index of GMO is substantially higher (1.463). However, when n-C_{10} was used as solvent the bilayer refractive index was only slightly less (1.429), although the refractive index of n-C_{10} itself is substantially lower (1.410). Hence it is likely that the refractive index of the membrane is largely determined by the lipid and is reduced from the bulk value by (i) anisotropic effects and (ii) hydration of the lipid polar groups. Similar effects were previously observed with egg lecithin bilayers (*4*).

6. Differential scanning calorimetric (DSC) measurements were made with a Perkin-Elmer model DSC-1B calorimeter for mixtures of monoglycerides and hexadecane in which the molar ratio of the components was approximately the same as those found experimentally (*3*) in bilayers generated from solutions (i), (ii), or (iii) (see text). Solutions were prepared for DSC by prior mixing of the components in the desired proportions in chloroform, evaporating off the chloroform at reduced pressure, adding an equal weight of distilled water, and thoroughly mixing the resulting paste.
7. We have compared the thickness obtained by the reflectance method with that calculated from the membrane capacitance for bilayers formed from GMO in n-decane, n-tetradecane, and n-hexadecane. The values are respectively 62, 62, and 49 Å from the optical technique, and 57, 49, and 41 Å from the capacitance data (*8*). A systematic error in the optical method was ruled out by verifying the previously published value of the membrane thickness for bilayers formed from egg lecithin in n-decane. Furthermore, the capacitance values of membranes formed in our laboratory were in essential agreement with those of Fettiplace *et al.* (*8*). Therefore, we conclude that the discrepancies in these two methods of determining membrane thickness must be due to the presence of microlenses of hydrocarbon solvent that are too thick to be measured in the membrane capacitance technique, but nevertheless contribute to the average bilayer thickness determined optically. The existence of such lenses has been inferred previously from electron micrographs (*9*) and composition studies (*3*) of the bilayer.
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10. Supported by the Science Research Council. R.E.P. acknowledges receipt of an EMBO short-term fellowship.

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5 January 1973; revised 15 March 1973

Hyperinnervation of Skeletal Muscle Fibers: Dependence on Muscle Activity

Abstract. *After the motor nerve to the rat soleus muscle was blocked reversibly by local anesthesia, individual muscle fibers became innervated by a transplanted motor nerve without losing their original innervation. Such cross-innervation of the denervated soleus muscle by the same foreign nerve was largely reduced by direct electrical stimulation of the muscle. The results demonstrate the importance of muscle activity for synapse formation by a foreign motor nerve.*

Normally innervated and denervated skeletal muscle fibers of adult mammals differ in their ability to accept additional nerves and in their sensitivity to acetylcholine (ACh). Normal muscle fibers are usually innervated by a single motor nerve fiber. They will not normally accept additional motor innervation from a transplanted foreign nerve (*1*), and their sensitivity to the transmitter ACh is sharply localized in the end plate region. In contrast, after denervation the sensitive region expands to cover the entire surface of the muscle fiber (*2*), and the muscle can become innervated by the original nerve or by a foreign nerve. In certain experimental situations skeletal muscle fibers can accept foreign motor nerves in addition to their original innervation. This has been demonstrated after botulinus poisoning (*3*) and after local injury of muscle fibers (*4*). In both these cases the formation of new synapses is associated with supersensitivity to ACh.

From observations on frog sartorius, Miledi (*5*) argued that the spread of ACh sensitivity was due to the absence of a neural "trophic factor" in denervated muscles. Recently, however, Lømo and Rosenthal (*6*) showed that the ACh-sensitive area in rat soleus muscle fibers could spread in the presence of innervation after a prolonged, reversible block of impulse conduction in the soleus nerve. Furthermore, they showed that denervation supersensitivity could be prevented by direct electrical stimulation of the muscle. These observations suggest that muscle activity per se is an important factor controlling the extent of ACh sensitivity over the surface of the muscle fiber. The series of experiments reported here were undertaken to see whether the ability of muscle fibers to accept additional motor innervation might be similarly controlled by muscle activity.

The experiments were performed on young white rats, weighing about 200 g. The superficial fibular nerve and its branches to the peroneal muscles were dissected free, cut distally, and transplanted onto the proximal dorsal surface of the soleus muscle. The fascia over the soleus muscle was left intact to avoid any injury to the muscle fibers. The transplanted nerve was fixed in its new location by fibrin (*7*). After 2 weeks, impulse conduction was blocked reversibly in the sciatic nerve by application of a cuff containing a local anesthetic (Marcaine, Bofors) (*6*). The effectiveness of the nerve block was evidenced by clear signs of paralysis in the ankle muscles. The block was checked in the final experiment 1 week after the application of the cuff, after the nerve and muscle had been isolated from the leg. In successful experiments muscle contractions were elicited by nerve stimulation distal to but not proximal to the block.

