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TRANSMISSION IN SQUID GIANT SYNAPSES

The Importance of Oxygen Supply and the Effects of Drugs

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ABSTRACT

Synaptic transmission was studied in giant synapses of the stellate ganglion of the squid. When bathed in air-saturated sea water, the synapses deteriorate in 10 to 20 min.; if the sea water is saturated with 100 per cent oxygen, they function steadily for up to 12 hours. Optimal results probably require a medium with lower magnesium and higher calcium than the sea water used.

Of eighteen compounds known to affect other synapses (Table I), none had stimulatory effects when applied to the preparation, but ten produced synaptic depression in concentrations of 10^{-3} gm. per ml. or higher. The only exception was procaine, which blocked at 6×10^{-5} gm. per ml.

Intracellular recording with microelectrodes near the synapse showed that the block was associated with a slower rise of the excitatory post-synaptic potential, without a change in the depolarization required to initiate the spike. Procaine was exceptional in also increasing the depolarization at which the spike occurred.

The dimensions of giant synapses of the stellate ganglion of the squid allow the insertion of pipettes and electrodes into the pre- and post-synaptic axons close to the synapse and hence suggest the possibility of relating pharmacological activity to changes in structure and function in elements of a single synapse. The morphology of these synapses has been described in considerable detail (Young, 1939). Using extracellular recording, Bullock (1948) determined many of their neurophysiological properties. Recently intracellular recording with microelectrodes with application of electrical currents through the synaptic membranes (Bullock and Hagiwara, 1955, 1957; Tasaki and Hagiwara, 1957) has led to the conclusion that electrical current flow from the pre- to the post-axons cannot account for impulse transmission across the giant synapse. The latter investigators as well as others (Fatt, 1954; Grundfest, 1957; and Eccles, 1957) have consequently postulated that chemical transmission must operate at this junction.

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The electrical evidence for chemical transmission depends mainly on the following findings: (1) There is a true delay after arrival of the pre-synaptic spike before onset of the excitatory post-synaptic potential (e.p.s.p.). (2) There is little direct electrical change in the post-axon due to the pre-axon action potential. (3) The e.p.s.p. can add to an antidromic spike during a period of probable electrical refractoriness. Several other properties such as unidirectional transmission and the characteristics of the e.p.s.p. are likewise compatible with the chemical transmission theory as generally conceived.

If transmission at this synapse is chemical, then a pharmacological characterization of the process would make this structure more useful as a model of synaptic transmission. Bullock (1948) showed that DFP blocked the giant synapses in about the same concentration which blocked axonal conduction. Apart from this observation there has been no systematic study of drug action upon them. With the latter in mind several substances known to be active at other synapses were applied externally to the stellate ganglion and their effects upon transmission were determined.

In previous investigations, the survival of the functioning, excised giant synapse has been brief. In the preliminaries of this investigation, it was demonstrated that a stable preparation may be made by saturating the bathing solution with oxygen.

Materials and Methods

Squid were obtained from the collection department of the Marine Biological Laboratory at Woods Hole, Massachusetts. Twelve adults and a few dozen freshly hatched squid were used in preliminary *in vivo* experiments. Almost all electrical recording procedures were carried out on excised ganglia. A total of ninety-five stellate ganglia with functioning giant synapses were excised from sixty-seven mature squid, *Loligo pealii*. The dissection technique with some modifications was essentially that of Bullock (1948).

For extracellular recording the stellate ganglia were removed bilaterally along with about 2 cm. of the preganglionic, pallial nerve, and 4 cm. of post-ganglionic, last stellar nerve, in a cooled (10–20°C.), running, tap–sea water trough. The other stellar nerves were cut at the border of the ganglion. The dissection time averaged about 15 min. for the first, and 20 min. for the second ganglion to be removed, from the time of decapitation. The extreme ends of the pre- and post-ganglionic nerve trunks were tied with lengths of silk thread which were used to transport the preparation. The fin nerve was usually allowed to remain attached the full length of the preparation for mechanical support; its presence did not interfere because of the ease of selective stimulation and recording from the giant axons.

