Intra-Axonal Injection of Biologically Active Materials[1]

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ABSTRACT

BRADY, ROSCOE O., CONSTANTINE S. SPYROPOULOS AND ICHIJI TASAKI. *Intra-axonal injection of biologically active materials.* Am. J. Physiol. 194(1): 207–213. 1958.—An improved method for injection of chemicals into the squid giant axon is described. A large number of metabolically active materials were found to be without any effect upon the action potential. Tetraethylammonium chloride produced a striking prolongation of the action potential. Cocaine and ethanol caused a transient reduction in the amplitude of the action potential. A filter paper eluate was found to contain materials which caused repetitive firing followed by a reduction in amplitude of the action potential and finally inexcitability.

THE INTERPRETATION of experiments on the extracellular application of chemicals is somewhat limited by the fact that there exists considerable doubt as to whether a given agent ever reaches its expected site of action. This difficulty may in part at least be circumvented by the intracellular introduction of the chemical in question. Several studies have been reported on the injection of materials into the giant axon of the squid (1–3) and lobster (4), into muscle fibers (5–8), motor neurones (9, 10) and other cells (11). In this communication, a modified method for the microinjection of materials into the squid giant axon is described, and the results of the first series of experiments are reported.

METHODS

The isolated giant axon was mounted on a 35-mm glass plate and the ends were secured with silk ligatures. Two small incisions were made through the membrane of the axon on each side of the microinjection chamber (fig. 1). A micropipette from 60 to 100 μ in diameter was mounted on a microinjection apparatus and inserted into the axon from one end of the axon. Another micropipette of about the same size, held by an independent micromanipulator, was introduced into the axon through the other incision. By a careful manipulation of the two micromanipulators, the two micropipettes were arranged in the axon in such a manner that there was an overlapping of at least 10 mm when the pipettes were in place (fig. 2). The second micropipette was filled with 0.57 M KCl and a fine silver wire was inserted into the flared end and sealed in place with wax. The silver wire was led to a cathode follower which utilized a Z729 Emitron tube in triode connection. The axon was stimulated near the site of the insertion of the microsyringe by means of two steel wires. The fluid surrounding the axon was grounded with a large Ag-AgCl (agar) electrode.

The injection of the material under investition was accomplished by maintaining a slight positive pressure on the fluid within the microsyringe while the latter was slowly withdrawn from the axon. When the injected fluid was colorless, 0.05% chlorphenol red dye was added to the injected fluid. By adjusting the rate of withdrawal and the applied pressure, the coloration of the axoplasm at the region of the receding tip of the microsyringe was kept constant. In this manner, an approximately uniform distribution of the injected agent was expected though not necessarily obtained (see DISCUSSION). The action potential was monitored throughout the period of injection. The

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[1] The work presented in this paper was carried out at the Marine Biological Laboratory, Woods Hole, Mass.

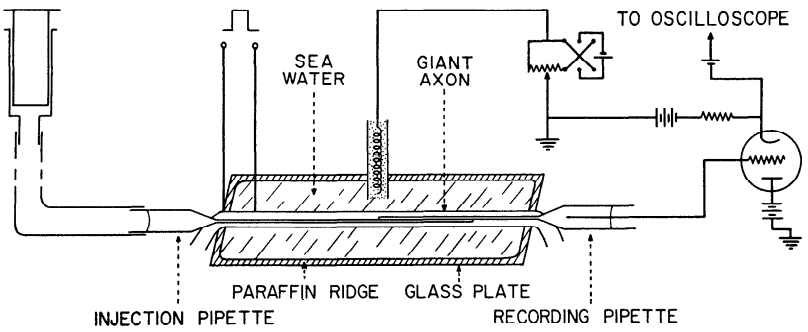

FIG. 1. Diagram of the injection apparatus.

fluid containing the materials to be examined was injected over a region of 15–35 mm.

