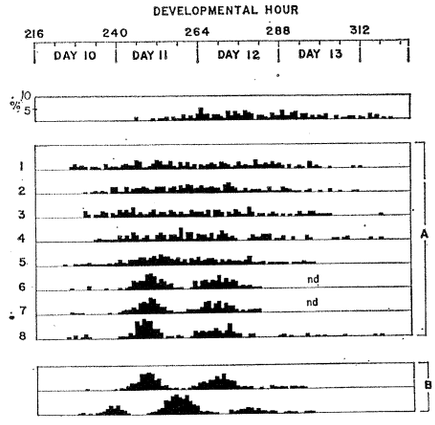

Fig. 5. (A) Effect of a single 12-hour temperature pulse (20°C/28°C/20°C) on the hatching of populations reared in continuous darkness. Numbers at left indicate day on which the pulse was given. Control (constant dark and constant 20°C) at top. (B) Distribution of hatching following a temperature pulse given on day 6 from 9 a.m. to 9 p.m. (upper), or from 9 p.m. to 9 a.m. (lower). Shading indicating constant darkness is omitted.

in *Pectinophora*, that a 12-hour temperature pulse induces rhythmicity when applied on day 6, or 7, or 8 but fails to do so on day 5 and earlier (Fig. 5A). Clearly the oscillator responsible for the ultimate timing of hatching, not its photoreceptor system, is what is lacking until the middle of the 6th day.

It seems probable to us that what remains undeveloped before day 6 is a specific element in the central nervous system which controls hatching. The absence of this specific "oscillator" before this day surely cannot imply a more generally inadequate complexity to sustain any circadian oscillation. Many protistan cells sustain them, and there is the remarkable and unique case (*6*) of circadian phase being transmitted from the mother through the egg of the Australian fruit fly *Dacus*. Kalmus (*7*) and Edwards (*8*) have reported the only other cases known to us of a circadian rhythm of hatching, but in neither case is it known that the responsible oscillation is present (that its phase can be set) before differentiation proceeds. Brett (*9*) reported that in *Drosophila* the phase of the rhythm of pupal eclosion can be set in early larval stages but not in the egg. The principal interest attaching to our observations is the potential they afford for further analysis of several correlations.

First, the fact that a circadian oscillation develops at about the same time that the pink pigment appears encourages the hypothesis that the pigment is involved in coupling the oscillation to the light cycle. That hypothesis can be tested by obtaining an action-spectrum for the oscillation's response to light, which can then be compared to the pigment's absorption.

Second, the photoperiodic induction of diapause in the fourth larval instar can be effected by treatment of the egg, a fact reported by Adkisson (*10*) and confirmed by us. Adkisson's data leave open the interpretation that such photoperiodic induction can, however, only be effected in the latter half of embryogenesis. Further data are needed before one can define the onset of photoperiodic inducibility as sharply as our data define the onset of the circadian oscillation.

Third, available data suggest that the effect of light on the rate of development, whether it be a genuine effect of light on growth rate or an indirect effect of temperature, is restricted to the latter half of development. The aperiodic distributions of hatching in cultures 1 to 10 in Fig. 3 show no clear tendency to be advanced the longer the light is on. This in itself renders implausible the hypothesis that increased temperature is involved. Among these populations there is variation in rate of development, measured by the onset of hatching or the median of the distribution. This is attributable to slight shifts in the temperature of the rearing boxes during the several months while the experiments were undertaken. Within each single experiment the agreement between populations was very close. For example, populations 1, 3, and 5 (a little slower) constituted one experiment; 7, 9, and 11 (a little faster) constituted another. It therefore remains possible, and testable, that the onset of the capacity to sustain a circadian oscillation is coincident with the onset of photoperiodic inducibility, and the onset of an effect of light, as such, on developmental rates.

