Here we shall be content to make the special canonical transformations differ only at $\tau_2$. The corresponding theorem is

$$tr\,\langle\tau_2|\,\tau_1\rangle^{x+x'} \times tr\,\langle\tau_1|\,\tau_2\rangle^{x+x''} = 2\pi\delta(q' - q'')\delta(p' - p'')$$

where

$$Q'(\tau) = -q'\delta(\tau - \tau_2),\ P'(\tau) = -p'\delta(\tau - \tau_2)$$

and similarly for $Q''(\tau)$, $P''(\tau)$. This statement follows immediately from the orthonormality of the $U(q'p')$ operator basis on evaluating the left-hand side as

$$tr\,e^{-i[p(\tau_2)q' - p'q(\tau_2)]}\,e^{i[p(\tau_2)q'' - p''q(\tau_2)]} = 2\pi\delta(q' - q'')\delta(p' - p'').$$

* Supported by the Air Force Office of Scientific Research (ARDC).

[1] These PROCEEDINGS, **46**, 883 (1960).

[2] These PROCEEDINGS, **46**, 570 (1960).

[3] This formulation is closely related to the algarithms of Feynman, *Phys. Rev.*, **84**, 108 (1951), *Rev. Mod. Phys.*, **20**, 36 (1948). It differs from the latter in the absence of ambiguity associated with noncommutative factors, but primarily in the measure that is used. See Footnote 5.

[4] These are directly useful as differential equations only when $G(qp)$ is a sufficiently simple algebraic function of $q$ and $p$. The kinematical, group foundation for the representation of equations of motion by functional differential equations is to be contrasted with the dynamical language used in these PROCEEDINGS, **37**, 452 (1951).

[5] In this procedure, $q(\tau)$ and $p(\tau)$ are continuous functions of the parameter $\tau$ and the Fourier coefficients that represent them are a denumerably infinite set of integration variables. An alternative approach is the replacement of the continuous parameter $\tau$ by a discrete index while interpreting the derivative with respect to $\tau$ as a finite difference and constructing $\delta[Q, P]$ as a product of delta functions for each discrete $\tau$ value. With the latter, essentially the Feynman-Wiener formulation, the measure $d[q, p]$ is the product of $dq(\tau)dp(\tau)/2\pi$ for each value of $\tau$, periodicity is explicitly imposed at the boundaries, and the limit is eventually taken of an infinitely fine partitioning of the interval $T = \tau_1 - \tau_2$. The second method is doubtless more intuitive, since it is also the result of directly compounding successive infinitesimal transformations but it is more awkward as a mathematical technique.

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## *OPPOSITE MECHANICAL RESPONSES OF TONIC MUSCLES TO ACETYLCHOLINE STIMULATION IN NON-IONIC AND IONIC SOLUTIONS**

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Studies of the links between the excitation of the muscle membrane and the activation of the contractile substance have often focused attention on the movement or change of state of certain metallic cations.[1-3] One hazard in interpreting these studies is that cardiac, smooth, and ordinary skeletal muscles are excited by action potentials passing along the cell membranes; conduction itself has specific ionic requirements[4] and, therefore, changing the external ionic environment may modify contraction by affecting conduction. Because of this we chose the frog rectus abdominus, a muscle which contains a high proportion of tonic fibers. Tonic fibers are excited at end-plates spaced over their entire length and never

conduct an action potential.[5, 6] The normal stimulus can be mimicked simply by bathing the muscle in solutions containing acetylcholine (ACh). We have studied the mechanical response of the rectus to ACh in isotonic, ion-free solutions and in solutions in which each of the cations of Ringer's solution was singly replaced. The results help to limit acceptable theories of excitation-contraction coupling, if a single hypothesis, applicable to all types of vertebrate muscle fibers, is desired.