This procedure offers a number of advantages over the methods employing transmembrane pipettes (e.g. 2) and the technique described by Hodgkin and Kenyes (3) using longitudinal pipettes. The transmembrane pipette technique is subject to the following limitations: *a*) the final concentration of the injected agent is uncertain; *b*) the bulk of the injected agent is localized within a small segment of the axon; *c*) when the recording pipette is the same as the injecting pipette, it may record potentials or changes in potentials not necessarily derived from the injected region. The advantages of the present method over the longitudinal pipette method previously employed reside primarily in the method of insertion of the pipette and in the manner in which the electrophysiological properties of the axon were tested. These advantages are as follows:[2] *a*) cleaning of the axon is not necessary; *b*) axons with normal responses (the amplitude of the action potentials ranging from 110 to 120 mv) can be obtained with facility and within a considerably shorter period of time; *c*) the possibility of damaging the nerve fiber due to repeated insertion and withdrawal is reduced; *d*) the resting and action potentials can be explored very easily over the entire injected and normal portions of the axon.

The solutions of all the chemicals tested were adjusted to pH 6.5–6.9 before injection. The experiments were carried out at room temperature (20–23°C). A total of 171 axons was employed in the present study.

[2] The method of insertion of longitudinal electrodes has been described in part in other communications (12, 13).

MATERIALS

Preparation of Enzymes. Apyrase was purified from an extract of potatoes as described by Krishnan (14). Phosphotransacetylase was obtained from an extract of lyophylized cells of *C. kluyveri* (15). Carbonic anhydrase was purchased from Worthington Biochemical Corporation. Diphosphopyridine nucleotidase, prepared from an extract of *Neurospora crassa* (16), was a gift from Dr. R. M. Burton. Purified acetyl cholinesterase from electric eel tissue was a gift from Dr. S. L. Friess.

Other Materials. Nucleotides and other chemicals were purchased commercially. The authors are indebted to Professor R. A. Peters for the gift of a sample of fluorocitrate which was synthesized by Dr. D. E. A. Rivett. Lysergic acid diethylamide was obtained from Sandoz Pharmaceuticals. The diisopropyl phosphofluoridate was a gift from Dr. Jules A. Gladner. Two preparations of tetraethyl ammonium chloride (TEA) were employed. One was Etamon purchased from Parke, Davis and Co., and the other was kindly supplied by Dr. Lorente de Nó. Succinyl coenzyme *A* was prepared from the acid anhydride (17) and fluoroacetyl coenzyme *A* from the mixed anhydride with ethyl formate (18). The filter paper eluates were prepared by the following procedure. A sheet of Whatman № 3 paper was exhaustively washed with water, dried, and an ascending chromatogram was run using 50% ethanol in 0.1 M potassium acetate buffer (pH 4.5) as the developing solvent (19). The chromatogram was dried in air and again washed with water, dried, and a second run in the ethanol-potassium acetate mixture was carried out. After drying, a portion of the chromatogram whose Rf values extended between 0.6 and 0.8 was eluted with a few milliliters of water. The eluate was

lyophylized and taken up in approximately one-tenth of the original volume of water.

RESULTS

The suitability of the microinjection technique employed in the present experiments was tested by showing that the injection of 17 axons with large volumes of 0.5 M KCl containing 0.05% chlorphenol red had no demonstrable effect upon the resting potential or the amplitude, duration and configuration of the action potential. The intra-axonal injection of a large number of enzymes, coenzymes, or enzyme inhibitors had little or no influence upon the action potential. These agents, the concentrations employed and volumes injected as well as the axon diameters, length of axon injected and observation times are listed in table 1.

The intra-axonal injection of a solution of 0.1% cocaine hydrochloride in 0.5 M KCl caused first a reduction in amplitude of the action potential followed by loss of ability to conduct the nerve impulse. The axon showed a spontaneous recovery (table 2). Comparable results were obtained with the injection of ethanol (table 2). The introduction of TEA resulted in a marked prolongation of the action potential without an appreciable change in the amplitude (table 2). Higher concentrations than are required to produce the prolongation caused a reduction in amplitude of the action potential. Under TEA, an undershoot (a positive phase) of the action potential was as a rule present; in a few instances, though, its amplitude was rather low. A more detailed description of the effect of TEA is presented elsewhere (13). The intra-axonal injection of an eluate from Whatman filter paper # 3 caused repetitive firing followed by a reduction in amplitude and finally inexcitability. Investigation of a number of products which may be obtained from lignin (21) revealed that compounds such as catechol, guiacol, resorcinol, etc. brought about similar changes upon the axon. A full account of these experiments will appear in connection with a different study (manuscript in preparation). The effects obtained by injection of acetyl coenzyme *A* are still under investigation; the results obtained to date with this chemical have not been consistent.