In all six rats with successful nerve blocks we found that stimulation of the transplanted fibular nerve elicited visible contractions in the soleus muscle. As suspected, some of this was due to fibular innervation of muscle fibers which had lost their original innervation due to mechanical pressure from the anesthetic cuff with subsequent nerve degeneration. But in each of five rats we found many muscle fibers that could be activated both by stimulation of the original soleus nerve

Table 1. Effect of direct muscle stimulation on innervation by transplanted fibular nerve: F, fibular twitch, peak twitch tension to supramaximal single shock stimulation of fibular nerve; D, direct twitch, peak twitch tension to supramaximal direct stimulation of muscle; F/D, percentage ratio of fibular to direct twitch; ACh, peak tension of the muscle when suddenly exposed to acetylcholine (10^{-5} g/ml) in the bathing solution. When ACh was used, the contractions rose to a peak within 10 seconds and subsided slowly over the subsequent 60 to 90 seconds. Three of the five control muscles gave observable contractions even with an ACh concentration of 10^{-6} g/ml.

Rat No.	Stimulated muscle				Control muscle			
	F (g)	D (g)	F/D (%)	ACh (g)	F (g)	D (g)	F/D (%)	ACh (g)
1	0.04	5.8	0.7	0	6.0	11.4	52.4	5.1
2	0	6.1	0	0	0.9	15.1	5.4	1.8
3	0.02	15.8	0.1	0	1.2	6.0	20.0	2.4
4	0.2	17.0	1.2	1.4	2.0	14.3	14.0	2.9
5	0	24	0	1.1	0.7	9.0	7.8	2.7

and by stimulation of the transplanted fibular nerve (Fig. 1A). As shown in Fig. 1B, these fibers had two distinct regions, one innervated by each nerve.

In three control experiments the fibular nerve was similarly transplanted onto the normally innervated, active soleus muscle. After 3 weeks we found no signs of hyperinnervation of soleus muscle fibers. This is in agreement with earlier reports (*1, 8*). Therefore, a week of reversible motor nerve paralysis appears to alter the properties of muscle fibers so that they will accept motor innervation in addition to their intact original supply.

The hyperinnervation of soleus muscle fibers after such a block of impulse conduction might still be due to the absence of a neural "trophic factor," if its release was coupled to impulse activity or if the local anesthetic also affected some required axonal transport function. However, if muscle activity per se was sufficient to prevent synapse formation by a foreign transplanted nerve, then it appears unnecessary to postulate such a trophic factor. We therefore examined the effect of direct electrical stimulation on synapse formation by the transplanted fibular nerve in the denervated soleus muscle.

The superficial fibular nerve was transplanted bilaterally on the soleus muscle in a series of rats. After 2 weeks the tibial nerve which contains all the soleus axons was cut in both hind legs, depriving the soleus of its original innervation. In one leg platinum stimulating electrodes were implanted, in the tibia and on the opposite side of the soleus in the gastrocnemius muscle. During the following 2 weeks the soleus muscle was stimulated directly at a rate of 10 hertz; the stimuli lasted for 8 seconds with 4-second intervals. This pattern of stimulation was chosen since it prevents the spread of ACh sensitivity in denervated soleus muscles (*6*). The shock intensity was supramaximal, and the efficiency of the stimulation was checked by inspecting ankle extension every day and by direct observation of soleus contraction initially during the final experiment. In the terminal experiment the peak twitch tension elicited from the fibular nerve and the maximal twitch to direct stimulation of the muscle were determined. In addition, we tested the sensitivity of the muscle to application of ACh in the bathing solution to obtain an independent assessment of the efficiency of direct electrical stimulation.

In five rats the soleus muscle was successfully stimulated throughout the 2-week period. Innervation of the soleus by the fibular nerve was much less extensive on the stimulated than on the control side in each of these rats. In two, fibular nerve stimulation resulted in no detectable contraction in the stimulated muscle. In the three others the fibular twitch on the stimulated side was only a small fraction (0.7 to 10 percent) of that in the control muscle. These results are summarized in Table 1, which also shows the twitch tensions to direct electrical stimulation.

