doses of mersalyl into rats, renal ATP-ase activity was unchanged in 4 hours; it was decreased after 24 hours. 3. Simultaneous microscopic and enzymatic studies suggest that the decrease in ATP-ase activity is a result and not a cause of renal injury produced by mersalyl.

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Acetylcholine Content of Tyrode Solution Perfused Through Muscles as Affected by Calcium and Procaine Hydrochloride.* (17847)

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There appear to be many similarities between muscle fibrillation caused by denervation and fibrillation produced by calcium lack(1,2,3,4,5). These experiments were performed in order to examine the possible rôle of acetylcholine in fibrillating dog muscle caused by these two conditions.

Method. A week to 10 days before an experiment a dog's left sciatic nerve was severed aseptically. On the morning of the experiment the animal was lightly anesthetized with pentothal sodium and prepared for cannulation of the right iliac artery and vein. The following solutions were in readiness: mammalian Tyrode solution containing sufficient heparin to provide the animal with a total of 10 mg/kg; Tyrode solution containing eserine in a concentration of 1:100,000. Two Ca^{++} free Tyrode solutions were also prepared and to one of them was added 1 millimol of procaine hydrochloride per liter. Provision was made for warming these solutions to body temperature by passage through a glass coil immersed in a tank maintained at about 40°C. Electromyograms were obtained from wire recordings made continuously throughout the experiment. We have recently described this technic and full details are readily available in another journal(6). The method is recommended as the wire recorder permits reproduction of the electrical activity of the muscle for photography at a later time when the experimenter is not preoccupied with operative and other technics. The standard procedure was as follows: the right sciatic nerve was cut, a concentric needle was inserted into the right gastrocnemius, and with the amplifier, wire recorder and loud speaker turned on, the right iliac artery was perfused with Tyrode solution containing heparin. Usually no electrical activity from the muscle was heard on the speaker. The iliac vein was then cannulated and the sanguinous perfusate collected. The muscle was then perfused with Tyrode solution containing eserine 1:100,000, calcium free Tyrode solution and lastly, calcium free Tyrode solution containing procaine hydrochloride 1 millimol per liter. Samples of perfusate were collected in separate flasks, additional eserine added, and the perfusates stored in the ice box for further use. A complete recording of the electrical activity of the perfused muscles was obtained. The whole procedure was then repeated on the denervated side and again separate eserinized samples of perfusate were

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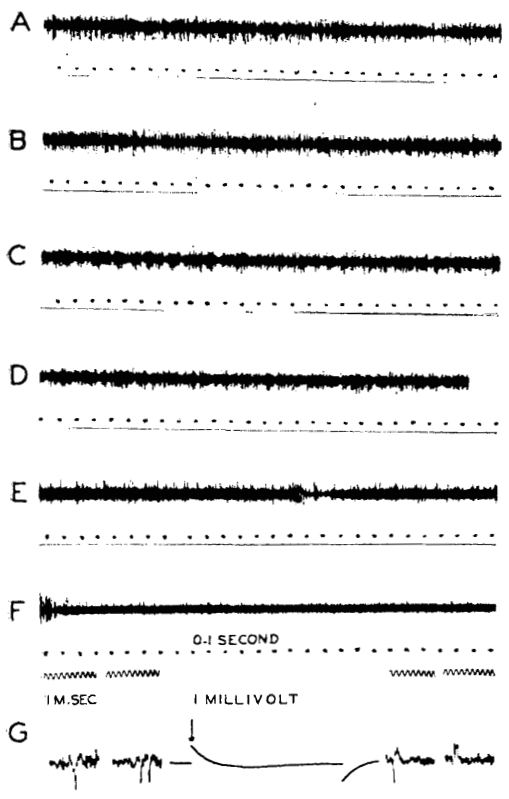

FIG. 1.

collected and stored.

Experimental results. The wire recording of muscle activity was fed into a suitable amplifier connected to a cathode ray oscilloscope and photographed on moving bromide paper. Fig. 1 contains representative samples of the recordings obtained from one of the 4 satisfactory experiments completed without complications. It will be seen from examination of the upper 4 records (A,B,C, D) that the electrical activity of the denervated muscle remained virtually constant throughout the experiment. However, long continued perfusion of a denervated muscle with Ca^{++} free Tyrode solution containing procaine hydrochloride usually caused a gradual decrease in electrical activity (higher concentrations of procaine hydrochloride, 10 millimolar, may interrupt the fibrillation relatively quickly). In contrast it was found that the electrical activity of the innervated muscle aroused by perfusion with Ca^{++} free Tyrode solution (record E) promptly ceased when 1 millimolar procaine hydrochloride was added to the perfusing fluid (record F). The last record (G) shows single spikes from denervated muscle (left) and single spikes from innervated muscle while being perfused with Ca^{++} free Tyrode solution (right).

