REPRIMING OF DELAYED POTASSIUM CONDUCTANCE IN FROG SKELETAL MUSCLE

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(Received 14 November 1979)

SUMMARY

A dissection of the semitendinosus muscle from Rana pipiens was developed for three-microelectrode voltage-clamp studies of the delayed potassium-selective conductance system. The delayed conductance inactivates in muscles bathed in high concentrations of potassium or rubidium, but can be reprimed by hyperpolarizing voltage pulses to membrane potentials beyond $-80 \text{ mV}$. The repriming time-course was studied by measuring the delayed conductance that could be activated following hyperpolarizing pulses of varying duration. Responses following 20–100 s pulses to potentials between $-90$ and $-140 \text{ mV}$ could not be reconciled with an exponential approach to the conductance present in normally polarized fibres. The sigmoid appearance of the early ($< 25 \text{ s}$) time course was exaggerated by cooling from 20 to 10 °C. This effect was described by a sequential model invoking two inactivated states with different temperature dependences. An explanation is suggested for differences in the kinetics and voltage dependence of repriming between briefly and chronically depolarized muscle cells.

INTRODUCTION

Hodgkin and Huxley (1952) proposed a mathematical scheme for describing the transient increase in sodium conductance responsible for nerve cell excitability. In their model, sodium conductance was proportional to the product of a rapidly developing (activating) third-order rate parameter and a more slowly declining (inactivating) first-order rate parameter, each of which was a function only of the membrane potential and time. This type of scheme has been successfully used to describe transient conductances in many different membrane systems (Cole, 1968). However, in the last decade there have arisen several difficulties in interpreting inactivation phenomena using the Hodgkin-Huxley model, notably in experiments on the transient sodium conductance in Myxicola axons. The kinetics of inactivation (Armstrong, 1970; Goldman & Schauf, 1972; Schauf & Davis, 1975; Bezanilla & Armstrong, 1977) and of recovery from inactivation (repriming) (Schauf, 1974) are sigmoid rather than exponential, the time course of inactivation depends on the activating test-pulse (Goldman

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& Schauf, 1972, 1973), the 'steady state' inactivation level varies with conditioning prepulses and also depends on the activating test-pulse potential (Goldman & Schauf 1972), and inactivation takes place more rapidly than repriming at the same membrane potential (Schauf, 1976). These deviations from the inactivation scheme proposed by Hodgkin and Huxley have been interpreted in terms of coupling between activation and inactivation (Hoyt, 1968; Goldman, 1975). An alternative is to postulate a sequential kinetic model (Armstrong, 1969, 1971; Moore & Cox, 1976) in which inactivation is subsequent to activation (a form of coupling in that activation must precede inactivation). It has been suggested that biphasic kinetics observed in multipulse experiments might be explained by multiple inactivated states (Argibay & Hutter, 1973; Hille, 1976).

Relatively few studies of the inactivating delayed conductance of skeletal muscle have been reported. Heistracher and Hunt (1969) observed a sigmoid repriming time course in snake fibres maintained in Ringer containing 100 mM-K. Adrian, Chandler & Hodgkin (1970) also observed a sigmoid repriming time course in frog fibres in high potassium, in contrast to the exponential repriming they found in briefly (< 10 s) depolarized fibres in 2.5 mM potassium Ringer. In a series of multi-pulse experiments on frog skeletal muscle fibres, Argibay & Hutter (1973) apparently detected multiple inactivated states. In one such experiment a brief repriming pulse was ineffective in eliciting recovery from a chronically inactivated membrane; after a long repriming pulse followed by a depolarization sufficient for full inactivation of conductance, the same brief repriming pulse did elicit some recovery of delayed conductance.

The purpose of this paper is to report voltage-clamp experiments on the repriming of frog skeletal muscle fibres with a chronically inactivated delayed conductance. In these experiments the temperature was varied in order to provide more detailed kinetic information. A dissection that permits good visualization of fibre terminations for use with the three-electrode voltage-clamp technique, using American frogs, is also described.

