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MECHANISM OF INHIBITION OF RAT BRAIN (Na + K)-ADENOSINE TRIPHOSPHATASE BY 2,2-BIS(*p*-CHLOROPHENYL)-1,1,1-TRICHLOROETHANE (DDT)

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Abstract—Inhibition of an (Na + K)-ATPase preparation from rat brain by DDT was five times as effective when the insecticide was particulate than when solubilized by the surfactant, Corexit 7664. The surfactant reduced binding of DDT to the membranes by about 5 fold. Inhibition correlated with the amount of DDT bound rather than with its concentration or physical state. In the absence of the surfactant, maximal inhibition and binding occurred when the membranes contained about 350 pmoles/μg of protein, an amount equal to 12 per cent of the protein content of the membranes. At low concentrations most of the DDT present is bound by the membranes, hence its effect decreases as membrane concentration increases. The kinetics of inhibition of the (Na + K)-ATPase by DDT, allethrin and DDE were examined by varying the concentrations of inhibitor, Na^+, or K^+, while holding the concentration of the others constant. Inhibition by all of these compounds was potentiated by increased K^+, and the increment in the inhibition caused by K^+ was reversible by increased Na^+ concentrations. Hence the effectiveness of DDT is dependent on the membrane concentration and the relative concentrations of Na^+ and K^+. The concentration of DDT required for maximal inhibition suggests that the inhibition is not caused by binding to a specific site on the enzyme, but is the result of indirect alterations of the membrane which interfere with allosteric transitions of the (Na + K)-ATPase mediated by Na^+ and K^+.

The (Na + K)-ATPase (ATP phosphohydrolase EC 3.6.1.3) is generally thought to be involved in the active transport of Na^+ and K^+ across animal cell membranes [1]. Several studies have reported that DDT [2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane] is a potent inhibitor of this enzyme in rat brain [2, 3], fish tissues [4–6] and lobster nerve [7]. Koch *et al.* [8–10], on the other hand, have reported that the (Na + K)-activated ATPase was only slightly inhibited in preparations from a variety of tissues, but the Mg-ATPase was more sensitive. The reasons for the differences between the sets of reports are not apparent.

The solubility of DDT in water is less than 1·2 ppb [11], and addition of ethanolic solutions of the pesticide to aqueous reaction solutions results in the formation of opalescent suspensions. Pocker *et al.* [12] have reported that DDT inhibition of carbonic anhydrase is the result of precipitation of the enzyme by the DDT crystals. The insecticide was not inhibitory when the assay was performed in media which solubilized it (34% dimethylformamide), and thus it is not a true inhibitor of this enzyme. A recent report by Jackson and Gardner [13] similarly reported that DDT and other organochlorine insecticides were not inhibitory to the (Na + K)-ATPase when solubilized by the addition of a surfactant, Corexit 7664. Co-precipitation seems inadequate to explain inhibition of the (Na + K)-ATPase, since preparations of the enzyme are composed of membrane fragments, i.e. are themselves particulate. An alternative suggestion proposed by Jackson and Gardner [13] is that Corexit competes with the membrane fragments for DDT. Part of this work is an attempt to learn more about the mechanism of inhibition of the (Na + K)-ATPase by correlating inhibition with the amount of DDT associated with the membranes in the presence and absence of Corexit.

Recent studies in this laboratory have also considered the kinetic interactions between DDT, Na^+ and K^+ in an (Na + K)-ATPase preparation from rat brain in an attempt to elucidate the mechanism of inhibition. The effects of DDT were compared with those of allethrin and DDE [2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene] in order to correlate neurotoxic activity and inhibition of the (Na + K)-ATPase, since these may have a similar mechanism. Allethrin is a synthetic pyrethrin insecticide which has little structural resemblance to DDT yet appears to cause similar electrophysiological effects on isolated nerves [14]; DDE is a metabolite of DDT which, in spite of its structural similarity to DDT, has little insecticidal activity or effect on the nerve action potential [7].

It is unlikely that inhibition of the (Na + K)-ATPase is the mechanism by which DDT causes Na^+ conductance changes in nerves [15], since the (Na + K)-ATPase does not participate directly in the action potential. Study of the nature of inhibition of this enzyme, however, may contribute to understanding of general aspects of effects of DDT on membranes and permeability phenomena.

