range of entrainment of the rhythm was estimated from the response curve to be from 21 to 27 h of light-dark cycles[9]. Individual differences in the phase response curve among 4 rats examined were small.

Discussion. Features of the present study, in which we obtained a phase response curve for the locomotor activity rhythm of the rat, are 2-fold: First, the instrument used in this study was to measure 'spontaneous' locomotor activity of the animal. In contrast, running wheel cages, previously used to obtain response curves in rodents[5-7], measured an activity accelerated by the wheel: i.e. rotation of the wheel itself affects an activity of the animal. Second, instead of the onset of activity, the acrophase of the rhythm calculated by the mathematical process was used as the phase reference point in this study. In this way, phase response curves were obtained even in rats which showed obscure onset of activity under free-running conditions. The extent of the phase shift, estimated by a difference of acrophases, reflects the changes of both phase and activity amount induced by a light signal.

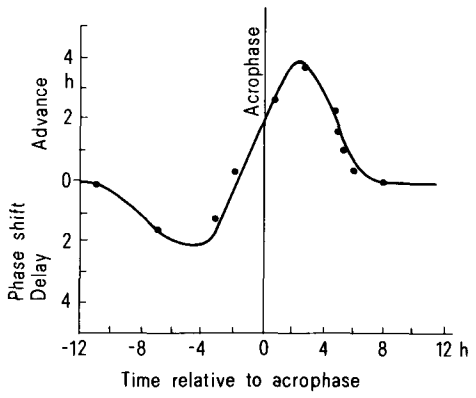

Fig. 2. A phase response curve for locomotor activity of a rat. Phases of the rhythm are expressed by the time relative to the acrophase which was located approximately at the midpoint of subjective night. The acrophase is indicated by a perpendicular line at the point of zero phase (a phase reference point). Before the acrophase, phases are indicated by negative sign and after the acrophase by positive sign. The point of switch-over is detected at about -2 h. The response curve is characterized by 2 distinct portions of phase delay and phase advance: the phase delay was observed in the early subjective night, followed by the phase advance. The maximum phase advancing effect of light signal (advance of about 4 h) was located 2 h after the acrophase.

The phase response curve obtained in the rat was very similar to those obtained in deer mice[5], flying squirrel[6] and hamster[7]. Intensity of the background illumination, intensity and duration of the light signal or kind of method used to obtain the response curve, all these factors were reported to affect the shape and the size of the phase response curve[1]. Nevertheless, essentially the same pattern of the response curve obtained in nocturnal rodents suggests an operation of a common phase control system in these species.

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Ca-electrogenesis in mealworm muscle: A voltage clamp study

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Summary. The membrane current in the muscle fibre of larval mealworm, *Tenebrio molitor*, was characterized by an early transient inward current followed by a late outward current. The results suggest that the inward current is associated with an inward movement of Ca ions down its electrochemical gradient, and Na ions have little to contribute to the inward current in this fibre.

During the past 6 years, substantial evidence has accumulated that Ca ion is the charge carrier of inward current at the non-synaptic membrane of insect skeletal muscle fibres[3-9]. One of the major paradoxes in the acceptance of this evidence is the reported Na-dependent electrogenesis in the muscle fibres of larval mealworm, *Tenebrio molitor*[10,11], in which increased Ca concentration of external medium depressed the directly evoked muscle spikes[12]. We wished to determine whether or not this particular ionic permeation of *Tenebrio* muscle fibre reflected a true phylogenic specialization. Here we report the use of the voltage clamp technique to solve this problem on ionic requirements for the electrogenesis in *Tenebrio* muscle. There has been no previous report on voltage clamp analysis of non-synaptic membrane in insect muscle fibres, except the preliminary note on TEA-treated locust muscle fibres[13].

We studied the segmental muscle of the body wall of immature larvae of *Tenebrio molitor*. The muscle fibres were clamped using a 2-microelectrode method, with a potential recording electrode connected to a current passing electrode through a high input impedance amplifier and a feedback amplifier (Dagan model 8500, Minneapolis, Minn.). The short muscle fibres, which have a length less than 800 µm and a diameter of about 100 µm, were used in the experiments to establish a good condition for space-clamp. A mean value for the length constant using the short cable model[14], was 1.4 mm. Thus, the efficiency of the clamp was greater than 96%.