**METHODS**

**Electrical measurements**

Adrian & Freygang (1962) first suggested measuring the potential difference between two points near the termination of a cylindrical cell to obtain an approximation of membrane current density. Refinements by Adrian et al. (1970) and Schneider & Chandler (1976) have made the three-electrode voltage clamp a standard technique in muscle electrophysiology. To apply the technique a cell is impaled by voltage-recording electrodes at distances \(x_1\) and \(x_2\) from the fibre terminus. A third microelectrode inserted at \(x_3 > x_2\) receives the output of a feedback circuit designed to maintain \(V_1\) (the potential difference across the membrane at \(x_1\)) at a chosen level.

The internal resistivity per unit length is estimated using linear cable theory:

\[
I_0 = \frac{V_1 e^{2x_1/k}}{\lambda \cosh x_1/\lambda}
\]

where \(I_0\) is the steady-state change in total current injected at \(x_3\) for a voltage step \(V_1\) at \(x_1\), \(\lambda = [V_1(x_2^2 - x_1^2)/2 \Delta V]^1/2\) is the membrane space constant, and \(\Delta V\) is the...
Membrane current density per unit cell length is approximately given by
\[ i_m \approx \frac{2 \Delta V}{(x_2^2 - x_1^2) r_i}, \]
(Almers, 1971).

Current density was referred to cell surface area by the conversion
\[ I_m = \frac{i_m}{2\pi a} = i_m \sqrt{\pi r_i G_i/2\pi}, \]  
(2)

where \( a \) is the cell radius; \( G_i \) was assumed to have a value of \( 2.56 \text{ mmho/cm} \) at \( 2^\circ \text{C} \) with a \( Q_{10} \) of 1.3 (Hodgkin & Nakajima, 1972).

Voltage-recording electrodes filled with 3 M-KCl had resistances of 6–15 M\( \Omega \) and tip potentials of less than 5 mV. Current passing electrodes filled with 2 M potassium citrate had resistances of 5–12 M\( \Omega \). Electrodes were shielded just before mounting using colloidal silver paint (Valdiosera, Clausen & Eisenberg, 1974). After successful impalement of a fibre with three microelectrodes, the potential difference across the membrane at position \( x_i \) was clamped to its initial value. Command pulses could then be imposed on \( V_l \), with 90% completion within about 0.5 ms.

Control studies confirmed the findings of Adrian et al. (1970), and Stanfield (1970), in showing that the ‘instantaneous’ conductance of the delayed rectifier is linear over the voltage range \( -80 \) to \( +20 \) mV. In a given experiment a fixed voltage step (typically from \( +10 \) to \( -40 \) mV) was used for all conductance measurements, and the instantaneous change in current divided by the size of the voltage step was taken to be the membrane conductance. Conductance was routinely measured both before and after a hyperpolarizing pulse, and the difference (having leakage current subtracted) was recorded as the ‘reprimed’ conductance \( (G_{re}) \). Inactivation was allowed to redevelop for five minutes following each repriming protocol.

**Preparation**

Accurate measurement of distances from the electrodes to the end of the cell is difficult in the sartorius muscle of *Rana pipiens*, so a new preparation using the semitendinosus muscle was developed. The two heads of the semitendinosus muscle are attached to opposite faces of the sheet-like distal tendon. Cutting away some of the fibres on the ventral side of this tendon resulted in excellent visualization of the fibre ends on the dorsal side of the sheet. A loose tie around the remaining ventral head fibres permitted effective spreading of the muscle in a small chamber. The region of muscle to be impaled by microelectrodes was supported by a Lucite pedestal coated with petroleum jelly. The muscle was usually mounted at about 1.3 times slack length.

The muscle in a cooling chamber was mounted under a Zeiss standard WL research microscope and viewed through a Leitz long working distance 20x objective at a total magnification of 250. Striation spacings, visible in normal Ringer, usually disappeared several minutes after changing to hypertonic sucrose solution.
Table 1. Composition of solutions

<table>
<thead>
<tr>
<th>Sol.</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Rb⁺</th>
<th>Ca⁺⁺</th>
<th>Cl⁻</th>
<th>H₂PO₄⁻</th>
<th>HPO₄²⁻</th>
<th>SO₄²⁻</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>118</td>
<td>2.5</td>
<td>—</td>
<td>1.8</td>
<td>121</td>
<td>0.42</td>
<td>1.08</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>2.6</td>
<td>82.5</td>
<td>—</td>
<td>8</td>
<td>—</td>
<td>0.42</td>
<td>1.08</td>
<td>49.2</td>
<td>463</td>
</tr>
<tr>
<td>C</td>
<td>113</td>
<td>—</td>
<td>80</td>
<td>7</td>
<td>—</td>
<td>0.42</td>
<td>1.08</td>
<td>102</td>
<td>350</td>
</tr>
</tbody>
</table>

