, however, calcium accumulation is prevented and we find a maximal ATPase activity of approx. 10 I.U. at 37°C in the presence of 2.5 μg/ml or greater A23187. Effects of lindane on coupled (Fig. 11) and uncoupled sarcoplasmic reticulum are very similar.

In the preceding paper [14] we showed that the hexachlorocyclohexanes quenched the fluorescence of a hydrophobic tryptophan derivative incorporated into lipid bilayers. The hexachlorocyclohexanes also quench the fluorescence of the tryptophan residues of the $(Ca^{2+} + Mg^{2+})$-ATPase. Quenching has no effect on the shape of the fluorescence emission spectrum. Fig. 12 presents a comparison of the effect of lindane on the fluorescence intensity of the ATPase and on the tryptophan analogue NPTH incorporated into lipo-

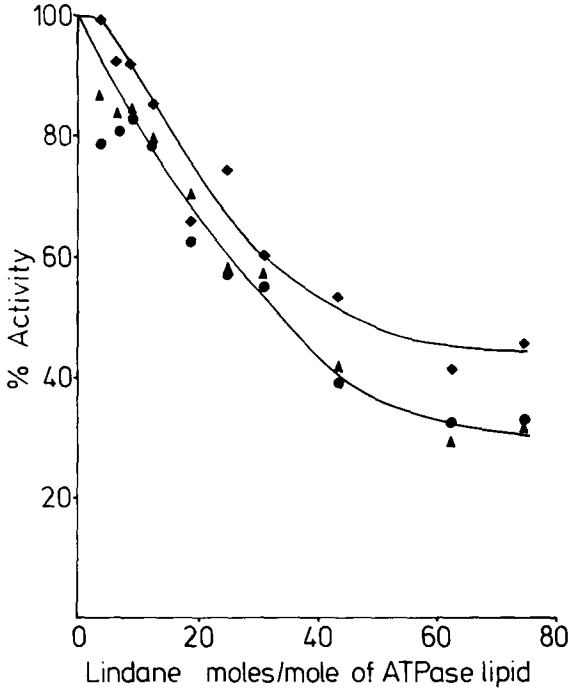

Fig. 8. Effect of lindane on ATPase activity at 37°C, at MgATP concentrations (μM) of: ◆, 2050; ●, 16.6; ▲, 1.7. Free Ca^{2+} = 1.2–1.4 μM, protein concentration 0.054 μM.

somes of dioleoylphosphatidylcholine, at equal lipid concentrations, based on a lipid/protein molar ratio of 30 : 1 for the ATPase system and a molecular weight of 115 000 for the ATPase. The

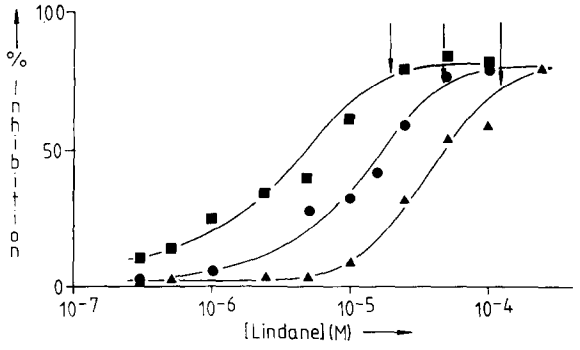

Fig. 9. Inhibition by lindane of the ATPase reconstituted with dioleoylphosphatidylcholine at (°C); ■, 10; ●, 20; ▲, 37. Protein concentration 0.036 μM, lipid concentration 21.4 μM. A concentration of 10^{-5} M lindane corresponds to a molar ratio of lindane to lipid of 0.5 : 1. Arrows indicate the calculated concentration of lindane at which the aqueous solubilities will be exceeded.

