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IN-VITRO METHODS FOR EVALUATING SIDE EFFECTS OF PESTICIDES AND TOXIC SUBSTANCES

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IN-VITRO METHODS FOR EVALUATING SIDE EFFECTS OF PESTICIDES AND TOXIC SUBSTANCES

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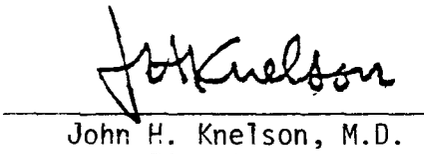
FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

The project described herein was initiated to meet program needs relevant to the development of *in vitro* test systems for pesticide toxicity. From a research point of view, the type of methodology developed can be used not only to predict the relative degree of toxicity but also to corroborate the results obtained using animal dose response models.



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ABSTRACT

Several skeletal muscle and smooth muscle preparations have been examined for their usefulness in evaluating the toxic effects of a variety of insecticides. The following preparations were found satisfactory for such test: guinea pig ileum for muscarinic receptors, guinea pig heart for β-adrenergic receptors, guinea pig vas deferens for α-adrenergic receptors, frog rectus abdominis for nicotinic receptors of tonic muscle, and rat diaphragm for nicotinic receptors of phase muscle. Five carbamate insecticides, four organophosphate insecticides and chlordimeform were studied. None of the insecticides tested had any direct and potent effect on these receptors except the effect on cholinergic receptors via cholinesterase inhibition. Carbofuran, propoxur and formetanate had potent stimulating actions on the guinea pig ileum, but these effects could entirely be attributed to the accumulation of acetylcholine in the synaptic cleft as a result of cholinesterase inhibition. Thus it can be concluded that these insecticides exert no direct action on cholinergic and adrenergic receptors.

I. INTRODUCTION

A variety of chemicals which are in use in agriculture, industry, and homes are potentially hazardous to humans. In addition, a large number of new chemicals are being developed for use, many of which are also potential hazardous compounds. Although the mechanism of major toxic action of some of these compounds is known, there could be a variety of side effects which might be responsible for acute and/or chronic toxicity caused by these substances.

In our previous study under the EPA Contract No. 68-02-1289, efficient, accurate and inexpensive methods have been developed to evaluate the neural toxicity of various pesticides and toxic compounds (Narahashi, 1976). The abdominal nerve cord preparation of the crayfish has been found to be most satisfactory among the various preparations examined. A variety of toxic compounds including organophosphate, carbamate, and pyrethroid insecticides exert very potent actions on this preparation.

In our second year study, we have developed methods whereby various side effects of toxic compounds can be evaluated. One of the most important examples is organophosphate and carbamate pesticides. It has been shown by a number of investigators that the inhibition of cholinesterases is the major mechanism whereby both mammals and insects are intoxicated by these pesticides. However, there is some notion that certain anticholinesterase pesticides do exhibit effects through the mechanisms other than the inhibition of cholinesterases. Such actions, sometimes called "direct actions", could pose serious problems when the aspects of safety, environment and treatment of intoxication by these compounds are considered. These possible side effects produced by the mechanisms other than cholinesterase inhibition are most probably responsible for a variety of systemic and behavioral effects which cannot be ascribed to excess stimulation of cholinergic receptors as a result of the

inhibition of cholinesterases. This problem has been largely ignored, and only very limited information is available despite its paramount importance. If there were serious side effects other than cholinesterase inhibition, routine approaches to the therapeutics such as those utilizing atropine and pralidoxime would not guarantee the complete safety. The same consideration applies to any other chemicals in use which are potentially hazardous and have multiple effects on human and other mammals.

The purpose of this study is to accomplish the following goals:

1. To compare various skeletal muscle and smooth muscle tissues for their usefulness in evaluating the toxic effects of a variety of substances including organophosphate and carbamate insecticides on cholinergic and adrenergic receptors.
2. To establish techniques whereby the toxic side effects other than those ascribed to cholinesterase inhibition can be evaluated with the tissues selected in project (1) above.
3. To study some toxic substances for their side toxic effects using the method established by project (2) above.

II. METHODS

A. Materials

One of the main purposes of this project was to test some skeletal and smooth muscle tissues for evaluation of toxic nature of various chemicals. Some muscles have nicotinic receptors but are devoid of muscarinic receptors and catecholamine receptors. Some other smooth muscles are innervated by both sympathetic and parasympathetic nerves, so that both muscarinic and adrenergic receptors exist. By using an appropriate blocking agent acting on one of the receptors, one should

be able to study the effect of a test compound on the other receptor of the tissue. In fact, this is a routine technique to study the interaction of a test compound with any particular type of receptors, and has been used very often. However, the data on pesticides and other environmental agents with respect to such interaction are very limited. The selection of the material was based on specificity for each type of receptor (such as nicotinic, muscarinic, α-adrenergic and β-adrenergic), easiness of handling, reproducibility of results, and costs. The following preparations were examined.

Guinea pig ileum

The isolated preparation of the ileum of the guinea pig contains the parasympathetic ganglia, the parasympathetic preganglionic and postganglionic fibers, the sympathetic postganglionic fibers, and the smooth muscle with both muscarinic and adrenergic receptors. With combination of appropriate blocking agents, it is possible to study the effect of a toxic substance on one of these systems. For example, with the preparation treated with an adrenergic blocking agent and a ganglionic blocking agent the effect of a test compound on the muscarinic receptor can be examined. If the test compound had a muscarinomimetic action, stimulation of the preparation would be manifest in the form of contraction. If it were a muscarinic blocking agent, the contractile response of the preparation to acetylcholine would be diminished.

For anticholinesterases such as organophosphate and carbamate insecticides, the direct effects other than those produced by cholinesterase inhibition can be studied with prior application of an inhibitor of transmitter release such as magnesium ions and black widow spider venom. An alternative method is to denervate the muscle.

Guinea pig heart

The guinea pig heart contains β-adrenergic receptors, and has been used for the study of sympathomimetic drugs with a specific affinity for β-receptors. Muscarinic blocking agents such as atropine can be used to block the muscarinic receptors on this heart preparation.

Guinea pig vas deferens

The guinea pig vas deferens contains α-adrenergic receptors, and has been used for the study of sympathomimetic drugs with a specific affinity for α-receptors. Muscarinic blocking agents such as atropine can be used to block the muscarinic receptors on this preparation.

Frog rectus abdominis

The frog rectus abdominis has been widely used for the study of drugs acting on nicotinic cholinergic receptors. It is routinely used to examine the potency of agonists acting on nicotinic receptors. There is no sympathetic innervation, so that no complication will arise as a result of the possible effect on the adrenergic receptors. This preparation is composed of tonic muscle fibers, and nicotinic agonists cause a contracture.

Rat diaphragm

The phrenic nerve-diaphragm preparation isolated from the rat has been used for the study of drugs acting on nicotinic cholinergic receptors. Unlike the frog rectus abdominis, the diaphragm is composed of phasic muscle fibers. Therefore, nicotinic agonists cause a transient depolarization of the end-plate membrane evoking tetanus. This phase is followed by a desensitization block.

B. Methods of Recording of Muscle Contractions

Guinea-pig ileum

The ileum was isolated from the male guinea pig weighing 450-550 g. The isolated tissue was cut in a length of 2 cm, tied off with silk threads at both ends, and mounted in a chamber of 15 ml capacity as illustrated in Fig. 1. One of the threads was connected with a force-displacement transducer (Grass model FT03C). The other thread was connected with a metal rod mounted on a micromanipulator which permitted adjustment of the muscle tension. Air was introduced from the bottom of the chamber, and the glass filter was effective in making fine air bubbles. Tyrode's solution (solution A, Table 1) was led into the chamber and was sucked up from the surface. The chamber was immersed in a water bath except for its top portion to maintain the temperature constant at 37°C. The output of the transducer was fed into a preamplifier (Grass model 5E) and contractions were recorded on a Grass Polygraph (model 5DWC.1).

Test solution (0.2 ml) was injected into the bath in the chamber (15 ml) while suspending perfusion. Thus the test solution was diluted by a factor of 75. The final concentration of test compound was given in this paper.

In some experiments, denervated preparations were used. Two methods were employed, one being turned the ileum inside out and the other being the one developed by Paton and Zar. In the former method, a piece of the ileum in about 10 cm long was turned inside out on a glass rod of 6 mm in diameter. The internal surface of the ileum, which was now faced outside, was stroked tangentially by means of a wisp of cotton wool so as to remove the circular muscle and the Auerbach's plexus. Thus only the longitudinal muscle layer remained on the glass rod.

The method of Paton and Zar is summarized as follows:

The ileum was stretched on a glass rod of 6 mm diameter, and was gently pulled upwards by applying traction at the proximal end to obtain a a stip of longitudinal muscle. Auerbach's plexus could be visible under

a dissecting binocular microscope, but most regions of the strip were free of the plexus. A stretch of the strip where no plexus was found was cut off and used as the nerve-free ileum preparation.

The denervated ileum was mounted in the chamber in the same manner as that for the intact ileum. The only differences were that a mixture of 95% O_2 and 5% of CO_2 instead of air was used to bubble the bathing medium, and that solution C was used instead of solution A (Table 1).

Guinea pig vas deferens

The vas deferens was isolated from the male guinea pig weighing 450 to 550 g, and cut in a length of 1.5 cm. The preparation was tied off at both ends with silk threads, and mounted in a 15 ml glass filter chamber as described in the preceding section for the guinea pig ileum. The methods of recording contractions and application of test compounds, and temperature were the same as those for the ileum. Solution C (Table 1) was used as the bathing medium.

Guinea pig heart

The heart was isolated from the male guinea pig weighing 450 to 550 g. The aorta was cannulated by a glass tubing of 2 mm in outer diameter which was in turn connected with the inlet polyethylene tubing (Fig. 2). The side arm of the cannula was capped with a rubber membrane through which test solution (0.2 ml) was injected into the perfusate. The reservoir of Tyrode-Locke solution (solution E, Table 1) was kept about 1.5 m above the heart, and was bubbled with a gas mixture of 95% O_2 and 5% CO_2. The solution was then passed through a water bath with a temperature of 45°C, and then perfused through the heart. The temperature of the heart was maintained at 33-40°C The ventricle of the heart was attached to a hook which in turn was connected with a force displacement transducer (Grass model FT03C), and contractions were recorded on a Grass Polygraph (model 5DWC.1) via a preamplifier (Grass model 5E).

Rat diaphragm

The diaphragm was isolated with a stretch (1.5 cm) of the phrenic nerve attached from the rat weighing 90 to 100 g (male and female mixed). One end of the muscle was tied off with a silk thread which in turn was connected with a force-displacement transducer (Grass model FT03C) (Fig. 3). The other end was tied off at a few points with silk threads which were connected with a metal rod. The rod was mounted on a micromanipulator which permitted adjustment of the tension of the muscle. The preparation was placed in a 60 ml glass filer chamber containing physiological saline solution. Solution D (Kreb's solution II) or solution B (Tyrode's solution II with a double amount of glucose) (Table 1) was used as the bathing medium. The perfusate was introduced to the glass filter in a water bath at a temperature of 37°C, and was sucked from the surface of the chamber. A gas mixture of 95% O_2 and 5% CO_2 was introduced from the bottom of the chamber, and was converted into fine bubbles when it passed through the glass filter. The cut end of the phrenic nerve was sucked into a suction electrode which consisted of a glass capillary of 0.1 mm inner diameter. The suction electrode was connected with a stimulator via an Ag-AgCl wire.

Electric pulses of 15-30 V in intensity and 0.3-10 msec in duration were applied to the nerve every 5 sec as supramaximal stimuli. The nerve evoked diaphragm contractions were recorded on a Grass Polygraph (model 5DWC.1) via a preamplifier (Grass model 5E). Test solution (0.3 ml) was injected into the bath (60 ml) while suspending the perfusion. Thus the test solution was diluted by a factor of 200. Dose was expressed as the final concentration in the bath.

Frog rectus abdominis

The rectus abdominis was isolated from the frog Rana pipiens about two inches in length. The methods of mounting in the chamber and recording contractions were the same as those for the guinea pig ileum. Air bubbled

frog Ringer's solution (solution F, Table 1) was used as the bathing medium which was kept at a constant temperature of 20°C.

C. Chemicals

The following chemicals acting on the autonomic nervous system were used in the present study. Unless otherwise stated, pure chemicals were used: Acetylcholine chloride (Sigma Chemical Co., St. Louis, Mo.), physostigmine salicylate (Sigma Chemical Co.) and Antilirium ampules from O'Neal, Jones and Feldman, Inc., St. Louis, Mo.), neostigmine mesylate (Prostigmin from Rocke Lab., Division of Hoffman-La Roche, Inc., Nutley, N. J.), atropine sulfate (Atropine from Elkins Sinn, Inc., Cherry Hill, N. J.), phentolamine mesylate (Regitine from CIBA Pharmaceutical Co., Division of CIBA-GEIGY Co., Summit, N. J.), propranolol hydrochloride (Sigma Chemical Co.), carbamylcholine chloride (Aldrich Chemical Co., Inc., Milwaukee, Wis.), isoproterenol hydrochloride (Isuprel from Winthrop Lab., Division of Sterling Drug Inc., New York, New York), norepinephrine hydrochloride (Sigma Chemical Co.), d-tubocurarine (Lilly Laboratories, Indianapolis, Ind.), diphenhydramine hydrochloride (Benadryl from Parke-Davis, Detroit, Michigan), hexamethonium bromide (Sigma Chemical Co.), nicotine dihydrochloride (J. T. Baker Chemical Co., Phillipsburg, N. J.), and histamine dihydrochloride (K & K Laboratories, Plainview, New York).

The insecticides examined were provided by the Environmental Protection Agency, National Environmental Research Center, Research Triangle Park, North Carolina. They were carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), carbaryl (1-naphthyl N-methylcarbamate), leptophos (O-(4-bromo-2,5-dichlorophenyl) O-methyl phenylphosphonothioate), monocrotophos (3-hydroxy-N-methylcrotonamide dimethyl phosphate), dichlofenthion (diethyl 2,4-dichlorophenyl phosphorothionate), dursban (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), propoxur (2-isopropoxyphenyl N-methylcarbamate), ferbam (ferric dimethyldithiocarbamate), formetanate (3-(((dimethylamino)methylene)amino)phenyl N-methylcarbamate hydrochloride), and chlordimeform (N'-(4-chloro-o-tolyl)-N-N-dimethylformamidine). Formetanate was

directly dissolved in aqueous solutions. Ferbam was first dissolved in dimethylsulfoxide (DMSO) to make up a stock solution which in turn was diluted with perfusate before experiment. The final maximum concentration of DMSO in test solutions was 0.0133% (v/v), and had no effect on the preparations used. All other insecticides were dissolved in ethanol to make up stock solutions which in turn were diluted with perfusate. The final maximum concentration of ethanol was 0.0133% (v/v), and had no effect on the preparations used.

III. RESULTS

A. Guinea Pig Ileum

Drugs acting on autonomic nervous system

A variety of drugs acting on the sympathetic and parasympathetic nervous systems were examined for their effects on the guinea pig ileum to establish the control picture of drug action. A representative drug was selected for each class of action.

a. Acetylcholine. Acetylcholine (ACh) was tested as a representative of agonists acting on muscarinic and nicotinic receptors. It caused a contraction of the ileum at low concentrations. An example of such a record is illustrated in Fig. 4. The effect of ACh was completely reversed after washing with drug-free medium. The dose-response relation is shown in Fig. 5. On the assumption of a one-to-one stoichiometric interaction between ACh and receptor, the apparent dissociation constant was estimated to be 2.67×10^{-7} M from the Lineweaver-Burk plot.

b. Physostigmine. At low concentrations ranging from 1.33×10^{-9} M to 1.33×10^{-8} M, physostigmine itself did not cause a sizable contraction of the ileum. However, the ACh contraction was potentiated by physostigmine at concentrations of 3.99×10^{-9} M to 3.99×10^{-8} M. An example of the potentiated contraction is illustrated in Fig. 4. In order to assess the optimal time for

physostigmine pretreatment, the contraction induced by ACh (1.55×10^{-8} M to 1.55×10^{-7} M) was measured after pretreatment with 1.33×10^{-8} M physostigmine for various periods of time. The results are summarized in Fig. 6. The physostigmine-induced potentiation appeared after 1 min of pretreatment, and after 5 min the potentiation was observed invariably. Therefore, the ileum was pretreated with physostigmine for 5 min in the subsequent experiments. Table 2 gives the results of two experiments in which the relative potency of various concentrations of physostigmine in potentiating the ACh response was examined. Physostigmine had no potentiating effect at 1.33×10^{-9} M, and the threshold concentration was estimated to be 4×10^{-9} M. The physostigmine-induced potentiation of ACh response is interpreted as being due to inhibition of acetylcholinesterase (AChE).

At high concentrations, physostigmine itself induced contractions. An example of such a record is illustrated in Fig. 7. Unlike the contraction induced by ACh, the physostigmine-induced response was characterized by a long latent period, a slow onset, and repetitive contractions. A slight sign of contractions or increasing tone was visible with 1.33×10^{-8} M physostigmine, and the response was clear at 3.99×10^{-8} M or higher concentrations. Data are given in Table 3. The mechanism underlying the direct stimulation of ileum by physostigmine will be discussed later.

c. Neostigmine. As is expected from the ability to inhibit AChE, neostigmine had a potentiating action on ACh response. An example of dose-response curves of ACh before and after treatment with 1.14×10^{-7} M neostigmine is illustrated in Fig. 8.

d. Atropine. Atropine is a competitive antagonist agains the ACh stimulation of muscarinic receptors. It suppressed the response of the ileum to ACh (Fig. 9). The time course of the action of atropine is shown in Fig. 10. At a concentration of 1.33×10^{-8} M, the atropine treatment for a period of 3-5 min was sufficient

to block the contraction caused by 7.74×10^{-7} M ACh. Therefore, atropine was applied for 5 min in the subsequent experiments. Dose-response curves for ACh contraction before and after treatment with 1.33×10^{-8} M atropine are illustrated in Fig. 11. The curve with atropine is shifted along the abscissa in the direction of higher ACh concentration indicating a competitive antagonism between these two drugs.

e. Phentolamine. Phentolamine is an α-blocking agent, and was tested on the guinea pig ileum for its possible action. It had no marked effect on the ACh-induced contraction at low concentrations up to 1.33×10^{-7} M, but suppressed the ACh response at higher concentrations (Fig. 12). The time course of action of a higher concentration of phentolamine (1.33×10^{-5} M) is illustrated in Fig. 13. The contraction induced by 7.74×10^{-8} M ACh was gradually suppressed as the phentolamine pretreatment was prolonged, and a near maximum suppression was observed with a 3 min pretreatment. Thus the phentolamine pretreatment was set at a 5 min period in the subsequent experiments. Dose-response curve of phentolamine to suppress the ACh contraction is illustrated in Fig. 14. The concentration of phentolamine to produce a 50% effect was of the order of 10^{-5} M. Fig. 15 shows an example of dose-response curves of ACh contraction before and after treatment with 1.33×10^{-5} M phentolamine. The curve is shifted in the direction of high concentration of ACh after pretreatment with phentolamine, indicating a competitive antagonism between the two drugs. The last observation strongly suggests that the phenotlamine-induced suppression of ACh contraction is not due to its effect on the α-receptor but due to its inhibitory effect on the muscarinic receptor.

f. Propranolol. Propranolol is a β-blocking agent, and was tested on the guinea pig ileum for its possible effect. It had no effect on the ACh contraction except when it was given at a very high concentration (Fig. 16).

Dose-response relation is illustrated in Fig. 17. The inhibitory effect of the high concentration of propranolol is presumably due to direct action on the muscarinic receptor.

Insecticides

Ten insecticides were examined for their direct effects on the guinea pig ileum and their ability to change the contractions induced by ACh and carbachol. Carbachol was used to distinguish the effect through the inhibition of AChE and the direct effect on the muscarinic receptor.

a. Carbofuran. Carbofuran caused little or no contraction at a concentration of 1.33×10^{-8} M (Fig. 18). However, potent contractions were induced at higher concentrations ranging from 1.33×10^{-7} M to 1.33×10^{-6} M (Fig. 18). Despite the strong stimulating action, carbofuran did not exhibit marked potentiating or depressing effect on the ACh- or carbachol-induced contraction (Fig. 19). The data are summarized in Table 4.

b. Carbaryl. An example of a record of muscle tension in the presence of 1.33×10^{-9}-1.33×10^{-6} M carbaryl is illustrated in Fig. 20. There was practically no effect on the ileum. Carbaryl exerted no direct effect on the ileum, and did not affect the ACh- or carbachol-induced contraction (Fig. 20B, Table 4).

c. Leptophos. In concentrations ranging from 1.33×10^{-9} M to 1.33×10^{-6} M, leptophos did not induce any sizable contraction by itself (Fig. 21), and had no effect on the ACh- or carbachol-induced contraction (Table 4).

d. Monocrotophos. Monocrotophos did not induce contraction by itself and had no influence on the ACh- or carbachol-induced contraction at concentrations ranging from 1.33×10^{-8} M to 1.33×10^{-6} M (Fig. 22, Table 4).

e. Dichlofenthion. Dichlofenthion did not induce contraction by itself and had no effect on the ACh- or carbachol-induced contraction at concentrations ranging from 1.33×10^{-8} M to 1.33×10^{-6} M (Fig. 23, Table 4).

f. Dursban. At concentrations ranging from 1.33×10^{-8} M to 1.33×10^{-6} M, dursban did not induce any contraction by itself (Fig. 24, Table 4). It had a slight tendency to decrease the ACh- or carbachol-induced contraction after 3 min pretreatment, but the effect eas small (Table 4).

g. Propoxur. At a concentration of 1.33×10^{-9} M, propoxur did not induce any measurable contraction (Table 4). At higher concentrations ranging from 1.33×10^{-8} M to 4×10^{-6} M, it induced slow contractions which increased in intensity with the concentration (Table 4). Examples of records of the propoxur-induced contractions are illustrated in Fig. 25. However, the effect of propoxur on the ACh- or carbachol-induced contraction was negligible (Fig. 26, Table 4).

h. Ferbam. Because of difficulty in solving ferbam in ethanol which was used as the solvent for the other insecticides tested excepting formetanate, methanol was first employed to dissolve ferbam. The ferbam solution thus prepared stimulated the ileum in producing contractions at concentrations ranging from 1.33×10^{-8} M to 1.33×10^{-6} M, but did not alter the ACh-induced contraction. However, when dimethylsulfoxide (DMSO) was used as the solvent, no such stimulating effect of ferbam was observed (Fig. 27, Table 4). The ACh-induced contraction was not affected by ferbam pretreatment (Fig. 27, Table 4). Thus the stimulating effect observed with ferbam solutions containing methanol as a solvent is due to the action of methanol.

i. Formetanate. Formetanate induced contractions at concentrations ranging from 1.33×10^{-8} M to 1.33×10^{-6} M, but had little or no effect on the ACh- or carbachol-induced contraction (Fig. 28, Table 4).

j. Chlordimeform. At concentrations ranging between 1.33×10^{-8} M and 1.33×10^{-6} M, chlordimeform did not induce any measurable contraction (Fig. 29, Table 4). The contraction induced by ACh or carbachol was not influenced by

chlordimeform at a concentration of 1.33×10^{-7} M, but was depressed somewhat by 1.33×10^{-6} M (Table 4).

Effects of specific inhibitors on insecticide-induced contractions

Of the ten insecticides that were examined, carbofuran, propoxur and formetanate had a potent stimulating action on the guinea pig ileum causing contractions. The effect could be exerted as a result of stimulation of the muscarinic receptor in the muscle, the nicotinic receptor in the muscle, the nicotinic receptor in the parasympathetic ganglia, or the histaminic receptor in the muscle. In order to determine the site of action of the three insecticides, experiments were carried out using specific inhibitors of these receptors. These inhibitors of these receptors. These inhibitors included atropine for the muscarinic receptor, hexamethonium for the nicotinic receptor, and diphenhydramine for the histaminic receptor.

a. Carbofuran. Pretreatment of the ileum with 1.33×10^{-8} M atropine abolished the carbofuran-induced contraction. Pretreatment with 1.33×10^{-4} M hexamethonium or with 1.33×10^{-7} M diphenhydramine was ineffective in preventing the contraction. Examples of contraction records under these conditions are illustrated in Fig. 30, and the data are summarized in Table 5. These results indicate that the carbofuran-induced contraction is the result of direct stimulation of the muscarinic receptor in the ileum, or stimulation of the muscarinic receptor in the parasympathetic ganglia which in turn stimulates the ileum via the activity of the postganglionic parasympathetic nerve fibers, or both.

b. Propoxur. Similar to the case of carbofuran, the propoxur-induced contraction was prevented by pretreatment with 1.33×10^{-8} M atropine, but not by 1.33×10^{-4} M hexamethonium or 1.33×10^{-7} M diphenhydramine (Fig. 31, Table 5). Thus the same conclusion as that for carbofuran applies to propoxur.

c. Formetanate. The formetanate-induced contraction was almost completely abolished by pretreatment with 1.33×10^{-8} M atropine, was slightly decreased by pretreatment with 1.33×10^{-7} M diphenhydramine, and was not affected by pretreatment with 1.33×10^{-4} M hexamethonium (Fig. 32, Table 5). Thus the major stimulating action of formetanate is exerted on the muscarinic receptor in the ileum or in the parasympathetic ganglia or both, and there is a minor stimulating action on the histaminic receptor.

Effects of low Ca^{++}-high Mg^{++} solutions on insecticide-induced contractions

As described in the preceding section, the contractions of the ileum induced by carbofuran, propoxur and formetanate are due to the stimulation of the muscarinic receptor in the muscle, the muscarinic receptor in the parasympathetic ganglia, or both. If the stimulation of the muscarinic receptor in the ganglia were the major cause of contraction, the effect would be abolished by preventing release of transmitter from nerve terminals. One way of inhibiting the transmitter release is to lower calcium concentration and raise magnesium concentration. Therefore, the effects of the three insecticides on the ileum were studied in low Ca^{++}-high Mg^{++} media. Prior to performing such experiments, control experiments were carried out to see whether low Ca^{++}-high Mg^{++} media had any effect on the ACh- and physostigmine-induced contractions.

a. ACh contraction. When the magnesium concentration in Tyrode's solution was increased from the normal level of 1.03 mM to 1.70-2.40 mM without changing the calcium concentration (1.80 mM), the contraction induced by ACh tended to decrease. The inhibitory effect was almost negligible in 1.7 mM Mg^{++}, but became more marked at 2.30-2.40 mM Mg^{++} (Table 6). In contrast, decreasing the calcium concentration from 1.80 mM to 0.18-0.36 mM with the magnesium concentration elevated to 1.70-2.20 mM had a marked depressant effect on the ACh-induced contraction (Fig. 33B, Table 6). Therefore, the effects of physostigmine and insecticides in

the modified Tyrode's solution were always normalized to the levels observed in the same Ca^{++} and Mg^{++} concentrations.

b. Physostigmine. Unlike the contraction induced by ACh, the response caused by physostigmine was characterized by a long latent period and slowly developing, repetitive contractions. It was therefore difficult to measure a maximum amplitude of the physostigmine-induced contraction as a measure of activity. Thus the latent period for the physostigmine-induced contraction to attain a level equivalent to the maximum amplitude of the contraction evoked by ACh was measured as an index of response. An example of a record is illustrated in Fig. 33. Decreasing the calcium concentration from 1.80 mM to 0.036-0.36 mM with the magnesium concentration elevated from 1.03 mM to 2.20-2.30 mM tended to prolong the latent period and to decrease the amplitude of the physostigmine response, but the difference was not marked (Table 7). Thus it can be said that the contraction induced by physostigmine is at least in part due to an increase availability of ACh released from the postganglionic nerve fibers as a result of inhibition of AChE.

c. Insecticides. The records of ileum contractions induced by carbofuran, propoxur and formetanate in modified Tyrode's solution are illustrated in Figs. 34, 35 and 36, respectively. For the three insecticides, the contraction was delayed in onset and decreased in amplitude by an increase in magnesium concentration from 1.03 mM to 2.20 mM and a concurrent decrease in calcium concentration from 1.80 mM to 0.36 mM (Table 8). Therefore it can be concluded that the contractions induced by the three insecticides are at least in part due to an increased availability of ACh released from the postganglionic nerve terminals as a result of AChE inhibition.

Effects of black widow spider venom on insecticide-induced contractions

Venom from the black widow spider is known to inhibit the release of transmitter from nerve terminals. Therefore, the venom was used in the present study in an

attempt to block the transmitter release. If the venom completely inhibited the transmitter release under the present experimental conditions, any contraction induced by the released ACh would be abolished.

Experiments were performed according to the following protocol. First the ileum preparation was soaked in Tyrode's solution containing black widow spider venom at various concentrations (0.2-4 glands/ml) and for various periods of time (5-60 min). Then the preparation was mounted in the chamber and washed with normal Tyrode's solution free of venom until the ileum regained the ability to respond to ACh. For some unknown reason, the ileum did not respond to ACh immediately after treatment with venom. Physostigmine was then applied to the preparation and the response was recorded.

Fig. 37 illustrates the contraction evoked by physostigmine application after treatment with black widow spider venom at a concentration of 0.4 glands/ml for 15 min at room temperature. The response was smaller than that in normal preparation, but was not abolished by venom treatment. Data from 13 ileum preparations in 6 series of experiments are summarized in Fig. 38. The ordinate represents the reciprocal of the time necessary for the contraction induced by 4×10^{-7} M physostigmine to reach the same level as the contraction induced by 6.65×10^{-8} M ACh. Thus increasing the ordinate value means greater response to physostigmine. The abscissa represents the time of venom treatment. It is clearly shown that the physostigmine response decreases with prolonging the time of venom treatment in 5 out of 6 series of experiments, but that venom fails to abolish the response. Thus black widow spider venom does not seem to be totally effective in abolishing transmitter release in the ileum preparation.

Denervated preparations

The most straightforward way to eliminate the effect of the transmitter ACh released from nerve terminals would be to remove the ganglia from the ileum

preparation. Two methods of denervation were attempted as described in the Methods section.

a. Denervation by inside-out. The ileum preparation in which its inside was turned over to the outside and the ganglia were removed by gently brushing them off was still responsive to physostigmine (Table 9). Thus the ganglia did not seem to be completely eliminated by this method, and this preparation was not used for the study of insecticides.

b. Denervation by Paton-Zar method. The ileum preparations denervated by the method developed by Paton and Zar (1968) did not respond to physostigmine (Fig. 39). This indicates that the ganglia were completely eliminated from the ileum. The ACh contraction was potentiated by physostigmine pretreatment as a result of inhibition of AChE.

