ciency of bleaching was also investigated. By changing the distance between the light source and the culture, the supplied intensities were varied. The light intensity measured by the spectroradiometer (ISCO SR) was 23 μW cm^{-2} at 120 cm and 8 μW cm^{-2} at 240 cm. The weaker the light intensity was, the more increased fraction of bleached cells was obtained in the growing culture (figure 2). Most cells were bleached ones when cultures were placed longer than 190 cm where light intensities were less than 10 μW cm^{-2}.

According to the action-spectrum of chlorophyll synthesis of *Euglena* studied by Nishimura and Hujishige[5], the least chlorophyll synthesis occurred at the wavelengths around 450–550 nm. When we examined the bleaching of the mutant cells with the growing culture under separate light sources (blue, green, yellow and red), it was found that the green light was most effective for the production of bleached cells (table 1). The green light supplied was a fluorescent lamp of type FL-20 GF which gave 80% intensity at 510–560 nm wavelength. The lamp was wrapped in a sheet of green and of blue cellophane papers for excluding the excess spectrum. No bleached cells were produced in the wild strain in any of these light sources.

When photosynthesis was inhibited by the addition of dichlorophenyl dimethyl urea (DCMU), at a final concentration of 10^{-4} M, 99% of bleaching was obtained within 4 generations (table 2).

Referring to these observations, it is plausible that the proplastids in U cannot multiply in darkness. A key point in the machinery of replication of the proplastids may have been genetically impaired. Product of photosynthesis, or any other function of mature chloroplast in U, would participate in the apparent accomplishment of chloroplast replication in the light.

Cell multiplication is required for bleaching, as was experienced in other type of experiments[6,7]. Rate difference produced between the multiplication of cell and that of plastids which was induced by environmental factors may give rise to the production of bleached cells.

Even in the light culture, U has always a certain amount of bleached cells. This would mean that the chloroplast-forming system may still be unstable also in the light. Although, by the lack of routine of genetic analysis in *Euglena*, the locus of the mutant gene cannot be determined, yet U seems to be available for the study of the regulation of chloroplast replication.

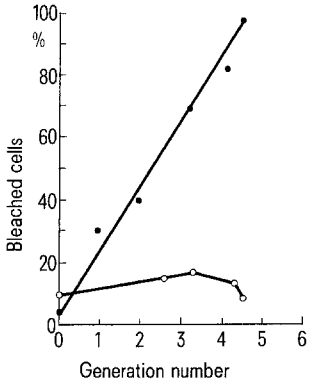

Fig. 1. Production of bleached cells in the growing culture. Cells of U, precultured in the light, were grown in the light or in the dark. At time intervals, frequencies of bleached cells were counted by means of plate culture. ○: Cells grown in the light, ●: cells grown in the dark. Mean generation time was 18 h for light culture, 30 h for dark culture.

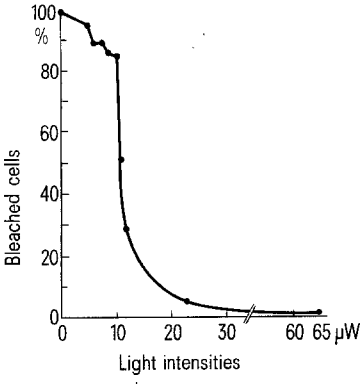

Fig. 2. Production of bleached cells by strain U under various light intensities. Cells of U, precultured in the light, were grown under various light intensities by changing the distance from the light source, a lamp of FL-20 PG. After 6 days growth, frequencies of bleached cells were counted by means of plate culture.

1 H.T. Epstein and J.A. Schiff, J. Protozool. *8*, 427 (1961).
2 Y. Ben-Shaul, J.A. Schiff and H.T. Epstein, Pl. Physiol. *39*, 231 (1964).
3 A.C. Zahalsky, S.H. Hutner, M. Keane and R.M. Burger, Arch. Mikrobiol. *42*, 46 (1962).
4 A.I. Stern, J.A. Schiff and H.T. Epstein, Pl. Physiol. *39*, 220 (1964).
5 M. Nishimura and H. Huzisige, J. Biochem. *46*, 225 (1959).
6 Y. Tsubo, in: Advance of Phycology in Japan, p. 180. Ed. J. Tokida and H. Hirose. Gustav-Fischer-Verlag, Jena 1975.
7 C.L. Hershberger, D. Morgan, F.A. Weaver and P. Pienkos, J. Bacteriol. *118*, 434 (1974).

