

Studies on the Mode of Action of Spinosad: The Internal Effective Concentration and the Concentration Dependence of Neural Excitation

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This is the second in a series of reports that define the mode of action of spinosyns, a novel class of naturally derived insecticidal macrocyclic lactones. The concentration dependence of central nervous system (CNS) stimulation was determined using ganglia isolated from cockroaches, house fly larvae, and tobacco hornworm larvae. The tobacco hornworm nervous system was most sensitive, with a steep dose–response relation and an ED₅₀ of 5 nM for spinosyn A. The response of the housefly CNS was comparable, showing no clear effect at 3 nM and near-maximal stimulation at 10 nM spinosyn A. The cockroach CNS also displayed a steep dose–response relation, with a threshold of 10 nM and an EC₅₀ of 32 nM. That the nerve cords of cockroaches are exposed to comparable levels of spinosyn A during poisoning was established by comparison of the concentration of spinosyn A taken up by cockroach nerve cords during exposure *in vivo*, in cockroaches treated with a threshold dose, with that in isolated nerve cords equilibrated with a solution of spinosyn A in saline. A saline concentration of 21 nM was estimated to give the same nerve cord concentration as found at the threshold dose for prostration. We can conclude that during poisoning, spinosyn A reaches a concentration inside the insect that is sufficient to directly excite the central nervous system. ©1998

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INTRODUCTION

Spinosad, the active ingredient in Tracer, Conserve, and Success insect control products for lepidopteran and thysanopteran pests, is a naturally occurring mixture of two active components, spinosyn A and spinosyn D (Fig. 1), produced by the soil actinomycete *Saccharopolyspora spinosa* (1). In addition, other natural spinosyns have been characterized (1, 2), and many synthetic derivatives, known as spinosoids have been made (3).

Although targeted primarily at lepidoptera, spinosad and spinosyns are inherently broad spectrum (4), and cockroaches, fruit flies, and tobacco budworm larvae all showed similar symptoms (5). Therefore, cockroaches were used as a model species for most mode of action studies.

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Preliminary results indicated that spinosad has a novel mode of action. The present study is the second in a series of reports that systematically define the novel mode of action of spinosad, spinosyns, and spinosoids, collectively referred to as spinosoids. In the first report (5), the poisoning symptoms were described in a variety of insects, and it was concluded that the first symptoms were caused by involuntary muscle contractions. Neurophysiological studies on poisoned insects were then used to determine that spinosyn A caused these contractions by exciting the central nervous system, and that it did so *in vitro* as well, when applied to isolated ganglia at submicromolar concentrations. Because of the prolonged hyperexcitation, insects eventually became paralyzed, apparently due to neuromuscular fatigue. In the present report, we focus on the central excitatory effects of spinosoids, measuring the concentration dependence of these effects in house flies, cockroaches, and

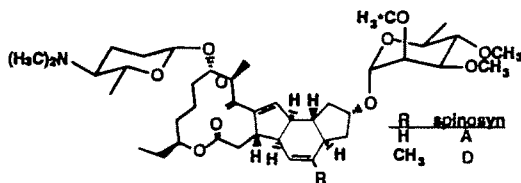


FIG. 1. Spinosad is a naturally occurring mixture of two active components, spinosyn A and spinosyn D. Radiotracer studies used spinosyn A labeled with ^{14}C in the 2'-methoxy group (asterisk).

tobacco hornworms. Furthermore, we use radiotracer methods to determine the aqueous concentration of spinosyn A needed to poison the cockroach nervous system to the same extent that it is poisoned in cockroaches treated with a threshold dose and find that it corresponds very well with the concentration needed to stimulate the cockroach central nervous system *in vitro*. Subsequent reports will examine the effects of spinosyns on receptors and ion channels of neurons in the central nervous system of cockroaches. A brief overview of spinosyn mode of action research has been published (6)