Although many insect muscle fibres cannot generate all-or-none action potentials in normal saline, *Tenebrio* muscle fibres responded to an outward current pulse with a spike in normal saline (NaCl 70 mM, KCl 30 mM, $CaCl_2$

5 mM, $MgCl_2$ 10 mM and glucose 445 mM; pH of the solution was adjusted to 7.2 with 5 mM HEPES-KOH) (figure 1, a). Figure 1, b illustrates membrane currents of the same fibre obtained by voltage clamp technique. Voltage control, as judged from the rise time and time course of the voltage records (upper trace), was satisfactory. The membrane current is characterized by an early transient inward current followed by a late outward current. The early current appeared at 0 to +10 mV and rapidly increased its amplitude to a maximum with positive shift of membrane potential about +15 mV. In some fibres, a 'hump' similar to that described previously as an early transient potassium current I_{kl} in crustacean muscle fibres[15-17] appeared just after the peak of inward current (not illustrated). Figure 1, c shows the current-voltage relation of the membrane at the peak of the early transient current and at the steady state obtained for 3 holding potentials, −60, −40 and −20 mV. It can be seen that different holding potentials do not change the size of the inward currents for any given pulses. A similar observation has been made with calcium type muscle membrane of a giant barnacle[18]. The reversal potential of the early currents determined from the current-voltage relation was about +54 mV. Figure 2 shows the dependence of the conductance for the early transient current upon the absolute membrane potential. The peak transient current and the reversal potential were measured in the same experiment. The membrane conductance for the peak inward current was calculated as $G_t = I_t/V - V_{rev}$ where V is the absolute membrane potential in mV, V_{rev} is the measured reversal potential in mV and I_t is the peak inward current in nA for each depolarization. This plot resulted in a sigmoidal relation similar to that found in other excitable membranes[19]. To elucidate the ionic dependence of the inward current, the effects of withdrawal of external Na ions were examined. When Na ions were replaced by choline, the inward current decreased to a lesser degree (21% reduction, mean of 9 experiments). In addition, the reversal potential for the inward current was not significantly changed in this saline. For example, the reversal potential measured in normal saline averaged +39 mV (SE = ±2.4 mV; N = 30) and that in Na-free saline, +43 mV (SE = ±4.1 mV; N = 9). Differences were statistically insignificant in t-test ($\beta < 0.2$). The most reliable method to measure the ionic selectivity of ionic channels to foreign cations is to compare the reversal potential of the ionic current in normal saline and in the test solution. Therefore, it can be argued that Na ions have little contribution, if any, to the inward current in *Tenebrio* muscle fibres. Also the effect of increasing Ca concentration on the current was tested in the choline-substituted Na-free saline. An increase in the external Ca concentration, by replacing a part of glucose in the medium with an osmotically equal amount of $CaCl_2$, resulted in an increase in the amplitude of the inward current (figure 3). As shown in the current-voltage relationship, the reversal potential for the inward current was shifted from 52 to 80 mV with 10-fold increase in the external Ca concentration. The facts strongly suggest that this current might be associated with an inward movement of Ca ions down its electrochemical gradient. Furthermore, pharmacological experiments showed that the inward current was not affected by the external application of tetrodotoxin, but $CoCl_2$ reversibly blocked the currents. This is further evidence for the existence of calcium channels in these muscles. The results presented here do not agree with reports in literature[10-12]. For example, Kusano and Janiszewski[12] suggested the existence of Na-dependent electrogenesis in muscle fibres of matured mealworm larvae and stated that Na conductance of electrically excitable membrane of the muscle fibre decreased as increasing Ca ions. Such a