*Materials and Methods.*—The rectus abdominis of *Rana pipiens* was prepared by splitting the muscle down the midline. When "paired muscles" were required, one half served as a control. The iliofibularis and sartorius were also used, as indicated later. Muscles kept in the experimental solution for several hr or more were held at 4°, and the volume of solution used was so large that outflow from the muscle did not appreciably alter the composition of the medium. For tension recording, the muscle was tied in a simple chamber and connected at one end to a Grass strain gauge which led to an inkwriter. All solutions were made from distilled water which had subsequently passed through a demineralizing column (Illinois Water Treatment Company). The Ringer's contained 117 mM NaCl, 4 mM $NaHCO_3$, 3 mM KCl, and 2.7 mM $CaCl_2$, and isotonic sucrose contained 78 grams/liter. All solutions containing a single salt at a specified concentration were made isotonic with sucrose. ACh was used at a concentration of 50 $\mu$g/ml, except in plotting the dose-response curve and in studying the effect of curare or of potentiating agents. All the results reported represent at least three experiments, although on most points many more muscles were studied.

*Results.*—1. *The removal of ions from the extracellular fluid:* When isotonic sucrose replaces Ringer's solution in the muscle chamber, the rectus undergoes a slow contracture.[7] The total increase in tension is between 1.5 and 4.0 gm and the time needed to reach the peak is as short as 30 min or as long as several hr. If the rectus is then challenged with ACh in sucrose, the muscle rapidly relaxes. While the ACh remains in the chamber, the muscle very slowly returns to the tension maintained before the challenge. Washing the muscle with isotonic sucrose permits a much more rapid return to the "baseline." This 'relaxation response' to ACh in sucrose is the mirror image of the abrupt contracture elicited by ACh in Ringer's (the response commonly measured in ACh assay). Relaxation is seen usually within 1 or 2 sec after adding ACh to the rectus bathed in sucrose and, in most instances, the maximum relaxation is reached smoothly in less than 30 sec. With an adequate pre-soak in sucrose, most of the muscles relaxed between one and two gm when challenged. Figure 1 shows the response of a rectus to ACh challenge in both Ringer's and sucrose.

The "relaxation response" can be elicited repeatedly from a rectus immersed in sucrose, although the amplitude may decline somewhat after several trials. The ability of the rectus to contract in response to ACh is rapidly restored by substituting Ringer's for sucrose in the muscle chamber; a contraction is obtained upon challenge one minute after the Ringer's is introduced, although a longer soak in Ringer's is necessary for maximum recovery. Full recovery, to the pre-sucrose level, is not seen. It should be emphasized that the conversion of a normal "contracting" rectus to a "relaxing" one by soaking in sucrose takes much longer than the return of the normal response by reimmersion in Ringer's.

The "relaxation response" could reflect the action of ACh at sites entirely distinct

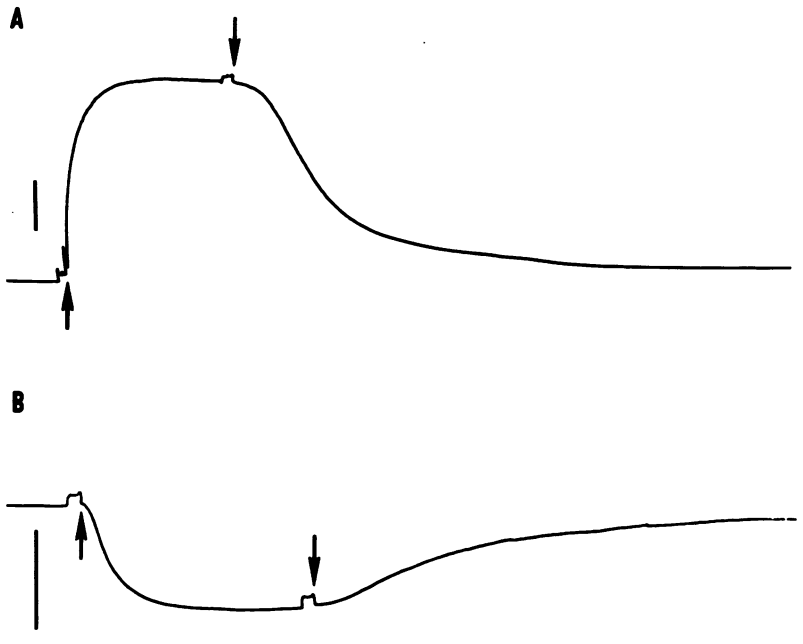


FIG. 1.—The response of a single rectus muscle to ACh (50 μg/ml) stimulation in Ringer's (A) and sucrose (B). ↑ indicates ACh addition to the muscle chamber and ↓ indicates a wash. An upward deflection reflects an increased muscle tension. Tension calibration one gm (note different scale in A and B) and time calibration one min.

