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PROPERTIES OF THE LARVAL NEUROMUSCULAR JUNCTION IN *DROSOPHILA MELANOGASTER*

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SUMMARY

1. The anatomy and physiology of the *Drosophila* larval neuromuscular junction were studied.

2. The dependence of muscle resting potentials on $[K^+]_o$ and $[Na^+]_o$ follows the Goldman–Hodgkin–Katz equation ($P_{Na}/P_K = 0{\cdot}23$). Chloride ions distribute passively across the membrane.

3. The mean specific membrane resistance of muscle fibres is $4{\cdot}3 \times 10^3$ Ω cm^2, and the mean specific membrane capacitance is 7·1 μF/cm^2. The muscle fibre is virtually isopotential.

4. Transmitter release is quantal. Both the miniature excitatory junctional potential and the evoked release follow the Poisson distribution.

5. Transmitter release depends on approximately the fourth power of $[Ca^{2+}]_o$. If Sr^{2+} replaces Ca^{2+}, it depends on approximately the fourth power of $[Sr^{2+}]_o$. Mg^{2+} reduces transmitter release without altering the fourth power dependence on $[Ca^{2+}]_o$.

INTRODUCTION

One approach to problems in neurobiology is to study perturbations in the function or development caused by single gene mutations. In much the same way as previously done for the biosynthetic pathways in microorganisms, specific components of neural activity or steps in neural development may be studied by altering specific genes.

Drosophila was chosen in our study of the neuromuscular junction because of its well developed genetics and its accessibility to experimental manipulation. Among the existing behavioural mutants (Benzer, 1971; Kaplan & Trout, 1969) and temperature-sensitive paralytic mutants (Grigliatti, Hall, Rosenbluth & Suzuki, 1973), some have abnormalities in neuromuscular transmission (Siddiqi & Benzer, 1976; Ikeda, Ozawa & Hagiwara, 1976; L. Y. Jan, Y. N. Jan and M. J. Dennis, in preparation). *Drosophila* larvae, in comparison to adult flies, are more suitable for

physiological studies, because the nerve–muscle preparation is thin and visible with Nomarski optics, and the nerve terminals are readily accessible to experimental manipulation. Here we will describe the neuromuscular junction of normal larvae to provide a basis for further work.

In many insects the relation between resting potential and ionic composition of the medium does not fit the Nernst equation for a potassium electrode or the Goldman–Hodgkin–Katz equation for a multi-ion electrode model, probably because of unfavourable anatomical features (Piek, 1975). In some cases the quantal nature of transmitter release has not been established, because of the unfavourable distribution of the multiple nerve terminals (Martin, 1966; Usherwood, 1972). These complications do not arise in the *Drosophila* larva. Here we show the larval muscle membrane resting potential obeys the Goldman–Hodgkin–Katz equation for a multi-ion electrode. Limited attenuation of excitatory junctional potential (e.j.p.s) and miniature excitatory junction potential (m.e.j.p.s) shows that the muscle fibre is best treated as a short cable, allowing the specific membrane capacitance and resistance to be calculated. Spontaneous and evoked release is quantal. Evoked release depends on approximately the fourth power of external Ca^{2+} concentrations.

METHODS

Preparation. For physiologial experiments late third instar larvae of the Canton-S strain were pinned to a layer of Sylgard (transparent silicone rubber) under 10 ml. saline in a glass dish (60 mm diameter). The larva was cut open along the dorsal mid line; the digestive and other internal organs were removed, the segmental nerves radiating from the ventral ganglion were cut close to the ganglion, and the larval brain and ventral ganglion were removed. Thus the preparation consisted of an array of muscle fibres attached to the larval cuticle, with one nerve bundle for each right and left half segment, containing the motor axons for the muscle fibres on that half segment. The anatomy of the larval nervous and muscular system was described by Hertweck (1931); a diagram (Pl. 1*A*) is reproduced from Hertweck's paper to illustrate the muscle distribution. Pl. 1*B* is a scanning electron micrograph of the preparation. The fibres studied most intensively were the ventral lateral longitudinal fibres first and second from the ventral mid line.

Physiological experiments. All experiments were done at room temperature ($21{\cdot}5 \pm 1{\cdot}0°$ C). Unless otherwise specified, standard deviations are given for all measurements.

Solutions. The 'normal saline solution' used in most experiments (solution A, Table 1) was a modification of the fly saline of Ikeda (Ikeda & Kaplan, 1970). Solutions with varying sodium concentrations were made by mixing solutions A and B in appropriate proportions. Chloride-free solutions with varied potassium and sodium concentrations were made by mixing different proportions of solutions D and E of Table 1 (Hodgkin & Horowicz, 1959). Solutions with a constant product of $[Cl^-]$ and $([K^+]+P_{Na}/P_K\ [Na^+])$ (see below) were made by mixing solutions C, D, E and F in appropriate proportions. Solutions with different calcium and magnesium concentrations were made by mixing either solutions G and H or solutions I

TABLE 1. Composition of solutions

Ref.	K^+	Cl^-	Na^+	Ca^{2+}	Mg^{2+}	$Tris^+$	SO_4^{2-}	Sucrose	HEPES	pH	Relative tonicity	Relative ionic strength
	<			(m-mole/l. solution)					>			
A	2	142	128	1·8	4	—	—	36	5	7	1·0	1·0
B	2	142	0	1·8	4	128	—	139	5	7	1·0	1·0
C	0	142	130	1·8	4	—	—	36	5	7	1·0	1·0
D*	0	0	85	1	4	—	48	162	5	7	1·0	1·0
E*	85	0	0	1	4	—	48	162	5	7	1·0	1·0
F	130	142	0	1·8	4	—	—	36	5	7	1·0	1·0
G	2	142	128	5·8	0	—	—	36	5	7	1·0	1·0
H	2	142	128	0	5·8	—	—	36	5	7	1·0	1·0
I	2	99	0	45	0	—	—	155	5	7	1·0	1·0
J	2	99	0	0	47	—	—	155	5	7	1·0	1·0
K	40	154	57	8	21	—	—	96	5	7	1·12	1·24

* The total concentration of $CaSO_4$ in solution D or E is 8 mM. But the concentration of ionized calcium is 1 mM (see Hodgkin & Horowicz, 1959).

and J. Solutions of various pH or tonicity were made by adjusting the pH or sucrose concentration of solution A.

Solution K of Table 1 approximates the *Drosophila* larval haemolymph as reported by Begg & Cruickshank (1962). It has the same concentrations of sodium, potassium, calcium and magnesium, the same pH and the same tonicity, but its chloride concentration is 154 mM instead of the reported 42·2 mM, to maintain charge neutrality.

All chemicals were analytical grade and glass-distilled water was used. HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffer was from CalBiochem, La Jolla, California. The pH of each solution was checked with a Beckman SS-2 pH meter.

Electrodes and electronics. Membrane potential was recorded differentially, using two glass micro-electrodes filled with 3 M potassium chloride or 4 M potassium acetate. Electrode resistances were usually between 15 and 30 MΩ. Similar electrodes were used for current injection. A chlorided silver wire in the bath was the ground. Suction electrodes of 10–20 μm inner diameter at the tip were used for stimulation of the nerve.

Two W-P Instrument M4A electrometers of unity gain and a Tektronix 565 oscilloscope were used for voltage recordings. Separate channels of the oscilloscope were used to monitor currents passed through the current injection or suction electrodes. A 100 MΩ resistor was in series with each of these electrodes to maintain constant current. A polaroid camera and a Brush 220 chart recorder were used to record events. For this preparation, m.e.j.p.s recorded by the chart recorder at sensitivity about 1 mV/cm were not decreased in amplitude by more than 10% compared with the oscilloscope.

Electron microscopy. Preparations dissected in saline were fixed in Karnovsky's fixative (Karnovsky, 1965) for 1 hr and then post-fixed in osmium overnight at 4° C. After dehydration with ethanol, the preparation was either critical-point dried, coated with gold, and studied under an ETEC scanning electron microscope, or embedded in Epon-Araldite (Ciba), sectioned and examined under a Philips 301 electron microscope. Thin sections were stained with uranyl acetate and lead citrate.

RESULTS

Anatomy

The full-grown larva is 3–4 mm in length, and has about 400 striated muscle fibres arranged in a constant pattern (Pl. 1). A muscle is a single fibre about 400 μm long, 80 μm wide, and 25 μm thick; its polytene nuclei (about 10–20 per fibre) are flattened and aligned in longitudinal rows on the innermost surface of the fibre. Sarcoplasmic reticulum and dyads are found among the filaments within each fibril; perforate Z-disks are also found which seem to be characteristic of supercontracting muscles (Osborne, 1967*b*). A tracheolar plexus is in close contact with each fibre to supply air. Each segmental nerve radiating from the ventral ganglion approaches the left or right segment at the ventral mid line, branches, and innervates fibres of that half segment. Examination with Nomarski optics shows nerve endings as clusters of boutons about 3–5 μm in diameter (Pl. 2).

