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HEPTACHLOR EPOXIDE: EFFECTS ON CALCIUM-MEDIATED TRANSMITTER RELEASE FROM BRAIN SYNAPTOSOMES IN RAT

ISAMU YAMAGUCHI, FUMIO MATSUMURA and ATEF A. KADOUS
Pesticide Research Center, Michigan State University, East Lansing, MI 48824, U.S.A.

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Abstract—The biochemical processes by which heptachlor epoxide affects transmitter release were studied, using rat brain synaptosomes. Heptachlor epoxide stimulated the release of preloaded [^{14}C]glutamate from isolated synaptosomes in the presence of externally available Ca^{2+} or a high concentration of K^+ (71 mM). This stimulatory effect appeared to be independent of the general membrane depolarization, since it could still be observed in a synaptosomal preparation suspended in a high K^+ concentration (71 mM). Heptachlor epoxide-treated synaptosomes were also found to take up more Ca^{2+} and to release less compared to untreated synaptosomes. By using subcellular preparations from disrupted synaptosomes, it was possible to demonstrate that an ATP-dependent Ca^{2+} uptake process was inhibited by heptachlor epoxide. Experiments *in vivo* also confirmed the above observation. It was noted that all these actions of heptachlor expoxide have the same effect of raising the intracellular level of free Ca^{2+}. A working hypothesis has been proposed, therefore, to explain the neurostimulatory effect of heptachlor expoxide on the basis of its effects on synaptic Ca^{2+} regulation, particularly that by Ca^{2+}–Mg^{2+} ATPase.

Though the details of the mechanism of action of chlorinated cyclodiene insecticides are still obscure, it is now generally agreed that they characteristically affect the synaptic region of the central nervous system in higher animals [1,2]. Electrophysiological evidence suggests that these insecticides promote the release of neurotransmitters, such as acetycholine, from pre-synaptic sites, causing typical excitation symptoms [3,4].

On the other hand, basic biochemical mechanisms of transmitter release are now becoming clear to the point that certain triggering processes may be defined. Here, the most crucial event appears to be the increase in the intracellular concentration of free Ca^{2+} following the general depolarization of the neuron. This increase signals the migration and exocytotic movement of synaptic vesicles which are the packets containing the transmitter. Within the synaptosome, free Ca^{2+} levels are regulated probably by the endoplasmic reticulum [5,6] and mitochondria, both of which are known to be capable of sequestering Ca^{2+}. However, from the viewpoint of affinity (i.e. effectiveness at low Ca^{2+} concentrations) and speed of response, the latter organelle appears to be inferior and, as such, the bulk of intricate Ca^{2+} regulation related to the transmitter release is believed to be carried out by the former. Another mechanism of controlling Ca^{2+} has been associated with the 'Ca-pump' at the plasma membrane, which extrudes excess Ca^{2+} from the cell at the expense of metabolic energy derived from ATP. Interestingly enough, both of these Ca^{2+} regulating activities are believed to be controlled by the Ca^{2+}–Mg^{2+} ATPase which is present in the synaptic components.

Recently, we found that the synaptic Ca^{2+}–Mg^{2+} ATPase is inhibited by heptachlor epoxide and other chlorinated cyclodiene insecticides *in vitro* [7]. In view of the proposed roles of Ca^{2+}–Mg^{2+} ATPase in the process of transmitter release, such an inhibitory action has at least the potential to explain some of the actions of these pesticides.

The purpose of this work is, therefore, to relate our previous finding to the biochemical processes of transmitter release and, thereby, to assess the significance of the inhibitory action of cyclodienes on the Ca^{2+}–Mg^{2+} ATPase.

EXPERIMENTAL

Animals. Adult male Sprague–Dawley rats, weighing 160–200 g, were supplied from Spartan Research Animals, Inc., Haslett, MI. The method of dissection and the preparation of the rat brain homogenate were identical to those reported previously by us [7]. In brief, the whole brains of Sprague–Dawley rats were excised and chilled in 0.32 M sucrose. The brains of two to four rats were used for a single preparation. After the removal of blood vessels, the brains were homogenized in cold 0.32 M sucrose with a glass Teflon homogenizer (a clearance of about 0.25 mm at 0°) at about 1000 r.p.m. for 3 min.

Preparation of synaptosomal fractions. Synaptosomes were prepared by the differential and sucrose gradient centrifugation procedure of Gray and Wittaker [8] and Hajos [9] from rat brain (mainly gray matter). To obtain subcellular fractions, the synaptosomes, suspended in 0.32 M sucrose, were disrupted with osmotic shock by adding 10 vol. of ice-cold distilled water. Further fractionation of disrupted synaptosomes was carried out by centrifugation in a discontinuous sucrose gradient (0.8–1.2 M sucrose) at 90,000 *g* (max. 90 min, 4°) using a Spinco

model L2-65B ultracentrifuge with an SW 25.1 rotor. They were separated into five fractions: top layer (0.03 M sucrose), fraction 1 (interlayer of top layer—0.8 M sucrose), fraction 2 (0.8 M sucrose), fraction 3 (0.8–1.2 M sucrose interlayer), fraction 4 (1.2 M sucrose), and fraction 5 (precipitate at the bottom).

