not useful for distinguishing between short regions of base-pairing and double-stranded RNA of high molecular weight (*2*).

Using serologically specific electron microscopy (*3*), I have been able to assay extracts of virus-infected tissue for double-stranded RNA, without prior purification or the use of phenol or detergent. Electron microscope grids with Parlodion-carbon films were floated on dilute antiserum to double-stranded RNA (*4*) for 30 minutes. During this time a layer of serum proteins was adsorbed to the film. Excess serum was removed by washing, and the grids were placed on tissue extracts or RNA preparations for 2 hours. If double-stranded RNA was present, it was specifically attached to the grid by reacting serologically with the adsorbed antiserum. The other components in the extract were removed by washing, and the double-stranded RNA was stained and rotary-shadowed (*5*) for examination with the electron microscope. Double-stranded RNA was readily detected in extracts of tobacco infected with tobacco mosaic virus (TMV) and, for comparison, in a solution of synthetic double-stranded RNA (Fig. 1). Double-stranded RNA was not found in control experiments using normal serum or antiserums to several different plant viruses and infected tobacco plants, or antiserum to double-stranded RNA and mock-inoculated or healthy tobacco plants.

Measurements of the molecules on several negatives, similar to the one used for Fig. 1 (left), indicated a number of long molecules, 1.5 to 1.7 μm in length, and some that were considerably shorter (Fig. 2). This variation in length is consistent with observations made on double-stranded RNA isolated from TMV-infected tissue (*6*). The long molecules compare favorably with the calculated length of 1.67 μm for the double-stranded form of the complete genome of TMV (*7*).

Antiserums produced to synthetic double-stranded RNA have been shown to react to a limited extent with DNA-RNA hybrids (*8*). The antiserum to double-stranded RNA used in this study does not react with ribosomal RNA, single-stranded RNA, or DNA (*4*); it has not been tested with DNA-RNA hybrids. There are several reasons for believing that the filamentous molecules observed in extracts of tobacco leaves inoculated with TMV are indeed double-stranded RNA associated with virus replication and not DNA-RNA hybrids: (i) The molecules are not observed in extracts of healthy or mock-inoculated tobacco leaves. (ii) They are present in relatively high concentrations a few days after inoculation with TMV, which is a time of rapid virus synthesis. They are present in low concentrations in older infections of systematically infected leaves where the rate of virus synthesis is low. (iii) The size of the molecules is as expected for double-stranded RNA associated with TMV replication.

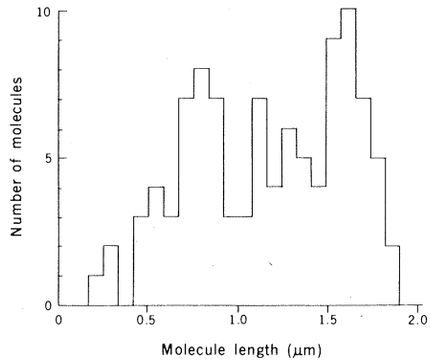

Fig. 2. Length distributions of double-stranded RNA from TMV-infected tobacco leaf. Electron microscope negatives were projected, and 100 linear molecules without overlaps of branching were selected at random and measured with a map measurer.

This technique appears to have several potential applications. In addition to demonstrating the existence of native double-stranded RNA, it may be useful in determining the concentration (*9*) and size of double-stranded forms as they occur in time-course experiments, and in studying infectious agents of unknown etiology that are suspected of being RNA viruses.

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9. The technique has been used to make quantitative assays of plant viruses [K. Derrick, *Virology* **56**, 652 (1973)].
10. Assays for double-stranded RNA can be done with extracts prepared with tris buffer. The addition of sucrose and NaCl decreases the amount of debris on the grids.

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Sodium Requirement for the Positive Inotropic Action of Isoproterenol on Guinea Pig Atria

Abstract. *Isoproterenol does not elicit its characteristic positive inotropic action in contracting guinea pig atria suspended in sodium-free media. However, the ability of isoproterenol to decrease the time to peak tension development during an individual contraction cycle is still present in sodium-free solutions. Removal of sodium diminished but did not eliminate the tissues' ability to elevate adenosine 3',5'-monophosphate in response to isoproterenol. The striking absence of an inotropic action by isoproterenol on atria in sodium-free media suggests that sodium (and possibly a sodium-calcium exchange across the sarcolemma) plays an important role in the inotropic action of catecholamines.*

