ELECTRICAL FLUCTUATIONS ASSOCIATED WITH ACTIVE TRANSPORT

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ABSTRACT Measurements were made of the spectrum of the voltage fluctuations developed in the 0.025–10 Hz band during active transport by frog abdominal skin with Ringer's solution on both sides. Decreasing the potential across the skin by an external supply of current diminishes the voltage fluctuations, but they do not disappear, reaching a minimum finite value. Thus, fluctuations in both the resistance of the skin and the electric current attendant to the active transport of sodium contribute to the voltage fluctuations. Ouabain eliminates the current fluctuations but not those of the resistance. At 20°C, the spectral intensities of the resistance and current fluctuations are nearly identical, varying as $1/f^a$, where f is frequency and a = 1.6–2.0. At 32°C, the spectrum of the voltage fluctuations is sigmoid shaped, evidencing a relaxation process with a time constant of 0.6 sec. The fluctuations can be accounted for by stochastic variations in the concentration of a complex formed between a carrier molecule, fixed or mobile, and the actively transported species, sodium.

INTRODUCTION

Although it has been the object of an extensive search, direct evidence of the actual mechanism of active, metabolically dependent transport has remained elusive. An approach to this problem which has had considerable success in the study of physical systems is through the analysis of the inevitable random electrical fluctuations accompanying the transport of electrically charged species. The statistical characteristics of this electrical noise are determined uniquely by the physicochemical details of the underlying transport mechanism, whether it be an active or passive process.

Frog abdominal skin has been an extremely convenient tissue for the study of active transport. When placed as a barrier separating two compartments of identical solution, it maintains a potential difference across itself provided that its metabolic activity remains intact. The potential difference is a direct and immediate manifestation of the rate of metabolically dependent transport of sodium from the anatomical outside surface to the inside one (Ussing and Zerahn, 1951). I have, therefore, used this tissue for an exploration and analysis of the electrical fluctuations associated with active transport.

METHODS

A piece of abdominal skin (area about 6 cm^2) removed from a decapitated frog (*Rana pipiens*) was held tautly sandwiched between two Plexiglas disks which separated two Plexiglas half-cells (15 cm^3 fluid volume each). The two halves of the skin carrier were pierced by a circular hole 0.64 cm in diameter. Grooves machined in the two pieces assured coincidence of the axes of the holes. These holes delimited the area of membrane under investigation to 0.32 cm^2. The carrier fit tightly into matching grooves in the two half-cells. The skin holder and the half-cells were clamped together by screws carefully tightened so as to minimize the damage to the skin. The chambers were filled with fluid and the levels equalized so that there was no hydrostatic pressure difference across the skin. Silicone grease applied to the various mating surfaces prevented fluid leakage around the skin edges or to the outside.

For the most part, the potential difference generated by the skin was detected via Ag-AgCl electrodes immersed in Ringer's solution and connected to the fluid in the half-cells by 4% agar-Ringer's solution bridges. Each half-cell also contained a 1 inch long $\times$ 0.1 inch diameter cylindrical Ag-AgCl electrode. These served as current electrodes when current was supplied; the electrode in the fluid in contact with the anatomical inside of the skin was the reference electrode for the potential measurements.

In some experiments, a second additional pair of potential electrodes was present. These were miniature calomel reference electrodes (Corning Glass Works, Laboratory Products Dept., Corning, N. Y., No. 476015) immersed in large volumes of Ringer's solution communicating with one of the half-cells via an asbestos fiber junction and a 1 mm bore glass capillary. The voltage developed across the calomels was monitored by a high input impedance ($>10^{11}$ ohms) DC-coupled differential amplifier (Burr-Brown Research Corp., Tucson, Ariz., model 3153/25). The difference in junction potential between the calomels was frequently measured during an experiment by noting the change in potential upon interchanging them. Measurements of the potential across the skin were corrected for the junction potential differences thusly obtained.

