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REVIEW ARTICLE

Pharmacological Testing Methods for Drugs Acting on the Peripheral Nervous System

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Keyphrases ☐ Pharmacological test methods—drug activity, peripheral nervous system ☐ Nervous system, peripheral—test methods, drug effects ☐ Observational studies, animals—drug effect, peripheral nervous system ☐ *In vivo* studies—drug effects, peripheral nervous system ☐ *In vitro* studies—drug effects, peripheral nervous system

CONTENTS

The goal of pharmacological testing of chemical agents is (*a*) to detect any biological activity that may be present. Following demonstration of activity the next steps are (*b*) to identify the type of activity and then (*c*) to compare the activity with the activity of a reference compound. To progress through steps *a* and *b* requires much experience and selective judgment. For comparison of activity, data from the experiments must be quantified. There is now a large number of excellent statistical procedures and a biologist must design his experiments so that one of the standard statistical procedures can be followed—otherwise an experimenter will end up with an experience and not an experiment. No reference to structure-activity relationships or even whether biological activity is present can be made until dose-response curves have been constructed and evaluated.

The hazards of pharmacological evaluation are many. The biologist must be aware and attempt constantly to eliminate as many variables as possible. He will always have fundamental questions that may be difficult to answer. Some of the points that must be considered are:

1. Selection of type of preparation is important. Preparations that are used widely are usually prudent choices.

2. The problem of choice of species is always present. To evaluate a particular type of pharmacological activity, choose a species that is highly sensitive to the particular agent. For example, one would not use the blood pressure of the rat to evaluate histamine-like compounds, *etc.*

3. It is necessary to determine the time-effect curves before attempting to compare the activity of two chemical agents. Agents will vary in time required for onset and duration of action. The agent's activity must be compared at time of peak effect.

4. The absorption, distribution, and excretion of agents in a given series may vary greatly. These factors

may be extremely important in determining the intensity and duration of biological activity.

5. In structure-activity relationship comparisons, the above variables make it highly desirable to evaluate compounds both *in vivo* and *in vitro*. If the relative activity of compounds evaluated in the two types of preparations remains constant, this is suggestive evidence that the difference in biological activity is related to differences in chemical structure and not related to factors such as plasma protein binding, metabolism, excretion, *etc.*

6. Perhaps an area where erroneous assumptions are sometimes made is in assuming that two compounds have the same mechanism of action. Unless two compounds have the same mechanism of action, one cannot compare statistically their biological activity. For example, it has recently been shown that choline is apparently devoid of muscarinic activity.[1] The literature contains many citations of the relative muscarinic activity of choline and acetylcholine and these comparisons are probably invalid. As for references to "mechanisms of action," this term means different things to different investigators. To a physiologist this term may refer to interactions at the organ level. A pathologist may be interested in cell damage or structural changes. A biochemist may be concerned with enzyme inhibition or activation. A molecular biologist may be interested in drug-receptor interactions, *etc.* All of these investigators using the techniques of their respective disciplines feel that they are determining the "mechanism of action." Each is for his particular biological level of integration, but one should remember that the "mechanism of action" at the molecular level awaits explanation for probably all drugs.

7. In comparing drugs one should also remember that although the end responses may look similar, the sites of action may be different. For example there could be a sequence of events as follows:

$$\underset{\text{drug}_1}{\overset{E_1}{S_1 \rightarrow}} \; \underset{\text{drug}_2}{\overset{E_2}{S_2 \rightarrow}} \; \text{response } (R)$$

where S = substrate, E = enzyme. Drug 1 may act on E_1 and drug 2 on E_2. Alteration of R may appear to be the same but the sites of action that altered the response are different.

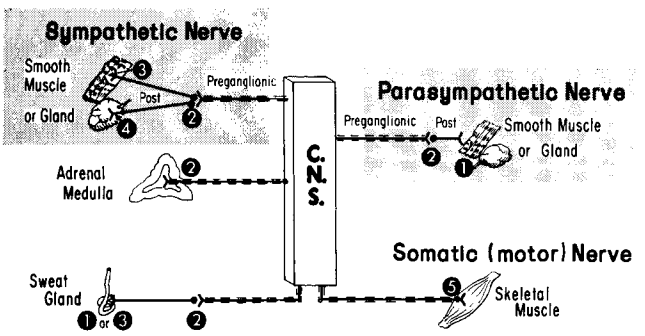

Figure 1—*A simplified diagram illustrating the sites of action of some standard drugs. The reference drugs that stimulate or inhibit at the various sites are listed in Table I.*

[1] When assayed using superfused guinea pig ilea, the intestinal stimulating action of choline is antagonized markedly by: (1) cooling the segment for 24 hr. at 2°, (2) treatment with triethylcholine, or (3) hexamethonium. None of these treatments alters the responsiveness of acetylcholine.

ANATOMY OF THE PERIPHERAL NERVOUS SYSTEM

Detailed outlines of the nervous system may be found in any standard physiology or pharmacology textbook. A simplified diagram illustrating the sites of action of some of the standard drugs is shown in Fig. 1 and Table I. Other anatomical points that should be considered in evaluating drug action are listed as follows:

1. Only postganglionic fibers are nonmyelinated.

2. Postganglionic sympathetic nerve fibers are usually long fibers with the ganglia outside the organs innervated. The ganglia and postganglionic parasympathetic nerve fibers usually lie within the organ innervated (the exceptions being the organs of the head and neck where the parasympathetic ganglia are on the surface or slightly removed from the organ that is innervated).

3. Various sympathetic fibers tend to be activated in unison. The activity of the parasympathetic nervous system tends to be much more localized—often serving as the efferent component of a reflex. For example, in a bright light miosis will occur, but the heart rate will not be slowed.

4. Acetylcholinesterase is found in high concentration where acetylcholine is the neurotransmitter. Inhibition of this enzyme with neostigmine or analogs or the organic phosphates will prolong the integrity of the acetylcholine molecule and increase its concentration at the transmitter site. The adrenergic enzymes, catechol-*O*-methyltransferase and monoamine oxidase, probably play a minor role in terminating the action of norepinephrine that has been liberated from the nerve terminal. Diffusion of norepinephrine from the receptor site appears to be the major terminating mechanism.

5. During the past 20 years evolution of the alpha and beta receptor theory has been seen along with the development of the respective stimulating and inhibiting agents. The major organs with their predominant type of receptor innervation are shown below:

Alpha Receptor	*Beta Receptor*
Vasoconstriction (cutaneous, renal, *etc.*)	Vasodilation (sk. muscle, *etc.*)
Myocardial ectopic excitation	Cardioacceleration
Splenic-capsule contraction	Myocardial augmentation
Myometrial contraction (rabbit, dog, human, *etc.*)	Myometrial relaxation (rat, nonpregnant cat, human)
Iris dilator contraction (mydriasis)	Bronchial relaxation
Nictitating membrane contraction	Intestinal relaxation
Intestinal relaxation	
Pilomotor contraction	
Glycogenolysis (?)	

6. The influence of drugs on the cholinergic nerve terminal has not been widely explored, but the future will probably see a marked increase of research activity. Evidence is appearing that many "nicotinic" agents may be acting by releasing acetylcholine from preganglionic or motor nerve terminals. These would be agents such as nicotine or choline. A number of agents have been reported that will inhibit or block the cholinergic nerve

Table I—Stimulants and Inhibitors at Various Sites in the Peripheral Nervous System

Site	Name	Neurotransmitter	Stimulants	Inhibitors
1	Postganglionic parasympathetic	Acetylcholine	Acetylcholine Methacholine Carbachol Neostigmine DFP	Atropine Scopolamine
2	Ganglionic	Acetylcholine	Acetylcholine Nicotine Tetramethylammonium Neostigmine	Hexamethonium (C_6) Nicotine (large concn.)
3	Postganglionic sympathetic	Norepinephrine	Norepinephrine (α) Epinephrine (α or β) Isoproterenol (β)	Phentolamine (α) Propranolol (β)
4	Sympathetic nerve terminal		Tyramine Ephedrine Cocaine	Reserpine Guanethidine Brethylium
5	Neuromuscular junction	Acetylcholine	Acetylcholine Nicotine	Curare Succinylcholine

terminal. These agents would include high Mg^{++} concentrations, hemicholinium (HC-3), triethylcholine, hexamethonium, or tetrodotoxin (puffer fish poison).

Since a drug or experimental chemical can only alter (stimulate or inhibit) the basal activity of the peripheral nervous system, one must understand the physiology of transmission. With this information one should have no difficulty in classifying autonomic agents.

The major influence of the autonomic nervous system innervation on the major organ system is outlined in Table II.

TERMINOLOGY

Often the same site of drug action is referred to by different names. For a large surplus of descriptive names, the autonomic drugs probably have a longer list than other classes of agents. The long list of synonyms used to describe autonomic drugs make the literature difficult to read for a nonpharmacologist. Table III lists those terms used commonly in the literature to describe actions of agents on the peripheral nervous system.

METHODS

The following portion of the manuscript discusses some of the procedures used to study the activity of chemical agents on the peripheral nervous system. The authors have tried to select preparations that are used quite widely and those with which they have had personal experience. Page space makes it necessary for the user of these preparations to refer to the original manuscripts for detailed procedures. All individuals interested in drug development must be familiar with a wide variety of preparations. One must clearly understand the uses and limitations of each preparation. In order to evaluate and explore new mechanisms, a biologist may need to develop his own special experimental procedures. The science of methodology is probably the pharmacologist's major tool that will lead to the discovery of new types of biologically active agents.