The excised ganglion preparations were kept in finger-bowls, 10 cm. in diameter by 4 cm. deep, which contained 200 ml. of the bathing medium. The threads were placed over the edge of the bowls in such a way as to keep the preparation free from contact with the sides. The bathing medium was either filtered Woods Hole sea water or an artificial "chloride sea water" with the following composition: NaCl, 440.0; KCl, 9.0; $CaCl_2$, 9.0; $MgCl_2$, 53.4 mM per liter. The sodium, potassium, and

calcium values (Steinbach, 1939), and the magnesium value (Page, 1927) were based on analyses of Woods Hole sea water. Control studies indicated no difference in synaptic function between natural and the chloride sea water media. In the magnesium and calcium studies, increases or decreases in these ions were compensated osmotically by changing the NaCl content of the artificial medium.

The bathing medium was kept at tap–sea water temperature by placing the finger bowl in the running sea water trough. The temperature did not vary more than a few degrees C. in the course of any one experiment. The solution in the finger-bowls was kept saturated with oxygen and stirred by constantly passing U.S.P. grade, 100 per cent oxygen into the solution at a high rate through fine gas dispersers. The effects of drugs were determined by transferring a preparation from control solution to another finger-bowl of similar temperature and oxygenation but containing the drug.

Synaptic transmission was tested by removing the preparation from the bathing medium to a lucite electrode strip containing parallel 5 mil silver wires in shallow grooves, for stimulating and recording. Pre-nerve stimulation was by 0.1 msec. square-wave pulses. Post-giant axon action potentials were displaced on a cathode ray oscillograph, and were easily distinguished from the small fiber action potentials. It was more difficult to distinguish pre-axon spikes from other pre-nerve trunk spikes.

A standard operational sequence was adhered to for the drug studies. The testing procedure took about 30 sec. "Transmission delay" was first measured with constant electrode position at 5 stimuli per sec. It includes conduction time in short lengths of pre- and post-axons, synaptic delay, and depolarization time of the e.p.s.p. Spontaneous activity became evident with some drugs during this test. The frequency was then increased to 50 per sec. for 5 sec., during which changes in delay and tendency to fatigue were noted. The frequency was then abruptly returned to 5 per sec. to observe any post-tetanic changes. The preparation was immediately replaced in the medium. This cycle of events could in the control situation be repeated for hours at 5- or 10-min. intervals, with little or no alteration in the observed quantities. When block occurred for any reason, pre- and post-axon conduction were always checked to insure that the blockade was not due to the failure of this and was therefore synaptic block.

For intracellular recording, the ganglia were removed with shorter pre- and post-nerves. Since it was not required that the pallial nerve be removed from inside the brain case, excision usually took only 5 to 10 min. from decapitation. Threads tied to the ends of the pre- and post-nerves were pressed into bits of clay on a microscope slide, stretching the preparation slightly. The slide was placed in cooled (10–15°C.) oxygen-saturated, sea water medium with the giant fiber lobe downward. The ganglion sheaths and other structures were stripped away from about the giant synapse under a binocular microscope using fine instruments. A critical combination of oblique and transmitted light was necessary for seeing the various parts of the giant synapse. The pre- and post-axons were horizontal. A 3 M KCl-filled, long taper, glass microelectrode was then inserted at a 45° angle into one of the synaptic elements, usually the post-axon at a point estimated to be center of the synaptic contact. The tissues above the synapse could be penetrated easily without breakage by tapping the micropositioner while slowly advancing the electrode tip. The micropipette tip diameters ranged from 0.2 to 5.0 micra. The microelectrode preamplifier was of the nega-

tive capacity type described by Haapanen and Ottoson (1954). Potentials were recorded at high and low gain simultaneously on a dual beam oscillograph.

RESULTS

1. Extracellular Recording

(*a*) *Influence of Oxygen Content of Medium.*—Of twenty-seven ganglia in which there was evidence that both pre- and post-axons were functional, eight were kept in cooled sea water (10.5–14.6°C.) and the remainder in warmer, running tap–sea water (18.3–20.0°C.). There was no apparent difference between cooled and uncooled preparations. Stimulation of the pre-nerves even at relatively low rates; *e.g.*, 5 per sec., almost always produced marked fatigue. Less than half (ten) of these preparations transmitted for longer than 10 min. Those ganglia which did transmit were highly unpredictable as to their maximum transmission rate and other measured properties and were too variable for the proposed drug studies.