The crude synaptic fraction was obtained by the method of DeRobertis *et al.* [10]. This preparation was considered to be heterogeneous and to contain membrane fragments and distrupted synaptosomes, as shown by Wittaker *et al.* [11]. The crude synaptic vesicle fraction was subjected to further fractionation by discontinuous sucrose density centrifugation as described above for the disrupted synaptosomes.

Uptake and release of [^{14}C]*glutamate by synaptosomes.* Synaptosomes (3–4 mg protein/ml) were incubated at 30° for 10 min with heptachlor epoxide in a physiological salt solution, 5 K^+ medium [12], which consisted of 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 5 mM mannitol and 20 mM Tris base (pH was adjusted to 7.65 by titration with maleic acid) in addition to 5 mM KCl and 132 mM NaCl, with or without 1.2 mM $CaCl_2$. The synaptosomes were preloaded with [^{14}C]glutamate by adding [^{14}C]-L-glutamic acid with gentle stirring (final concentration, 4–5 μM). The release of [^{14}C]glutamate was studied by changing the ionic conditions (see Table 1). A high K^+ medium, 71 K^+ medium [12], was obtained by mixing 5 K^+ medium with the same volume of high K^+ saline which contained 137 mM KCl in place of 5 mM KCl and 132 mM NaCl. After incubation, synaptosomal suspensions were filtered through a millipore filter (HA 0.45 μm was used throughout) following by washing with ice-cold incubation medium. The filters were dried by air and radioactivity was assessed by dissolving the entire filter in 10 ml of liquid scintillation counting solution.

Uptake and release of $^{45}Ca^{2+}$ *by synaptosomes.* $^{45}Ca^{2+}$ uptake by synaptosomes was studied by the method of Blaustein and Ector [12]. The synaptosomes (1.0–1.5 mg protein/ml) were preincubated with chemicals in Ca^{2+}-free medium at 30° for 10 min, and the reaction was initiated by the addition of $^{45}Ca^{2+}$ (final concentration, 1.2 mM). The incubation was carried out for 1.0 min, and $^{45}Ca^{2+}$ uptake was terminated by adding one fifth volume of ice-cold stopping solution, which contained 30 mM Tris–EGTA* in place of glucose and mannitol in Ca^{2+}-free 5 K^+ medium. Ice-cold Ca^{2+}-free 5 K^+ solution (3 vol.) was added to the suspension, and synaptosomes were precipitated by centrifugation at 9000 *g* for 4 min. After washing with cold 5 K^+ medium, the pellets were dissolved in 1 N NaOH at 50° and the radioactivity of a neutralized aliquot was assayed by liquid scintillation spectrometry.

The release of $^{45}Ca^{2+}$ was examined (see Table 3) using synaptosomes preloaded with $^{45}Ca^{2+}$ in the same way as described above, except that the synaptosomes were suspended in 50 K^+ medium, which was obtained by adding high K^+ saline to 5 K^+ medium. After preloading, synaptosomes were resuspended in ice-cold 5 K^+ medium, and they were treated with heptachlor epoxide or gramicidin D at 2° for 10 min. The incubation was carried out at 30° for 10 min. The samples were quickly chilled in an ice bath, filtered through a millipore filter (HA 0.45 μm), and washed with ice-cold 5 K^+ medium. The released amount of $^{45}Ca^{2+}$ was assessed from the difference of radioactivity in synaptosomes (and also in filtrate if necessary) before and after incubation.

$^{45}Ca^{2+}$ *binding by disrupted synaptosomes.* Freshly prepared synaptosomes (containing 0.9 mg protein/0.1 ml 0.32 M sucrose) were disrupted by osmotic shock as above. Heptachlor epoxide was added and incubated with the system before (pre-treated) or after (post-treated) disruption (see Table 4). The disrupted synaptosomes were incubated with EGTA-equilibrated $^{45}Ca^{2+}$ (free ion concentration, 5×10^{-6}M; Portzehl *et al.* [13]) at 30° for 10 min in the reaction mixture containing 145 mM KCl, 2 mM KH_2PO_4, 0.1 mM ouabain, 0.2 mM sodium azide, 0.2 mM 2,4-dinitrophenol, 0.7 μg/ml oligomycin and 10 mM Tris–HCl (pH 7.6), with or without 2 mM ATP or 3 mM $MgCl_2$. The suspensions were cooled and filtered through a millipore filter and the radioactivity on the filter was assayed as above.

ATPase assay method. The ATPase activities were measured in terms of the liberation of ^{32}Pi from γ-[^{32}P]ATP [14]. The insecticide was added with 5 μl of ethanol to the assay mixture containing a 0.1-ml aliquot of enzyme source (20–50 μg protein) and 0.85 ml of the assay buffer (the details of the incubation conditions are given under Results). The reaction was initiated by the addition of 50 μl [^{32}P]ATP (final concentration, 0.5 mM), and stopped with 0.2 ml of 10% trichloroacetic acid. The enzyme protein was coprecipitated with bovine serum albumin (1 mg/0.1 ml, dissolves in 100 mM KH_2PO_4), and the remaining [^{32}P]ATP in the reaction mixture was eliminated by active charcoal (50 mg). The charcoal on the wall of the test tubes was rinsed with 0.2 ml ethanol and was removed by low speed centrifugation (1000 r.p.m., 5 min). The radioactivity in an aliquot of the supernatant fraction was assayed. The Ca^{2+}–Mg^{2+} ATPase activity was defined as enzyme activity stimulated by 5×10^{-6} M Ca^{2+} over the basal activity (Ca^{2+}, 10^{-8}M). EGTA (0.5 mM) was used to buffer Ca^{2+} [13].