Observational Studies of Animals

Available methods for detecting drug actions in the intact unanesthetized animal are convenient and valuable but are less reliable and nonspecific in the analysis of individual drug effects. They can be used only for preliminary confirmation of drugs affecting the peripheral nervous system. Therefore, no attempt will be made to describe or to discuss these methods in detail. For definitive studies on the sites of drug actions, the *in vivo* and *in vitro* methods described in this paper should be employed.

Pupil Size—Mice are widely used in this experiment but dogs, cats, and rabbits can also be utilized. The pupil size is controlled primarily by tonic activity of the cholinergic nervous system. An increase in cholinergic activity or a blockade of adrenergic activity produces

Table II—Usual Responses Seen with Autonomic Function Alteration (Stimulation or Inhibition)[a]

Organ	Sympathetic: Stimulation	Sympathetic: Inhibition	Parasympathetic: Stimulation	Parasympathetic: Inhibition
Heart				
Rate	↑	↓ or 0	↓[b]	↑ or 0
Force	↑	↓ or 0	↓[b]	↑ or 0
Arterioles	↑	↓	—[c]	—
Veins	↑	↓	—[d]	—
Blood pressure	↑	↓ or 0	↓[e]	↑ or 0
Eye				
Miosis (constriction)	↓	↑	↑	↓
Mydriasis (dilatation)	↑	↓	↓	↑
Intraocular pressure	↓	0	↓	↑
Bronchi	↓	↑ or 0	↑	↓ or 0
Salivary glands	↑[f]	0	↑[g]	↓
Gastrointestinal tone	↓	↑ or 0	↑	↓ or 0
Sweat glands	↑	↓ or 0	↑	↓

[a] ↑, increased, stimulated; ↓, decreased, inhibited; 0, no change; —, not relevant. [b] Acetylcholine in the presence of atropine will produce a positive inotropic and chronotropic action. [c] Though there are no parasympathetic nerve fibers to arterioles, acetylcholine may be a powerful dilator (for example the limbs or the vascular beds of skeletal muscle). In some vascular beds ACh will induce constriction—aortic strips, rabbit ear, renal artery. [d] The cephalic vein is constricted by large doses of ACh. [e] Large doses of ACh administered intravenously will produce a pressor response. This response will be enhanced by prior treatment with atropine. [f] Small volume, high viscosity. [g] Large volume, low viscosity.

Table III—Synonymous or Related Terms Used in Autonomics

Site	Stimulant	Blocking Agent
Ganglia (sympathetic & parasympathetic)	Ganglionic stimulant Nicotinic Preganglionic stimulant	Ganglionic blocking agent Ganglionic depressant Preganglionic depressant
Postganglionic parasympathetic neuroeffector junction	Postganglionic parasympathetic stimulant Cholinergic Muscarinic Parasympathetic stimulant Parasympathomimetic Cholinomimetic	Postganglionic parasympathetic blocking agent Anticholinergic Antimuscarinic Parasympathetic blocking agent Parasympatholytic Cholinolytic
Postganglionic sympathetic neuroeffector junction	Postganglionic sympathetic stimulant Adrenergic stimulant Sympathetic stimulant Sympathomimetic Releasing agents	Postganglionic sympathetic blocking agent Adrenergic blocking agent Sympathetic blocking agent Sympatholytic Antirelease agents Depleting agents "False transmitters" Myoneural blocking agents
Myoneural junction neuromuscular junction	Myoneural stimulants Nicotinics	Myoneural depressants Neuromuscular blocking agents Relaxants Muscle relaxants

miosis, whereas an increase in adrenergic activity or a blockade of cholinergic activity produces mydriasis. The pupil size of mice can be measured with a dissecting microscope *via* a calibrated eyepiece (1–3). The drugs are administered parenterally (3) or topically on the cornea (1, 2).

Tone of the Nictitating Membrane—The position of the nictitating membrane is controlled primarily by the tonic activity of the adrenergic nervous system. Adrenergic agents cause contraction of this membrane while adrenolytic agents produce relaxation. Although cats are most satisfactory in this test, dogs and rabbits may also be used (4).

Respiratory Arrest—Subsequent to drug administration, respiratory arrest prior to cardiac arrest indicates that either the respiratory center is inhibited or that the neuromuscular junction of the phrenic nerve diaphragm is blocked. Mice can be used satisfactorily for this test (5). Central and peripheral effects can be differentiated by using isolated preparations.

Blood Pressure and Heart Rate—The overall peripheral vascular resistance is regulated by the adrenergic nervous system which can be blocked by adrenolytic agents or physiologically antagonized by cholinergic agents to produce hypotension. The heart rate is controlled primarily by the cholinergic mechanism which can be blocked by cholinolytic agents or physiologically antagonized by adrenergic agents to induce tachycardia. The indirect methods for blood pressure determination in dogs are described by Prioli and Winbury (6) and those in rats are reviewed by Van Proosdij-Hartzema (7) and Boura and Green (8). The advantages and disadvantages of these methods are discussed by Fregly (9).

In Vivo Study

Dog Blood Pressure Preparations—Mongrel dogs are anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of barbital sodium administered intravenously. The trachea is cannulated and the vagi are sectioned to eliminate the vagal reflexes. The arterial pressure is measured with a pressure transducer (10–13) or mercury manometer (11, 14). The blood pressure can be measured at the common carotid artery, the femoral artery, or any other large artery. All compounds are injected *via* a polyethylene catheter inserted into the femoral vein. The injection is followed immediately by a wash of isotonic saline. In all cases the total volume of the injected solutions is kept constant. However, drugs can be injected directly into the femoral vein without using the polyethylene catheter. In this case, the isotonic saline wash is not necessary. The species of animal, the routes of administration, the procedures of cannulation, and the methods of recording may all be varied to meet the needs of the investigator. This preparation can also be done with rats, cats, and rabbits. Depressor responses that are blocked by atropine sulfate are regarded as muscarinic and pressor reponses after 1–2 mg./kg. of atropine sulfate are regarded as nicotinic. However, due to the multiple factors affecting the mean systemic pressure, interpretation of the pressure change is difficult. If the blood flow in the large vessels is recorded by use of an electromagnetic flowmeter (4) simultaneously with the pressure change, then the interpretation of the experimental results is facilitated.

The catecholamines released by ganglionic stimulants can be estimated by collecting blood samples at the time of peak pressor response. Blood cells and protein in the blood samples are removed (15) and the catecholamines in the final samples are assayed fluorometrically using the method described by Chang (16). The sites of catecholamine release, either from the adrenal medulla (mainly epinephrine release) or from sympathetic nerve terminals (mainly norepinephrine release), have thus been differentiated (14).

The blood pressure preparation is widely used to determine dose-response curves and activities of drugs (10–12, 14). The eviscerated cat blood pressure preparation is quite sensitive to acetylcholine and can readily detect 0.002 mcg. ACh/kg. (17). The rat preparation is also widely used for bioassay of minute amounts of acetylcholine and the analogs from tissue extracts (11, 18, 19).

Recently more attention has been given to the direct measurement of blood pressure using unanesthetized animals. This preparation enables investigators to study the drugs with a very long duration, to repeat measurements of blood pressure over a long period of time, and to avoid the untoward interaction between anesthetics and drugs to be studied. The advantages and disadvantages of both preparations have been discussed by Freyburger (20). For dogs, the polyethylene catheters are implanted chronically in various parts of the circulation (21), mostly into thoracic aorta (22, 23) and the femoral artery (20). The transcutaneous needle puncture of a femoral artery immediately before experimenting is also used and is the most simple method to be performed with satisfactory results. Hypodermic needles (19–21 gauge) are widely used to obtain full pulse pressure measurements. However, smaller needles are recommended to avoid vessel hardening and hematomas (20, 24). The polyethylene catheters or hypodermic needles are connected to the pressure transducer for recording the blood pressure described previously. The direct measurements of blood pressure on unanesthetized rats are described by Weeks and Jones (25) and Fujita and Tedeschi (26).

Cat Superior Cervical Ganglia Preparation—Cats are anesthetized with 30–35 mg./kg. of pentobarbital sodium administered intrathoracicly. The trachea is cannulated as usual and the carotid artery is exposed. The lymphatic gland is removed and all the arterial branches, except those running to the tissue around the superior cervical ganglion, are tied and divided. The small internal carotid artery and veins are divided, leaving only the vein from which perfusion fluid is to be collected, usually the internal jugular vein. A length of cervical sympathetic nerve, sufficient for placement of the electrodes, is freed and the cat's head is then fixed in position so that the contraction of the nictitating membrane can be recorded. The common carotid artery is then ligated and perfused. A small cannula is then tied into the internal jugular vein to collect the perfusion fluid. The electrodes are placed on pre- and postganglionic fibers (27–29). In order to prevent blood coagulation during manipulation, 0.5 ml./kg. of 8% solution of chlorazal fast pink or 5 mg./kg. of heparin sodium is injected intravenously (30). The nerve is stimulated with monophasic pulses, 6 msec. in duration, with maximal voltage of 10–15 v. and frequencies of 10–25 c.p.s. (31). A viable preparation will respond to single shock stimulation. This is an extremely useful preparation for detecting the release of acetylcholine from ganglia, either by preganglionic nerve stimulation (32, 33) or by close arterial injection of the drugs (34, 35). The effects of drug and nerve stimulation can be detected from the responses of nictitating membrane. An ingenious experiment was devised by Collier *et al.* (36) in which the ACh in the ganglia was replaced with ^{3}H-ACh through perfusion of the ganglia with Locke solution containing ^{3}H-choline during continuous preganglionic stimulation. Both preganglionic stimulation and close arterial injection of carbachol released radioactive materials and contracted the nictitating membrane, indicating that endogenous ACh was released during these treatments. The close arterial injection of ACh, however, contracted the nictitating membrane without releasing appreciable amounts of radioactive substance. This suggests that ACh might not be involved in the release of endogenous ACh from the preganglionic site.