Because the maximal direct twitch reflects the remaining contractile force of the muscle after 2 weeks of denervation and atrophy, it is perhaps more meaningful to compare the ratio of innervated to noninnervated fibers (F/D) in the stimulated and control muscles. On the average this "index of cross-innervation" was 60 times higher on the control side than on the stimulated side, and in no case was it less than 12 times higher in the control muscles (Table 1).

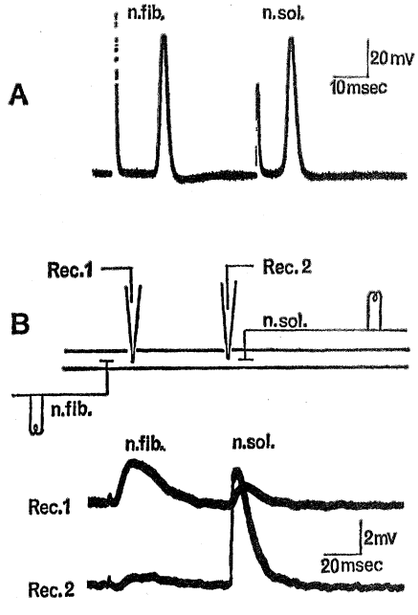

Fig. 1. Hyperinnervated single muscle fibers. (A) Action potentials of a muscle fiber elicited by stimulation of transplanted fibular nerve (*n. fib.*) and the original soleus nerve (*n. sol.*). Intracellular record. (B) End plate potentials from hyperinnervated muscle fiber. Neuromuscular transmission was blocked by magnesium (8 m*M*). Two microelectrodes were inserted in the same muscle fiber (see sketch). One electrode (*Rec. 1*) was close to the new end plate formed by the implanted fibular nerve, and the other (*Rec. 2*) was close to the original end plate of the soleus nerve. The first recorded an attenuated end plate potential to fibular nerve stimulation and a focal end plate potential from the soleus nerve. The relation between the two end plate potentials was reversed when recorded by the second electrode.

It is possible that the mere presence of the implanted electrodes on the stimulated side affected the muscle and prevented cross-innervation. In two rats muscle stimulation failed on the first day after the denervation and implantation of the electrodes. These were kept as controls and showed fibular innervations of 48 and 27 percent of the direct maximal twitch. Thus, the presence of implanted electrodes did not significantly reduce the degree of innervation by the fibular nerve.

As a control of possible side effects of the stimulating current on nerve growth in general, we crushed the soleus nerve in three rats and stimulated the soleus muscle directly in the usual manner during the next 14 days. The soleus muscles were extensively reinnervated at the end of this time (80 percent or more of the direct twitch tension was obtained by nerve stimulation in all three cases). This result demonstrates that muscle stimulation did not prevent growth of nerve fibers. Furthermore, reinnervation of the muscle by its own nerve was not prevented by muscle activity. This is not necessarily due to a real difference in the ability of the original and foreign nerves to innervate the muscle. After the crush of the original nerve the regenerating soleus axons will reach the original end plate regions, which retain their sensitivity to ACh in spite of muscle stimulation (*6*).

The results reported here demonstrate that innervation of a denervated muscle by a foreign nerve can be prevented by maintaining the muscle activity by direct stimulation. It suggests that muscle activity per se is a principal factor regulating the innervation of mammalian skeletal muscle, and that there is no need for postulating a neural trophic factor to explain the observations. A similar conclusion was reached with regard to the spread of ACh sensitivity of denervated muscle (*6*).

In its effect on hyperinnervation the muscle activity might act by reducing the supersensitivity. It has been suggested earlier that ACh receptors themselves may induce the develop-

ment of neuromuscular synapses (*3*). However, in other experimental situations—for example, in studies of the effects of neural tissue on muscle in organ culture (*9*), the effects of colchicine treatment on motor nerves (*10*) and the control of ACh sensitivity of frog muscles (*11*)—the participation of a neural trophic factor may be required.

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12. We thank Dr. Eric Frank for valuable comments.

20 February 1973; revised 27 March 1973

Exocytosis-Endocytosis Coupling in the Pancreatic Beta Cell

Abstract. *The stimulation of the release of insulin by glucose is accompanied by an enhanced uptake of cytochemically demonstrable horseradish peroxidase into endocytotic vesicles within the beta cells. An exocytosis-endocytosis coupling might represent a mechanism by which membrane constituents are recycled within the beta cells under conditions of increased secretory activity.*

As first described by Lacy and Hartroft (*1*), emiocytosis (or exocytosis) probably represents the physiological route by which insulin is released by the pancreatic beta cell into the extracellular space (*2*). In such a process, the Golgi-derived membranous sacs surrounding the insulin secretory granule cores fuse with and are eventually incorporated into the plasma membrane. However, the ultimate fate of this membranous material remains unknown. The present report aims at demonstrating that emiocytosis in glucose-stimulated beta cells is coupled with a compensatory movement of membrane from the cell surface into the cytoplasm.

