Inhibition by DDT of Calcium Accumulation in Isolated Cell Fractions From Rat Muscle[1]

Wayne N. Yunghans, John H. Elder, Bruce Wintersteen[2] and D. J. Morré
Department of Botany and Plant Pathology
Purdue University, West Lafayette, Indiana 47907

Abstract

DDT inhibits the accumulation of calcium ions by a sarcoplasmic reticulum fraction of rat muscle. Accumulation was observed in electron micrographs as electron dense deposits of calcium oxalate in membrane vesicles. Calcium accumulation and DDT inhibition were measured using radioactive $^{45}Ca++$ and spectrophotometric assay techniques. Inhibition of calcium accumulation by DDT occurred at concentrations greater than 0.1 micromolar with maximum inhibition at concentrations greater than 5 uM. Inhibition was most pronounced during the first minute of the reaction. The inhibition of calcium accumulation by DDT may be partly responsible for the loss of muscle control that is characteristic of DDT-poisoned animals.

The toxic effects of DDT [2, 2-bis (p-chlorophenyl)-1, 1, 1-trichloethane] have been related to alterations in monovalent ion transport (7, 10, 17, 18) especially that mediated by ATPases stimulated by Na^+ and K^+ (12, 13, 16, 19). DDT also interferes with processes which involve the transport of Ca^{++} such as egg shell thickening (1, 9, 11) and calcification of surface scales by a marine alga (5). Since fragments of sarcoplasmic reticulum of skeletal muscle accumulate calcium (3, 4), and because this accumulation can be quantitated, we examined the effect of DDT on this system.

Materials and Methods

Cell fractions from the gastrocnemius muscles of male rats (Holtzman Co., Madison, Wis.) were prepared by differential centrifugation according to the method of Martonosi and Feretos (15). Muscle tissue was minced with razor blades and then homogenized with a Polytron homogenizer in a ratio of 5 g tissue per 20 ml of homogenization medium (0.1M KCl, 5 mM histidine pH 7.0, 5 mM dithiothreitol) (22). Myofibrils were sedimented by centrifugation at 1,000 x g for 20 min and the supernatant was centrifuged at 8,000 x g for 20 min. The 8,000 x g pellet was used for the calcium accumulation experiments after resuspension in a solution containing 0.3 M sucrose, 5 mM dithiothreitol, and 5 mM histidine (pH 7.0) to a protein concentration of 2-3 mg/ml (6).

The spectrophotometric measurement of calcium accumulation was according to Fairhurst and Jenden (6). The reaction mixture contained 5 mM potassium oxalate, 20 mM imidazole pH 7.0, 30 mM KCl, 5 mM

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$MgCl_2$, 5 mM ATP and approximately 50 μg protein per assay in a volume of 3 ml. After 2 min preincubation, the reaction was initiated by the addition of $CaCl_2$ (final conc 2 x 10^{-4} M) and the absorbance change at 350 nm was followed with time at 26° C on a Bausch and Lomb 505 recording spectrophotometer. DDT was added in ethanol or methyl cellosolve (2-methoxy ethanol) solutions with a final concentration of solvent of less than 1%.

Experiments with radioactive calcium ion ($^{45}Ca^{++}$, 0.5 mCi/mM) were performed with the same reaction mixture as the spectrophotometric assays except that the amount of protein was 0.2 to 0.3 mg per reaction mixture. The reaction was started by the addition of a mixture of radioactive and nonradioactive $CaCl_2$ and was terminated by filtration through 0.45-μ Millipore filters followed by five 1-ml washes with nonradioactive 0.1 M $CaCl_2$. The filter containing the washed sarcoplasmic reticulum fragments was then placed in Bray's solution (2) and radioactivity was measured on a liquid scintillation counter. Protein was measured according to the method of Lowry *et al.* (14).

Pellets of sarcoplasmic reticulum were prepared for electron microscopic examination by subjecting reaction mixtures with and without $CaCl_2$ to centrifugation. The membrane pellets were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer pH 7 followed by 1% OsO_4 in the cacodylate buffer. The pellets were then dehydrated in an acetone series and embedded in Epon 812 (21). Sections were either stained with lead citrate (20) or left unstained and examined and photographed with a Phillips EM 200 electron microscope.