The effects of carbofuran, propoxur and formetanate on the denervated preparations are illustrated in Fig. 39. None of them caused any contraction. The results are summarized in Table 10. Nicotine was also tested to insure the absence of ganglia. There was no effect of nicotine (Table 10).

Thus it can be concluded that carbofuran, propoxur and formetanate have no direct stimulating action on the muscarinic receptor in the ileum. The contractions induced by these insecticides in the intact ileum preparation are due to an increased availability of ACh released from the postganglionic nerve fibers as a result of inhibition of AChE.

B. Guinea Pig Heart

Drugs acting on autonomic nervous system

The guinea pig heart was chosen as material to study the effects of insecticides on β-adrenergic receptors. Before examining the insecticide action, a few drugs known to act on muscarinic and β-adrenergic receptors were tested to establish the

basic pattern of responses of the heart preparation.

a. Carbachol and atropine. Carbachol is an agonist acting on both nicotinic and muscarinic receptors. As expected from the muscarinic stimulating action, carbachol exerted a potent negative inotropic effect on the heart. An example of a record is illustrated in Fig. 40, and numerical data are given in Table 11. The amplitude of the contraction became smaller with increasing the dose, and the contraction was stopped after an 0.2 ml injection of 5×10^{-6} M carbachol (Fig. 40).

Atropine itself had no effect on the contraction even at a very high dose (1×10^{-4} M, 0.2 ml) (Fig. 41, Table 11). However, atropine effectively eliminated the negative inotropic action of carbachol (Fig. 41, Table 11). It should be noted that the effect of atropine lasted for a long period of time, and the muscarinic blocking action was quite evident even after a 60 min washing with atropine-free medium.

b. Isoproterenol and propranolol. Isoproterenol is an agonist acting on β-adrenergic receptors. Since isoproterenol is relatively unstable in aqueous solutions, its stability was first examined under various experimental conditions. The results are illustrated in Fig. 42. When isoproterenol was simply dissolved in Locke's solution, the potency of the test solution declined with time. Keeping the test solution in dark or adding $Na_2S_2O_5$ was not particularly effective in preventing degradation of isoproterenol. Ascorbic acid was satisfactory in this regard, and was added to isoproterenol solutions at a concentration of 1×10^{-4} M in all the subsequent experiments. The pH of the solution was adjusted to 4.0.

As expected from the β stimulating action, isoproterenol exerted a positive inotropic effect on the heart (Fig. 43, Table 12). In order to exclude the possibility that isoproterenol acts on the muscarinic receptor of the heart, the effect of atropine on the isoproterenol stimulation was examined. Atropine did not modify the positive inotropic action of isoproterenol (Fig. 43, Table 12).

Propranol is a β-adrenergic blocking agent. It had no effect on the

contraction when applied alone, but effectively suppressed the propranolol-induced positive inotropic effect (Fig. 44, Table 13).

Insecticides

Carbofuran, propoxur and formetanate had a potent stimulating action on the guinea pig ileum as described in Section A, and were chosen to study the possible action on the heart β-adrenergic receptor. Examples of records of the heart contraction in the presence of carbofuran, propoxur and formetanate are illustrated in Figs. 45, 46 and 47, respectively, and numerical data are given in Table 14. These experiments were performed according to the following protocol: First 0.2 ml of 1×10^{-4} M atropine solution was added to the perfusate to block the muscarinic receptor. One minute later, 0.2 ml of 154 mM NaCl solution with or without an insecticide was added to see the direct insecticide action on the β-adrenergic receptor. Three minutes later, 0.2 ml of 1×10^{-6} M isoproterenol solution was added to see the suppressive effect of the insecticide on the β-adrenergic receptor. None of the three insecticides tested had any direct stimulating action or suppressive action in the β-adrenergic receptor.

Thus it can be concluded that carbofuran, propoxur and formetanate have no effect on the β-adrenergic receptor of the guinea pig heart.

C. Guinea Pig Vas Deferens

Drugs acting on autonomic nervous system

The guinea pig vas deferens was chosen as a material to study the insecticide action on α-adrenergic receptor. Stimulation of this receptor causes contractions in the vas deferens. Norepinephrine was effective in causing contractions at a concentration of 2.66×10^{-5} M, and the effect was abolished by 1.33×10^{-6} M phentolamine, an α-blocker (Fig. 48, Table 15).

Insecticides

Carbofuran, propoxur and formetanate, insecticides capable of stimulating the guinea pig ileum at low concentrations, were examined for their effects on the α-adrenergic receptor of the guinea pig vas deferens. Figures 49, 50 and 51 represent examples of records in carbofuran, propoxur and formetanate, repectively, and numerical data are summarized in Table 16. The protocol of experiments is as follows: First 1.33×10^{-8} M atropine was applied for 2 minutes to block the muscarinic receptor. Second an insecticide solution was applied for 3 minutes to see direct stimulating effect. Then 2.66×10^{-5} M norepinephrine was introduced to examine the blocking action of the insecticide on the α receptor. None of the three insecticides had any stimulating or blocking effect on the α-adrenergic receptor of the guinea pig vas deferens.

D. Rat Diaphragm

Drugs acting on nicotinic receptors

The rat diaphragm was used to study the effect of insecticides on nicotinic receptors. As control experiments, the effects of physostigmine was first examined. At low concentrations (5×10^{-7} M, 1.5×10^{-6} M), physostigmine potentiated the contractile response evoked by nerve stimulation, whereas at a higher concentration (5×10^{-6} M) it suppressed the contraction (Figs. 52 and 53, Table 18). This dual action is interpreted as being due to an increased availability of the release ACh as a result of inhibition of AChE by physostigmine.

α-Tubocurarine suppressed the nerve evoked contraction of the diaphragm (Fig. 54, Table 17).

Insecticides

a. Carbofuran. Carbofuran, at concentrations of 5×10^{-9} M and 5×10^{-8} M, had no effect on the diaphragm contraction evoked by nerve stimulation (Table 18). At higher concentrations (5×10^{-7} M, 5×10^{-6} M), however, it potentiated the contraction (Table 18). Examples of records are illustrated in Fig. 55.

b. Propoxur. At concentrations ranging from 5×10^{-9} M to 5×10^{-7} M, propoxur had no effect on the diaphragm contraction. At a higher concentration of 5×10^{-6} M, the contraction was unaffected in most cases (Fig. 56), but potentiated in others to make the average value slightly higher than the control (Table 18).

c. Formetanate. Formetanate had no effect on the contraction at concentration ranging from 5×10^{-9} M to 5×10^{-7} M. It potentiated the contraction at 5×10^{-6} M (Fig. 57), but the effect was much less at a higher concentration of 5×10^{-5} M (Table 18).

In summary, all of the three insecticides tested potentiated the nerve evoked contractile response of the rat diaphragm at concentrations beyond a certain threshold level. This effect is presumably due to the inhibition of AChE which in turn would cause an accumulation of ACh in the end-plate region. If this is the case then prior inhibition of AChE by an anticholinesterase would abolish this effect. This was tested by using physostigmine as an anticholinesterase.

d. Effects on physostigmine-treated diaphragm. As is given in Table 19, the rat diaphragm pretreated with physostigmine at a concentration of 1×10^{-6} M did not respond to carbofuran, propoxur or formetanate in the form of potentiated contraction (Figs. 58, 59 and 60). Thus it can be concluded that the potentiating effect of the three insecticides tested on the rat diaphragm is due to the AChE inhibition which in turns causes an accumulation of ACh.

E. Frog Rectus Abdominis

Physostigmine

The frog rectus abdominis contains nicotinic receptors, and responds to nicotinic agonists to produce contracture. Therefore, this is another convenient preparation to examine the effects of insecticides on nicotinic receptors.

Examples of ACh contractions with and without physostigmine are illustrated in Fig. 61. The contraction caused by ACh was potentiated by physostigmine at concentrations ranging from 1×10^{-5} M to 1×10^{-4} M (Figs. 62 and 63). At a higher concentration of 3.99×10^{-4} M, physostigmine tended to inhibit the ACh contraction. Both the potentiation and inhibition by physostigmine can be interpreted as being due to the accumulation of ACh in the end-plate region.

Insecticides

When applied to the preparation pretreated with physostigmine to inhibit AChE, carbofuran, propoxur and formetanate did not evoke any contraction by themselves and did not modify the ACh contraction at any concentrations used ranging from 1.33×10^{-8} M to 1.33×10^{-5} M or 1.33×10^{-4} M (Figs. 64, 65 and 66, Table 20).

It can be concluded that none of the three insecticides has any direct effect on the nicotinic receptor of the frog rectus abdominis.

IV. SUMMARY AND CONCLUSIONS

1. A variety of skeletal muscle and smooth muscle tissues have been examined for their usefulness in evaluating the toxic effects of environmental agents, organophosphate and carbamate insecticides in particular. Methods have been established whereby the toxic side effects on various receptors caused by cholinesterase inhibition and by other mechanisms can be evaluated. Several insecticides were studied for their effects on various receptors using these methods.

2. The guinea pig ileum is a convenient preparation for the study of drug action on muscarinic receptors. Acetylcholine caused contractions at concentrations in the order of 10^{-7} M. Physostigmine potentiated the acetylcholine-induced contraction at low concentrations ($\sim 10^{-8}$ M), and induced contractions by

itself at higher concentrations (> 4 x 10^{-8} M). The potentiation is interpreted as being due to the increased availability of acetylcholine as a result of acetylcholinesterase inhibition. Neostigmine (10^{-7} M) also potentiated the acetylcholine contraction. Atropine (10^{-8} M) effectively antagonized the acetylcholine-induced contraction.

The α-adrenergic blocking agent phentolamine competitively antagonized the contraction by acetylcholine only at high concentrations (10^{-5} M). Thus the phentolamine suppression is not due to its effect on the α-adrenergic receptor but due to its inhibitory effect on the muscarinic receptor. The β-adrenergic blocking agent propranolol suppressed the acetylcholine contraction only at a very high concentration (10^{-5} M). This effect is presumably due to direct action on the muscarinic receptor.

3. Among the ten insecticides tested on the guinea pig ileum, the following seven had no direct effect and did not affect the acetylcholine- and carbachol-induced contraction at concentrations indicated: carbaryl (10^{-9} - 10^{-6} M), lepthophos (10^{-9} - 10^{-6} M), monocrotophos (10^{-8} - 10^{-6} M), dichlofenthion (10^{-8} - 10^{-6} M), dursban (10^{-8} - 10^{-6} M), ferbam (10^{-8} - 10^{-6} M), and chlordimeform at a higher concentration (10^{-6} M) slightly depressed the acetylcholine- and carbachol-induced contractions.

4. Three insecticides had a potent stimulating action on the guinea pig ileum. Carbofuran induced potent contractions at concentrations of 10^{-7} to 10^{-6} M. However, it did not exhibit any potentiating or depressing effect on the acetylcholine- or carbachol-induced contraction. Propoxur and formetanate induced contractions at concentrations of 10^{-8} - 10^{-6} M, but had no effect on the acetylcholine- or carbachol-induced contraction. The contraction induced by carbofuran or propoxur was abolished by pretreatment with atropine (10^{-8} M) but not by hexamethonium (10^{-4} M) or diphenhydramine (10^{-7} M). Thus the

contraction by either of these two insecticides is the result of stimulation of the muscarinic receptor. The formetanate-induced contraction was abolished by pretreatment with atropine (10^{-8} M), was slightly suppressed by diphenhydramine (10^{-7} M), but was not affected by hexamethonium (10^{-4} M). This formetanate stimulates the muscarinic receptor and slightly stimulates the histaminic receptor.

5. The chemicals effective on the guinea pig ileum may exert effects through inhibition of acetylcholinesterase or may directly act on the muscarinic receptors of the muscle. In an attempt to distinguish these two possibilities, use was made of low Ca^{++} - high Mg^{++} media which were known to inhibit transmitter release from nerve terminals. The onset of the contraction induced by physostigmine, carbofuran, propoxur and formetanate was delayed by decreasing Ca^{++} concentration from 1.80 mM to 0.036-0.36 mM and simultaneously increasing Mg^{++} concentration from 1.03 mM to 2.20-2.30 mM, but was not abolished. Thus the contraction induced by these drugs is at least in part due to an increased availability of acetylcholine released from nerve terminals as a result of acetylcholinesterase inhibition.

6. Black widow spider venom has been known to inhibit transmitter release from nerve terminals. The use of the venom in the guinea pig ileum still failed to distinguish the two possible mechanisms described in the preceding paragraph (5), since the venom suppressed the acetylcholine response itself and also since physostigmine produced contractions after venom treatment.

7. Denervation of the guinea pig ileum was successful in locating the site of action of the chemicals effective in producing contractions. Turning over the ileum inside out to brush off the ganglia did not give satisfactory results, because physostigmine was still effective. The method developed by Paton and Zar (J. Physiol. 194, 13 [1968]) was satisfactory, and physostigmine became ineffective.

Carbofuran, propoxur and formetanate also failed to stimulate the denervated ileum. Thus it can be concluded that these insecticides have no direct stimulating action on the muscarinic receptor in the ileum, and that the contractions induced by them in intact ileum preparations are due to an increased availability of acetylcholine released from the postganglionic nerve fibers as a result of inhibition of acetylcholinesterase.

8. The guinea pig heart is a suitable preparation to study drug action on β-adrenergic receptors. Carbachol had a potent negative inotropic effect through the stimulation of the muscarinic receptor, and the effect was antagonized by atropine. Isoproterenol had a positive inotropic effect through the stimulation of the β-adrenergic receptor, and the effect was antagonized by propranolol, a β-blocker. Carbofuran, propoxur and formetanate had no effect on the atropinized heart, and did not alter the positive inotropic effect of isoproterenol. Thus it can be concluded that these three insecticides have no effect on the β-adrenergic receptor of the guinea pig heart.

9. The guinea pig vas deferens was chosen as a material to study the drug action on α-adrenergic receptor. Norepinephrine (10^{-5} M) was effective in causing contractions through the stimulation of α-receptors, and the effect was antagonized by phentolamine (10^{-6} M), an α-blocker. Carbofuran, propoxur and formetanate (up to 10^{-5} M) did not cause contraction in the atropinized vas deferens, and did not alter the norepinephrine-induced contraction. It can be concluded that none of the three insecticides has any stimulating or blocking effect on the α-adrenergic receptor of the guinea pig vas deferens.

10. The rat diaphragm was used as a representative of nicotinic receptors in mammalian phasic muscles. Physostigmine potentiated the nerve evoked contraction at low concentrations ($\sim 10^{-6}$ M), but suppressed it at higher concentrations (5×10^{-6} d-Tubocurarine (10^{-6} M) suppressed the contraction. Carbofuran potentiated the nerve evoked contraction at high concentrations (5×10^{-7} M, 5×10^{-6} M). Propoxur and

formetanate also potentiated the contraction at a high concentration (5×10^{-6} M). However, none of the three insecticides had any effect on the contraction in the muscle pretreated with physostigmine (10^{-6} M). Thus it can be concluded that the potentiating effect of these insecticides on the rat diaphragm is due to the inhibition of acetylcholinesterase which in turn causes an accumulation of acetylcholine.

11. The frog rectur abdominis was chosen as a representative of nicotinic receptors in the tonic muscle of cold blooded animals. Physostigmine potentiated the acetylcholine-induced contraction at 10^{-5} - 10^{-4} M, but suppressed it at a higher concentration (4×10^{-4} M). Carbofuran, propoxur and formetante (up to 10^{-5} M) did not evoke any contraction and did not modify the acetylcholine contraction in the muscle pretreated with physostigmine (10^{-4} M). It can be concluded that none of the three insecticides has any effect on the nicotinic receptor of the frog rectus abdominis.

12. For the purpose of studying the side effects of various insecticides and other environmental agents on postsynaptic receptors, the guinea pig ileum (muscarinic receptor), the guinea pig heart (β-adrenergic receptor), the guinea pig vas deferens (α-adrenergic receptor), the rat diaphragm (nicotinic receptor in phasic muscle), and the frog rectus abdominis (nicotinic receptor in tonic muscle) have proved quite satisfactory. The potent stimulating action of carbofuran, propoxur and formetanate on the guinea pig ileum is due to acetylcholinesterase inhibition, and they have no direct effect on the muscarinic, nicotinic, α-adrenergic and β-adrenergic receptors.

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V. REFERENCES

Narahashi, T. (1976) In vitro screening methods evaluating the neurotoxic potential of pesticides. Environmental Health Effects Research Series, EPA-600/1-76-005, Environmental Protection Agency, Office of Research and Development.

Paton, W. D. M. and M. A. Zar. (1968) The origin of acetylcholine released from guinea pig intestine and longitudinal muscle strips. J. Physiol. (London) 194, 13-33.

TABLE 1

Compositions of Bathing Media (mM)

	A Tyrode I	B Tyrode II	C Krebs I	D Krebs II	E Tyrode-Locke	F Ringer
NaCl	136.9	136.9	113	94.1	154	111.2
KCl	2.68	2.68	4.7	4.69	5.63	1.88
$CaCl_2$	1.80	1.80	2.5	2.52	2.16	1.08
$MgSO_4$	1.03	1.03	1.2	0.45	------	------
$NaHCO_3$	11.9	11.9	25	25	1.99	2.38
NaH_2PO_4	0.36	0.36	-----	-----	------	0.07
KH_2PO_4	------	------	1.2	1.18	------	------
Glucose	5.55	11.1	11.5	11.1	5.55	11.1
Na pyruvate	------	-----	-----	2.45	------	------
Na fumarate	------	-----	-----	4.49	------	------
Na glutamate	------	-----	-----	2.66	------	------
pH	8.0-8.1	7.6	7.4	7.4	8.1	7.9

TABLE 2

Effects of 5-Minute Pretreatment with Physostigmine on Acetylcholine-Induced Contraction in Guinea Pig Ileum

Physostigmine (M)	Maximum Contraction by Acetylcholine	
	7.74×10^{-8} M[a]	1.55×10^{-7} M[b]
0	1	1
1.33×10^{-9}	0.97	1.00
3.99×10^{-9}	1.12	1.15
1.33×10^{-8}	1.33	1.23
3.99×10^{-8}	1.24	1.77

[a]Preparation 6-16-75.

[b]Preparation 5-30-75.

TABLE 3

Contraction Induced by 3.99×10^{-7} M Physostigmine in Guinea Pig Ileum

Preparation	Contraction after exposure to physostigmine (min)									
	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
1	0	0	±	+	+	++	++	++	++	++
2	0	0	0	0	±	+	+	++	++	++
3	0	0	±	+	+	++	++	++	++	++

[a]0, no contraction; ±, slight contraction; +, contraction; ++, strong contraction.

TABLE 4

Effects of Insecticides on Guinea Pig Ileum

Insecticides	Concentration 1.33×10^{-X} (X)	Insecticide-induced Contraction	Insecticide pretreatment on			
			Acetylcholine-induced contraction		Carbachol-induced contraction	
		3 min	1 min	3 min	1 min	3 min
Carbofuran	8	±,±,0	1.12 0.96	1.06	0.93 1.00	1.04
	7	+,+,+,±	1.35 1.03	0.89[C]	1.05 0.93	
	6	+,+,+	1.00[C]	1.03[C]	1.71[C]	1.07
Carbaryl	9	0	0.84 0.89		1.00	
	8	0,0,0	1.13 0.95	1.08	1.05 1.02[C]	1.09[C] 0.80[C]
	7	0,0,0	1.23 1.15 0.89	0.81 0.95	0.95 1.08[C]	1.04[C]
	6	0,0,±	1.30 0.81 0.82 0.85	1.35	0.46 0.96[C] 0.89	0.99 1.12
Leptophos	9	0	1.02			
	8	0,0,±	1.00 0.93	0.93[C]	0.85	0.73[C]
	7	0,0,±	1.10 1.00	0.92	0.83	0.85[C]
	6	0,0,±	1.02 1.13 1.02	0.93	0.89 0.93 0.95[C]	1.07[C]

(continued)

TABLE 4 (continued)

Insecticides	Concentration 1.33×10^{-X} (X)	Insecticide-induced Contraction	Insecticide Pretreatment on			
			Acetylcholine-induced contraction		Carbachol-induced contraction	
		3 min	1 min	3 min	1 min	3 min
Monocrotophos	8	0,±,0	1.07	0.93	1.03	1.02[C]
	7	0,0,0	0.98	0.68 0.96	0.86 1.04[C]	0.96[C]
	6	0,0,0,0	0.78 1.08 0.99	0.79 1.00	0.97[C] 1.01[C]	0.94[C] 1.08[C]
Dichlofenthion	8	0,0,0	0.95	0.92 0.90		1.38
	7	0,0,0	1.00	0.79		1.02[C]
	6	0,0,0,0,0	0.94	0.87[C] 0.87	0.97[C] 1.02[C] 0.97	1.24[C] 0.79[C] 1.00
Dursban	8	0,0	1.00	0.88	0.96[C]	0.88[C]
	7	0,0	1.04	0.85	0.94[C]	1.89[C]
	6	0,0,0,0	0.91 0.93	0.87	0.88[C] 0.94[C] 0.96[C] 0.74[C]	0.71[C] 0.81[C] 0.86[C]
Propoxur	9	0				
	8	±,0,±	0.98			
	7	±,±	0.95			
	6	+,0,+,+,±,±	0.93	0.98 1.03 1.11	0.94 1.07 0.91[C]	1.14[C] 0.97[C] 1.09[C]

(continued)

TABLE 4 (continued)

Insecticides	Concentration 1.33×10^{-X} (X)	Insecticide-induced Contraction	Insecticide Pretreatment on			
			Acetylcholine-induced contraction		Carbachol-induced contraction	
		3 min	1 min	3 min	1 min	3 min
Ferbam[d]	8	0,0,0		0.82		0.69[c] 0.98
	7	0,0,0		0.95		0.84[c] 0.86[c]
	6	0,0,0		1.05[c]		0.89[c] 0.90[c]
Formetanate	8	±,±		0.96		0.90
	7	±,±,±		1.06		1.34 1.03
	6	+,+,+,+		0.96		1.19
Chlordimeform	8	0,0				
	7	0,0,0,0,0		1.12 1.00		0.92[c] 0.91 0.94[c]
	6	0,0,0,0,0		0.80 0.82		0.79[c] 0.60 0.86[c]

[a]Insecticide-induced contraction was measured 3 min after treatment. Each observation was made with different preparation. 0, no effect; ±, slight contraction; +, contraction.

[b]Effects of 1-min and 3-min pretreatment with insecticides on the contractions induced by acetylcholine (1.33×10^{-7} M or 7.65×10^{-8} M) and by carbachol

(continued)

TABLE 4 (continued)

(1.33×10^{-7} M). The amplitude of the maximum contraction is given in a value relative to the control before treatment with insecticides. Each measurement was made with different preparation

[c]Contraction slowly increased with time.

[d]Dimethylsulfoxide was used as solvent instead of ethanol.

TABLE 5

Effects of 4-Minute Pretreatment with Atropine, Hexamethonium and Diphenhydramine on Insecticide-Induced Contraction of Guinea Pig Ileum[a]

Pretreatment	Insecticide-induced contraction[b]		
	Carbofuran 1.33×10^{-6} M	Propoxur 3.99×10^{-6} M	Formetanate 1.33×10^{-6} M
None	+	±	+
	+	±	+
	+	+	++
	++	+	++
	++		
Atropine 1.33×10^{-8} M	0	0	0
	0	0	0
	0	0	±
Hexamethonium 1.33×10^{-4} M	++	±	+
	++	+	+
	++	+	+
Diphenhydramine 1.33×10^{-7} M	+	±	±
	+	+	±
	+	+	±
			+

[a]Each measurement represents response of individual ileum preparation.

[b]0, no contraction; ±, slight contraction; +, contraction; ++, strong contraction.

TABLE 6

Effects of Mg^{++} and Ca^{++} Concentrations on Contraction Induced by 6.67×10^{-8} M Acetylcholine in Guinea Pig Ileum

Mg^{++} (mM)	Ca^{++} (mM)	Relative contraction[a]	Mean
1.70	1.80	1.00 0.70 0.90 1.00 0.96 1.29 1.07	0.99
1.80	1.80	0.80	0.80
1.90	1.80	0.94	0.94
2.00	1.80	0.84	0.84
2.03	1.80	0.90 0.56	0.73
2.10	1.80	0.92	0.92
2.20	1.80	0.88 1.35 1.05 1.00	1.07
2.30	1.80	0.73	0.73
2.36	1.80	0.73 0.68	0.71
2.40	1.80	0.61	0.61

(continued)

TABLE 6 (continued)

Mg^{++} (mM)	Ca^{++} (mM)	Relative contraction[a]	Mean
1.70	0.36	0.71	0.71
1.70	0.18	0.67 0.63	0.65
2.20	0.36	0.39 0.35 1.00	0.58
2.20	0.18	0.48	0.48

[a]Amplitude of maximum contraction in modified Tyrode's solution relative to that in normal Tyrode's solution containing 1.03 mM Mg^{++} and 1.80 mM Ca^{++}.

TABLE 7

Effects of Mg^{++} and Ca^{++} Concentrations on Physostigmine-Induced Contraction in Guinea Pig Ileum[a]

Mg^{++} (mM)	Ca^{++} (mM)	Latent time (Min., Sec.)	Mean
1.03	1.80	2'40" 2'30"	2'35"
2.20	1.80	3'10" 2'40"	2'55"
2.20	0.36	2'50" 5'30" 3'30"	4'00"
2.20	0.18	5'30" 3'00"	4'15"
2.20	0.09	3'00"	3'00"
2.20	0.036	4'20" 3'40"	4'00"

[a]Data are given in latent time for the contraction induced by 4.00×10^{-7} M physostigmine to attain the same amplitude as the maximum contraction induced by 6.67×10^{-8} M acetylcholine.

TABLE 8

Effects of Mg^{++} and Ca^{++} Concentrations on Insecticide-Induced Contraction in Guinea Pig Ileum[a]

Insecticide (Concentration)	Mg^{++} (mM)	Ca^{++} (mM)	In insecticide (min)					Washing[b] (min)	
			1	2	3	4	5		
Carbofuran	1.03	1.80	±	+	++	++	++	++	(5)
(1.33×10^{-6} M)			0	±	±	+	+	+	(5)
	2.20	0.36	0	±	+	+		++	(5)
			0	0	±	±	+	++	(5)
			0	0	0			++	(3)
			0	0	±			++	(3)
Propoxur	1.03	1.80	0	0	±	+	+	+	(5)
(3.99×10^{-6} M)	2.20	0.36	0	0	0	0	±	+	(5)
			0	0	0	±	±	+	(5)
			0	0	0	0	±	++	(5)
Formetanate	1.03	1.80	0	0	+	+	++	++	(5)
(1.33×10^{-6} M)			0	0	±	+	+	+	(5)
	2.20	0.36	0	0	±	±	±	++	(5)
			0	0	0	0	±	+	(5)
			0	0	0	±	±	++	(5)
			0	0	0	±	+	++	(5)

[a] 0, no contraction; ±, slight contraction; +, contraction; ++, strong contraction.

[b] Washing with insecticide-free normal Tyrode's solution containing 1.03 mM Mg^{++} 1.80 mM Ca^{++}.

TABLE 9

Effects of Drugs and Insecticides on the Guinea Pig Ileum Denervated by Inside-Out Method

Drug	Concentration (M)	Contraction[a]		
Acetylcholine	6.67×10^{-8}	+	+	+
Nicotine	6.67×10^{-6}	0	0	±
Physostigmine	3.99×10^{-7}	±		
Carbofuran	1.33×10^{-6}	±	0	+
Propoxur	1.33×10^{-6}	0		
Formetanate	1.33×10^{-6}	+	+	±

[a]Individual symbols represent contractile responses of individual preparations. 0, no contraction; ±, slight contraction; +, contraction.

TABLE 10

Effects of Drugs and Insecticides on the Guinea Pig Ileum Denervated by Paton-Zar Method

Drug	Concentration (M)	Contraction[a]		
Acetylcholine	6.67×10^{-8}	+	+	+
Nicotine	6.67×10^{-6}	0	0	0
Physostigmine	3.99×10^{-7}	0	0	0
Carbofuran	1.33×10^{-5}	0	0	0
Propoxur	1.33×10^{-5}	0	0	0
Formetanate	1.33×10^{-4}	0	0	0

[a]Individual symbols represent contractile responses of individual preparations. 0, no contraction; +, contraction.

TABLE 11

Effects of Carbachol and Atropine on the Contraction of Guinea Pig Heart[a]

Drug	Concentration[b] (M)	Relative[c] contraction
Carbachol	1×10^{-6}	0.43
		0.68
		0.59
		0.56
	2×10^{-6}	0.17
		0.20
		0.16
	5×10^{-6}	0
Atropine	1×10^{-7}	0.94
	1×10^{-6}	0.97
	1×10^{-5}	0.97
	1×10^{-4}	1.05
Atropine	1×10^{-4}	
+	+	0.98
Carbachol	2×10^{-6}	

[a]Preparation 9-22-75.

[b]0.2 ml test solution added to the perfusate.

[c]Amplitude of contraction relative to the control before application of drugs.

TABLE 12

Effects of Isoproterenol and Atropine on the Contraction of Guinea Pig Heart

Preparation	Drug	Concentration[a] (M)	Relative[b] Contraction	After atropine / Before atropine
9-23-75	Isoproterenol	1×10^{-5}	4.40	
	Atropine	1×10^{-4}	1.29	
	Isoproterenol	1×10^{-5}	2.88	0.66
9-24-75	Isoproterenol	1×10^{-5}	8.00	
	Atropine	1×10^{-4}	0.80	
	Isoproterenol	1×10^{-5}	9.25	1.16
9-25-75A	Isoproterenol	1×10^{-6}	1.56	
	Atropine	1×10^{-4}	0.90	
	Isoproterenol	1×10^{-6}	1.44	0.92

[a]0.2 ml test solution added to the perfusate.

[b]Amplitude of contraction relative to the control before application of drugs.

TABLE 13

Effects of Isoproterenol and Propranolol on the Contraction of Guinea Pig Heart

Preparation	Drug	Concentration[a] (M)	Relative[b] Contraction	After propranolol
				Before propranolol
9-23-75	Isoproterenol	1×10^{-5}	4.25	
	Propranolol	1×10^{-4}	1.00	
	Isoproterenol	1×10^{-5}	0.88	0.21
9-24-75	Isoproterenol	1×10^{-6}	5.50	
	Propranolol	1×10^{-5}	0.89	
	Isoproterenol	1×10^{-6}	1.13	0.21

[a]0.2 ml test solution added to the perfusate.

[b]Amplitude of contraction relative to the control before application of drugs.