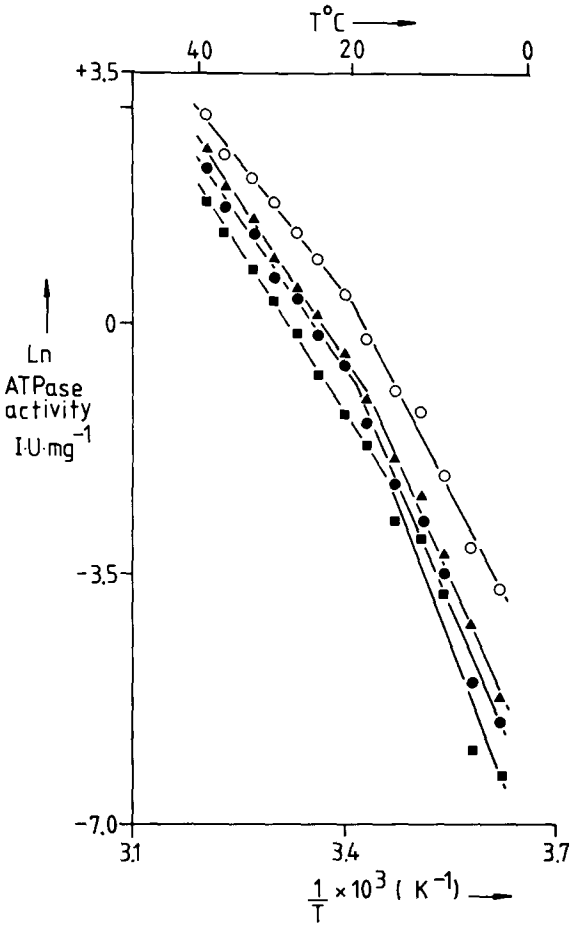

Fig. 10. Temperature dependence of ATPase activity in the absence (○) or presence of the α-(▲), γ-(●) and δ-(■) isomers of hexachlorocyclohexane at a molar ratio of hexachlorocyclohexane to lipid of 20 : 1. Protein concentration 0.083 μM.

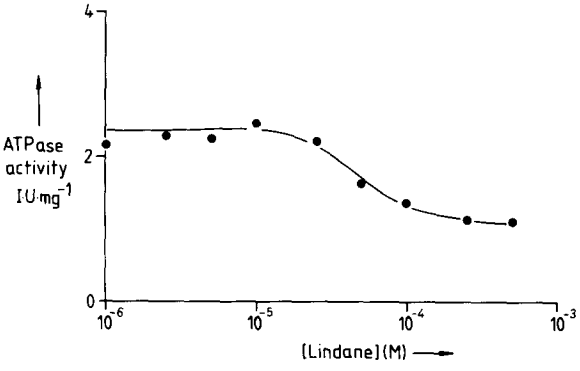

Fig. 11. Effects of lindane on ATPase activity of coupled vesicles of sarcoplasmic reticulum, at 37°C. Protein concentration 0.072 μM.

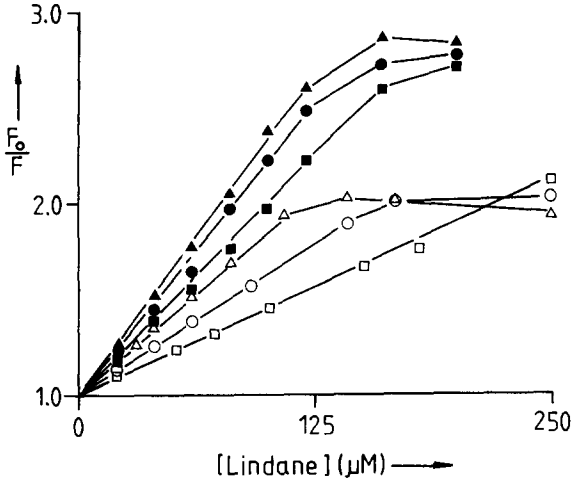

Fig. 12. A comparison of fluorescence quenching of NPTH in liposomes of dioleoylphosphatidylcholine (filled symbols) and of ATPase (open symbols) by lindane at 37°C. Lipid concentrations (μM); ▲ △, 15; ● ○, 60; ■ □, 150.