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EXPERIMENTAL

The brains used as a source of the (Na + K)-ATPase for this study were from Wistar rats weighing 200–400 g and the microsomal preparation used was the R-2 fraction as described by Ahmed and Judah [16]. The preparation was stored frozen (−20°) in 1·5-ml portions for up to 3 weeks with loss of less than 10 per cent of original activity. The reaction mixture for the measurement of ATPase activity consisted of 40 mM Tris–HCl (pH 7·4), 5 mM $MgCl_2$, 5 mM ATP (Tris salt), 10–40 μg of microsomal protein, and various concentrations of NaCl and KCl in a volume of 1 ml. The (Na + K)-ATPase activity was obtained by subtracting the ATPase activity measured in the absence of either Na^+ or K^+ from that measured in the presence of Na^+, K^+ and Mg^{2+}. The ATPase activity which required only Mg^{2+} was about 10 per cent of the maximal activity measured in the presence of Na^+, K^+ and Mg^{2+}. The pesticides were added to the reaction mixtures in 10 μl ethanol 15 min prior to the addition of ATP. Controls also contained 10 μl ethanol. After 30 min of incubation with ATP, the reaction was stopped by placing the tubes in an ice bath and adding 1 ml of 7·5% trichloroacetic acid. Inorganic phosphate was measured by the Fiske and Subbarow method [17] or, when Corexit was present, by the Lowry and Lopez method [18], in order to avoid the formation of a precipitate caused by the detergent in the more acidic solution used by Fiske and Subbarow. The ATPase data reported in this paper are averages of triplicate assays. Where Corexit 7664 was present in the assay mixture, it was routinely used in a concentration of 0·2% (v/v) and was added prior to the addition of ATP and DDT. As reported by Jackson and Gardner [13], this concentration of Corexit 7664 had no detectable effect on ATPase activity; further, the kinetic dependence on Na^+ at K^+ concentrations of 10 and 150 mM was identical in preparations which contained no Corexit.

DDT binding to the membranes was measured by incubating the membranes and pesticide at 37°, then separating membranes and unbound DDT by centrifugation on a discontinuous sucrose density gradient. About 0·1 to 0·3 mg of membrane protein was incubated with [^{14}C]-DDT for 1 hr at 37° in 1 ml of a solution identical to that used to assay the (Na + K)-ATPase. Higher concentrations of membranes were used for measuring binding than for ATPase assays in order to have sufficient protein for analysis in the fractions collected after centrifugation. The usual concentration of DDT was 40 nmoles/ml with a specific activity of 846 cpm/nmole. At the end of the incubation, the DDT and membranes were mixed with 5 ml of 2·3 M sucrose at room temperature and transferred to 13-ml nitrocellulose centrifuge tubes. Five ml of 1·55 M sucrose was layered on the 6 ml in the tubes without mixing, followed by 3 ml of 0·05 M K^+-phosphate buffer (pH 7·5). The tubes were centrifuged in a Beckman model L2-65 (SW 41 Ti rotor) for 2 hr at 20° and 28,500 rev/min (100,000 *g*). The centrifuged tubes were then sampled with a Beckman gradient fractionator in 0·5-ml fractions starting at the bottom of the tube; samples of the fractions were analyzed for protein and [^{14}C]-DDT. The membranes formed an obvious band at the boundary of the 1·55 M sucrose and the phosphate buffer. Controls containing [^{14}C]-DDT but no membranes were run with each experiment. The amount of radioactivity in the appropriate fractions was subtracted from that in the same fractions which contained membranes to give the amount of DDT associated with the membranes.

The radioactive DDT was measured by liquid scintillation using Multisol (Isolab Inc., Akron, Ohio) as solvent and scintillator. Protein was analyzed by the method of Lowry *et al.* [19]. The ATP was purchased as the disodium salt (Sigma Chemical Co., St. Louis) and converted to the Tris salt by passing it through a column of Bio-Rad AG 50 W-X4 (H^+-form) cation-exchange resin and neutralizing with Tris. The pp-DDT (>99 per cent purity) was obtained from Aldrich Chemical Co. (Milwaukee), radioactive DDT [*p*-chlorophenyl-^{14}C-(U)] from New England Nuclear (Boston, and Corexit 7664 was provided by the Esso Research and Engineering Co. (Florham Park, N.J.).

RESULTS

Effect of Corexit on inhibition and binding. A comparison of the inhibitory effects of solubilized and particulate suspensions of DDT shows that DDT was a more effective inhibitor when presented as a particulate suspension (Fig. 1). However, in contrast to the report of Jackson and Gardner [13], DDT partially inhibited the enzyme in the presence of 0·2% Corexit 7664.