The inotropic effects of isoproterenol and other beta-adrenergic agonists appear to be mediated by adenosine 3',5'-monophosphate (cyclic AMP) (*1*). Several aspects of cardiac calcium metabolism are affected by cyclic AMP. Isoproterenol, dibutyryl cyclic AMP, and the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (MIX) influence the cardiac action potential in a manner which suggests that these agents increase the rate of transmembrane calcium influx during excitation (*2*). Cyclic AMP also enhances the phosphorylation of isolated cardiac sarcoplasmic reticulum (*3*) associated with stimulation of calcium-activated adenosinetriphosphatase activity and enhanced calcium uptake (*4*). Isoproterenol characteristically increases tension development and decreases time to peak tension. We now report that in sodium-free solutions, guinea pig atria respond to isoproterenol or MIX with a decrease in developed ten-

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sion. Time to peak tension is decreased in a normal manner, suggesting a loss in the ability of these agents to enhance calcium movement across the sarcolemma while their ability to influence calcium uptake into the sarcoplasmic reticulum remains.

Guinea pig left atria were removed from male animals which had been injected intraperitoneally with 5 mg of reserpine per kilogram of body weight 16 to 20 hours before they were killed. The atria were equilibrated in normal-sodium media maintained at 32°C and paced through platinum point electrodes with 1-volt monophasic pulses. Diastolic tension was 0.5 g and contractions were continuously monitored with a network of interconnected devices consisting of an oscillographic recorder, a memory oscilloscope, and a PDP-11 computer system. Cyclic AMP was measured by radioimmunoassay (*5*) after acetylation of the samples (*6*).

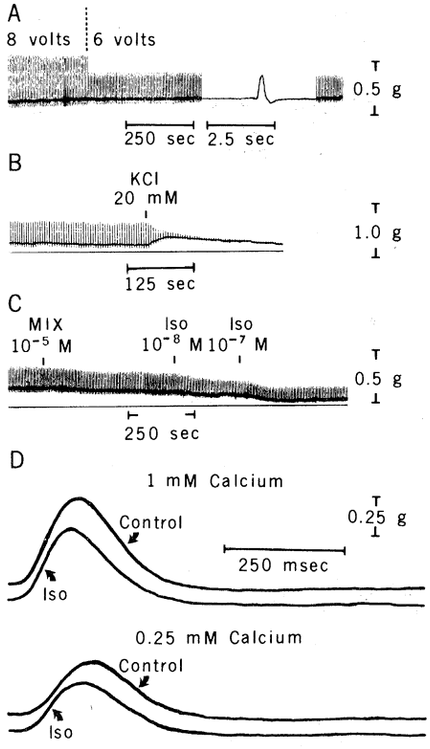

Fig. 1. Records of contractions from guinea pig atria produced in the absence of sodium. Before transfer to sodium-free medium, atria were superfused for at least 1 hour in buffer containing (in millimoles per liter) NaCl, 118; KCl, 4.74; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.0; and glucose, 5.0; maintained at 32°C; and oxygenated with 95 percent O_2 and 5 percent CO_2, *p*H 7.4. The sodium-free medium was identical except that choline salts were substituted for sodium salts and 1 μM atropine was added. Atria were paced at 0.2 hertz. (A) Effect of a reduction in voltage to cause an immediate reduction in tension. The high-speed tracing displays a typical beat and afteroscillation. (B) Cessation of contractions and transient rise in diastolic tension on addition of 20 mM KCl. (C) Response of a typical atrium to MIX and l-isoproterenol (*Iso*). There is a dose-dependent decrease in developed tension and diastolic tension. (D) Oscilloscope tracings of single contractions from a typical atrium paced with 10 volts. Twitch tension decreased in response to a reduction in calcium from 1 to 0.25 mM while diastolic tension was unaltered. Isoproterenol (1 μM) caused a decrease in twitch tension, a decrease in time to peak tension, and a reduction in diastolic tension at both calcium concentrations.