METHODS

Insects

American cockroaches, *Periplaneta americana*, were reared in the dark at 27°C and 50% RH, in 5-gal. buckets, on Purina dog chow and water. Compounds were injected with a Hamilton 10- μl syringe, fitted with a 30-gauge needle (Hamilton Company, Reno, NV). The needle was inserted ventrolaterally, through an intersegmental membrane in the posterior third of the abdomen, and the tip was advanced to the next anterior segment before the dose was discharged. The vehicle, dimethyl sulfoxide (DMSO), had no effects at injection volumes up to 3 μl . House fly, *Musca domestica*, larvae were raised on fly chow (Kern, Kitley and Herr, Lebanon, IN) at 27°C and 60% R.H., on a 14:10 h light:dark cycle. Tobacco hornworm, *Manduca sexta*, eggs were obtained from Carolina Biological (Burlington, NC) and larvae were reared on hornworm medium at 27°C, on a 12:12-h light:dark cycle. Fifth instar larvae were used for all experiments.

Electrophysiology

All recordings were carried out on insect ganglia from which the perineurial sheath was removed to speed penetration of spinosyn A. For *in vitro* measurements on synganglia of wandering house fly larvae, the ganglia were isolated, desheathed with fine forceps, and placed in bicarbonate-buffered house fly saline (Table 1). From cockroaches, third thoracic or sixth abdominal ganglia were isolated, desheathed with fine forceps, and placed in cockroach bicarbonate saline (Table 1). From late fifth instar *M. sexta* larvae, abdominal ganglia were isolated, desheathed with fine forceps, and placed in *Manduca* saline (Table 1).

Activity in one of the segmental nerves was recorded with a suction electrode, at a passband of 30 to 4000 Hz. Spike rate was measured online as the number of spikes in 1-s intervals, with EGAA software (R.C. Electronics, Goleta, CA). Traces were smoothed offline with a digital Gaussian filter, using IGOR data analysis software (Wavemetrics, Lake Oswego, OR).

Concentration Measurements

To measure the nerve cord content of spinosyn A after *in vivo* exposure, adult male American cockroaches were injected with the approximate LD_{50} , 1.9 μg of 2'-O-[^{14}C -methyl]spinosyn A (Fig. 1; >99% pure, sp act 51.6 mCi/mmol) in 1.5 μl of DMSO, as described above. After 64 h, the nerve cord was dissected intact from each insect and digested in 400 μl of Protosol (E.I. DuPont de Nemours and Company, Boston, MA) for 1 h at 50°C. The amount of radioactivity contained in the nerve cord was measured by scintillation spectrophotometry using 4 ml of Ultimate Gold scintillation cocktail (Packard Instrument Company, Meriden, CT).

To measure the metabolism of spinosyn A, 10 adult American cockroaches were injected as described above, with 1.9 μg (0.13 μCi) of [^{14}C] spinosyn A, and after 48 h the nerve cords were removed, combined, and homogenized in water, using a glass/glass tissue grinder. The ground tissue was extracted three times with 5-ml aliquots of methylene chloride. Both the organic and the aqueous fractions were evaporated to

TABLE 1
Composition of Salines Used

Saline component	HF saline	bicarbonate roach saline	Low Ca ⁺⁺ roach saline	HEPES ^a roach saline	Manduca saline
NaCl	172	210	210	205	140
KCl	13.3	3.1	3.1	13.3	5
CaCl ₂	4	5.4	0.5	5.4	4
MgCl ₂	6	—	20	—	—
NaH ₂ PO ₄	0.6	0.5	0.5	—	—
NaHCO ₃	10	2.1	2.1	—	—
Na-Hepes	—	—	—	5	5
α-D-Glucose	—	—	—	20	28
Sucrose	30	—	—	—	—
pH	7.2	7.2	7.2	7.2	7.4

Note. Concentrations are in millimolar.