data is presented as a Stern-Volmer plot of F_0/F where F_0 and F are fluorescence intensities in the absence and presence of quencher, respectively. Quenching in the ATPase system reaches a maximum level beyond which further addition of lindane has no further effect. The concentrations of lindane where the quenching saturates are similar

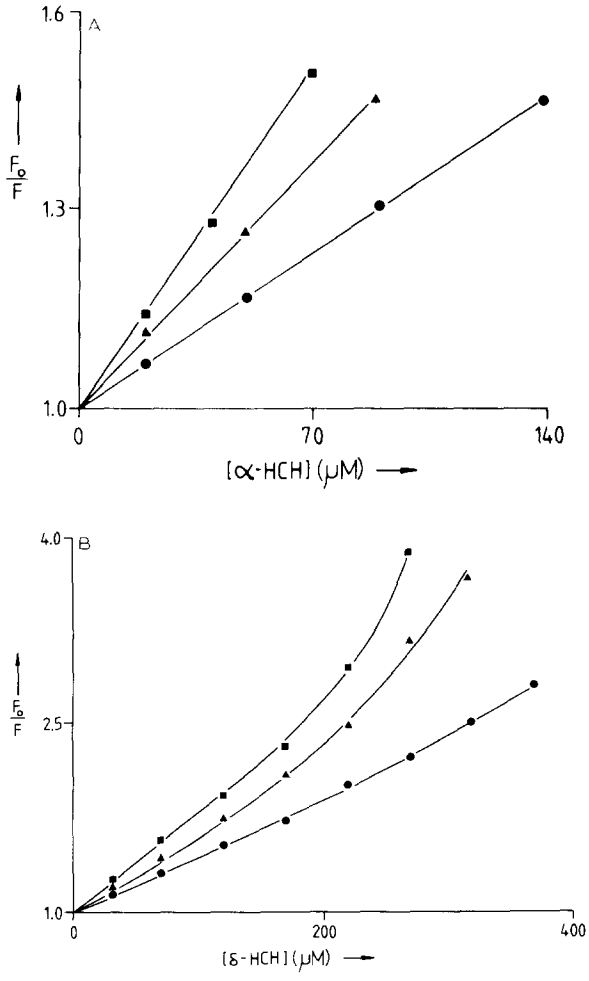

Fig. 13. Quenching of ATPase fluorescence by α-(A) and δ-(B) isomers of hexachlorocyclohexane (HCH) at 37°C. Lipid concentrations (μM): ■, 15; ▲, 60; ●, 150.

TABLE I

PARTITION COEFFICIENTS AND STERN-VOLMER QUENCHING CONSTANTS FOR HEXACHLOROCYCLOHEXANES IN THE (Ca^{2+} + Mg^{2+})-ATPase SYSTEM

Isomer	Temp. (°C)	K_p	K_{sv} (M^{-1})
γ	10	14000	0.80
	20	12000	0.90
	30	10500	0.95
	37	9000	1.00
α	10	14000	0.55
	20	12000	0.62
	30	10500	0.72
	37	9500	0.75
δ	10	13500	0.68
	20	10000	0.90
	37	7000	0.55 [a]

[a] With a static quenching constant $V = 0.35$.

in the lipid and ATPase systems taken at equivalent lipid concentrations. Stern-Volmer plots for quenching by α- and δ-hexachlorocyclohexane are presented in Figs. 13A, B.

In the previous paper [14] we analysed the linear regions of the Stern-Volmer plots for NPTH in lipid bilayers according to the equations of Omann and Lakowicz [24] to obtain partition coefficients for the hexachlorocyclohexanes. We have analysed the data for the ATPase in the same way to obtain the partition coefficients given in Table I. As for NPTH in lipid bilayers, quenching by the δ-isomer gave curved Stern-Volmer plots,

particularly at higher temperatures and so was analysed in terms of mixed static and collisional quenching [14]: partition coefficients for this isomer are therefore likely to be less reliable than for the other isomers.