At these sites, extracellular recording shows excitatory junctional current. Electron microscopy shows these boutons contain microtubules,

mitochondria, clear vesicles and presynaptic dense projections. They are separated from the muscle membrane by a 200 Å cleft, and are surrounded by complex folds known as the subsynaptic reticulum (Osborne, 1967*a*) (Pl. 3).

The resting potential

(*a*) *Effects of pH and osmolarity*

The effect of pH on membrane potential was studied by using solution A (Table 1) without sucrose, adjusted with sodium hydroxide to various pH. HEPES buffer was used because of its negligible divalent cation binding and the stability of its pKa with temperature ($\Delta p\mathrm{Ka}/^\circ \mathrm{C} = -0{\cdot}014$) (Good, Winget, Winter, Connolly, Izawa & Singh, 1966). The resting potential at pH 5·9 was -47 ± 7 mV (twenty-six fibres, two larvae), at pH 6·8 was -55 ± 6 mV (twenty-four fibres, two larvae), at pH 7·0 was -52 ± 7 mV (twenty-three fibres, two larvae), at pH 7·3 was -53 ± 5 mV (twenty-four fibres, two larvae), and at pH 8·0 was -63 ± 8 mV (twenty-eight fibres, two larvae). Thus, the resting potential was not very sensitive to changes in pH between 6·8 and 7·3.

The effect of osmolarity was studied by using solution A with different sucrose concentrations. The three values of osmolarity tested corresponded to solution A (relative tonicity 1·00), *Drosophila* larval haemolymph (relative tonicity 1·12), and frog Ringer (relative tonicity 0·88). The resting potential was -47 ± 6 mV (thirty-six fibres, two larvae) at relative tonicity 1·00, -47 ± 4 mV (thirty-one fibres, two larvae) at relative tonicity 1·12, and -52 ± 7 mV (twenty-three fibres, two larvae) at relative tonicity 0·88. Since the m.e.j.p., the e.j.p., as well as the resting potential were not very sensitive to changes in osmolarity within this range, nor to changes in pH between 6·8 and 7·3, all solutions used subsequently were pH 7 and with the osmolarity of solution A.

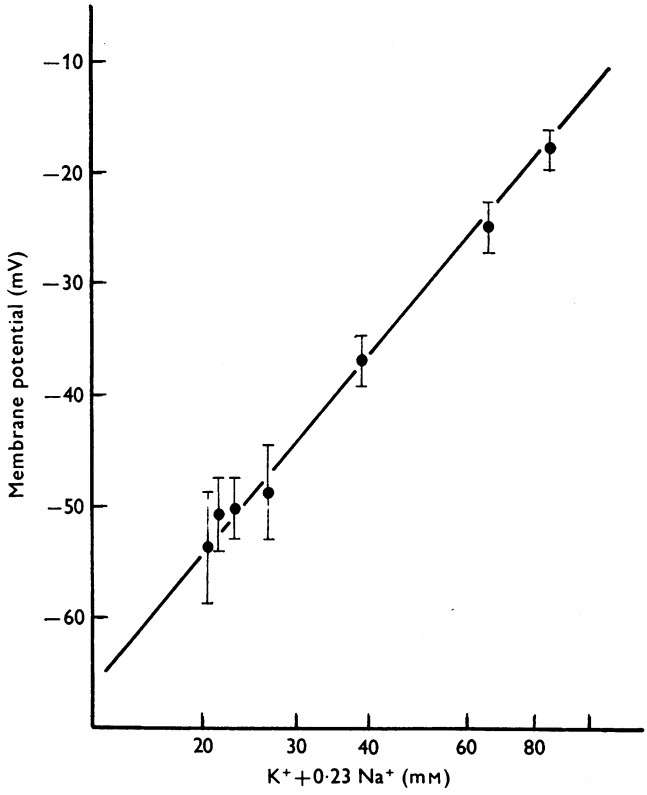
(*b*) *Effects of changes in* $[K^+]_o$ *and* $[Na^+]_o$ *in the absence of* $[Cl^-]_o$

The effect of monovalent cations on membrane potential was studied using chloride-free test solutions, prepared by mixing solution D and E (Table 1) (Hodgkin & Horowicz, 1959).

When a chloride-free solution was introduced to replace solution A, the muscle fibres depolarized by 10–20 mV, but this depolarization was transient. Fig. 1 shows the equilibrated resting potentials at various $[K^+]_o$ and $[Na^+]_o$.

The change of the resting potential with $[K^+]_o$ and $[Na^+]_o$ was largely reversible. If a preparation was bathed in solution A after equilibrating for 60 min in a chloride-free solution containing 85 mM potassium and no sodium, the resting potential recovered to 80–90 % of its original value in

solution A within 10 min. In order to check any possible effect of sulphate, the resting potential in solution A was compared to that in a solution substituting 39 mM-Tris (Tris (hydroxymethyl) aminomethane) sulphate for the 36 mM sucrose. After 90 min equilibration, the average potential for twenty-one fibres was -46 ± 7 mV, compared to -45 ± 5 mV (twenty-two fibres) for the same preparation in solution A.



Text-fig. 1. Relation between muscle resting potential and log ($[K^+]_o + 0{\cdot}23\ [Na^+]_o$). Chloride-free solutions containing 8 mM calcium sulphate (1 mM ionized calcium) were used. Each point is the average resting potential of about twenty ventral lateral longitudinal fibres, first and second from the ventral mid line, of a larva, which was dissected in solution A and equilibrated for 30–50 min in a chloride-free solution. Error bars represent standard deviations. All solutions had the same ionic strength and tonicity as those of solution A. Potassium concentrations used ranged from 0·85 to 85 mM; sodium concentrations ranged from 0 to 84 mM.

(*c*) *The Goldman–Hodgkin–Katz equation*

If a membrane is permeable only to potassium and chloride, it behaves as a potassium electrode and the membrane potential at equilibrium obeys the Nernst equation (Boyle & Conway, 1941):

$$E_m = \frac{RT}{F} \ln \frac{[K^+]_o}{[K^+]_i} = \frac{RT}{F} \ln \frac{[Cl^-]_i}{[Cl^-]_o}. \quad (1)$$

This is true for frog muscle only at high $[K^+]_o$ (Hodgkin & Horowicz, 1959). The departure from the simple potassium electrode hypothesis at low $[K^+]_o$ can be explained by the presence of a small permeability for sodium ions. Thus, the frog muscle membrane behaves as a multi-ion electrode and obeys the Goldman–Hodgkin–Katz equation (Goldman, 1943).

$$E_m = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o}, \quad (2)$$

where P_K, P_{Na} and P_{Cl} are permeability coefficients (Hodgkin & Katz, 1949). Furthermore, Hodgkin & Horowicz (1959) showed that chloride ions were distributed passively across the membrane, and the Goldman–Hodgkin–Katz equation for the resting potential could be re-written as

$$E_m = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o}{P_K[K^+]_i + P_{Na}[Na^+]_i} = \frac{RT}{F} \ln \frac{[Cl^-]_i}{[Cl^-]_o}, \quad (3)$$

where $P_{Na}/P_K = 0{\cdot}01$ for frog muscle fibres.

The resting potential of *Drosophila* larval muscle does not obey the Nernst equation for a potassium electrode even at high $[K^+]_o$. Therefore, eqn. (2) for a multi-ion electrode was used to fit the resting potential. The average resting potential in a chloride-free solution containing 2 mM potassium and 83 mM sodium was -51 ± 3 mV (nineteen fibres), close to the average resting potential of -47 ± 4 mV (thirty-one fibres) in solution containing 142 mM chloride, 2 mM potassium and 128 mM sodium ions. Therefore, $[Cl^-]_o$ did not seem to influence E_m to any large extent. This implied that either P_{Cl} was very small or chloride ions were distributed passively across the membrane. The latter was most plausible because of the transient depolarizations observed immediately after the preparation was changed from the chloride-containing solution A to a chloride-free solution (section (*b*)). Similar transient depolarization has been observed in frog muscle and analysed in detail by Hodgkin & Horowicz (1959). Therefore eqn. (3) was used for the *Drosophila* larval resting potential. Since the resting potential was measured in six different chloride-free solutions, five independent determinations of P_{Na}/P_K could be made. The average of the five estimates for P_{Na}/P_K was $0{\cdot}23 \pm 0{\cdot}023$. Thus, if the

resting potential was plotted on a semilogarithmic scale *vs.* $[K^+]_o + 0{\cdot}23\,[Na^+]_o$, the points fell along a straight line with a slope close to 58 mV (Fig. 1), indicating that the resting potential obeys the equation

$$E_m = \frac{RT}{F} \ln \frac{[K^+]_o + 0{\cdot}23\,[Na^+]_o}{[K^+]_i + 0{\cdot}23\,[Na^+]_i} = \frac{RT}{F} \ln \frac{[Cl^-]_i}{[Cl^-]_o}. \quad (4)$$