TABLE 14

Effects of Insecticides on the Contraction of Guinea Pig Heart[a]

Insecticide	Concentration[b] (M)	Direct[c] Effect	In the presence[d] of isoproterenol	N
Carbofuran	6	0	0.96 ± 0.02	3
	5	0	1.12 ± 0.17	4
	4	0	0.94 ± 0.02	7
Propoxur	6	0	0.99 ± 0.04	3
	5	0	1.02 ± 0.04	3
	4	0	1.00 ± 0.04	4
Formetanate	6	0	0.94 ± 0.03	3
	5	0	0.96 ± 0.03	3
	4	0	0.97 ± 0.04	4
	3	0	0.98 ± 0.06	3
	2	0	0.87 ± 0.07	5

[a]Protocol: 0.2 ml of 1×10^{-4} M atropine; 1 min later, 0.2 ml of 154 mM NaCl without (control) or with (test) insecticide; 3 min later, 0.2 ml of 1×10^{-6} M isoproterenol.

[b]Concentration of test solution added to the perfusate.

[c]0, no effect.

[d]Ratio of the contractions $\left(\frac{\text{isoproterenol + insecticide}}{\text{insecticide}}\right)/\left(\frac{\text{isoproterenol + control solution}}{\text{control solution}}\right)$ in the mean ± S.E.M.

TABLE 17

Effects of d-Tubocurarine on the Rat Diaphragm Contraction Evoked by Nerve Stimulation

Perfusate	Concentration (M)	Relative contraction	
		Without physostigmine	With physostigmine 4×10^{-7}M
Solution E	0	1	
	5×10^{-8}	0.93	
	5×10^{-7}	0.95	
	2.5×10^{-6}	0.35	0.71
		0.20	0.68
Solution F	0	1	
	3.33×10^{-9}	1.00	
	3.33×10^{-8}	0.83	
	3.33×10^{-7}	0.76	
	1.67×10^{-6}	0	
	3.33×10^{-6}	0	

TABLE 18

Effects of Physostigmine and Insecticides on the Rat Diaphragm Contraction Evoked by Nerve Stimulation

Insecticide	Concentration (5×10^{-X} M)	Relative contraction	N
Physostigmine	7	1.12 ± 0.04	3
	6	3.01 ± 0.48	
Carbofuran	9	1.15 ± 0.05	4
	8	0.96 ± 0.04	4
	7	2.25 ± 0.78	4
	6	2.27 ± 1.33	3
Propoxur	9	0.84 ± 0.12	5
	8	0.96 ± 0.06	4
	7	0.95 ± 0.06	5
	6	1.46 ± 0.41	8
Formetanate	9	0.89 ± 0.07	6
	8	0.94 ± 0.03	4
	7	0.92 ± 0.02	7
	6	3.32 ± 0.52	10
	5	1.19 ± 0.28	5

TABLE 19

Effects of Insecticides on the Nerve Evoked Contraction of Rat Diaphragm Pretreated with 1×10^{-6} M Physostigmine for 10 Minutes

Insecticide	Final concentration (M)	Relative contraction	Mean ± S.E.M.
Carbofuran	5×10^{-9}	1.04 1.02 1.00 1.03	1.02 ± 0.01
	5.5×10^{-8}	1.04 1.02 1.06 1.08	1.05 ± 0.01
	5.55×10^{-7}	1.03 0.97 1.00 0.98	1.00 ± 0.01
	5.56×10^{-6}	0.97 0.97 0.94 0.93	0.95 ± 0.01
Propoxur	5×10^{-9}	1.17 1.03	1.10
	1×10^{-8}	1.04 1.12	1.08
	6×10^{-8}	1.02 1.02	1.02
	1.1×10^{-7}	1.04 1.00	1.02
	5×10^{-7}	1.00	
	6.1×10^{-7}	1.02 1.00	1.01

(continued)

TABLE 19 (continued)

Insecticide	Final concentration (M)	Relative contraction	Mean ± S.E.M.
Propoxur	1×10^{-6}	1.00	
	1.11×10^{-6}	1.07	
	5.61×10^{-6}	0.98	
	6×10^{-6}	0.94	
	6.11×10^{-6}	1.08	
	1.1×10^{-5}	0.89	
Formetanate	5×10^{-9}	1.00 1.00 1.09 1.09	1.05 ± 0.03
	5×10^{-8}	1.00 1.00	1.00
	5.5×10^{-8}	1.09 1.09	1.09
	5×10^{-7}	1.00 1.06	1.03
	5.55×10^{-7}	1.05 1.00	1.03
	5×10^{-6}	1.00 1.00	1.00
	5.56×10^{-6}	1.02 0.92	0.97
	5×10^{-5}	0.77 0.70	0.74
	5.56×10^{-5}	1.05 0.94	1.00

TABLE 20

Effects of Insecticides on the Contraction of Frog Rectus Abdominis Pretreated with Physostigmine

Experiment[b]	Insecticide	Concentration (1.33×10^{-X} M)	Direct[c] Effect	In the presence[d] of acetylcholine	N
I	Carbofuran	8	0	0.96 ± 0.06	4
		7	0	1.01 ± 0.01	4
		6	0	1.03 ± 0.02	4
		5	0	1.07	2
II	Carbofuran	8	0	0.89 ± 0.04	6
		7	0	1.00 ± 0.05	4
		6	0	0.83 ± 0.06	8
		5	0	1.07 ± 0.04	3
	Propoxur	8	0	0.88 ± 0.05	8
		7	0	0.90 ± 0.06	8
		6	0	0.91 ± 0.02	8
		5	0	1.00 ± 0.02	9
	Formetanate	8	0	0.97 ± 0.02	4
		7	0	0.93 ± 0.04	4
		6	0	0.96 ± 0.07	8
		5	0	1.02 ± 0.06	7
		4	0	0.89 ± 0.08	7

(continued)

TABLE 20 (continued)

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[a]Protocol: Physostigmine for 5 min; insecticide for 3 min; acetylcholine 3-4 min.

[b] I, 3.99×10^{-6} M acetylcholine and 8.07×10^{-5} M physostigmine.

II, 1×10^{-6} M acetylcholine and 1.33×10^{-4} M physostigmine.

[c]0, no effect.

[d]Ratio of the contraction in acetylcholine + insecticide to that in acetylcholine in the mean + S.E.M.

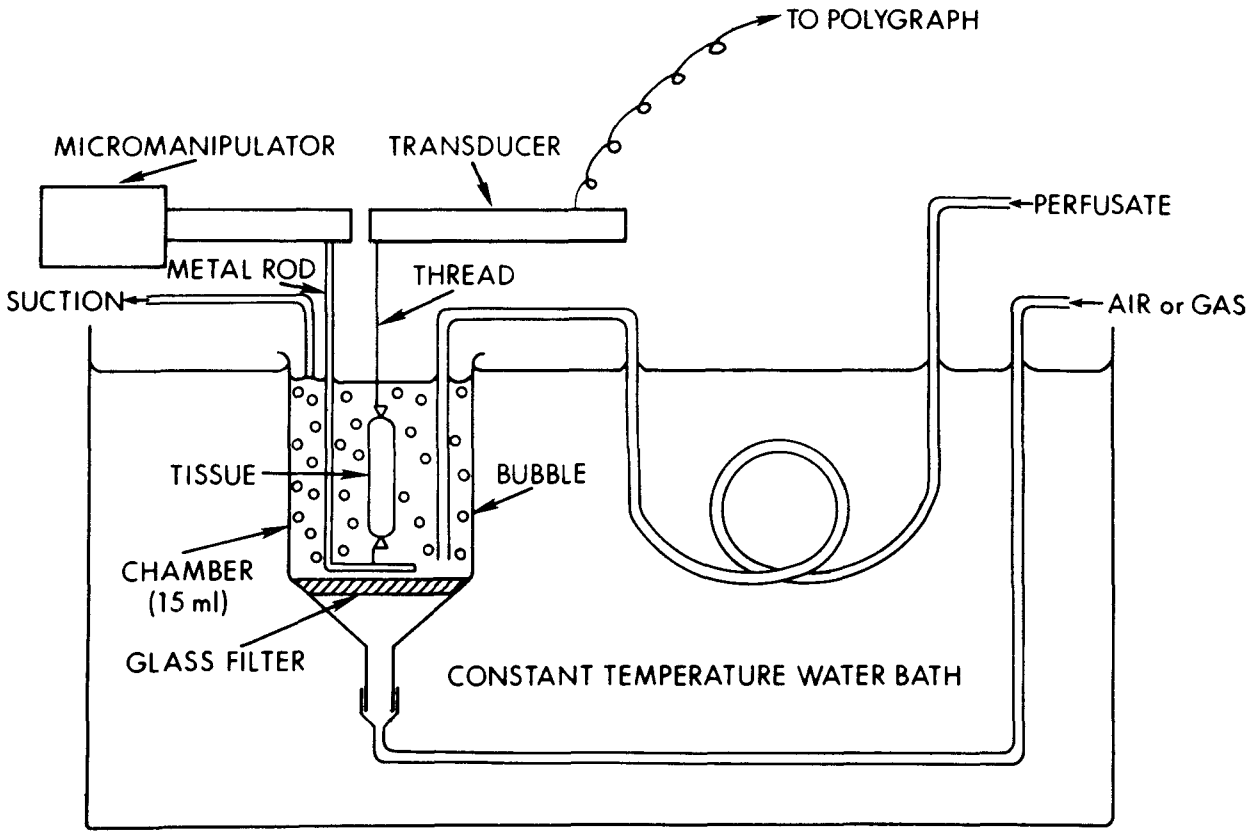

Figure 1. Diagram of experimental set-up for guinea pig ileum.

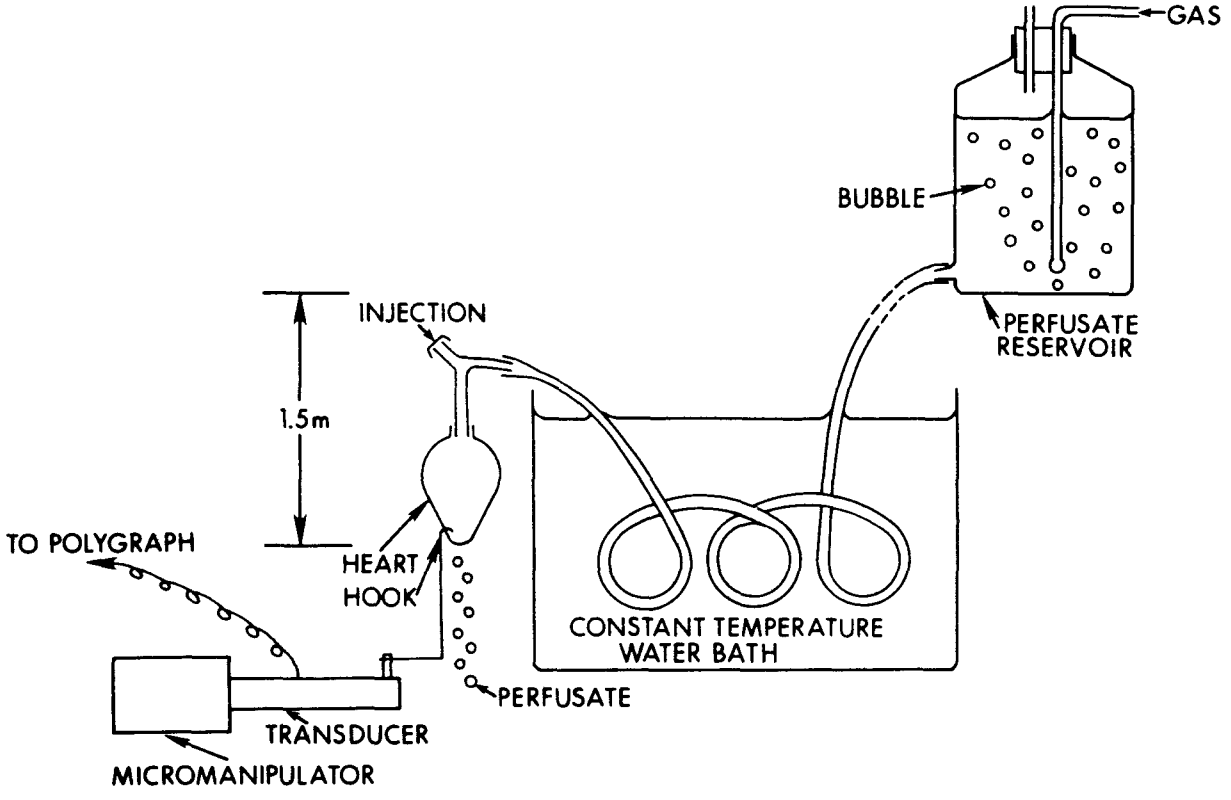

Figure 2. Diagram of experimental set-up for guinea pig heart.

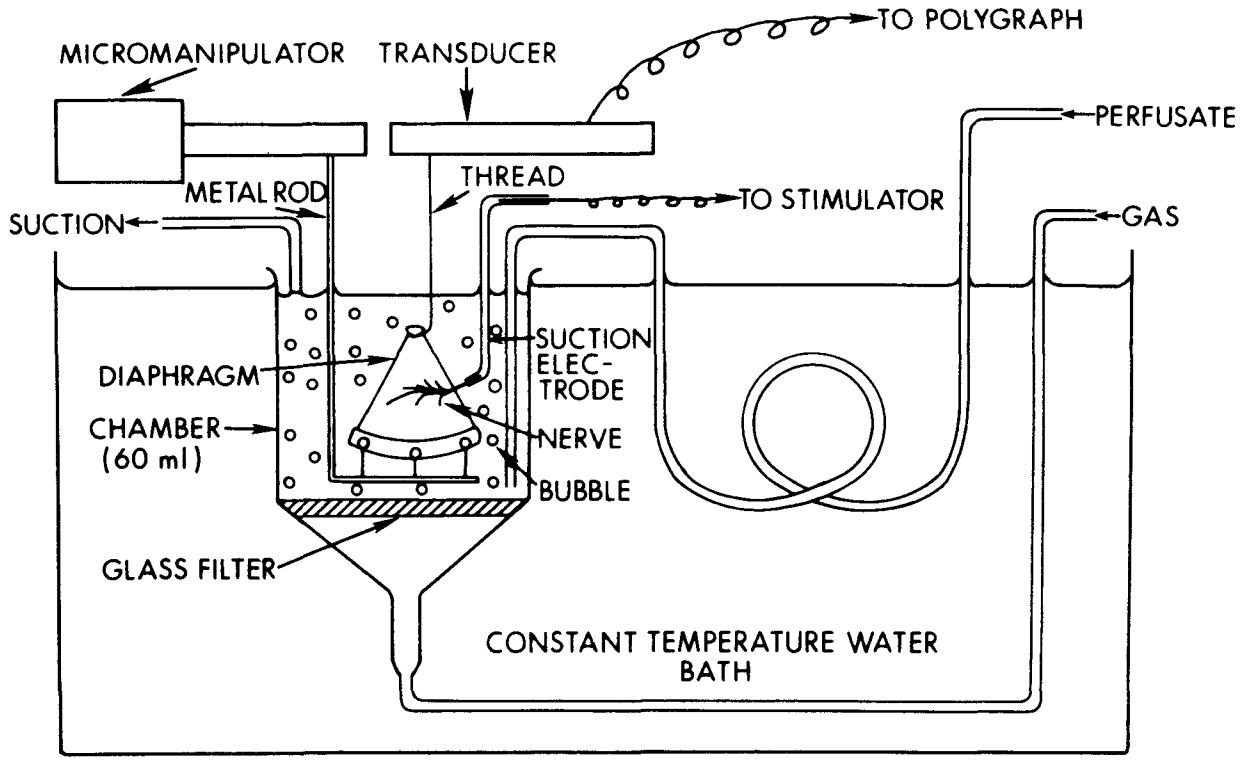

Figure 3. Diagram of experimental set-up for rat diaphragm.

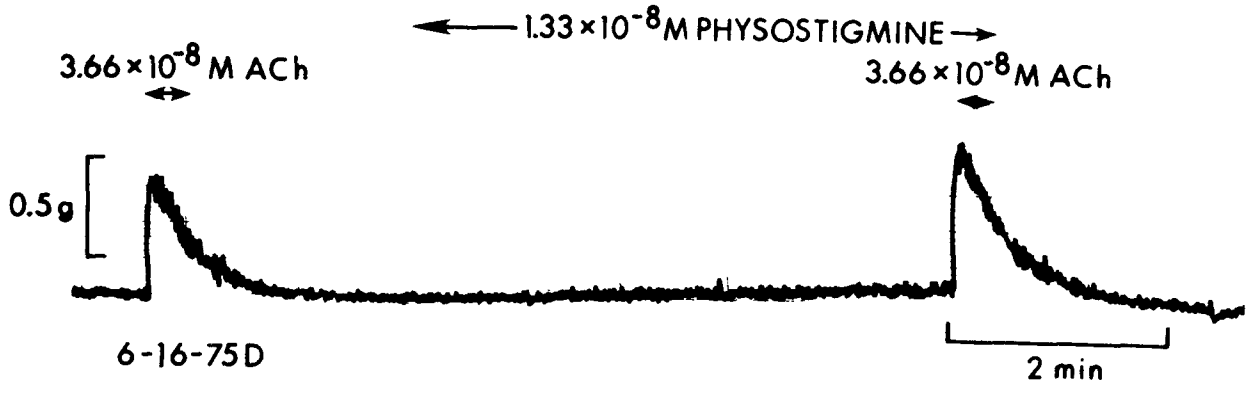

Figure 4. Contraction of guinea pig ileum induced by acetylcholine (ACh) (3.66×10^{-8} M) and potentiation by a low concentration of physostigmine 1.33×10^{-8} M).

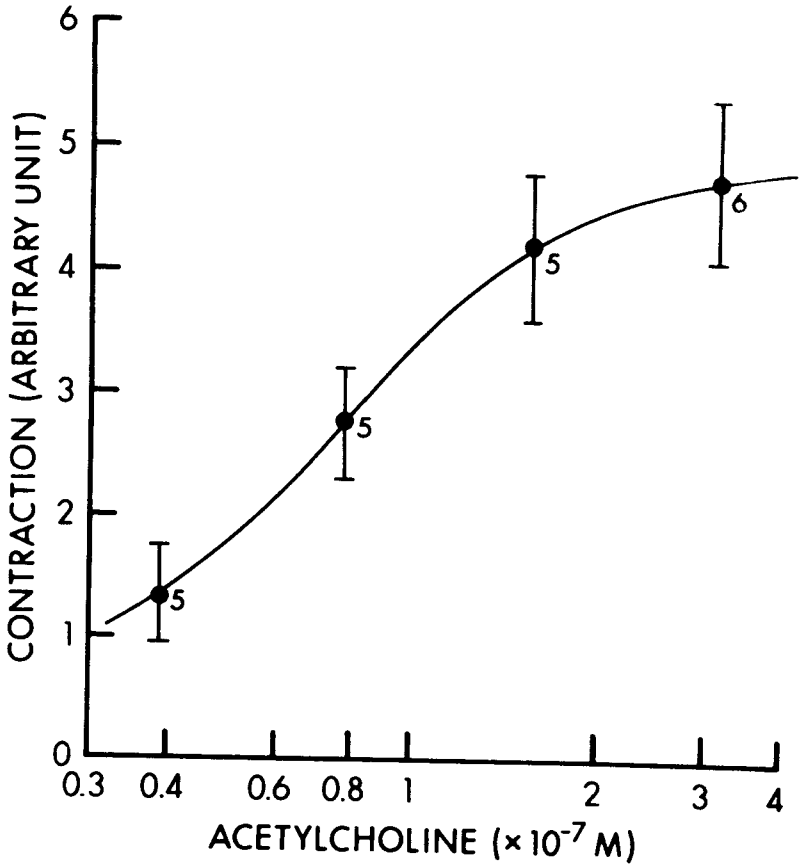

Figure 5. Dose-response relation for contraction of guinea pig ileum by acetylcholine. Data are given in the mean ± S.E.M. with the number of experiments beside the symbol.

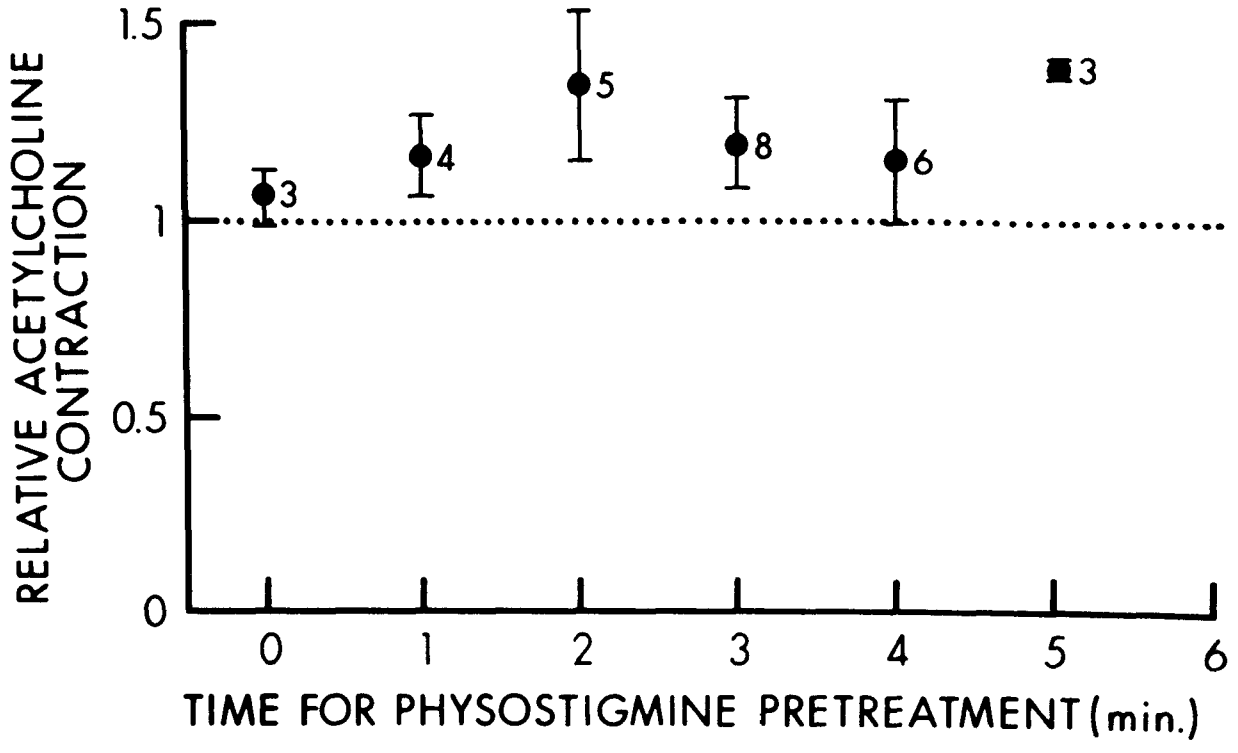

Figure 6. Potentiation of acetylcholine (1.55×10^{-8} - 1.55×10^{-7} M) contraction of guinea pig ileum by pretreatment with physostigmine (1.33×10^{-8} M) for various periods of time. Data are given in the mean $\pm$ S.E.M. with the number of experiments beside the symbol. The ordinate represents the amplitude of acetylcholine contraction relative to that without physostigmine.

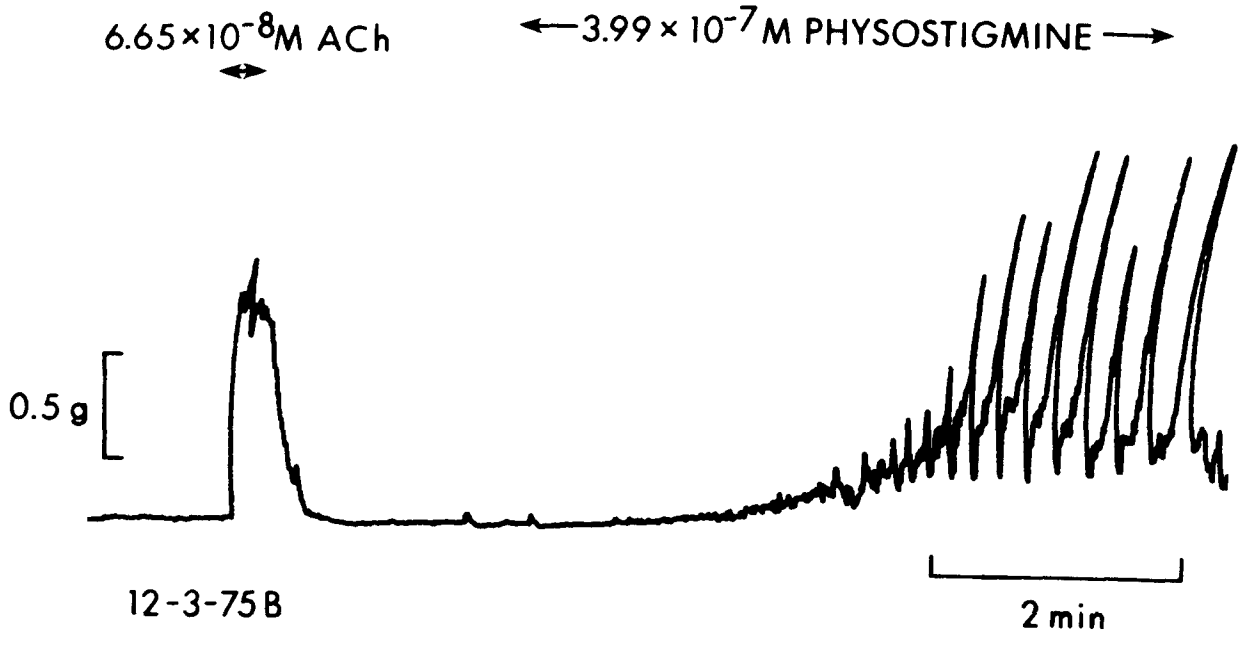

Figure 7. Contraction of guinea pig ileum induced by 6.65 x 10^{-8} M acetylcholine (ACh) and repetitive contractions induced by a high concentration of physostigmine (3.99 x 10^{-7} M).

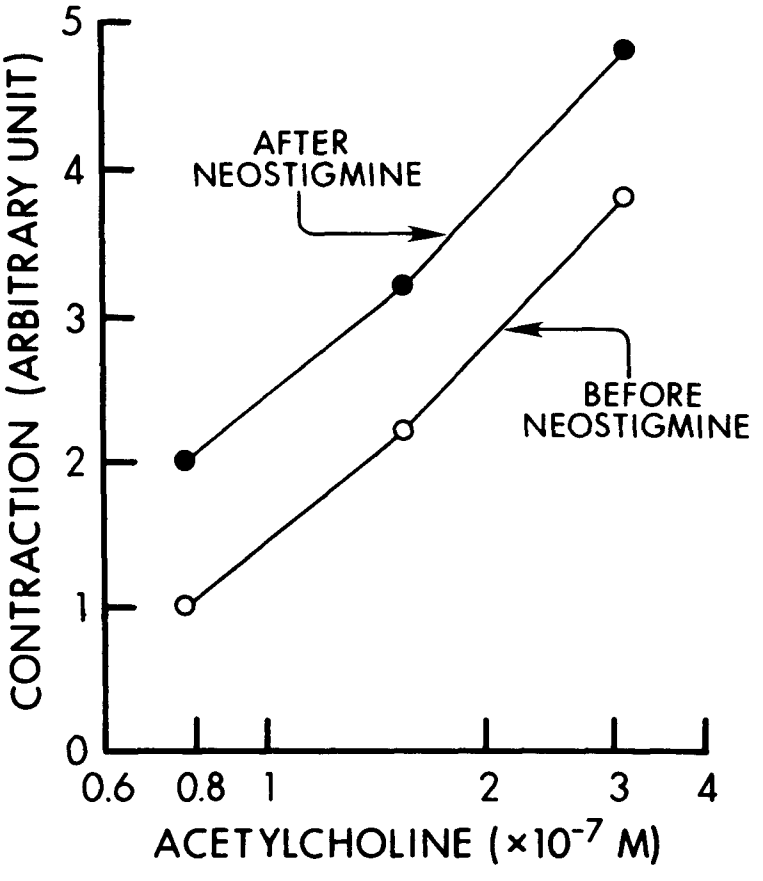

Figure 8. Dose-response relations for contraction of guinea pig ileum by acetylcholine before and after treatment with neostigmine (1.14×10^{-7} M) for 6 min.

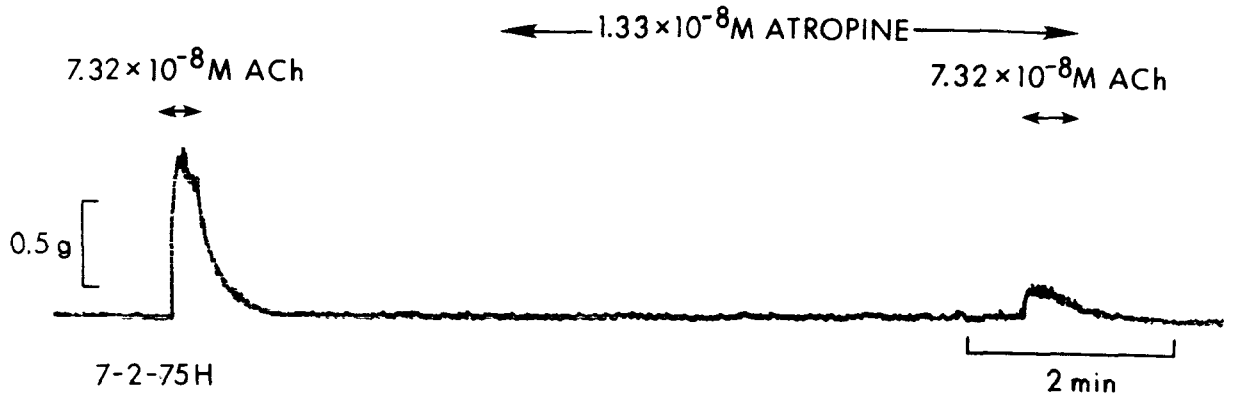

Figure 9. Contraction of guinea pig ileum by 7.32 x 10^{-8} M acetylcholine (ACh) and suppression by 1.33 x 10^{-8} M atropine.