^a(N- [2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])

dryness, combined, and brought up to a volume of 150 μ l in ethanol. The entire sample was analyzed by normal phase TLC using Whatman Silica Gel PLK5 1000- μ m TLC plates (20 \times 20 cm) and developed with methylene chloride:methanol (9:1) plus 0.1% ammonium hydroxide in a fully lined developing tank. Location and quantification of the material was done using a Bioscan System 200 imaging scanner in 2D analysis mode using a high-energy 10 mm collimator. Ten lanes of 0.5 cm width were counted for 10 min each. Factor A had an R_f of 0.70 under these conditions.

To measure the uptake of spinosyn A by nerve cords *in vitro*, nerve cords dissected from adult male American cockroaches were incubated at 22°C in HEPES cockroach saline containing 100 nM [¹⁴C]spinosyn A. At various time points between 0 and 8 h, nerve cords were removed from the incubation medium and quickly rinsed three times in saline. The radioactivity of each nerve cord was determined as described above for nerve cords treated *in vivo*.

RESULTS

Concentration Dependence of Neural Excitation by Spinosyn A

In a previous study, it was found that 100 nM spinosyn A increased the average spike rate in

the house fly larval CNS (5). Figure 2 shows a series of similar measurements, with the addition of the indicated concentration of spinosyn A or the 0.1% DMSO solvent control at the broken line. While spike rate was measured as the number of spikes in 1-s samples of nerve activity, the records were filtered offline with a digital Gaussian filter. The nerve activity varied greatly between preparations and within a single preparation over time. In most experiments, the rate was 20 Hz or lower for much of the time, with bursts up to 100 Hz or more lasting for periods of a few seconds to several minutes. Occasionally, however, the rate remained high throughout the experiment, as seen in the bottom trace. Also, the rate sometimes spontaneously became continuously high, as seen in the top trace. Spinosyn A, at concentrations of 10 nM and above, caused the spike rate to increase to a high constant level within 10 to 15 min and then subsequently decline to low levels. Three nanomolar spinosyn A was not effective in increasing spike rate in house fly ganglia.

As with house fly ganglia, application of spinosyn A to desheathed cockroach ganglia led to increased efferent activity. Unlike in house fly, however, this excitatory effect on cockroach ganglia persisted, without a consistent decline of activity. As seen in Fig. 3, the threshold for this effect was approximately 10 nM, with a

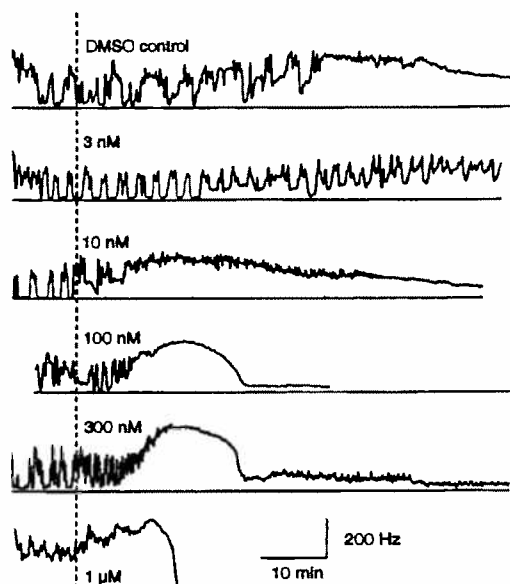


FIG. 2. Activity in segmental nerves of third instar house fly larvae, with each trace representing a different ganglion. The indicated concentrations of spinosyn A or the 0.1% DMSO solvent control were added at the broken line. Spike rate was measured as the number of spikes in 1 second samples of nerve activity, and the records were filtered off-line with a digital gaussian filter. The traces shown are representative of at least three experiments at each concentration.

sharp increase at 30 and 100 nM. If the assumption is made that the effect produced by 1000 nM was maximal, the estimated EC_{50} for the increase in firing rate was 32 nM.