Concentrations given in mM throughout. Tetrodotoxin was present in C and sometimes B at a concentration of 1 μM (32 μg/100 ml). Actual ionized calcium in chloride-free solutions is about 1 mM (Hodgkin & Horowicz, 1959).

Solutions

Extracellular bathing solutions are listed in Table 1. Muscles were dissected and mounted in normal Ringer, solution A (Adrian, 1956). Experimental solutions B and C had sulphate substituted for chloride to eliminate chloride conductance and 350 mM added sucrose to abolish movement. Solution B had the same ionic strength as A (Hodgkin & Horowicz, 1959). Rubidium-containing solution C was introduced at a later stage in the investigation to block currents through the ‘inward rectifier’ (Adrian, 1964); sucrose was reduced and ionic strength increased to retard the deleterious effects of sucrose, and so that the results would be more directly comparable to recent experiments of Adrian & Rakowski (1978).

Data analysis

Oscilloscope traces were photographed by a Grass Camera on Recordak film (Kodak). Results were projected on graph paper and measurements made directly on the projected image or on tracings. Instantaneous currents were measured by determining the current time course 5-15 ms after a voltage step and linearly extrapolating back to the end of the step, as the current was obscured by the capacity transient during the first few milliseconds.

Curve-fitting was carried out on a PDP 11/40 computer (Digital Equipment Corporation) using a random direction search method (Bremmerman, 1970).

RESULTS

Repriming of delayed conductance

The delayed potassium conductance in skeletal muscle fibres is activated in normally polarized fibres by voltage-clamp steps to membrane potentials above -40 mV, and conductance reaches a maximum at positive membrane potentials (Adrian et al. 1970). With prolonged depolarization the system inactivates, and recovery at a polarized potential (repriming) is necessary before conductance can again be activated by depolarization (Heistracher & Hunt, 1969; Adrian et al. 1970; Stanfield, 1970). In the present experiments, fibres placed in solutions containing 80 mM potassium or rubidium spontaneously depolarized to membrane potentials of -30 to -20 mV. In these fibres the delayed conductance system is chronically inactivated. Voltage-clamp steps to membrane potentials of -140 to -90 mV resulted in recovery of the delayed conductance.

Fig. 1 shows records taken from a cell in solution C with a holding potential of -20 mV. A small deflection in the current (ΔV) trace accompanies the voltage st
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Fig. 1. Conductance repriming. Membrane conductance was taken to be the ratio of current change to voltage change at the times indicated by the arrows. Test pulses to $V_t = +3.5$ mV, post-pulses to $V_p = -5.45$ mV. 50 mV of $\Delta V$ corresponds to a membrane current density of 0.65 mA/cm$^2$. (a) Conductance measurement without repriming. $G(t_0 = 0) = 2.8$ mmho/cm$^2$. (b) Conductance measurement after 4 s repriming at $-108$ mV. $G(t_0 = 4) = 25.0$ mmho/cm$^2$. Holding potential $V_h = -20$ mV. Solution C, 20 °C. Fibre 27104: length constant $\lambda = 2.5$ mm, $r_i = 2.9$ M$\Omega$/cm, $x_1 = 213$ um, $x_2 = 470$ mm, $x_3 = 515$ um.

to +4 mV, and very little 'tail' current is seen with a post-pulse to $-54$ mV (Fig. 1a). With an identical voltage sequence immediately following a 4 s repriming pulse to $-108$ mV, a large delayed current accompanies the test-pulse to +4 mV, and a large tail current is seen with the post-pulse to $-54$ mV (Fig. 1b). Conductance was measured as the ratio of the 'instantaneous' current change to voltage change at the time of the step from the test-pulse voltage ($V_t$) to the post-pulse voltage ($V_p$) (arrows). The difference in conductance before and after a hyperpolarizing pulse, the 'reprimed conductance', was 22.2 mmho/cm$^2$.