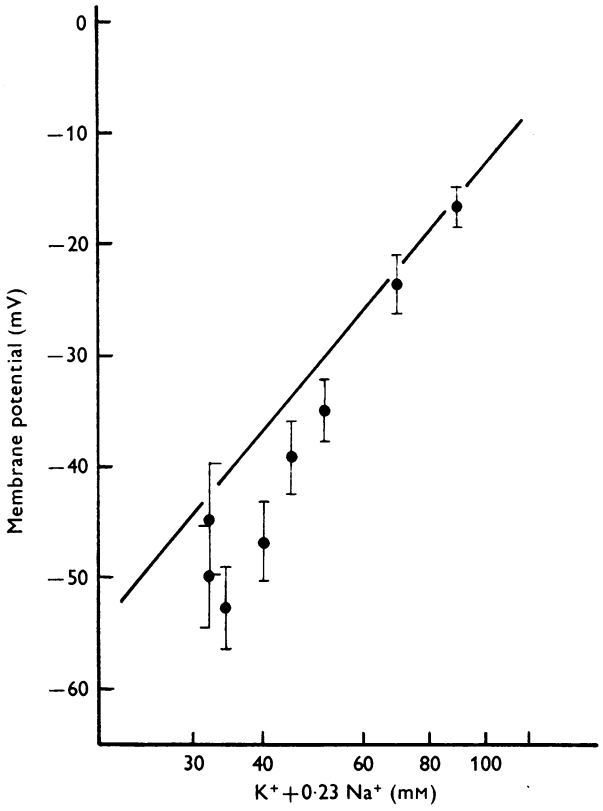
From the best fit of the data in Fig. 1 $\{[K^+]_i + 0{\cdot}23\,[Na^+]_i\}$ was estimated to be about 173 mM.

(d) Effect of changes in $[Cl^-]_o$ and $\{[K^+]_o + 0{\cdot}23\,[Na^+]_o\}$ at constant product

If chloride is distributed passively across the muscle membrane, and if the membrane potential is governed by $[K^+]_o$ and $[Na^+]_o$ according to eqn. (4), there should be no movement of chloride ions when the preparation is flushed with various test solutions in which the product $[Cl^-]_o \{[K^+]_o + 0{\cdot}23\,[Na^+]_o\}$ is kept constant. Hence, in such an experiment, the membrane potential should respond rapidly and reversibly to alterations in $[Cl^-]_o$ and $[K^+]_o + 0{\cdot}23\,[Na^+]_o$.

Complete solution changes required 1–2 min in our apparatus. Within that interval, the membrane potential reached its new value, remained constant, and did not change with further washes of the same test solution. The change of resting potential with $[K^+]_o + 0{\cdot}23\,[Na^+]_o$ was reversible. Fig. 2 shows that the relation between membrane potential is predicted reasonably well by eqn. (4). This agreement, along with the rapid response of the membrane potential in these experiments, compared to the transient depolarization and slow equilibration observed in the experiments described in section (*b*), suggest that chloride distributes passively across the muscle membrane.

The fact that the measured resting potentials were more hyperpolarized than the prediction may be due to the different calcium concentrations used. The straight line in Fig. 2 is the prediction of eqn. (4), where $[K^+]_i + 0{\cdot}23\,[Na^+]_i$ is estimated from the best fit of data obtained in chloride-free solutions (Fig. 1). The chloride-free solutions were mixtures of solutions D and E, which contained 1 mM ionized calcium, whereas the solutions used in this section (Fig. 2) were mixtures of solutions C, D, E and F and the calcium ion concentrations in solutions C and F were 1·8 mM. Thus, in Fig. 2, $[Ca^{2+}]_o$ varied from 1·3 mM at $[K^+]_o + 0{\cdot}23\,[Na^+]_o = 90$ mM to 1·8 mM at $[K^+]_o + 0{\cdot}23\,[Na^+]_o = 31$ mM. As shown in the sections to follow, a higher calcium concentration in the external medium often resulted in a more hyperpolarized membrane potential. Such an effect of $[Ca^{2+}]_o$ might account for the small discrepancy between the results in this section (Fig. 2) and those in chloride-free solutions (Fig. 1). The reason why higher calcium concentration results in more hyperpolarized membrane potential is unknown. One possible explanation may be that higher calcium concentration causes better sealing around the recording electrode, thus reduces the non-specific membrane conductance that tends to bring the membrane potential to a more depolarized value.



Text-fig. 2. Relation between muscle membrane potential and log ($[K^+]_o + 0{\cdot}23\ [Na^+]_o$) when using solutions with constant product $[Cl^-]_o \cdot ([K^+]_o + 0{\cdot}23\ [Na^+]_o)$ (about 4450 mM²). Each point represents the average potential of about twenty ventral lateral longitudinal fibres in the abdominal segments of a larva. All test solutions had the same ionic strength, tonicity, magnesium concentrations and product $[Cl^-]_o \cdot ([K^+]_o + 0{\cdot}23\ [Na^+]_o)$ of solution A. Potassium concentrations ranged from 2 to 86 mM. Sodium concentrations ranged from 15 to 128 mM. Chloride concentrations ranged from 50 to 142 mM. Calcium concentrations ranged from 1·3 to 1·8 mM. The straight line is the prediction from eqn. (4), where $[K^+]_i + 0{\cdot}23\ [Na^+]_i$ is assumed to be 173 mM.

(*e*) *Effects of changes in* $[Na^+]_o$ *at constant* $[K^+]_o$

A P_{Na}/P_K value of 0·23 predicts a large hyperpolarization of membrane potential with decreasing $[Na^+]_o$, if $[K^+]_o$ is kept constant. To test this prediction various amounts of sodium ion had to be replaced by some impermeable and inert cation to keep the ionic strength and tonicity of the solution constant. Choline was unsuitable, since it caused vigorous muscle

contraction, often resulting in damage. As noted above, replacing the sucrose in solution A by 39 mM-Tris sulphate did not change the membrane potential. Therefore Tris was chosen.

In Table 2 the average resting potentials in solutions with different $[Na^+]_o$ were compared with the values predicted from eqn. (4). When $[Na^+]_o$ was reduced to 127, 102, or 64 mM by $Tris^+$ substitution (expts. 1, 2, 3 in Table 2), the measured resting potential agreed reasonably well with the predicted value. When $[Na^+]_o$ was further reduced to 26 or 6 mM (expts. 4, 7 in Table 2), however, the measured resting potential deviated from the predictions of eqn. (4).

One reason the resting potential at low $[Na^+]_o$ might be less hyperpolarized than predicted is possible membrane damage caused by high concentrations of Tris. This was tested by using sucrose, instead of Tris chloride, to substitute for sodium chloride, but this did not alter the average resting potential (expts. 6, 9, in Table 2). Thus the deviation from eqn. (4) was probably not caused by Tris.

Another reason the resting potential did not hyperpolarize further at lower $[Na^+]_o$ would be a small calcium or magnesium permeability, which would contribute significantly only at low $\{[K^+]_o + 0{\cdot}23\,[Na^+]_o\}$. To test this, the resting potentials at different $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ were measured. At 26 or 6 mM-$[Na^+]_o$, reducing $[Mg^{2+}]_o$ from 4 to 0 mM did not alter the average resting potential (expts. 5, 8 in Table 2), thus a permeability for magnesium could not account for the deviation at low $[Na^+]_o$. At 128 mM-$[Na^+]_o$, reducing $[Ca^{2+}]_o$ to 0·1 or 0 mM (expts. 15, 16 in Table 2) resulted in depolarization, while increasing $[Ca^{2+}]_o$ to 5·8 mM (expt. 14 in Table 2) resulted in hyperpolarization. Such an effect is of the wrong sign to be due to a membrane permeability for calcium ions, but could be explained by a membrane stabilizing effect of calcium (Stefani & Steinbach, 1969). Thus we were unable to assess the importance of a calcium permeability in its contribution to the resting potential at low $[Na^+]_o$.

The effect of large concentrations of divalent cations on the resting potential can be studied by substituting these divalent cations for sodium. The average resting potential was about -55 mV, whether Tris, sucrose, or magnesium was used to substitute for sodium (expts. 7, 9, 13 in Table 2). If calcium were substituted for some of the sodium, however, the membrane was much more hyperpolarized (expts. 10, 11, 12 in Table 2). Indeed, membrane potentials as negative as -94 to -97 mV were recorded for several fibres under these conditions. In frog muscles, the input resistance increases and the membrane seals more readily in isotonic calcium chloride (Stefani & Steinbach, 1969). A similar effect of calcium on the larval muscle membrane may explain the results described here.