Enhanced excitability in locust muscle fibres induced by calcium free saline

D.A. Mathers[1,2] and S. Thesleff

Department of Pharmacology, University of Lund, S-223 62 Lund (Sweden), 16 August 1978

Summary. The membrane of locust muscle fibres normally exhibits a graded electrical response to outward current pulses of increasing strength. On removal of Ca^{++} ions from the external medium, these fibres are shown to exhibit depolarizing membrane responses of variable time course and duration. These responses are abolished in Na^{+}-free solutions, and by the addition of Mn^{++} ions.

The membrane of arthropod skeletal muscle fibres normally exhibits a graded electrical response to outward current pulses of increasing strength[3,4]. All-or-none excitability can, however, be produced by addition of Ba^{++}, Sr^{++} or tetraethylammonium ions to the perfusion medium[5-7]. Divalent cations appear to be major carriers of inward current during action potential generation in many arthropod muscles[3-7].

The ionic basis of graded and all-or-none responses of locust muscle fibres is as yet unclear, although both Na^{+} and Ca^{++} ions have been proposed as inward current carriers[6,8,9]. In this paper, the role of Na^{+} and Ca^{++} ions in the electrically excitable response of locust muscle fibres has been the subject of further studies.

Methods. The experiments were performed on the metathoracic extensor tibiae muscle of the locust *Schistocerca gregaria.* Following dissection[10], the preparation was perfused continuously at 1–2 ml/min with standard locust saline of composition NaCl, 200; KCl, 10; $CaCl_2$, 2; NaH_2PO_4, 4; Na_2HPO_4, 6 mM. Fibres innervated by the

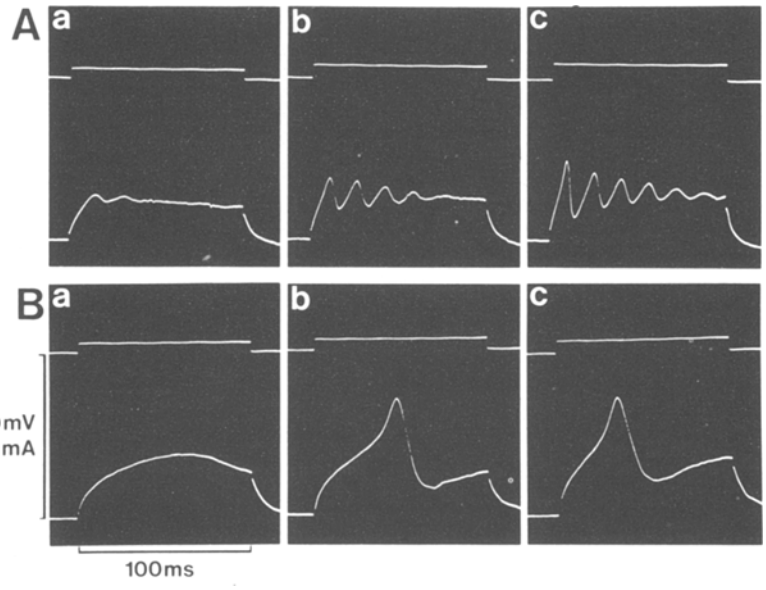

Fig. 1. Intracellularly recorded membrane responses (lower traces, mV) of a single locust extensor tibiae muscle fibre to outward current pulses of increasing strength (upper traces, nA). *A* a, b, c. Graded oscillatory responses in standard locust saline. *B* a, b, c. Constant amplitude responses recorded in same fibre after 15 min in Ca^{++}-free saline.
In figures 1–3, the base line of the current trace indicates the zero potential level of the cell.