On transfer to sodium-free media (with sodium replaced by choline or sucrose) there was a progressive increase in developed tension for 2 to 3 minutes followed by asystole. The atria, which were washed in sodium-free medium every 5 minutes for 30 minutes, developed contractures which peaked in 10 to 15 minutes before subsiding slightly to reach a plateau tension of 1.01 ± 0.04 g (mean ± standard error of the mean) in 20 to 25 minutes. Unlike contractures produced by elevated potassium solutions, contractures caused by the absence of sodium are not accompanied by a change in the membrane resting potential (*7*). Twitches could be restored by raising the stimulating voltage. The resulting contractions were voltage-dependent, peaking at 20 to 25 volts, and usually displayed after oscillations (Fig. 1A). Atria stimulated to contract with 10 volts at 0.2 hertz in sodium-free medium containing 1 mM calcium had twitch tensions one to two times those observed in normal-sodium solutions. The twitches did not display a frequency-force relationship at frequencies between 0.02 and 1 hertz, and tension declined slowly with time as a function of the stimulation frequency. For example, at 0.2 hertz a decline of 1 percent per minute was observed. Changing from a normal-sodium to a sodium-free medium increased the time to peak tension from 103 ± 3 to 179 ± 5 msec and the relaxation half-time (time from peak tension to 50 percent relaxation) from 42 ± 2 to 109 ± 6 msec. Contractions qualitatively similar to these were observed in preliminary experiments with rat and rabbit atria and have been observed in sodium-free ventricular preparations from goldfish, trout, rabbits, dogs, and cows (*8*) and atrial preparations from frogs (*9*). Changes in membrane potential in voltage-clamped Purkinje fibers of sheep or calf suggest that there is a voltage-dependent slow inward current carried by calcium ions in sodium-free solutions (*10*). Evidence suggesting transsarcolemmal calcium influx in guinea pig atria in sodium-free solutions is shown in Fig. 1B. The addition of 20 mM KCl in sodium-free solutions could obliterate the contractions within several beats. Increased voltage was unable to restore contractions in the presence of elevated KCl. Potassium-depolarized tissues in the absence of sodium have been shown to lack slow inward calcium currents (*2*). We also found, in other experiments not shown here, that contractions were reduced in sodium-free solutions by lanthanum ion, which is known to interfere with transmembrane calcium fluxes (*11*). Tetrodotoxin (23 μM), which blocks fast sodium channels, did not affect the contractions in sodium-free medium.

The addition of MIX and isoproterenol to guinea pig atria in sodium-free media did not elicit a positive inotropic response in 20 of 20 preparations but decreased diastolic tension in a dose-dependent manner (Fig. 1C). Normal contractions and positive inotropic responsiveness to isoproterenol could be restored by returning the atria to normal sodium. Removal of sodium caused the atria to be more sensitive to extracellular calcium ion. Transfer from 118 mM sodium to zero sodium decreased the concentration of extracellular calcium ion

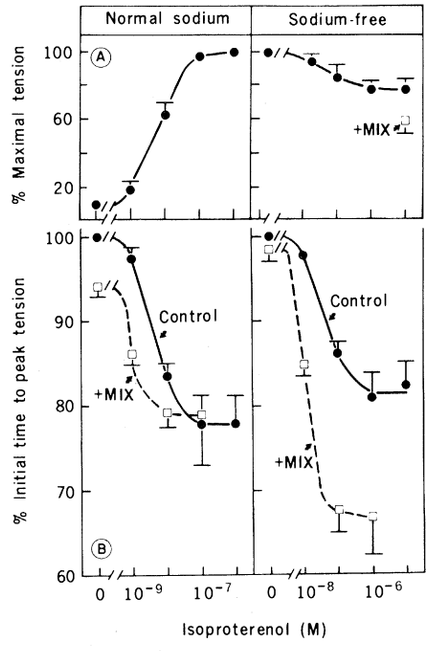

Fig. 2. Effect of cumulative doses of l-isoproterenol on peak tension development (A) and time to peak tension (B) in normal and sodium-free solutions. Solution contents are described in the legend to Fig. 1. The addition of MIX (10 μM) 10 minutes before the first dose of isoproterenol had no effect on maximal tension development in normal sodium. The KCl concentration was elevated to 9 mM to prevent automaticity in normal sodium. In the absence of sodium there was a 1.1 ± 0.1 percent decline in twitch tension per minute. This decline was subtracted from the results to obtain the data shown. Each point represents the mean ± standard error of the mean for six atria.

required for maximum tension development from 7 to 1.5 m*M*. Isoproterenol (1 μM) failed to elevate contractile tension in the absence of sodium even when the calcium concentration was reduced below 1 m*M* (Fig. 1D).

The action of isoproterenol on tension development and time to peak tension in normal and sodium-free solutions is shown in Fig. 2. In normal sodium the characteristic inotropic action and decrease in time to peak tension development was observed, while in sodium-free medium isoproterenol caused a dose-dependent decline in twitch tension. However, in sodium-free solutions isoproterenol still decreased the time to peak tension. In addition, the ED_{50} (dose of isoproterenol necessary for a 50 percent decrease in time to peak tension) increased by an order of magnitude on transferring atria from a normal to a sodium-free environment.