TABLE 2. Relation between $[Na^+]_o$ and the resting potential

Expt.	External concentrations (mM)							Relative ionic strength	Relative tonicity	Average resting potential (mV)	Predicted from eqn. (4) (mV)
	Na^+	$Tris^+$	K^+	Cl^-	Ca^{2+}	Mg^{2+}	Sucrose				
1	127	1	2	142	1·8	4	36	1·0	1·0	−50 ± 5 (16)*	−43
2	102	26	2	142	1·8	4	56	1·0	1·0	−51 ± 4 (16)	−48
3	64	64	2	142	1·8	4	87	1·0	1·0	−59 ± 6 (16)	−59
4	26	102	2	142	1·8	4	118	1·0	1·0	−54 ± 8 (12)	−78
5	26	102	2	134	1·8	0	118	0·9	1·0	−59 ± 5 (20)	—
6	26	0	2	40	1·8	4	210	0·3	1·0	−57 ± 3 (24)	—
7	6	122	2	142	1·8	4	138	1·0	1·0	−54 ± 4 (14)	−99
8	6	122	2	134	1·8	0	138	0·9	1·0	−54 ± 5 (22)	—
9	6	0	2	20	1·8	4	242	0·2	1·0	−55 ± 2 (22)	—
10	0	0	2	99	45	4	155	1·0	1·0	−81 ± 4 (20)	−112
11	0	0	2	99	32	17	155	1·0	1·0	−88 ± 4 (20)	−112
12	0	0	2	99	19	30	155	1·0	1·0	−75 ± 5 (19)	−112
13	0	0	2	99	1·8	47	155	1·0	1·0	−56 ± 3 (20)	−112
14	128	0	2	142	5·8	0	36	1·0	1·0	−57 ± 3 (16)	−43
15	128	0	2	139	0·1	4	36	1·0	1·0	−40 ± 4 (17)	−43
16	128	0	2	142	0	5·8	36	1·0	1·0	−34 ± 4 (14)	−43

* The number in each parenthesis indicates the number of fibres studied in each experiment.

To summarize, reducing $[Na^+]_o$ from 128 to 102 or 64 mM produced a slight hyperpolarization, as predicted from eqn. (4). Reducing $[Na^+]_o$ to 26 mM or less produced no further hyperpolarization, unless $[Ca^{2+}]_o$ was raised at the same time. This deviation from the prediction is not explained. One remaining possibility is that the muscle membrane is unstable and does not seal well after micropipette penetration, especially when the fibre is hyperpolarized by reducing $[Na^+]_o$, unless more calcium is introduced to improve the membrane stability and the micropipette penetration.

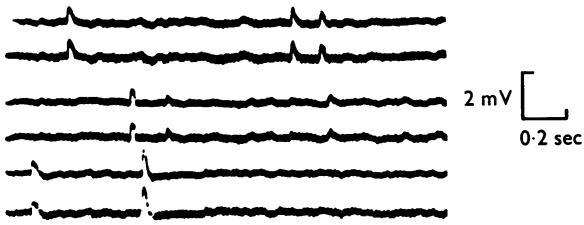
(f) Resting potential in a haemolymph-like solution

Begg & Cruickshank (1962) reported that *Drosophila* larval haemolymph contains 56·5 mM sodium, 40·2 mM potassium, 8 mM calcium, 20·8 mM magnesium, 42·2 mM chloride, and 2·8 mM phosphate. The apparent anion deficit was presumably accounted for by amino acids, organic acids, and proteins. Thus, for a solution that had the same concentrations of sodium, potassium, calcium and magnesium, the same pH and the same tonicity as that in the haemolymph (solution K), the chloride concentration had to be adjusted to 154 mM. The average resting potential in solution K was -37 ± 3 mV (twenty-two fibres), which was somewhat more hyperpolarized than the prediction of -30 mV from eqn. (4). This could be due to the higher calcium and magnesium concentrations in solution K, which may stabilize the membrane. Indeed, if the calcium concentration was reduced to 1·8 mM and magnesium to 4 mM in solution K, the average resting potential was -32 ± 3 mV (twenty-two fibres) as predicted.

Resistance and capacitance of the fibres

The ventral lateral longitudinal fibres second from the ventral mid line are about 400 μm long, 100 μm wide, and 25 μm thick. M.e.j.p.s and e.j.p.s could be monitored with micro-electrodes anywhere in the fibre. (Electrical coupling between fibres in the same segment is negligible, and the coupling between corresponding ventral lateral longitudinal fibres in neighbouring segments is much less than 10 %.) Since the nerve endings are located near the middle of the fibre (Pl. 2), maximum attenuation should be observed by comparing m.e.j.p.s recorded simultaneously with two micro-electrodes 200 μm apart, one near the middle of the fibre, the other near one end. Fig. 3 shows the results. The error of measurement due to noise was about 5 % of the m.e.j.p. amplitude and the attenuation was 5 % or less. To obtain the resistance and capacitance of a muscle fibre, two micro-electrodes, one for recording the membrane potential and one for injecting currents, were used. Since the m.e.j.p. is attenuated more

than the steady-state response to current injection, the attenuation of recorded membrane potential changes, caused by current pulses injected through the current electrode, should be even less than 5–10 %.



Text-fig. 3. M.e.j.p.s recorded simultaneously with two micro-electrodes 200 μm apart, one near the middle of the fibre and the other near one end. There is essentially no attenuation of m.e.j.p.s along the fibre.

With the infinite cable model (Hodgkin & Rushton, 1946), 5 % attenuation corresponds to a length constant of 4 mm, 10 % attenuation to a length constant of 2 mm. In either case the length constant is much greater than the fibre's length. Thus the basic assumption in the infinite cable model does not hold, and a short cable model (Weidmann, 1952) has to be used to estimate the length constant, and the specific membrane resistance and capacitance.

For the short cable model the following equations hold:

$$\text{at} \quad x = 0; \frac{V_o}{I_o} = 0{\cdot}5\, r_i\, \lambda \coth\left(\frac{0{\cdot}5\, L}{\lambda}\right) \tag{5}$$

$$\text{at} \quad x \neq 0; \frac{V_x}{V_o} = \cosh\left(\frac{0{\cdot}5\, L - x}{\lambda}\right) \Big/ \cosh\left(\frac{0{\cdot}5\, L}{\lambda}\right) \tag{6}$$

if the current electrode is inserted in the middle of the fibre, x is the distance between the current and voltage electrodes, L is the length of the fibre, r_i the internal resistance of the fibre per unit length (Ω/cm), $\lambda = (r_m/r_i)^{\frac{1}{2}}$ and r_m is the transverse membrane resistance per unit length (Ω cm). (Since the fibre is kept in a large volume of saline, r_o, the external resistance per unit length is small compared to r_i.) In cases when $0{\cdot}5\, L/\lambda$ is much smaller than unity, the short cable eqn.(5) can be transformed into the asymptotic form.

$$\frac{V_o}{I_o} = 0{\cdot}5\, r_i \lambda \left(\frac{\lambda}{0{\cdot}5\, L}\right)\left(1 + \tfrac{1}{2}\left(\frac{0{\cdot}5\, L}{\lambda}\right)^2 + O\left(\left(\frac{0{\cdot}5\, L}{\lambda}\right)^4\right)\right)_{L \ll 2\lambda} \simeq \frac{r_m}{L}\left(1 + \frac{L^2}{8\lambda^2}\right). \tag{7}$$

From eqn. (6), 5 % attenuation at $x = 200$ μm corresponds to a length constant of about 600 μm and 10 % attenuation corresponds to $\lambda \simeq 450$ μm. In the former case, $L^2/8\lambda^2$ is about 6 %; in the latter case, $L^2/8\lambda^2$ is about 10 %. Thus, one can obtain r_m to within 10 % from the equation:

$$\frac{V_o}{I_o} = \frac{r_m}{L}. \tag{8}$$

The specific membrane resistance R_m can then be obtained using the equation $R_m = l \cdot r_m$, where l is the circumference of the fibre (Weidmann, 1952). For the fibre studied here, $L = 400\ \mu m$ and $l = 2 \times (100\ \mu m + 25\ \mu m) = 250\ \mu m$. Therefore $R_m \cong 250\ \mu m \times 400\ \mu m \times V_o/I_o$.