'fast' excitatory axon only[11] were impaled centrally by 2 glass micropipettes (10–20 MΩ resistance, 2 M KCl filled) positioned less than 50 µm apart. Graded responses or action potentials were elicited by cathodic current pulses of 100-msec duration delivered from a micropipette coupled to a constant current source[12]. The remaining micropipette, coupled to a DC preamplifier, was used to record the resulting membrane potential change. The effective membrane resistance of single muscle fibres was estimated from the amplitudes of electrotonic potentials evoked by 20–35 nA anodic current pulses of 500-msec duration. Test salines of the following compositions could be admitted to the experimental chamber: 1. Ca^{++}-free saline, $CaCl_2$ omitted from standard saline; 2. Na^+- and Ca^{++}-free saline, $CaCl_2$ omitted, NaCl and phosphates replaced by equimolar amounts of choline chloride (sigma) and Tris HCl, respectively. EGTA (Sigma), tetrodotoxin (Sankyo Ltd., Tokyo) and manganese chloride (Sigma) were dissolved in the appropriate saline as indicated below. All salines were buffered to pH 6.7–6.8 and experiments were made at room temperature (21–25 °C).

Results. On exposure to Ca^{++}-free saline after equilibration for 1 h in standard locust saline, extensor tibiae muscle fibres depolarized by a few mV and fibre input resistance fell by 28±1% (mean±SEM, n=8). These changes were normally reversible on return to standard saline. Similar results have been reported in the case of locust coxal adductor muscle fibres[9].
In standard solution, the response of the locust muscle fibre membrane to outward current pulses consisted of graded, oscillatory potential changes which failed to reach the zero membrane potential level, as previously reported[6,8,9] (figure 1, A). Within 0.5 h of exposure to Ca^{++}-free saline, however, outward current pulses of suitable strength evoked a membrane response of constant amplitude (figure 1, B).
In further experiments, the preparation was bathed in Ca^{++}-free saline, or in Ca^{++}-free saline containing 1 mM EGTA for 1–2 h prior to recording. Following either of these treatments, outward current pulses evoked a variety of membrane responses. In many fibres, a response of 35–80-msec duration was seen which normally reached a maximum amplitude close to the zero potential level (figure 3, A). In other fibres, the appearance of persistant membrane depolarizations outlasting the duration of the stimulus current was observed (figure 2, A). In some fibres

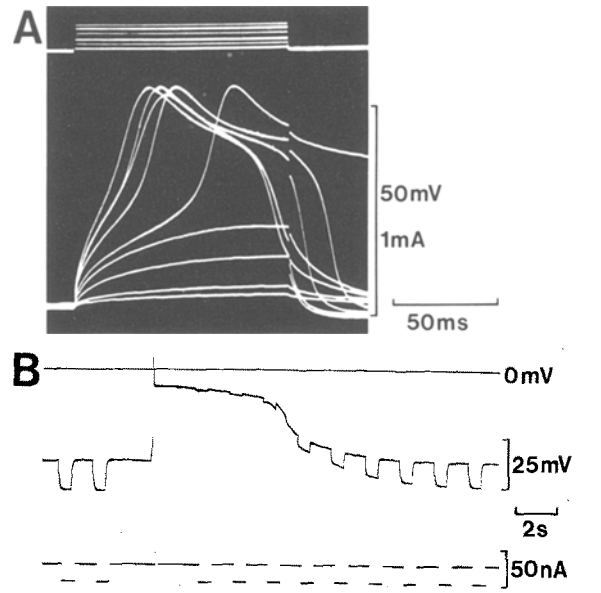

Fig. 2. Intracellularly recorded responses from muscle fibres exposed to Ca^{++}-free saline for 1 h. *A* Responses of a single muscle fibre (lower traces, mV) to outward current pulses of increasing strength (upper traces, nA). Current and voltage traces are superimposed from successive oscilloscope sweeps. Note appearance of membrane responses outlasting duration of current pulse at higher intensity stimulation levels. *B* Response (middle trace, mV) of a second muscle fibre, from same preparation as in A above, to a single brief outward current pulse (lower trace, upward deflection). The upper trace shows the zero membrane potential level. Fibre input resistance was monitored by injection of anodic current pulses (lower trace, downward deflections). The response was evoked at the resting membrane potential level.