Dog Chorda Tympani–Wharton's Duct Preparation—Dogs weighing 12 kg. or more are anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of the barbital sodium administered intravenously. The trachea is cannulated. Wharton's ducts are located directly beneath the mylohyoideus and extend across the lingual nerve between the chorda tympani nerve and the submandibular ganglion. Both Wharton's ducts and the chorda tympani nerve are ligated. The chorda tympani nerve is cut free from the lingual nerve with a thread attached to the nerve end. One of the Wharton's ducts is then cannulated with polyethylene tubing (PE-60) which is attached to a reservoir filled with physiological saline. The outflow of the reservoir is connected to a dropping tube and a Becker signal magnet is connected to a relay which is activated by each drop. A shielded silver electrode is attached to the chorda tympani nerve for stimulation. The nerve is stimulated supramaximally with 15 v. for a duration of 2 msec. with a single biphasic impulse and with a frequency of 20 c.p.s. for 1 sec. every 10 sec. (37). After 10–20 min. the saliva flow will remain quite constant for at least 90 min.

Salivation from the Wharton's duct can be stimulated either by drugs such as pilocarpine, acetyl-β-methylcholine, and acetylcholine injected intravenously into the femoral vein or by nerve stimulation. The salivation is effectively blocked by atropine. This preparation is useful for studying drugs which act on or block the cholinergic system causing salivation. Hemicholinium has been shown to block gradually the salivation induced by chorda tympani nerve stimulation, suggesting that its blocking effect is due to the inhibition of ACh synthesis in the nerve tissues (37).

Rabbit Sciatic Nerve–Gastrocnemius Muscle Preparation—Dutch rabbits, weighing 1–2 kg., are anesthetized with 200 mg./kg. of phenobarbital sodium administered slowly (at least 2 min.) into the marginal ear vein. The sciatic nerve is ligated and cut, and a shielded electrode is placed on the peripheral portion of the nerve. The gastrocnemius muscle is freed as completely as possible from surrounding muscles and a thread is attached to the tendon of the muscle. The twitches of the muscle are elicited by supramaximal stimulation and are recorded through a force transducer. The parameters for interrupted tetanic stimulation are 250 c.p.s. with pulse duration of 1 msec. at 15 v. applied for 0.2 sec. every 10 sec. Single shock stimulation is performed with pulse duration of 5 msec. and a supramaximal voltage of 15 v. is applied every 10 sec. The drugs are administered intravenously into the marginal ear vein. This is a convenient and simple preparation for studying drugs acting at the neuromuscular junction *in vivo* (37–41). Although there are some *in vitro* neuromuscular preparations available, this preparation has the advantage of studying the drug effects under physiological conditions. For example, hemicholinium and its derivatives have been shown to block neuromuscular transmission in all preparations. In this preparation, blockade can be efficiently reversed by choline (39–41)

but this is difficult using *in vitro* preparations. Animals other than rabbits, such as cats and rats, can be used satisfactorily.

There are two other preparations, the cat soleus muscle preparation and the cat tibialis anticus muscle preparation which are very useful and similar to the gastrocnemius muscle preparation. These can be prepared in a method similar to that described for gastrocnemius muscle except that close arterial injection can be made using the tibialis anticus preparation. Also, soleus muscle consists primarily of slow muscle, whereas tibialis anticus is fast muscle (42). The experimental procedures for these two preparations are described by some investigators in detail (42–44). The characteristics of slow and fast muscles are analyzed anatomically, histochemically and biochemically by some investigators. The fast muscle is composed of three distinct fibers (45) whereas the slow fiber is relatively homogeneous, consisting primarily of Type B fibers (46). Also, the fast muscle contains higher activities of glycolytic enzymes and lower activities of oxidative enzymes, while the reverse is true for the slow muscle (47, 48). The effects of drugs on slow and fast muscles will be discussed further in the following sections.

In Vitro Study

The organ bath method is the most widely used technique for studying isolated organs. The equipment is rather simple and is available commercially. Usually, the organ bath is made of glass and placed inside a water bath so that the temperature can be well controlled. The physiological salt solution is warmed before it is added to the organ bath, so that there will be no temperature change when the tissue is washed. The physiological salt solution can be warmed either by putting the reservoir bottle in a water bath or by passing the physiological solution through coils connected to the organ bath within the same water bath. The isolated tissue is mounted in such a way that one end of the tissue is fixed at the bottom of the organ bath while the other is fixed to the recording lever or a force transducer for isotonic or isometric recording. The organ bath varies both in size and volume depending on the specific purposes and the size of tissues. The volume of the drug solutions to be added to the organ bath should be less than 10% of the total volume of the bath fluid to avoid possible decrease of fluid temperature in the organ bath.

The superfusion method was first described by Finkleman (49) and modified by Gaddum (50). It is more rapid and more sensitive than the organ bath method for bioassay. This technique reduces the volume of solution to an absolute minimum by replacing the organ bath with a slow flow of bathing fluid over the external surface of the tissue. Consequently, the total amount of drug required to induce a response in the tissue is very small. Recently, this technique has been used successfully to study the fate and release of vasoactive hormones in the circulation (51–53). In this application the heparinized blood was continuously removed from the anesthetized animal, assayed for its hormone content by superfusion over a series of isolated muscle preparations, and then returned to the animal body intravenously.

For superfusion, the isolated tissue is mounted in air in the middle of a wide glass tube immersed in a warm water bath. The physiological salt solution is oxygenated and kept in a bottle immersed in a warm water bath. It is then pumped through a Holter motor pump to the top of the isolated tissue. The rate of flow of the superfusion fluid is adjusted to 1–5 ml./min. depending on the size of the tissues; the drug solutions are injected into the stream of the superfusion fluid in volumes of not more than 0.1 ml. Of course drugs may be added directly to the perfusing solution. Isometric recordings are preferred to isotonic recordings because the changes in the length of the tissue may dislocate the superfusion fluid in some cases and cause dryness of part of the tissue.

Skeletal Muscle Preparations—*Frog Rectus Abdominis Muscle Preparation*—The frog rectus abdominis muscle is usually obtained from *Rana pipiens* weighing approximately 20 g. The frog is stunned and decapitated. The spinal cord is destroyed with a long needle. The rectus abdominis muscles are dissected from the pelvic girdle to their insertion in the cartilage of the pectoral girdle. The threads are attached to both ends of the muscle before they are dissected from the body. An initial tension of 0.5–1.0 g. is placed on the rectus muscle. The preparation is kept at room temperature (25°) in frog Ringer solution (NaCl, 6.5; KCl, 0.14; NaH_2PO_4, 0.005; glucose, 2.0; $NaHCO_3$, 0.4; and $CaCl_2$, 0.12 g./l.) and oxygenated with 95% O_2 and 5% CO_2. At least 30 min. is allowed for the preparation to stabilize. This preparation was orginally described by Burn (54). Both the organ bath method (5–10 ml.) (54–56) and the superfusion technique (10, 39) can be used to perform experiments. The frog Ringer solution can also be made by diluting 1 l. of Ringer solution to 1.4 l. with distilled water.

The rectus abdominis muscle preparation is extremely useful because it develops slow contracture in responding to acetylcholine. This contracture is blocked by curare and its derivatives. Depolarizing agents, such as decamethonium or succinylcholine, will induce contracture that is similar to acetylcholine. Although the semispinalis muscle of the chick has the same useful properties, the simplicity and economy of the rectus preparation are widely appreciated by many investigators.

After contraction, the rectus muscle does not relax rapidly. It is, therefore, difficult to obtain the straight base line which is essential for bioassay. Thus, in order to obtain this straight base line, the muscle must be stretched gently by increasing the tension. This disadvantage can be eliminated by using isometric recording instead of isotonic recording. Also since the rectus muscle does not relax rapidly, it takes at least 6-min. intervals between doses in the organ bath method to assay acetylcholine. The superfusion technique is considerably faster, since it requires only 1–2-min. intervals between doses.

The rectus muscle preparation can also be used for assaying acetylcholine and its derivatives, although its sensitivity is less than that of the guinea pig ileum, chick semispinalis muscle, and leech dorsal muscle treated with physostigmine. The sensitivity of

the preparation can be increased markedly by using the superfusion technique instead of the organ bath method and by treating the rectus muscle with physostigmine or neostigmine. The frog rectus muscle preparation, treated with 1.5×10^{-6} mole of physostigmine, can detect 0.01 mcg./ml. of acetylcholine chloride (1×10^{-7} mole) in the organ bath method (55) or 0.01 mcg. of acetylcholine chloride per injection in the superfusion method (10, 39).