The acetylcholine content of the perfusates. The perfusates were rendered protein free by treatment with trichloroacetic acid; after filtration they were neutralized with $NaHCO_3$. The protein free filtrates were divided into two portions. One of each pair was rendered alkaline (pH 8-8.5), briefly heated, cooled and neutralized with CO_2. Finally all filtrates including those hydrolyzed by the above procedure were tested for ACh using the inhibition of the heart of *Venus mercenaria* as an indicator. Records from 4 clam hearts are presented in Fig. 2. The upper row consists of cardiograms obtained from a series of controls in which Tyrode solution, Tyrode solution treated with trichloroacetic acid and then neutralized, Ca^{++} free Tyrode solution and Ca^{++} free Tyrode solution containing 3 millimols of procaine hydrochloride (3 times the concentration used for muscle perfusion) were added successively in quantities of one milliliter to the sea water at A,B,C, and D respectively. The heart was not washed between these 4 tests. The initial bath volume was 35 cc. At E, 1 cc of sea water containing 0.1 γ of ACh bromide was added. Between F and G 0.1 γ of ACh in 1 cc of sea water containing 3 millimols of procaine hydrochloride was gradually added and after washing with sea water, 0.1 γ of ACh in 1 cc of sea water was added at H. It will be seen that although the amplitude of the contractions gradually declines, the effect of a final concentration of 0.0025 γ of ACh per cc of sea water was plainly visible. The first record in the second row was obtained from another heart in which 1 cc of protein free Tyrode solution, obtained after perfusion through an intact muscle *at rest* was added to the bath at I. Note the increase in amplitude of the heart contraction (see discussion below). The record at J was obtained after the addition to

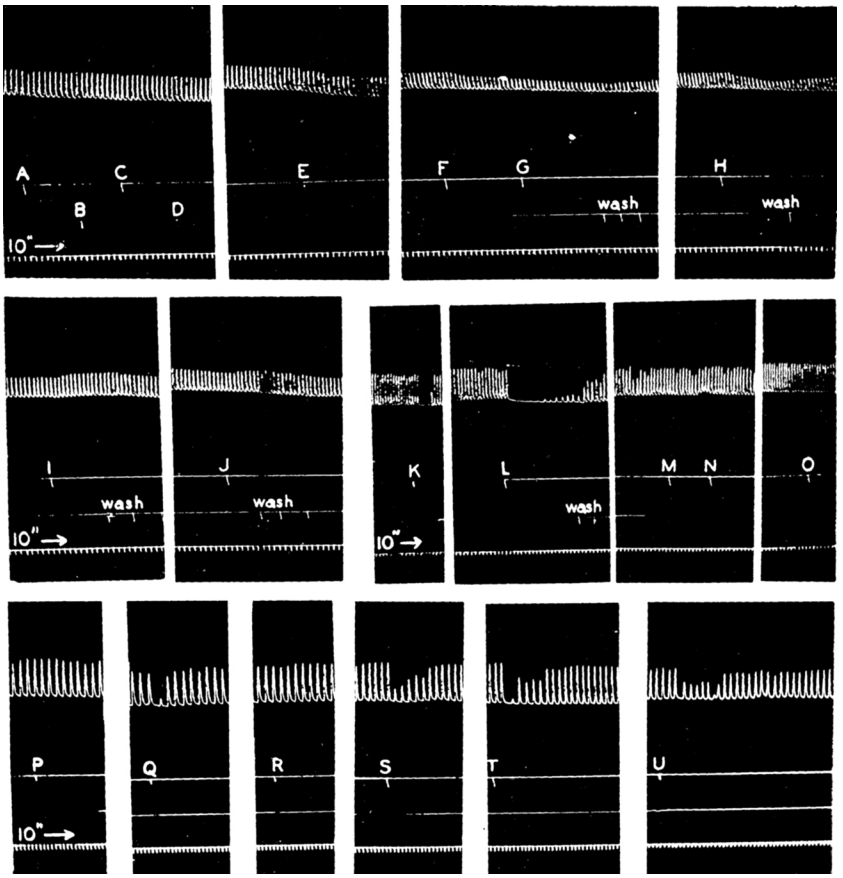

FIG. 2.