Discussion

The activity of the $(Ca^{2+} + Mg^{2+})$-ATPase purified from sarcoplasmic reticulum has been shown to be very sensitive to the chemical structure of the surrounding, annular, phospholipid both to the structure of the headgroup and the length of the fatty acyl chain. There is an optimal chain length for activity of about 18 or 20 carbons, and chain lengths either longer or shorter than this optimal length support sub-maximal activities [8–10]. It has been shown that a wide variety of hydrophobic molecules, including alkanes, fatty acids and sterols, can overcome the effects of sub-optimal phospholipids on the activity of the ATPase [9,11]. For molecules such as the fatty acids and sterols which will be anchored at the membrane/water interface, effects on activity have been suggested to follow from binding to a set of sites on the ATPase distinct from the annular sites at the lipid/protein interface: at higher concentrations these molecules bind at annular sites as well as at non-annular sites, and produce inhibition of activity. However, it has also been suggested that stimulation of the ATPase in bilayers of short chain phospholipids by short-chain alkanes such as decane follows from a thickening of the bilayer to an optimal value, with inhibition at higher concentrations following from an over-thickening of the bilayer [9]. X-ray studies have been interpreted in terms of partitioning of short chain alkanes into the centre of the bilayer with membrane thickening [6], although from neutron-diffraction studies it has been suggested that shorter chain alkanes have no effect on membrane thickness but rather increase the surface area per phospholipid molecule [33]. X-ray studies have also suggested that a variety of other hydrophobic molecules such as cyclopropane have no effect on membrane thickness [5] although measurements of membrane impedance have been interpreted in terms of membrane thickening [14].

Our results show that the pattern of effects of a variety of alkanes and isomers of hexachlorocyclohexanes on ATPase activity are similar. For the ATPase reconstituted with the short-chain phospholipid dimyristoleoylphosphatidylcholine, all molecules cause stimulation at low concentrations followed by inhibition at higher concentrations. For the ATPase reconstituted with dioleoylphosphatidylcholine, stimulation, if present, is small and inhibition is thus more marked.

Although the effect of the long chain alkane hexadecane is less marked than that of decane, it does cause a significant stimulation of the ATPase (Fig. 2), comparable to that caused by oleic acid [11]. This argues against an effect following from partition into the bilayer centre. The effect of nonane and its positional isomer, 2,2,4-trimethylhexane are comparable (Fig. 2), so that if their effects were to follow from effects on membrane thickness it would be alkane volume rather than chain length that is important. The effects of the hexachlorocyclohexanes vary markedly between isomers (Figs. 3, 4), although the volumes of the isomers would be expected to be similar. Maximum stimulation by lindane occurs at a molar ratio of lindane to lipid of approx. 3:1 (Fig. 3) compared to 1:1 for decane (Fig. 1). With a density for lindane of 1.89 g/ml [25], the volume occupied by lindane in the membrane will be about double that occupied by decane at the point of maximum stimulation, assuming that comparable fractions of the additives have partitioned into the membrane.

The similarities between the patterns of stimulation and inhibition observed for the hexachlorocyclohexanes and those observed for other hydrophobic molecules such as fatty acids and sterols argues for a similar mode of action. It has been suggested that inhibition follows from binding at annular sites with consequent displacement of phospholipid and that activation follows from binding to other, non-annular sites [11–13]. The observation that the hexachlorocyclohexanes quench the fluorescence of tryptophan residues in the ATPase demonstrates that the hexachlorocyclohexanes can bind to the ATPase, since quenching by chlorines is likely to be a short-range, contact phenomenon [26]. Detailed interpretation of the quenching results is not possible. However, the observation that maximal activations reached