Leech Dorsal Muscle Preparation—The Leech, *Hirudo medicinalis*, is pinned on its back through the mouth and the tail sucker and a cut is made along the two pale lateral lines to get two parallel strips from the dorsal body wall. The threads are attached at both ends of each piece. The muscle is suspended at room temperature (25°) in frog Ringer solution, or in leech Locke solution. These solutions are made by diluting 1 l. of Ringer solution or Locke solution (NaCl, 9.0; KCl, 0.42; glucose, 1.0; $NaHCO_3$, 0.5; and $CaCl_2$, 0.12 g./l.) to 1.4 l. with distilled water. The solution is oxygenated with 95% O_2 and 5% CO_2. An organ bath with a capacity of 5–10 ml. is used. The preparation must be stabilized for about 3 hr. before an experiment is begun. The contraction responses are very slow. However, unlike the frog rectus muscle, it should not be stretched by increasing the tension. Consequently, the responses are slow and it is necessary to allow at least 20 min. between doses (56). Physostigmine (3×10^{-5} mole) is preferred to neostigmine (3×10^{-4} mole) in order to sensitize this preparation (57). The muscle can be sensitized about 1000-fold to acetylcholine responses by physostigmine. This preparation is highly sensitive and is suitable for detection of acetylcholine (less than 10^{-8} mole or 0.001 mcg./ml.) released from nerve tissue (58–60). However it is not suitable for studying the pharmacology of drugs because of the length of response time involved. It is much better to choose the guinea pig ileum, chick semispinalis muscle, or frog rectus abdominis muscle for studying the pharmacology of the drugs since a larger quantity of results can be obtained in a shorter period of time.

Chick Semispinalis Cervicis Muscle Preparation—A chick, 2–3 weeks old, is sacrificed with chloroform. A longitudinal incision is made in the skin at the back of the neck from the base of the skull to the region of the thoracic vertebrae. The two biventer cervicis muscles can be seen on either side of the midline just underneath the surface skin. Those located beneath the biventer cervicis muscles are the two semispinalis cervicis muscles. The semispinalis cervicis muscle is dissected away from the surrounding muscles and the threads are tied around each end of the preparation. The muscle can be mounted either in an organ bath (5–10 ml.) (61–63) or on a superfusion apparatus (64). An initial tension of 1 g. is placed on the tissue. Tyrode solution (NaCl, 8.0; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.26; NaH_2PO_4, 0.05; glucose, 1.0; $NaHCO_3$, 1.0; and $CaCl_2$, 0.2 g./l.) at 40°, oxygenated with 95% O_2 and 5% CO_2, is used for the bathing fluid.

Like the frog rectus abdominis muscle, this preparation produces a slow contracture in response to acetylcholine and acetylcholine-like substances. Because it is more sensitive to drugs mimicking acetylcholine and because the muscle contracts and relaxes more rapidly this preparation is more advantageous than the frog rectus abdominis preparation (61, 62). Consequently, artificial stretching of the muscle is not required. This preparation is capable of detecting 0.004 mcg./ml. of acetylcholine chloride (2.4×10^{-8} mole). Edrophonium chloride is preferred to physostigmine, neostigmine, diisopropylfluorophosphate, and tetraethylpyrophosphate for inhibition of the cholinesterases in the tissue because these anticholinesterases either produce too little increase in sensitivity or cause a partial contracture of the muscle. Edrophonium chloride (final concentration of 8 mcg./ml. or 4×10^{-5} mole) is injected into the organ bath before each addition of acetylcholine. It is not included in the Tyrode solution, since, if used in this way, it will cause long lasting contracture even at extremely low concentrations (62).

Rat Phrenic Nerve–Diaphragm Preparation—A rat is decapitated and the blood is drained. The skin is removed from the middle of the chest. The thorax is opened and the front part of the left thoracic wall is removed. The phrenic nerve can be seen quite distinctly. The nerve is cut just below the thymus and a thread is attached to the cut end. The nerve is then freed carefully from the attached tissue. However, no attempt is made to clean the nerve completely from the tissues attached to it. An incision is made in the left abdominal wall just below the diaphragm. Two converging cuts are made through the diaphragm and the ribs towards the tendinous part of the diaphragm with the phrenic nerve attached to the center of the diaphragm. The fan-like preparation is about 3 mm. wide at the tendinous end and is about 15 mm. wide at the costal margin. A thread is attached to the tendinous part of the diaphragm. The preparation is fixed by a stainless steel rod with a pair of pins hooked on the rib. It is lowered into the organ bath and the thread from the muscle is attached to the writing lever or the force transducer. The nerve is stimulated with a pair of electrodes with a hole about 1 mm. wide. The right phrenic nerve–diaphragm preparation is isolated in the same manner. The organ bath should be 30–40 mm. in diameter and 30–40 ml. in capacity. Tyrode solution at 37° containing double the amount of dextrose or Krebs solution (NaCl, 6.9; KCl, 0.35; $MgSO_4 \cdot 7H_2O$, 0.14; KH_2PO_4, 0.16; glucose, 2.0; $NaHCO_3$, 2.1; and $CaCl_2$, 0.28 g./l.) is used as bathing fluid. The solution should be well oxygenated with 95% O_2 and 5% CO_2. The nerve is usually stimulated about 12 times per minute by rectangular-wave pulses of about 0.5-msec. duration at 3–5 v.

This preparation was originally described by Bülbring (65) and was modified and widely used thereafter by many investigators for studying drugs affecting the neuromuscular transmission (55, 66–68). The drugs are left in the organ bath either for quite short periods of time (3–8 min.) or for as long as the maximum effect can be observed. This preparation is composed mainly of fast muscle and is excellent for determining the drug potency to block or facilitate neuromuscular transmission. However, it is not a good preparation for differentiating between depolarizing and nondepolarizing neuromuscular blocking agents because, in many cases, depolarizing blocking agents fail to demonstrate initial facilitation and fail to reverse the effect of nondepolarizing blocking agents. For differentiating between depolarizing and nondepolarizing blocking agents the

chick biventer cervicis nerve muscle preparation and the chick sciatic nerve–tibialis anticus muscle preparations are preferred.

Chick Biventer Cervicis Nerve Muscle Preparation—This preparation is isolated by the same method described in the section on the chick semispinalis cervicis muscle. A loop is tied around the caudal belly of the muscle and hooked on the bottom of the electrode assembly. The oral end of the tendon is passed through the electrode and attached to a lever or an isometric force transducer. The whole assembly is placed in the organ bath (63). Superfusion technique has been applied to this preparation by Chiou and Long (64). The electrode assembly is similar to the one used in the organ bath method described by Ginsborg and Warriner (63) except that a small cup with a hole for superfusion is attached above the electrode. The small cup over the electrode is essential for superfusion of the biventer cervicis nerve muscle preparation because it eliminates the mechanical stimuli of the drops of superfusion fluid on the motor nerve, which cause irregular twitching of the muscle (64).

Tyrode or Krebs solution, at 37°, is used as bathing fluid and oxygenated with 95% O_2 and 5% CO_2. The initial tension placed on the muscle is 1 g. The organ bath is similar to that used for the rat phrenic nerve–diaphragm preparation. The nerve is stimulated about 6–12 times per minute by rectangular-wave pulses of about 0.5-msec. duration at 100 v. Supramaximal interrupted tetanic stimulation can also be applied (64). This preparation is extremely useful for easily differentiating between depolarizing and nondepolarizing neuromuscular blocking agents because the former agents contract slow fiber while the responses of fast fiber to the nerve stimuli are blocked, whereas the latter agents block the responses of fast fiber to the nerve stimuli without disturbing the tonus of the slow fiber. In addition, this preparation has been used successfully to demonstrate the acetylcholine releasing effects of some nicotinic agents which were originally believed to act directly at the acetylcholine receptors on the muscle membrane (64). The sensitivity of this preparation to acetylcholine and nicotinic agents can be increased markedly with 7×10^{-7} mole of physostigmine. Neostigmine is not a good agent to be used since it causes long-lasting contracture with concentrations at 1.5×10^{-6} mole or higher.

Chick Sciatic Nerve–Tibialis Anticus Muscle Preparation—Chicks, 3–8 days old, are decapitated and the skin of the legs is rapidly removed. The leg is removed from the body by cutting through the hip joint, and is suspended in a Petri dish containing Krebs-Henseleit solution equilibrated with 95% O_2 and 5% CO_2 at 4°. The muscles of the thigh are dissected and the sciatic nerve with the superficial peroneal branch is freed from the upper leg tissue. The fascia is removed from the lower leg and the tibialis anticus tendon identified. A thread is attached to the tendon of the muscle and the tibialis anticus muscle is freed towards but not up to the knee joint attachment where the nerve enters into this region. The upper and lower leg bones are then cut off leaving the muscle with its nerve attached to the knee joint. The tendon is fastened by the hook to a ring in the bottom of the organ bath. A thin steel rod is attached to the knee joint by means of a pointed clamp, partially driven into the bone. The contraction of the muscle is recorded isometrically. The nerve is passed through an electrode similar to the one used in the rat phrenic nerve–diaphragm or the chick biventer cervicis nerve muscle preparations. The nerve is stimulated six times per minute for 0.5-msec. duration at supramaximal voltage. The organ bath is filled with 20 ml. Krebs-Henseleit fluid, at 37°, and is oxygenated with 95% O_2 and 5% CO_2.