The decreased sensitivity of the enzyme in 0·2% Corexit could be the result of competition between surfactant and membranes for the DDT or simply the result of solubilization of the pesticide. In order to choose between these alternatives, a method was developed to measure the amount of DDT bound by the membrane fragments in the presence and absence of Corexit. Centrifugation procedures which sediment the membranes do not permit their separation from particulate DDT, since unbound DDT is sedimented along with them. Figure 2A shows that DDT which had been incubated in medium containing Corexit remained in the 6 ml of 1·9 M sucrose at the bottom of the centrifuge tube due to lack

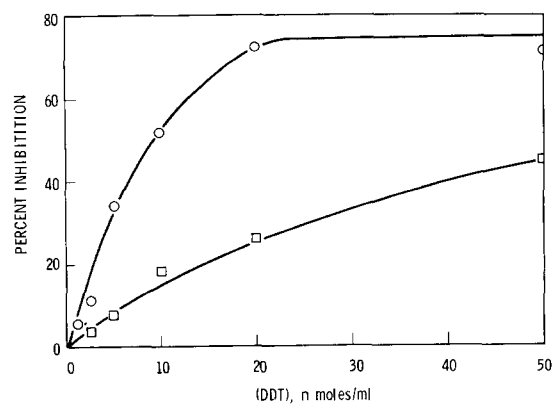

Fig. 1. Effect of Corexit 7664 on inhibition of (Na + K)-ATPase activity by DDT. The incubation mixtures contained 20 mM NaCl, 100 mM KCl and were incubated in the absence (○——○) and presence (□——□) of 0·2% Corexit 7664. The concentration of membrane protein was 40 μg/ml; the specific activity of controls was 0·26 μmoles mg^{-1} min^{-1}.

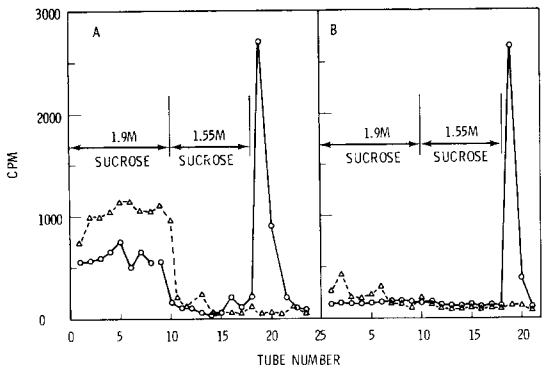

Fig. 2. Isolation of [^{14}C]-DDT bound to membranes by centrifugation in a sucrose density gradient. The DDT was incubated for 1 hr at 37° in the presence (O——O) and absence (△---△) of rat brain microsomal membranes. Fractions 1–10 contained 1·9 M sucrose; fractions 11–18 contained 1·55 M sucrose and 19–23 contained 0·05 M K^+ phosphate buffer (pH 7·5). Membrane protein was detected in fractions 19 and 20 only. Fractions (0·5 ml) started from the bottom of the tube. (A) Incubation mixture contained 625 μg membrane protein and 0·2% Corexit; (B) incubation mixture contained 125 μg protein and no Corexit.

of mixing, while the membrane fragments and associated DDT moved through and floated on the 1·55 M sucrose layer. When DDT was incubated in the absence of surfactant (Fig. 2B), it sedimented on the bottom of the tube and was not recovered unless it was bound to membranes. Assay of all fractions for protein and (Na + K)-ATPase activity after incubation with and without Corexit revealed that they were detectable only at the top of the 1·55 M sucrose layer, suggesting that the enzyme was not solubilized by the surfactant. Time course studies showed that the amount of DDT bound was constant with 5–60 min of incubation. In order to examine the tightness of binding, membranes containing DDT bound in the absence of surfactant were recovered after a centrifuge run, suspended in 1·9 M sucrose and recentrifuged as before. The membranes recovered after the second centrifuging contained 92 per cent of the DDT measured after the first analysis, showing that the complex formed between DDT and the membrane fragments is stable in aqueous solutions.

Table 1. Effect of Corexit, Na^+ and K^+ concentrations on binding of DDT to rat brain microsomal membranes*

NaCl concn (mM)	KCl concn (mM)	Corexit concn (%, v/v)	DDT bound (pmoles/μg protein)
60	20	0	255
60	20	0·2	60
60	20	0·5	33
60	20	1·0	23
0	0	0	238
150	10	0	247
10	150	0	248

* The membranes (250 μg/ml) were incubated with [^{14}C]-DDT (40 nmoles/ml) for 1 hr at 37° in: 5 mM ATP, 40 mM Tris–HCl (pH 7·5), 5 mM $MgCl_2$ and the indicated concentrations of NaCl, KCl and Corexit.

The presence of 0·2% Corexit in the incubation mixture reduced the binding of DDT to the membranes (Table 1) to about the same extent that it reduced inhibition (Fig. 1). Increasing the concentration of Corexit beyond 0·2% further decreases the binding. If the association of DDT with the membranes were the result of co-precipitation, one would expect that increasing the concentration of Corexit above that required to solubilize the DDT would have no further effect on binding. In fact, as Corexit increased from 0·2 to 1%, binding was reduced almost 3-fold, suggesting that Corexit competes with the membranes for DDT or affects the membranes and somehow reduces their ability to bind the pesticide. The effect of Na^+ and K^+ concentrations was examined because the activity of the (Na + K)-ATPase is dependent on them. The binding of DDT evidently was not influenced by the changes in salt concentration.