In order to examine whether synaptic transmission was required for excitation of the cockroach nervous system by spinosyn A, ganglia were bathed in low Ca^{2+} (0.5 mM)/high Mg^{2+} (20 mM) saline to block neurotransmitter release. The effect of this saline on baseline neural activity in ganglia was variable. In some cases, activity was decreased (mean decrease of $63 \pm 15\%$ (SEM, $n = 6$), but in others it was either unchanged ($n = 2$) or increased ($n = 2$). Spinosyn A was applied after activity had stabilized in the new saline (25–40 min) and could still increase efferent activity in these ganglia, but the dose–response relation was shifted far to the right (Fig. 3; $EC_{50} = 490$ nM) and the maximal response appeared to be decreased.

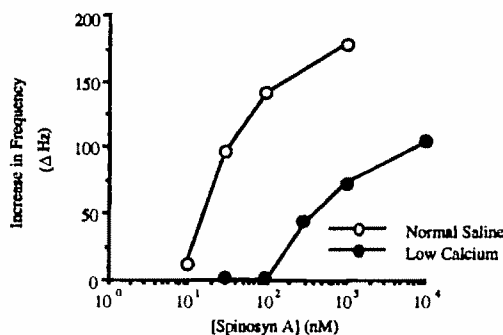


FIG. 3. Dose–response relations for spinosyn A-induced increases in efferent activity from *P. americana* ganglia in normal cockroach saline and in low Ca^{2+} /high Mg^{2+} saline. Each point represents the mean of independent observations from two to four ganglion preparations.

As with house fly and cockroach ganglia, application of spinosyn A to isolated *Manduca* abdominal ganglia led to an increase in efferent activity (Fig. 4). The threshold for this effect was approximately 3 nM, with a near maximal firing rate increase seen at 10 nM. The EC_{50} for the excitatory action of spinosyn A on *Manduca* abdominal ganglia was 5 nM. As with cockroach ganglia, spike rate did not consistently decline with prolonged exposure to spinosyn A. These results showed that spinosyn A has similar neural effects in three insect orders. Although the

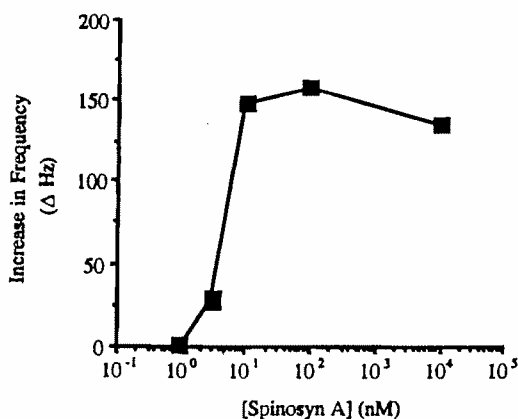


FIG. 4. Dose–response relation for spinosyn A-induced increase in *M. sexta* motor nerve firing. Each point represents the mean of independent observations from two to four ganglion preparations.

cockroach nervous system was the least sensitive of the three tested, it was used for the subsequent studies because of its large size and because the higher physiological concentrations required would facilitate measurement of the nerve cord content of spinosyn A at toxicologically relevant doses.

Concentration of Spinosyn A in Nerve Cords of Treated Insects

Ten adult male American cockroaches were each injected with 1.9 μg of [^{14}C]spinosyn A. Mortality continued to increase up to 48 h after injection, but did not change between 48 and 64 h, so at 64 h after injection, the insects were segregated into three groups. Group one contained six insects that were asymptomatic and could quickly right themselves after being placed on their backs. Group two contained two insects that showed poor motor coordination and could not right themselves when placed on their backs. Group three contained two insects that were prostrate. The average amount of radioactivity contained in the nerve cords of these insects corresponded to 5, 11, and 30 pmol equivalents, respectively (Table 2).