Having obtained R_m, one can measure the time constant τ_m and calculate the specific membrane capacitance $C_m = \tau_m/R_m$. For the infinite cable model, $V_o(t)$ is an error function of t, and the time taken for $V_o(t)$ to reach 0·84 of its steady value, V_o, is a measure of τ_m (i.e. $V_o(\tau_m) = 0{\cdot}84\ V_o$). In a spherical core conductor, $V_o(t)$ is an exponential function of t, and $V_o(\tau_m)/V_o$ is 0·63. For the short cable model, $V_o(\tau_m)/V_o$ lies between 0·63 and 0·84, and has been estimated for various L/λ values using the total electrical reflexion model (Stefani & Steinbach, 1969). In the *Drosophila* larval fibres, the observed $V_o(t)$ fits an exponential function, but not an error function, of t, indicating that $V_o(\tau_m)/V_o$ approximates 0·63. Using our rough estimate of λ based on the upper limit for attenuation of m.e.j.p.s recorded in the fibre, $V_o(\tau_m)/V_o$ lies between 0·63 (no attenuation) and 0·675 (10 % attenuation). 0·63 was used as an approximation of $V_o(\tau_m)/V_o$ in measuring τ_m. This implies that the specific membrane capacitance may be underestimated by about 10 %. Note that by using eqn. (8) to estimate R_m, additional underestimation of C_m by 10 % or less may result.

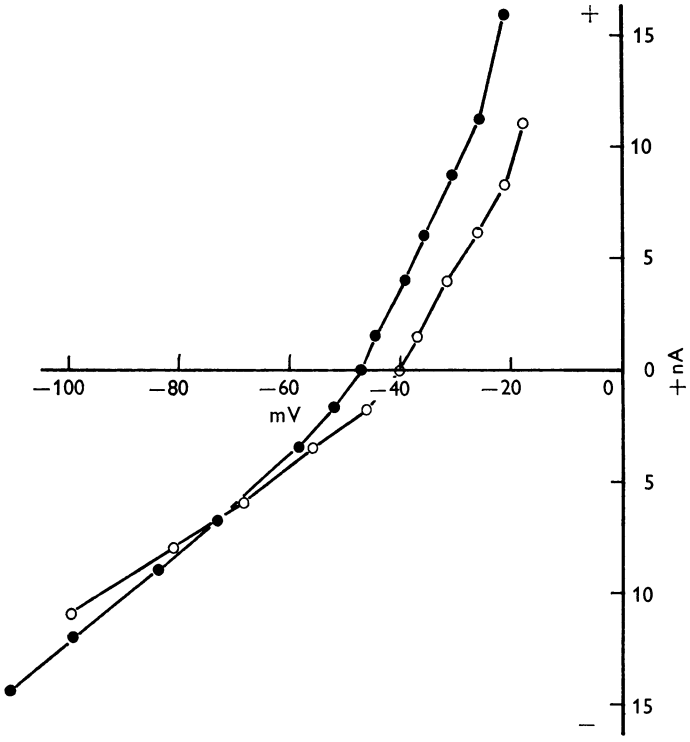
The measured mean specific membrane capacitance was $7{\cdot}1 \pm 0{\cdot}8\ \mu F/cm^2$ for eight fibres in solution A (see Table 1 for compositions). Since the specific membrane capacitance for a lipid bilayer or a biological membrane without proliferation is about $1\ \mu F/cm^2$ (Katz, 1949; Fatt & Katz, 1951), this large specific membrane capacitance here suggests large areas of membranous structures, continuous with the outer membrane, such as the T systems (Falk & Fatt, 1964). When the muscle fibre was hyperpolarized, the membrane potential was approximately a linear function of the current injected, allowing an estimate of membrane resistance from eqn. (8). The average specific membrane resistance for six fibres was $(4{\cdot}3 \pm 0{\cdot}9) \times 10^3\ \Omega\ cm^2$, similar to the value obtained for frog twitch muscle (Stefani & Steinbach, 1969).

Fig. 4 shows the I–V curves for two fibres. These fibres did not show anomalous rectification when hyperpolarized. They did show delayed rectification when depolarized. However, in solution A (Table 1), they did not fire action potentials upon nerve stimulation or upon direct depolarization of the membrane to -5 mV. Nerve stimulation elicited only e.j.p.s; these increased in amplitude if the fibre was hyperpolarized.

The excitatory junctional potential

Thresholds. If an abdominal nerve is cut at the end near the ventral ganglion, it can be stimulated with a suction electrode, generating e.j.p.s in muscle fibres within the corresponding half segment. Neuromuscular transmission has been studied extensively in the two most medial ventral lateral longitudinal fibres. As the intensity of the stimulus pulse applied through the suction electrode was gradually increased, two thresholds were often observed, indicating innervation by at least two motor axons.

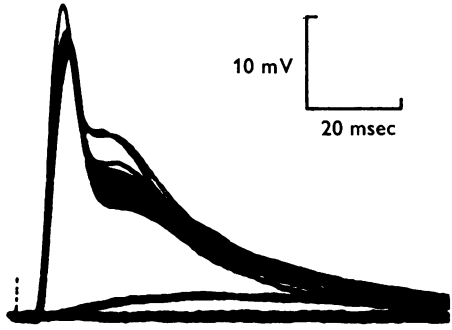
The lower threshold axon generated an e.j.p. that is small (about 4 mV peak amplitude) and slow rising (about 30 msec to peak). The second axon, with a threshold slightly higher than and sometimes overlapping the first one, produced an e.j.p. that is larger (about 34 mV peak amplitude) and faster (about 4 msec to peak). Usually this fast e.j.p. is accompanied by a slower component (about 15 msec to peak) (Fig. 5). This slower component



Text-fig. 4. Current–voltage relation in two ventral lateral longitudinal fibres of 3rd instar larvae in solution A.

decayed to 37 % of its peak value in about 20 msec, a time course comparable to the time constant of the muscle fibre measured with current injection, while the fast e.j.p. decayed to 37 % of its peak amplitude more quickly, in less than 10 msec. Similar observations have been made in crustacean limb muscle (Fatt & Katz, 1953), and may be explained by a two-time constant model (Falk & Fatt, 1964) which assumes that, in addition to the parallel elements of resistance and capacitance of the muscle surface membrane, the impedance between the inside and the outside of the fibre contains another path of a resistance in series with a capacitance, probably owing to the presence of the T system and the triads. For the

Drosophila larval fibres, extracellular recording from a single bouton also revealed an e.j.p. with both fast and slow components, indicating the two components are one e.j.p. with complicated time course. An end-plate potential with similar time course has been observed in frog skeletal muscles treated with xylocaine (Steinbach, 1967).



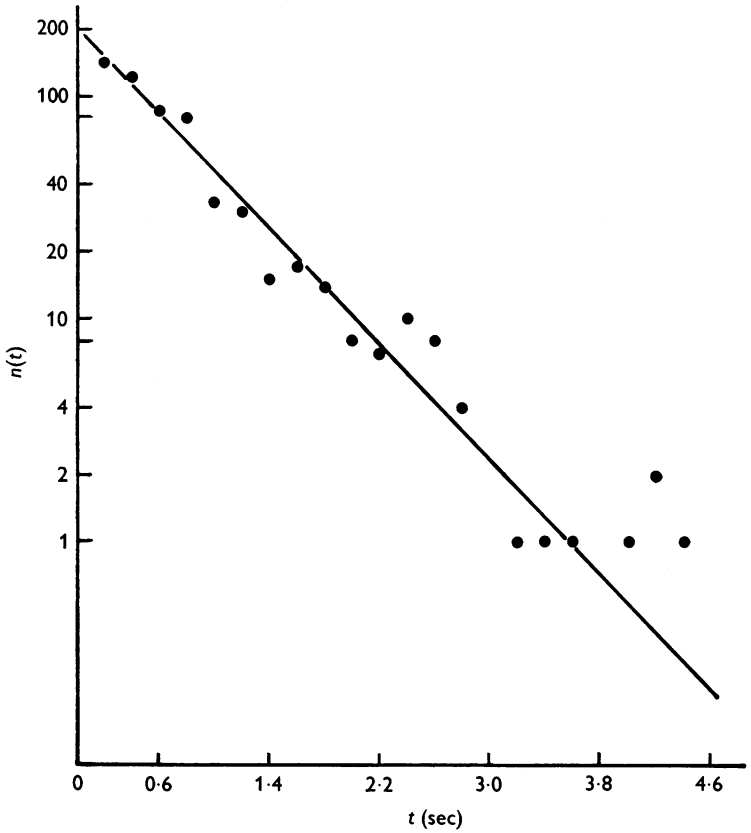
Text-fig. 5. Excitatory junctional potentials of a ventral lateral longitudinal fibre in solution A at various stimulus intensities, showing two thresholds which indicate innervation by at least two motor axons.

The latency of the e.j.p. following stimulation increased with the length of the nerve, from 3 msec for a nerve length less than 200 μm to 6 msec for a roughly 800 μm nerve. This would correspond to a conduction velocity of about 0·2 m/sec.