The association of membranes and insoluble DDT shows that the membranes are saturated at about 350 pmoles/μg of membrane protein (Fig. 3). This is equivalent to 123 ng DDT/μg of protein or about 12 per cent of the protein content of the membrane. The relative amounts of bound and unbound DDT cannot be measured directly, since the unbound DDT is lost on the bottom of the nitrocellulose centrifuge tube and the protein was not quantitatively recovered from the gradient. Since no protein was detectable in regions other than the 1·55 M sucrose–phosphate buffer interface, the concentration of DDT in the samples of membranes reflects the overall concentration. With this assumption, the total amount of bound DDT can be calculated by multiplying the DDT concentration in the recovered membranes by the total quantity of protein added to the assay system. The calculation demonstrates that in the absence of Corexit about 90 per cent of the DDT added was bound at initial DDT concentrations of 20 nmoles/ml and less, 80 per cent at 40 nmoles/ml, and 40 per cent at 100 nmoles/ml. In the presence of Corexit, however, only 40 per cent was bound

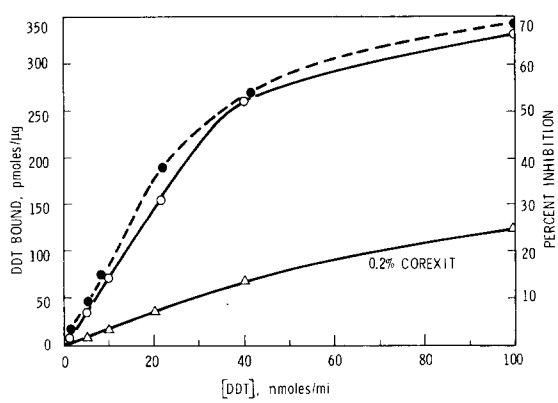

Fig. 3. Concentration dependence of DDT binding to microsomal membranes and inhibition of the (Na + K)-ATPase. Binding in absence of surfactant (O——O) was measured by incubating membranes (125 μg protein) with [^{14}C]-DDT in 1·0 ml. Binding in the presence of 0·2% Corexit 7664 (△——△) was measured by incubating 250 μg membrane protein in 1·0 ml with the labeled DDT. The (Na + K)-ATPase assays (●---●) contained 125 μg protein, no Corexit, 20 mM NaCl and 100 mM KCl in 1 ml and were incubated for 10 min at 37°.

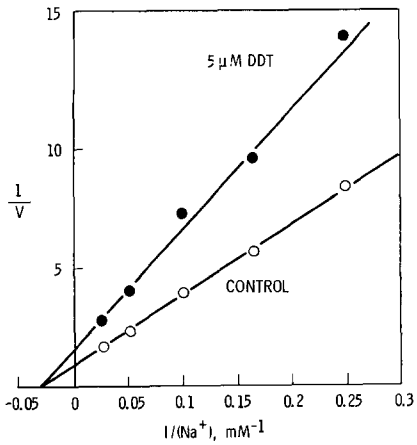

Fig. 4. Lineweaver–Burk plot of the kinetics of activation of the (Na + K)-ATPase in the presence (●——●) and absence (○——○) of 5 μmoles/l. of DDT. The concentration of KCl was kept at 1 mM, while NaCl was varied as shown. The protein concentration was 20 μg/ml.

at 20 and 40 nmoles/ml, and saturation was not evident even at 100 nmoles/ml DDT. Without Corexit, binding was limited by the amount of DDT present, not by its concentration, and the plot is not a real illustration of the affinity of the membranes for DDT. The membranes can be viewed as a separate phase containing a solvent for DDT rather than as proteins which contain a limited number of specific binding sites. In the absence of surfactant, the partitioning of DDT strongly favors association with the membranes.

In the absence of Corexit and at DDT concentrations of less than 40 nmoles/ml, essentially all of the DDT in the system was bound by the membranes. It seems likely that with less membrane material in the incubation mixture the concentration of DDT in the membranes would be higher; thus saturation would occur at lower DDT concentrations. In fact, the concentration vs inhibition curve with 40 μg protein/ml (Fig. 1) showed maximal inhibition at 20 nmoles DDT/ml and half-maximal at 5 nmoles DDT/ml, whereas with 125 μg membrane protein/ml (Fig. 4), half-maximal binding occurred at about 20 nmoles/ml. The effect of DDT on the enzyme when the membrane protein concentration was 125 μg/ml is also shown in Fig. 4. The concentrations of DDT which caused maximal and half-maximal inhibition were about the same as those which caused the same relative amount of binding. This suggests that the concentration of DDT in the membranes is a more important variable than its original concentration in the medium. The failure to achieve complete inhibition is explained by the observed saturation of the membranes at about 350 pmoles/μg of protein. Inhibition can be related to membrane DDT content in Fig. 4 by comparing the two ordinates, e.g. the concentration of DDT which causes 50 per cent inhibition is about 250 pmoles/μg of protein.