The nature of the radioactivity in nerve cords 64 h after injection of cockroaches with 1.9 μg of [^{14}C]spinosyn A was determined as described under Methods. The parent molecule, spinosyn A, represented approximately 38.5% of the recovered radioactivity (Fig. 5). The remainder of the radioactivity was contained in a broad,

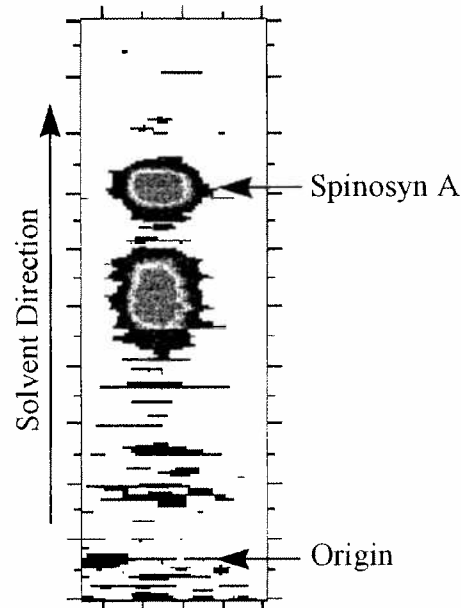


FIG. 5. TLC separation of [^{14}C]spinosyn A and metabolites in nerve cords taken from cockroaches treated 64 h earlier with 1.9 μg spinosyn A. Spinosyn A was 38.5% of total radioactivity. The other radioactivity consisted of a broad poorly resolved band of radioactivity with an R_f of approximately 0.50, which appeared to consist of several more polar metabolites.

poorly resolved band at $R_f = 0.50$, which appears to consist of multiple metabolites that are more polar than spinosyn A. Using 38.5% as the portion of nerve cord radioactivity at 64 h that was spinosyn A, the threshold nerve cord dose of

TABLE 2
Symptomology and Nerve Cord Content of Adult Male *P. americana*, 64 h after Injection of 1.9 μg [^{14}C]Spinosyn A

Symptom	No. of insects/10	Radioactivity (pmol-equivalent)	Spinosyn A (pmol)	Calculated aqueous conc (nM)
None	6	5 (2.1–9.9)	2.0	10
Difficulty righting	2	11 (8.9–12.8)	4.2	21
Prostrate	2	30 (15.9–45.1)	11.17	60

Note. The numbers of insects showing each symptom are shown in the second column. The third column shows the mean radioactivity in the nerve cords, in picomole equivalents, and this is converted to spinosyn A content in column 4, using 38.5 as the percentage of radioactivity that is spinosyn A (from Fig. 5). The fifth column shows the calculated aqueous concentration of spinosyn A that would be in equilibrium with nerve cords containing that amount of spinosyn A (see text). Ranges shown in parentheses.

spinosyn A, defined as that which caused difficulty righting, was calculated to be 4.2 pmol (Table 2, column 4).

The threshold-equivalent aqueous concentration of spinosyn A is defined as the aqueous concentration needed to give the threshold nerve cord dose of spinosyn A at steady state. In Fig. 6, 100 nM spinosyn A gave a steady-state nerve cord content of 19.4 pmol. Assuming that nerve cord uptake is proportional to concentration, the aqueous concentration that would give the measured spinosyn A content associated with each level of symptoms was calculated by proportionality and is shown in column 5 of Table 2. For insects showing difficulty righting, this concentration is the threshold-equivalent aqueous concentration and was calculated to be 21 nM.

DISCUSSION

In the preceding paper (5), spinosyn poisoning symptoms were described in a variety of insects, and it was concluded that the first symptoms were due to involuntary muscle contractions caused by excitation of the central nervous system. Furthermore, it was shown that spinosyn A had a direct excitatory effect on isolated house fly ganglia. In the present report, we studied the concentration dependence of central nervous