Batches of 30 to 40 islets of Langerhans isolated from the pancreas of fed rats by collagenase digestion (*3*) were incubated for 60 to 90 minutes at 37°C in bicarbonate-buffered medium containing albumin (0.5 percent, weight to volume). Glucose (3.0 mg/ml) and horseradish peroxidase (2.0 mg/ml) (*4*), a macromolecular marker of endocytotic activity (*5*), were incorporated into the incubation medium at various periods of incubation. After incubation, the islets were centrifuged into a pellet, fixed in 2 percent glutaraldehyde in phosphate buffer (0.1*M*, *p*H 7.2) for 90 minutes, rinsed in the same buffer, embedded in agar gel, and sliced in sections 40 μm thick. The slices were treated according to the procedure of Graham and Karnovsky (*5*) to demonstrate sites of peroxidase activity. The fragments of islets were then postfixed with osmium tetroxide in phosphate buffer, dehydrated in graded dilutions of alcohol, and embedded in Epon 812. Thin sections, obtained with a diamond knife on a LKB microtome, were mounted on uncoated copper grids and examined with a Philips EM 300 electron microscope. Islets not exposed to peroxidase at any stage of incubation and processed in order to reveal endogenous peroxidase (*5*, *6*) served as controls. Such preparations contained no peroxidase-tagged structures.

In the first series of experiments, islets were incubated with peroxidase for 1 hour in the absence (Fig. 1a) or presence (Fig. 1b) of glucose. In both conditions, the macromolecular marker easily penetrated the extracellular space, the reaction product delineating sharply the islet cells. Reaction product could also be found in flask-shaped invaginations of the plasma membrane of beta cells, as well as in membrane-bounded vesicles free in the cytoplasm. A comparison of a and b in Fig. 1 clearly shows that the number of such peroxidase-containing vesicles was dramatically increased in beta cells incubated at a high concentration of glucose, a condition known to promote insulin release. However, because of the cell to cell variability of such a phenomenon, we have applied the stereological principles of morphometry (*7*) for the measurement of the surface density of peroxidase-positive

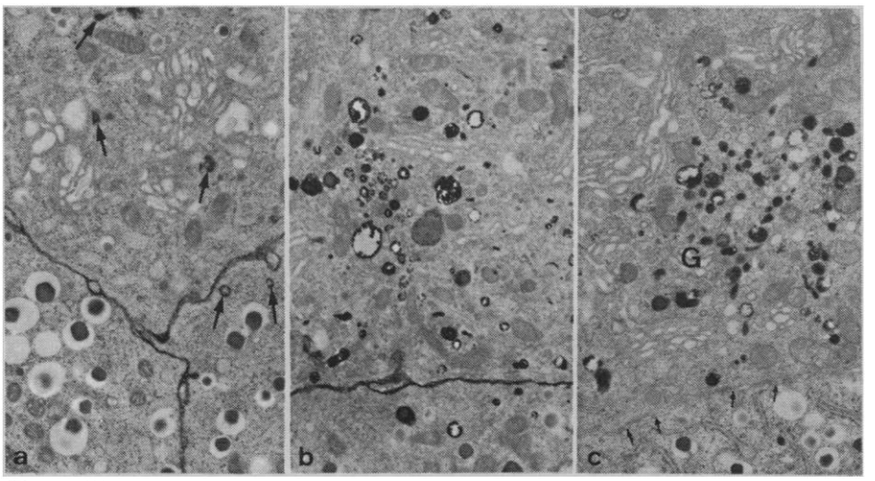

Fig. 1. Electron micrographs of beta cells incubated for 60 minutes in the presence of horseradish peroxidase (2.0 mg/ml). (a) Islet incubated in the absence of glucose. The intercellular space is filled with reaction product. Deposits of reaction product are also present in a few vesicles (arrows) (× 9500). (b) Islet incubated at a high glucose concentration (3.0 mg/ml). The reaction product is present in the intercellular space as well as in many vesicles of varying size (× 9500). (c) After incubation for 60 minutes with peroxidase at a high glucose concentration (3.0 mg/ml), the islet was further incubated for 30 minutes at the same glucose concentration but in the absence of peroxidase. Vesicles with the reaction product are present in large numbers in the Golgi area (*G*), but no reaction product is found in the extracellular space (arrows) (× 9500).