The fact that a few impulses were transmitted immediately before complete failure suggested that block was a failure to recover from a previous impulse rather than a result of tissue trauma. The possibility of some metabolic deficiency in the recovery process seemed likely. Saturation of the bathing medium with pure oxygen was tried and resulted in a profound improvement in the ability of the giant synapse to transmit impulses. The first two preparations thus treated transmitted at much higher rates than previously with very short delays for spike initiation. Both these preparations were tested frequently and were found to remain in the same relatively good condition for over 12 hours, at which time the experiment was terminated. The ability of high oxygen to maintain transmission for long periods was repeatedly confirmed. Excised ganglia placed in oxygen-saturated sea water usually showed decreasing fatigability and shortening delays for the first few minutes, presumably due to the restoration of oxygen after the ischemia during dissection. Solutions saturated with oxygen were used in all drug studies reported below.

In an attempt to quantify the oxygen effects, two preparations were tested while being transferred alternately between media saturated with air or 100 per cent oxygen. Fig. 1 illustrates the results. Within a short time (15 min.) after placing the preparation into the oxygen-saturated medium, it was able to transmit all test impulses at 50 per sec. presented to it in the period of 5 sec. The transmission delay also fell to a lower value during this period. When placed in the air-sturated medium the situation was reversed; the preparation transmitted a small fraction of the test burst and the transmission delay increased. The actual oxygen concentrations as determined at 20°C. by the Winkler manganous acid–iodide method were 4.7 ml. per liter of sea water for the air-saturated medium and 21.8 ml. per liter for the oxygen-saturated medium.

Before the effects of drugs could be determined criteria for normal transmission were necessary. Transmission delay was between 1.5 and 2.1 msec. in control preparations and increased to 2.8 to 6.0 msec. during development of synaptic blockade. In oxygen-saturated sea water with few exceptions the synapses could be depended on to transmit each response at a pre-axon stimulation rate of 50 per sec. up to 20 or more sec. before increase in transmission

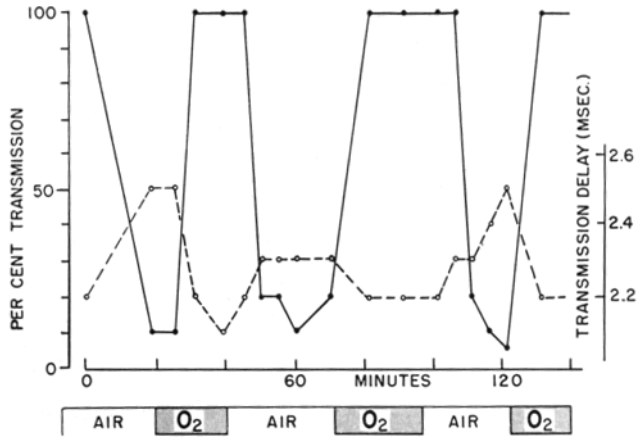

FIG. 1. Effects of alternate transfer between air-saturated and oxygen-saturated sea water on synaptic transmission in the giant synapse of the squid. Broken line, transmission delay. Unbroken line, per cent of impulses at 50 per sec. transmitted in a 5 sec. period.

delay occurred. Return to normal could then immediately be achieved by dropping the frequency to 5 per sec. It was usually possible to raise the frequency again to 50 per sec. and see fatigue after fewer stimuli than with the first burst. After replacement in oxygenated medium for about 5 min., the fatiguing process was similar; and this could usually be repeated several times. Since with this procedure there was sometimes a tendency toward cumulative block, the less strenuous challenge described under Method was offered to the synapse; the 50 per sec. bursts were limited to 5 sec. every 5 to 10 min.

(*b*) *Effects of Drugs.*—Table I summarizes the action of eighteen compounds on giant synapse transmission tested as described.[1] With these agents no evidence of synaptic excitation *per se* was ever observed. With tubocurarine, tetra-

[1] Gamma-aminobutyric acid, meprobamate, morphine, betaine, chlorpromazine, and carbachol at relatively high concentrations (3×10^{-4} to 1×10^{-3} gm. per ml.) had no effect on the controls up to 60 minutes. Cyanide and 2,4-dinitrophenol blocked the synapse at the lowest concentrations (1×10^{-5} gm. per ml.) of any agents employed thus far. Potassium produced reversible block, probably by depolarization, at concentrations between 18 and 36 mM per liter.

ethylammonium, and physostigmine at high concentrations there occasionally occurred some spontaneous activity in the post-axon. Since diphasic recording indicated that impulse generation was occurring from the tied end of the axon to the same extent as from the synaptic end, this effect is concluded to be axonal rather than synaptic. The only other effect observed within 60 minutes' exposure was synaptic depression. The pattern of synaptic depression as observed with extracellular recording was similar for all these agents and resembled the depression produced by prolonged tetanization or anoxia. The earliest event to occur was the lengthening of "transmission delay." With further exposure this increased, transmission block appeared during the 50 per sec. burst, and eventually blockade occurred with low frequency or even with single shocks. There was no noticeable change in pre- or post-axon threshold except with procaine, which produced an increase. Procaine did, however, produce synaptic block before axonal conduction block occurred.