system excitation in house flies, cockroaches, and tobacco hornworms and used radiotracer methods to estimate the equivalent aqueous concentration of spinosyn A in the hemolymph of cockroaches treated with a threshold dose. Spinosyn A activated tobacco hornworm nerve activity with an ED_{50} of 5 nM. The response of the house fly CNS was comparable, showing no clear effect at 3 nM and maximal excitation at 10 nM spinosyn A. The cockroach CNS was somewhat less sensitive, with a threshold of 10 nM and an EC_{50} of 32 nM, assuming that the effect produced by 1000 nM was maximal. While we do not know exactly how much of an increase in neural activity is needed to interfere with the insect's righting ability, it is likely to be less than 50% of the maximal increase and therefore between 10 and 32 nM. The latter result was corroborated very well by our estimate from radiotracer measurements that 21 nM spinosyn A is needed to poison cockroach nerve cords to the same extent that they are poisoned in cockroaches treated with a threshold dose. We conclude that spinosyn A attains an internal concentration in treated insects that is sufficient to cause the observed symptoms by direct action on the central nervous system.

The threshold physiological concentration was estimated indirectly as the aqueous concentration needed to give the steady-state nerve cord concentration attained during poisoning at a threshold dose, because this value is more meaningful than whole hemolymph concentration, and direct measurement of hemolymph aqueous concentration in poisoned insects was not practical, due to the difficulty of removing cells and proteins from whole hemolymph without also removing a portion of the hydrophobic spinosyn from the aqueous phase.

The equivalent aqueous concentration defined and measured here can be equated theoretically to the concentration in the aqueous phase of the hemolymph. Hemolymph is a complex tissue, composed of cells suspended in a complex solution of salts and organic molecules, including high concentrations of proteins. A solute such as a spinosyn would partition between hemolymph cells, proteins and the aqueous phase, as shown

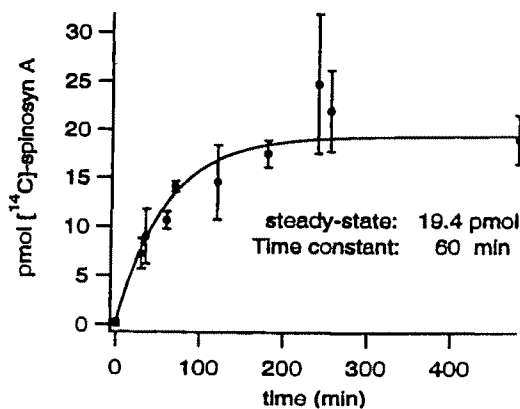


FIG. 6. Content of [14 C]spinosyn A in nerve cords from adult male *P. americana* after incubation with 100 nM [14 C]spinosyn A at room temperature for various times. The curve shows an exponential function fitted to the data, with each point weighted as $1/\text{standard deviation}$. Bars show standard deviation.

on the left in Fig. 7. Furthermore, the solute would partition between the aqueous phase and the nerve tissue, with a steady-state partition coefficient of $P_{HA} = C_{TH}/C_{HA}$, where C_{TH} is the concentration in the nerve tissue and C_{HA} is the concentration in the hemolymph aqueous phase. In *in vitro* physiological experiments, the tissue is exposed to the compound at a concentration C_S in saline, and the steady-state concentration in the nerve tissue is determined by $P_S = C_{TS}/C_S$, where C_{TS} is the concentration achieved in the tissue. The assumption that $P_S = P_{HA}$ leads directly to the relation

$$C_{HA} = \frac{C_{TH}}{C_{TS}} C_S. \quad (1)$$

Thus, in a particular pair of experiments in which C_{TH} is determined for a dose of interest, such as a threshold dose, and C_{TS} is determined at a particular saline concentration, C_S , this equation can be used to calculate the equivalent aqueous concentration, as was done in column 5 of Table 2.

The threshold equivalent aqueous concentration measured here is a valuable reference for future *in vitro* studies of spinosyn A on isolated cells and receptors. Assuming that the steady-state distribution of compound within the nerve

cord is the same whether the nerve cord is in hemolymph or saline, receptors within nerve cords soaked in saline containing a compound would be exposed to the same concentration of compound as would those in nerve cords from poisoned insects, when the nerve cords contain the same total amount of compound. Furthermore, assuming no specific transport mechanisms for the compound into the nervous system or to the receptors, the equivalent aqueous concentration determined in this way is also the aqueous concentration to which the receptors are expected to be exposed.