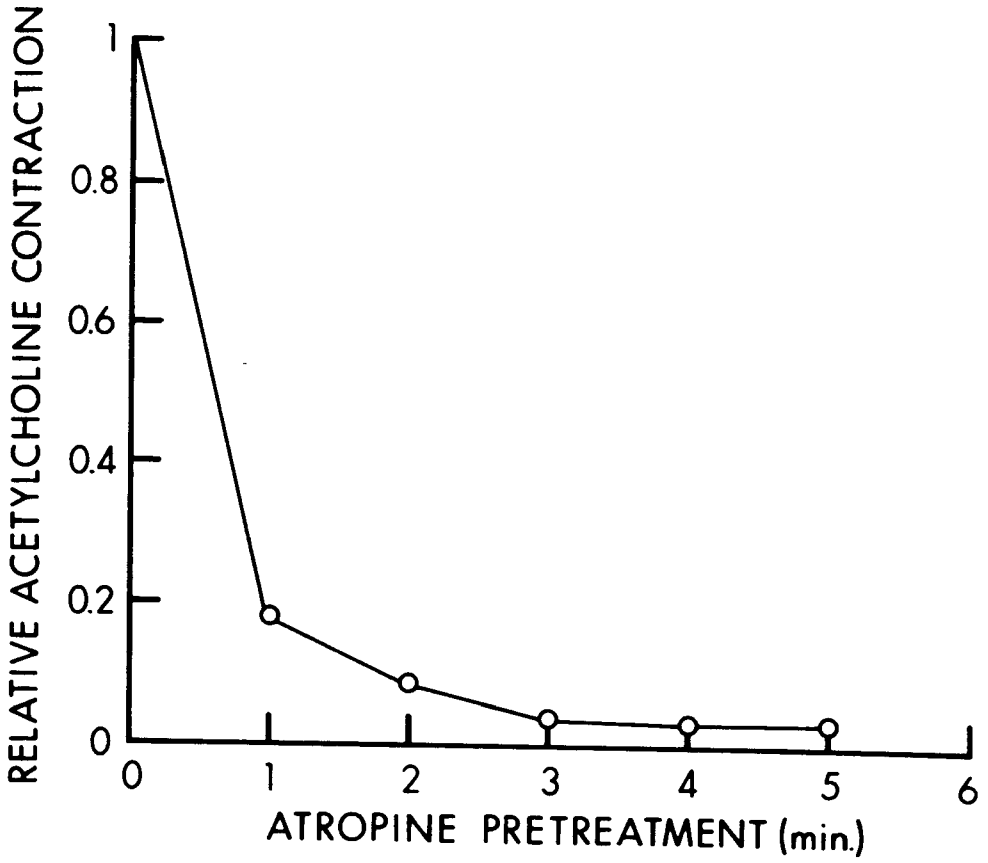

Figure 10. Suppression of acetylcholine (7.74×10^{-7} M) contraction of guinea pig ileum by pretreatment with 1.33×10^{-8} M atropine for various periods of time.

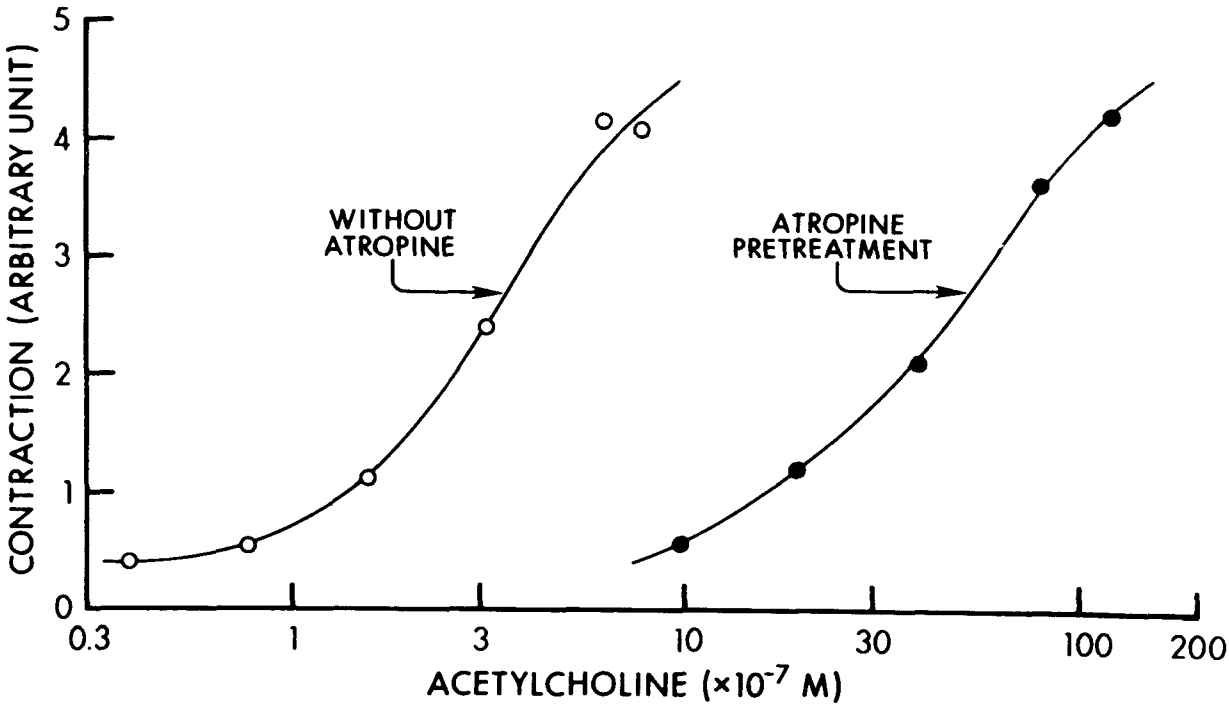

Figure 11. Dose-response relations for contraction of guinea pig ileum by acetylcholine with and without pretreatment with 1.33×10^{-8} M atropine for 4 min.

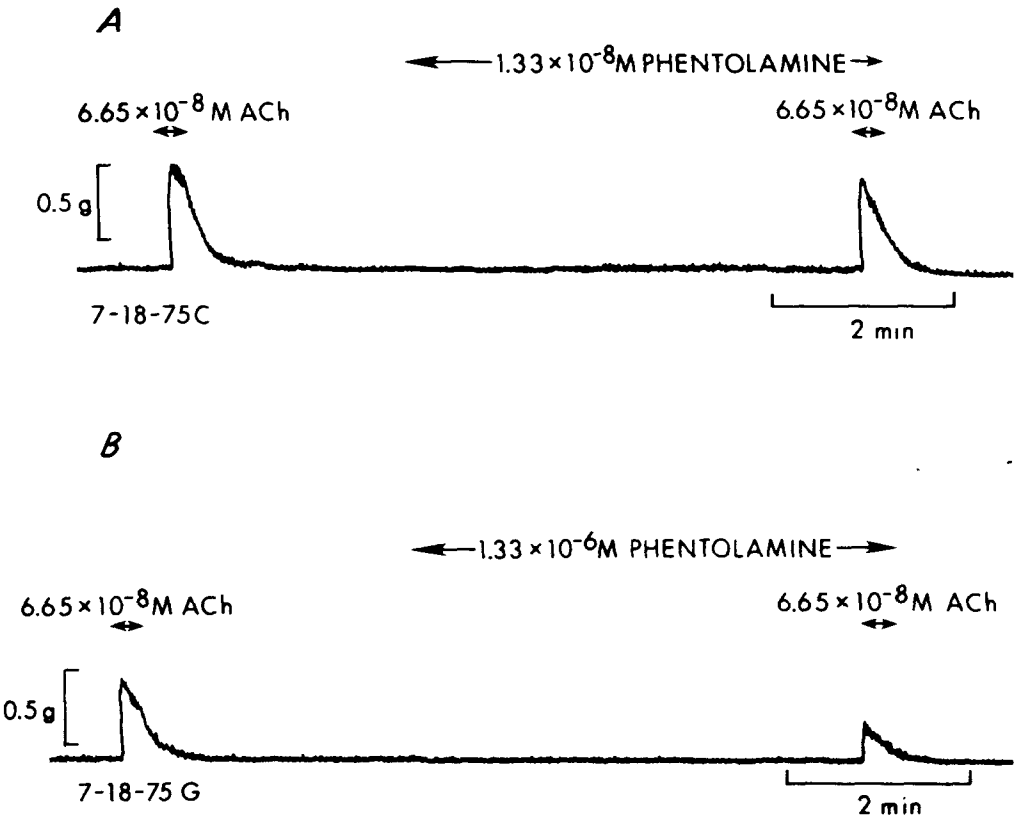

Figure 12. Ineffectiveness of a low concentration of phentolamine (1.33×10^{-8} M) in suppressing acetylcholine (ACh)-induced contraction in guinea pig ileum (A), and suppression by a high concentration of phentolamine (1.33×10^{-6} M) (B).

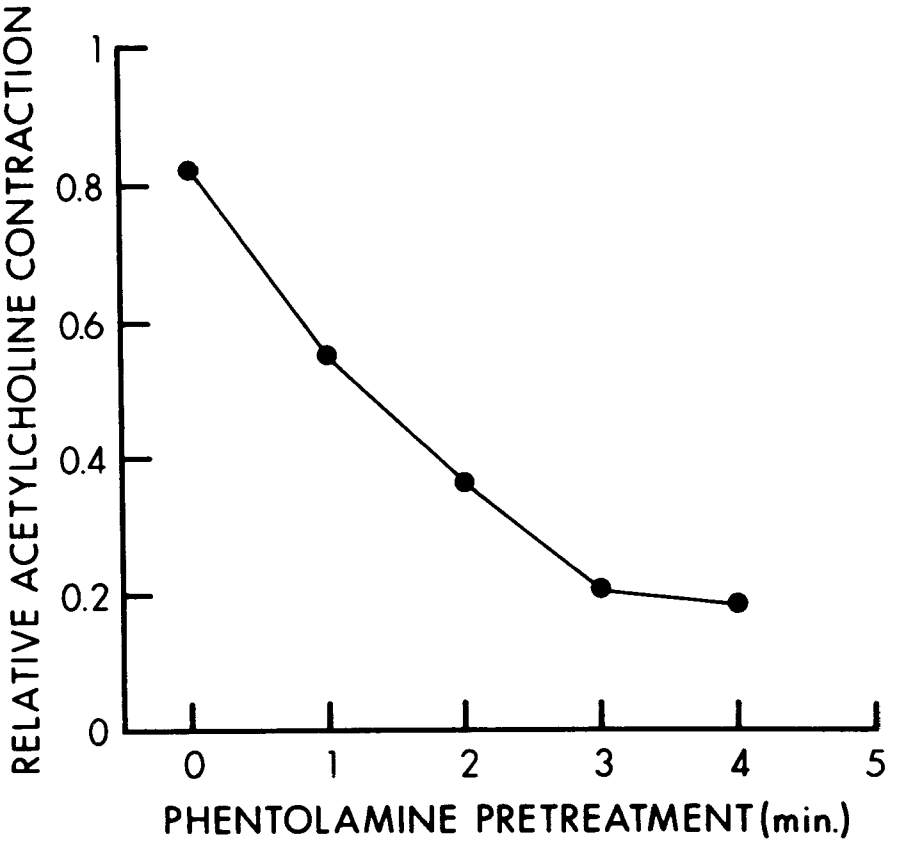

Figure 13. Suppression of acetylcholine (7.74×10^{-8} M) contraction of guinea pig ileum by pretreatment with 1.33×10^{-5} M phentolamine for various periods of time.

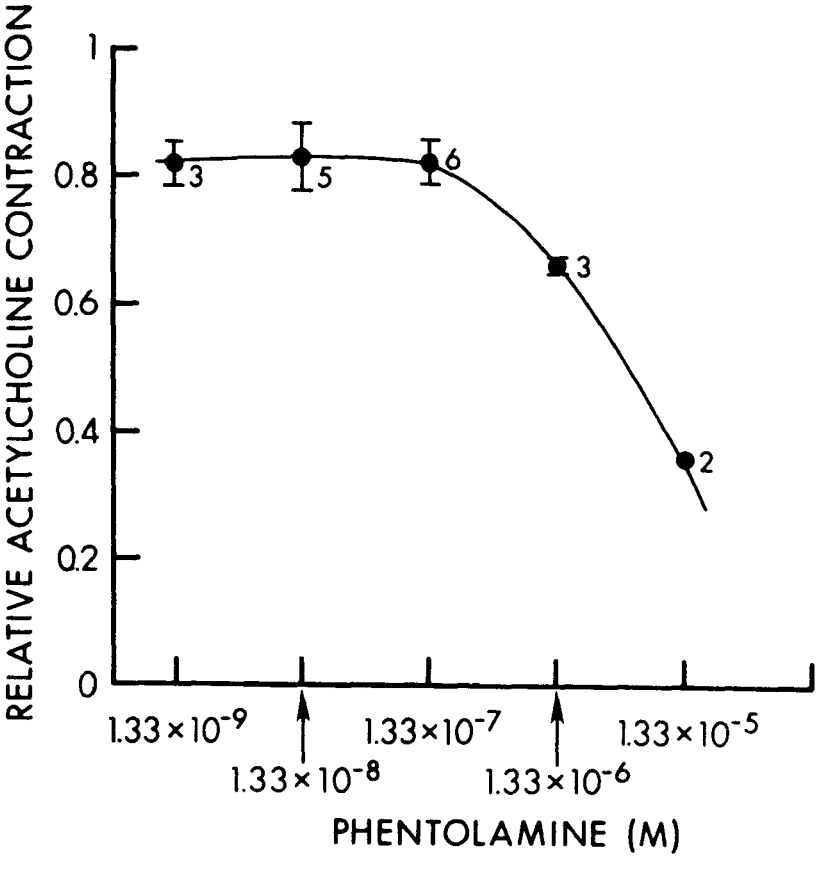

Figure 14. Dose-response relation for phentolamine suppression of acetylcholine (6.65 and 7.74 x 10^{-8} M) contraction in guinea pig ileum. Tissue is treated by phentolamine for 5 min prior to acetylcholine application. Data are given in the mean ± S.E.M. with the number of experiments beside the symbol.

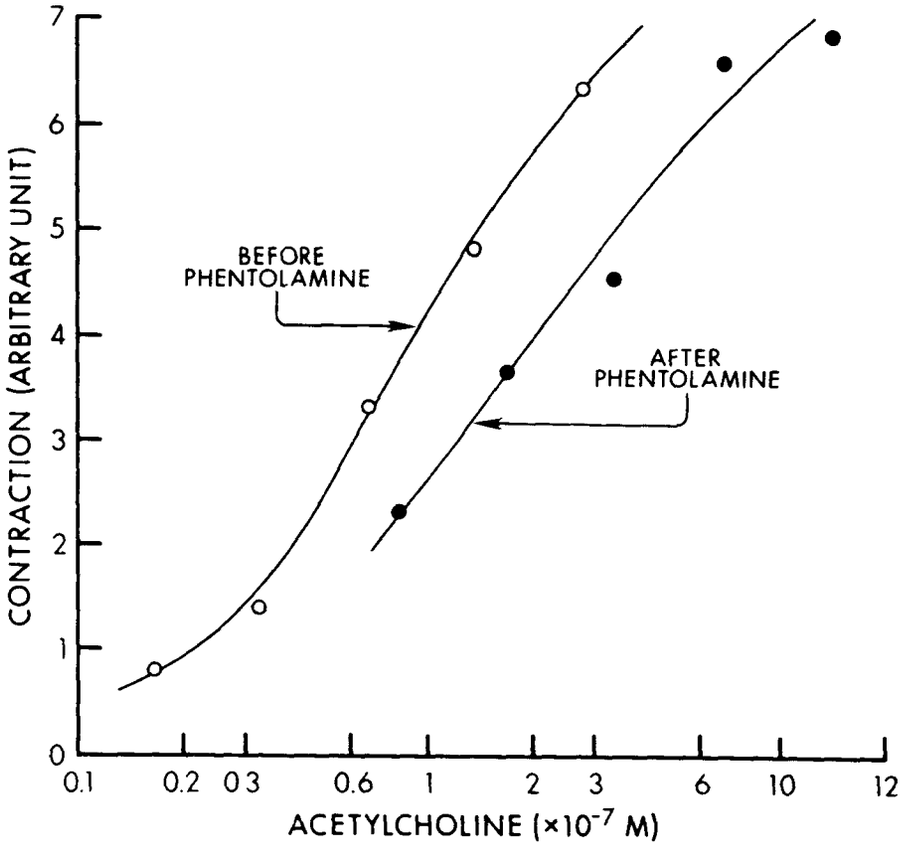

Figure 15. Dose-response relation for contraction of guinea pig ileum by acetylcholine before and after treatment with 1.33×10^{-5} M phentolamine for 4 min.

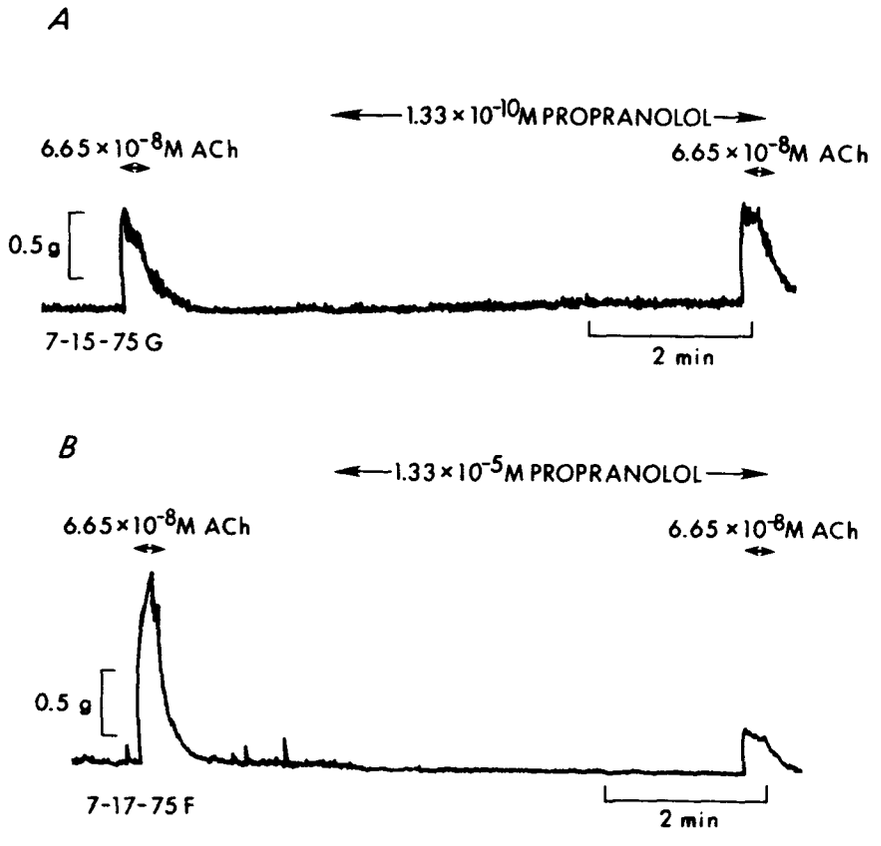

Figure 16. Ineffectiveness of a low concentration of propranolol (1.33×10^{-10} M) in suppressing acetylcholine (ACh)-induced contraction in guinea pig ileum (A), and suppression by a high concentration of propranolol (1.33×10^{-5} M) (B).

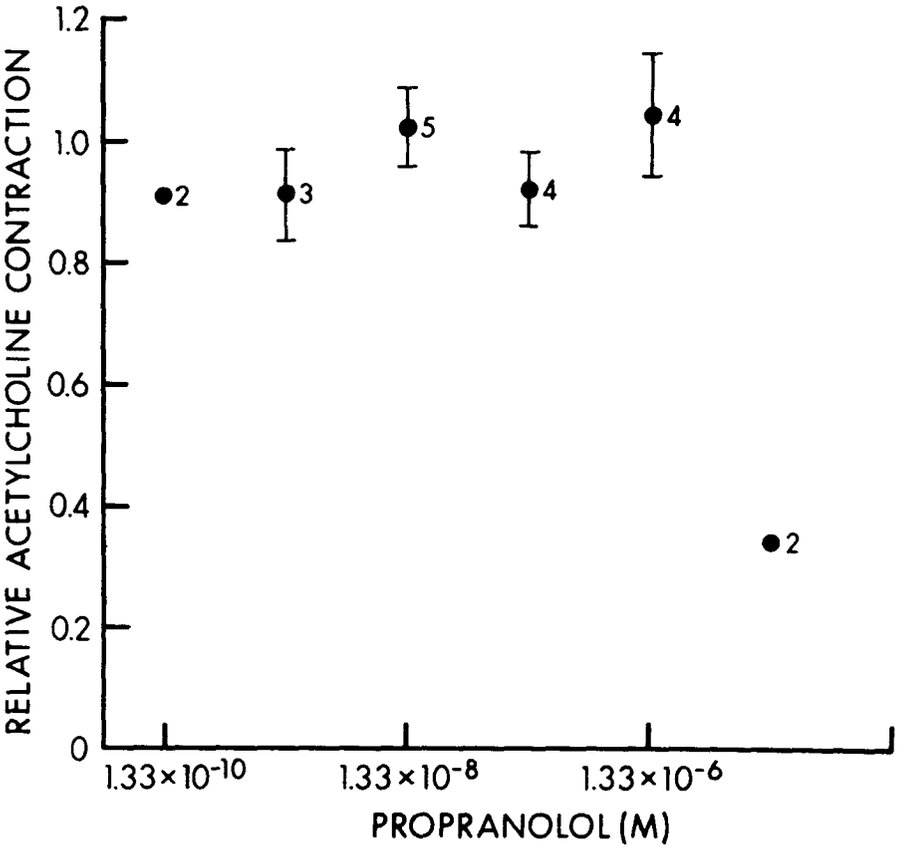

Figure 17. Effects of various concentrations of propranolol on acetylcholine (6.65×10^{-8} M) contraction of guinea pig ileum. Tissue is pretreated with propranolol for 5 min prior to acetylcholine application. Data are given in the mean $\pm$ S.E.M. with the number of experiments beside the symbol.

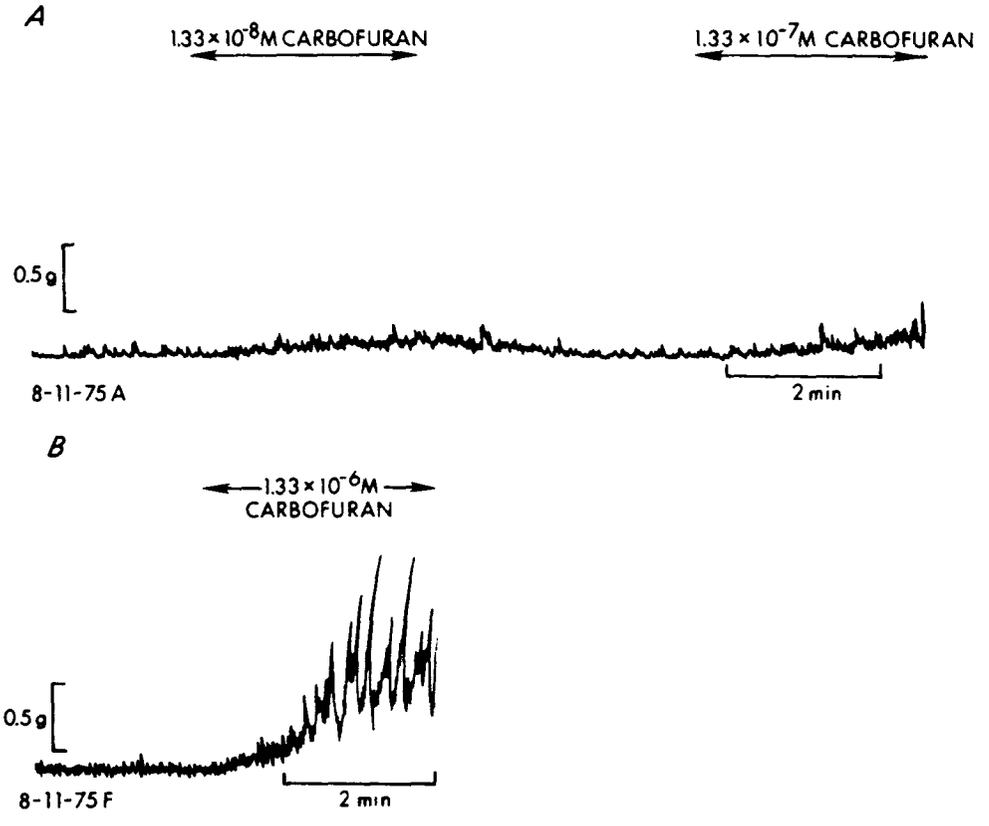

Figure 18. Contractions of guinea pig ileum caused by various concentrations of carbofuran. The contractions are much slower in onset than the acetylcholine (ACh)-induced contraction, and are repetitive in nature at a high concentration (1.33×10^{-6} M). The contraction induced by acetylcholine (ACh) (6.65×10^{-8} M) is not affected by pretreatment with carbofuran.

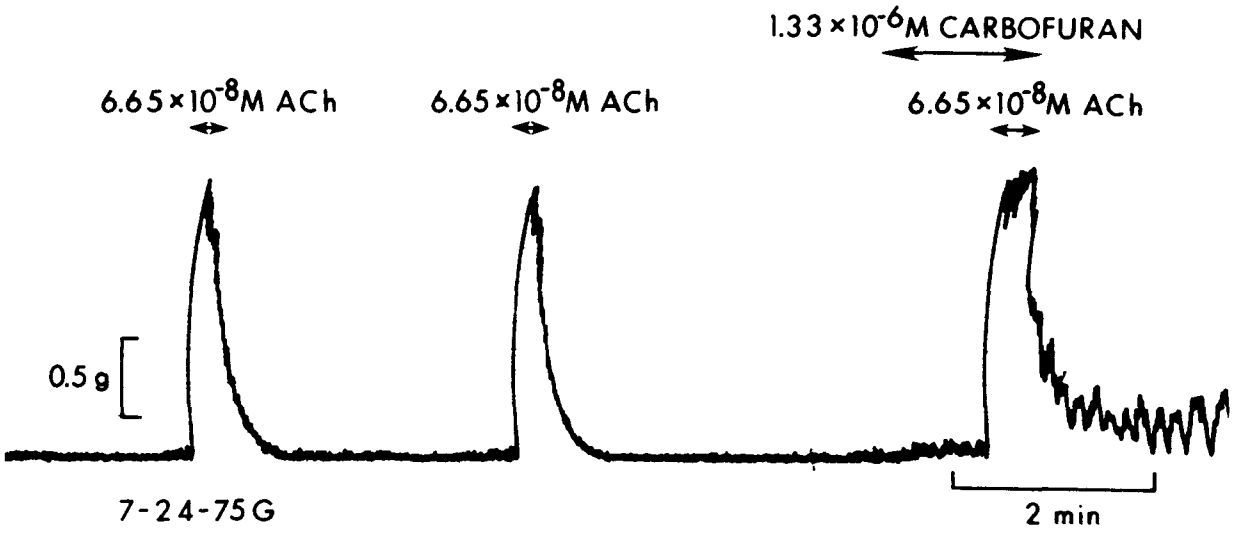

Figure 19. Absence of the effect of carbofuran (1.33×10^{-6} M) pretreatment on the acetylcholine (ACh)-induced contraction in guinea pig ileum. Small repetitive contractions are caused by the high concentration of carbofuran itself (see Fig. 18).

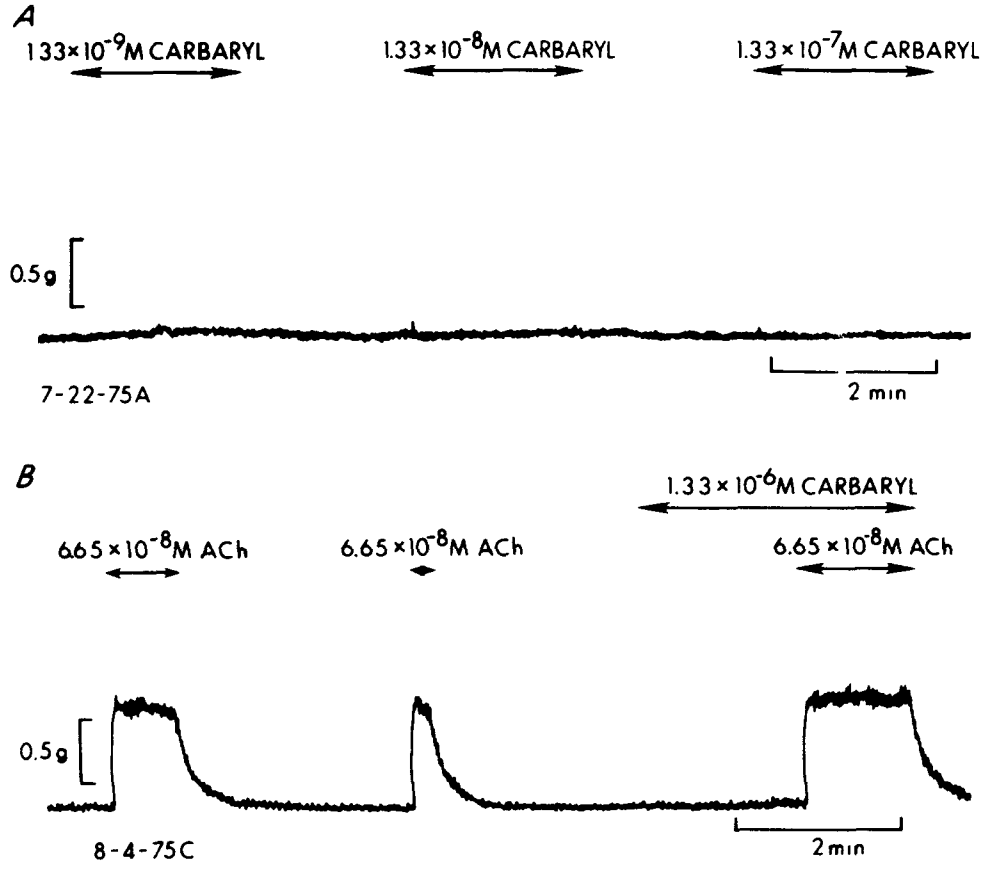

Figure 20. A, absence of direct stimulating action of carbaryl (1.33×10^{-9} - 1.33×10^{-7} M) in guinea pig ileum. B, it also fails to modify acetylcholine (ACh)-induced contraction.