from those involved in normal membrane excitation. If ACh does act in such a peculiar fashion when external ions are lacking, it will not be possible to correlate the mechanical responses to ACh challenge with the composition of the extracellular fluid. A pharmacological comparison of sites of action of ACh was made by studying the dose-response curves and the effects of curare and of potentiating agents on paired muscles in sucrose and Ringer's. The minimum ACh concentration adequate to elicit relaxation of the muscle in sucrose was between 0.5 and 1 μg/ml, while the muscles in Ringer's had thresholds between 0.1 and 0.5 μg/ml. Both contraction and relaxation increased in amplitude as the ACh concentration was increased to 50 μg/ml. In sucrose, challenges at this concentration tend to have a deleterious effect on subsequent responses, so it is difficult to plot the curve past this point. The dose-response curves obtained for a typical pair of muscles are shown in Figure 2.

Agents known to block or potentiate ACh were studied on paired muscles in sucrose and Ringer's in the following way: the dose-response curves to ACh were determined, the blocking or potentiating drug was added, and the response to different concentrations of ACh was measured again in both solutions. By comparing the records, the dose of ACh required to produce the same response before and after the addition of the drug was calculated. The potentiating drug used was 3-hydroxyphenyldiethylmethylammonium;[8] this drug made ACh two to three times more effective in both Ringer's and sucrose. Physostigmine also appeared to potentiate ACh in both solutions. d-Tubocurarine so reduced the effectiveness of

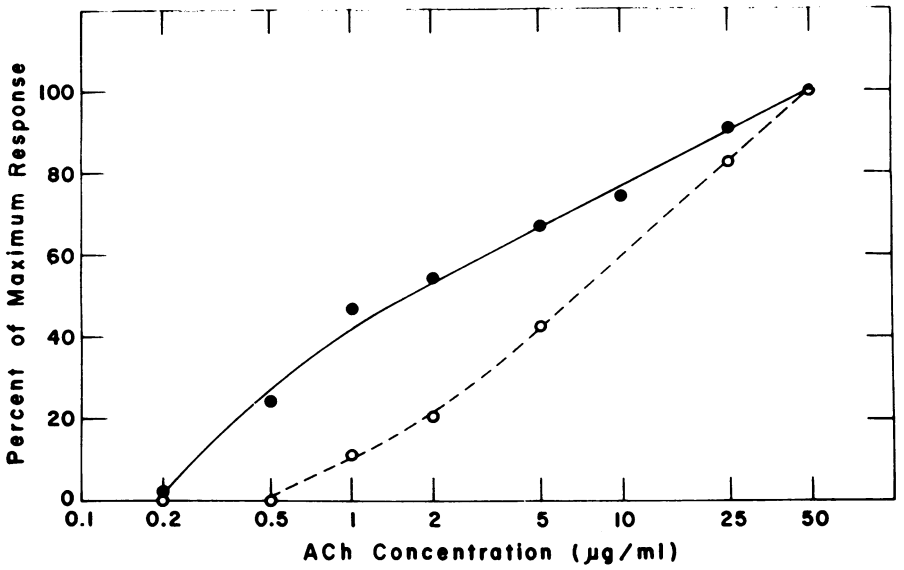


FIG. 2.—The ACh dose-response curves for a single pair of muscles in sucrose (open circles) and Ringer's (closed circles). The values plotted for the muscle in sucrose are the percentage of the maximum *relaxation* amplitude, while the responses of the Ringer's muscle are plotted as percentage of the maximum contraction.

ACh in sucrose that 25 to 50 times as much was needed to produce a given relaxation; in Ringer's the same treatment raised the ACh requirement only 1.5 to 4 times. It seems that d-tubocurarine is more effectively bound to its receptor on the muscle in solutions of low ionic strength, as would be predicted by the Debye-Hückel-Onsager theory of electrolytes.[9] The results with blocking and potentiating drugs suggest that ACh is acting on the same membrane receptors regardless of whether contraction or relaxation is elicited.