In several of the experiments discussed below the enzyme assay mixtures contained 5 nmoles/ml of DDT, 11–20 μg/ml of membrane protein and no Corexit. Although the DDT content of the membranes was not measured, it can be estimated using the data shown in Fig. 3. An assay mixture containing 5 nmoles DDT and 11 μg protein would contain about 300 pmoles DDT/μg of protein, since about 454 pmoles/μg would be available for binding. Assays containing 20 μg protein would be expected to bind about 80 per cent of available DDT, and the membranes would contain about 200 pmoles/μg of protein.

Effect of Na^+ and K^+ on DDT inhibition. In order to study the kinetic interactions between Na^+ and DDT, the concentration of NaCl was varied while holding the concentration of KCl at 1 mM and DDT at 5 μmoles/l. (Fig. 4). At this relatively low concentration of K^+, the double reciprocal plots yield straight lines, suggesting a conventional hyperbolic relationship between activity and Na^+ concentration. It is clear that DDT changed both the intercept and the slope of the lines: their intersection on the abscissa indicates that the interaction of DDT and Na^+ was noncompetitive.

A Lineweaver–Burk plot of the effect of DDT on K^+ activation of the (Na + K)-ATPase is shown in Fig. 5. In this experiment, K^+ was varied in the range from 0·075 to 1·0 mM, i.e. below saturation of the K^+-activation site. The data show that the relationship between activity and the K^+ concentration was hyperbolic and that the inhibition by DDT was noncompetitive. At low K^+ concentrations then, DDT inhibition was independent of Na^+ and K^+ concentrations.

At higher concentrations of K^+ it was obvious that DDT inhibition was dependent on the concentration of Na^+ and K^+. Both cations are required for activation and both inhibit the enzyme when present in supraoptimal concentrations [20, 21]. The specific activity of the (Na + K)-ATPase preparation in response to Na^+, K^+ and Li^+ at concentrations used to examine their interactions with DDT is shown in Fig. 6. As the concentration of K^+ is increased above 5 mM, in a constant concentration of NaCl, the enzyme is increasingly inhibited. This effect is more pronounced in 20 mM than in 150 mM NaCl. The response of enzyme activity to Na^+ is hyperbolic at low K^+ (Fig. 4) but becomes sigmoidal at higher K^+ concentrations (Fig. 6) [20–22]. As shown in Fig. 6, Li^+ inhibits the enzyme, presumably by acting as a competitive inhibitor of Na^+, K^+ or both [20, 22].

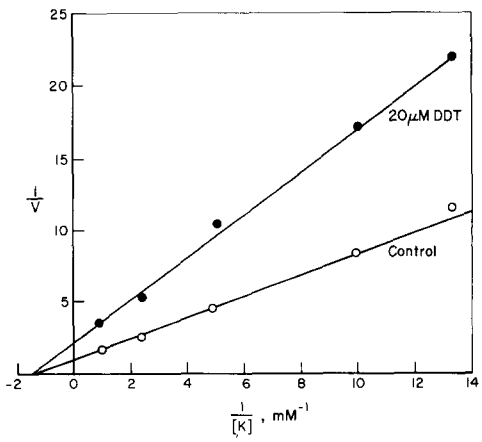

Fig. 5. Lineweaver–Burk plot of activation of the (Na + K)-ATPase by KCl in the presence (●——●) and absence (○- - -○) of 20 μmoles/l. of DDT. The concentration of NaCl was kept constant at 10 mM. The protein concentration was 20 μg/ml.

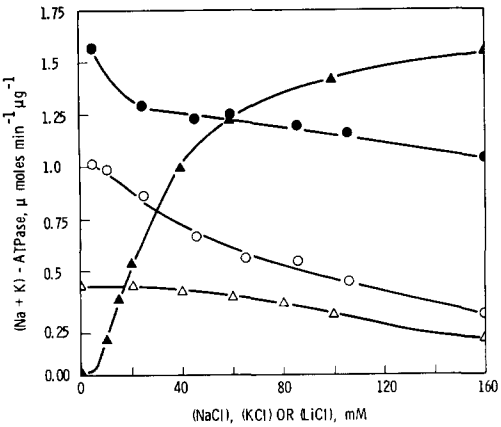

Fig. 6. Dependence of the (Na + K)-ATPase on the concentration of Na^+, K^+ and Li^+. The independent variables and corresponding symbols are: NaCl in constant 150 mM KCl (▲——▲); KCl in 20 mM NaCl (○——○); KCl in 150 mM NaCl (●——●); and LiCl in 2 mM KCl and 20 mM NaCl (△——△). The protein concentration was 11·4 μg/ml.