Determination of heptachlor epoxide levels in various nerve components. Heptachlor epoxide was extracted with ether (5 ml) three times from brain homogenate or synaptosomal fractions (2.5 ml) which were treated with the pesticide *in vivo* or *in situ* (i.e. isolated synaptosomes). Ether layers for each sample were pooled and the solvent was evaporated under N_2. The residue was dissolved in 2.5 ml *n*-hexane, and heptachlor epoxide was extracted from the hexane layer with acetonitrile (2 ml, three times). Acetonitrile was removed by bubbling N_2 at 70°. The residues were dissolved in acetone and were subjected to gas liquid chromatographic (g.l.c.) analysis. A Varian Aerograph Series 2400 equipped with an electron-capture detector was used. The metal column (4 ft) was packed with 30% SE on gaschrom Q (column temp. 225°). Injection and detector temperatures were 280 and 250°, respec-

* EGTA = ethyleneglycolbis(aminoethylether)tetra-acetate.

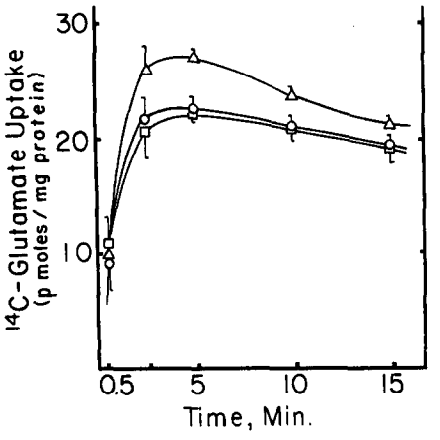

Fig. 1. Uptake of [^{14}C-]glutamate by synaptosomes. Synaptosomes (3.2 mg protein/ml medium) were incubated in 5 K^+ medium (Ca^{2+}-free) with or without heptachlor epoxide for 10 min. at 30°. [^{14}C]-L-Glutamic acid (0.5 μCl; final concn. 4.4 μM) was added and further incubation was carried out. Aliquots (0.5 ml each) were removed periodically from the incubation mixture. They were transferred to chilled Ca^{2+}-free 5 K^+ medium containing ice-cold 5 μM glutamate, and immediately filtered through a millipore filter. The synaptosomes on the filter were washed by 5 ml of Ca^{2+}-free 5 K^+ medium with non-labeled glutamate (5 μM) for three times, and the radioactivity on the filter was assayed. Key: (○) control, (□) 10^{-7} M heptachlor, and (△) 10^{-5} M heptachlor epoxide.

tively. The flow rate of carrier gas (N_2) was adjusted to 25 ml/min. The levels of heptachlor epoxide in various brain components were expressed as the amount per mg protein.

Chemicals. Heptachlor epoxide was supplied by the Velsicol Chemical Co., Chicago, IL, and was free from any impurity as judged by g.l.c. analyses. ATP (Tris salt and disodium salt), EGTA, mersalyl, ruthenium red, sodium pentobarbital, gramicidin D, oligomycin and bovine serum albumin were purchased from the Sigma Chemical Co., St. Louis, MO. γ-[^{32}P]ATP and [^{45}Ca]Cl_2 were from New England Nuclear, Boston, MA. All other reagents were of the highest grade commercially available, and distilled–deionized water was used throughout the study.

RESULTS

Release of [^{14}C]glutamate from the isolated synaptosomes. Glutamate was chosen as a model transmitter since this amino acid is stable and is not readily degraded upon release. It is known to be present in the rat brain and is regarded as an excitatory transmitter [15]. In addition, its release has been shown to be controlled by Ca^{2+} at the level of the synaptic vesicle [16].

In the first experiment, isolated synaptosomes were incubated with [^{14}C]glutamate in a low K^+ (5 mM) medium according to a modified method (see Fig. 1 caption) of Takagaki [17]. The rate of uptake of [^{14}C]glutamate reached a peak at 5 min at 30° and showed a slight decline in the next 10 min (Fig. 1). In the presence of a high concentration of heptachlor epoxide (10^{-5} M), the rate of uptake of glutamate was slightly more (about 10 per cent at 10 min). To preserve the freshness of the synaptosome, 10 min was adopted as a standard preloading time period. In the second experiment, the composition of the external medium was changed to study the nature of the heptachlor epoxide-induced increase in the transmitter release (Table 1). Heptachlor epoxide stimulated the release of glutamate at low K^+ (5 mM) in the presence of Ca^{2+}. Increase of the external K^+ concentration to 71 mM clearly stimulated the transmitter release as expected from the general depolarization effect of K^+ [12]. It was unexpected, however, that heptachlor epoxide would still retain the ability to stimulate the release of [^{14}C]glutamate in this high K^+ medium (Table 1). This must mean that heptachlor epoxide stimulated both external Ca^{2+}-dependent and independent transmitter release mechanisms.