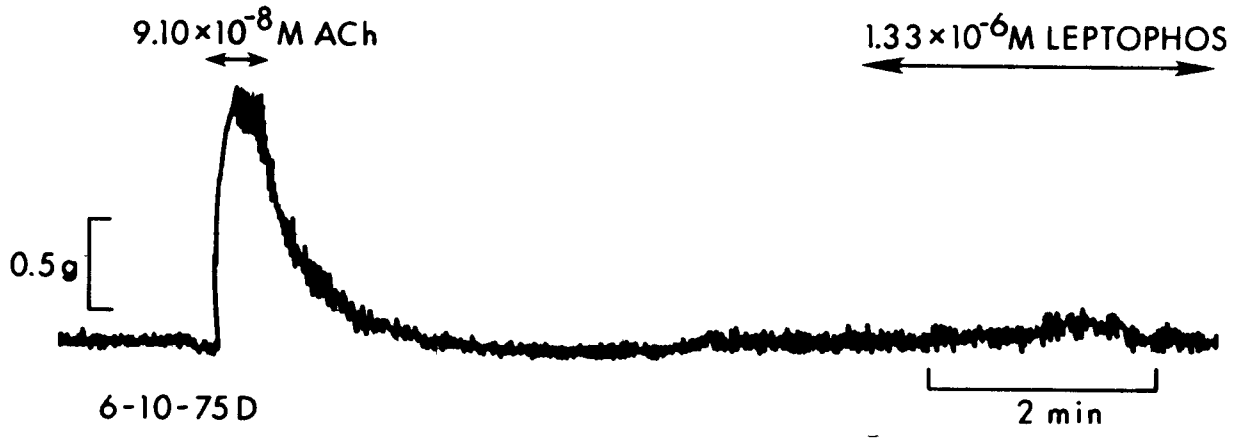

Figure 21. Acetylcholine (ACh)-induced contraction in guinea pig ileum, and absence of direct stimulating action of 1.33×10^{-6} M leptophos.

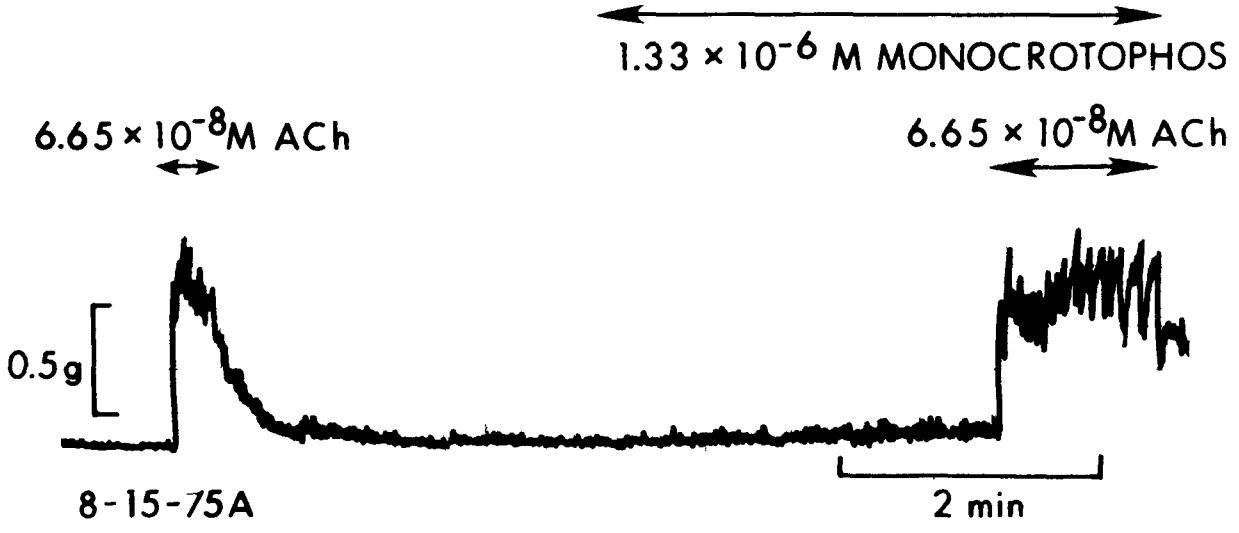

Figure 22. Absence of direct stimulating action of 1.33×10^{-6} M monocrotophos in guinea pig ileum. Acetylcholine (ACh)-induced contraction is not appreciably affected by pretreatment with monocrotophos.

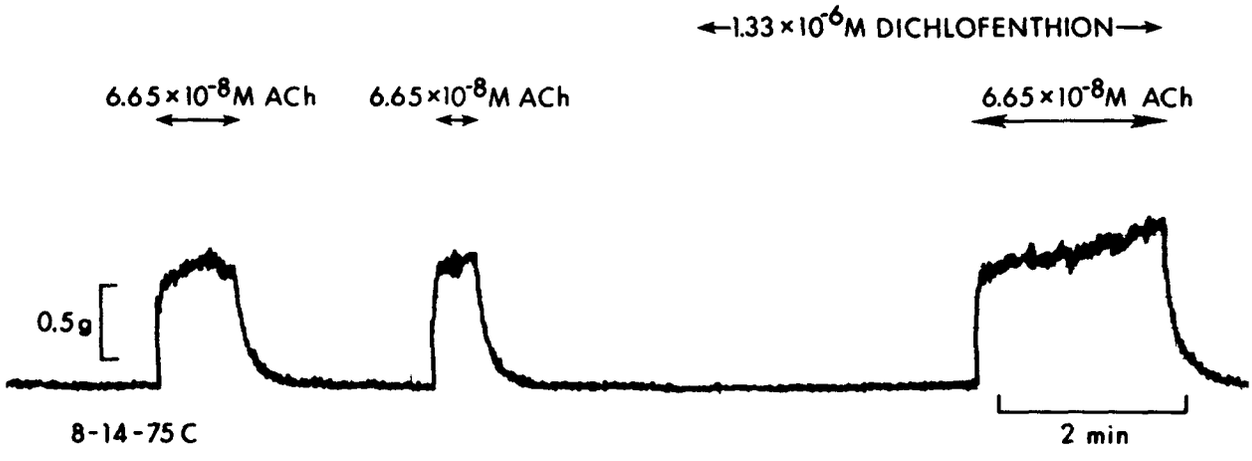

Figure 23. Absence of direct stimulating action of 1.33 x 10^{-6} M dichlofenthion in guinea pig ileum. Acetylcholine (ACh)-induced contraction is not affected by pretreatment with dichlofenthion.

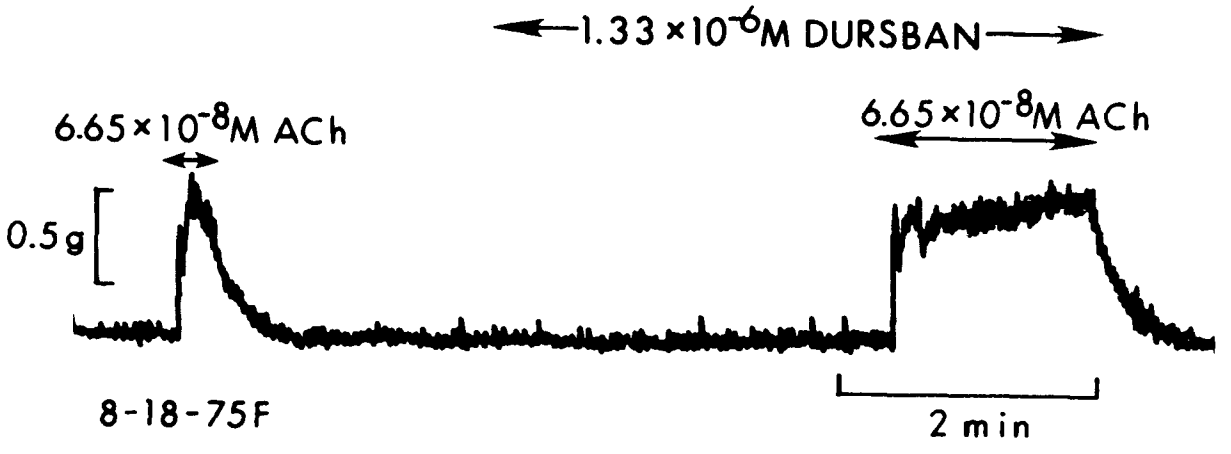

Figure 24. Absence of direct stimulating action of 1.33 x 10^{-6} M dursban in guinea pig ileum. Acetylcholine (ACh)-induced contraction is only slight reduced by pretreatment with dursban.

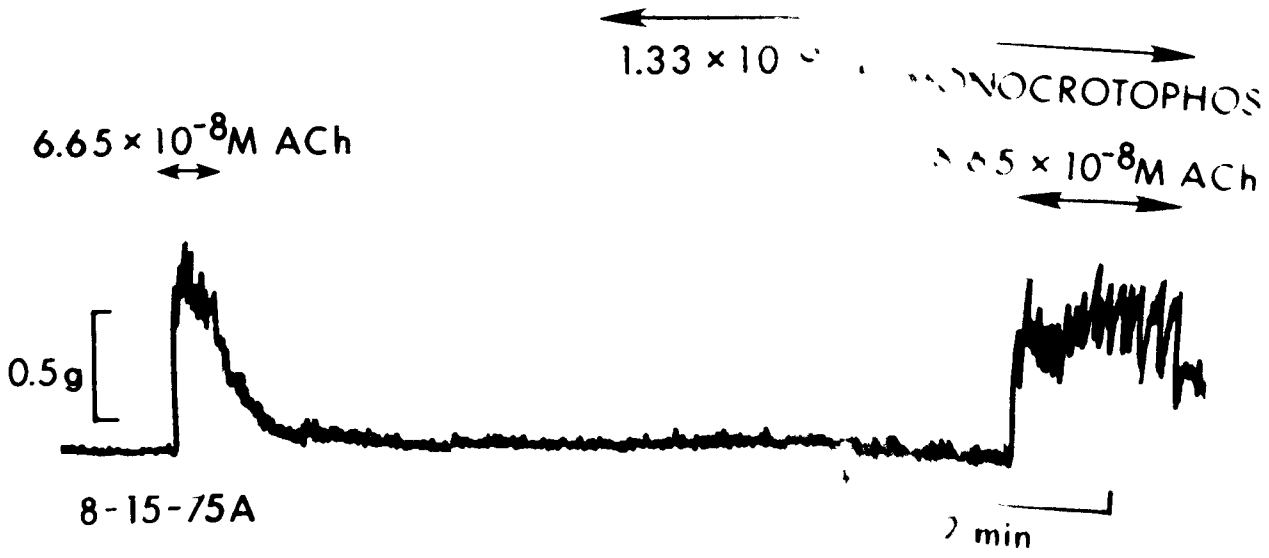

Figure 22. Absence of direct stimulating action of
in guinea pig ileum. Acetylcholine (ACh)-induced co
affected by pretreatment with monocrotophos.

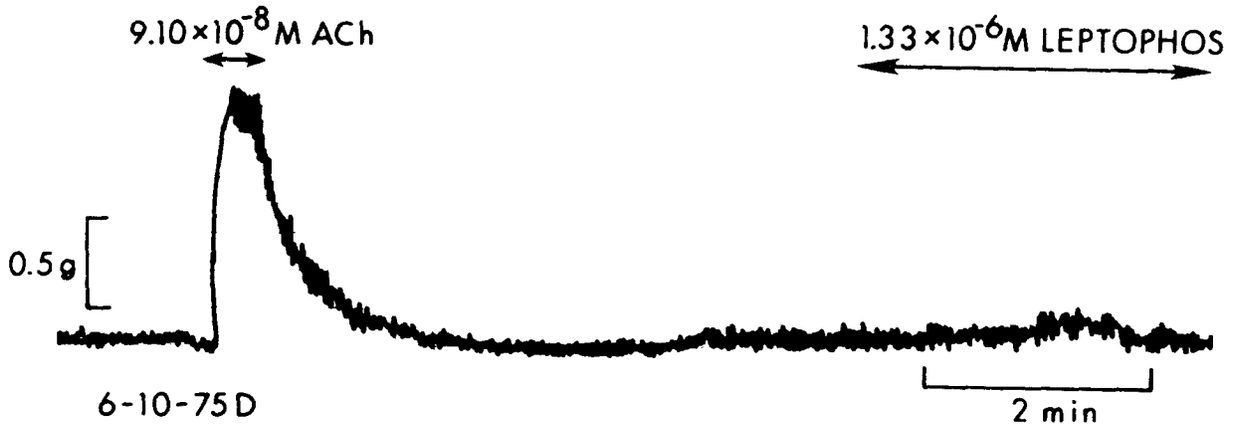

Figure 21. Acetylcholine (ACh)-induced contraction in guinea pig ileum, and absence of direct stimulating action of 1.33×10^{-6} M leptophos.

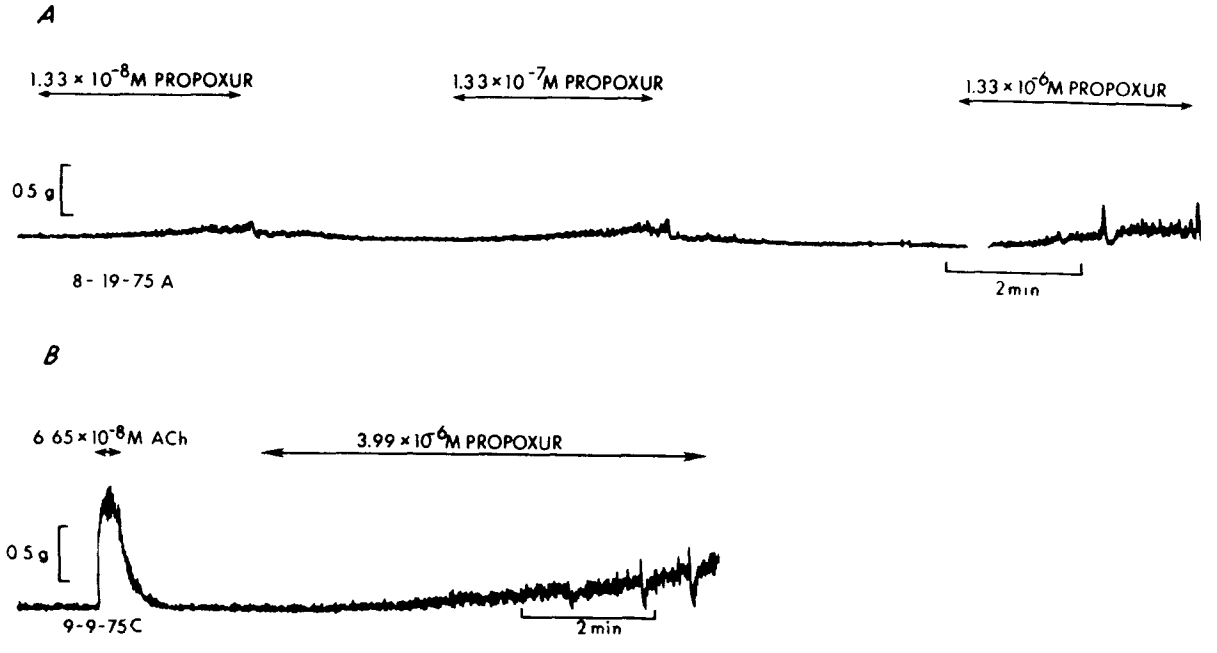

Figure 25. Contractions induced by various concentrations of propoxur in guinea pig ileum. The contractions are small in amplitude and slow in onset compared to the acetylcholine (ACh)-induced contraction.

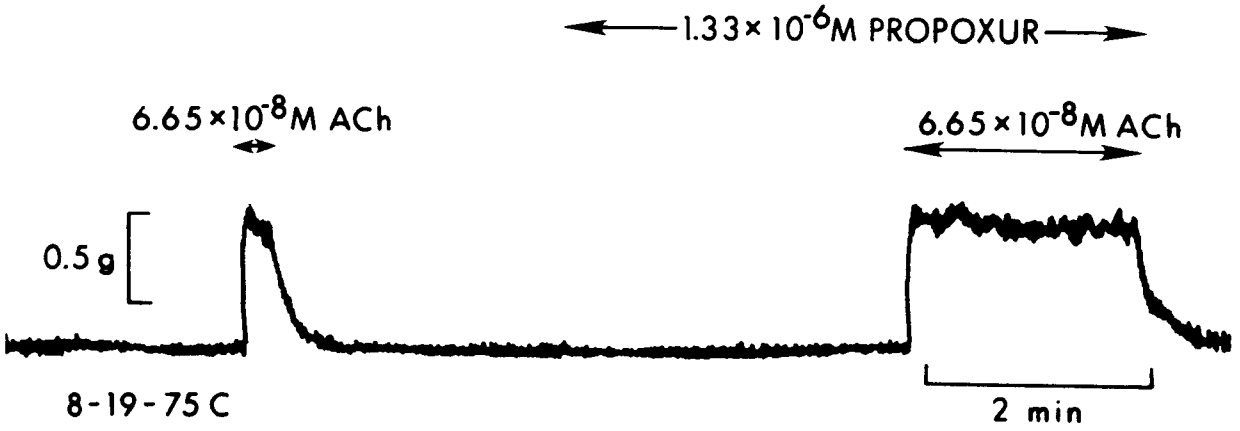

Figure 26. Absence of the effect of propoxur (1.33×10^{-6} M) pretreatment on the acetylcholine (ACh)-induced contraction in guinea pig ileum.

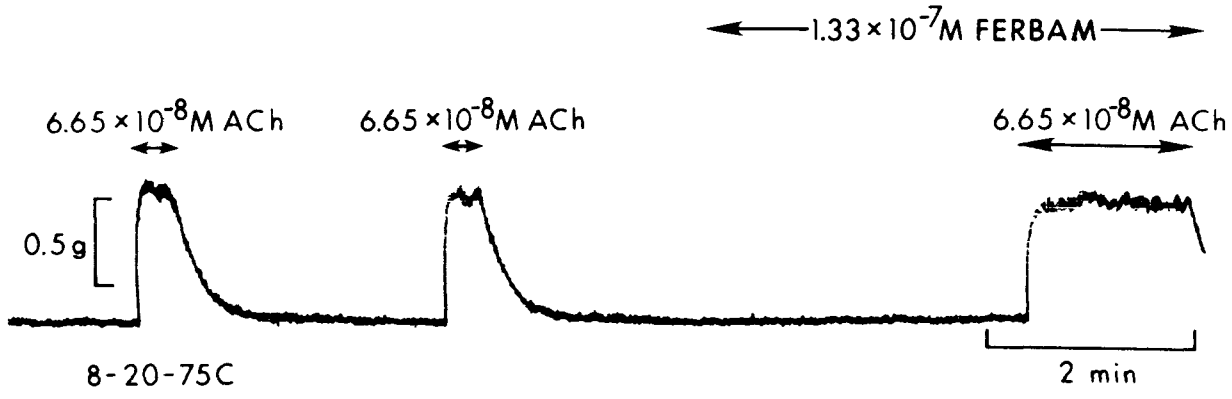

Figure 27. Absence of direct stimulating action of 1.33 x 10^{-7} M ferbam in guinea pig ileum. Acetylcholine (ACh)-induced contraction is not affected by pretreatment with ferbam.

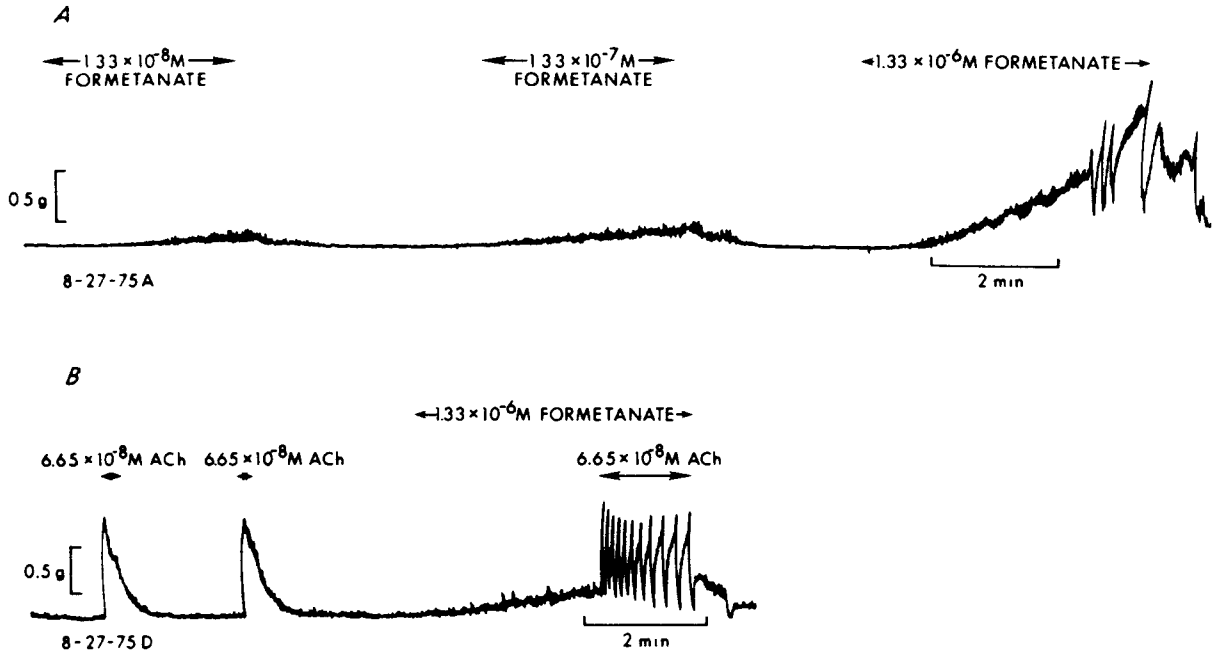

Figure 28. A, contractions induced by formetanate (1.33×10^{-8} - 1.33×10^{-6} M) in guinea pig ileum. The contractions become more intense with increasing the contraction, but are slow in onset. B, absence of the effect of 1.33×10^{-6} M formetanate pretreatment on the acetylcholine (ACh)-induced contraction. Repetitive contractions are caused by formetanate itself.

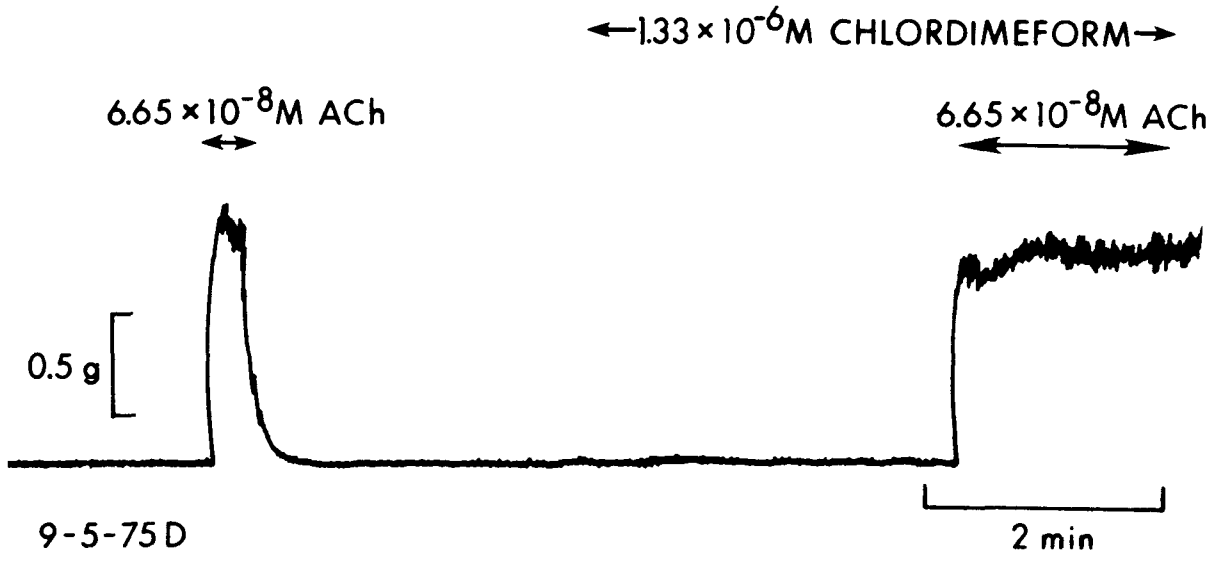

Figure 29. Absence of direct stimulating action of 1.33 x 10^{-6} M chlordimeform in guinea pig ileum. Acetylcholine (ACh)-induced contraction is slightly suppressed by pretreatment with chlordimeform.

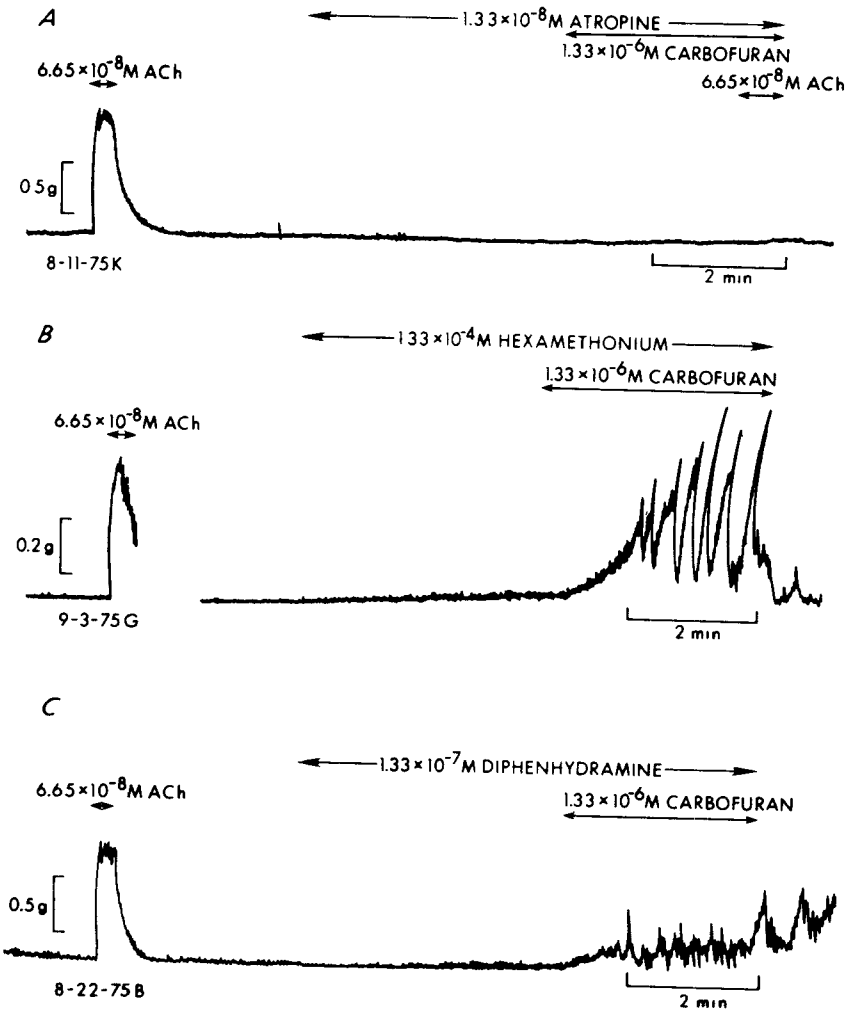

Figure 30. Effects of pretreatment with atropine (1.33×10^{-8} M), hexamethonium (1.33×10^{-4} M), diphenhydramine (1.33×10^{-7} M), and physostigmine (1.33×10^{-8} M) on the contractions induced by carbofuran (1.33×10^{-6} M) in guinea pig ileum. None of these pretreatments themselves causes contraction. The carbofuran contractions are completely abolished by atropine (A), but not appreciably affected by hexamethonium (B), or diphenhydramine (C). Acetylcholine (ACh)-induced contractions are also illustrated for comparison. See Fig. 18 for the contractions in the presence of carbofuran alone.

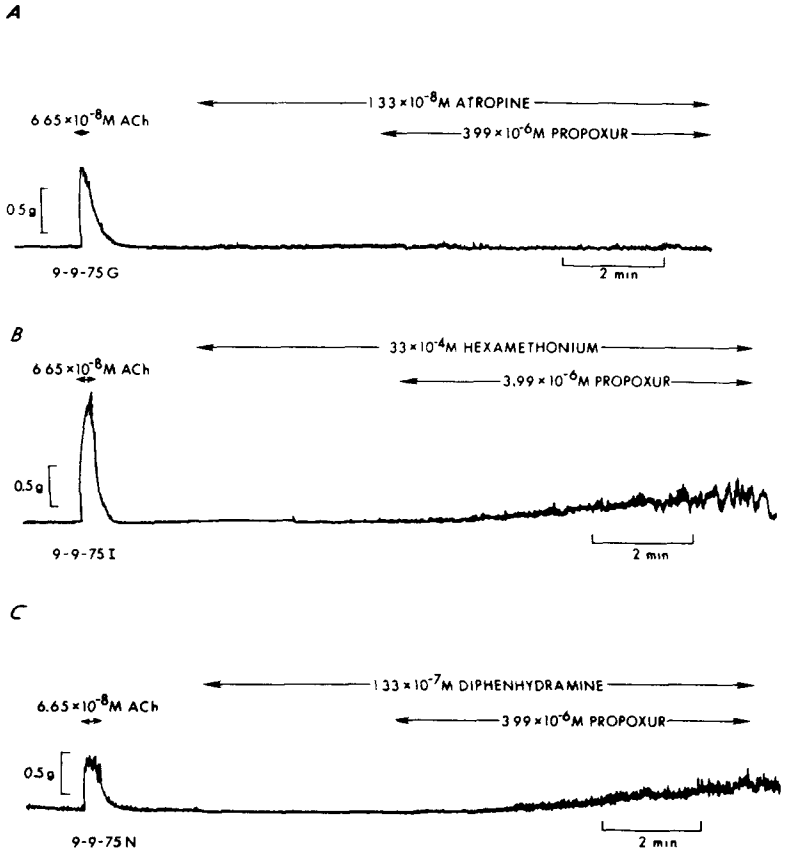

Figure 31. Effects of pretreatment with atropine (1.33×10^{-8} M), hexamethonium (1.33×10^{-4} M), and diphenhydramine (1.33×10^{-7} M) on the contractions induced by propoxur (3.99×10^{-6} M) in guinea pig ileum. The propoxur contractions are completely abolished by atropine (A), but not appreciably affected by hexamethonium (B) or diphenhydramine (C). Acetylcholine contractions are also illustrated for comparison. See Fig. 25 for the contractions in the presence of propoxur alone.