This preparation was originally described by Van Riezen (69, 70) and is similar to the chick biventer cervicis nerve muscle preparation. It is a simple and useful preparation for measuring drug potency and for classifying the neuromuscular blocking agents into depolarizing and nondepolarizing agents. The curare-like drugs produce a neuromuscular blockade whereas the decamethonium-like drugs induce a contracture of the slow fiber when the neuromuscular transmission is blocked.

Smooth Muscle Preparations—*Guinea Pig Ileum Preparation*—Guinea pigs, weighing 300–500 g., are stunned by a blow on the head. The abdomen is opened, the cecum is lifted forward and the ileum which joins to the back portion of the cecum is identified. The terminal portion of the ileum is excised and placed in a dish containing Tyrode soluton. The mesentery is trimmed away and pieces are cut from the length of ileum as required. Threads are tied around both ends of the ileum. Both the organ bath method (5–10 ml. capacity) (55, 56, 71–73) and the superfusion technique can be used satisfactorily (10, 49, 74, 75). Tyrode solution at 37°, oxygenated with 95% O_2 and 5% CO_2, is used. This is a simple, sensitive, and accurate preparation for the detection of acetylcholine (0.0025 mcg./ml. or 1.5×10^{-8} mole with organ bath method and 0.0025 mcg./injection with superfusion technique). Occasionally the spontaneous contraction of the ileum makes it difficult to establish a straight base line which is essential for bioassay. Antihistamines (71) or morphine (72, 73) may be added to the Tyrode solution to eliminate these spontaneous contractions. The longitudinal smooth muscle of the guinea pig ileum is preferred to the whole ileum in this respect because it produces very little if any spontaneous contractions (55). Three minutes should be allowed between doses in the organ bath method and 1 min. between doses in the superfusion technique.

A superfused cooled guinea pig ileum preparation has been developed particularly for biosassay of endogenous acetylcholine released by acetylcholine releasers (74, 75).

Longitudinal Smooth Muscle of Guinea Pig Ileum—The guinea pig ileum, up to 15.24 cm. (6 in.) in length, is excised according to the method described in the section on the guinea pig ileum preparation. The ileum is gently freed of excess mesentery and pulled over a glass rod immersed in Tyrode solution in a large shallow container at room temperature (25°). Dissection of the longitudinal muscle layer is performed by making the initial incision along the site of attachment of the mesentery. The mesenteric remnant is held with forceps to minimize crush damage and the longitudinal muscle is separated from the underlying structure by gentle tension with forceps in a direction parallel to the underlying circular muscle fiber (55, 76–80). A piece of longitudinal smooth muscle, about 2 cm. in length, tied at both ends,

is placed in an organ bath with a capacity of 1–5 ml. or in a superfusion assembly. The Tyrode solution, at 32°, is oxygenated with 95% O_2 and 5% CO_2. The preparation shows minimum spontaneous contractions when the Tyrode solution is kept at 32° (55, 81).

This preparation is preferred to the whole ileum for the following reasons (55): (*a*) The preparation consists of fairly pure longitudinal smooth muscle (about 82%) and the amount of noncontractile tissues such as nervous elements, serosa, *etc.*, is small compared to that of the whole ileum. (*b*) The preparation is very thin so that the drugs can easily contact the cell membrane and can easily be washed away. Therefore, the preparation lasts longer and gives more consistent responses to the drugs. (*c*) The preparation shows little, if any, spontaneous contraction, whereas spontaneous contraction of the whole ileum makes it difficult to establish a uniform base line. (*d*) The preparation is much smaller than the whole ileum so that a much smaller organ bath can be used to perform the experiment. The capacity of 1 ml. has been used in this preparation, whereas 5 ml. is the minimum capacity for whole ileum preparation. The sensitivity of this preparation to acetylcholine is about the same as that of whole ileum preparation.

Peristalsis Movement of Guinea Pig Ileum—A piece of guinea pig ileum, about 4–6 cm. in length, is suspended in an organ bath with a capacity of 50 ml. The oral end of the ileum is tied to the short end of a "J" tube while the caudal end is attached to an inverted "U" tube which is fitted with a valve made from flat drainage tubing. The inverted U tube is suspended from a lever or a force transducer which records the longitudinal contractions. The long limb of the J tube is used to transfer the pressure changes in the intestinal lumen by air transmission to a float recorder. Tyrode solution from a Marriotte bottle enters the intestinal lumen through a polyethylene tube connected to the short end of the J tube. The peristaltic reflex is initiated by raising the pressure in the lumen by 2–3 cm. of Tyrode solution, at 37°, which is oxygenated with 95% O_2 and 5% CO_2.

This preparation was described by Bülbring *et al.* in detail (82). It can be used to study the intestinal peristalsis, longitudinal contractions, intraluminal pressure, and volume of fluid expelled. The peristaltic movements can be inhibited effectively by catecholamines. The doses of catecholamines required for complete inhibition of peristalsis are: 0.1 mcg./ml. of epinephrine HCl, 0.5 mcg./ml. of norepinephrine HCl, and 30 mcg./ml. of *dl*-isoproterenol sulfate. The inhibitory effect of the catecholamines on the peristaltic movement can be reversed by the adrenergic blocking agents (83). The drugs can be applied to the inside of the intestine as well as to the outside. This preparation can also be used to test local anesthetics, ganglionic blocking agents, and atropine-like compounds (84–86). However, the sensitivity of the tissue fluctuates considerably. Thus, it is more suitable for qualitative than quantitative work. This method is modified from those described by Bülbring and Lin (87), and Trendelenburg (88).

Coaxial Stimulation of Guinea Pig Ileum—A loop of guinea pig ileum is suspended in Krebs solution, at 37°, and oxygenated with 95% O_2 and 5% CO_2. The lower end of the intestine is tied to glass tubing into which an electrode protrudes so that the electrode can move freely with the movements of the intestine. The upper end of the ileum is tied to a fine polyethylene tubing which encloses the upper part of the platinum wire from its emergence from the intestine, up above the surface of the bath fluid. A second platinum electrode is dipped into the bath fluid and makes the whole bath a diffuse external electrode. The arrangement permits a uniform excitation over the whole ileum and ensures that all the stimulation current applied traverses the intestinal wall. The preparation is stimulated with shocks of 0.5-msec. duration. The threshold voltage is about 1 v. and the maximal voltage is about 5–25 v. (89).

This preparation is more suitable for qualitative than for quantitative study and was originaly described by Paton (89). It has been suggested that the postganglionic cholinergic fibers are selectively stimulated through coaxial electrodes in this preparation, which has been used to differentiate between direct and indirect drug actions on the guinea pig ileum (86, 90–92).

Nerve–Jejunum Preparation of Rabbit—The rabbit is stunned by a blow on the head. The abdomen is opened and the jejunum which has the most mobile mesenteric attachment is taken. The nerve which lies in the mesentery along with the arterial blood supply is identified. Great care is taken not to stretch or otherwise damage the nerve in the mesentery. The intestine, about 2–3 cm. in length, is mounted in the organ bath of 30-ml. capacity or on a superfusion assembly. The mesentery is threaded and passed through an electrode similar to that used in the rat phrenic nerve–diaphragm preparation. The nerve is stimulated with rectangular-wave pulses of 0.5-msec. duration and about 10 v. The slow rate stimulation (2–4 pulses/min.) may produce parasympathetic effects, whereas a high rate of stimulation (30–50 pulses/min.) produces sympathetic effects. To avoid fatigue, the stimulus should not be applied for more than 30 sec. and at least 1.5–2 min. should be allowed for recovery. Tyrode solution at 37°, oxygenated with 95% O_2 and 5% CO_2, is used to perform the experiment.

This preparation was originally described by Finkleman (49) and is suitable for qualitative rather than quantitative studies of adrenergic blocking agents, cholinergic blocking agents, and local anesthetics. The preparation is especially useful for evaluating agents acting on the adrenergic nerve terminals. Physiological antagonism between catecholamines and parasympathetic effects can also be shown in this preparation (56).

Rat Fundus Strip Preparation—A rat is decapitated and is left to bleed. The abdomen is opened. The fundal part of the stomach is dissected, opened out longitudinally and placed in a dish containing Krebs solution. Suitable transverse cuts are made to obtain a strip about 4–5 cm. long. Threads are attached at both ends and the strip is mounted in an organ bath of 5–10-ml. capacity. An initial tension of 1 g. is placed on the preparation. Krebs solution, at 37°, is oxygenated with 95% O_2 and 5% CO_2. Since the muscle does not relax spontaneously after contraction by drugs, it must be stretched to assist its recovery to the original length. Isometric recording is better than isotonic recording since the muscle length

can be kept constant. The preparation should be left in the organ bath at least 30 min. before use and 6 min. or longer should be allowed between doses.

This method was originally described by Vane (93). It is extremely sensitive to 5-hydroxytryptamine which contracts the muscle at dose levels as low as 0.4–0.8 ng./ml. For bioassaying 5-hydroxytryptamine in the tissue extracts, atropine or hyoscine must be added to the Krebs solution. The sensitivity of this preparation to acetylcholine is lower than that of the leech dorsal muscle treated with physostigmine and is about the same as that of the guinea pig ileum. This muscle is rather insensitive to nicotine and histamine.