Table 1. Effects of heptachlor epoxide (HE) and ionic conditions on the release of [^{14}C]glutamate from synaptosomes*

Conditions before release	Releasing agents added	[^{14}C]Glutamate released (c.p.m./mg protein/min)†
5K	0	0
5K	Ca^{2+} (1.2 mM)	3070 ± 270
5K	K^+ (71 mM)	4930 ± 130
5K	K^+ (71 mM) + Ca (1.2 mM)	5810 ± 65
5K + HE (10^{-7} M)	0	0
5K + HE (10^{-7} M)	Ca^{2+} (1.2 mM)	4025 ± 205‡
5K + HE (10^{-7} M)	K^+ (71 K)	6065 ± 115§

* Synaptosomes (0.95 mg protein/ml medium) were preloaded with [^{14}C]glutamate in Ca^{2+}-free 5 K^+ medium containing 132 mM Na, as shown in Fig. 1. The released amounts of [^{14}C]glutamate were determined by the difference of radioactivity remaining in the synaptosomes before and after 1 min of incubation at 30°. The preloaded amount of [^{14}C]glutamate was 11,000 ± 300 c.p.m./mg protein.

† Since the amounts of endogenous glutamate in these synaptosomal preparations were not determined, it is not possible to express the amount released in a molarity unit. Each value is the mean of nine determinations ± standard error.

‡ Difference against 5K + 1.2 mM Ca^{2+} significant at 0.025.

§ Difference against 5K + 71K significant at < 0.005.

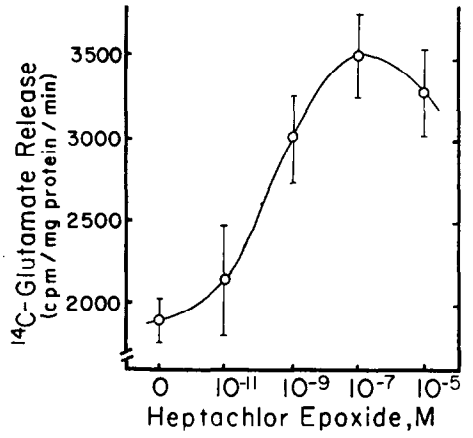

Fig. 2. Effect of heptachlor epoxide on the rate of release of [^{14}C]glutamate from synaptosomes. Conditions of preincubation of synaptosomes with heptachlor epoxide and preloading of [^{14}C]glutamate are as indicated in the legend of Fig. 1. The released amounts of [^{14}C]glutamate from synaptosomes were determined by the difference of radioactivity remaining in the synaptosomes before and after 1 min of incubation at 30° in Ca^{2+}-free 71 K^+ medium. Each point represents the mean with standard error.

In the next experiment, synaptosomes preloaded with [^{14}C]glutamate in 5 K^+ medium with or without heptachlor epoxide were incubated for an additional 1.0 min at 30° in the 71 K^+ medium. The amount of radioactivity released to the medium during the change of K^+ concentration was measured by removing the intact synaptosomes by millipore filtration. The results (Fig. 2) clearly show that heptachlor epoxide had a significant stimulator power on the process of glutamate release. Even at as low as 10^{-9} M the level of stimulation was highly significant. In this time span, about 30 per cent of the total radioactivity loaded in the synaptosome was released in the control. The level of stimulation ranged from 11 to 50 per cent over the control values.

$^{45}Ca^{2+}$ *uptake by synaptosomes.* To test the possibility that an increase in Ca^{2+} uptake by the intact synaptosomes is one of the causes of the stimulatory effect of heptachlor epoxide, $^{45}Ca^{2+}$ was added to the external medium (5 K^+ medium). After incubation, synaptosomes were harvested by a millipore-filtration method [12], and the amounts of $^{45}Ca^{2+}$ picked up by them were assessed. The results shown in Table 2 indicate that the rate of $^{45}Ca^{2+}$ uptake was stimulated by 10^{-7}M of externally applied heptachlor epoxide *in situ*. On the other hand, pentobarbital which counteracts K^+-induced release of transmitter did not have a significant effect under these experimental condition. Since this drug is known to reduce transmitter release by inhibiting only the portion of Ca^{2+} uptake related to depolarization [12], the above result supports the view that this aspect of the effect of heptachlor epoxide is related to the increase in Ca^{2+} uptake unrelated to general depolarization.

On the other hand, stimulation of Ca^{2+} uptake does not necessarily lead to a high level of intracellular Ca^{2+}, since a parallel increase in Ca^{2+} release from the synaptosome would cancel out the uptake effect. To examine this possibility, synaptosomes preloaded with $^{45}Ca^{2+}$ in 50 K^+ medium were suspended in 5 K^+ medium, and were treated with heptachlor epoxide or gramicidin D. The system was transferred to a water bath maintained at 30° and incubated for 10 min. The system was chilled, synaptosomes were removed by millipore-filtration, and the amounts of Ca^{2+} discharged were assessed from the radioactivity in the cell-free medium. The result (Table 3) shows that heptachlor epoxide also inhibited the process of Ca^{2+} release in addition to its stimulation of uptake. The overall result must be that the Ca^{2+} concentration in the synaptosome would increase unless an efficient system of Ca^{2+} sequestration is still operative in the synaptosome. Gramicidin D, on the other hand, clearly stimulated the release of $^{45}Ca^{2+}$, indicating that for this chemical the overall effect must be an increase in Ca^{2+} permeability for both directions.