DISCUSSION

In attempting to evaluate the results obtained by the intra-axonal injection of materials, several limitations of this procedure must be kept in mind. When it is stipulated that a given agent had no effect upon the axon being examined, it is implied that there was no change in the resting potential greater than 15%, in the amplitude of the action potential greater than 10%, in the duration of the action potential greater than 30%, or in the configuration of the action potential which could be detected without a critical examination of the recorded pattern. Furthermore, even though an agent did not affect the action or resting potential, other properties of the axon such as the threshold, accommodation, etc. may have been altered.

It must be emphasized that the effect produced by a particular agent may be quite different if the concentration, pH, or period of observation were changed. These factors were not varied to any large extent in these experiments. The choice of concentrations was sometimes made arbitrarily, sometimes determined

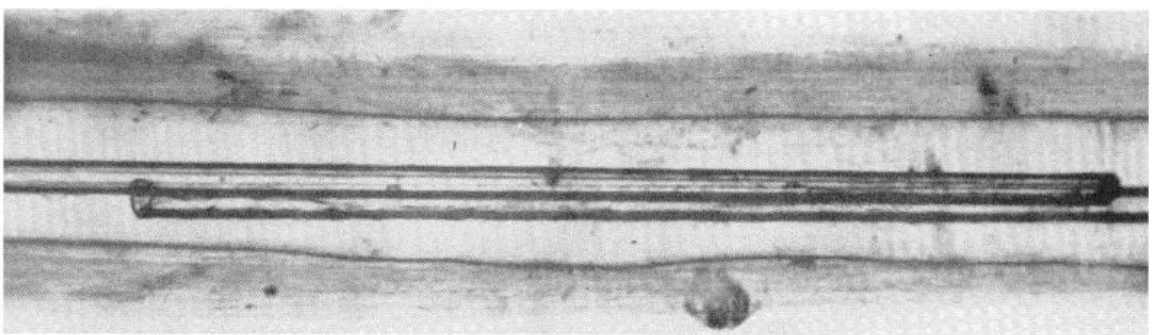

FIG. 2. Photomicrograph of the intra-axonal position of the injection and recording pipettes prior to the introduction of the test solution. Magnification 25.7 ×.