TABLE I

Drug	Total No. experiments	Maximum concentration ineffective in 55 or more min.		Minimal concentration producing synaptic blockage in less than 60 min.	
		gm./ml.	*min.**	*gm./ml.*	*min.**
d-Tubocurarine chloride	5	1×10^{-2}	55	—	—
Atropine sulfate	3	3×10^{-3}	60; 70	—	—
Tetraethylammonium bromide	5	1×10^{-2}	100	3×10^{-2}	16; 18
Hexamethonium chloride	2	5×10^{-3}	60	1×10^{-2}	60
Acetylcholine chloride	3	1×10^{-3}	70	—	—
Nicotine hydrochloride	5	1×10^{-4}	70	3×10^{-3}	40‡; 42‡
Neostigmine methylsulfate	2	3×10^{-4}	66; 78	—	—
Physostigmine	2	3×10^{-4}	109; 111	—	—
Epinephrine bitartrate	4	1×10^{-4}	122; 133	1×10^{-3}	30; 40
Levarterenol bitartrate	2	1×10^{-4}	150; 150	—	—
Tyramine hydrochloride	1	—	—	3×10^{-3}	40
Procaine hydrochloride	3	—	—	6×10^{-5}	60
Serotonin creatinine sulfate	1	—	—	1×10^{-3}	60‡
Histamine diphosphate	4	1×10^{-3}	100; 100	3×10^{-3}	50
Caffeine	9	3×10^{-3}	120	6×10^{-3}	30; 28; 30; 28‡
Strychnine sulfate	3	1×10^{-4}	60	3×10^{-3}	15
Barbital sodium	1	—	—	3×10^{-3}	60‡
Pyrexin (Menkin, 1945)	1	3×10^{-4}	120	—	—

* Each number represents one experiment at this concentration.

‡ Incomplete block to 20 per cent transmission.

Upon return of the blocked preparation to drug-free medium, complete recovery occurred within 50 min. when the block had been produced by tetraethylammonium (one of two preparations), hexamethonium, procaine, and

caffeine. In the rest of the experiments of Table I, recovery was absent or incomplete in 30 to 75 min.

(*c*) *Changes in Magnesium, Calcium, pH, and Osmotic Pressure.*—The effects of varying the concentration of magnesium and calcium in the medium were also studied. If the normal magnesium concentration of the medium (53.4 mM per liter) were doubled, synaptic transmission was depressed in the same manner previously described for the agents in Table I. Block was produced in 30 min. and recovery was effected within 20 min. in normal medium. In either magnesium-free or in 10 per cent (5.3 mM per liter) magnesium media there was much spontaneous activity. Rapid bursts of spikes occurred after a single orthodromic stimulus. There was also spontaneous antidromic post-axon activity, indicating that this was not a purely synaptic phenomenon.

The normal calcium concentration (9.0 mM per liter) was tripled in two preparations. This resulted in improvement in transmission as indicated by decreased transmission delay and less tendency to fatigue for at least 35 min. Decreases in calcium concentration or changes in the other ions of the medium were not attempted.

Since many of the test solutions (section *b*) were in high concentration and unbuffered, controls for pH and osmotic pressure were made. No effects due to pH were noted in a range from pH 6 to pH 9; nor were effects seen within 60 min. when the osmotic pressure was changed by plus or minus 20 per cent.

2. Intracellular Recording

To characterize better the action of several agents intracellular recordings were made from the post-axon as close to the synaptic center as possible. Control studies with orthodromic stimulation without drugs gave intracellular recordings similar to those reported by Bullock and Hagiwara (1955, 1957). Resting potentials of about 50 mv. with action potentials of about 100 mv. were usually obtained. Synaptic delay could be evaluated in a few experiments in which it was possible to see the pre-spike artefact in the recording, and it amounted to between 0.5 and 1.0 msec. at 20°C. In the unfatigued preparation the inflection point at which the spike develops out of the e.p.s.p. was difficult to discern because of the rapid rise of the e.p.s.p. In the slightly fatigued or partially blocked preparation (no drugs) the inflection point could be seen and indicated a critical depolarization of approximately 10 to 15 mv. Occasionally the spike undershoot was very small in the synaptic region with both orthodromic and antidromic stimulation. An orthodromically induced e.p.s.p. could be superimposed on an antidromic spike at about the middle of its falling phase; *i.e.*, at 0.5 msec. from the beginning of the spike, as previously shown by Bullock and Hagiwara (1957).