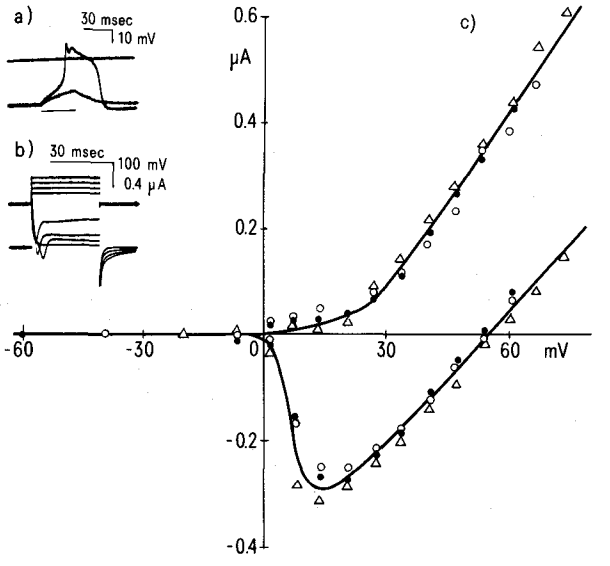

Fig. 1. *a* Membrane potential changes (lower trace) elicited by outward current pulses (indicated by a bar below the record). 1 action potential and 1 subthreshold electrotonic potential are superimposed. The upper trace represents reference potential level. *b* Superimposed current traces (lower trace) for increasing depolarizing steps (upper trace) from a holding potential of −60 mV. *c* Current-voltage relations at the peak of the early transient current (lower line) and at the steady state (upper line). Vertical axis represents the membrane current in μA and the horizonal axis represents the absolute membrane potential in mV. Each voltage clamp series was run at a different holding potential: −60 mV (filled circles); −40 mV (open circles); and −20 mV (open triangles).

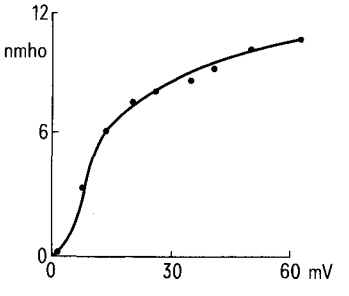

Fig. 2. Conductance for the early transient current as a function of absolute membrane potential (mV). Vertical axis represents the conductance (nmho) of a *Tenebrio* muscle fibre. The reversal potential was obtained in figure 1, c.

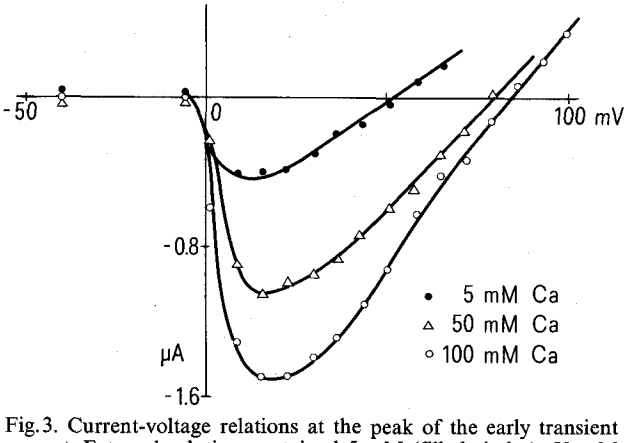

Fig. 3. Current-voltage relations at the peak of the early transient current. External solution contained 5 mM (filled circles), 50 mM (triangles) and 100 mM Ca (open circles) but was devoid of Na ions. The steady state current is not shown. Vertical axis represents the membrane current in μA and the horizontal axis represents the absolute membrane potential in mV.

marked difference in the nature of ion channels in the same species is difficult to rationalize. It is, however, possible that the discrepancy may arise from the difference in the developmental stages of the animals used. Different ionic requirements for the regenerative responses have been reported during the stage of development in the embryonic excitable cell membrane[20]. In our experiments, immature larvae smaller than 10 mm in their total length were used exclusively.

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Butanol extracts from myelin fragments. III. SDS-urea gel electrophoretic studies

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Summary. The butanol extracts from myelin fragments showing 5-HT binding capacity were studied by SDS-urea gel electrophoresis. 5 main bands were observed and their molecular weights were determined by the method of Ferguson relationship, and revealed that these extracts contained proteolipid protein, DM-20 and basic proteins. Furthermore, the reconstituted fraction with crude basic proteins and lipids showed the saturable binding capacity for C^{14} · 5-HT.