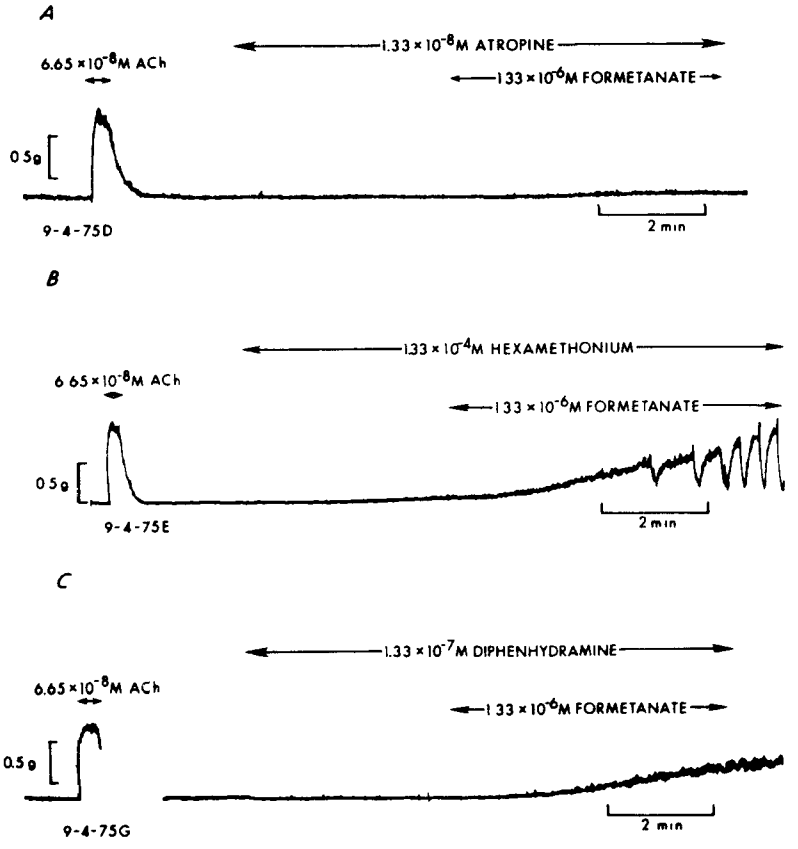

Figure 32. Effects of pretreatment with atropine (1.33×10^{-8} M), hexamethonium (1.33×10^{-4} M), and diphenhydramine (1.33×10^{-7} M) on the contractions induced by formetanate (1.33×10^{-6} M) in guinea pig ileum. The formetanate contractions are completely abolished by atropine (A), slightly suppressed by diphenhydramine (C), but not affected by hexamethonium (B). Acetylcholine contractions are also illustrated for comparison. See Fig. 28 for the contractions in the presence of formetanate alone.

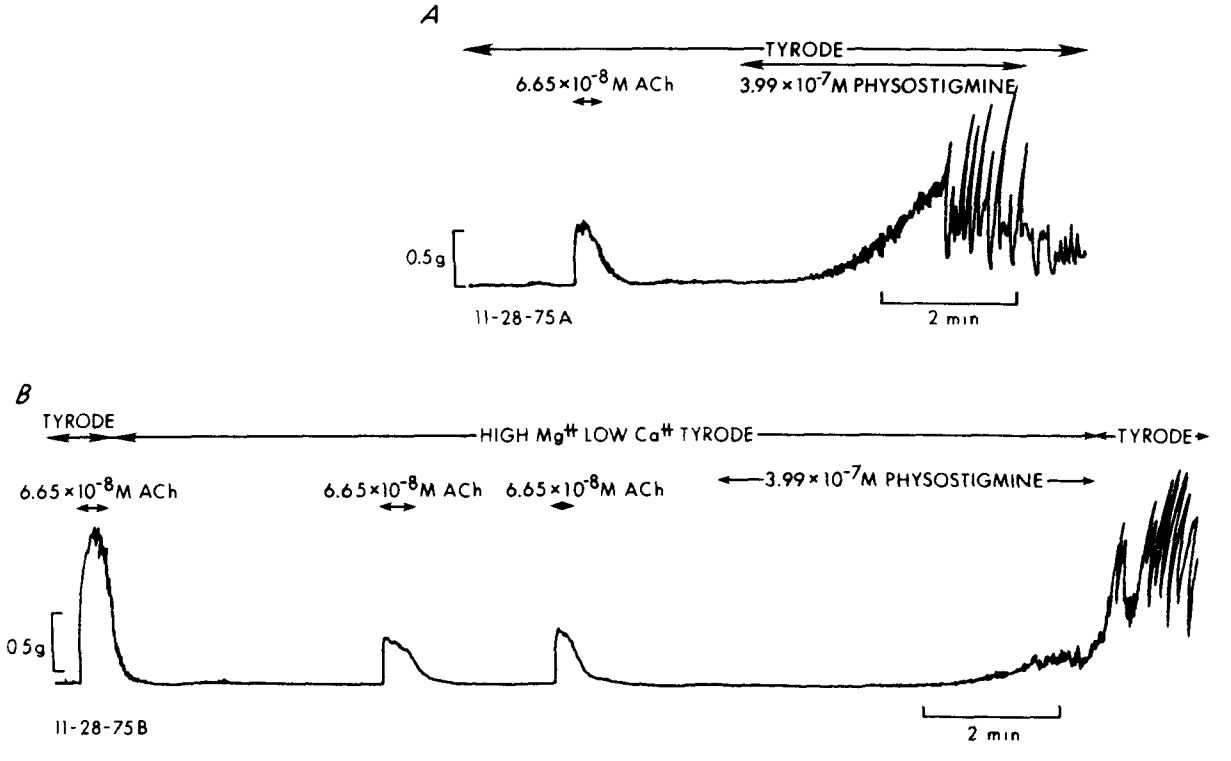

Figure 33. Effect of high Mg^{++} - low Ca^{++} (2.2 mM Mg^{++}-0.36 mM Ca^{++}) Tyrode's solution on the contractions of guinea pig ileum induced by acetylcholine (ACh) (6.65×10^{-8} M) and by physostigmine (3.99×10^{-7} M). The ACh contraction is smaller in amplitude and the physostigmine contraction is smaller in amplitude and longer in latency in the modified Tyrode than in normal Tyrode.

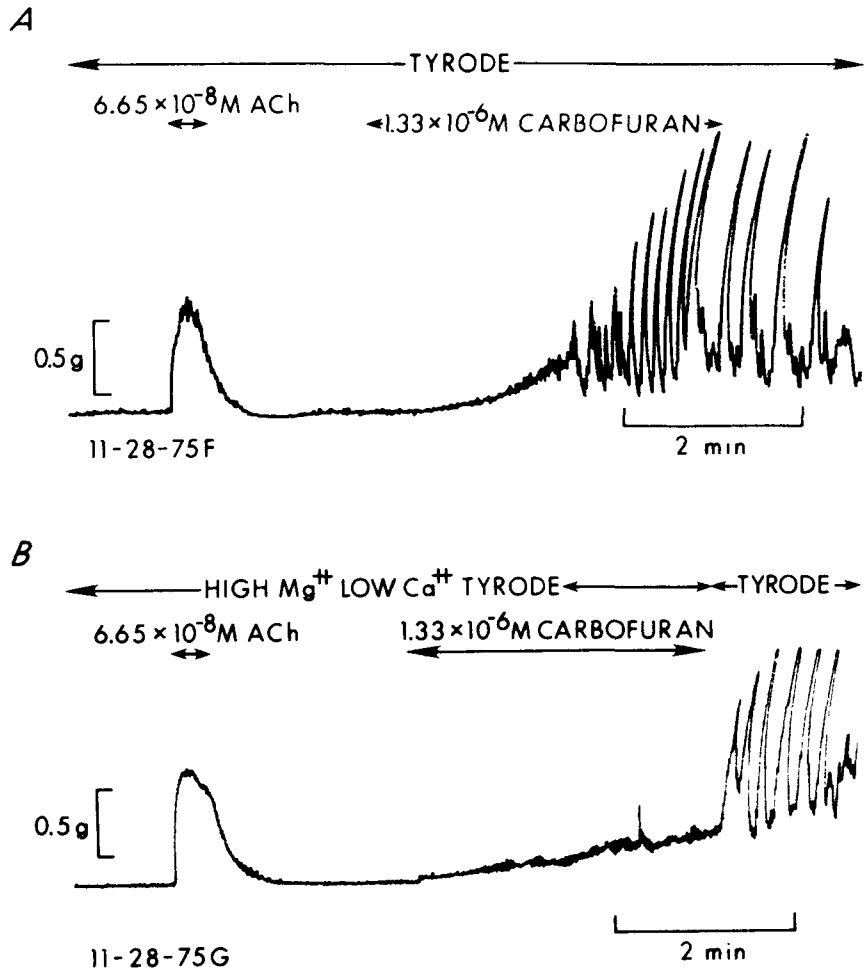

Figure 34. Effect of high Mg^{++} - low Ca^{++} (2.2 mM Mg^{++}-0.36 mM Ca^{++}) Tyrode's solution on the contractions of guinea pig ileum induced by carbofuran (1.33×10^{-6} M). The carbofuran contractions are smaller in amplitude and longer in latency in the modified Tyrode than in normal Tyrode.

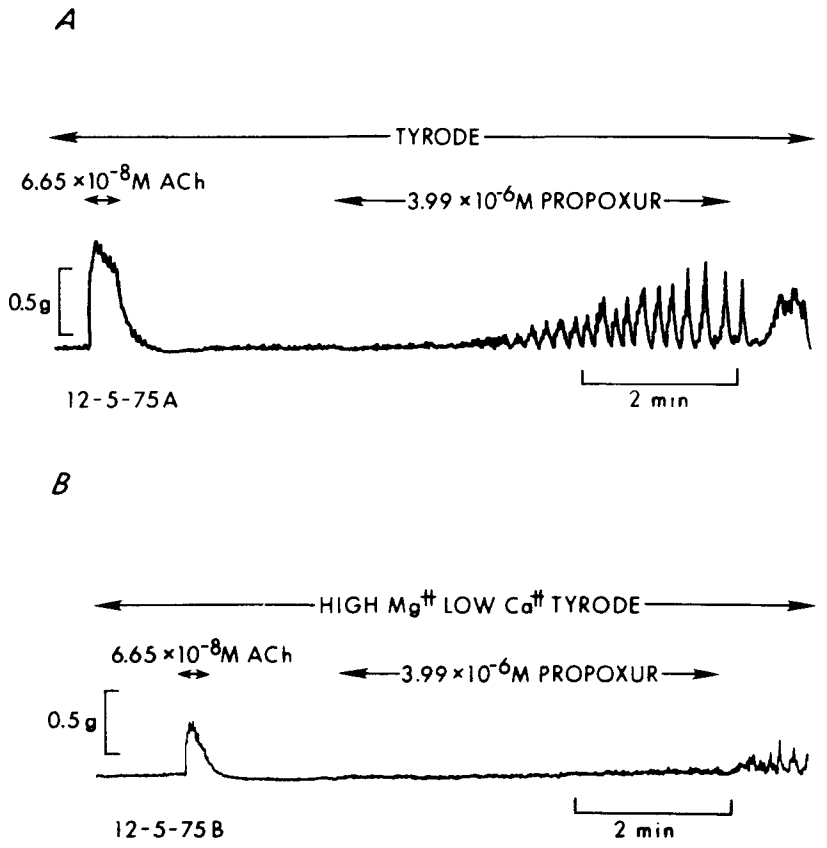

Figure 35. Effect of high Mg^{++} - low Ca^{++} (2.2 mM Mg^{++}-0.36 mM Ca^{++}) Tyrode's solution on the contractions of guinea pig ileum induced by propoxur (3.99×10^{-6} M). The propoxur-induced contractions are smaller in amplitude and longer in latency in the modified Tyrode than in normal Tyrode.

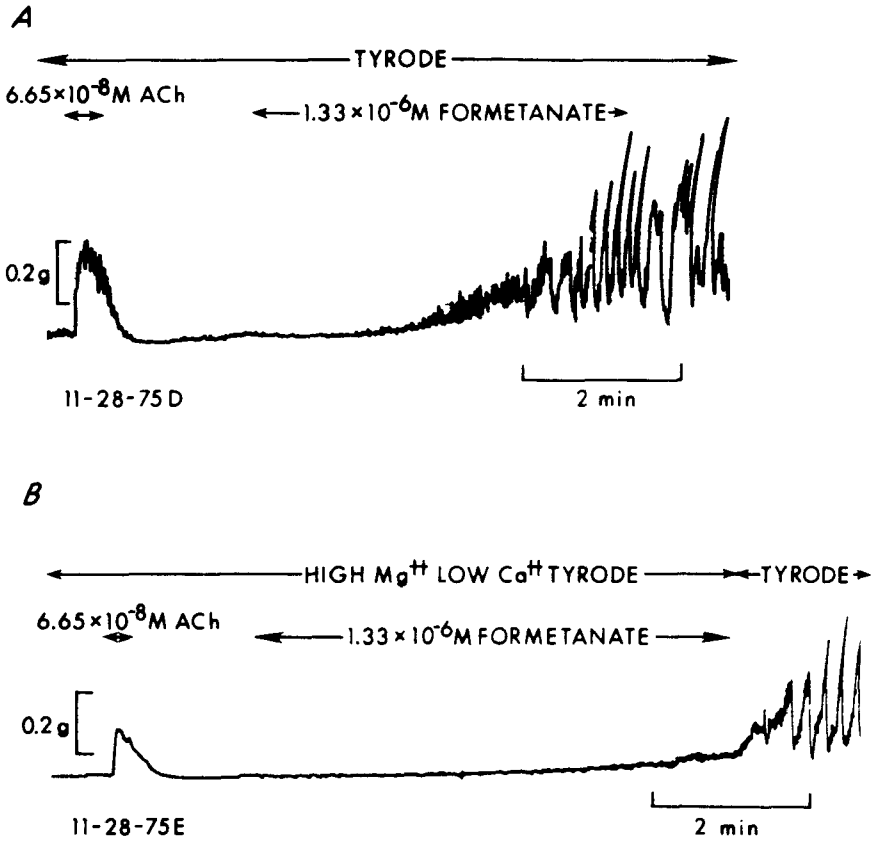

Figure 36. Effect of high Mg^{++} - low Ca^{++} (2.2 mM Mg^{++}-0.36 mM Ca^{++}) Tyrode's solution on the contractions of guinea pig ileum induced by formetanate (1.33×10^{-6} M). The formetanate contractions are smaller in amplitude and longer in latency in the modified Tyrode than in normal Tyrode.

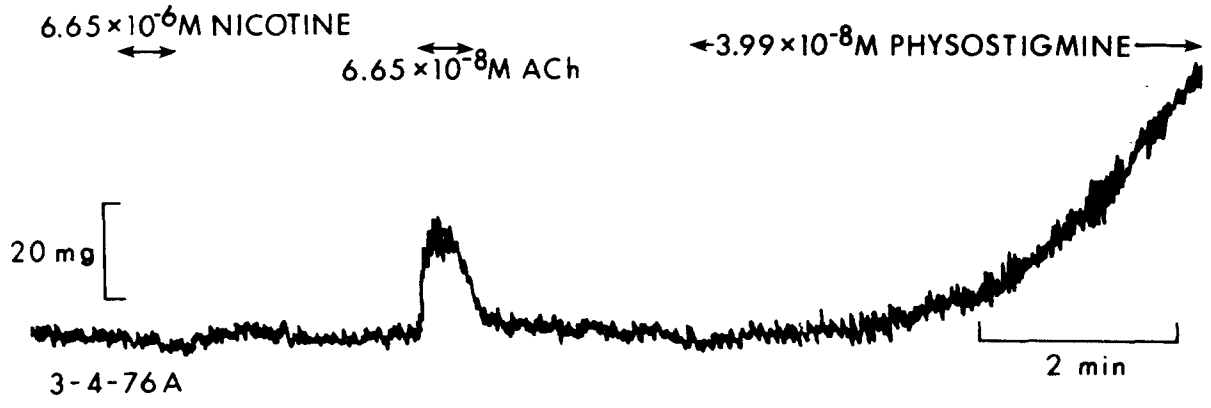

Figure 37. Effects of 6.65 x 10^{-6} M nicotine, 6.65 x 10^{-8} M acetylcholine (ACh) and 3.99 x 10^{-8} M physostigmine on the guinea pig ileum treated with black widow spider venom (0.4 glands/ml) for 15 minutes. Although nicotine fails to stimulate the preparation, acetylcholine response is small and physostigmine still induces contractions.

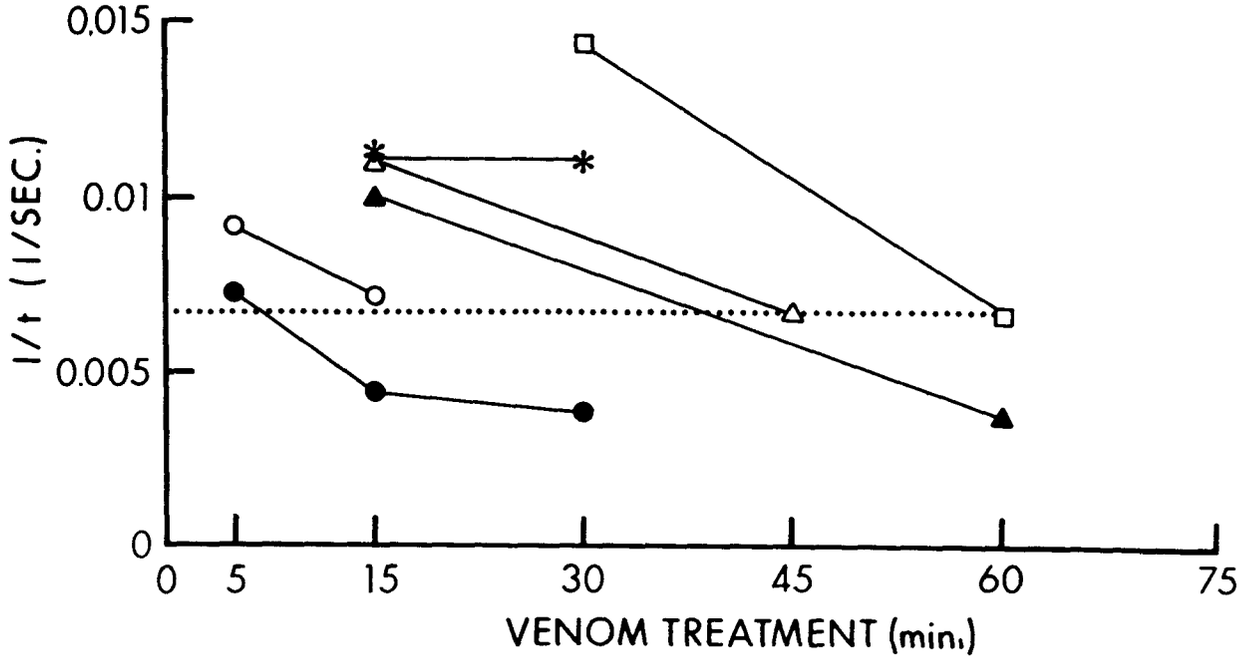

Figure 38. Effect of pretreatment of guinea pig ileum with black widow spider venom at various concentrations (0.2-4 glands/ml) at 24°C or 37°C on physostigmine contraction. Each symbol represents each preparation. The ordinate represents the reciprocal of the time for the physostigmine (3.99×10^{-7} M) contraction to attain the same level as the acetylcholine (6.65×10^{-8} M) contraction in the same preparation. The dotted line shows the mean control value without venom treatment.

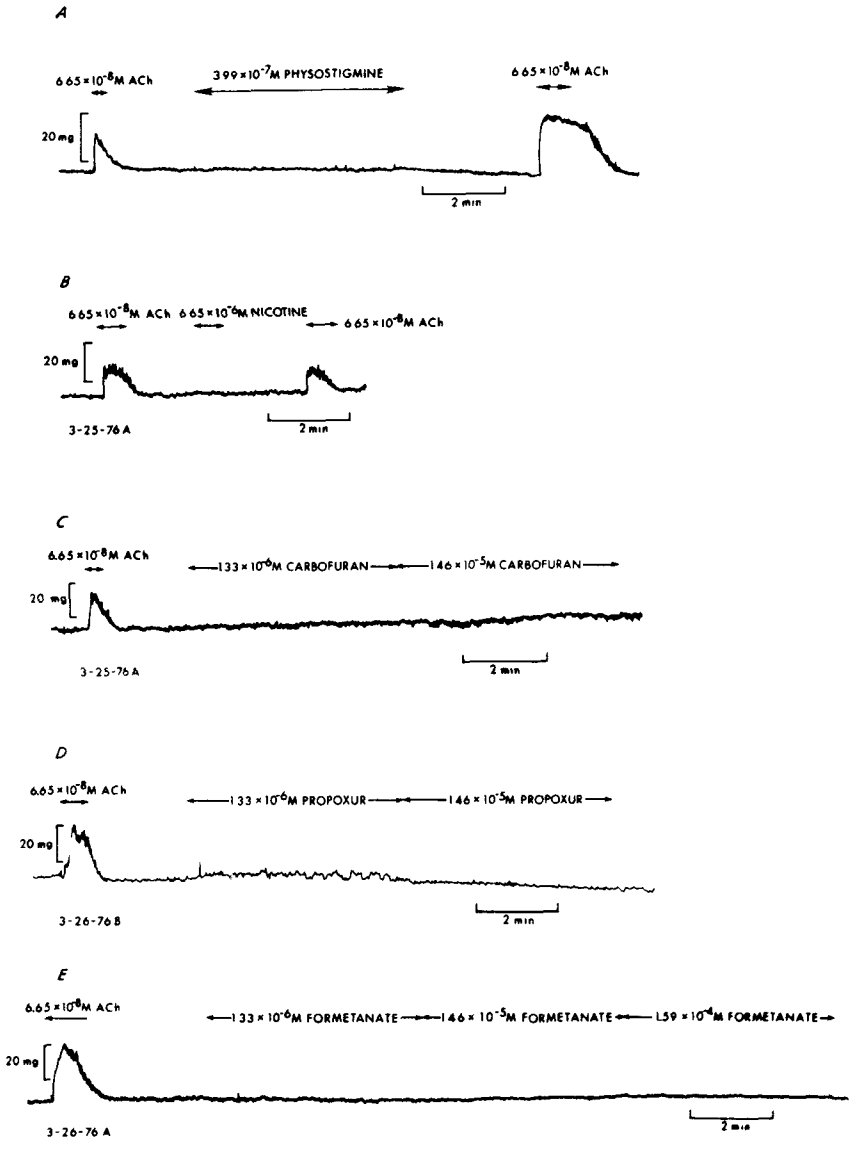

Figure 39. Responses of the denervated guinea pig ileum (Paton-Zar method) to drugs and insecticides. A, physostigmine (3.99×10^{-7} M) itself causes no contraction, but potentiates the acetylcholine (ACh)-induced contraction. B, Nicotine (6.65×10^{-6} M) fails to induce contraction. C, carbofuran (1.33×10^{-6} M and 1.46×10^{-5} M) fails to stimulate the ileum. D, propoxur (1.33×10^{-6} M and 1.46×10^{-5} M) fails to stimulate. E, formetanate (1.33×10^{-6} M, 1.45×10^{-5} M, and 1.59×10^{-4} M) fails to stimulate.

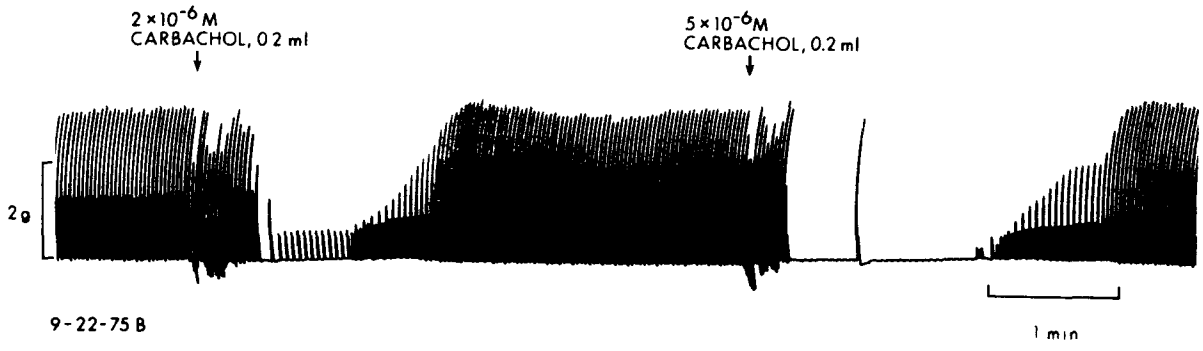

Figure 40. Negative inotropic effect of carbachol on the guinea pig heart. Carbachol solutions are injected to the perfusate at arrows in the amount and concentrations indicated.

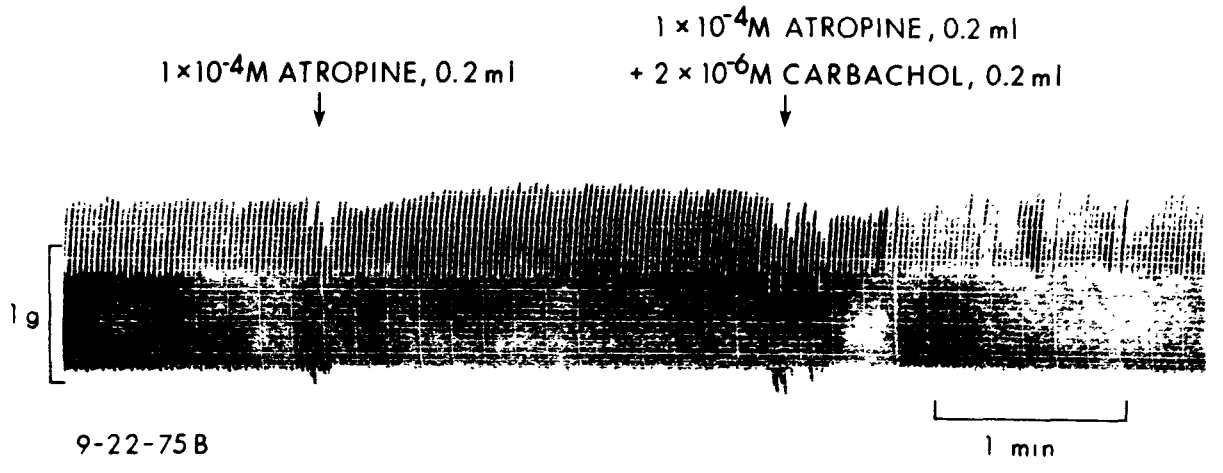

Figure 41. Effect of atropine on the contraction of the guinea pig heart. Drug solutions are injected to the perfusate at arrows in the amount and concentrations indicated. Although atropine itself has no effect on the contraction, it abolishes the negative inotropic action of carbachol seen in Fig. 40.

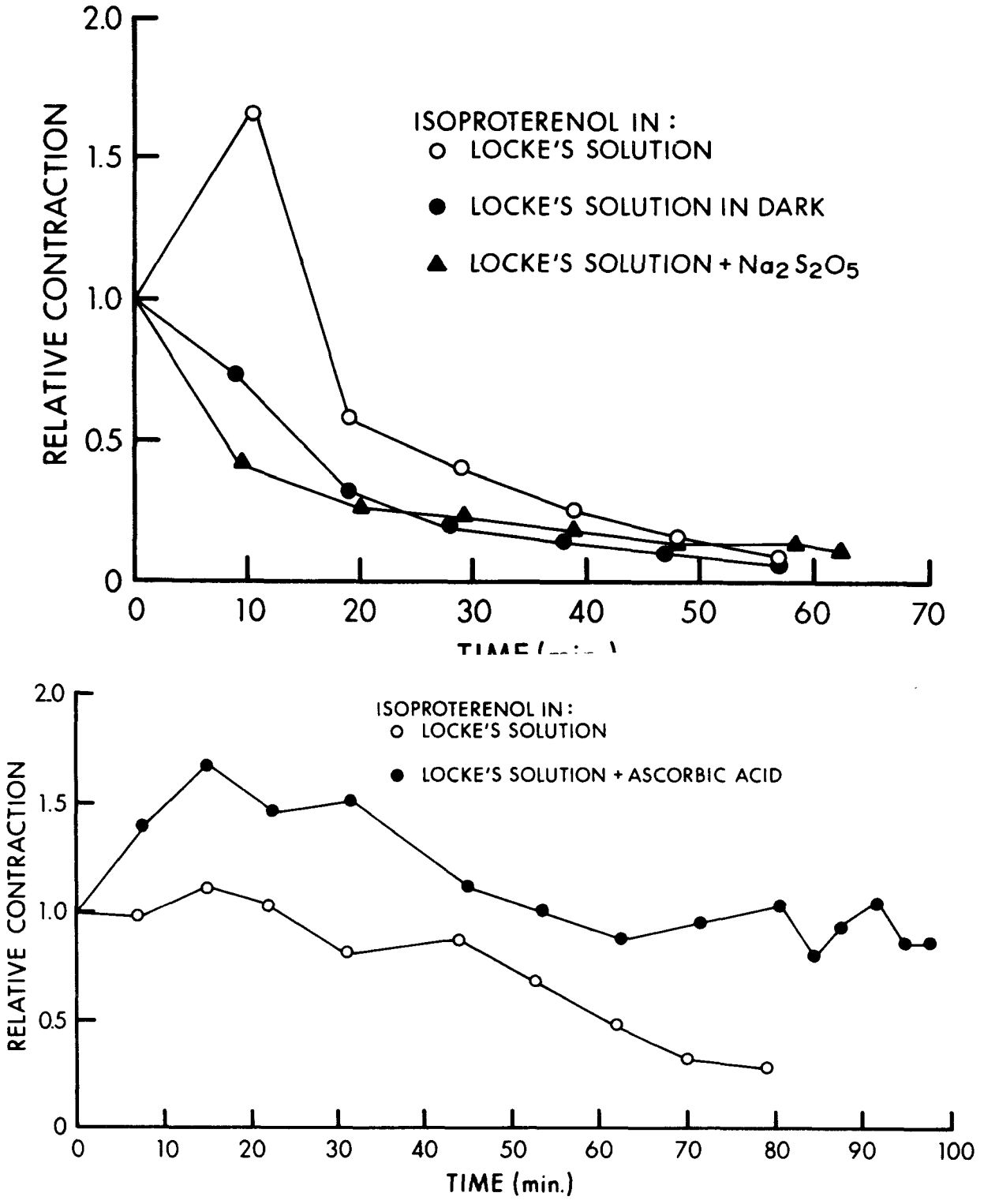

Figure 42. Effects of storage of isoproterenol solution under various experimental conditions on its potency in causing a positive inotropic action on guinea pig heart. The ordinate represents the amplitude of contraction relative to that observed in freshly prepared solution, and the abscissa is the storage time. Concentrations of the test solutions (0.2 ml) injected are 1×10^{-6} M for isoproterenol, 4.26×10^{-4} M for $Na_2S_2O_5$ and 1×10^{-4} M for ascorbic acid.