Rat Uterus Preparation—Young female rats, weighing 150–200 g., are decapitated and the abdomen opened. The animal should be in oestrus which can be induced by injecting subcutaneously 0.1 mg./kg. of stilbestrol 24 hr. before the animal is sacrificed. The two horns of uteri are transferred to a dish containing warm DeJalon solution (NaCl, 9.0; KCl, 0.42; glucose, 0.5; $NaHCO_3$, 0.5; and $CaCl_2$, 0.03 g./l.) or Krebs bicarbonate solution. Each horn is cut open and divided longitudinally so that four pieces can be obtained from one animal. The threads are tied to each end of each piece (about 2 cm. in length *in situ*), which is then mounted in an organ bath of 10 ml. capacity. The DeJalon solution, at 30–32°, is oxygenated with 95% O_2 and 5% CO_2, which maintains the pH at about 7.4. An initial tension of 0.5 g. is applied to the muscle and the contraction is measured isometrically. The preparation should be left for 30 min. before use (94).

The responses obtained with this tissue are quicker than those obtained with the rat fundus strip preparation but are slower than those produced by the guinea pig ileum preparation. Spontaneous contractions are quite obvious in this preparation when usual physiological salt solutions are used as bathing fluid. The calcium concentration in DeJalon solution is therefore lower than in other physiological salt solutions. Three minutes or longer should be allowed between doses.

This preparation is extremely useful for studying the cholinergic effects of acetylcholine and its derivatives for the following reasons (56): (*a*) The slope of the log dose-response curve obtained with acetylcholine and its derivatives is very steep and thus is convenient for analysis of the results. (*b*) It is remarkably insensitive to histamine. However, it is not suitable for bioassay of acetylcholine and its derivatives obtained from tissues because the sensitivity of this preparation is lower than those of the guinea pig ileum and the leech dorsal muscle treated with physostigmine. Though the preparation is less sensitive than the rat fundus strip for 5-hydroxytryptamine assays, it has the definite advantage of readily returning to basal tension following exposure.

This preparation is historically very important because it has β-adrenergic receptors. Therefore, it is very sensitive to epinephrine (10^{-7} mole) and *dl*-isoproterenol (2×10^{-8} mole) but is relatively insensitive to norepinephrine (2×10^{-6} mole) and ephedrine (2×10^{-5} mole) (94–96). The bioassay of catecholamines has been largely replaced by the sensitive fluorometric method. However, this preparation is still very useful for pharmacologic studies on catecholamines. For pharmacologic study of α-adrenergic agents, the rabbit aortic strip preparation can be used.

Rabbit Aortic Strip Preparation—A rabbit is sacrificed by a blow on the head and cutting the throat for bleeding. The descending aorta is removed and is placed in a dish containing Krebs solution at room temperature. The excised aorta is cut spirally with a small sharp-pointed scissors to produce a strip about 3 mm. wide and 2–4 cm. long. The uncut portion of the aorta is held gently between thumb and fingers of the free hand and is moved gradually forward to the scissors to permit a continuous spiral incision. It is not recommended to pull the aorta over a glass rod or a wooden rod for faster cutting because the aorta strip does not respond well to drugs because of injury by stretching on the rod. Threads are attached to both ends of the preparation which is then mounted in an organ bath of 10-ml. capacity. The Krebs solution, at 37°, is oxygenated with 95% O_2 and 5% CO_2. An initial tension of 3–4 g. is placed on the muscle. The preparation should be allowed to equilibrate for 30 min. or more before use (97, 98).

Since drug response is slow and recovery is long, 30 min. or more is necessary between doses. However, if isometric recording is used, a much shorter period of time is required for recovery and more accurate results can be obtained, due to the small changes in length during muscle contraction. In contrast to the rat uterus preparation, this preparation contains α-adrenergic receptors. Therefore, the effects of catecholamines on this preparation are not blocked by β-adrenergic blockers such as propranolol (99).

This preparation is extremely sensitive to epinephrine (5×10^{-9} mole) and norepinephrine (5×10^{-9} mole) but is less sensitive to *dl*-isoproterenol (2×10^{-7} mole), histamine (5×10^{-8} mole), 5-hydroxytryptamine (5×10^{-8} mole), and acetylcholine (2×10^{-6} mole) (98, 100, 101). Because of the long duration of the experiments, it is important to check the solutions of catecholamines for deterioration. If necessary, a freshly made solution should replace the original.

Dog Mesenteric Artery Preparation—Mongrel dogs are anesthetized with 15 mg./kg. of thiopental sodium and 250 mg./kg. of barbital sodium. The bundles of nerves which follow the vessels through the mesentery are freed at the central end of the arteries. The superior mesenteric artery is freed of mesenteric attachment and cannulated with polyethylene tubing (PE 100). Distally, the branching fans of smaller arteries are cut at the sites of entrance into the small intestine. The isolated preparation is quickly mounted in an organ bath with 100 ml. of Krebs bicarbonate solution at pH 7.3. It is oxygenated with 95% O_2 and 5% CO_2 and is maintained at 37°. The mesenteric arteries are perfused with a constant flow of Krebs bicarbonate solution (30–50 ml./min.). An arterial pressure transducer is attached to the cannula which leads from the pump to the artery. Changes of perfusion pressure are then proportional to changes in arterial resistance. The drugs are injected into the rubber tubing close to the mesenteric artery with a volume of not more than 0.1 ml. The nerve is pulled through the electrodes and is held in place by a ligature. The nerve is stimulated with 1-msec. duration, 50 c.p.s. frequency and 10–15 v. supramaximal voltage (102, 103).

This preparation gives reproducible pressor responses to injected catecholamines and to nerve stimulation for periods of several hours (102). The responses to nerve stimulation are postganglionic α-adrenergic in nature (103). Various adrenergic and adrenolytic agents have been studied with this preparation (103). This is a good preparation for qualitative study of the pharmacology of α-adrenergic and adrenolytic agents on the blood vessels innervated with sympathetic nerves.

Guinea Pig Tracheal Chain Preparation—Adult guinea pigs are sacrificed by a blow on the head. The trachea is dissected and transferred to a dish containing Krebs solution. The trachea is then sectioned into rings by cutting transversely between the segments of cartilage. The rings are tied together to form a chain with the muscular parts of the rings arranged alternatively at two sides of the tracheal chain. The Krebs solution, at 37°, is oxygenated with 95% O_2 and 5% CO_2. The capacity of the organ bath is about 10–15 ml. An initial tension of 0.2–0.5 g. is placed on the tracheal chain.

This preparation was originally described by Castillo and DeBeer (104). The tracheal muscle of the calf can also be used (105, 106). It is a slow responding preparation. Thus at least 15 min. should be allowed between doses for responses induced by small doses and 30 min. or longer for responses induced by large doses of agonists such as acetylcholine and histamine. This preparation can be used to study the pharmacologic effects and dose-response curves of catecholamines. In this case the preparation is contracted with acetyl-β-methylcholine first and then relaxed with various doses of catecholamines. Ariens added cumulative doses of catecholamines to the organ bath to get satisfactory cumulative dose-response curves (105, 106). The threshold doses of epinephrine, norepinephrine, and *dl*-isoproterenol are about 10^{-7} mole, 10^{-7} mole, and 5×10^{-8} mole, respectively.

Guinea Pig Vas Deferens Preparation—Male guinea pigs, weighing 400–800 g., are sacrificed by a blow on the head. The abdomen is opened in the midline and the intestines displaced to the right. The vas deferens is freed from connective tissue and cut from the epididymis. The vas deferens is held with small forceps near the cut end and is freed from the adjacent tissue. The right and left hypogastric nerves can be seen in the middle of the mesentery of the colon. One nerve is tied, and cut about 5 cm. from the vas deferens; this is cleaned to within 0.5 cm. of the vas deferens. The remainder of the nerve which is fine and diffuse is preserved by isolating the piece of peritoneum which contained it. The vas deferens is then cut from the urethra and removed together with its nerve and small piece of peritoneum and is placed in a dish containing Krebs solution. An organ bath containing 20 ml. of Krebs solution at 32°, oxygenated with 95% O_2 and 5% CO_2, is used. The preparation is set up as described below and an initial tension of 0.75–1.0 g. is applied.

For stimulating the hypogastric nerve, the vas deferens is set up in a method similar to that of the rabbit nerve jejunum preparation (Finkleman preparation) (107, 108). The vas deferens is tied by its proximal end to the bottom of the organ bath and the distal end to a lever or a force transducer. The hypogastric nerve is stimulated submaximally with rectangular pulses of 2–3 v. and of 2 msec. duration for 2–3 sec. every minute at a frequency of 80 c.p.s.

For transmural stimulation one end of the vas deferens is tied to a supporting hook and the other (upper end) to the recording lever. The electrodes are two parallel lengths of platinum wire, 0.05-cm. (0.02-in.) diameter, cemented to the edges of a Perspec gutter so that the vas deferens can be suspended between them. Stimulation is performed for periods of 15 sec. at 3-min. intervals at a frequency of 25 c.p.s. with a pulse duration of 0.1 msec. at supramaximal voltage, usually 90–120 v. (109, 110). The transmural stimulation can also be performed by Paton's method (89) described in the section of the coaxial stimulation of the guinea pig ileum.

