blockage of inhibition could be obtained in an anatomically more complex system we injected similar doses of strychinine and observed the effect on striatal inhibition.

In ten cats the effect was uniformly the same at all three levels of the neuraxis (association cortex, intralaminar thalamus, and brainstem reticular formation). The intravenous injection of 0.1 to 0.2 mg/kg of strychnine sulfate completely blocked the inhibition produced by stimulating the head of the caudate nucleus. With the doses used, no increases in spontaneous background activity, or in the amplitude of the evoked response were observed after drug administration; hence, no general hyperexcitability could account for the blocking of inhibition.

In the centrum medianum the slight response to caudate stimulation (Fig. 1, *B2, C2*) may reflect the discharge of those cells which microelectrode recordings have shown to be excited by striatal stimulation (*9*). After strychnine administration, this response, though enhanced, does not inhibit the peripherally evoked potential. This would be consistent with the notion that these cells may represent interneurons in the inhibitory pathways and that their synaptic action has been blocked by strychnine. It also shows that occlusion cannot be the cause of the inhibition, because, if it were, the increased response to the caudate volley (Fig. 1, *C2*) would have resulted in even more effective occlusion of the peripherally evoked potential.

Little is yet known about this finding, that strychnine blocks the inhibition of impulses in supraspinal polysynaptic systems (*10*). Not all types of postsynaptic inhibition are susceptible to strychnine (*11*), but the small amounts of this substance used in the present experiments suggest a postsynaptic mechanism of response. Also, the transmitter is probably similar to that of spinal motoneurons; this would be consistent with the notion of Eccles (*12*), that there are relatively few inhibitory transmitters in the mammalian central nervous system.

The cortex does not appear to be an active site of inhibition since subcortical inhibition was still observed after acute and chronic hemidecortication. The inhibitory effect on the cortex (Fig. 1, *B1*) is thus merely the passive reflection of an active subcortical process.

It is probably that the intralaminar thalamus represents only one of several regions which show postsynaptic inhibition in response to striatal stimulation; other thalamic structures such as the nucleus ventralis lateralis, certain reticular regions, and even regions in the spinal cord, may generate postsynaptic inhibition to striatal stimulation (*13*).

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Cytochrome Function in Relation to Inner Membrane Structure of Mitochondria

Abstract. *Projecting subunits of an average diameter of 80Å are found on the cristae of mitochondria prepared from the muscle of* Ascaris lumbricoides. *A spectroscopic examination of the cytochrome content of these mitochondria shows no detectable cytochrome* c_1, a, *or* a_3 *and does reveal cytochromes of types* c *and* b. *Subunits in the same size range are found in cytochrome c deficient mitochondria of the emergent bee, while the frequency of their occurrence along the cristae is decreased relative to the adult bee. Apparently, the cytochrome content of the respiratory chain is not related to the size of the subunits, but may be related to the frequency of occurrence of the subunits.*

Spectrophotometric studies of the stoichiometry and reaction kinetics of components of the cytochrome chain indicated, some time ago, that macromolecular assemblies containing approximately one each of the cytochrome respiratory enzymes might be sufficient for the function of electron transfer and oxidative phosphorylation (*1*). Models of such a system have been shown to be consistent with mitochondrial structure by Estabrook and Holowinski (*2*) and by Lehninger (*3*). More recently, the term "oxysome" has been introduced to represent the concept of a macromolecular assembly of enzymes capable of carrying out all the functions of electron transport and oxidative phosphorylation and specifically including the coupling factors of oxidative phosphorylation and the pathways of energy-linked reversal of electron transfer (*4*).

Observation by electron microscopy of the fine structure units of the inner membrane (IMS), in the size range, 80 to 100 Å, by Fernandez-Moran (5), and more recently by Parsons (*6*), Stoeckenius (*7*), Smith (*8*), and Sjostrand (*9*) has led Green and his coworkers to suggest that these units consist either of the entire assembly of respiratory enzymes (*5, 10*), a "lumped oxysome or "elementary particle" (*10*), or of one or more components of the respiratory chain (*4*), a "distributed" oxysome. There are differences of opinion whether these fine-structure units

are present in intact cristae or whether they arise because of special treatment of the specimen, as for example, the extrusion of protein that ensues in the negative staining procedure in the course of drying with phosphotungstic acid (*9*).