an adequate stimulus current evoked greatly prolonged depolarizations of characteristic shape and time course (figure 2, B). The initial spike-like phase of this response normally overshot the zero membrane potential level (mean peak depolarization, +6±1 mV, n=10), and was ended by a rapid phase of repolarization (5–10 msec half decay time). The plateau depolarization which followed, invariably occurred at a membrane potential negative to the zero potential (mean −8±1 mV, n=10). The plateau was terminated by a further repolarization phase. The

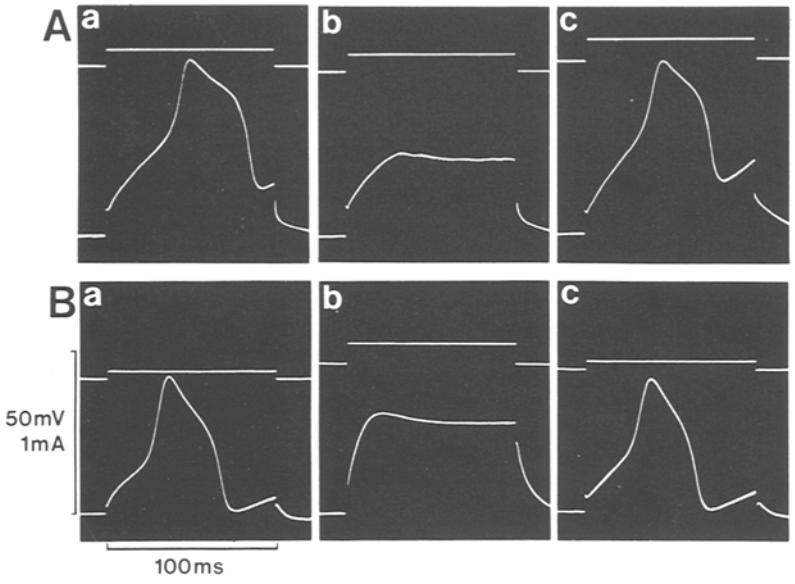

Fig. 3. Membrane responses (lower traces, mV) evoked by outward current pulses (upper traces, nA) in muscle fibres bathed in Ca^{++}-free saline for 1 h prior to start of experiment. *A* a. Response recorded in Ca^{++}-free saline. b. Abolition of response after 20 min exposure to Ca^{++}-free, Na^{+}-free saline. c. Recovery of response 10 min after reapplication of Ca^{++}-free saline. *B* a. Response recorded in a second muscle fibre, after 1 h in Ca^{++}-free saline. b. Abolition of response in this cell 10 min after application of Ca^{++}-free saline containing 20 mM Mn^{++} ions. c. Recovery of response 20 min after return to Ca^{++}-free saline only.

mean duration of these membrane responses measured at half plateau amplitude was 7 ± 0.5 sec. During the response, the amplitude of electrotonic potentials evoked by anodic current pulses was reduced, showing that the membrane depolarization was accompanied by a transient decrease in fibre input resistance (figures 2, B). The peak amplitude of the depolarization seen during both brief and prolonged responses in Ca^{++}-free saline was not significantly affected by applying stronger outward current pulses (see figures 2, A). The effect of removal of Na^{+} ions from the perfusion medium on membrane responses of locust muscle fibres exposed to Ca^{++}-free saline is shown in figure 3, A. This procedure reversibly abolished depolarizing electrogenesis. Tetrodotoxin (TTX) suppresses Na^{+}-dependent action potentials in most[13,14] but not all tissues[15]. At concentrations up to 5×10^{-6} M, TTX failed to block the membrane responses evoked in locust muscle fibres by exposure to Ca^{++}-free saline. Manganese ions (Mn^{++}) are believed to inhibit the passage of charged species through Ca^{++} channels in a wide variety of tissues[9,16,17]. The addition of 20 mM manganese chloride to the Ca^{++}-free saline reversibly abolished membrane responsiveness to outward current pulses (figure 3, B).