Metabolism of spinosyn A in the cockroach was significant by 64 h; only 38.5% of the radioactivity in the nerve cord at that time was spinosyn A. This is the first demonstration that spinosyn A is metabolized by insects. A previous study had found no significant metabolism of spinosyn A (LY-232105) in tobacco budworm (7), although at a much shorter incubation time of only 6 h. It is unlikely that significant metabolism occurred in the *in vitro* nerve cord uptake experiment (Fig. 6), because the incubation time was only 8 h, and insect nerve cord is relatively low in microsomal oxidases (8).

Spinosyn A increased neural activity in all species examined. In house fly and *Manduca* ganglia, the concentration-response relations were very steep. The potency of spinosyn A was similar in the three species, with the EC_{50} varying less than 10-fold, from 5 nM in *Manduca* to 32 nM in the cockroach. The greater sensitivity of the *Manduca* nervous system to spinosyn A is consistent with the higher efficacy of spinosad on lepidoptera in general and suggests that the target site is intrinsically more sensitive in this lepidopteran than in the cockroach or the house fly, although more direct studies are needed to establish this with certainty.

It is interesting that all concentrations of spinosyn A that were effective in increasing the activity of house fly ganglia also subsequently depressed activity. *In vivo* measurements of nerve activity in cockroaches poisoned with spinosyn A showed that activity remained high for many hours and even days after prostration, well into paralysis. In fact, the paralysis that

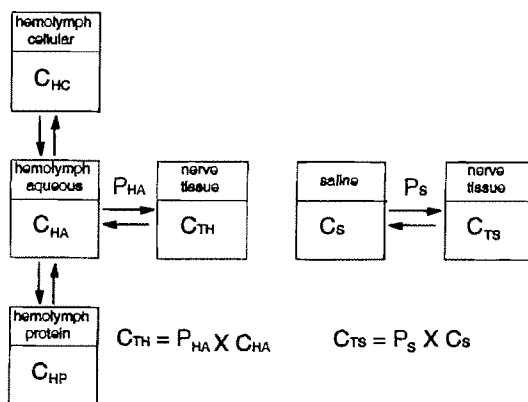


FIG. 7. Left, partitioning of a compound between the protein, cellular, and aqueous phases of hemolymph and between the aqueous phase and the nerve tissue. Right, partitioning of a compound between saline and nerve tissue. See Discussion for description.

occurred in the late stages of spinosyn poisoning was found to be due to neuromuscular block rather than to a decrease in neural activity (5). If neural activity declined in the same way in poisoned house flies, the insects would be expected to become paralyzed soon after prostration by spinosyns. This was not seen in adult house flies or in fruit flies (5). Thus, it is likely that the delayed decrease in nerve activity caused by spinosyn A in house fly ganglia is an experimental artifact, perhaps due to nutrient or oxygen deficiency.

Reduction of $[Ca^{+2}]$ and elevation of $[Mg^{+2}]$ is a standard means of blocking chemical synaptic neurotransmission. However, since Ca^{+2} is involved in numerous intracellular and extracellular processes (e.g., Refs. 9, 10), the block of synaptic neurotransmission is clearly not the only consequence of this altered divalent state. Moreover, from our cockroach ganglion experiments, there is no means of confirming whether synaptic neurotransmission was indeed blocked under these conditions. Nonetheless, spinosyn A was less active on cockroach ganglia equilibrated in decreased $[Ca^{+2}]$ /elevated $[Mg^{+2}]$. This suggests that the most potent excitatory actions of spinosyn A require synaptic transmission in order to be expressed in cockroach ganglia. Further studies will be necessary to confirm this suggestion.

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