In order to examine interaction between ion concentrations and DDT, the concentration of Na^+, K^+ or both, was held constant and the concentrations of K^+, Na^+ and Li^+, respectively, were varied in the presence and absence of DDT (Fig. 7). The data, which are presented as per cent inhibition by DDT, were calculated relative to controls lacking DDT at each ion concentration. The specific activity of the control values for these data are shown in Fig. 6. Although the (Na + K)-ATPase activity of the control assays in both Na^+ concentrations decreased as the concentration of K^+ increased, in 20 mM Na^+ the inhibition attributable to DDT was not a constant proportion of remaining activity. In 20 mM Na^+ therefore, K^+ and DDT did not act independently, but interacted in such a way that K^+ increased the relative effectiveness of DDT. On the other hand, DDT inhibition in the presence of 150 mM Na^+ was independent of the concentration of K^+ and at about the same level as with 5 mM K^+ and 20 mM Na^+.

In the presence of 150 mM K^+, addition of Na^+ reduced inhibition by DDT. Since Na^+ and K^+ have opposite effects the potentiating effect of K^+ was not a general effect of increased ionic strength or Cl^-. The data from an experiment designed to test further the specificity of the potentiating effect of K^+ on DDT inhibition are also shown in Fig. 7. The concentration of LiCl was varied in the presence of 20 mM Na^+, with and without DDT. Increasing the concentration of Li^+ partially inhibited the enzyme as did K^+, but did not increase the inhibition by DDT. Although Li^+ inhibits the (Na + K)-ATPase, it does not mimic K^+ in that it does not increase the sigmoidicity of the response to Na^+ [22].

Unlike the effects of Na^+ and K^+, protection of the enzyme from DDT inhibition by Corexit can be attributed to decreased association of the pesticide with the membranes. If the effect of Corexit is simply to decrease the availability of DDT, one would expect the mechanism of inhibition to remain the same in the presence of Corexit, i.e. K^+ should increase and Na^+ should decrease inhibition. An experiment designed to test this prediction (Fig. 8) shows that the nature of the kinetic interaction with NaCl and KCl resembled that of particulate DDT. As the KCl concentration was increased from 10 to 150 mM, inhibition due to DDT increased from 25 to 63 per cent in 10 mM NaCl but remained constant at about 20 per cent in 150 mM NaCl. As the NaCl concentration increased from 10 to 150 mM in 150 mM KCl, inhibition decreased from 65 per cent to about 20 per cent. This pattern is identical to that observed with 5 μmoles/l. of particulate DDT, suggesting that Corexit changes the degree of inhibition but not its mechanism.

Effect of allethrin and DDE. The same kind of kinetic experiments used to study the effect of DDT on the (Na + K)-ATPase were used to examine the

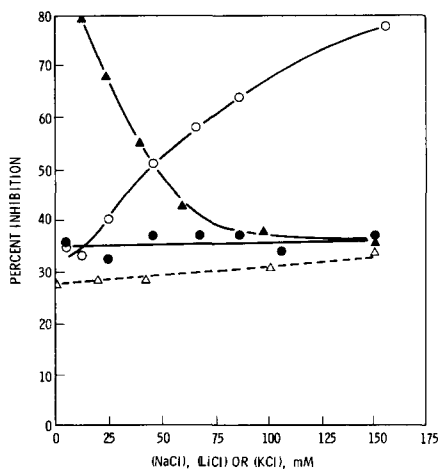

Fig. 7. Effect of NaCl, KCl and LiCl on inhibition of the (Na + K)-ATPase by DDT. The independent variables and symbols are: KCl in 20 mM NaCl (○——○); KCl in 150 mM NaCl (●——●); NaCl in 150 mM KCl (▲——▲); and LiCl in 2 mM KCl and 20 mM NaCl (△---△). The per cent inhibition was calculated relative to controls at each different cation concentration. The control values for each of these points are shown in Fig. 6. The protein concentrations used were 11·4 μg/ml.

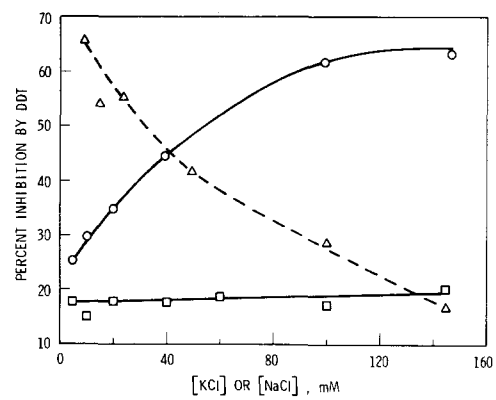

Fig. 8. Effect of NaCl and KCl concentration on inhibition of the (Na + K)-ATPase by 50 nmoles/ml of DDT in the presence of 0·2% Corexit 7664. The per cent inhibition was calculated relative to controls lacking DDT at each concentration of NaCl and KCl. The independent variable of data represented by solid lines is KCl concentration, with 10 mM (○——○) and 150 mM NaCl (□——□); that of the dashed line is NaCl concentration with 150 mM KCl (△---△). The protein concentration was 20 μg/ml.