The presence of more than one threshold suggests that these ventral lateral longitudinal fibres are polyinnervated. Polyinnervation in many systems becomes a major obstacle in the quantal analysis of transmitter release, because the post-synaptic response may contain components of varying quantal content and with different attenuation (Martin, 1966) and the size distribution of the extracellularly recorded m.e.j.p.s may have more than one peak (Usherwood, 1972). This is not the case here because: (1) the larval fibres are short and essentially isopotential. Although produced by more than one axon, the e.j.p.s are not attenuated in the fibres; (2) the m.e.j.p. size histogram has a unimodal profile similar to a Gaussian distribution (described below); and (3) the stimulation used in all studies involving statistical analysis always exceeded the maximum threshold. At low $[Ca^{2+}]_o$, when only one or a few quanta are released by each nerve impulse, the unit response has a single-peaked size distribution similar to the m.e.j.p. size distribution. Therefore, if the Ca^{2+} sensitivity and statistical nature of release are the same for all terminals of all motoneurones, one can study quantal release despite the polyinnervation.

Spontaneous m.e.j.p.s. The average m.e.j.p. amplitude is 0·6 mV. The

size distribution is approximately Gaussian, but is slightly skewed in the direction of large amplitude (Fig. 8*A*). Fig. 6 shows that $n(t)$, the number of intervals between adjacent m.e.j.p.s that were smaller than t but greater than $t-200$ msec, is an exponential function of t, as predicted by the

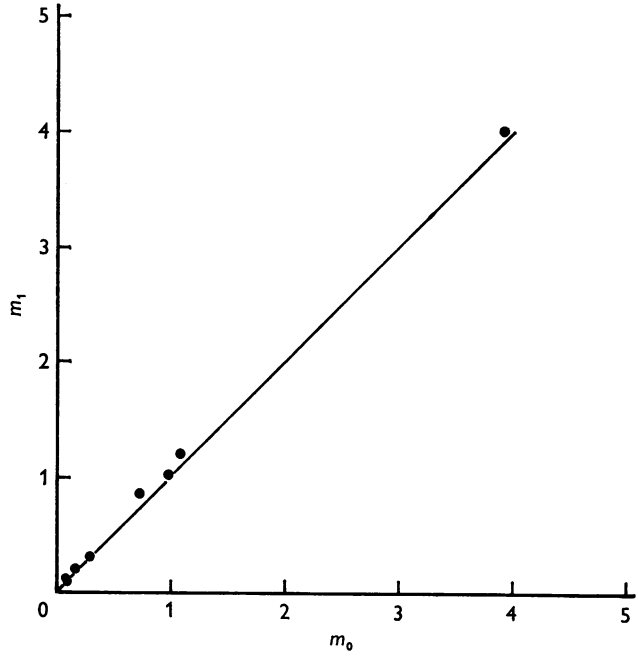


Text-fig. 6. Distribution of time intervals between adjacent m.e.j.p.s in a series of 578 m.e.j.p.s recorded from a ventral lateral longitudinal fibre in a solution containing 0·1 mM calcium chloride. In this Figure log $n(t)$ is plotted against t, $n(t)$ being the number of intervals between t and $t-200$ msec. Note that $n(t)$ is an exponential function of t, as predicted by a Poisson distribution. The mean interval between adjacent m.e.j.p.s is 668 msec.

Poisson distribution for random, non-interacting events (Fatt & Katz, 1952).

Quantal nature of transmitter release. Transmitter release at the nerve terminal was strongly dependent on $[Ca^{2+}]_o$. At low $[Ca^{2+}]_o$, the e.j.p. amplitude was greatly reduced. Failures were often observed, indicating that the release was quantal in nature. The quantal content m_1 of evoked

e.j.p.s is the ratio of average e.j.p. amplitude to average m.e.j.p. amplitude. If the release of each quantum is random, and if the total number of quanta available is large, the release should follow a Poisson distribution and m_1 should equal $m_0 = \ln N/n_0$, where N is the total number of responses and n_0 is the number of failures (del Castillo & Katz, 1954; Boyd & Martin, 1956). Fig. 7 shows that the independent estimates m_0 and m_1 agree at



Text-fig. 7. Comparison of mean quantal content m estimated from e.j.p. amplitudes, $m_1 = \bar{v}/\bar{v}_{\text{m.e.j.p.}}$ with an m estimated from transmission failures, $m_0 = \ln N/n_0$, where n_0 is the number of failures and N is the total number of responses. The eight points in the Figure represent data from four ventral lateral longitudinal fibre in three larvae. The divalent cation concentrations used were: 4 mM magnesium and 0·1 mM calcium, 4 mM magnesium and 0·15 mM calcium, 4 mM magnesium and 0·2 mM calcium, 2 mM magnesium and 0·05 mM calcium, 2 mM magnesium and 0·1 mM calcium. For each point in the Figure, the fibre was stimulated once every 4 sec for 200 times. From 100 to 200 m.e.j.p.s were recorded to give $\bar{v}_{\text{m.e.j.p.}}$.

low calcium concentrations. (We did not test whether release will conform to Poisson statistics at calcium concentrations that would result in more than 5 quanta released per impulse.)

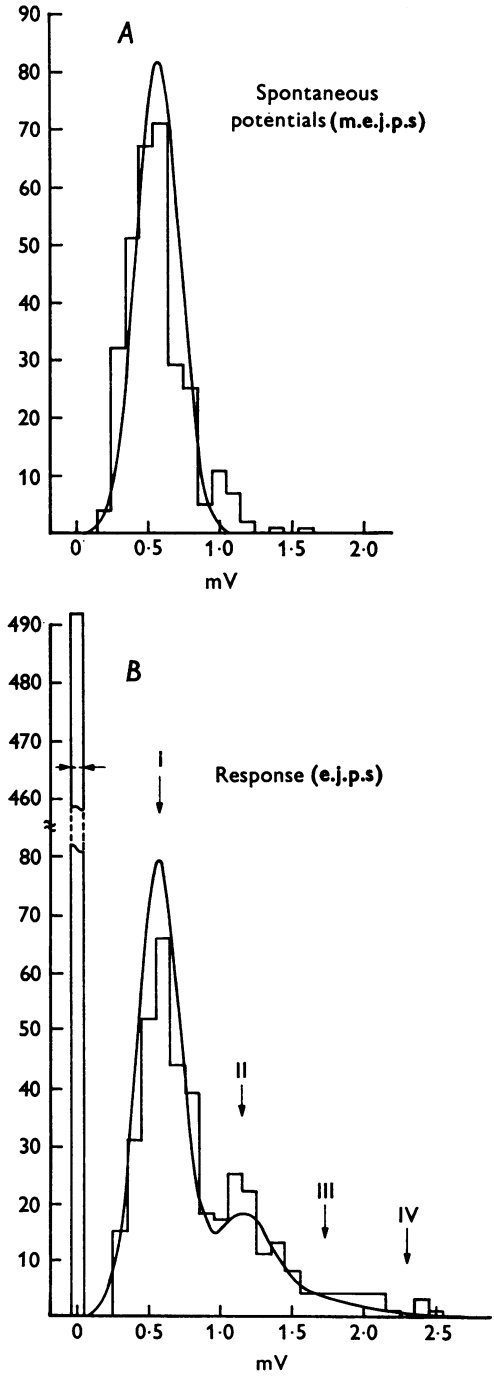
Another test for the applicability of the Poisson distribution is to compare the theoretically expected distribution of e.j.p. amplitude with that observed experimentally (del Castillo & Katz, 1954). Fig. 8 shows that

the prediction derived from a Poisson distribution and the distribution of m.e.j.p. amplitudes agrees with the observed e.j.p. amplitude distribution, similar to the observations in frog and crayfish (del Castillo & Katz, 1954; Dudel & Kuffler, 1961).