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Vascular Smooth Muscle: Quantitation of Cell Thickness in the Wall of Arterioles in the Living Animal in situ

Abstract. *The wall of microarterial vessels, in the living animal* in situ, *was examined at magnification up to × 6500 on the television screen. The optical resolution af the cell components of the wall was sufficiently clear to permit image formation and splitting for permanent recording and quantitation. Thickness of single smooth muscle cells at rest was estimated to be 2.08 microns (S.D. ± 0.24 micron) and 2.78 microns (S.D. ± 0.59 micron) by two different approaches. Changes in cell thickness during activity were also recorded and quantitated.*

Control of blood flow and volume distribution by the microvessel depends primarily on the effector smooth muscle cell in the wall. Yet reliable information relative to the events of the contractile process of the muscle cell at this level of the circulation is scanty. Except for measurements of intracellular potentials in microvessels made in the living animal *in situ* (*1*), most of the data concerning the contractile process in vascular smooth muscle are derived from studies in vitro of tissue samples obtained from the larger segment of the circulatory system. The aim of the experiments reported here was to determine and measure a geometric variable in single smooth muscle cells in the wall of precapillary arterioles, both under steady state and during activity of the vessel, in the living animal *in situ*.

The experiments were carried out in arterioles of mesentery and skeletal

(cremaster) muscles of the anesthetized rat. The tissues were prepared and maintained under controlled conditions of moisture and temperature according to procedures already described (*2*). For observation and measurements, the image-splitting television microscope recording system was used (*3*). The system allows for viewing on the television screen of the formation of a double image of an object and simultaneous accurate electrographic recording of the amount of image shearing. Measurements with the image-splitting device can provide an accuracy "better by a factor of 10 or more than the resolving power of the optical system" (*4*).

Initial optical magnifications up to × 1760 and × 2400, respectively, were obtained with a Bausch and Lomb zoom microscope equipped with × 55 and × 75 water immersion lenses, a × 10 ocular, and the × 1.6 additional correction factor afforded by the prisms of the incorporated image-splitter. Further electronic magnifications (up to × 6500) achieved on the video screen were determined as follows: $n = r/\alpha$, where n is the unknown quantity of object magnification, r is the dimension of the image on the video screen, in millimeters, and α is the object size in microns, derived from image-splitting.

At lower magnification (× 3000) the image of the wall of an arteriole having a lumen 26.0 μ in diameter, when it is in the focal plane, appears to be formed by a series of "beaded" structures (Fig. 1A). At higher magnification (× 6500) each bead appears to be formed by segments of two cell bodies, separated from each other by a dense line (Fig. 1B, between arrows). The inner components of such beads correspond anatomically to the inlying endothelium, and the outer, to the media of the microvessel wall, which at this level of the circulation is known to be formed by a single layer of smooth muscle cells (*5*). The beads are elongated and tapered at their poles and have their greatest diameter perpendicular to the longitudinal axis of the vessel. In this study, in instances in which a clear-cut separation of the cells was obtained, the measurements were made by forming and splitting the image of the smooth muscle cell alone. In cases in which such cell outline is lacking, measurements were made by splitting the image of the whole wall (Fig. 1C). In the latter event,