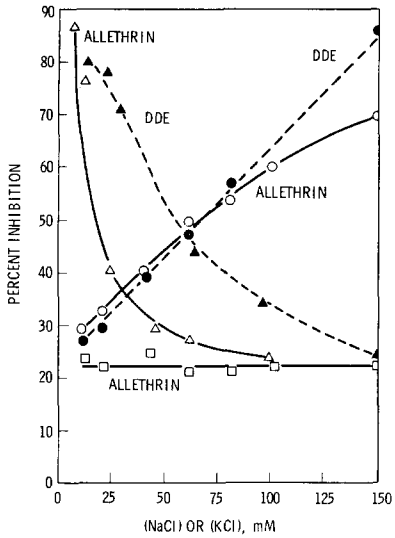

Fig. 9. Effect of NaCl and KCl concentration on inhibition of the (Na + K)-ATPase by allethrin and DDE. The inhibitors, independent variables and corresponding symbols are; 25 μM allethrin and KCl in 20 mM NaCl (○——○) and 150 mM NaCl (□——□); NaCl in 40 mM KCl (△——△); 5 μM DDE, KCl in 20 mM NaCl (●---●); and NaCl in 100 mM KCl (▲---▲). The protein concentration was 20 μg/ml.

effects of allethrin and DDE. Both formed opalescent suspensions in the assay mixtures and both inhibited the enzyme. Allethrin was less potent than DDT, since 20 μmoles/l. caused about one-half maximal inhibition, whereas only 5 μmoles/l. of DDT was required for the same level of inhibition. The effectiveness of DDE, however, was about equal to that of DDT.

Allethrin and DDE inhibition, like that of DDT, was determined by the concentration of Na^+ in the assay mixture (Fig. 9); as its concentration increased from 10 to 100 mM, inhibition decreased. The inhibition by both compounds also decreased to a minimum of about 25 per cent in the presence of high Na^+ concentrations. Thus, the kinetics of interaction between Na^+ and these compounds strongly resemble that of DDT. In 20 mM Na^+, the effectiveness of both allethrin and DDE increased with the K^+ concentration (Fig. 9). In the presence of 150 mM Na^+, however, allethrin inhibition remained constant and at the same level as in 20 mM Na^+ and 10 mM K^+. The results of this experiment were what one would expect if the mechanism of inhibition of the two compounds were the same as that of DDT.

Table 2. Combined inhibition of rat brain (Na + K)-ATPase by DDT and allethrin*

Inhibitor	Inhibition (%) Observed	Predicted
5 μM DDT	45	
50 μM Allethrin	37	
5 μM DDT + 50 μM allethrin	41	60

* The assays were performed in the standard assay mixture containing 20 mM NaCl and KCl, and 20 μg/ml of membrane protein. The inhibitors were added in 10 μl ethanol 15 min before the addition of ATP. The specific activity of controls was 0·31 μmole mg^{-1} min^{-1}. Calculation of the predicted cumulative inhibition, assuming separate noninteracting sites, was by the method of Woolfolk and Stadtman [23].

When two inhibitors act independently and at different sites on the same enzyme, their cumulative inhibition can be predicted from their individual effects. The total per cent of inhibition should be the sum of the individual inhibition of one plus the individual inhibition of the other acting on the portion of activity not blocked by the first inhibitor [23]. Table 2 shows that DDT and allethrin acting together are less inhibitory than would be predicted, assuming separate sites of inhibition. The combined inhibition is, in fact, no more than the individual inhibition of DDT alone. These data, then, support the premise that DDT and allethrin act on the (Na + K)-ATPase by a common mechanism. It seems likely that DDE and DDT also inhibit in the same way because the dependence of inhibition by either compound on the concentration of cations is identical and they are structurally related compounds.

DISCUSSION

The inhibition of the (Na + K)-ATPase demonstrated in the presence of 0·2% Corexit rules out coprecipitation of the enzyme by DDT crystals as the only mechanism of inhibition. Inhibition by particulate DDT increases in high K^+ and decreases in high Na^+ concentrations, and since inhibition resulting from solubilized DDT responds to Na^+ and K^+ in exactly the same manner, it seems likely that the mechanism of inhibition by the insecticide is not dependent on its physical form. These data disagree with the earlier report that the enzyme from fish brain is insensitive to DDT when its precipitation is prevented by surfactant [13]. Comparison of effects of DDT on the (Na + K)-ATPase with results of other studies is difficult, since the inhibition is influenced by ion levels, the concentration of membranes, and probably by variability in the binding properties of different membrane preparations.