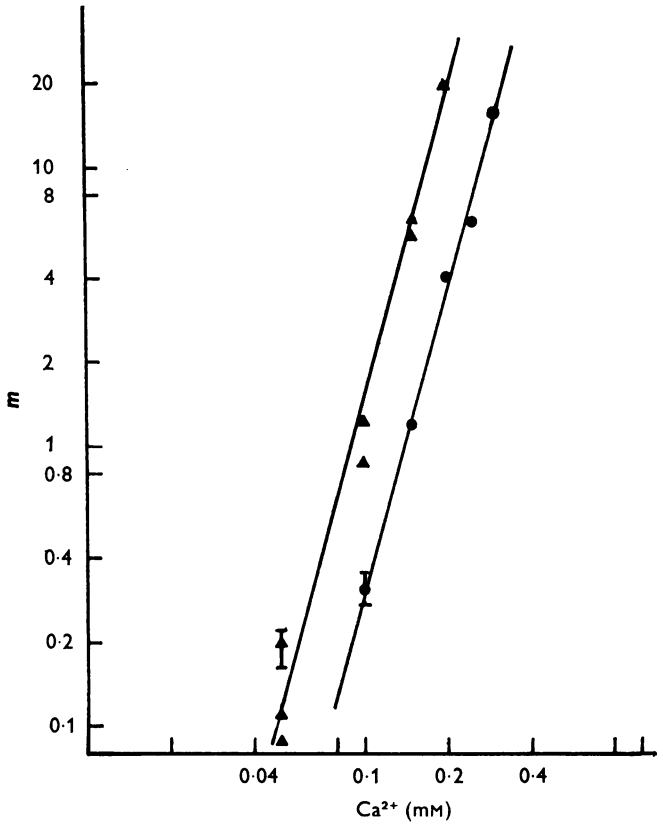
Calcium dependence of transmitter release. The calcium dependence of transmitter release was studied by measuring the quantal content in solutions in which magnesium ions were constant while calcium ions was varied. This was done by altering the calcium chloride and magnesium chloride concentrations of solution A (Table 1). The change in osmolarity introduced was negligible (less than 2 %). For these experiments a nerve was stimulated at 0·25/sec and about 200 responses were averaged at each calcium concentration. In Fig. 9 the calcium dependence of transmitter release is shown by plotting the logarithm of the quantal content, measured from the average e.j.p. and m.e.j.p. amplitude and corrected for non-linearity (Martin, 1955), against log calcium concentration. The slope of this double logarithmic plot is 3·64, similar to the values obtained for the frog sartorius muscle by Dodge & Rahamimoff (1967). Reducing magnesium concentration from 4 to 2 mM increased the quantal content, but did not change the slope of the double logarithmic plot. These results can be explained by assuming that magnesium competes with calcium for the same binding sites and that transmitter release requires the co-operative action of four bound calcium ions.

In calcium-free solutions, strontium mimicked the effect of calcium on transmitter release. It also greatly enhanced delayed release (Fig. 10), as has been described for the frog neuromuscular junction (Miledi, 1966; Rahamimoff & Yaari, 1973). The order of the strontium dependence of immediate release was similar to that of the calcium dependence (Fig. 11). Due to the temporal overlap of immediate and delayed release, these measurements may be in error by a factor of three. Such an error, however, would have little effect on the slope of the double logarithmic plot.

The calcium dependence of transmitter release in *Drosophila* larvae was similar to that in the frog but different from the linear dependence found in crayfish (Zucker, 1974). One possible explanation for the linear dependence in crayfish is that the calcium dependence of excitatory transmitter release is complicated by the action of a tonic release of inhibitory transmitters either on the muscle membrane or on the excitatory axon's terminal (Parnas, Rahamimoff & Sarne, 1975). Since there did not seem to be any effect of known inhibitory transmitters on the *Drosophila* larval neuromuscular junction (Jan & Jan, 1976), it might be possible that pre- and post-synaptic inhibition was not present or was not important; this would explain the similarity between the calcium dependence of transmitter release in the frog and that in *Drosophila* larva.

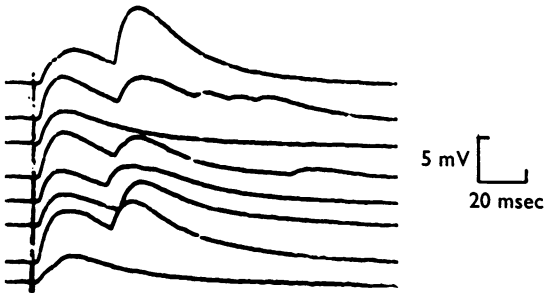


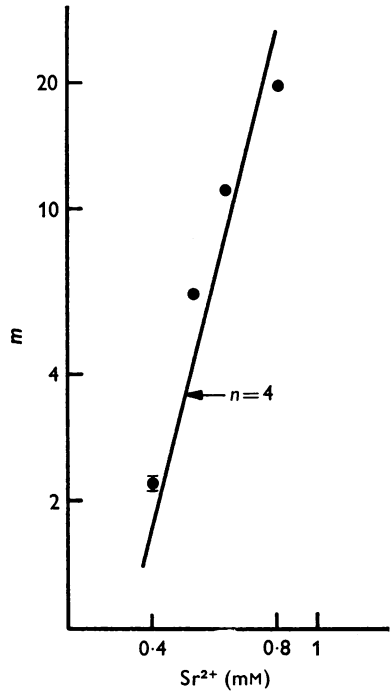
Text-fig. 8. For legend see facing page.



Text-fig. 9. Calcium dependence of transmitter release. The quantal content was calculated as $m_1 = \bar{v}/\bar{v}_{\text{m.e.j.p.}}$ and correction was made for the non-linear behaviour of e.j.p.s (Martin, 1955). A single ventral lateral longitudinal fibre second from the ventral mid line was used to obtain the five points (filled circles) at 4 mM magnesium. Four fibres in two larvae were used for the nine points (triangles) at 2 mM magnesium. The error bars for the standard deviations of the mean are shown in the Figure only when they are larger than the symbols used. Eight points with the lowest quantal contents in this Figure were plotted against m_0 in Fig. 7, to test the applicability of Poisson distribution.

Text-fig. 8. Histogram showing distribution of m.e.j.p. (*A*) and e.j.p. (*B*) amplitudes of a ventral lateral longitudinal fibre in a solution containing 0·11 mM calcium chloride. Continuous curve in *A* is a Gaussian distribution. Continuous curve in *B* is calculated on the hypothesis that the e.j.p. responses are built up statistically of units whose mean size and amplitude distribution are identical with those of the spontaneous m.e.j.p.s (del Castillo & Katz, 1954). In *B*, first bar is the number of failures. Expected number of failures are indicated by two horizontal arrows. The four vertical arrows indicate multiples of mean m.e.j.p. amplitude. Ordinates: number of observations. Abscissae: amplitude of m.e.j.p. (*A*) or e.j.p. (*B*) responses.





Text-fig. 10

Text-fig. 11

Text-fig. 10. Delayed release of transmitter following the initial immediate release, observed in calcium-free solution containing 0·6 mM strontium. Concentrations of sucrose and ions other than calcium are as in solution A.

Text-fig. 11. Strontium dependence of immediate transmitter release in calcium-free solutions. The quantal content was calculated as $m_1 = \bar{v}/\bar{v}_{\text{m.e.j.p.}}$, and correction was made for non-linear behaviour of e.j.p. (Martin, 1955). For each point, 160–200 measurements were averaged. The error bars for standard deviation of the mean are not given when they are smaller than the diameter of the dot.

DISCUSSION

In cockroach, locust, grasshopper and stick insect, the recorded resting potentials fit those calculated from the internal and external potassium concentration for a potassium electrode (Hoyle, 1955; Wood, 1957). In moth and mealworm larva, however, the relation between resting potential and ionic composition of the medium did not fit either a potassium electrode or a multi-ion electrode model using an extended constant-field equation for mono- and divalent ions (Belton & Grundfest, 1962*a*, *b*; Huddart, 1966; Piek, 1975). In moth an ouabain-insensitive electrogenic pump was apparently responsible for most of the resting potential (Reuben,

1972). In addition to active transport processes, the existence of a barrier of some sort which separates the extracellular fluid (inside the transverse tubular system) from the haemolymph in some insects might account for discrepancies between the observed resting potentials from values predicted by the Nernst equation. For extensive reviews and discussion on this subject, see Usherwood (1969) and Piek (1975).

Such complications are apparently unimportant in the *Drosophila* larva. The resting potentials in haemolymph-like or other solutions of varying ionic composition were predicted by the Goldman–Hodgkin–Katz equation, assuming that chloride ions distributed passively across the membrane and using a ratio of 0·23 for sodium : potassium permeabilities.

The neuromuscular junctions in the *Drosophila* larva and the frog are similar in a number of ways. Transmitter release in both cases depends on approximately the fourth power of $[Ca^{2+}]_o$. Strontium can mimic the effect of calcium and greatly enhance delayed release (Miledi, 1966; Rahamimoff & Yaari, 1973). In the absence of calcium, transmitter release depends on roughly the fourth power of $[Sr^{2+}]_o$, supporting the notion that strontium and calcium act on the same site (Meiri & Rahamimoff, 1971). The order of calcium and strontium dependence is also unaltered in a mutant, although facilitation and many other aspects of neuromuscular transmission are abnormal (L. Y. Jan, Y. N. Jan and M. J. Dennis, in preparation).