Recovery following long repriming pulses

Repriming of conductance by 90 s pulses to $-109$ mV, between 2.5 and 20 °C, is shown in Fig. 2. Conclusions based on these data are limited since they reflect temperature effects on both the maximum conductance $G_K$ and the recovery rate. However, the absence of an abrupt drop in this range rules out a phase change effect such as has been reported in end-plate tissue culture (Lass & Fischbach, 1976), and suggests a reasonably smooth temperature dependence of both the maximum conductance and the recovery rate.

A repriming time course for relatively long pulses is shown in Fig. 3. Most of the pulses delivered to this fibre were to $-95$ mV; at this potential, an exponential fit
Fig. 2. Repriming temperature dependence with long pulses. Ordinate is reprimed conductance, the difference between conductance measured with and without a hyperpolarizing pulse. Repriming duration $t_{Re} = 90 \text{s} (\bullet), 60 \text{s} (\Delta)$, repriming potential $V_{Re} = -109 \text{mV}, V_h = -22 \text{mV}, V_i = +10 \text{mV}, V_p = -45 \text{mV}$. Solution C. Fibre 03091: $\lambda = 1.0 \text{ mm}, r_i = 5.6 \text{ MO/cm}, a_t = 36 \mu\text{m}, x_2 = 258 \mu\text{m}, x_3 = 470 \mu\text{m}, x_8 = 549 \mu\text{m}$. Line drawn by eye. Value of leakage conductance subtracted before plotting ranged from 0.77 to 1.43 mmho/cm$^2$ except for the points at 2.8 and 5°C where it was 3.73 and 5.46 mmho/cm$^2$.

Fig. 3. One hundred seconds of repriming at 12.5°C. Ordinate is reprimed conductance, the difference between conductance measured before and after a hyperpolarizing pulse having duration indicated on the abscissa. Repriming potentials ($V_{Re}$) of $-95 \text{mV} (\bullet), -105 \text{mV} (\Delta)$, and $-114 \text{mV} (\square)$. $V_h = -17 \text{mV}, V_i = +13 \text{mV}, V_p = -37 \text{mV}$. Solution C. Fibre 04082: $\lambda = 1.12 \text{ mm}, r_i = 22.65 \text{ MO/cm}, a_t = 20.5 \mu\text{m}, x_2 = 224 \mu\text{m}, x_3 = 448 \mu\text{m}, x_8 = 493 \mu\text{m}$. The curve is an exponential with a time constant of 75.9 seconds and steady state value 1.62 mmho/cm$^2$. The leakage conductance subtracted before plotting ranged from 0.27 to 0.36 mmho/cm$^2$, except for the measurement at $-114 \text{mV}$ for which the control subtracted was 0.64 mmho/cm$^2$. 
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Fig. 4. Repriming time course at 20 °C. Ordinate is reprimed conductance, the difference between conductance measured with and without a repriming pulse having duration indicated on the abscissa. Abscissa is time reprimed at —108 mV. Same fibre as in Fig. 1. Line is the least-squares fit to equation 3. Model parameters for the fit: $G = 24.1$ mmho/cm$^2$, $\alpha = 1.62 \text{ s}^{-1}$, $\beta = 0.80 \text{ s}^{-1}$. Leakage conductance ranged from 0.70 to 3.1 mmho/cm$^2$.

Fig. 5. Sigmoid time course at 10 °C. $V_{a1} = -107$ mV, $V_a = -27$ mV, $V_f = +11$ mV, $V_p = -35$ mV. Solution B. Fibre 15043: $\lambda = 1.11$ mm, $r_i = 8.7 \text{ M}\Omega/cm$, $a = 34 \mu$m, $x_a = 224 \mu$m, $x_2 = 470 \mu$m, $x_3 = 526 \mu$m. Model parameters for the fit to equation 3: $G = 3.93$ mmho/cm$^2$, $\alpha_1 = 0.128 \text{ s}^{-1}$, $\beta_1 = 0.128 \text{ s}^{-1}$. Leakage conductance ranged from 0.07 to 1.03 mmho/cm$^2$.

resulted in a time constant of 75 seconds and an asymptote of 1.6 mmho/cm$^2$. Two measurements at more negative potentials indicate that this was not a true steady state value, however, and suggest the presence of a component requiring many minutes to develop at —95 mV (Adrian & Rakowski, 1978). Other experiments using 20–100 s pulses to membrane potentials between —90 and —140 mV confirmed the multi-state character of repriming at long times, and demonstrated the difficulty of taking recovery to completion within a reasonable time.