It has not been proved that these fine structure units represent any enzymatic function at all. Nevertheless, Green and his co-workers (*10, 11, 12*) have actively pursued the hypothesis that the fine-structure units represent respiratory assemblies (*4*); they put forward as evidence, comparisons of the volume of the fine structure with the total known components of the respiratory chain or with a selection of these components (*11, 12*). One view is that the correlation is dubious (*4*). On the other hand, a selected list of components of the oxysome reduces the aggregate molecular weight to a size consistent with that of the fine structure (IMS) to within a factor of two (*12*). Further assumptions with respect to the degree of shrinking of the fine-structure units and with respect to the assymetry of the subunits leads to conclusions that the dimensions are those of a spheroid or cylinder of 114 Å in diameter and 182 Å in length (*13*), instead of the approximately 90 Å shapes that appear in electromicrographs. These assumptions also aid in bringing the sizes into agreement. Further comparisons have been made on the basis of the size of particles derived from the mitochondrial structure, either by partial purification or by reconstitution of partially purified components of the respiratory chain (*12*). On this point, however, it should be remembered that cytochrome *c* (molecular weight 12,800) appears as long as 38 to 40 Å (*14*) and cytochrome oxidase ($a + a_3$) [estimated molecular weight 530,000 (*15*)] as large as 110Å for a protein density of 1.3 (see also *16*).

In this report, a new method is proposed for evaluating the possible function of fine structure subunits (IMS) by correlating the size and frequency of occurrence with natural variations in the cytochrome content of the mitochondria of the flight muscle of the honey bee, *Apis mellifera L.* (*17*), and of the longitudinal muscle of the worm, *Ascaris lumbricoides* (*18–20*). If it is assumed for the purposes of argument, that the fine structures do contain cytochrome, these natural deficiencies of cytochrome in these mitochondria would be expected to decrease their size or their spacings or both. Also, such an assumption allows us to consider whether all the electron transport components could be located in single units of the fine structure, a lumped oxysome (*10, 11, 12*), or whether the function of electron transfer and oxidative phosphorylation must be distributed over the inner membrane surfaces of the mitochondria, a distributed oxysome (*4*).

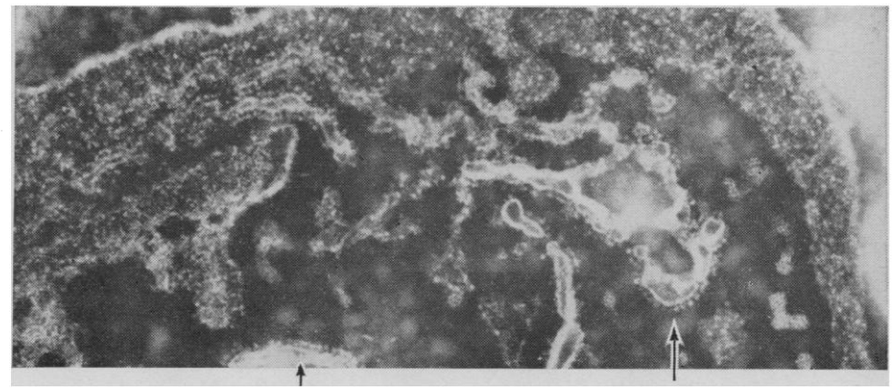

Fig. 1. An electron micrograph of *Ascaris* mitochondria obtained by negative staining. The size of the units of the fine structure is indicated in the histogram of Fig. 2 (*31*).