$^{45}Ca^{2+}$ *binding by the total subcellular components of the disrupted synaptosomes.* The above experiment established that one of the effects of heptachlor epoxide is to increase the synaptosomal uptake of external Ca^{2+}. However, it has been shown previously (Table 1) that another effect of heptachlor epoxide is to increase the rate of transmitter release that is triggered by a high K^+ concentration (i.e. general depolarization) in the absence of externally added Ca^{2+}. Since the transmitter release is actually increased by the increase in internal Ca^{2+} which can be induced by both the increase in uptake of the external Ca^{2+} and by the release of internal Ca^{2+} by Ca^{2+}-sequestering organelles, we decided to examine the effect of heptachlor epoxide on Ca^{2+} uptake by subcellular components of the synaptosome. To study the binding pattern of $^{45}Ca^{2+}$ to subcellular components, freshly prepared synaptosomes were either incubated with heptachlor epoxide for 10 min at 2° and disrupted by osmotic shock (i.e. 'pretreated'), or they were first disrupted and then

Table 2. Effects of heptachlor epoxide and pentobarbital on $^{45}Ca^{2+}$ uptake by synaptosomes

Conditions	$^{45}Ca^{2+}$ uptake* (μmoles/g protein/min)	No. of independent experiments
Control	0.0290 ± 0.0060	9
Heptachlor epoxide (10^{-7}M)	0.0338 ± 0.0014†	6
Heptachlor epoxide (10^{-7}M) + pentobarbital (10^{-3}M)	0.0345 ± 0.0003†	3

* All data are expressed as mean ± standard error.
† Difference against control significant at 0.25.

Table 3. Effect of heptachlor epoxide on the release of $^{45}Ca^{2+}$ from synaptosomes

	Relative amounts of $^{45}Ca^{2+}$ released from synaptosomes (Control = 100)
Control	100.0 ± 1.6*
Heptachlor epoxide (10^{-9} M)	90.1 ± 4.7†
Heptachlor epoxide (10^{-7} M)	89.8 ± 1.8‡
Heptachlor epoxide (10^{-5} M)	85.1 ± 1.7‡
Gramicidin D (10 μg/ml)	165.6 ± 1.6‡

* Mean ± standard errors.
† Difference against control significant at 0.05.
‡ Difference against control significant at < 0.005.

Table 4. Effect of heptachlor epoxide on ATP-dependent $^{45}Ca^{2+}$ binding by disrupted synaptosomes

Conditions		ATP-dependent $^{45}Ca^{2+}$ binding* (pmoles/mg protein/10 min)
Control		1305 ± 24
Heptachlor epoxide		
(pre-treated)	10^{-8} M	1129 ± 21
	10^{-6} M	986 ± 130†
	10^{-4} M	961 ± 9‡
(post-treated)	10^{-5} M	974 ± 120†
Mersalyl§	5×10^{-5} M	82 ± 19‡

* ATP-dependent $^{45}Ca^{2+}$ binding is defined as the amount of the increase in $^{45}Ca^{2+}$ binding due to the addition of ATP to the medium containing all other constituents including Mg^{2+}. The value for ATP-independent $^{45}Ca^{2+}$ binding was 337 pmoles/mg protein/10 min (average of four determinations). Data are expressed as mean ± S.E.
† Difference against control significant at 0.05.
‡ Difference against control significant at < 0.005.
§ Mersalyl was added after disruption of synaptosomes.

Table 5. Effects of heptachlor epoxide on Ca^{2+}–Mg^{2+} ATPase and Mg^{2+} ATPase activity in disrupted synaptosomes*

	(nmoles/mg $^{32}P_i$ protein/10 min)	
	Ca^{2+}–Mg^{2+} ATPase	Mg^{2+} ATPase
Control	44.2 + 22.0†	65.4 + 8.2†
Heptachlor epoxide (10^{-5} M)	0.5 ± 6.0‡	77.1 ± 17.2

* Disrupted synaptosomes (50 μg protein/reaction mixture) were incubated with heptachlor epoxide (10^{-5} M) at 2° for 10 min in a medium containing 145 mM KCl, 3 mM $MgCl_2$, 2 mM KH_2PO_4, 0.1 mM ouabain, 3 mM sodium azide, 0.7 μg/ml oligomycin, 10 mM Tris–HCl (pH 7.6), and EGTA-equilibrated Ca^{2+}, 10^{-8} M (basal medium) and 5×10^{-6} M (medium for activation). The reaction was started by the addition of [^{32}P]ATP (0.5 mM; 0.05 μCi), and the incubation was carried out at 30° for 10 min.
† Mean ± standard error.
‡ Difference against control significant at 0.05.

Table 6. Effects of heptachlor epoxide on distribution of $^{45}Ca^{2+}$ in the synaptosomes*

Centrifugal fractions	Ratio = [c.p.m./mg protein] treated / [c.p.m./mg protein] control
Top layer	1.03 ± 0.07
Fraction 1 (0.03 M–0.8 M sucrose)	0.98 ± 0.05
Fraction 2 (0.8 M sucrose)	0.82 ± 0.06
Fraction 3 (0.8 M–1.2 M sucrose)	1.16 ± 0.02
Fraction 4 (1.2 M sucrose)	1.12 ± 0.03
Fraction 5 (precipitate at bottom)	1.05 ± 0.06