with the hexachlorocyclohexanes are considerably less than for decane (Figs. 1, 3) suggests that binding to the (inhibitory) annular sites on the ATPase is considerable and thus that such binding should make the major contribution to fluorescence quenching. Although quenching by molecules such as fatty acids anchored at the membrane/water interface is largely static [11], quenching by the non-polar hexachlorocyclohexanes is likely to contain a large dynamic component. If this is the case, then quenching can be analysed in terms of Stern-Volmer plots, as for the quenching of the hydrophobic tryptophan analogue, NPTH [14]. Treating the lipid phase of the membrane as homogeneous, a partition coefficient can be defined as:

$$K_p = Q_m / Q_a$$

where Q_m and Q_a are concentrations of quencher per litre of lipid and per litre of water, respectively. Partition coefficients and Stern-Volmer quenching constants obtained in this way are given in Table I. Partition coefficients for the ATPase system differ by only a factor of two from those for simple lipid bilayers [14]. It is also noticeable that partition coefficients increase with decreasing temperature in the ATPase system as in the lipid system. Detailed interpretation of the differences between the ATPase and lipid systems is probably not justified. However, the data suggests a preferential partition of the quencher into the annulus around the ATPase. If the ATPase system is treated as a two-phase system with partition both into the lipid phase and into the lipid/protein interface, with quenching being dominated by quencher at the lipid/protein interface, then the partition coefficient, K_p^{obs} calculated from the quenching data is given by:

$$K_p^{obs} = K_p^{lipid} K_A$$

where K_p^{lipid} is the partition coefficient for the lipid phase and K_A is the partition coefficient between lipid and lipid-protein interface. The value of K_a would then be close to 2.

Stern-Volmer constants for quenching in the ATPase system are markedly smaller than those for quenching of NPTH in lipid systems (Table I, Ref. 14). This could reflect, in part, a reduced accessability of tryptophans in the ATPase to collisional quenching and, in part, a reduced rate of diffusion. ESR studies show that annular lipid is immobilised relative to phospholipid in the bulk phase [27].

As with simple lipid systems, quenching of ATPase fluorescence by hexachlorocyclohexanes is limited by solubility in the aqueous phase. For all isomers, concentrations are reached beyond which further addition of quencher causes no further increase in quenching: at these concentrations sample turbitidy increases. Effects of hexachlorocyclohexanes on the activity of the ATPase are also limited by aqueous solubility. Aqueous solubilities of the hexachlorocyclohexanes in buffer at 37°C were estimated from light scattering to be 60, 120 and 230 μM for the α-, γ- and δ-isomers, respectively. Solubilities were then estimated by light scattering in the presence of native ATPase or ATPase reconstituted with dioleoylphosphatidylcholine, under the conditions of the activity measurements in Figs. 4 and 5. As shown in Table II, the observed saturation points agree well with those calculated on the basis of the partition coefficients given in Table I. Concentrations corresponding to aqueous saturation are marked by arrows in Figs. 3–5. For the α- and γ-hexachlorocyclohexanes, limited solubility prevents ob-

TABLE II

OBSERVED AND CALCULATED CONCENTRATIONS FOR SATURATION OF THE AQUEOUS PHASE BY HEXACHLOROCYCLOHEXANES AT 37°C

System	Isomer	Saturation concentration	
		Observed (μM) [a]	Calculated (μM) [b]
Native-ATPase [c]	α	62	61
	γ	120	122
	δ	230	233
Reconstituted ATPase [d]	α	70	69
	γ	135	137
	δ	250	255

[a] Determined from light scattering breakpoints.
[b] Calculated from partition coefficients in Table I.
[c] Conditions as in legend to Fig. 5.
[d] Conditions as in legend to Fig. 4.

servation of the total activity curve whereas the greater water solubility of the δ-isomer allows observation of the curves to total inhibition.