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EXPLANATION OF PLATES

PLATE 1

A, diagram of the distribution of nerves and muscles in a *Drosophila* larva, reproduced from Hertweck's paper (1931). The ventral ganglion and two hemispheres are located at the ventral mid line. The larva consists of one head segment, three thoracic segments, and eight abdominal segments.

B, distribution of muscles of a 3rd instar larva as seen under the scanning electron microscope. The larva was opened along the dorsal mid line, and the larval brain and ventral ganglion were removed.

In both *A* and *B*, the star and triangle mark the ventral lateral longitudinal fibre second and first from the ventral mid line, respectively, on the left half of the second abdominal segment. The muscle arrangements in the second through the seventh segment are identical.

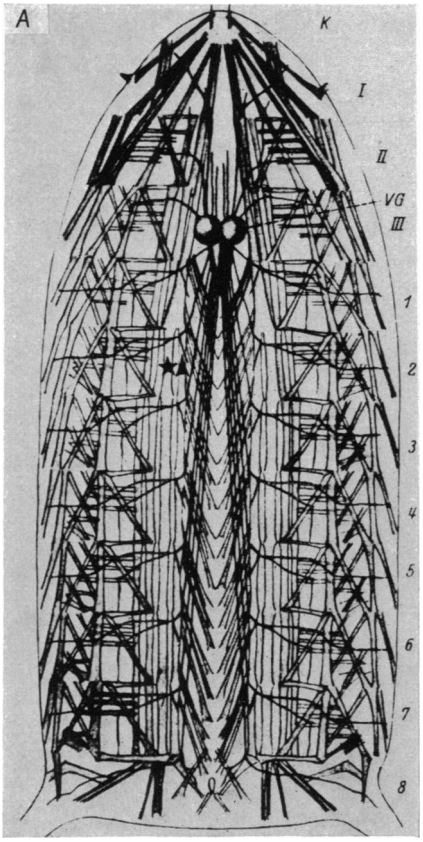
PLATE 2

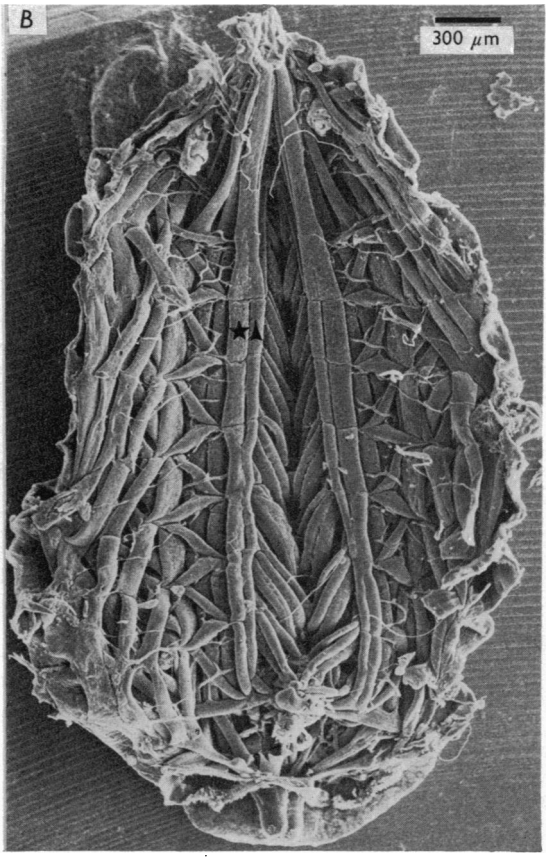
A, ventral lateral longitudinal fibre first (left) and second (right) from the ventral mid line, as viewed with Nomarski optics. *b*, boutons; *n*, nerve; *nu*, nucleus; *tr*, trachea.

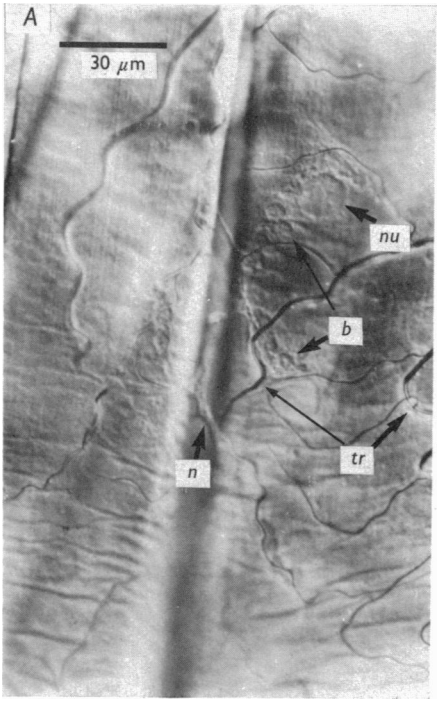
B, transverse section showing a small portion of the ventral lateral longitudinal fibres first (left) and second (right) from the ventral mid line, and a nerve branch located between these fibres. *a*, axon; *d*, diad; *g*, glial cell; *ne*, nerve ending; *m*, mitochondria; *z*, *z*-disk.

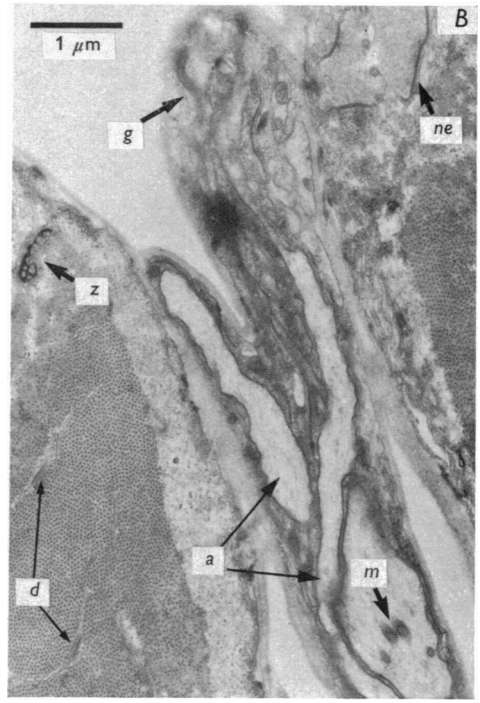
PLATE 3

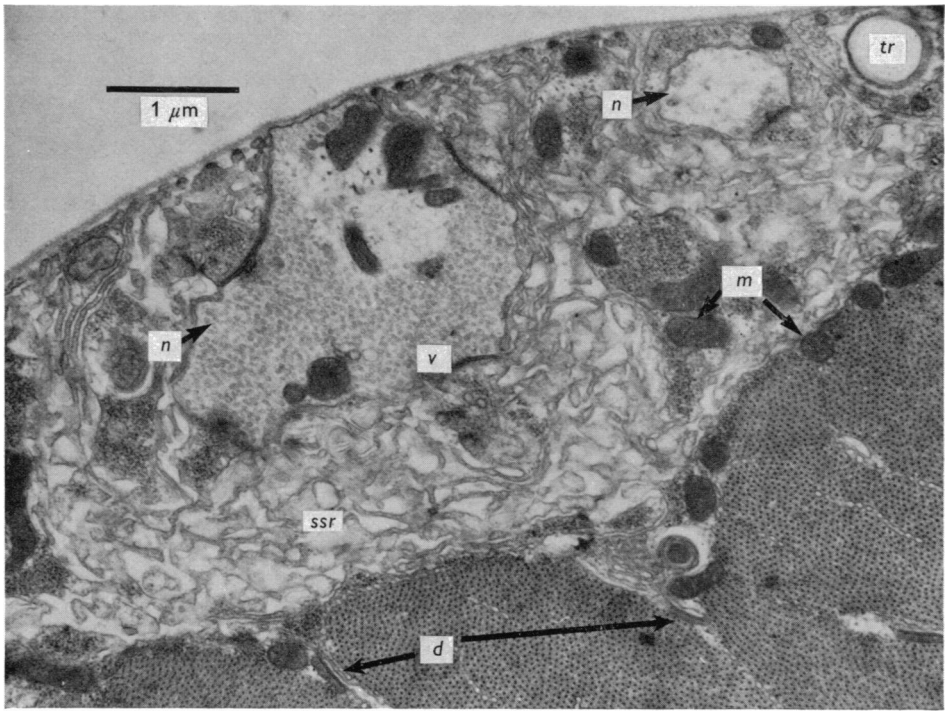
Neuromuscular junction of a ventral lateral longitudinal fibre second from the ventral mid line: *d*, diad; *m*, mitochondria; *n*, nerve ending; *ssr*, subsynaptic reticulum; *tr*, trachea; *v*, vesicles.


A
K
I
II
VG
III
1
2
3
4
5
6
7
8


B
300 μm


A
30 μm
nu
b
tr
n


B
1 μm
g
ne
z
a
m
d


1 μm
tr
n
m
n
v
ssr
d