The "relaxation response" in sucrose is not limited to the rectus. The iliofibularis, known to contain many tonic fibers, also relaxes upon ACh challenge in sucrose, although the amplitude of response is smaller. The sartorius, studied because tonic fibers are almost entirely absent from it, has not shown the relaxation response. This may reflect, simply, the low density of end-plates in a "twitch" muscle.

The "relaxation response" of the rectus is obtained after soaking the muscle in isotonic glucose or in $\alpha$-methyl d-glucoside, although the amplitude is much less in glucose than it is in sucrose. This may explain why Vanremoortere[10] did not report it.

Muscles soaked in sucrose for 48 hr still relax upon ACh challenge—when Ringer's is introduced into the muscle chamber, contractions can then be elicited.

2. *The return of ions to the extracellular fluid:* In this series of experiments, muscles were challenged in Ringer's, then soaked in sucrose for 5 or 6 hr. During the last hr of the sucrose soak, the muscle was held under tension at room temperature. The sucrose was then replaced with a solution containing either NaCl, KCl, or $CaCl_2$ and within 4 min the muscle was challenged with ACh made up in the same solution. When several concentrations of a salt were tested on the same

muscle, 15 min of soaking in sucrose separated the tests. Control challenges in the sucrose always gave a "relaxation response."

a. *Potassium:* Following a 5-hr soak in sucrose, the introduction of 3, 20, 60, or 120 mM KCl always elicited a rapid contraction. When the muscle had relaxed to a stable tension, ACh challenge brought about either no mechanical response or, in a few instances, a slow, gradual relaxation.

b. *Calcium:* The addition of a solution containing 3, 20, 60, or 80 mM of $CaCl_2$ caused a rapid relaxation by the rectus, the tension decreasing by as much as 2.5 gm. In 3 or 20 mM $CaCl_2$, ACh challenge gave either a full relaxation response, a diminished relaxation, or, in a few instances, a slight contraction. With 60 or 80 mM $CaCl_2$ present, a slight contraction was always seen. The amplitude of the contraction never exceeded 0.3 gm and was never more than 10 per cent of the tension produced by the same muscle in Ringer's.

c. *Sodium:* The addition of a solution containing 2, 5, 10, or 125 mM NaCl almost always caused a slight relaxation; after one min the muscle tension was stable. With 2 mM NaCl present in the chamber all of the muscles relaxed slightly to ACh challenge, while with 5, 10, or 125 mM NaCl present definite contractions were always elicited. Isotonic $Na_2SO_4$ was also effective in restoring contractility. After 1 min in 125 mM NaCl the contractions averaged 40 per cent of the initial Ringer's response, in 10 mM NaCl the average was 17 per cent, and in 5 mM NaCl the average was 14 per cent. Apparently the contraction is graded according to the concentration of sodium in the external medium.

Since NaCl alone was so effective in restoring the contraction of the rectus, several muscles were soaked overnight in isotonic NaCl to determine how long the capacity to contract was maintained with only $Na^+$ and $Cl^-$ in the bathing fluid. After the muscles were challenged in NaCl, the solution was replaced by Ringer's and a second ACh contraction was elicited for comparison. Muscles soaked 16 to 19 hr in isotonic NaCl gave responses which averaged 51 per cent of the amplitude of the same muscles responding in Ringer's; the contractions in NaCl ranged from 1.5 to 6.1 gm. After 23 to 25 hr in isotonic NaCl, the response of 7 muscles averaged 17 per cent of the response subsequently obtained in Ringer's. It is clear that the ability to contract is retained in NaCl solutions, but that there is a slow decrease with time in the amplitude of the response which can be obtained.