Discussion. The present experiments have shown that on exposure to Ca^{++}-free saline, locust muscle fibres exhibit depolarizing membrane responses with variable time course and duration. The graded responsiveness seen in normal saline is not present in the absence of external Ca^{++} ions. Although insensitive to TTX and sensitive to Mn^{++} ions, the membrane responses seen in Ca^{++}-free solution are dependent on the presence of external Na^{+} ions. The Ca^{++}-dependent action potential observed in locust muscle fibres in the absence of external Na^{+} has also been reported to be sensitive to Mn^{++} ions, but to be unaffected by TTX[9]. These findings suggest the existence in the locust muscle fibre membrane of channels which allow entry of both Na^{+} and Ca^{++} ions, but which are fundamentally of the Ca^{++} permeable type as defined by their sensitivity to Mn^{++} ions. The recently reported Mn^{++}-sensitive Na^{+} current in the membrane of snail neurones bathed in Ca^{++}-free EGTA solution has also been attributed to the entry of Na^{+} ions through Ca^{++}-permeable channels[18].

During the prolonged type of membrane response reported in the present work, the plateau depolarization was observed to be much more negative than the expected value of the Na^{+} equilibrium potential (+55 mV[19]). This fact, in addition to the very low membrane resistance at this stage of the response, suggests that ions other than Na^{+} may participate in the plateau phase. Indeed, the presence of anomalous rectification in Ca^{++}-free treated fibres could conceivably account for the time course of the short duration responses. The persistence of membrane depolarization long after the stimulating current pulses has ended, seen in many fibres, and the abolition of all Ca^{++}-free saline induced responses by removal of Na^{+} ions are, however, difficult to explain solely in terms of outward membrane currents. Clearly, further experiments under voltage clamp conditions will be necessary to determine the site or sites of action of Ca^{++} ions in the control of membrane excitability in locust muscle fibres.

1 D.A.M. was the recipient of a Royal Society and Science Research Council European Exchange Fellowship. We thank the Centre for Overseas Pest Research, Kensington for supplying the locusts.
2 Present address: Physiologisches Institut der Technischen Universität München, Biedersteiner Strasse 29, 8000 München 40, Federal Republic of Germany.
3 J. Del Castillo, G. Hoyle and X. Machne, J. Physiol., Lond. *121*, 539 (1953).
4 R. Werman and H. Grundfest, J. gen. Physiol. *44*, 997 (1961).
5 P. Fatt and B.L. Ginsborg, J. Physiol., Lond. *142*, 516 (1958).
6 R. Werman, F.V. McCann and H. Grundfest, J. gen. Physiol. *44*, 979 (1961).
7 J.W. Deitmer and W. Rathmayar, J. comp. Physiol. *112*, 123 (1976).
8 P.N.R. Usherwood, Comp. Biochem. Physiol. *6*, 181 (1962).
9 H. Washio, J. gen. Physiol. *59*, 121 (1972).
10 G. Hoyle, Proc. Rog. Soc. *B 143*, 343 (1955).
11 P.N.R. Usherwood and H. Grundfest, J. Neurophysiol. *28*, 497 (1965).
12 F. Dreyer and K. Peper, Pflügers Archiv Physiol. *348*, 263 (1974).
13 T. Narahashi, J.W. Moore and W.R. Scott, J. gen. Physiol. *47*, 965 (1964).
14 Y. Nakamura, S. Nakajima and H. Grundfest, J. gen. Physiol. *48*, 985 (1965).
15 P. Redfern and S. Thesleff, Acta physiol. scand. *82*, 70 (1971).
16 S. Hagiwara and S. Nakajima, J. gen. Physiol. *49*, 793 (1966).
17 P.F. Baker, D.L. Hodgkin and E.B. Ridgeway, J. Physiol., Lond. *218*, 709 (1971).
18 P.G. Kostyuk and O.A. Krishtal, J. Physiol., Lond. *270*, 509 (1977).
19 R. Anwyl, J. Physiol., Lond. *273*, 367 (1977).