For mitochondrial preparations, emergent bees were collected from the comb over an interval of several hours. Adult bees were those which were emergent for roughly a week, or were actually flying from the hive. Mitochondria were prepared from the thoracic muscle of the bee. (*Apis mellifera L.*) (*20*). The bees were obtained through the kindness of Drs. H. Borei, R. C. Herold (*17*), and of N. Meadows. The *Ascaris* were obtained from pigs at local slaughter houses in Philadelphia and in Baltimore with the help of Dr. Saz. After washing, the muscle was carefully dissected in order to avoid contamination with bacteria or other organs. Thereafter, the procedure for preparation of the mitochondria was essentially that of Chance and Hagihara (*21*). Electron micrographs showed a large number of intact mitochondria in the preparation with morphology similar to that observed previously (*22*).

The technique of low temperature spectroscopy (*23*) was used to determine the relative concentration of cytochromes *c* and ($a + a_3$) in the emergent and adult bees. In this case, the mitochondria were frozen in the mannitol-sucrose tris reaction medium (*21*). Under these conditions, cytochrome c_1 could normally be observed with adequate clarity, cytochrome *c* was measured at 547 mμ, cytochrome c_1 at 551 mμ, cytochrome *b* at 561 mμ and cytochrome a at 600 mμ. Possible sources of error in this method have been discussed (*24*).

In *Ascaris* mitochondria, two cytochrome peaks are observed at liquid-nitrogen temperatures, one at 547 mμ and the other at 557 mμ.

The protein concentrations were determined by the biuret method (*25*).

For examination by electron microscopy, the isolated mitochondria were lysed by treatment for 10 to 30 minutes with 1m*M* phosphate buffer, *p*H 7.4, if not previously so treated, and the sample was negatively stained by the surface spreading technique (*6*). The negative stain was 2 percent potassium phosphotungstate, *p*H 6.8. The dried preparations were examined in the Siemens Elmiskop I at a microscope magnification of × 40,000. Micrographs were obtained at the University of Pennsylvania through the courtesy of Dr. Frank Pepe.

Size of the subunits. Previous electron micrographic studies reveal mitochondria in *Ascaris* muscle with relatively few cristae (*26*). We find inner membrane subunits located

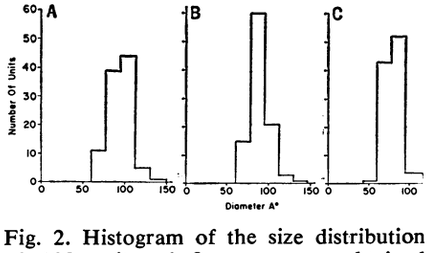

Fig. 2. Histogram of the size distribution of 100 units of fine structure obtained from negative-stained electron micrographs similar to Figs. 1 and 3. **A** and **B**, Bee thoracic muscle (*A*, adult; *B*, 3-hour emergent). *C*, *Ascaris* muscle.

Table 1. Correlation of fine structures appearing in electron micrographs with stoichiometries of the cytochrome in thoracic muscle of emergent bees and in the longitudinal muscle of *Ascaris lumbricoides*.

Item	Bee		*Ascaris* (adult)
	Adult	Young	
	Cytochrome (mμ mole/mg protein)		
a	0.87	0.30	
c			0.13
	Stoichiometry of cytochromes		
c_1/a	1.3	1.3	
c/a	1.6	0.7	
b/a	1.5	1.4	
a_3/c			<.05
a/c			<.05
b/c			1.6
c_1/c			<.1
	**IMS (Å)*		
Diameter	90	90	80
Spacings	115	174	139

* From electron micrographs.

on the cristae of such mitochondria (Fig. 1). The size and spacing of these subunits is illustrated by Figs. 2 and 3. The average diameter is 80 Å, in the middle of the range determined for mitochondria of a wide variety of tissues (*6*). The frequency of occurrence of the subunits illustrated by Fig. 3 is intermediate between that of the emergent and adult bee-flight muscle.