The vas deferens can also be stimulated alternatively through the hypogastric nerve and transmurally. For both stimulations, a frequency of 25 c.p.s. and a pulse width of 0.1 msec. with supramaximal voltage (90–120 v. for transmural and 30–60 v. for hypogastric nerve stimulation) are used (110).

This preparation was originally described by Huković (111) and modified by other investigators (107–110, 112). It is extremely useful for stimulating separately, preganglionic and postganglionic fibers; the former through the hypogastric nerve and the latter through transmural stimulation. The electrodes must be placed within 2 mm. of the tissue to obtain primarily postganglionic hypogastric nerve activation. The usual 3–5-cm. distance on the hypogastric nerve from the tissue will activate primarily preganglionic fibers. The preparation can also be done successfully using rats, rabbits, and mice (112, 113).

For studying the pharmacologic mechanism of adrenergic systems, this is an extremely useful preparation because when the vas deferens is removed without the hypogastric nerve and stimulated transmurally, the contractions produced are primarily due to the excitation of postganglionic adrenergic nerves (109, 110). It is also useful for studying the pharmacology of cholinergic agents and local anesthetics. This preparation can be contracted both by cholinergic and adrenergic agents. The greatest advantage of this preparation for studying adrenergic agents, as compared with the guinea pig tracheal chain preparation and rabbit aortic strip preparation, is that it responds and relaxes to the drugs rapidly. Consequently, much data can be obtained within a short period of time. However, the sensitivity of this preparation to most of the drugs in comparison with the other preparations is rather low and thus is not suitable for quantitative bioassay.

Heart Muscle Preparations—*Rabbit Heart Preparation*—A rabbit is sacrificed by a blow on the head. The heart is removed as quickly as possible. It is placed in a bath containing Ringer-Locke solution (NaCl, 9.0; KCl, 0.42; glucose, 1.0; $NaHCO_3$, 0.5; and $CaCl_2$, 0.12 g./l.) at room temperature and is squeezed gently to remove the blood. The aorta is identified, freed, and cut just below the point where it divides. The aorta is then tied onto the glass cannula of the perfusion apparatus. The Ringer-Locke solution, oxygenated with 95% O_2 and 5% CO_2, at 37° is perfused through the heart with constant pressure (30 mm. Hg). The fluid passes only through the coronary vessels and escapes from the in-

Table IV—Methods for Bioassay of Cholinergic Agents

Preparation	Speed of Assay	Technique	Threshold Dose of Acetylcholine	References
Leech muscle	Slow	Organ bath	6×10^{-9} mole (0.001 mcg./ml.)	17
Guinea pig ileum	Rapid	Organ bath	1.5×10^{-8} mole (0.0025 mcg./ml.)	55
Chick semispinalis	More rapid	Organ bath	2.4×10^{-8} mole (0.004 mcg./ml.)	62
Frog rectus muscle	Adequate	Organ bath	6×10^{-8} mole (0.01 mcg./ml.)	55
Cat blood pressure	Rapid	*in vivo*	0.002 mcg./kg.	17
Guinea pig ileum	Very rapid	Superfusion	0.0025 mg./injection	10, 75
Frog rectus muscle	More rapid	Superfusion	0.01 mcg./injection	10, 39

ferior vena cava since the aortic valve is closed by the pressure of the perfusion fluid. The perfusate is collected by a funnel and the rate of flow can be measured with a graduated cylinder and a stopwatch. A thread is attached to the ventricle by a hook and connected to spring levers or a force transducer to record the amplitudes of the heart contractions. Readings of the heart rate and of the coronary flow are usually taken over a period of 30 sec. Drugs are injected through the rubber cap into the perfusion fluid. The heart rate and the flow rate of the fluid should be taken approximately every 3 min.

This preparation is well known as the Langendorff preparation (114, 115) and can be used to study coronary dilators, coronary constrictors, and drugs affecting the inotropic and chronotropic effects of the heart (116, 117). For example, coronary vessels are constricted by vasopressin (8×10^{-11} mole/injection) and dilated by amyl nitrite (2×10^{-5} mole/injection). The rate and force of the heart are consistently increased by epinephrine (2.5×10^{-9} mole/injection), norepinephrine (3×10^{-9} mole/injection) or *dl*-isoproterenol (7.5×10^{-10} mole/injection) which are blocked effectively by β-adrenergic blocking agents such as propranolol (7.5×10^{-9} mole/injection). Ventricular fibrillation may be observed when chloroform (1×10^{-5} mole/injection) is given and followed by catecholamines. This preparation is used mainly for qualitative pharmacologic studies.

Guinea Pig Auricle Preparation—Guinea pigs are killed by a blow on the head. The heart is removed as quickly as possible and placed in Feigan solution (NaCl, 9; KCl, 0.42; $CaCl_2$, 0.62; glucose, 1.0; and $NaHCO_3$, 0.6 g./l.) at room temperature. All other tissue is cut away, leaving only the auricles. Threads are tied to the tips of each auricle and the preparation is mounted on a superfusion assembly or in an organ bath with a capacity of 25 ml. The auricle is maintained with a resting tension of 1 g. and is allowed to stabilize for at least 30 min. before use. Feigan's solution is oxygenated with 95% O_2 and 5% CO_2 at 30°.

For transmural stimulation in the organ bath, platinum electrodes are placed on each side of the auricle at a distance of 1 cm. from the auricle (118, 119). The duration of the pulse is held constant at 20 msec., the voltage at 150 v., and the period of the stimulus at 15 sec. A frequency of 2 c.p.s. is used to elicit a parasympathetic response and a frequency of 15 c.p.s. is used to induce both sympathetic and parasympathetic response (119). The preparation can have the vagus nerve attached, which can be stimulated with biphasic stimulation of 35 c.p.s., 6-msec. duration, at 3 v. (120).

For transmural stimulation of the auricle in the superfusion assembly, platinum wire electrodes are placed above and below the auricle, so that they are in continuous contact with the bathing fluid, but not with the tissue. The stimulus parameters are the same as in the organ bath method (121).

Both Feigan solution and Locke-Ringer solution (NaCl, 9.0; KCl, 0.42; $CaCl_2 \cdot 2H_2O$, 0.24; $NaHCO_3$, 0.3; and glucose, 2.0 g./l.) can be used as bathing fluid. However, the former solution is preferred due to the higher concentration of $CaCl_2$ (0.62 g./l.) which produces a heart beat that is stronger, more consistent, and longer lasting. The pH of the solution should be kept at 7.4. This preparation can be done using the frog, turtle, chicken, rat, and rabbit as well (119).

The normal preparation is used primarily for qualitative studies of drugs affecting heart function, such as acetylcholine and its derivatives, catecholamines, atropine-like compounds, and adrenergic blocking agents. However, it can also be used to determine the affinity constants of competitive antagonists of acetylcholine. The transmurally stimulated and the vagus nerve

Table V—Methods for Bioassay of Adrenergic Agents

Preparation	Speed of Assay	Technique	Threshold Dose of Catecholamines[a]	References
Rat uterus	Adequate	Organ bath	2×10^{-8} mole Iso 1×10^{-7} mole Epi 2×10^{-6} mole N.E.	94, 95, 96
Rabbit aorta strip	Slow	Organ bath	2×10^{-7} mole Iso 5×10^{-9} mole Epi 5×10^{-9} mole N.E.	97, 98
Guinea pig tracheal chain	Slow	Organ bath	5×10^{-8} mole Iso 1×10^{-7} mole Epi 1×10^{-7} mole N.E.	104
Guinea pig vas deferens	Adequate	Organ bath	3×10^{-5} mole N.E.	111

[a] Iso, isoproterenol; Epi, epinephrine; and N.E., norepinephrine.

Table VI—Methods for Bioassay of Histaminergic Agents

Preparation	Speed of Assay	Technique	Threshold Dose of Histamine	References
Rabbit aorta strip	Slow	Organ bath	5×10^{-8} mole	97, 98
Guinea pig tracheal chain	Slow	Organ bath	5×10^{-7} mole	104

stimulated preparations are used primarily for the qualitative studies of the mechanisms of heart functions and drugs affecting heart functions (119, 120).

Kitten Papillary Muscle Preparation—Kittens are killed by a blow on the head and the heart is dissected as quickly as possible. The papillary muscles which are usually 0.5–0.7 mm. wide and 5–8 mm. long are dissected. The preparation is mounted on the apparatus described by Blinks (122, 123). The tension of the muscle is adjusted initially to approximately half the level associated with maximally developed tension and the length kept constant thereafter. The papillary muscle can also be obtained from the guinea pig.

This preparation is used primarily for qualitative studies of the drugs affecting heart function. The field stimuli can affect the heart function *via* sympathetic effects and parasympathetic effects. The heart function is also affected by synchronization of the contractions of the various parts of the preparation when the conduction in the muscle is slow. Field pulses of 50-ma. strength and 2-msec. duration are found to be nearly maximally effective in eliciting both sympathetic and parasympathetic effects (123).