the water bath of 1 cc of *hydrolyzed* Ca^{++} free filtrate, collected during intense muscle-activity. (It will be noted that again the amplitude of contraction was slightly augmented, see discussion.) The remaining records on the second row were obtained from a third clam heart and show (K) the inhibition following the addition of 1 cc of Ca^{++} free Tyrode solution containing 1 γ of ACh. At L is shown the effect of 1 cc of Ca^{++} free Tyrode solution after perfusion through an active innervated muscle. The next record shows the effect obtained when filtrate prepared from Ca^{++} free perfusate containing procaine hydrochloride, and obtained during the cessation of muscle activity, was gradually added to the bath between M and N. In the next record, at O, a similar filtrate but obtained after muscle activity had ceased was added to the bath. Note the absence of cardiac inhibition. The remaining records (bottom row) were obtained respectively from: the addition of 1 cc of Tyrode solution containing a large excess of K^{+}, (3 times the normal content) to the bath (P); the addition of 1 cc of Tyrode solution containing a microgram of ACh (Q). R shows the absence of inhibition obtained from a *hydrolyzed* protein free perfusate obtained during active fibrillation of a denervated muscle; (compare this record with J). The remaining 3 records were obtained from solutions perfused through the same denervated fibrillating muscle. S, shows the presence of a cardio-inhibitor in Tyrode solution, T, the continued presence of this substance in a Ca^{++} free Tyrode perfusate and U, the continued presence of this cardio-

inhibitor in a Tyrode solution containing 1 millimol of procaine hydrochloride.

Discussion. The high degree of specificity of *Venus mercenaria* hearts for ACh has been established by many(7,8,9,10,11,12,13). The nature of the substance frequently seen in hydrolyzed perfusates from muscles, which causes the augmentation of cardiac amplitude, seen in Fig. 2 at I,J and to a less extent at R, has not been determined. It occurs in protein free filtrates prepared by trichloroacetic acid preparation and survives heating at pH 8. The possibility of the presence of ATP in the perfusates, so prepared, cannot be excluded. Our experiments reveal that ACh appears in the eserinized perfusates from muscles undergoing spontaneous fibrillation induced by a deficiency of calcium or as a consequence of denervation. They also show that procaine arrests spontaneous muscle activity due to calcium lack in the innervated preparation, but has no immediate effect upon the spontaneous activity of denervated muscle.

It might be thought that procaine arrests spontaneous activity caused by calcium lack by simply blocking ACh access to muscle effectors. This conclusion is, however, erroneous because the cessation of spontaneous activity occurs within a few seconds and at that time the muscle will respond to indirect excitation, and the failure of procaine to arrest this spontaneous fibrillation of the denervated muscle appears to preclude the possibility of block. Furthermore the work of Jaco and Wood(14) clearly demonstrated that the intra-arterial injection of procaine (in comparable concentrations to those used by us) did not interrupt indirect excitation. These workers found that when a muscle was indirectly stimulated by maximal spike shocks applied to the nerve the intra-arterial administration of prostigmine greatly increased the amplitude of the muscle contraction; however, when procaine was then injected intra-arterially the normal muscle response to indirect stimulation promptly returned and continued without further decrement. They also found block of indirect excitability of the muscle induced by the intra-arterial injection of 25 γ of ACh was prevented by the intra-arterial injection of cocaine. They point out that curarine, unlike procaine, fails to antagonize prostigmine. Hence the actions of procaine and curarine at the neuro-muscular junction differ: procaine depresses the production of acetylcholine at the neuromuscular junction; curarine, on the other hand, blocks acetylcholine. Consequently the failure of indirect excitation caused by procaine reported by Harvey(15) is brought about by separate mechanisms. It is believed that the observations reported here and those of others all are capable of explanation by the following hypothesis. Under normal conditions acetylcholine production at the neuromuscular junction results as a consequence of depolarization of the membrane at the termination of the motor axon and the destruction of acetylcholine is associated with the repolarization of the same structure. Any acetylcholine that "escapes" from the immediate vicinity of the motor-nerve endings is destroyed by the cholinesterase associated with the "palisade region" described by Couteaux(16) and possibly by the cholinesterase of the R.B. cells(17). Under these conditions repetitive responses of muscle to a single nerve stimulus are impossible, neither can spontaneous