* $^{45}Ca^{2+}$ preloaded synaptosomes were treated with 10^{-5} M heptachlor epoxide (10^{-8} moles/mg protein) for 10 min at 2° in 50 K^+ medium. They were transferred to 5 K^+ medium and incubated for 10 min at 30°. The suspensions were chilled in ice water and centrifuged at 10,000 *g* for 10 min. After the pellets were rinsed with 5 K^+ medium and 0.32 M sucrose, they were disrupted with osmotic shock. The disrupted synaptosomes were centrifuged in a discontinuous sucrose gradient at 90,000 *g* for 90 min, and they were fractionated as shown. Data are expressed as the ration of specific radioactivities found in treated fractions against control (the mean from four experiments ± standard error).

incubated with heptachlor epoxide at 2° for 10 min (i.e. 'post-treated'). In either case, $^{45}Ca^{2+}$ was added to the disrupted synaptosomal suspension, incubated, and the uptake of $^{45}Ca^{2+}$ was measured by millipore-filtration. The result clearly shows that heptachlor epoxide had inhibitory effects on ATP-dependent $^{45}Ca^{2+}$ binding in the presence of mitochondrial toxicants (Table 4). Mersalyl (mercuramide), a known inhibitor of -SH enzymes including ATPases, clearly inhibited the $^{45}Ca^{2+}$ binding process, supporting the contention that the process studied here involves -SH proteins.

Effect of heptachlor epoxide on Ca^{2+}–Mg^{2+} *ATPase of subcellular components of disrupted synaptosomes.* The above inhibitory action of the insecticide on the ATP-dependent $^{45}Ca^{2+}$ uptake may be related to its similar action on Ca^{2+}-regulating ATPases. To test this possibility, Ca^{2+}–Mg^{2+} ATPase and Mg^{2+}-ATPase of the same preparation were examined. Heptachlor epoxide was found to inhibit Ca^{2+}–Mg^{2+} ATPase, but not Mg^{2+} ATPase (Table 5).

Studies on heptachlor epoxide inhibition of $^{45}Ca^{2+}$ *binding and* Ca^{2+}–Mg^{2+} *ATPase in individual subcellular components of synaptosomes.* The above findings suggest that some component of the synaptosome is affected by heptachlor epoxide so that its internal Ca^{2+} sequestering ability is impaired. The fresh synaptosomes were loaded with $^{45}Ca^{2+}$, treated with heptachlor epoxide, and disrupted by means of osmotic shock. Various intracellular components were separated by discontinuous sucrose-density centrifugation. Each subcellular fraction was collected and the distribution of $^{45}Ca^{2+}$ was determined. The ratios of $^{45}Ca^{2+}$ level found in heptachlor epoxide-treated synaptosomes to that of the control was calculated for each fraction; the results are summarized in Table 6. There were two fractions in which $^{45}Ca^{2+}$ levels were lower in the heptachlor epoxide-treated synaptosomes than in the control. Fraction 1 is known to contain synaptic vesicles, myelin, axon, and other membranous components, and fraction 2 mainly consists of endoplasmic reticulum and synaptosomal ghosts.

Studies on the effects of in vivo *administered heptachlor epoxide on synaptosomal ATPases.* The most crucial test in relating such an inhibition of Ca^{2+}–Mg^{2+} ATPase by heptachlor epoxide to its mode of action in live organisms, is to prove that the inhibition actually takes place *in vivo*. Four rats, two for control and two for the treatment, were used for this purpose. Heptachlor epoxide was intraperitoneally injected (200 mg/kg) and symptoms were closely observed as they developed. At the onset of the violent convulsive symptoms (5 hr post-treatment), the rats were killed and the synaptosomes were isolated as before. Disruption by osmotic shock and discontinuous sucrose centrifugation gave the same subcellular fractions as the previous experiment (Table 7). As a result of ATPase assay, it was found that the Ca^{2+}–Mg^{2+} ATPase of the same two fractions previously shown to be susceptible *in vitro* to heptachlor epoxide was found to be inhibited also *in vivo*.

The levels of heptachlor epoxide in the brain and subcellular fractions were determined using a g.l.c. technique. The total level of heptachlor epoxide in the brain was 3.15 p.p.m. on a wet weight basis, which corresponds to 10.5 ng/mg protein. On the other hand, approximately four times as much heptachlor epoxide was found in synaptosomes (48.4 ng/mg protein). Within the synaptosomal components, fractions 1 and 2 had the highest level of heptachlor epoxide (160 and 199 ng/mg respectively) (Table 7).

Since it was observed that the Ca^{2+}–Mg^{2+} ATPase of fractions 1 and 2 was specifically inhibited, while the ATPase activity was also high in other fractions, particularly in fraction 3, a question must be raised as to why only the ATPase of the former two fractions was inhibited. This discrepancy can be understood if it is assumed that the Ca^{2+}–Mg^{2+} ATPase in fractions 1 and 2 is more accessible to heptachlor epoxide than that of fraction 3 *in vivo*. The residue data on the distribution pattern of heptachlor epoxide generally agrees with the above assumption. To prove the above point, that the Ca^{2+}–Mg^{2+} ATPase in fraction 3 is not qualitatively different from the one in fractions 1 and 2, an inhibition experiment was conducted wherein the inhibitor was added directly to

Table 7. Effect of *in vivo* administered heptachlor epoxide on synaptosomal Ca^{2+}–Mg^{2+} ATPase activity and its distribution in synaptosomal subfractions*