As shown in Fig. 2, MIX decreased the dose of isoproterenol necessary for a 50 percent effect on time to peak tension in atria suspended in either normal or sodium-free solutions. It did not alter the effect of maximal concentration of isoproterenol in normal sodium, while in the absence of sodium the efficacy of isoproterenol was enhanced by MIX. In addition, MIX increased the amount of relaxation seen with maximal amounts of isoproterenol in the absence of sodium but had no effect on the peak positive inotropic response in normal sodium. An alteration in cyclic AMP metabolism was observed in sodium-free preparations treated with isoproterenol. The ability of isoproterenol to elevate cyclic AMP was significantly reduced in sodium-free medium when compared to atria in normal sodium (Table 1). When sodium was omitted, 1 μM isoproterenol (which is a maximal dose with or without sodium) elevated cyclic AMP only 108 percent compared to 420 percent in the presence of sodium. In the presence of MIX in sodium-free medium, 1 μM isoproterenol elevated cyclic AMP only to about 10 pmole per milligram of protein. In normal sodium 1 μM isoproterenol alone elevated cyclic AMP to about 22 pmole/mg. It has been demonstrated that the maximal inotropic action of isoproterenol is correlated with about a threefold elevation of myocardial cyclic AMP (*12*). Thus the lack of an effect by MIX on the dynamics of contraction in normal sodium is consistent with the fact that isoproterenol had maximally saturated the cyclic AMP binding sites. On the other hand, the ability of MIX to alter contractions in the absence of sodium even after the addition of 1 μM isoproterenol corresponds to its ability to further raise cyclic AMP when isoproterenol alone failed to elevate cyclic AMP enough to saturate binding sites.

Table 1. Intracellular cyclic AMP concentrations of atria in normal sodium and sodium-free media (described in the legend of Fig. 1). All tissues were paced at 0.2 hertz and exposed to 1 μM 1-isoproterenol for 30 seconds or 10 μM MIX for 10 minutes before clamping between tongs precooled in liquid nitrogen. Cyclic AMP was determined in trichloroacetic acid extracts of the tissues. Each value is the mean ± standard error of the mean for four to six atria. Significance was determined by Student's *t*-test.

Experiment	Cyclic AMP (pmole/mg protein)	
	Normal sodium	Sodium-free
Control	5.1 ± 0.4	3.4 ± 0.6
1-Isoproterenol	22.0 ± 4.1*	7.1 ± 1.0*
MIX	6.4 ± 0.6	5.2 ± 0.6
1-Isoproterenol plus MIX	33.3 ± 7.1*	9.7 ± 0.6*†

*$P < .0025$ compared to control. †$P < .025$ compared to isoproterenol alone.

The effects of isoproterenol in reducing time to peak tension and the relaxant effects observed in sodium-free medium appear to be mediated by or correlated with an increase of intracellular cyclic AMP, since MIX potentiates both the mechanical response and the generation of cyclic AMP. We cannot yet determine whether the lack of an inotropic action by isoproterenol in the absence of sodium is due to diminished cyclic AMP generation or the inability of the tissue to respond to the cyclic AMP produced. It is likely that the sarcoplasmic reticulum remains sensitive to isoproterenol in the absence of sodium since catecholamine-induced decreases in time to peak tension are still observed. If it is assumed that time to peak tension is controlled by the rate at which the sarcoplasmic reticulum can pump calcium, then the site for both isoproterenol-induced positive inotropism and its requirement for sodium may reside in the sarcolemma.

Alteration in cyclic AMP metabolism in the absence of sodium may result from increased diastolic levels of calcium, which are implied by the observed increase in diastolic tension. Such a relationship between tissue levels of cyclic AMP and intracellular free calcium has been proposed to account for the inverse correlation between tension development and cyclic AMP concentration in rabbit papillary muscle (*13*). Calcium ion has been reported to influence the activities of both phosphodiesterase and adenylate cyclase (*14*).

The influence of sodium on cardiac contractility has been interpreted in terms of a carrier which exchanges one calcium for two sodium ions. The equilibrium for this process may shift to favor calcium influx during membrane depolarization, which precedes each beat (*15*). A reduction of extracellular sodium has been associated with a net gain in tissue calcium and a reduction in the rate of calcium efflux in mammalian cardiac preparations (*16*). The contracture, increase in time to peak tension, and increase in relaxation half-time caused by lack of sodium are consistent with the hypothesis that in the absence of a means for sodium-calcium exchange, the efflux of free intracellular calcium is impaired. In contracting tissues in which the sodium current is blocked by tetrodotoxin or a slight depolarization with KCl (27 m*M*), isoproterenol, MIX, and dibutyryl cyclic AMP retain their abilities to increase contractility (*2*). Thus there is a fundamental difference between preparations lacking sodium current and those which lack sodium altogether. The absence of an inotropic action by isoproterenol when sodium is removed raises the possibility that isoproterenol increases contractile tension in normal sodium by influencing the transsarcolemmal equilibrium between sodium and calcium.

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