Low temperature spectroscopy of the cytochrome components of mitochondria prepared from *Ascaris* muscle shows, however, a vast difference when compared with mitochondria containing a full complement of cytochromes (Fig. 4). Even in the region of the Soret band where the detection of the 442 mμ (77°K) peak of cytochrome oxidase is optimal, no indication of cytochrome oxidase is recorded, and its content must be considerably less than the usual value for the respiratory chain. Table 1 gives limiting values for cytochromes a_3, *a*, and c_1 contents as being less than 5 percent of the cytochrome *c* content. The amount of cytochrome *c* in the mitochondria is low relative to that of the bee thoracic muscle (*18, 25*) but is within the range of values for liver mitochondria (*2*). The two prominent bands at 557 and 547 mμ are respectively attributed to cytochromes of type *b* and *c* on the basis of their band position and the kinetics of their reduction and oxidation (*27*). There is no evidence for a cytochrome of type c_1 (552 mμ), nor is there any measurable interference from *Ascaris* myoglobin which is found in the supernatant fraction from the preparation of these mitochondria. The trough in the region of 460 mμ is attributed to a flavoprotein component of these mitochondria, which is about two times the flavoprotein to cytochrome *c* content of pigeon-heart mitochondria (*21*).

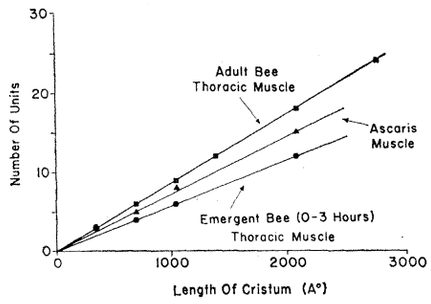

Fig. 3. Evaluating the frequency of occurrence of the fine-structure units on negatively stained electron micrographs of adult emergent bees similar to those in Fig. 4. The frequency of occurrence of fine structure units on the cristae of the *Ascaris* mitochondria is also included.

A deficiency of cytochrome oxidase should have a considerable impact upon the size of a subunit which is expected to contain all the cytochrome components of the respiratory chain. Taking 530,000 for the molecular weight of cytochrome oxidase (*15*), we find that a decrease in the size of the fine structure subunits of over 100 Å would be expected on the basis of the protein density of 1.3. Cytochrome c_1, also missing, would correspond to a diameter of over 40 Å. The experimental result is clear; subunits of the normal diameter (80 Å) are found on the cristae of mitochondria which are highly deficient in the cytochrome oxidase and cytochrome c_1 components of diameters respectively over 100 Å and over 40 Å. These results make it unlikely that each subunit contains all the cytochromes in the normal mitochondria. Although the possibility that the 100 Å space left vacant by the absence of cytochrome oxidase is filled with some unknown substance could be considered, the burden of proof would rest clearly upon those who propose the theory.

Frequency of occurrence of subunits. Electron microscopy of the inner membrane structure of the emergent and the adult bee shows fine-structure subunits in both cases. However, the occurrence of the subunits in the emergent bee is highly irregular (Fig. 5). Figure 2 shows that the size distribution of both the subunits in the adult and emergent bee is very nearly the same (90 Å), although the distribution in sizes of the adult bee covers a somewhat wider range and might eventually be shown to be bimodal (*6*). A comparison of the frequency of occurrence of the subunits illustrated by Fig. 3 shows a 35 percent decrease in the frequency of their occurrence (the spacing of 115 Å compared with 174 Å).

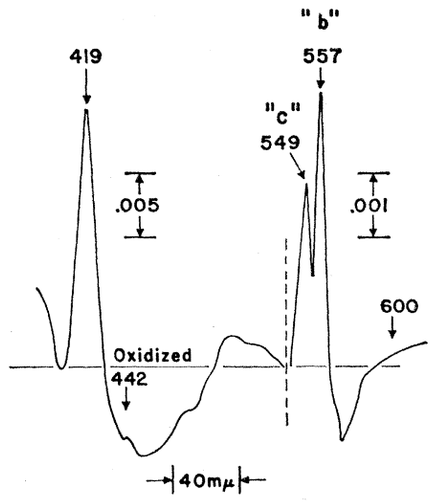

Fig. 4. Oxidized-reduced spectrum of the mitochondrial fraction of the *Ascaris* muscle at 77°K. The wavelengths are measured in mμ; the optical densities are per cm.

Low-temperature spectroscopy shows a deficiency of cytochrome *c* in the mitochondria prepared from the thoracic muscle of the emergent bee. Only 43 percent of cytochrome *c* relative to cytochrome *a* is present in the emergent bee as compared with the adult bee (*17*).