TABLE 1. MATERIALS WHICH HAD NO APPRECIABLE EFFECT ON THE ACTION POTENTIAL

Axon No.	Axon Diam.	Material Injected	Conc.	Inject. Medium	Vol. Inject.	Length of Axon Inject.	Observ. Time
	μ			M KCl	10^{-6} ml/mm	mm	min.
1–17	350–550	Chlorphenol red	0.05%	0.5	40–60	15–35	15–75
18	500	Cholinesterase (800 U*/mg prot.)	550 U/ml	0.5	42	20	20
19	500	Cholinesterase (800 U*/mg prot.)	550 U/ml	0.5	56	20	45
20	500	Cholinesterase (800 U*/mg prot.)	5,500 U/ml	0.4†	42	20	30
21	350	Diisopropyl phosphofluoridate	0.001 M	0.5	42	20	30
22	400	Diisopropyl phosphofluoridate	0.001 M	0.5	50	25	40
23	450	Eserine sulfate	0.005 M	0.5	60	25	22
24	500	Eserine sulfate	0.005 M	0.5	50	25	15
25	400	Eserine sulfate	0.01 M	0.5	40	20	20
26	300	Acetylcholine	0.0001 M	0.5	50	20	43
27	300	Acetylcholine	0.001 M	0.5	42	19	30
28	350	Acetylcholine	0.025 M	0.5	45	20	30
29	300	Acetylcholine	0.10 M	0.4	30	20	30
30	350	Acetylcholine	0.10 M	0.4	50	25	60
31	400	Choline chloride	0.10 M	0.4	42	15	15
32	350	Choline chloride	0.20 M	0.3	60	25	60
33	300	Adenosine triphosphate	0.02 M	0.5	60	15	30
34	350	Adenosine triphosphate	0.05 M	0.5	54	20	30
35	380	Adenosine triphosphate	0.05 M	0.5	57	20	30
36	300	Potassium acetate	0.02 M	0.5	60	25	60
37	300	Potassium acetate	0.02 M	0.5	55	20	30
38	270	Reduced coenzyme A	0.006 M	0.5	50	30	20
39	275	Reduced coenzyme A	0.01 M	0.5	50	25	25
40	400	Reduced coenzyme A	0.02 M	0.5	48	19	30
41	400	Reduced coenzyme A + Potassium acetate + adenosine triphosphate	0.016 M 0.060 M 0.016 M	0.3	50	25	32
42	350	Potassium butyrate	0.03 M	0.5	45	20	30
43	350	Potassium butyrate	0.03 M	0.5	40	20	15
44	400	Succinyl coenzyme A	0.005 M	0.5	25	20	30
45	400	Succinyl coenzyme A	0.01 M	0.5	30	20	30
46–51	400–500	Fluoroacetyl coenzyme A	0.005 M	0.5	25–40	20	20–40
52–56	400–500	Fluoroacetyl coenzyme A	0.008 M	0.5	30–42	20	20–30
57	400	Reduced glutathione	0.10 M	0.4	60	25	30
58	400	Reduced glutathione	0.10 M	0.4	55	15	30
59	450	Phosphotransacetylase (900 U/mg prot.)	700 U/ml	0.4‡	42	20	25
60	500	Phosphotransacetylase (900 U/mg prot.)	700 U/ml	0.4‡	60	25	30
61	400	Phosphotransacetylase (900 U/mg prot.)	700 U/ml	0.4‡	50	25	40
62	450	Potassium arsenite	0.024 M	0.5	60	25	30
63	260	Potassium arsenite	0.027 M	0.5	56	25	30
64	300	Carbonic anhydrase (120 U/mg prot.)	1500 U/ml	0.5	56	20	50
65	450	Carbonic anhydrase (120 U/mg prot.)	1500 U/ml	0.5	50	20	30
66	500	Acetazoleamide	0.03 M	0.5	57	25	20
67	400	Acetazoleamide	0.02 M	0.5	60	25	30
68	450	Sulfanilamide	0.01 M	0.5	42	30	30
69	400	Sulfanilamide	0.01 M	0.5	50	35	15
70	450	Diphosphopyridine nucleotidase (21 U/mg prot.)	340 U/ml	0.5	60	23	50
71	450	Diphosphopyridine nucleotidase (21 U/mg prot.)	340 U/ml	0.5	45	22	60
72	360	Reduced diphosphopyridine nucleotide	0.09 M	0.5	50	25	30
73	350	Reduced diphosphopyridine nucleotide	0.09 M	0.5	50	25	15
74	360	Reduced triphosphopyridine nucleotide	0.035 M	0.5	50	18	30
75	400	Reduced triphosphopyridine nucleotide	0.025 M	0.5	25	20	20
76	550	Apyrase (6500 U/mg prot.)	14,000 U/ml	0.5	50	20	70
77	450	Apyrase (6500 U/mg prot.)	14,000 U/ml	0.5	56	22	30
78	440	Adenosine diphosphate	0.05 M	0.5	60	25	20
79	400	Adenosine diphosphate	0.05 M	0.5	35	20	10
80	440	Adenosine monophosphate	0.05 M	0.5	60	25	20
81	400	Adenosine monophosphate	0.05 M	0.5	30	20	20

TABLE 1—(*continued*)