Subcellular Preparations—*Synaptic Vesicles*—Rats, weighing about 300 g., are decapitated and the cerebral cortex or any other portion of the brain tissue is isolated as quickly as possible. All procedures described here should be performed at 0–4°. The brain tissue is weighed and homogenized in 0.32 *M* sucrose solution at 1200 r.p.m. for four strokes in a glass homogenizer with a Teflon plunger. The homogenate is diluted to 10% with 0.32 *M* sucrose and centrifuged in the cold for 10 min. at 900 $\times g$. The supernate is saved and the sediment is washed once by rapid rehomogenization in sucrose solution and centrifuged as above. The two supernates are combined and centrifuged for 20 min. at 11,500 $\times g$. The pellet is treated with distilled water (10 ml. per 1 g. tissue) containing 1×10^{-6} mole of Ca^{++} and 1.5×10^{-5} mole of physostigmine. The material is rehomogenized and centrifuged at 11,500 $\times g$ for 20 min. The supernatant is recentrifuged at 100,000 $\times g$ for 30 min. The synaptic vesicle is localized mainly at the pellet portion. This procedure is described by DeRobertis *et al.* (124–126). The procedure described by Whittaker *et al.* is essentially the same as that described by DeRobertis *et al.*, except that the final supernatant is subjected to density gradient purification in order to obtain a purer preparation (127, 128).

This is a convenient preparation to be used to study the drugs which release acetylcholine from the nerve terminals (129). Recently, it has been found that most of the nicotinic agents act through release of acetycholine from the nerve terminals (64, 75). These nicotinic agents can also release acetylcholine effectively from the synaptic vesicle preparation. The acetylcholine-releasing effects of these nicotinic agents are markedly blocked by triethylcholine on the isolated guinea pig ileum and baby chick biventer cervicis nerve muscle preparations (64, 75), but are not blocked on the synaptic vesicle preparation (129). This indicates that the nicotinic agents are taken up by the nerve terminals before they can release acetylcholine from the synaptic vesicles. The uptake of nicotinic agents by the nerve terminals is possibly blocked by triethylcholine at the membrane site of the nerve terminals. This hypothesis can be further illustrated using the synaptosome preparation described in the following section. The acetylcholine released from the synaptic vesicles or synaptosomes can be bioassayed with superfused, cooled guinea pig ileum described in the section on the guinea pig ileum preparation. This preparation can also be obtained from the brains of animals such as guinea pig, rabbit, cat, mouse, and others.

Synaptosome Preparation—Rats, weighing about 300 g., are sacrificed by decapitation and the brain tissue is removed as quickly as possible. All manipulations are performed at 0–4°. A 10% homogenate of brain tissue is prepared in 0.25 *M* sucrose using a glass and Perspex homogenizer rotating at 1200 r.p.m. with a difference in diameter of 0.025 mm. between pestle and mortar. The homogenate is centrifuged at 1000 $\times g$ for 15 min. The supernatant is recentrifuged at 12,500 $\times g$ for 15 min., and the sediment is resuspended in 0.25 *M* sucrose (2 ml./g. of original tissue). Five milliliters of this crude mitochondrial fraction is carefully layered on the top of the synthetic polymer (Ficoll) gradient and the contents are centrifuged at 90,000 $\times g$ for 60 min. The synaptosomes are obtained mainly from the second and third fractions of the six distinct layers. These fractions are diluted with 0.25 *M* sucrose and centrifuged at 120,000 $\times g$ for 20 min. to remove the polymer gradient. The pellets are suspended in 0.25 *M* sucrose and aliquots are used for assay or incubation. The polymer gradients are made by successive layering of 5 ml. each of 20, 16, 12, 8, and 2% polymer solutions made with 0.25 *M* sucrose into a 2.54 × 7.62-cm. (1 × 3-in.) cellulose tube. The

Table VII—Methods for Bioassay of Serotonin

Preparation	Speed of Assay	Technique	Threshold Dose of Serotonin	References
Rat fundus	Adequate	Organ bath	1×10^{-9} mole	93
Rabbit aorta strip	Slow	Organ bath	5×10^{-8} mole	97, 98

Table VIII—Methods for Study of the Sites of Action of Drugs on the Peripheral Nervous System

Cholinergic Nervous System			Adrenergic Nervous System	
Muscarinic or Postganglionic Site	Nicotinic or Preganglionic Site	Neuromuscular Junction	Postganglionic Site	Preganglionic Site
Dog blood pressure	Dog blood pressure after atropine	Rabbit sciatic nerve–gastrocnemius (fast muscle)	Cat nictitating membrane	Cat superior cervical ganglia
Dog chorda tympani–Wharton's duct	Dog chorda tympani-Wharton's duct	Cat soleus muscle (slow muscle)	Peristalsis movement of guinea pig ileum	Guinea pig vas deferens–hypogastric nerve
Guinea pig ileum	Frog rectus abdominus muscle	Cat tibialis anticus muscle (fast muscle)	Rat fundus (for β-adrenergic receptor)	Rabbit nerve–jejunum (fast stimulation)
Longitudinal smooth muscle of guinea pig ileum	Leech dorsal muscle	Rat phrenic nerve diaphragm (fast muscle)	Rabbit aortic strip (for α-adrenergic receptor)	
Coaxial stimulation of guinea pig ileum	Chick semispinalis cervicis muscle	Chick biventer cervicis (fast and slow muscles)	Guinea pig tracheal chain	
Rat fundus	Rabbit nerve–jejunum (slow stimulation)	Chick sciatic-tibialis anticus (fast and slow muscles)	Guinea pig vas deferens (transmural stimulation)	
Rabbit aortic strip			Dog mesenteric artery	

tubes are allowed to stand at room temperature for 60 min. and at 4° for 30 min. (130, 131).

The synaptosomes can also be obtained by methods described by Whittaker *et al.* (127, 132, 133) or De-Robertis *et al.* (124). However, since these methods are performed with the density gradient in hypertonic sucrose solution, the synaptosomes show characteristic morphological changes (134), decrease in oxidative phosphorylation (135), and spontaneous release of bound acetylcholine (131).

The synaptosome preparation can be used to study the drugs which release acetylcholine from the nerve terminals and the drugs which block the release of acetylcholine. It is a convenient method and gives direct evidence to demonstrate the turnover, kinetics, and pharmacology of cholinergic mechanisms at the nerve terminals. It is hoped that combined use of the synaptic vesicle and synaptosome preparations may facilitate the clarification of cholinergic mechanisms which are much less understood than are adrenergic mechanisms. Methods for determining the quantity of drugs taken-up into synaptosomes and the volume of synaptosomes are described by Marchbanks (136, 137).

CONCLUSION

Bioassay is a technique developed entirely by the pharmacologists (138). A good bioassay technique must be sensitive, accurate, and rapid in responding to the drugs administered. For years the leech dorsal muscle and the frog rectus abdominis muscle preparations were the only skeletal muscle preparations widely used for bioassay of nicotinic agents (54, 57). The introduction of the chick semispinalis cervicis muscle preparation furnishes a more rapid technique for the bioassay of these agents (62).

The superfusion technique was first described by Finkleman (49) and later modified by Gaddum (50). This is an extremely useful technique for bioassay because it can be performed with more speed, greater accuracy, and higher sensitivity than the organ bath technique (Table IV). It is highly recommended for bioassay of drugs of trace quantity. It is also a good technique for obtaining ample amounts of data within a short period of time. The methods for bioassay of drugs acting at the peripheral systems are summarized in Tables IV–VII.

Besides being used for bioassay, the biological preparations described are also widely used to study the sites of drug effects on the peripheral nervous system. The methods for studying the specific sites of the peripheral nervous systems are classified in Tables VIII and IX. The rat phrenic nerve–diaphragm muscle preparation has long been used as the sole preparation for *in vitro* study of neuromuscular transmission (65). The chick biventer cervicis nerve muscle preparation (63, 64) and chick sciatic–tibialis anticus preparation (69, 70) were introduced later. They have the advantages of consisting of both fast and slow muscles. They are easy to prepare and can survive with interrupted tetanic stimulation (74). The dog mesenteric artery preparation is an interesting one with sympathetic nerve attached to the artery. It is extremely useful for studying the pharmacology of drugs and nerve regulations on the blood vessels (102, 103).

The synaptic vesicle and synaptosomes are newly developed preparations with high potential for solving

Table IX—Methods for Study of the Sites of Drug Actions at Heart and Nerve Terminals

Heart	Nerve Terminals
Rabbit heart	Synaptic vesicles
Guinea pig auricle	Synaptosome
Kitten papillary muscle	

the mechanism of drug actions at the nerve terminals. For example, nicotinic agents such as nicotine, trimethylamino alcohols, decamethonium, and others have been reported to release acetylcholine from the nerve terminals, which can be blocked by triethylcholine (64, 75). The action mechanism of these findings are explored by using synaptosome and synaptic vesicle preparations. Nicotine and choline have been found to be taken into the intracellular site of superior cervical ganglia (139) and synaptosomes (137), which can be blocked by hexamethonium and hemicholinium, respectively. It has also been found that nicotinic agents are capable of releasing acetylcholine from the synaptic vesicles, which is not blocked by triethylcholine nor by hemicholinium (129). It is thus concluded that the nicotinic agents are probably taken into nerve terminals for releasing endogenous acetylcholines whereas the uptake of nicotinic agents is blocked by triethylcholine or hexamethonium at the membrane site of the nerve terminals (64, 75, 129).

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