Early repriming time course

Experiments at 20 °C were performed with a fixed repriming potential while varying pulse duration ($t_{Re}$). Figure 4 illustrates the time course of repriming at —108 mV.
The least-squares fit of the data in Fig. 4 to a rising exponential (not shown) resulted in a time constant of 3·9 s. Data from another cell studied at $-117$ mV was fitted by a rising exponential with a 3·1 s time constant. Both fits deviated from the data at short times, however, since conductance appeared following an initial delay.

Cooling to 10 °C considerably slowed the early recovery from inactivation. The cell illustrated in Fig. 5 required hyperpolarizing pulses of several seconds in length to begin to elicit repriming, compared with the delay of 0·1 s seen in Fig. 4. Data from another cell studied at three different temperatures are shown in Fig. 6. With cooling from 14·5 to 9·2 °C the initial delay appeared to become more pronounced.

**DISCUSSION**

*Use of American frogs for the 3-electrode clamp*

The three microelectrode voltage-clamp has emerged as a convenient and reliable technique for measuring membrane current density at a site of controlled membrane potential in preparations where space clamping is not feasible. Previous work using this method has mainly utilized the pelvic end of the sartorius muscle from the English frog, *Rana temporaria*, since pelvic terminations of sartorius muscle cells from *Rana pipiens* are poorly visualized (Schneider & Chandler, 1976). However, terminations of cells of the semitendinosus muscle from *Rana pipiens* are easily exposed and provide a convenient voltage-clamp preparation for investigators in the United States.
Measurement of reprimed conductance

Although the instantaneous conductance is linear in this preparation, several difficulties interfere with simple application of Ohm's law to determine membrane conductance from total current. Problems include current-dependent changes in concentration of permeant ions in the transverse tubules, the unreliability of absolute potential measurements using microelectrodes, and the uncertainty of the zero current level in the presence of gradually developing electrode leaks. The technique described in the Methods depends only on changes in current (ΔV) simultaneous with a fixed voltage step. Uncertainties due to changes in ionic driving forces are eliminated since the method does not require determination of the channel reversal potential. Electrodes were chosen for small tip potentials to minimize errors in potential measurements.

The method of data analysis was chosen to minimize errors due to leakage current at the electrode impalement sites. The scaling factor (1/ναι, (x₂³ — x₁²)) to convert ΔV/V₁ to conductance per unit surface area was determined by using small pulses at the beginning of each experiment. Since only this scaling factor is affected by leaks at x₂ and x₃, changing leakage at these sites will not affect measured conductances. Leakage at x₁, which does result in ΔV errors, was corrected for in the procedure used to calculate reprimed conductance by subtracting leakage conductance (with no repriming) from the total conductance following the hyperpolarization protocol, to obtain reprimed conductance (G₆). This correction assumes that leakage at x₁ is unchanged during the repriming protocol.

Effects of temperature on repriming

The present data do not reveal the full time course of repriming kinetics (Fig. 3). Reduced survival time of fibre subjected to strenuous repriming protocols made complete determinations impossible. Lacking steady-state measurements of reprimed conductance, data cannot be normalized and quantification of early sigmoidicity can only be regarded as tentative. However, some useful inferences can still be drawn from observations of recovery from inactivation made at short and intermediate times (< 25 s).