Some investigators[2,3] reported the butanol extracts of central nervous tissue having 5-HT binding affinity, but their materials were the total particulate fractions. In previous experiments[4], we demonstrated that the particulate fraction of Godwin and Sneddon[3] contained a considerable number of myelin fragments and, moreover, butanol extracts from myelin fragments showed binding capacity for C^{14} · 5-HT. Pasquini and Soto[5] have reported that the butanol extracts of bovine brain grey and white matter contain a complex mixture of phospholipids, galactolipids, cholesterol and proteolipid protein of Folch-Lees[6]. They also indicated the results of acrylamide gel electrophoresis, but detailed observations were not made. The objective of the present work is to analyze the binding components present in the butanol extracts of myelin by SDS-urea polyacrylamide gel electrophoresis.

Materials and methods. The details of preparation of butanol extracts from the myelin fragments of rat brain stems were as reported previously[7]. Briefly, the brain stems were homogenized in 0.32 M sucrose (10%). The myelin fragments were isolated from the homogenate by the method of Whittaker et al.[8] and extracted with butanol-water mixtures. The butanol phase thus obtained was concentrated under N_2 to about one-third of its original volume (TE). TE was treated with water (14%, v/v) to dissolve the insoluble materials (water-treated TE), and C^{14} · 5-HT binding capacity was checked by Sephadex LH_{20} column chromatography as described previously[4,7]. SDS-urea polyacrylamide gel electrophoresis was carried out according to the method of Chan and Lees[9]. The water-treated TE was dried under N_2 and emulsified in a medium containing 300 mg of SDS, 450 mg of dithiothreitol, and 800 mg of sucrose per 10 ml of water (mg protein/ml). To obtain complete solubilization, the mixtures were heated at 60 °C for 10 min, then 8 M urea was added and heated 10 min more at same temperature, and 50 µl of samples including 0.005% Bromophenol blue were loaded onto SDS-urea gels. Electrophoresis was performed for 18 h at a constant current of 1 mA/tube, and then gels were stained in 0.25% Coomassie blue. Crude basic protein fractions (0.1 N HCl extracts) were prepared from myelin fragments by the method of Martenson et al.[10] and lipid mixtures were obtained from the water-treated TE by the method of Mokrasch[11].

Results and discussion. Typical gel profile of the butanol extracts from myelin fragments showing C^{14} · 5-HT binding capacity is shown in figure 1. 5 main bands (A-E) were identified, and sometimes a fast anode component (lipid) was lightly stained. Low mobility components (i.e., high mol.wt proteins = HMW) were also observed. From the studies of synaptic membrane proteins, Banker and Cotman[12] pointed out the risk of depending on a single gel concentration for mol.wt determination without prior validation of the procedures by the method of Ferguson relationship. Theoretical treatment of the Ferguson relationship and its application to SDS-urea system have been thoroughly reported[9,13]. On a plot of log R_F (relative mobility) vs. T (% of total acrylamide concentration = 7, 8.5, 10, 11.5 and 13%), i.e., Ferguson plot, lines of 5 protein bands fall into 3 Y_0 (y intercept). Lines of A and B have the same Y_0 (1.65); C has a Y_0 of 1.45. Lines D and E have a Y_0 of 1.03. These results indicate that 5 bands are separating by size and charge differences, and thus their mol.wt can be determined only from the K_R (slope of line) vs. mol.wt relationship as shown in figure 2. Based on this method, we obtained the mol.wt of A, B, C, D, and E is 12,180±1650, 18,330±2520, 19,830±2260, 22,410±1110 and 31,460±1700, respectively (mean±SEM of 6 experiments). The relative ratio of C to E was approx. 1. Chan and Lees[9] obtained 30,600, 24,300 and 18,900 for proteolipid protein (PLP), DM-20 and basic protein (BP) from bovine white matter