*Discussion.*—The 'relaxation response' of the rectus to ACh in ion-free solutions is probably 'physiological' since it is rapidly reversed by restoration of a more normal external medium. ACh is as effective in stimulating the muscle in isotonic sucrose as it is in Ringer's solution. The mechanical response to ACh challenge in sucrose shows that ACh excites the membrane even when ions are absent from the extracellular fluid. In addition, the fact that the mechanical response is reversed rather than abolished by the removal of external ions suggests that these ions have a rather specific role in the activation of muscle contraction, a proposal often made and still not confirmed. It is intriguing to conjecture that the 'relaxation response' reflects the operation of the excitation-contraction coupling in a direction opposite to the customary one. If this is true, ideas about the relation of physical and chemical events to the mechanical response of muscle fibers can be put to a simple test in the rectus preparation with ACh stimulation, a system not subject to extreme alterations in excitability and independent of action potential

conduction. If, for example, the movement of a specific ion in a certain direction is thought to be necessary for physiological contraction, it should be possible to show that this ion moves in the opposite direction during ACh-sucrose relaxation.

One event common to the activation of all muscle fibers is a momentary increase in membrane permeability, either a selective increase during the action potential in twitch and cardiac fibers,[11] or a relatively non-specific increase in permeability during the period of ACh action at the end-plate of twitch[12] and tonic fibers.[13] When this permeability increase occurs, ions such as $Na^+$ and $Ca^{++}$ which have been restrained are now free to flow inward; it might be thought that one of these ions is the essential "trigger" for contraction. In the rectus-ACh system, it might seem that "trigger ion" is $Na^+$ since the muscle contracts in response to ACh with only sodium salts added in the extracellular fluid. However, external $Na^+$ is *not* needed for contraction in all instances; a rectus soaked for hours in sucrose will contract when KCl is introduced into the muscle chamber (ref. 10, Par. 2*a*).

Extracellular $Ca^{++}$ has been shown to be necessary for conduction in frog nerve[4] and also for the contraction of some muscles under certain conditions.[1, 14] It is important to emphasize that $Ca^{++}$ need not be added to the external medium in the simple rectus-ACh system. This observation has been made before.[15] However, it cannot be emphatically stated that extracellular $Ca^{++}$ is unimportant, because some $Ca^{++}$ must be contributed to the extracellular fluid by diffusion from the muscle, and only 0.01 mM of external $Ca^{++}$ is sufficient to maintain conduction in an isolated single nerve fiber.[4]

The sodium ion appears to have a specific role in the stimulation of tonic fibers by ACh. This role can conveniently be described in terms of the gradient of sodium concentration across the membrane. Normally the relation of $(Na^+)_o$ to $(Na^+)_i$ is such that sodium flows in during membrane excitation. It is proposed that the sodium influx drives an event in the membrane *in a specific direction*, and that this leads to contraction. When the $(Na^+)_o$ is very low, the increase in membrane permeability during ACh stimulation leads to a flow of Na *outwards*; the hypothetical membrane event is driven in the opposite direction, and the muscle rapidly *relaxes*.

It is possible that the role of the sodium ion in this system is simply to change the potential on the membrane: inflow causing depolarization and outflow hyperpolarization. The possibility that membrane depolarization is *always* correlated with contraction and hyperpolarization with relaxation *in this system* is being investigated by intracellular electrical recording.

In general, it is noteworthy how long the frog rectus abdominis can be maintained in isotonic ion-free solutions or in solutions of peculiar composition and still respond to appropriate stimulation. It reminds us again that the components of the medium Ringer devised to support the rhythmic beat of the heart are not important in the same way in *sustaining* the responsiveness and contractility of an inactive muscle. This distinction may be of general importance in understanding the role of the physiological ions in the functioning of normal excitable cells.

*Summary.*—1. In ion-free, isotonic solutions, the frog rectus abdominis and other 'tonic' muscles undergo a slow contracture. When the muscles are then stimulated with acetylcholine (ACh), they exhibit a rapid relaxation in which the tension decreases by as much as two grams.

2. The relaxation response, like contraction, is blocked by tubocurarine and increased by ACh-potentiating drugs.

3. Returning $Na^+$ to the external fluid immediately restores the capacity of the rectus to contract to ACh. None of the other cations or anions of Ringer's solution in physiological concentrations restores this contraction. However, muscles will contract to KCl stimulation when $Na^+$ is not present in the extracellular fluid.

4. If the relaxation response of the rectus in ion-free solutions is the reverse of normal contraction, ideas about the relation between chemical and physical events and muscle contraction can be easily tested in this simple experimental preparation.

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