We may compare the 57 percent decrease of the cytochrome *c* content with the 35 percent decrease in the frequency of occurrence of the subunits on the basis of a "distributed" oxysome hypothesis. In this case, cytochrome *c* would constitute only one of a number of units of the oxysome structure, probably about one in seven and thus, the decrease in the frequency of occurrence of subunits would be expected to be only 56/7 or 13 percent, as compared with the 35 percent observed. It is very likely, however, that components of the oxysome in addition to cytochrome *c* are deficient in the flight muscle of the emergent bee (*17*).

The results of the emergent bee also have some bearing on the "lumped" oxysome hypothesis. If, indeed, the cytochrome *c* has a diameter in the mitochondrial structure as large as that observed by Levin (about 45 Å), a deficiency of 57 percent would suggest that over half the subunits should lack a molecule of diameter equivalent to 45 Å. This suggests that a bimodal dis-

tribution of subunit diameters would be more likely in the subunits of emergent bees than of adult bees. Just the opposite result is observed in Fig. 2, adding to the evidence unfavorable to the "lumped" oxysome hypothesis.

Figure 3 and Table 1 indicate that the spacing and the frequency of occurrence of the subunits in the *Ascaris* muscle is somewhat less than that of the intact flight muscle. While this result is in agreement with the cytochrome deficiency, no reference material is available for the *Ascaris* mitochondria by which it might be determined how frequently the subunits would appear if cytochrome oxidase and c_1 were present in their normal relation to cytochrome *c*. It is apparent, however, from the diameter of the subunits (Table 1) that the spacing could be reduced to 80 Å, a decrease of 70 percent, which is more than enough to accommodate two additional components of the respiratory chain as subunits on the cristae.

Inner membrane subunits very nearly the same diameter as those which contain a full complement of cytochromes are found in mitochondria that have a complete deficiency of cytochrome oxidase and a partial deficiency of cytochrome c_1. This result suggests that there is no detectable correlation between the diameter of the subunits and the cytochrome content of these mitochondria. For this reason, we feel that the current hypothesis (compare footnote in reference *28*) proposed by Green (*10, 11, 12*) that these subunits contain the *entire* cytochrome complement of the respiratory chain must be abandoned, or at least revised.

Actually, there is no direct experimental evidence contrary to our conclusion; Green's current hypothesis is based upon plausible qualitative relationships between the size of the subunits and the total size of components needed for electron transfer functions (*10, 11, 12*). It has been apparent, however, that this relationship does not stand the test of a detailed quantitative analysis, particularly when cytochrome oxidase itself requires a subunit of over 100 Å in diameter. A number of assumptions, both with respect to dimensions of the subunits and with respect to the molecular weight and the cytochrome complement of the electron transfer system chain, are necessary to achieve any agreement of the chemical and structural data (*11, 12*). It has further been assumed by Green *et al.* (*13, 29*) that two dimensions are 20 percent greater than those observed and that a third dimension is nearly 200 percent greater. The increase of the first two dimensions is a somewhat unlikely possibility and the third is seemingly unreasonable, particularly in view of the electron micrographic views of the subunits not only from the side but also from the top; for example, in insect mitochondria (*8*). In addition, the close (100 Å) packing of the cristae of mitochondria of intact flight muscle (*8*) does not permit the increase of the third dimension of the subunit essential to the "lumped oxysome" hypothesis. The decreased frequency of occurrence of the subunits in the cytochrome *c* and cytochrome *a*, a_3, and c_1 deficient mitochondria is evidence in favor of, but by no means proof of, the distributed oxysome hypothesis. The general idea of the oxysome as the functional unit of electron transfer and oxidative phosphorylation is, of course, not bound to a particular structure of the mitochondria.

These results suggest that the size of the inner membrane subunit is not related to the cytochrome content of the electron transport system. A correlation has been found, however, between the cytochrome content and the frequency of occurrence of subunits in the membranes, supporting the idea that if these subunits have any role in cytochrome function, they participate in an oxysome function (*4*) that is distributed over the surface of the mitochondrial membrane and involves a number of subunits of the fine structure.