Axon No.	Axon Diam.	Material Injected	Conc.	Inject. Medium	Vol. Inject.	Length of Axon Inject.	Observ. Time
	μ			*mKCl*	10^{-6} *ml/mm*	*mm*	*min.*
82	300	Cytidine triphosphate	0.05 M	0.5	42	25	25
83	350	Cytidine triphosphate	0.05 M	0.5	25	15	10
84	300	Cytidine diphosphate	0.05 M	0.5	50	25	25
85	400	Cytidine diphosphate	0.05 M	0.5	30	20	10
86	300	Cytidine	0.10 M	0.4	55	25	30
87	400	Cytidine	0.10 M	0.5	40	20	10
88	225	Phosphocreatine	0.037 M	0.4	42	25	35
89	300	Phosphocreatine	0.10 M	0.4	60	25	25
90	400	Dinitrophenol	0.009 M	0.5	60	25	80
91	300	Dinitrophenol	0.01 M	0.5	36	20	25
92	450	Potassium cyanide	0.40 M	0.1	31	20	40
93	300	Potassium cyanide	0.25 M	0.25	60	25	65
94	450	Sodium azide	0.05 M	0.45	60	25	60
95	500	Sodium azide	0.05 M	0.45	60	25	15
96	360	Potassium iodoacetate	0.05 M	0.45	60	25	90
97	400	Potassium iodoacetate	0.05 M	0.45	60	25	30
98	500	Parachloromercuribenzoate	0.00083 M	0.5	56	25	60
99	400	Parachloromercuribenzoate	0.0075 M	0.5	60	25	60
100	440	Potassium fluorocitrate	0.01 M	0.5	60	25	20
101	460	Potassium fluorocitrate	0.01 M	0.5	50	25	30
102	380	Potassium fluoride	0.05 M	0.5	60	18	25
103	350	Potassium fluoride	0.20 M	0.3	56	20	25
104	350	Hydroxylamine	0.05 M	0.5	55	25	25
105	500	Hydroxylamine	0.05 M	0.5	42	20	30
106	500	Versene	0.01 M	0.5	60	25	30
107	400	Versene	0.01 M	0.5	60	25	40
108	400	Ethylamine	0.09 M	0.4	45	20	30
109	500	Ethylamine	0.10 M	0.4	50	20	14
110	500	Triethanolamine	0.10 M	0.4	55	25	30
111	400	Triethanolamine	0.10 M	0.4	50	20	28
112	350	Tris(hydroxymethyl)aminomethane	0.13 M	0.37	50	20	15
113	350	Tris(hydroxymethyl)aminomethane	0.13 M	0.37	35	20	20
114	400	Lysergic acid diethylamide	0.0005 M	0.5	55	19	75
115	400	Lysergic acid diethylamide	0.0005 M	0.5	60	30	60
116	350	Thiamine hydrochloride	0.01 M	0.5	36	20	15
117	400	Thiamine hydrochloride	0.01 M	0.5	45	20	15
118	500	Bovine serum albumin	10 mg/ml	0.6	45	20	90
119	500	Bovine serum albumin	10 mg/ml	0.5	50	20	30
120	450	Parahydroxybenzoic acid	0.005 M	0.5	21	30	25
121	450	Parahydroxybenzoic acid	0.005 M	0.5	30	30	50
122	400	Para-amino benzoic acid	0.005 M	0.5	40	30	20
123	400	Para-amino benzoic acid	0.005 M	0.5	55	25	20

* Micromoles of acetylcholine hydrolyzed per hour (20). † M$(NH_4)_2SO_4$. ‡ M$K_2HA_sO_4$.

by the solubility of the agent, and in other instances by a knowledge of the concentration required to produce an effect in other systems. It is sometimes difficult to estimate the final concentration of an injected agent in the vicinity of the excitable membrane; this problem has been oversimplified in most previous studies. Upon injection, an agent may diffuse longitudinally along the axis of the fiber or transversely up to or across the membrane. It may be bound to materials in the fiber, or it may be metabolized. The rate of diffusion and final distribution of the agent may be similar or different from that of the accompanying dyestuff. In some instances it appears to be different.

Ethanol and cocaine produced a transient reduction or loss of excitability. The reversible nature of this effect is probably due to a reduction of the intra-axonal concentration by