Results

Membrane vesicles of the sarcoplasmic reticulum fraction showed only scattered accumulations of electron-dense materials when incubated in the absence of calcium (Fig. 1A, lead stained). In the presence of calcium, holes bounded by a membrane appeared in the sections (Fig. 1B, lead stained). When the sections were not lead-stained, some membrane vesicles contained electron-dense deposits (Fig. 1C). The presence of the insoluble calcium oxalate precipitates inside the vesicles provided the basis for the isotope and spectrophotometric assay techniques used in this study and identified the vesicles as sarcoplasmic reticulum.

The accumulation of calcium (as $^{45}Ca^{++}$) by sarcoplasmic reticulum membranes required adenosine-5′-triphosphate (ATP) as an energy source (Fig. 2). Almost no activity was measured during the first minute of the reaction with guanosine-5′-triphosphate (GTP) or uridine-5′-triphosphate (UTP); whereas accumulation was extensive in the presence of ATP. Calcium accumulation was markedly inhibited during the first minute of the reaction by 8.4 μM DDT and the lag in uptake was more pronounced with 17 μM DDT (Fig. 3). After the initial lag, accumulation occurred and eventually reached the control level (data not shown).

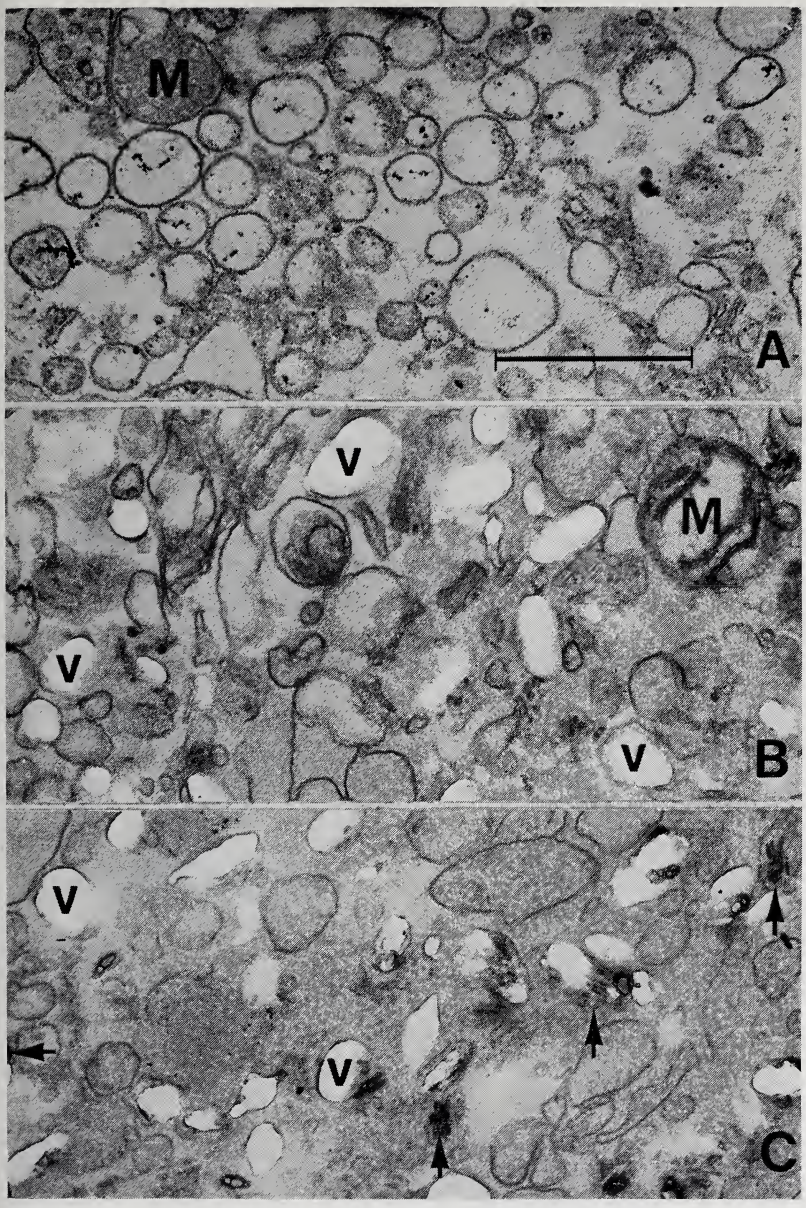

FIGURE 1. *Electron micrographs of sarcoplasmic reticulum fractions sedimenting at 8,000 X g. A. Fraction incubated in the absence of* $CaCl_2$. *Section stained with alkaline lead citrate. B. Fraction incubated in the presence of 0.2* mM $CaCl_2$. *Section stained with alkaline lead citrate. Holes surrounded by membranes result from loss of calcium oxalate deposits from the sarcoplasmic reticulum vesicles (V). C. As in B except the section was not stained with alkaline lead citrate. Here, electron-dense deposits (arrows) of calcium oxalate are seen inside some of the membrane vesicles. M=mitochondrion. Bar=0.5 u.*