Fig. 5 shows that inhibition of native ATPase by the hexachlorocyclohexanes mirrors fluorescence quenching, suggesting that inhibition could follow from binding to the lipid-protein interface. In Fig. 14 effects of hexachlorocyclohexanes on the activity of native ATPase and ATPase reconstituted with dioleoylphosphatidylcholine are plotted as a function of the mole fraction of hexachlorocyclohexane in the membrane, calculated from the partition coefficients given in Table I. It is clear that, expressed in this way, effects in both systems occur over very similar concentration ranges, although in terms of aqueous concentrations or in terms of total hexachlorocyclohexane to lipid ratios, the concentrations required to cause inhibition are very different in the native and reconstituted systems. It is also clear that effects of the α- and γ-isomers are very similar. Effects of the δ-isomer are apparently greater, but the partition coefficient used for the δ-isomer is rather suspect. Stern-Volmer plots for the δ-isomer are curves, as for simple lipid systems, where it was suggested that the partition coefficient might be underestimated by a factor of ca 2. If the partition coefficient of the δ-isomer were increased to 19000 at 37°C for the ATPase system, then inhibition by all three isomers would be more comparable.

The inhibition curves of Fig. 14 can be interpreted in terms of a very simple model. It is known that the activity of the ATPase is independent of the molar ratio of lipid to protein down to 30 : 1 but that activity then decreases linearly with decreasing molar ratio to approx. 15 : 1 at which point activity is zero [28]. As shown in Fig. 14, inhibition is close to being linearly related to mole fraction of hexachlorocyclohexane in the bilayer, assuming zero activity at a mole fraction of 0.5. The above analysis is of course oversimplified and ignores the activation that occurs at low mole ratios of hexachlorocyclohexane, particularly in the presence of dimyristoleoylphosphatidylcholine and which is believed to follow from binding to other, non-annular sites.

As shown in Figs. 9 and 10, the inhibitory effects of the hexachlorocyclohexanes increase as the temperature is decreased. These effects corre-