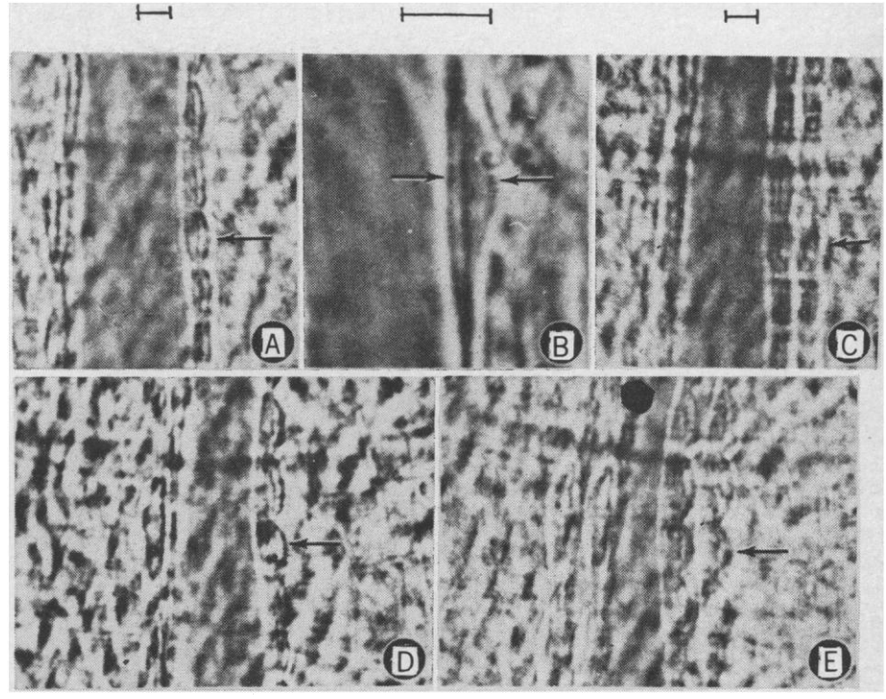

Fig. 1. Microphotograph (unretouched) of an arteriole (26.0 μ in lumen size) in the mesentery of the living rat (A) before and (D) during vasoconstriction. Split double image of the vessel wall *in toto* under both circumstances (C and E). Note that at higher image magnification each bead (B, between arrows) exhibits a dense line, parallel to the long axis of the wall, separating the bead into two components. Markers, 10 μ. (A, C, D, and E, × 3000; B, × 6500).

since the corresponding thicknesses of the segment of the endothelium and muscle cells were approximately the same, the quantitated value of the electrogram was divided by 2. Inasmuch as the exact pattern of arrangement of the muscle cell in the wall of the living microvessel was unknown and the measurements were made at the site of greatest wall thickness, shearing of the image over the object in a direction perpendicular to the long axis of the vessel measures the thickness of the cell.

The results of 44 measurements in 11 arterioles, ranging from 14.8 to 24.0 μ in lumen diameter, gave an average single muscle cell thickness of 2.08 μ (S.D. ± 0.24 μ) when the former procedure was employed. When the procedure of splitting the image of the vessel wall *in toto* was used, a value of 2.78 μ (S.D. ± 0.59 μ) for the single muscle cell thickness was derived from 68 measurements in 17 vessels, of the same type and range in lumen size.

Such numerical values (average 2.08 and 2.78 μ) obtained here for the media of arterioles (14.8 to 24.0 μ lumen size) are obviously greater than those derived (average 1.0 μ) from electron microscopical studies of longitudinal sections of arterioles of comparable size (*6*). This discrepancy is apparent rather than real and is to be expected if one bears in mind the nonuniformity of the thickness of the media (single cell–layered) of arterioles under consideration. The larger values, derived by image-splitting at the site of greatest wall thickness, represent an average of the upper limits of the media and maximal values for the thickness of the single smooth muscle cell. The lower estimates represent an average thickness of the media, including also the thinnest (tail) segments of the muscle cell as they appear in the plane of the longitudinal sections of the vessel wall.

Measurements of the changes in the thickness of the effector smooth muscle cell during several (three to four) gradients of drug-induced vasoconstriction were made possible by the method of rapid (20 to 25 times per minute) accurate splitting of the image of the vessel wall (Fig. 1, D and E). Figure 2 shows the electrographic recordings of changes in thickness of a single smooth muscle cell in the wall of an arteriole (14.8 μ in lumen diameter) undergoing active constriction in re-

sponse to the stimuli of a vasoactive drug. Topical application (0.05 ml) of norepinephrine solution, at 10-minute intervals, in increasing strengths of 0.1, 0.2, and 1.0 μg/ml, resulted in a proportionate increase in the response of the effector smooth muscle cell, in terms of both the maximum percent increase in cell thickness and the duration (Fig. 2, top) of the response.