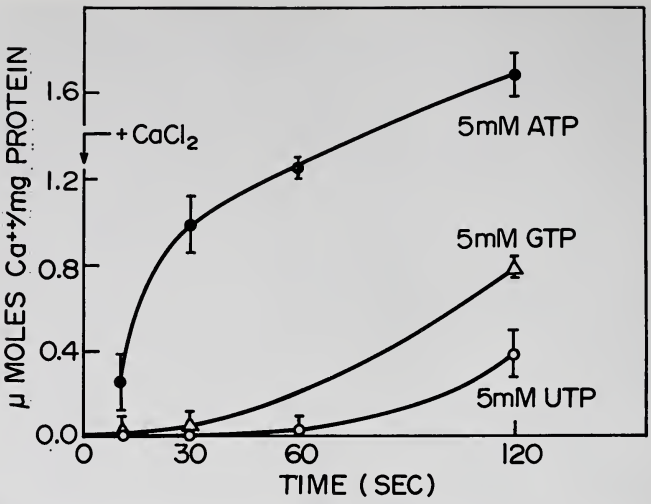

FIGURE 2. *Calcium accumulation by the sarcoplasmic reticulum fraction in the presence of 5 mM ATP, GTP or UTP. $CaCl_2$ containing $^{45}CaCl_2$ was added at t=o. Samples were removed and the membranes were collected by Millipore filtration. Results are the average of two determinations ± mean absolute deviations.*

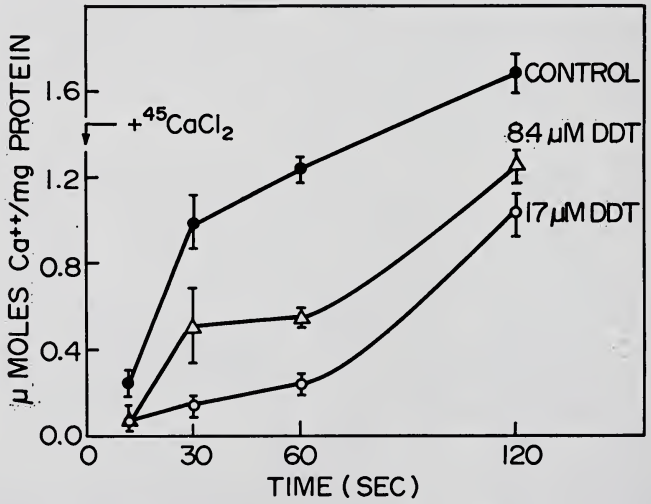

FIGURE. 3. *Calcium accumulation by the sarcoplasmic reticulum fraction. The procedure was as in Figure 2 except that DDT was added as indicated.*

Spectrophotometric analyses verified that DDT inhibited accumulation of calcium by sarcoplasmic reticulum vesicles (Fig. 4). Again, inhibition by DDT was most pronounced during the first minute of the reaction. A plot of the calcium accumulation (△O.D. after 1 min.) *versus* the logarithm of the DDT concentration (Fig. 5) showed no

inhibition at concentrations below 0.1 μM (10^{-7} M). Inhibition occurred between 0.1 μM and 5 μM DDT and reached a maximum near 5 μM DDT. Increasing the DDT concentration above 5 μM did not increase the degree of inhibition.