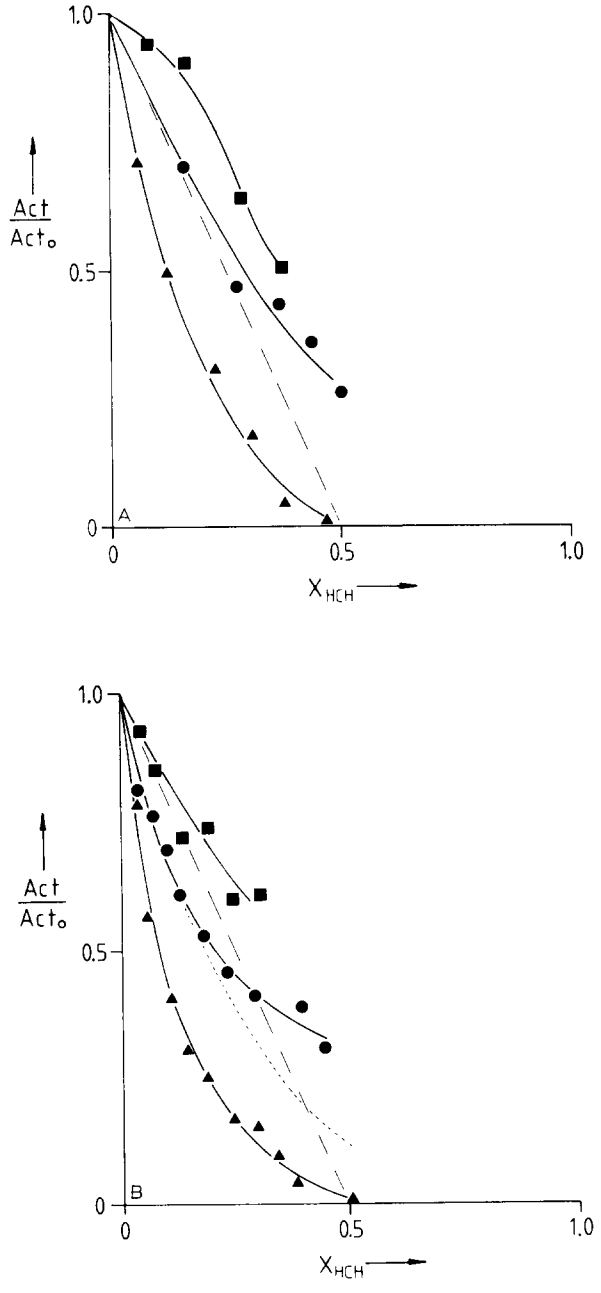

Fig. 14. Activities of native ATPase (A) and ATPase reconstituted into dioleoylphosphatidylcholine (B) as a function of the calculated mole fraction of hexachlorocyclohexane in the lipid bilayer. Details of activity measurements as in legends to Figs. 4 and 5. Isomers of hexachlorocyclohexane: ■, α; ●, γ; ▲, δ. In (B) the dotted line (· · · · · ·) presents calculations based on a K_p of 19000 for the δ-isomer (see text). The broken line is the concentration dependence of activity expected for the model presented in the text.

late well with the negative temperature coefficient for partition into the ATPase system (Table I) and into lipid bilayers. Arrhenius plots of activity of the native ATPase show a breakpoint at 20°C, as reported previously [29]. In the presence of α- and δ-hexachlorocyclohexane this breakpoint is shifted to lower temperatures.

The reaction step (or steps) affected by the hexachlorocyclohexanes is (are) unclear. None of the isomers had any significant effect on the Ca^{2+} dependence of ATPase activity (see Fig. 6) and so presumably did not affect the Ca^{2+} affinity of the ATPase. Effects of ATP concentration on ATPase activity are complex and suggest either two ATP sites on the ATPase (catalytic and regulatory) or one site which can exist in two states of differing affinity. The data for lindane presented in Figs. 7 and 8 suggest that lindane has no marked effect on ATP affinity and that inhibition by lindane is comparable at both high (2 mM) and low (1.7 μM) ATP concentrations. Dupont [34] has suggested that the dependence of ATPase activity v on concentration of MgATP can be fitted to the equation:

$$\frac{v}{V_{max}} = \frac{[S]^2 + [S]K_{reg}\alpha^{-1}}{[S]^2 + [S]K_{reg} + K_{cat}K_{reg}}$$

where [S] is the concentration of MgATP, K_{cat} and K_{reg} are dissociation constants for MgATP at the presumed catalytic and regulatory sites, respectively, and α is a linking constant. In the absence of lindane, the data fits reasonably well to this equation, with $K_{cat} = 2$ μM, $K_{reg} = 125$ μM and $\alpha = 6.5$. In the presence of lindane (Fig. 7), the data fits to $K_{cat} = 2$ μM, $K_{reg} = 120$ μM and $\alpha = 5.0$. It is unlikely, therefore that lindane binds directly at the ATP site in the way that, for example, halothane binds at the adenine binding site in adenylate kinase [31]. It has been suggested that the conformation change of the ATPase between the E and E* forms could be particularly sensitive to change in lipid environment [30] and it is possible that it is steps involving such changes which are sensitive to the presence of hexachlorocyclohexanes.

Effects of lindane on the purified and reconstituted ATPase are comparable to effects on the ATPase in both coupled and uncoupled vesicles of sarcoplasmic reticulum (Fig. 11). These results disagree with those of Antunes-Madeira and Madeira [32] who report a slight stimulation of ATPase activity in vesicles of sarcoplasmic reticulum.

It is difficult to compare the concentrations of hexachlorocyclohexanes required to effect the activity of the ATPase with those required for insecticidal activity, although it is clear that the concentrations used in our experiments are high. However, there is no simple relationship between occupancy of a receptor site and a pharmacological effect as complex as death. If, for example, the effects of lindane were to follow from an effect on neurotransmitter release [3], then a small effect on synaptic Ca^{2+} levels would have a large effect on the pattern of neurotransmitter release.

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