Fractions	Ca^{2+}–Mg^{2+} ATPase activity† (nmoles $^{32}P_i$/mg protein/20 min)		Distribution of heptachlor epoxide (ng/mg protein)
	Control	Treated	
1	150 ± 42	0‡	160
2	504 ± 98	64 ± 48‡	199
3	1775 ± 134	1642 ± 186	156
4	1563 ± 96	2054 ± 100	130
5	286 ± 102	292 ± 78	33

* Heptachlor epoxide dissolved in corn oil was intraperitoneally injected into the rats (200 mg/kg). When convulsions were observed, the rats were killed and the synaptosomes were prepared by the method of Hajos [9]. The synaptosomes were disrupted and fractionated as in the legend of Table 6. Ca^{2+}–Mg^{2+} ATPase activity was assayed in a medium containing 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM ouabain, 30 mM imidazole–HCl, pH 7.2, and EGTA-equilibrated Ca^{2+}, 10^{-8} M (basal medium) or 5×10^{-6} M (medium for activation). [^{32}P]ATP (0.5 mM) was used as substrate. The determination of the level of heptachlor epoxide in the brain and subcellular fractions was carried out by gas liquid chromatography after extraction by ether.

† Expressed as the average of two independent experiments (each experiment involving two duplicate determinations) ± standard error.

‡ Difference against control significant at < 0.005.

each discontinous sucrose gradient fraction obtained from the crude synaptic vesicle fraction, and the levels of inhibition were determined. The results shown in Table 8 clearly indicate that the Ca^{2+}–Mg^{2+} ATPase in fraction 3 was as sensitive to heptachlor epoxide as that in fractions 1 and 2, while its sensitivity to ruthenium red was different. Ruthenium red at this concentration is known to inhibit Ca^{2+}–Mg^{2+} ATPase and other Ca^{2+} ATPases [5,6].

DISCUSSION

Since we have found in this work that heptachlor epoxide induces a variety of effects on biochemical processes of the synapse, we feel it is necessary to make an effort to integrate all the information leading to the increased transmitter release. As shown in Fig. 3, heptachlor epoxide clearly stimulated external Ca^{2+} uptake and reduces Ca^{2+} release by the synaptosome and, therefore, the net result was an increase in the total Ca^{2+} content in the synaptosome. This aspect of the heptachlor epoxide effect would become important when the uptake process of external Ca^{2+} is the rate-limiting reaction in the whole process of transmitter release. Within the synaptosome, heptachlor epoxide reduced Ca^{2+} uptake by the endoplasmic reticulum, the proposed Ca^{2+} sequestering organelle. This could explain the phenomenon that heptachlor epoxide stimulated the high K^+-induced transmitter release even in the absence of externally added Ca^{2+}. At any rate, these two actions added together should clearly increase the availability of Ca^{2+} to other intracellular organelles. The result observed (Table 6) is consistent with the above expectation.

The mechanism by which the increase in the intrasynaptic concentration of Ca^{2+} resulted in an increase in transmitter release is not completely clear. One

Table 8. Sensitivity of Ca^{2+}–Mg^{2+} ATPase to heptachlor epoxide and ruthenium red*

	(nmoles $^{32}P_i$/mg protein/10 min)		
	Fraction		
	1	2	3
Control	954 ± 6	582 ± 9	1044 ± 45
Heptachlor epoxide (10^{-5} M)	420 ± 3‡	354 ± 75§	345 ± 71§
Ruthenium red (10^{-5} M)	40 ± 12‡	273 ± 110§	836 ± 23§

* A crude synaptic vesicle fraction suspended in 0.32 M sucrose (5 mg protein/2 ml) was centrifuged at 100,000 *g* for 2 hr in discontinuous sucrose gradient as in Table 6. The resulting layers were separated by aspiration.
ATPase activity was determined using [^{32}P]ATP as in Table 7.

† Expressed as mean ± standard error.

‡ Difference against control significant at <0.005

§ Difference against control significant at 0.025–0.05.

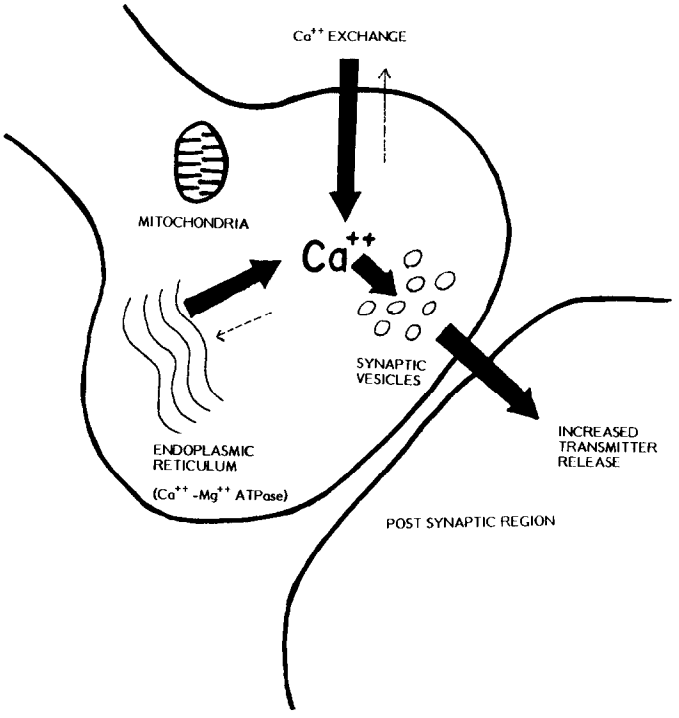

Fig. 3. Schematic illustration of the overall effects of heptachlor epoxide on the processes of transmitter release. Arrows represented by solid lines show that the process is stimulated by heptachlor epoxide. Those represented by dotted lines show its inhibitory effects. The overall effect of heptachlor epoxide is the increase of internal free Ca^{2+} which triggers the release of the transmitter. The role of mitrochondria in Ca^{2+} regulation was not examined in this work.