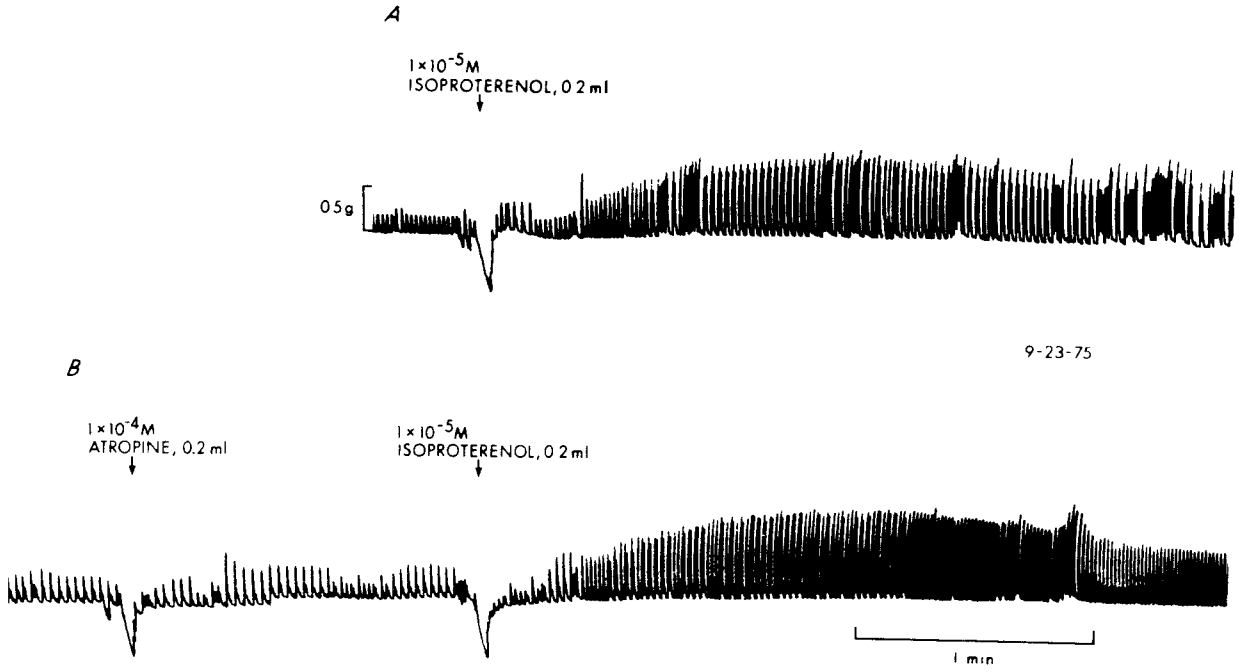

Figure 43. Positive inotropic effect of isoproterenol on the guinea pig heart (A). Drug solutions are injected to the perfusate at arrows in the amount and concentrations indicated. Atropine does not affect the isoproterenol action (B).

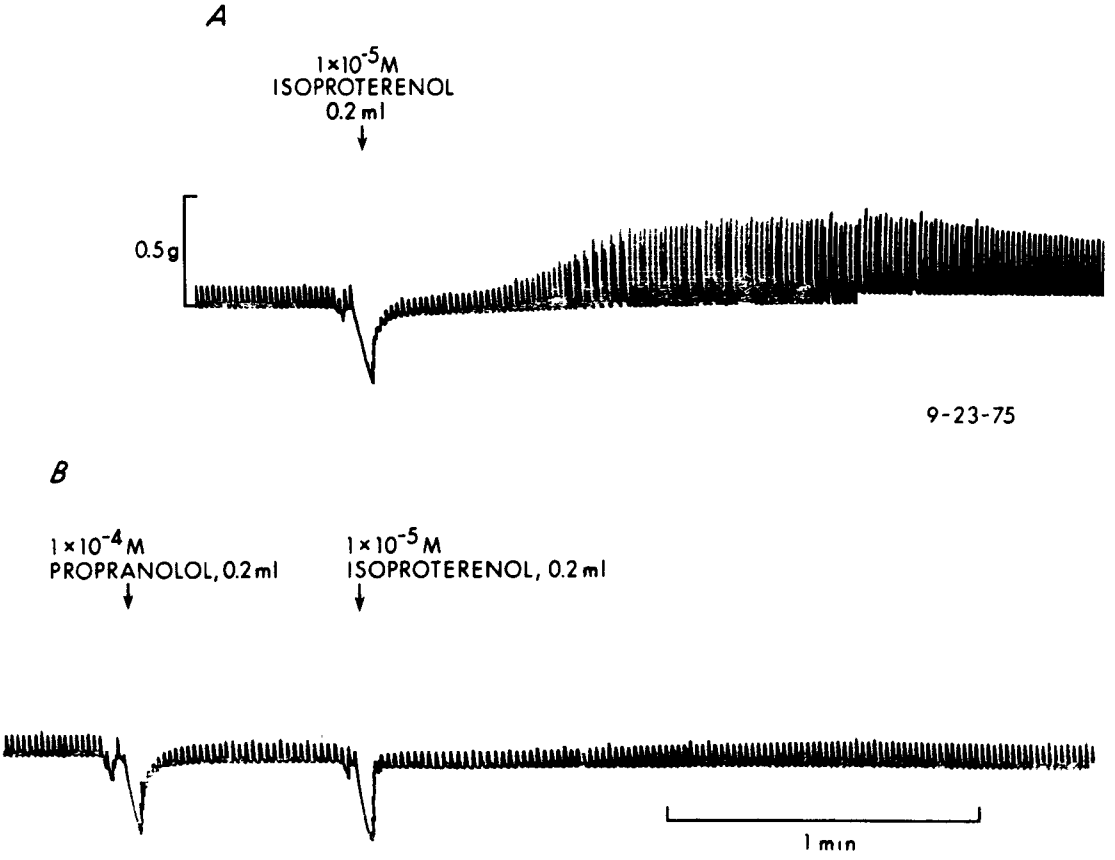

Figure 44. Abolition of the positive inotropic action of isoproterenol on the guinea pig heart (A) by propranolol (B). Drug solutions are injected to the perfusate at arrows in the amount and concentrations indicated.

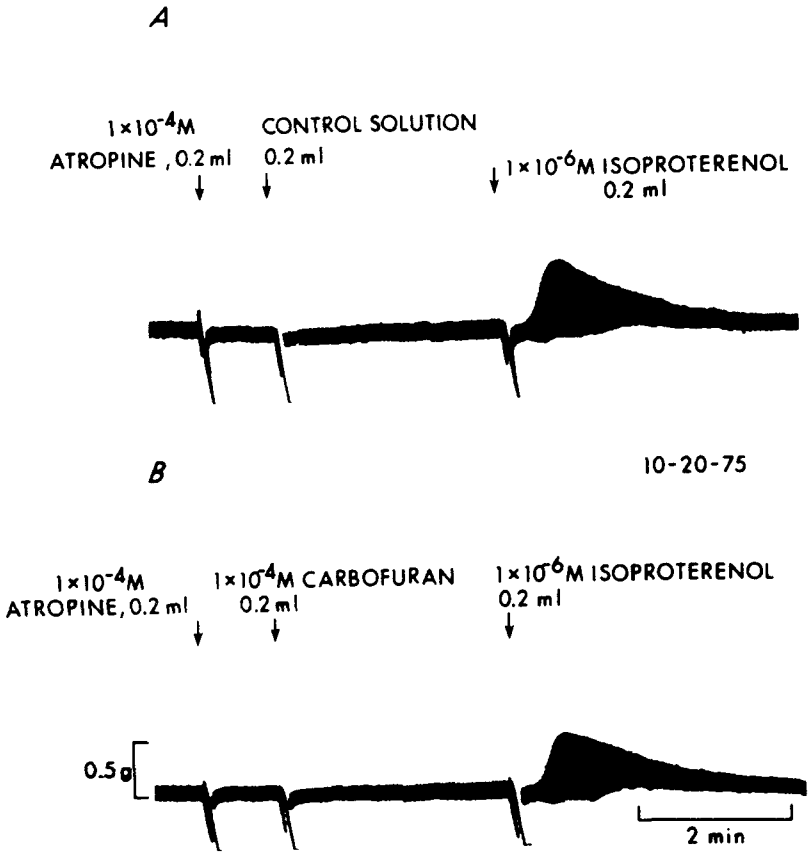

Figure 45. Effect of carbofuran on the contraction of the guinea pig heart. The positive inotropic action of isoproterenol on the atropinized preparation (A) is not affected by carbofuran (B). Carbofuran itself has no direct effect on the contraction (B). Drug solutions are injected to the perfusate at arrows in the amount and concentrations indicated.

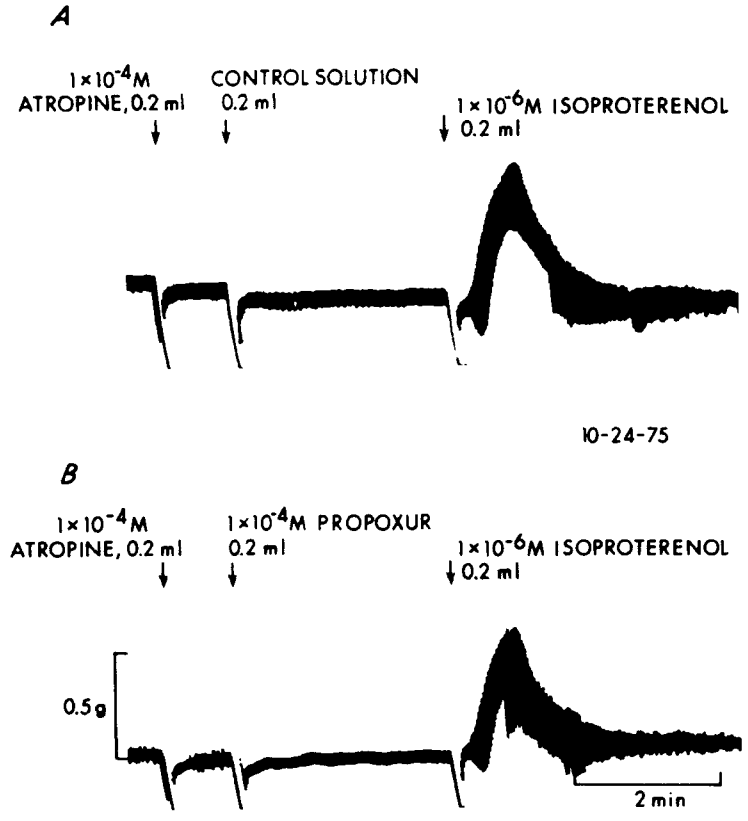

Figure 46. Effect of propoxur on the contraction of the guinea pig heart. The positive inotropic action of isoproterenol on the atropinized preparation (A) is not affected by propoxur (B). Propoxur itself has no direct effect on the contraction (B). Drug solutions are injected to the perfusate at arrows in the amount and concentrations indicated.

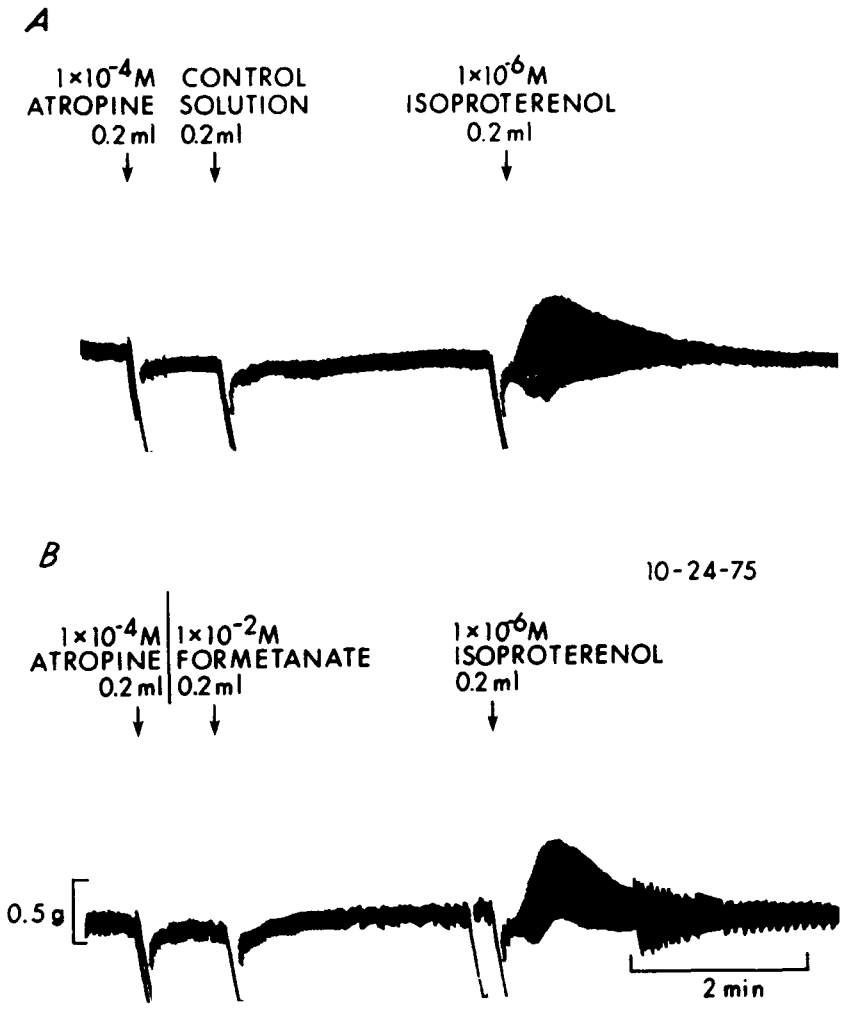

Figure 47. Effect of formetanate on the contraction of the guinea pig heart. The positive inotropic action of isoproterenol on the atropinized preparation (A) is not affected by formetanate (B). Formetanate itself has no direct action on the contraction (B). Drug solutions are injected to the perfusate at arrows in the amount and concentrations indicated.

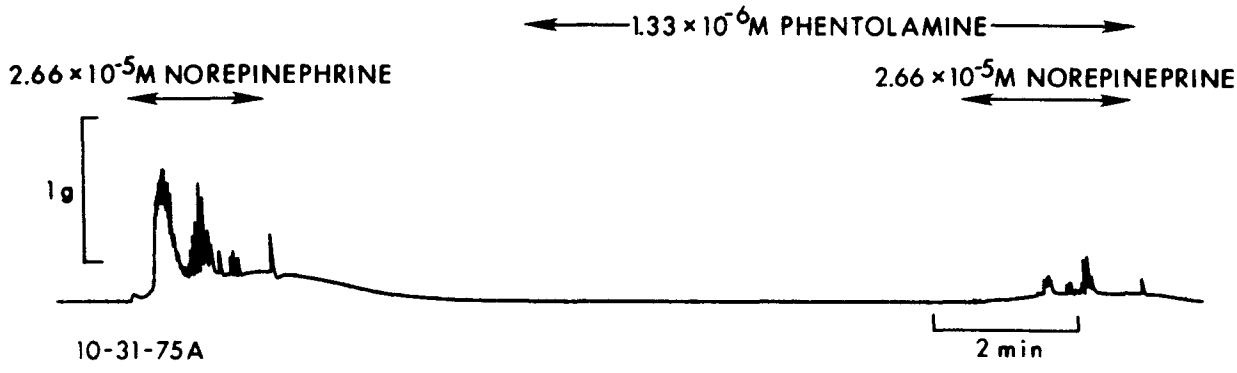

Figure 48. Contractions of the guinea pig vas deferens induced by norepinephrine (2.66×10^{-5} M), and suppression by phentolamine (1.33×10^{-6} M).

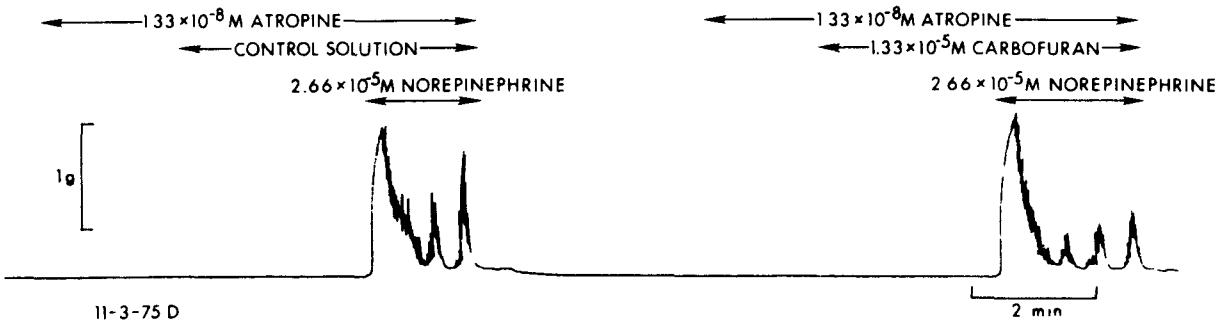

Figure 49. Effect of carbofuran on the atropinized guinea pig vas deferens. Carbofuran (1.33×10^{-5} M) does not stimulate, and fails to affect the norepinephrine-induced contraction.

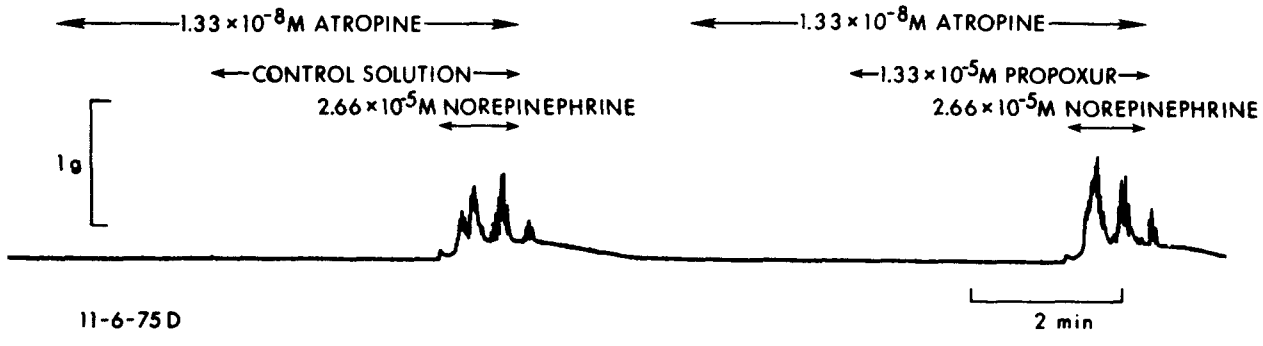

Figure 50. Effect of propoxur on the atropinized guinea pig vas deferens. Propoxur (1.33×10^{-5} M) does not stimulate, and fails to affect the norepinephrine-induced contraction.

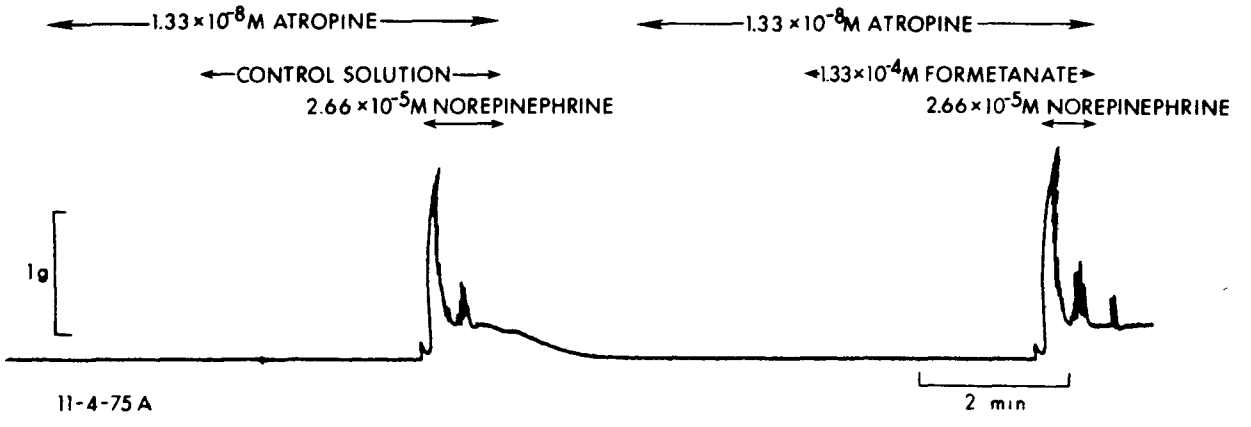

Figure 51. Effect of formetanate on the atropinized guinea pig vas deferens. Formetanate (1.33×10^{-4} M) does not stimulate, and fails to affect the norepinephrine-induced contraction.

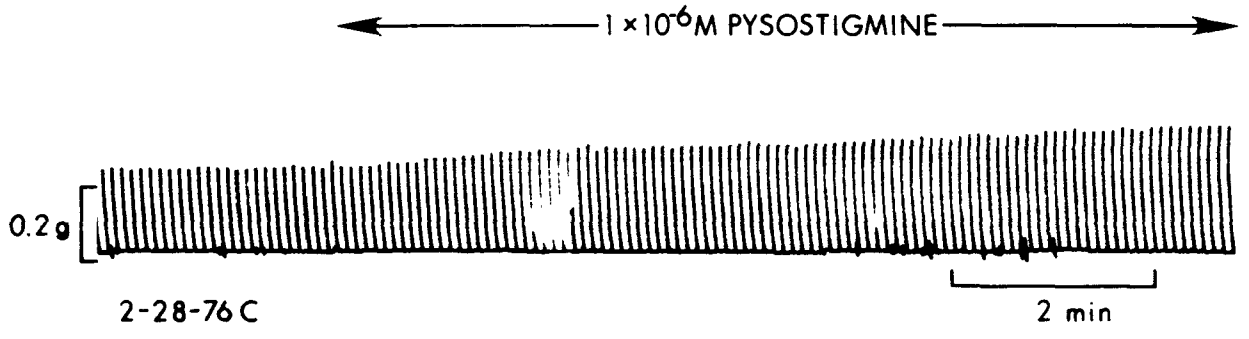

Figure 52. Potentiation of the contractions of the rat diaphragm evoked by nerve stimulations by 1 x 10^{-6} M physostigmine.

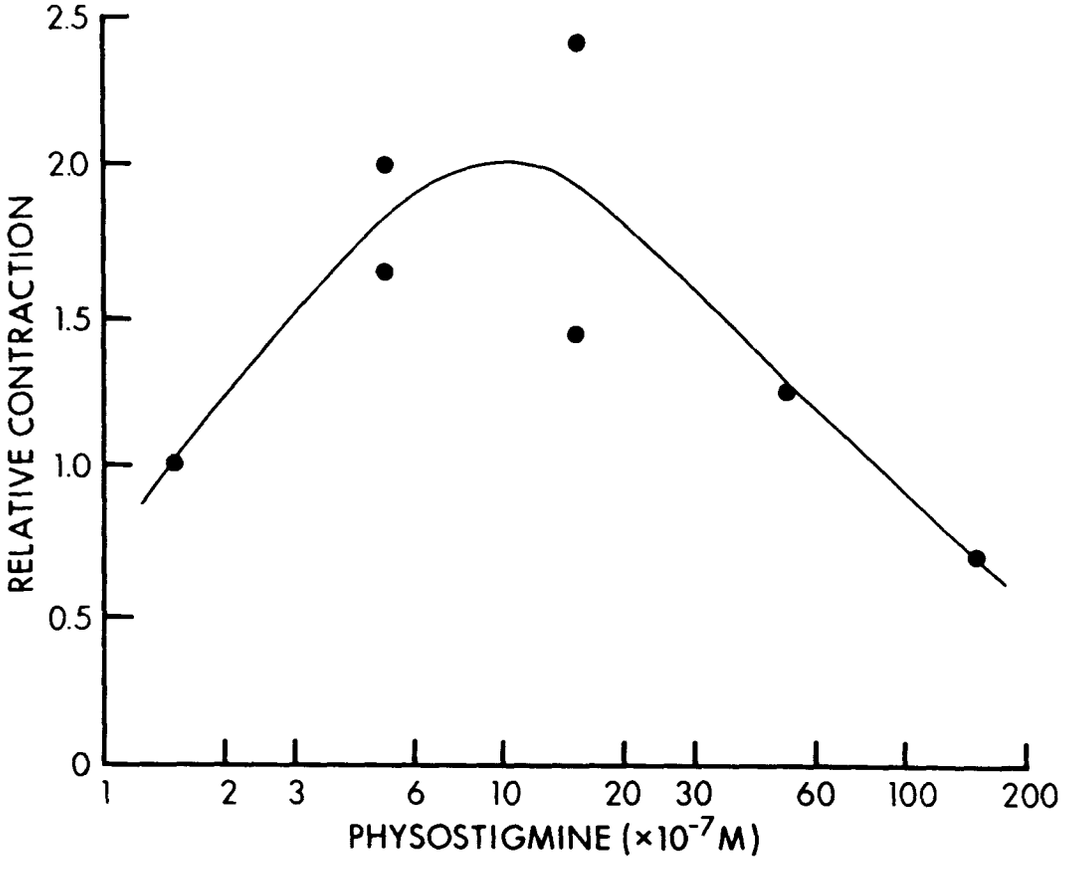

Figure 53. Effects of physostigmine pretreatment for 5 min on the contraction evoked by nerve stimulation in rat diaphragm. The ordinate represents the height of contraction relative to that without physostigmine.

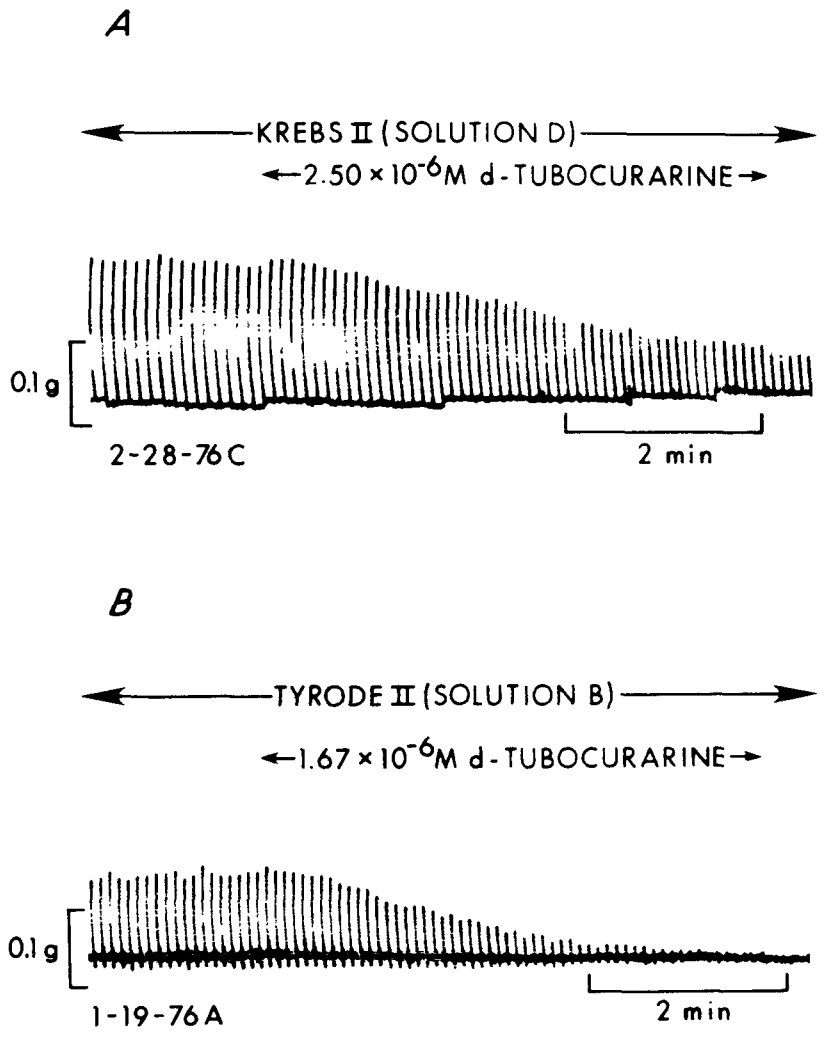

Figure 54. Suppression of the contractions of the rat diaphragm evoked by nerve stimulations by 2.50 x 10^{-6} M d-tubocurarine in solution D (record A) and by 1.67 x 10^{-6} M d-tubocurarine in solution B (record B).

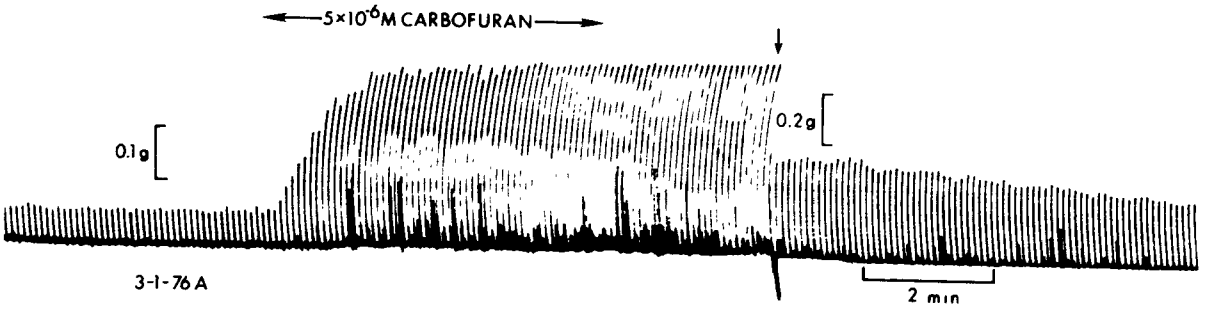

Figure 55. Potentiation of the nerve evoked contraction of rat diaphragm by 5×10^{-6} M carbofuran. The sensitivity of recording system is reduced to one-half at vertical arrow.

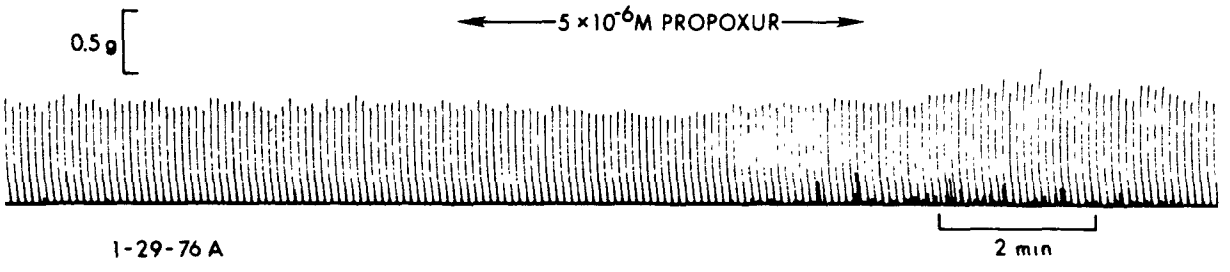

Figure 56. Absence of the effect of 5 x 10^{-6} M propoxur on the nerve evoked contraction of rat diaphragm.