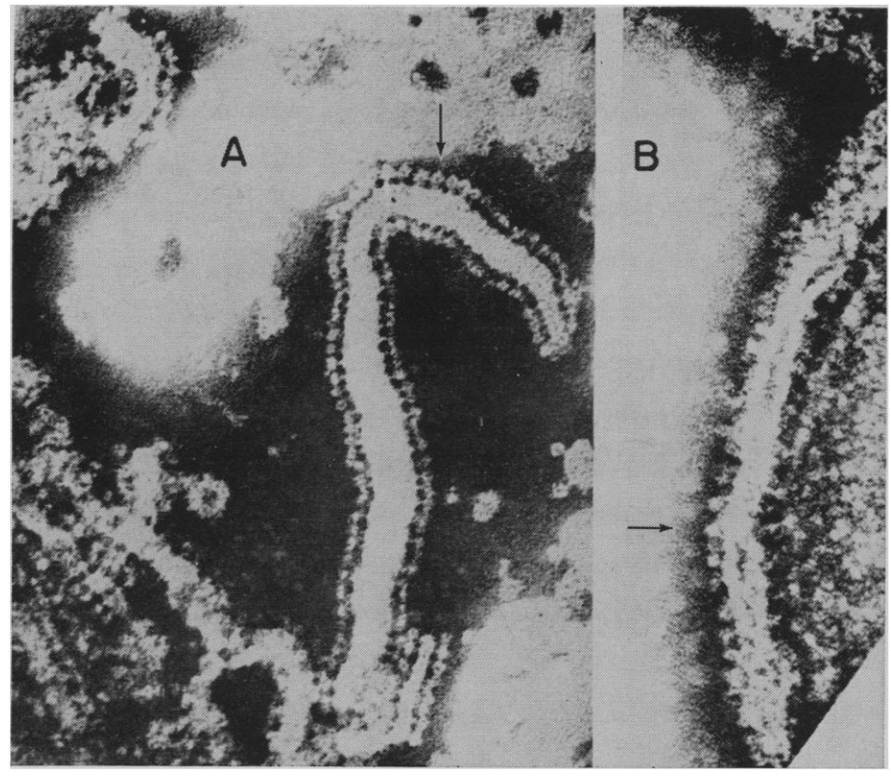

Fig. 5. Electron micrographs of portions of the cristae of the adult bee and the 0- to 3-hour emergent bee obtained by negative staining methods. The size of the units is indicated in the histogram of Fig. 2.

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31. For complete protocols on figures our file numbers are as follows: Fig. 1 (Parsons 5); Fig. 2 (2–4^{IV}); Fig. 3 (2–4^{IV}); Fig. 4 (4–3^{IV}); Fig. 5 (Parsons 6A and B).

10 October 1963

Separation of Transducer and Impulse-Generating Processes in Sensory Receptors

Abstract. *New evidence is presented that spike and transducer processes in sensory receptors are independent events; impulse activity in the crustacean stretch receptor neuron and the mammalian pacinian corpuscle was selectively blocked by a compound (tetrodotoxin) without affecting any of the parameters of the generator potential.*

The two components of electrical activity in sensory receptors, the generator and the all-or-none action potential, have a number of distinctive features (*1*, *2*), suggesting that they may arise in different membrane components. It would be difficult, however, to provide direct evidence for such a segregation with the available techniques. Even in those favorable cases, such as the crustacean stretch receptor organ and the pacinian corpuscle, in which a coarse spatial segregation of the components appear to exist (*3*, *4*) there are uncertainties. In the first case, absence of impulse activity in the dendrites, where the generator potential arises (*5*), might be the consequence of geometrical factors; and in the latter case, spatial restrictions for excitations (*2*) and other factors (*6*) may possibly prevent all-or-none activity at the nonmyelinated nerve terminal. One has to rely, therefore, on the more indirect method of blocking selectively one of the two electrical components to demonstrate the independence of their underlying mechanisms. A variety of techniques have been used previously to this effect (*7*), but all have the disadvantage that in blocking the all-or-none component they also affect to some extent the generator potential. In the present experiments we used tetrodotoxin. This toxic substance obtained from the puffer fish has already been shown to block impulse activity in nerve and muscle fibers (*8*, *9*). Here, in two receptors, the crustacean stretch receptor neuron and the pacinian corpuscle, tetrodotoxin will be seen to act selectively on the all-or-none component, blocking the action potential, but leaving the generator potential unaffected.