The effects of an active concentration of tetraethylammonium (3×10^{-2} gm. per ml.), caffeine (6×10^{-3} gm. per ml.), nicotine (3×10^{-3} gm. per ml.),

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procaine (1×10^{-3} gm. per ml.), and 106.8 mM per liter magnesium were observed with intracellular recording. After control recordings, the drug was added to the bathing medium. Oscillograph recordings were made frequently until synaptic block occurred. The response to single shocks as well as to tetanic bursts was observed. The drug was then washed away and recovery followed.

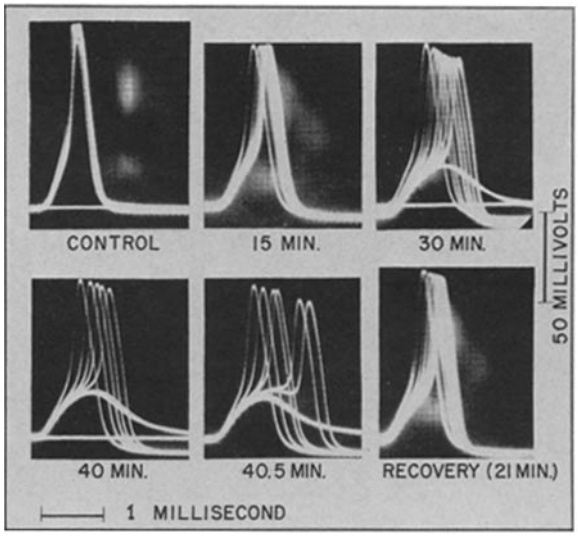

FIG. 2. Effect of procaine on the giant synapse of the squid. Intracellular recording. Each record represents a short burst of stimulation at 50 per sec. before (control) or at the times indicated in relation to exposure to procaine hydrochloride (1×10^{-3} gm. per ml.).

All the agents in this group with the exception of procaine followed a similar pattern while transmission blockade developed. The development of synaptic block with tetraethylammonium was typical. With increasing exposure to tetraethylammonium there was produced a decrease in slope of the e.p.s.p. accompanied by increased depolarization time before spike initiation and increased tendency to fatigue with the tetanizing burst. There was little change in critical membrane depolarization required to initiate the spike and no significant change in the synaptic delay; *i.e.*, the time from pre-spike artefact to the beginning of the e.p.s.p.

The exception to this group, procaine, is illustrated in Fig. 2. Procaine decreased the rising slope of the e.p.s.p. as did the other compounds. It also produced a progressive increase in membrane depolarization required for spike initiation from about 15 to 30 mv. as block developed. Thus with procaine

transmission fails despite a relatively large e.p.s.p. The resting membrane potential measurements would be of value in determining whether the induced blockade was accompanied by slow membrane potential changes. Resting membrane potentials recorded at the synapse over long periods were unsatisfactory because of anomalous electrode changes and drift which occurred if the electrode was merely left in; or because of progressive injury if the synapse was repeatedly punctured.

In two preparations, while recording from the post-axon at the synapse, several cubic millimeters of a 10 per cent solution of acetylcholine chloride were directed externally to the synapse through a small pipette which had penetrated the connective tissues immediately over the synapse. No effects were observed either in membrane resting potential, e.p.s.p., or action potential.

DISCUSSION

Our electrophysiological findings confirm those of Bullock and Hagiwara (1957). Previous investigations have not recognized the vital role of oxygen in maintaining constant the properties of the giant synapse. The relative indifference of the function of the giant axon with respect to oxygen supply is in striking contrast to the dependence of the synapse. Bullock (1948) showed that the giant synapse responded to anoxia with increased synaptic delay and depression of the slope of the e.p.s.p., with consequent delayed origin of the spike. These effects were not quantified but were said to be similar to the effect of cooling the ganglion. The survival of the preparation was not considered in relation to the oxygenation of the medium. In the more recent studies of Bullock and Hagiwara (1957) cooling and aeration probably increased survival of the preparation; presumably to the level shown for air-saturated media in Fig. 1. Failure of their preparations may have been associated with hypoxia rather than with trauma as they suggested.