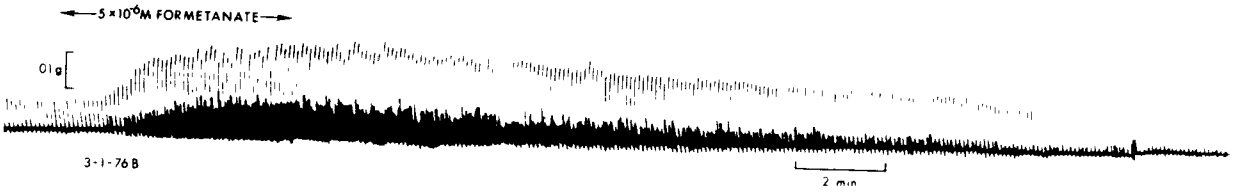

Figure 57. Potentiation of the nerve evoked contraction of rat diaphragm by 5×10^{-6} M formetanate.

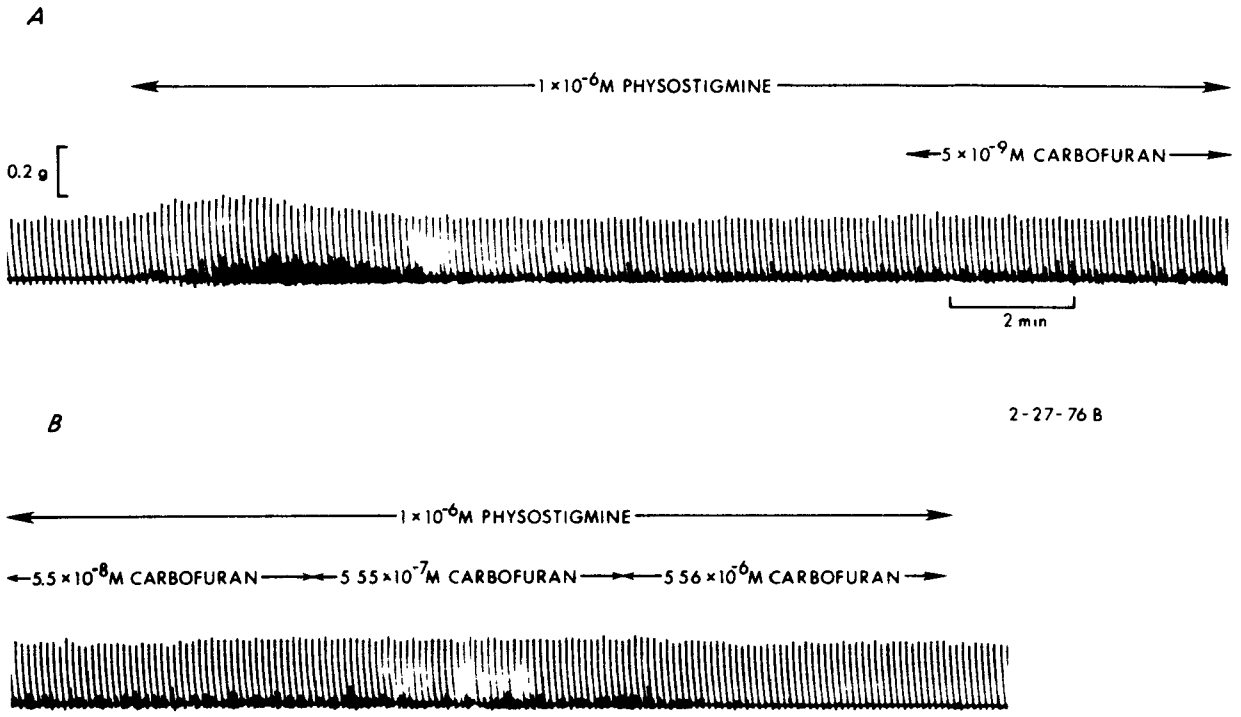

Figure 58. Absence of the effects of carbofuran (5×10^{-9} M to 5.56×10^{-6} M) on the nerve evoked contractions of the rat diaphragm pretreated with 1×10^{-6} M physostigmine.

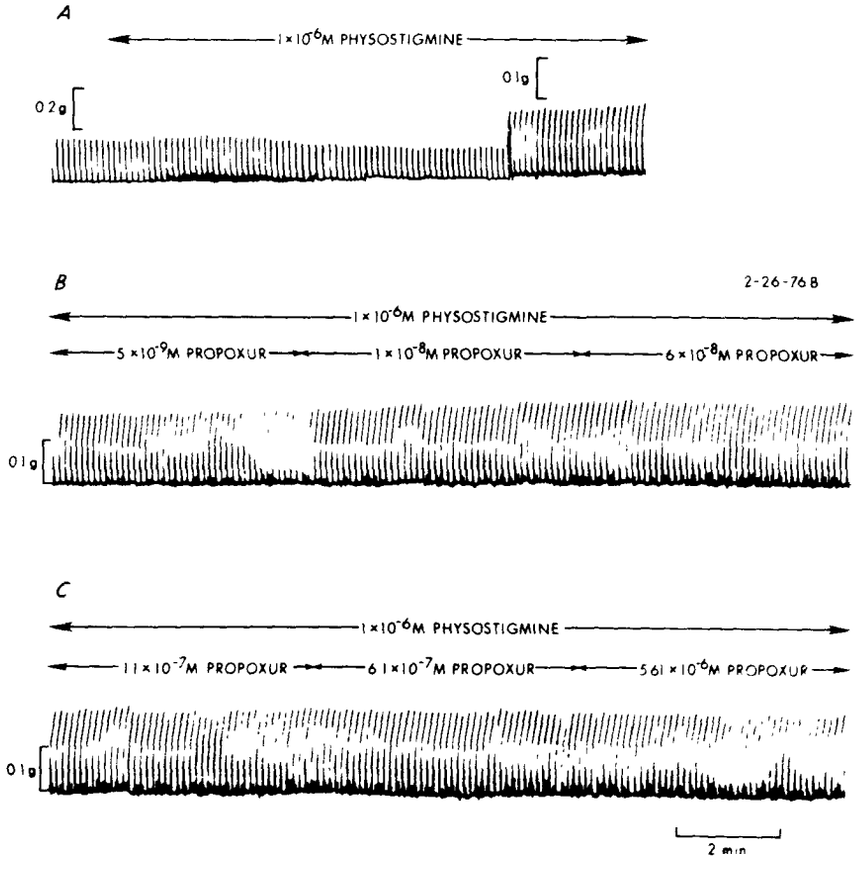

Figure 59. Effects of propoxur (5×10^{-9} M to 5.61×10^{-6} M) on the nerve evoked contractions of the rat diaphragm pretreated with 1×10^{-6} M physostigmine. The contractions are potentiated only slightly.

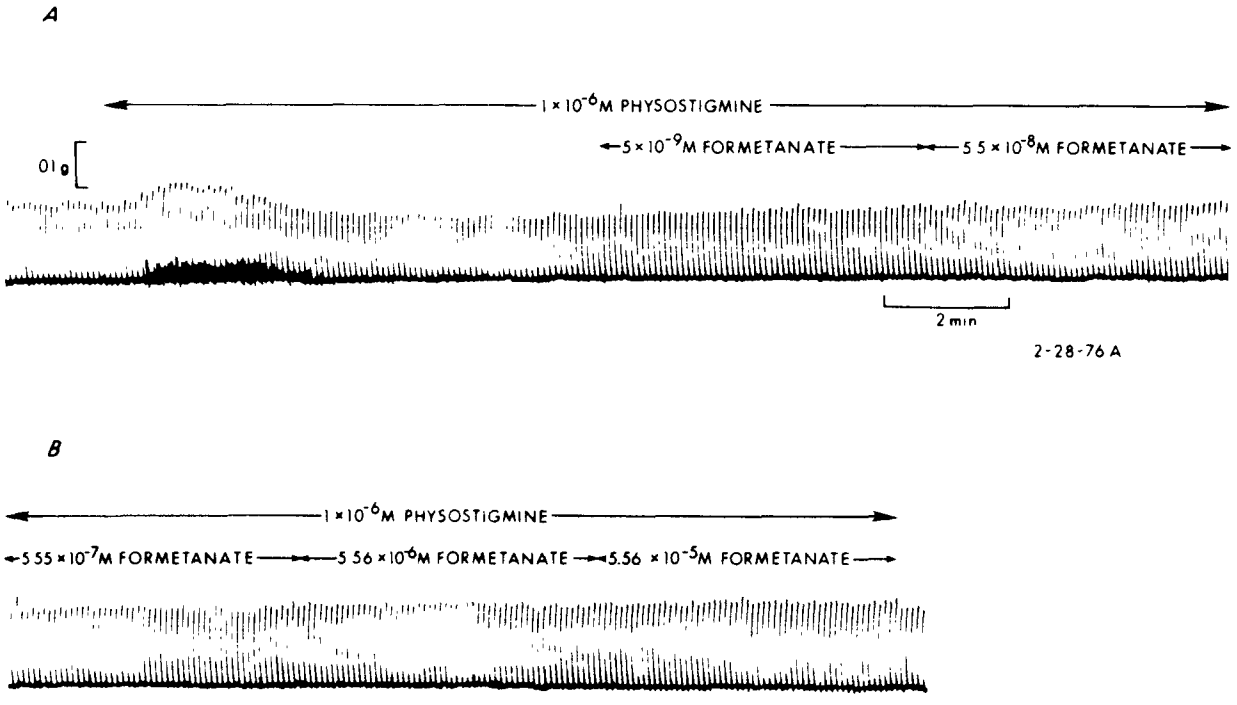

Figure 60. Effects of formetanate (5×10^{-9} M to 5.56×10^{-5} M) on the nerve evoked contractions of the rat diaphragm pretreated with 1×10^{-6} M physostigmine. The contractions are potentiated only slightly.

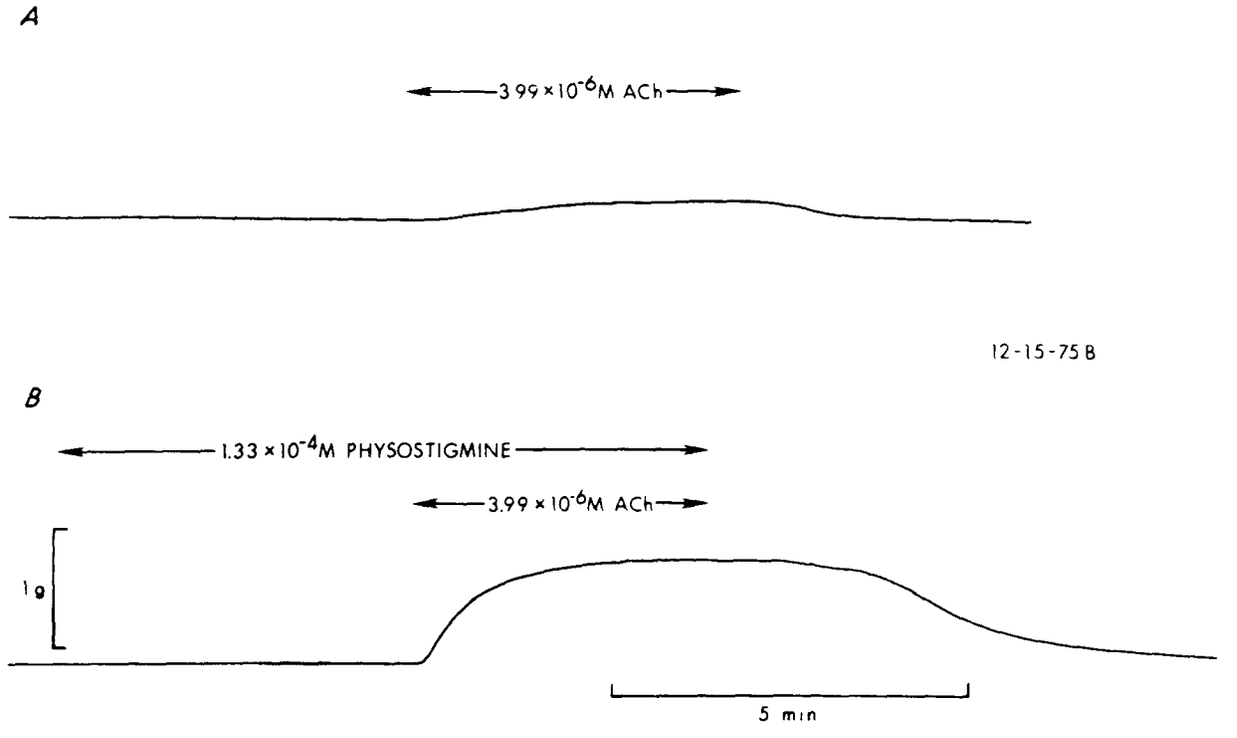

Figure 61. Contraction of the frog rectus abdominis by 3.99×10^{-6} M acetylcholine (ACh) (A), and potentiation by 1.33×10^{-4} M physostigmine (B).

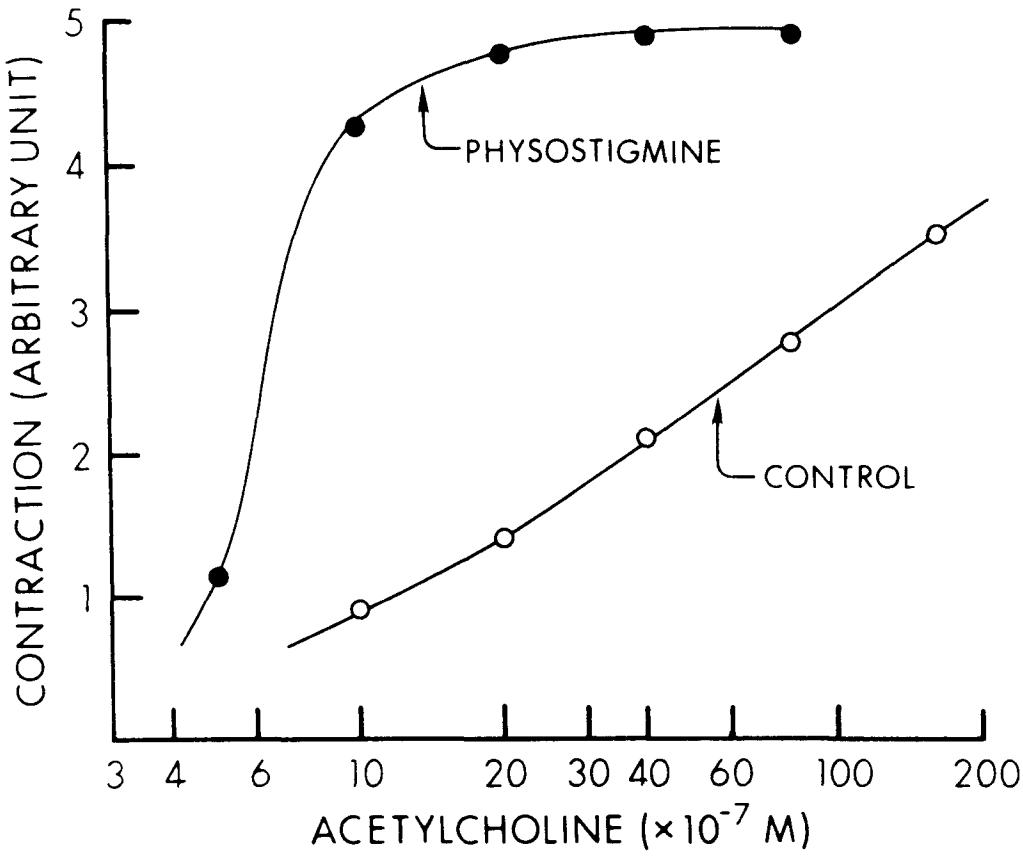

Figure 62. Dose-response relation for acetylcholine contraction of frog rectus abdominis with and without pretreatment with 8.07×10^{-5} M physostigmine for 5 min.

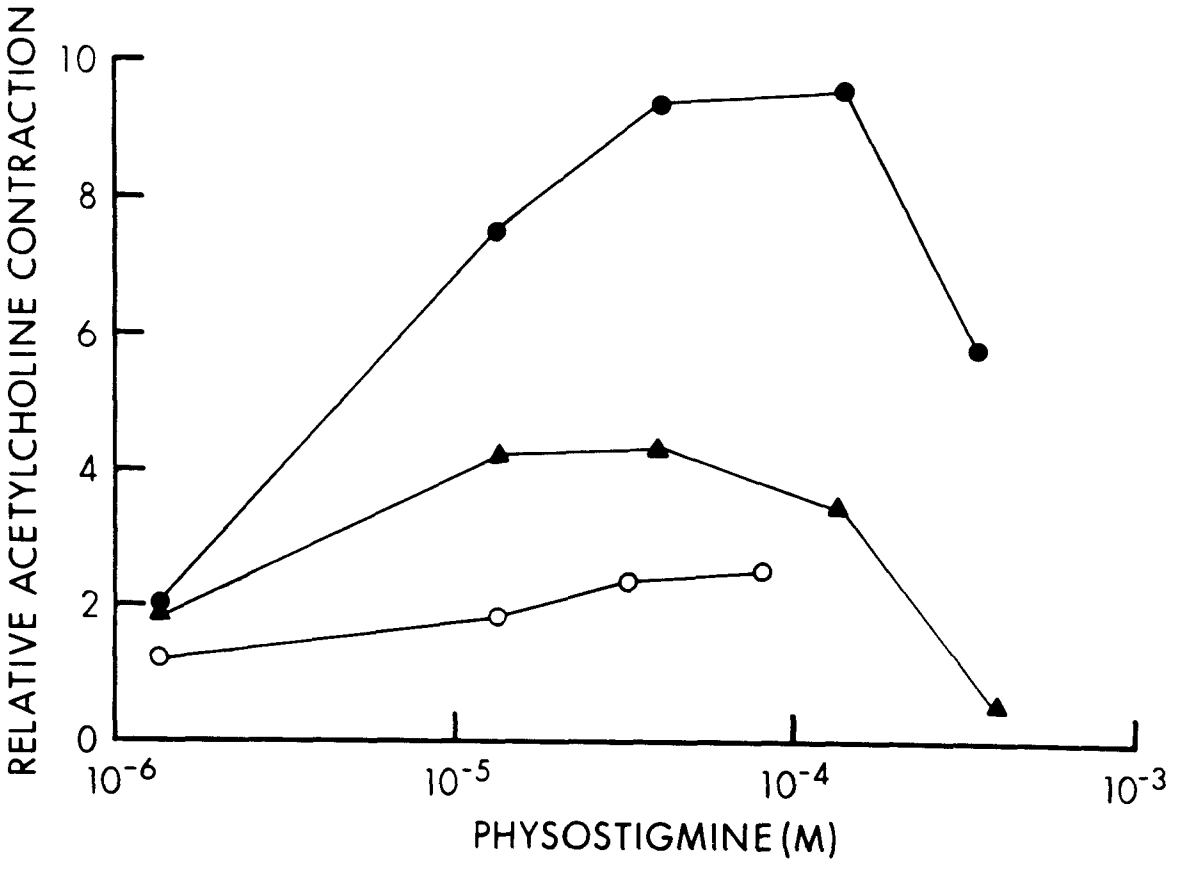

Figure 63. Effects of pretreatment with various concentrations of physostigmine for 5 min on acetylcholine (3.99×10^{-6} M) contraction of frog rectus abdominis. Each symbol represents each preparation.

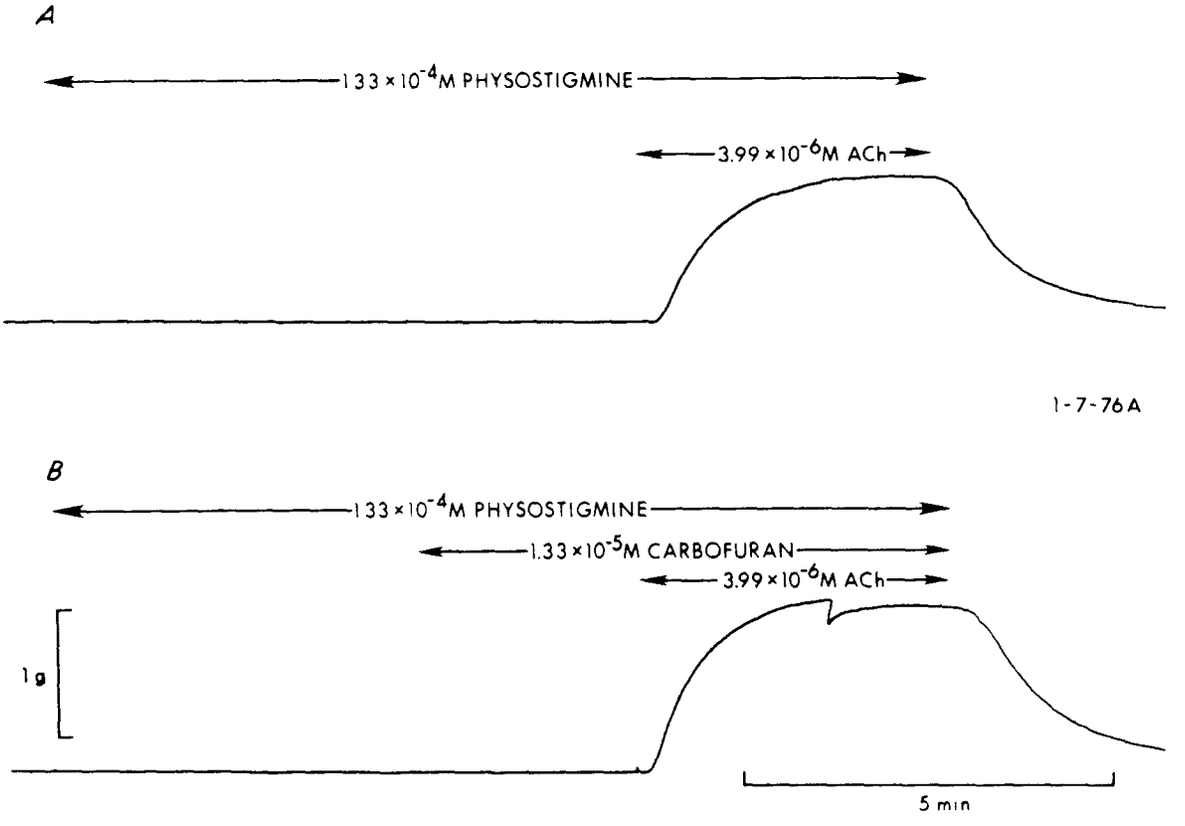

Figure 64. Effect of 1.33×10^{-5} M carbofuran on the frog rectus abdominis pretreated with 1.33×10^{-4} M physostigmine. Carbofuran does not cause contraction itself, and fails to potentiate markedly the contraction evoked by 3.99×10^{-6} M acetylcholine (ACh).

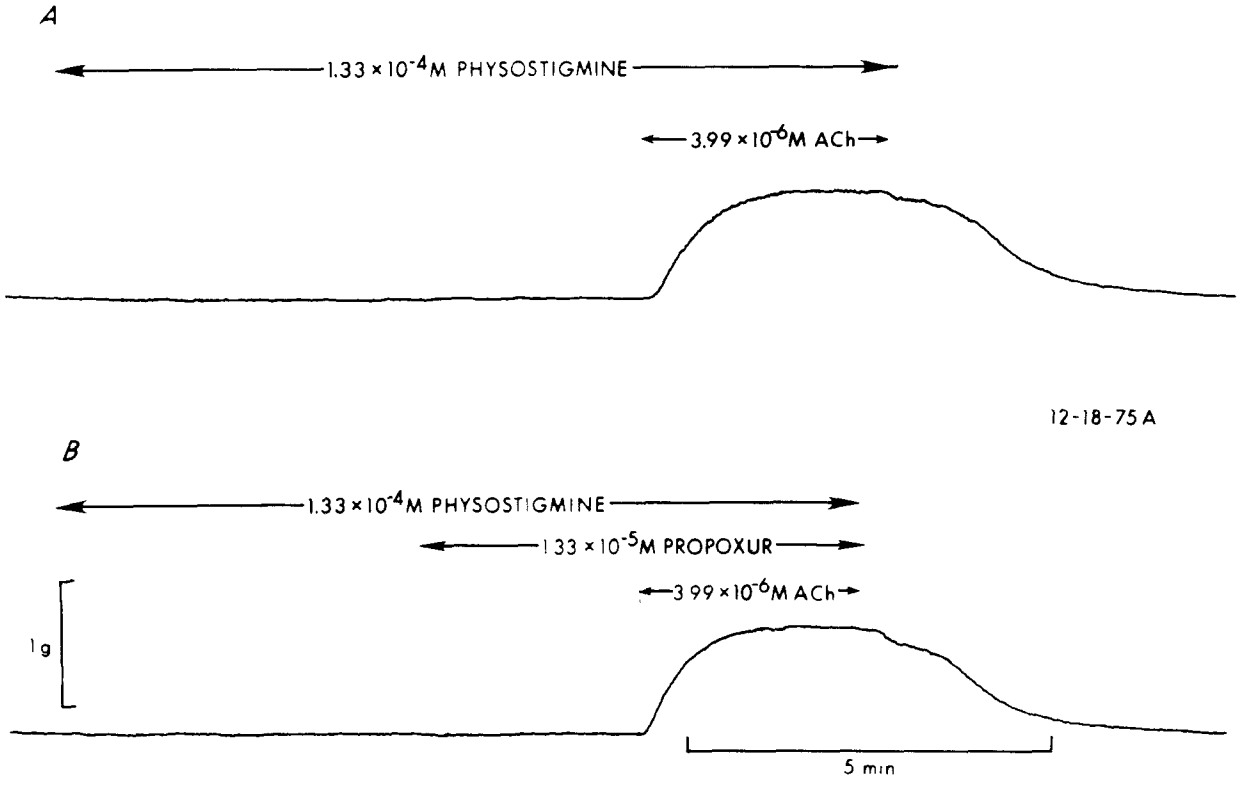

Figure 65. Effect of 1.33 x 10^{-5} M propoxur on the frog rectus abdominis pretreated with 1.33 x 10^{-4} M physostigmine. Propoxur does not initiate contraction by itself, and fails to potentiate the contraction evoked by 3.99 x 10^{-6} M acetylcholine (ACh).

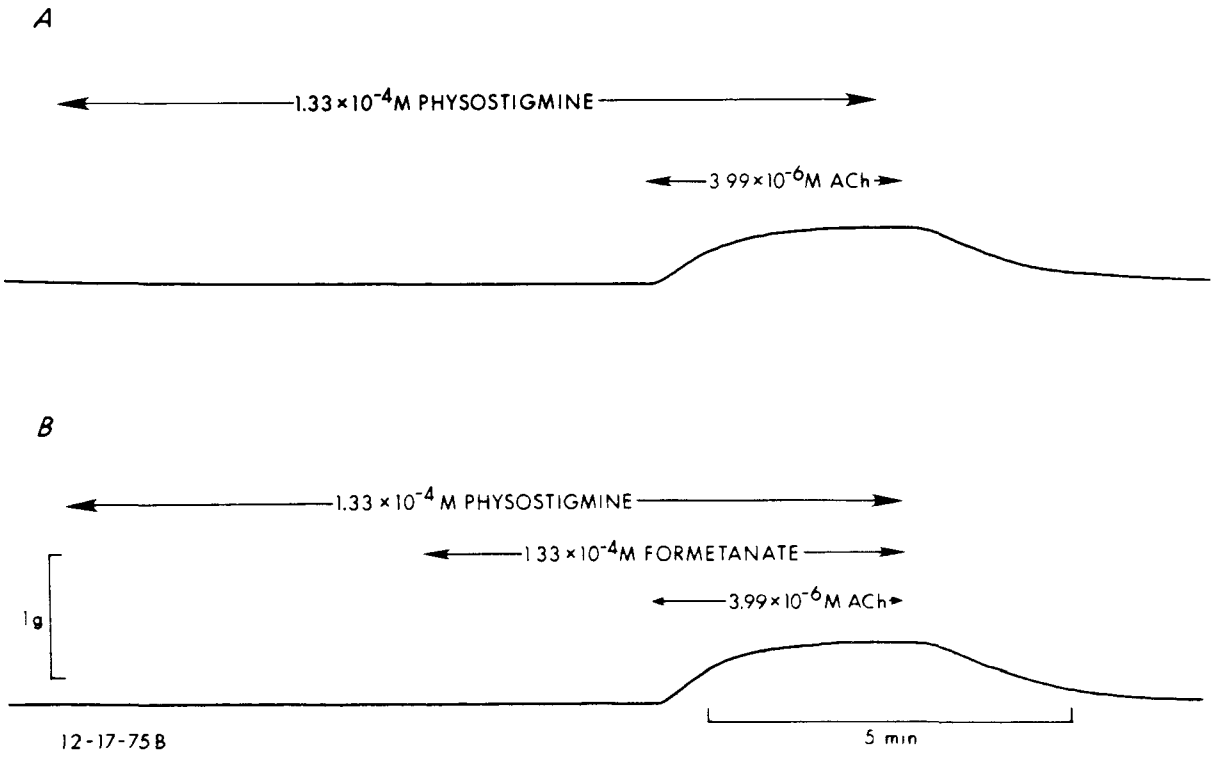

Figure 66. Effect of 1.33×10^{-4} M formetanate on the frog rectus abdominis pretreated with 1.33×10^{-4} M physostigmine. Formetanate does not initiate contraction by itself, and fails to potentiate the contraction evoked by 3.99×10^{-6} M acetylcholine (ACh).

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16. ABSTRACT

Several skeletal muscle and smooth muscle preparations have been examined for their usefulness in evaluating the toxic effects of a variety of insecticides. The following preparations were found satisfactory for such test: guinea pig ileum for muscarinic receptors, guinea pig heart for β-adrenergic receptors, guinea pig vas deferens for α-adrenergic receptors, frog rectus abdominis for nicotinic receptors of tonic muscle, and rat diaphragm for nicotinic receptors of phase muscle. Five carbamate insecticides, four organophosphate insecticides and chlordimeform were studied. None of the insecticides tested had any direct and potent effect on these receptors except the effect on cholinergic receptors via cholinesterase inhibition. Carbofuran, propoxur and formetanate had potent stimulating actions on the guinea pig ileum, but these effects could entirely be attributed to the accumulation of acetylcholine in the synaptic cleft as a result of cholinesterase inhibition. Thus, it can be concluded that these insecticides exert no direct action on cholinergic and adrenergic receptors.

17. KEY WORDS AND DOCUMENT ANALYSIS		
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