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muscle activity occur. In the presence of eserine or prostigmine any ACh that escapes destruction as a consequence of repolarization of the axon membrane accumulates and results in: (A) a larger muscle response to single indirect stimuli as described by Jaco and Wood (see above), (B) repetitive muscle responses as found by Eccles, Katz and Kuffler(18) and (C) neuromuscular block as described by Briscoe(19). These three phenomena can be duplicated by the intra-arterial injection of increasing concentrations of ACh. Jaco and Wood (see above) showed that ACh block was inhibited by cocaine and this effect is readily understandable when it is remembered that cocaine inhibits the depolarization of nerve(20) and hence not only diminishes the production but also augments the rate of destruction of ACh. The cause of spontaneous muscle activity in normal preparations exposed to calcium free solutions is, according to this hypothesis, the result of the accumulation of ACh as a consequence of partial depolarization of the motor axon terminal membrane resulting from the withdrawal of calcium ions. Direct measurements of the resting end-plate potential are difficult; a decrease in resting potential of nerve upon exposure to calcium free solutions has been observed by us. Kuffler (l.c.) also has reported negative resting potentials developing in muscles immersed in calcium free solutions and augmentation of muscle responses to single indirect stimuli, with the appearance of repetitive muscle responses and finally block. This is the same sequence of events which occurs when increasing concentrations of ACh are applied to the neuromuscular junction(21). The cholinesterase content of denervated muscle is not markedly lower than that of normal muscle(22) hence the increase in sensitivity of denervated muscle to ACh is not explainable on the basis of a lack of this enzyme, and some other explanation is required. It is believed that the hypothesis presented fills this need. In conclusion it should be pointed out, that with larger concentrations, or more prolonged exposure to 1 millimolar procaine, there may be direct effects upon muscles; and prolonged exposure to low calcium concentrations may also affect the muscles; under these abnormal conditions the hypothesis would not apply.

Summary. 1. Dogs' denervated fibrillating muscles when perfused with Tyrode solution, Ca^{++} free Tyrode solution, or Ca^{++} free Tyrode solution containing 1 millimol of procaine hydrochloride per liter, continue to fibrillate and the perfusate from such muscles contains a heat labile material capable of inhibiting the heart of *Venus mercenaria in vitro*.

2. Intact muscle perfused with Tyrode solution does not usually exhibit electrical activity and the perfusate from such inactive muscles does not inhibit the heart of *Venus mercenaria*.

3. Innervated muscle perfused with eserinized Ca^{++} free Tyrode solution displays electrical activity similar to the fibrillation of denervation and the perfusate from such muscles contains a heat labile material capable of inhibiting the heart of *Venus mercenaria in vitro*.

4. Perfusates subjected to alkaline hydrolysis obtained from denervated fibrillating muscle, or from innervated muscle rendered active by perfusion with Ca^{++} free solutions, do not contain a cardio-inhibitor substance when tested for such an inhibitor on the heart of *Venus mercenaria in vitro;* these solutions often augment the contractions of the isolated clam heart.

5. Electrical activity of innervated muscle perfused with Ca^{++} free Tyrode solution ceases when procaine hydrochloride is added to the perfusing fluid and the perfusate from the muscle then contains no heat labile cardio-inhibiting substance.

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6. A new hypothesis is discussed in which it is postulated that motor nerve endings at rest are in part responsible for the removal of acetylcholine at the neuromuscular junction.

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"Spontaneous" Ovulation in the Rabbit Following Combined Estrogen-Progesterone Treatment. (17848)

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Unlike the human and a large majority of other mammals, the rabbit seldom, if ever, ovulates spontaneously; normally ovulation occurs only after neurogenic stimulation of the hypophysis attendant on coitus. Estrogen has been reported to facilitate the release of ovulating hormone from the rabbit hypophysis in response to copper salts(1), to coitus during pseudopregnancy(2), and to mechanical stimulation of the vagina(3), but progesterone has always been considered inhibitory to ovulation in this species(4,5). In such widely unrelated spontaneously-ovulating forms as the rat(6,7,8) the hen(9) and probably the monkey(10), however, ovulation has been stimulated by the appropriately timed injection of progesterone. Everett(11) has suggested that once the critical factor of timing is understood, progesterone facilitation of LH release may be disclosed in other species. We have recently found that progesterone administration, independently of extrinsic estrogen, conditions the estrous rabbit in such a way that mechanical stimulation of the vagina induces ovulation(12). The present work reveals that, in a significant number of rabbits, combined treatment with estrogen and progesterone dispenses with the requirement of a coital stimulus and allows ovulation to occur "spontaneously."

This preliminary study employed 30 mature female rabbits, all apparently in estrus as judged by hyperemia of the vaginal orifice. To avoid the possibility of mechanical stimulation the vagina was not examined after the first hormone injection. The estrogen used was α-estradiol benzoate.* The rabbits were injected subcutaneously on 2 consecutive mornings with doses of 85 μg estrogen in 0.1 ml peanut oil and/or 2 mg progesterone in 0.2 ml peanut oil. In the animals receiving both hormones the progesterone was administered within 4 hours after the second estrogen injection. The ovaries were examined for ruptured follicles at laparotomy about 48 hours after the last injection.

The results are summarized in Table I. It is apparent that whereas neither estrogen nor progesterone alone was followed by ovulation, 4 out of 10 rabbits ovulated after the combined treatment.

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