The isolated crustacean neuron provided an appropriate preparation since, first, its dendrites are readily accessible to water-soluble molecules at least as large as sucrose (*10*); second, the generator potential is readily measured by intracellular recordings; and third, the stretch-generator potential relationship and the "equilibrium potential" of the generator potential are known (*3*).

The soma of neurons of the slowly adapting stretch receptor was impaled with microelectrodes (3*M* KCl) mounted on a bridge circuit to stimulate the cell directly and to measure membrane resistance (*3*). Potential changes (generator and action potentials) produced by different degrees of stretch were recorded. The bathing solution (van Harreveld) was then exchanged for one containing tetrodotoxin, and the measurements were reported.

Tetrodotoxin in concentrations of 1 to 5 g in 10^6 ml abolished the action potential produced by direct or antidromic stimulation, as well as the sustained impulse activity in response to a steady stretch (Fig. 1*A*). The resting membrane potential remained unchanged and the membrane resistance decreased only slightly. The amplitude of the initial and steady phases of generator potentials, just threshold for impulse initiation, was not affected by the drug, even when the concentration

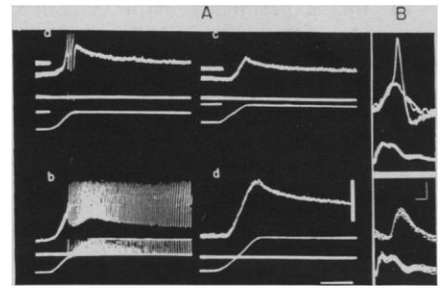

Fig. 1. (*A*) Crayfish slowly adapting stretch receptor organ. In each record from top to bottom: intracellular recording, record from the axon (2 mm or more from the soma), stretch. Response to two degrees of stretch before (*a*, *b*) and after (*c*, *d*) treatment with tetrodotoxin (5×10^{-6} wt/vol). Calibration: 2 sec; 10 mv. (*B*) Decapsulated pacinian corpuscle. Responses to a threshold stimulus before (upper record) and after (lower record) application of 5×10^{-4} wt/vol tetrodotoxin. Lower beam, photoelectric record of mechanical pulses. Calibration: 1 msec; 15 μv.

was increased by an order of magnitude above that sufficient to block impulse activity. The relationship between generator potential amplitude (steady phase) and applied stretch was similar to that of the untreated receptor (Fig. 2*A*). Moreover, in two receptors, in which delayed rectification was negligible, the equilibrium potential of the generator potential after application of tetrodotoxin was found to be similar to that previously obtained in untreated neurons (*3*).

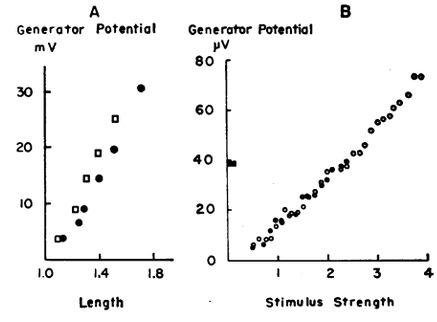

Fig. 2. (*A*) Relation between generator potential (steady phase amplitude) and relative length of the muscle bundle in two crustacean slowly adapting stretch receptors after treatment with tetrodotoxin. Maximum length at which no impulse activity was present was taken as unity. The relationship is simular to that previously described in normal receptors (compare with Fig. 1 of Ref. *3*). (*B*) Generator potential–stimulus strength relation in an intact pacinian corpuscle before (●) and after (o) application of tetrodotoxin. Bar on ordinate marks level for impulse initiation before drug application.