The existence of a sigmoid response (having an initially increasing slope, followed by a decreasing slope) is incompatible with the single-state model of inactivation (see also Argibay & Hutter, 1973). If all the inactivation processes had identical temperature dependences, temperature changes would have the same effect as scaling the axes, with no change in the shape of the time-course curves. However, inspection of the data taken at different temperatures suggested that scaling would not result in superposition. A simple two-state model for recovery from inactivation was solved in order to quantitatively describe the sigmoid character of early recovery. I₂ is the chronic inactivated state, I is the intermediate inactivated state, and R is the reprimed condition:

\[
\begin{align*}
R & \xrightarrow{a₁} I \xrightarrow{β₁} I₂.
\end{align*}
\]

This mechanism can account for exponential onset of inactivation, exponential recovery after brief inactivation, and sigmoid recovery after long-term inactivation if
the following relationships among the rate constants are assumed: at $-20 \text{ mV}$ \( \alpha_1 > \alpha_2, \beta_1 > \beta_2 \) and \( \alpha_1 > \beta_1 \); and at $-110 \text{ mV}, \beta_2 > \beta_1, \alpha_2 > \alpha_1, \) and \( \alpha_2 \) is of the same order of magnitude as \( \beta_2 \). A cell in depolarizing solution will have a fully populated state \( I_2 \). Voltage-clamp hyperpolarization to $-110 \text{ mV}$ will elicit recovery after the initial delay required to repopulate state \( I \). With a step in hyperpolarization, the solution for the recovery time-course is

$$R = \mathcal{G} \left( 1 - \frac{\beta_2 e^{-\alpha_2 t} - \alpha_2 e^{-\beta_2 t}}{\beta_2 - \alpha_2} \right).$$

The curves shown in Figs. 4 and 5 were obtained by adjusting the parameters \( \mathcal{G}, \alpha_2, \) and \( \beta_2 \) to minimize the sum of the squared deviations of the data from the calculated curves.

The sigmoid character of equation 3 is monotonically related to the ratio of the smaller to the larger rate constant, with the largest delay for \( \beta_2 / \alpha_2 = 1 \). This is the case in Fig. 5 (10 °C), where the data would be better fit by a model allowing even more sigmoidicity. In Fig. 4 (20 °C) a good fit was obtained for \( \beta_2 / \alpha_2 = 0.49 \). The fit to another time-course at $20^\circ$ resulted in \( \beta_2 / \alpha_2 = 0.059 \). These observations quantitatively support the suggestion that sigmoid time courses are more pronounced at lower temperatures.

Fits to repriming time courses at several temperatures (Fig. 6) were calculated by including temperature coefficients for \( \mathcal{G}, \alpha_2, \) and \( \beta_2 \) as adjustable parameters. Parameter values resulting from these fits are of limited use since there were only about twenty data points and six adjustable parameters. Nevertheless, in Fig. 6, the ratio \( \beta_2 / \alpha_2 \) ranged from 0.054 at $14.5^\circ$ to 0.105 at $9.2^\circ$, corroborating the trend seen in experiments on separate fibres studied at different temperatures.

The results of this curve-fitting exercise show that for the limited data available, the sigmoid descriptions did exhibit increasing delays as the temperature was lowered. Delays were determined by comparing the ratios of two rate constants, a method that is independent of axis-scaling. It is therefore concluded that the repriming mechanism consists of at least two rate processes with different temperature dependences.

Relation to previous results

The present finding of a sigmoid repriming time course is consistent with the results of Adrian et al. (1970), Heistracher and Hunt (1969), and Argibay & Hutter (1973). Adrian and Rakowski (1978) observed an exponential repriming time course under similar conditions; however, their experiments were more concerned with steady-state recovery and the early time-course was not investigated in detail. Although the latter authors described repriming as a single-state exponential process, they also observed fibres that did not achieve a steady level of recovery after several minutes. Their interpretation in terms of a separate very slowly repriming conductance is supported by the present results (Fig. 3).

Adrian & Rakowski (1978, fig. 10) also found that the voltage dependence of repriming showed a marked shift towards more negative potentials compared to results obtained by Adrian et al. (1970, fig. 16) and Almers (1976, pp. 19–20) in normally polarized cells. A shift in this direction is predicted by the proposed model. The kinetic differences between repriming in briefly and in chronically inactivat
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Wells reported by Adrian et al. (1970) and Heistracher and Hunt (1969) are also predicted.

I would like to thank Dr R. F. Rakowski for suggestions and advice, Dr W. F. Pickard and the Biomedical Engineering Program for flexibility and support, and Dr C. B. Ballou for continued encouragement. This work constituted partial fulfillment of the requirements of the Doctor of Science degree in Electrical Engineering at Washington University, and was supported by P.H.S. grant number 5-To1-GM01827 to the Biomedical Engineering Program.

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