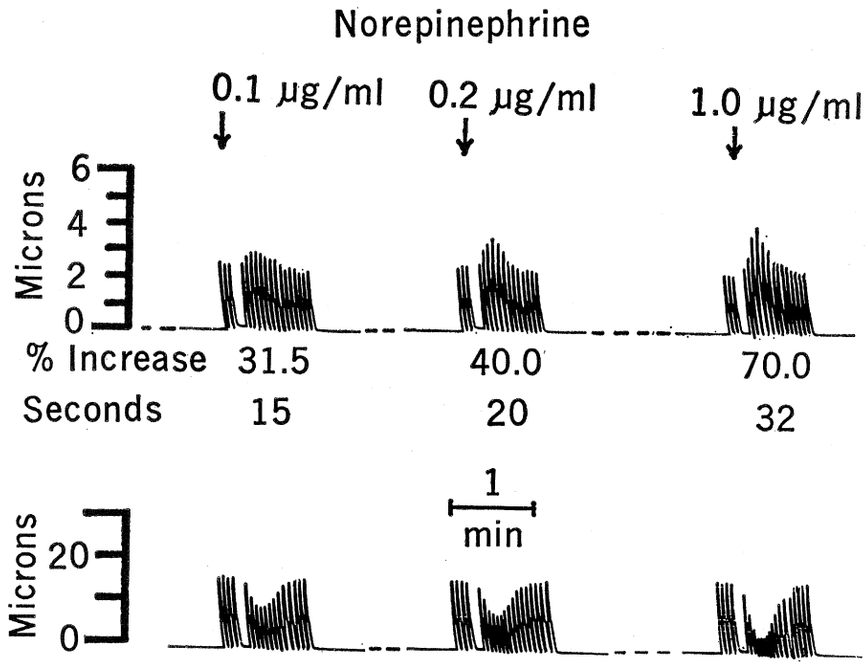

Fig. 2. Electrographic recordings of (top) the thickness (2R) of single smooth muscle cells in the wall of an arteriole and (bottom) of the vessel lumen size (2Ri), before and during the action of graded concentrations of norepinephrine. Each electrographic deflection is a value of the thickness of a single muscle cell or of the vessel lumen diameter, in microns, at the time of measurement. The gap in the electrogram (immediately after arrows) results from the time required by the observer to initiate image-splitting following topical application of the stimulating drug. (Data same as in experiment 1, Table 1.)

Table 1. Effects of graded strength of norepinephrine concentrations on a geometric variable (thickness) of single smooth muscle cells in the wall of microarterial vessels. Experiments Nos. 1 and 5 are from striated (cremaster) muscle vascular bed; Nos. 2, 3, and 4, in mesentery microvascular system. Muscle cell thickness (2R) refers to single muscle cell thickness.

Arteriole lumen (μ)	Norepinephrine (μg/ml)	Muscle cell thickness (μ)		Thickness increase (%)	Duration (seconds)
		Control	Experimental		
		Experiment 1			
14.8	0.1	1.9	2.5	31.5	15
14.8	.2	2.0	2.8	40.0	20
14.8	1.0	2.0	3.4	70.0	32
		Experiment 2			
19.0	1.0	2.0	2.8	40.0	40
19.0	2.0	2.1	3.4	61.9	48
19.0	5.0	2.1	3.9	85.7	60
19.0	10.0	2.1	4.4	109.5	68
		Experiment 3			
22.4	10.0	2.5	4.4	76.0	60
22.4	5.0	2.4	3.6	50.0	45
22.4	2.0	2.4	3.2	33.3	23
22.4	1.0	2.4	3.0	25.0	20
		Experiment 4			
16.0	1.0	2.4	2.9	20.8	17
16.0	2.0	2.4	3.1	29.1	26
16.0	5.0	2.4	3.5	45.8	40
16.0	10.0	2.3	4.6	100.0	60
		Experiment 5			
24.7	0.1	2.1	2.4	14.3	28
24.7	.2	2.0	2.7	35.0	36
24.7	1.0	2.1	3.4	61.9	60
24.7	2.0	2.1	3.8	80.9	140

The changes in the geometric parameter measured were not limited to the target cell alone. This was attested to by both the concomitant changes in thickness of neighboring smooth muscle cells, up and downstream (in the vessel wall) from the site of the one selected for the measurement (Fig. 1D), and also by the progressive stepwise decrease in the vessel's lumen diameter (2 Ri) in response to topical application of the same amount of norepinephrine, in the same order of increments in strengths and time intervals of application (Fig. 2, bottom). The results of this and four other similar experiments on microarterial vessels of the mesentery and the striated (cremaster) muscle, using norepinephrine as the stimulating drug, are tabulated (Table 1). With slight variation from one experiment to another, there was in all cases a remarkable proportionality in the degree (maximum percent) and duration (in seconds) of the changes in the thickness of the target single smooth muscle cell. Such a step-wise modality in the increment of muscle cell thickness is in close dependence on the amount of the stimulating drug used and unrelated to the order of its application (Table 1, experiments 3 and 4). The above quantitation of a geometric variable (thickness) of single smooth muscle cells in vivo, made possible by rapid repetitive image-splitting and simultaneous analog recording, affords novel and reliable information of general interest about the nature of the contractile process of the smooth muscle in microvessels.

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