The data describing the effect of Na^+ and K^+ on DDT, allethrin and DDE inhibition of the rat brain (Na + K)-ATPase can be explained by postulating that inhibition occurred by two distinct kinetic modes. Inhibition by one of these modes was independent of K^+ and Na^+ concentration and inhibited the enzyme to a maximum of about 35 per cent, whereas inhibition by the other mode was dependent on K^+ and reversed by Na^+. This hypothesis explains the observation that the apparent minimum of inhibition in 20 mM Na^+ and 5 mM K^+ corresponds closely with the level of inhibition observed in 150 mM Na^+ at all K^+ concentrations. Binding of DDT to the enzyme preparation was independent of changes in Na^+ and K^+ concentrations, suggesting that they did not interfere with the availability of the DDT, but affected the enzyme directly. Although it is possible that a small, critical fraction of the binding was increased by K^+ and reduced by Na^+, this seems unlikely due to the close correlation between DDT levels in the membranes and inhibition (Fig. 3).

A considerable body of evidence suggests that Na^+ and K^+ are allosteric effectors of the (Na + K)-ATPase. Plots of (Na + K)-ATPase activity as a function of Na^+ concentration are sigmoidal in high concentrations of K^+ [20–22]. An increase in Na^+ concentration also causes an increase in the apparent order of the dependence on K^+ [24]. These observations are consistent with the notion that the (Na + K)-ATPase exhibits both homotropic and heterotropic allosteric interactions and that binding of the ions causes specific conformational changes. Several studies of kinetic interactions between Na^+, K^+ and inhibitors support this concept [25–27].

Recent reports [22, 28, 29] have shown that the sigmoidal response to Na^+ of active Na^+ efflux in red cells and (Na + K)-ATPase in red cell ghosts was caused by intracellular K^+. This effect of K^+ was explained by a model in which intracellular K^+ acts as an allosteric inhibitor of Na^+ efflux in erythrocytes [22]. The K^+ concentration range required to cause a sigmoidal response of efflux and (Na + K)-ATPase activity resembles that of the physiological intracellular concentration and also the range required to increase the effectiveness of DDT, i.e. 20–150 mM. Similarly Li^+ does not affect the sigmoidicity of the response to Na^+ and has no effect on DDT inhibition. The response of the rat brain (Na + K)-ATPase to Na^+ is dependent on K^+ in the same way as the erythrocyte enzyme, hence this also seems allosteric.

DDT probably does not inhibit by classical binding to a specific site, since the concentration required in the membrane to maximally inhibit the enzyme was 12 per cent of the membrane protein. It seems more likely that it dissolves in the lipid phase of the membrane and indirectly inhibits by altering the properties of this region. Alterations of the lipid part of membranes are known to have dramatic effects on the allosteric properties of the (Na + K)-ATPase and other membrane-bound enzymes [30–32]. Kimelberg and Papahadjopoulos [33] demonstrated that the fluidity of fatty acids associated with the (Na + K)-ATPase affects the temperature dependence of the enzyme. Further, cholesterol inhibits the enzyme, presumably by decreasing the fluidity of its local environment. Since DDT is a planer lipophilic molecule of similar size and is accumulated to high concentrations in the membrane, it seems likely it also decreases the fluidity of at least some regions of the membrane lipid. The local environment of the (Na + K)-ATPase is lipid and an increase in viscosity of this phase would be expected to alter its allosteric transitions. The effect of DDT can be explained by proposing that the enzyme is stabilized in the K^+ (inhibited) conformation by the presence of high concentrations of DDT in the lipid environment of the enzyme.

The data shown here suggest that DDT, DDE and allethrin inhibit the enzyme in a similar manner and apparently at the same sites. Matsumura and Narahashi [7], using lobster nerves, observed that chemicals which cause electrophysiological effects similar to those caused by DDT were also ATPase inhibitors and that DDE, which is not neurotoxic, did not inhibit the enzyme. In contrast to their work, but in agreement with that of Akera *et al.* [34], the data presented here show that DDE was as effective as DDT in inhibiting the (Na + K)-ATPase. Further, the nature of the inhibition by DDE was virtually identical to that of DDT inhibition. The correlation between neurotoxic activity and inhibition of the (Na + K)-ATPase is strong for allethrin but weak for DDE.

These results suggest a mechanism for the neurotoxic activity of DDT and allethrin, which apparently act by prolonging the falling phase of the action potential, i.e. delaying the restoration of membrane impermeability to Na^+ [35, 36]. They may interfere with a protein conformational change required to decrease the permeability of the axon membrane to Na^+ during the action potential by the same indirect mechanism proposed for inhibition of the (Na + K)-ATPase. This explanation, however, does not account for the lack of correlation between DDE inhibition and neurotoxic activity. Perhaps the solubility properties of DDE cause its distribution among blood, other tissues and nerves to differ from that of DDT so that effective concentrations are not achieved in the nerve cell membranes *in vivo*.

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