Baglioni (1905) and Frölich (1910) described and quantified a similar high oxygen requirement for transmission in the stellate ganglion of the octopus. The fact that the octopods lack giant axons or synapses in their stellate ganglia (Young, 1936) makes these findings difficult to relate to the squid problem. A host of observations indicates the relatively great oxygen need of the squid for integrated activity (Redfield and Goodkind, 1929; Prosser, 1950). It is not surprising to find such need reflected in the activity of the giant synapses.

Lowering the magnesium concentration of the sea water medium increased axonal excitability and improved synaptic transmission up to the point at which spontaneous axon spikes appeared. Doubling the magnesium concentration resulted in synaptic block. These results suggest that a magnesium concentration lower than normal would allow better synaptic function. It is possible that *in vivo* the magnesium concentration outside the giant synapses is lower than that of the sea water used here. Considering the improvement

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with raised calcium concentration perhaps a better medium would also contain more of this ion.

Although electrical studies support the notion that a chemical mediator is involved in transmission through the giant synapses, as yet no pharmacological data support the proposal. In these studies the synapses were neither excited nor blocked by any of the well known synaptic agents in concentrations one would expect to be released at a synapse. The drugs were, however, applied externally, and the lack of immediate effects at low concentrations may indicate the presence of a penetration barrier. The fact that a heterogeneous group of compounds almost all had the same depressant effect in high concentration offers little encouragement toward identifying a chemical mediator. Although the block was in all cases clearly synaptic rather than axonal, the manner in which it developed was the same with the drugs as with such changes as hypoxia, damage, cooling, or fatigue.

The largest group of agents tested was that active at cholinergic synapses: acetylcholine, tubocurarine, atropine, tetraethylammonium, hexamethonium, nicotine, neostigmine, and physostigmine. In spite of the proposed role of acetylcholine in the function of the giant axon (Nachmansohn, 1955), the agents which stimulate cholinergic synapses did not stimulate the giant synapse. Some of those which block cholinergic synapses did block here, but only in high concentrations. In preliminary experiments, large amounts of tetraethylammonium or nicotine were injected into the blood vessels of whole squid. The mantle contractions and the heart beat soon ceased. The animals were thereupon opened, and the pre-axons were stimulated. Mantle contractions resulted and in some preparations post-axonal spikes were found to be present. Hence these drugs had not interfered with the function of the giant synapses and must be concluded to have acted more centrally. In juvenile squid, nicotine can cause "mantle convulsions" (Moore, 1919). This action appeared to be selective for the head ganglion and not for the stellate ganglion, as we have confirmed. Moore also found atropine to affect head ganglion but not stellate ganglion in juvenile squid. Here again, though reaching the head ganglion, these compounds may have been excluded from the giant synapse.

Serotonin, epinephrine, levarterenol, tyramine, and histamine are other candidates for the role of "transmitter substance" not supported by these experiments. Serotonin has been identified in the nervous system of cephalopods by many workers (Welsh, 1955) and has been proposed as an *Aktionssubstanz* in these forms (Florey and Florey, 1954). One or more of the adrenergic agents have been shown to stimulate heart action (Östlund, 1954); the chromatophore system (Sereni, 1930); or visceral muscle (Ungar, 1937) in cephalopods. Though the identification of adrenergic substances in the squid (*Loligo*) has not been satisfactory, amine oxidase activity is present to a large extent in its nervous tissue (Blaschko and Himms, 1954).

The remaining substances affect the mammalian central nervous system in various ways. In squid, caffeine (1×10^{-4} gm. per ml.) stimulates the head ganglion, whereas strychnine (2×10^{-6} gm. per ml.) seemed to stimulate both head and stellate ganglia of small squid (Moore, 1917, 1919). Although we confirmed Moore's findings in immature squid, strychnine did not stimulate the adult excised ganglia. It is not clear that the giant synapses are involved in the mantle excitation produced by strychnine. If they are, perhaps the absence of integrated control through the various neuronal pathways in the excised preparation prevents seeing the strychnine effect. This factor may operate for many of the other agents as well.

Procaine was the most potent of the blocking agents. The high potency may arise from the fact that procaine adds to the action of the other agents an increase in electrical threshold of the post-axon to the e.p.s.p. This effect is consistent with the known actions of local anesthetics on axonal excitability. Because of the increased threshold, block appeared before much decrease in the slope of the e.p.s.p. had occurred.

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