theory is that availability of Ca^{2+} to synaptic vesicles induces a myosin (on the synaptic vesicle)–actin (on the pre-synaptic membrane) type interaction leading to exocytotic movement [16]. Whatever the actual biochemical mechanisms are, it is clear from much experimental evidence that the increase in Ca^{2+} availability to synaptic vesicles and other intracellular organelles is the key to triggering transmitter release [5,6,16,18].

As for the question of a Ca^{2+} sequestering organelle within the synaptosome, we are aware of the existence of two schools of thought. One theory considers the function in the endoplasmic reticulum to be similar to the mechanism controlling Ca^{2+} in muscle sarcoplasmic reticulum [5,6]. The other theory is the one advocated by Rahamimoff (e.g. Ref. 18) attributing the major Ca^{2+}-regulating activity to mitochondria. In the present study, we eliminated mitochondrial function by the use of 0.2 mM sodium azide and 0.2 mM 2,4-dinitrophenol and, thereby, concentrated only on the effect of endoplasmic reticulum. It is known that mitochondria play a strong role in this regard, and that various cyclodiene insecticides (e.g. heptachlor epoxide) inhibit mitochondrial ATPases (e.g. Refs. 19 and 20). However, Blaustein *et al.* [5] have shown that brain mitochondria pick up little Ca^{2+} when the ionized Ca^{2+} level is as low as 0.3 μM, the concentration range where the active Ca^{2+} sequestering organelle should be operating to control the transmitter releasing event. Moreover, it is also known that mitochondrial poisons such as 2,4-dinitrophenol and oligomycin do not induce typical neuro-excitatory symptoms in insects. They are classified among the general respiratory poisons. Certainly the whole question of Ca^{2+} sequestration in the synaptosome must be resolved. Meanwhile, as far as the mechanism of action of heptachlor epoxide is concerned, the question of the mitochondrial role appears to be best studied as a separate case involving other tissues more sensitive than the nervous system.

Another key question is the inhibition of Ca^{2+}–Mg^{2+} ATPase by heptachlor epoxide [7] as related to its effects on Ca^{2+} regulatory function in the synaptosome. There are two possible roles of Ca^{2+}–Mg^{2+} ATPase: one is the role in the endoplasmic reticulum as suggested by Blaustein *et al.* [5], and the other is at the pre-synaptic plasma membrane where Ca^{2+}–Mg^{2+} ATPase acts as a 'Ca-pump' as suggested by Duncan [21]. Since both of them are expected to be present in fractions 1 and 2 [11], there is no easy way to distinguish them. For the sake of Ca^{2+} regulation, however, the question may be regarded as moot, inasmuch as their roles are the same: i.e. to lower the internal Ca^{2+}. Indeed, membranes of endoplasmic reticulum are generally regarded as the extension of the outer cell membrane, at least in origin, and the intracellular lumen of the reticulum may be considered to be equivalent of the extracellular space. Thus, inhibition of either function would lead to a higher internal level of Ca^{2+}.

Finally, the meaning of heptachlor epoxide stimulation of glutamate release must be discussed. Shank-

land and Schroeder [3] and Uchida *et al.* [4] have shown, by electrophysiological means, that dieldrin and γ-BHC, respectively, stimulate the release of acetylcholine. The Ca^{2+}-mediated mechanisms of release of these two transmitters are expected to be identical. Indeed, Waller and Richter [22] have demonstrated, using the method of Blaustein and Ector [12] (the same method used in the current work), that the release of ACh, serotonin, glutamic acid, gamma-aminobutyric acid, aspartic acid and glycine was stimulated by Ca^{2+}, while the release of alanine was not. Also, Puszkin and Kochwa [16] showed that glutamate release from the synaptic vesicle in the presence of synaptic membrane is Ca^{2+} dependent. Thus, at this stage, there is no reason to suspect that the basic mechanisms of release between glutamate and ACh are different. Indeed, Gowdey *et al.* [23] have shown that aldrin induces parasympathomimetic symptoms that most likely are due to the increase of ACh release at the parasympathetic ganglia, and Hathway *et al.* [24] have demonstrated that dieldrin increased the level of gamma-aminobutyric acid.

In summary, it has been established that heptachlor epoxide indices an elevation of intracellular Ca^{2+} in the synaptosome. This increase most likely plays a significant role in the heptachlor epoxide-induced increase of the transmitter release. As for the cause of the intracellular Ca^{2+} increase, a working hypothesis has been proposed that it is related to the inhibitory action of heptachlor epoxide on Ca^{2+}–Mg^{2+} ATPase, as shown by both *in vitro* and *in vivo* experiments.

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