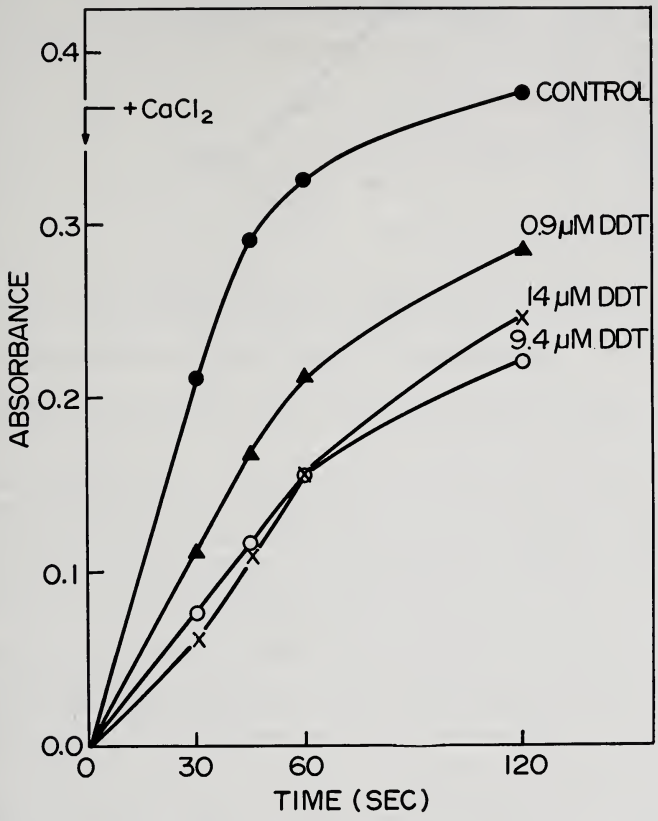

FIGURE 4. *Calcium accumulation by the sarcoplasmic reticulum with and without DDT. Activity was monitored by the change in absorbance at 350 nm.*

Discussion

The electron micrographs of the sarcoplasmic reticulum fraction incubated in the presence of calcium show that electron-dense precipitates formed inside many of the membrane vesicles (Fig. 1). We interpret the micrographs as showing that the membrane vesicles accumulate calcium ions which form insoluble deposits of calcium oxalate. The calcium oxalate is responsible, in turn, for the electron dense deposits (Fig. 1C), and the holes in the sections from which deposits have been lost (Figs. 1B and 1C, see also ref. 5).

The results of the radioactive and spectrophotometric assays show that DDT inhibits the accumulation of calcium by sarcoplasmic reticulum fractions. The major inhibition appears in the early part of the re-

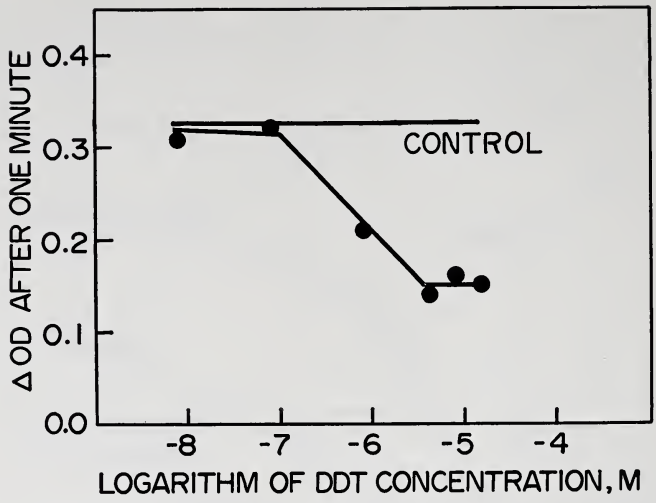

FIGURE 5. *Change in absorbance at 350 nm after 1 min vs. the logarithm of DDT concentration. Control activity is after one minute in the absence of DDT.*

action (Figs. 3 and 4). The initial lag in accumulation may be significant since proper muscle contraction and relaxation are dependent upon rapid transport of calcium ions by the sarcoplasmic reticulum (3, 4, 8). If the rate of accumulation of calcium ions were reduced, relaxation of the muscle would also be slowed sufficiently to potentiate the muscle twitching symptom of DDT toxicity.

The inhibition of calcium accumulation by DDT is characterized by threshold dose dependency (Fig. 5). At concentrations below 0.1 μM there is no apparent effect and at concentrations of 5 μM or greater inhibition is maximal. This range of inhibition is similar to that for the inhibitory effect of DDT on ATPases of synapses noted by Matsumura and Patil (16). Thus the active, membrane-associated transport of calcium by sarcoplasmic reticulum of muscle cells is inhibited by DDT. The inhibition is expressed as a lag in the initial uptake of calcium and occurs at DDT concentrations similar to those which inhibit other ion transport systems. The results suggest that DDT interferes with membrane associated calcium transport to cause ataxia in poisoned animals.

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