TABLE 2. MATERIALS EXAMINED WHICH AFFECTED THE ACTION POTENTIAL

Axon		Material Injected*	Conc.	Volume Inject., 10^{-6} ml/mm	Length of Axon Inject., mm	Effect	Recovery	Observ. Time
No.	Diam., μ							
124	500	Cocaine HCl	0.1%	60	25	Decr. ampl. and eventual inexcit.	Yes	20
125	500	Cocaine HCl	0.1%	30	25	Decr. ampl. and eventual inexcit.	Yes	15
126	500	Ethanol	25%	30	20	Decr. ampl. and eventual inexcit.	Yes	25
127	500	Ethanol	25%	35	25	Decr. ampl. and eventual inexcit.	Yes	20
128	500	Ethanol	20%	15	15	Decr. ampl. and eventual inexcit.	Yes	20
129	450	Ethanol	20%	30	20	Decr. ampl. and eventual inexcit.	Yes	30
130	450	Ethanol	15%	20	20	Decr. ampl. and eventual inexcit.	Yes	15
131	500	Ethanol	10%	35	25	None		15
132–159	400–500	TEA	0.5 M	10–33	20	Marked prolongation of falling phase	No	15–60
160	350	TEA	0.5 M	40	20	Reduced amplitude	No	30
161	450	TEA	0.5 M	50	20	Reduced amplitude	No	30
162	500	TEA	0.5 M	60	25	Reduced amplitude	No	30
163–171	400–500	Filter paper eluate		5–20	20–30	Repetitive firing followed by reduced amplitude and inexcitability	No†	30–60

* Injection fluid was adjusted to 0.5 M KCl and pH 6.7 ± .1 with the exception of the experiments with TEA in which the KCl was omitted from the injection solution. † In 2 axons recovery was observed.

diffusion through the membrane. This explanation is consistent with the fact that these agents are effective when applied externally and that their effects are reversible. The material in the filter paper eluate was also effective when applied externally and in such experiments the effect could be diminished by washing the axon with sea water. Although spontaneous recovery following injection of the filter paper eluate was observed in only two out of nine experiments, the distribution of this material in the axon may be similar to that of ethanol and cocaine. Chlorphenol red applied externally will not promptly color the axoplasm. Applied internally, it does not appear to diffuse through the membrane. The diffusion rate within the gelatinous axoplasm of many of the enzymes investigated may well be significantly different from that of the dye. On the other hand, the lack of an effect of TEA when applied externally and the permanency of the effect when applied internally suggest that the distribution of this agent may be similar to that of the dye. Finally, assuming that the distribution of an agent is similar to that of the dye, a barrier of some sort beyond the resolution of the ordinary microscope may still be imposed between the agent and the reactive site.

The finding that the injection of acetyl choline over a wide range of concentrations was without any significant effect upon the action potential is in contrast with the results obtained by another group of investigators (22). Attempts to inactivate acetyl cholinesterase by injecting diisopropyl phosphofluoridate or eserine sulfate were ineffective unless exceptionally high concentrations of eserine were employed. At these higher levels, it might be expected that ancillary effects of this nitrogenous compound may play a role other than that of solely inhibiting acetyl cholinesterase. In other experiments designed to attempt to reduce the concentration of acetyl choline, purified acetyl cholinesterase was injected intra-axonally. Similarly, the enzyme phosphotransacetylase and potassium arsenate were injected to catalyze the destruction of acetyl coenzyme *A* (15). Apyrase, which catalyzes the hydrolysis of adenosine triphosphate, was also injected. None of these enzymes or enzyme inhibitors exhibited a significant effect on the action potential. These results do not lend support to the hypothesis of Nachmansohn and others (e.g., 23, 24) regarding the indispensable role of acetyl choline in the immediate process of excitation and conduction.

It is also unlikely that interfering with glycolysis or the tricarboxylic acid cycle had any immediate pronounced effect upon the production of the action potential since potent metabolic inhibitors such as iodoacetate, potassium fluoride, fluorocitrate, cyanide, azide

and dinitrophenol were without effect. Grundfest (25) has previously mentioned the lack of an effect of certain sulfhydryl inhibitors and the present experiments with *p*-chloromercuribenzoate and potassium arsenite are in accord with his observations.

The marked prolongation of the falling phase of the action potential caused by TEA is comparable to the prolongation of the response of the node of Ranvier caused by alkaloids such as brucine, emetine, sinomenine and heroine (26), hypertonic NaCl (26), repetitive stimulation (27), as well as hypertonic urea, glycerol or sucrose, a number of metal ions such as Ni^{++}, Co^{++} and Be^{++}, and certain derivatives of morphine (Spyropoulos, C. S. and R. O. Brady, manuscript in preparation). In many respects, these prolonged responses of both the giant axon and the node of Ranvier show similarities to the normal response of the heart muscle (13, 27 and Spyropoulos, manuscript in preparation).

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