The Ag-AgCl potential electrodes were connected to the inputs of a low noise, differential input preamplifier (Princeton Applied Research Corp., Princeton, N. J., model 113). For the measurement of total skin potential, the electrodes were DC coupled to the input (input impedance $>10^9$ ohms) and the gain of the amplifier set to 10; for the measurements of the fluctuating component of the potential, the electrodes were AC coupled (input impedance 0.1 μF in series with 10^8 ohms) to the amplifier and the gain was increased to 10^3–10^4. The low frequency, -3 db, cutoff was set to either 0.1 or 0.01 Hz; the upper one to either 10 or 10^4 Hz. The output of the preamplifier was connected to an operational amplifier (Philbrick/Nexus Research, Boston, Mass., model P85AU) set for a gain of 1–10.

The analysis of the spectral content of the voltage fluctuations was carried out either in the real time domain or was performed on a magnetic tape recording of the signals. In the first case, the output of the operational amplifier was applied to a band-pass filter (Dytronics Co., Inc., Columbus, Ohio, model 719) of variable center frequency with its nominal bandwidth set to either ± 6 or $\pm 18\%$ of the center frequency. The filter output was converted to a pulse train by a voltage-to-frequency converter (Hybrid Systems Corp., Burlington, Mass., model 403) the output of which (0–2 kHz) is accurately proportional to the absolute value of the input. The pulses were counted (Anadex Instruments Inc., Van Nuys, Calif., model CF-600R); the time average of the converter output, so obtained, is proportional to the average of the absolute value of the output of the filter. Measurements were made with various settings of the center frequency of the filter in order to obtain the spectrum of the voltage fluctuations developed by the skin. The respective averaging periods for the filter center frequencies employed were as follows: 50 sec (10, 5 Hz), 100 sec (2.5, 1.0, 0.5 Hz),

200 sec (0.25 Hz), 300 sec (0.1 Hz). The low frequencies and small filter bandwidths necessitated the long averaging periods for reasonable accuracy (Ziel, 1954). The spectral intensity is obtained by expressing the measurements in the form: (mean absolute value of voltage)^2/filter bandwidth, in radians (seconds)$^{-1}$. The power spectrum, so determined, is not materially different from that which would be obtained if the filter output were measured directly with a square law detector, rather than with a linear one, as here (Rice, 1954). The power spectra presented below are the average of two immediately consecutive runs in the order, first, 10–0.1 Hz and, then, 0.1–10 Hz. Only one measurement was made at 0.1 Hz. After a change in the center frequency of the filter, sufficient time was always allowed for the filter output to achieve a new steady state. Except where specifically noted, the power spectra were obtained by this real time domain method.

At a later stage of the investigation, it became desirable to extend the frequency analysis below the lower limit of the filter, 0.1 Hz. For this purpose, an FM tape recorder, permitting a fourfold compression of the real time scale, was assembled of laboratory instruments. The amplified noise signal was mixed with an ultrastable DC bias signal by an operational amplifier (P85AU) connected as an adder. The gain of the system was adjusted such that the instantaneous value of the noise voltage did not exceed $\pm 25\%$ of the bias signal, as more excessive deviations introduced subsequent signal distortions. The signal-plus-bias was then converted to a pulse train by the voltage-to-frequency converter, the output of which was amplified and formed into a standard pulse by a pulse generator (Tektronix, Inc., Beaverton, Ore., type 161). These pulses were recorded on magnetic tape by a tape recorder (Tandbergs Radiofabrikk SA, Norway, model 3000X) at 1⅞ in (seconds)$^{-1}$. The recorded signal was monitored continuously by amplifying it, pulse forming it with a pulse generator, and converting it back into a continuous analogue signal by means of an Anadex model PI-408 frequency-to-voltage converter. This signal, together with the original one, were both displayed by a storage oscilloscope (Tektronix type RM564). In this fashion, it was possible to be assured that the tape system was making an accurate undistorted record of the voltage fluctuations of the skin. Instrumental noise generated by the system was found to be negligible, as determined by performing a spectral analysis on a recording of the DC bias alone.

In an experiment, the noise voltage was recorded for a period of 48 min at 1⅞ in (seconds)$^{-1}$. The tape was then played back at 7½ in (seconds)$^{-1}$, and the analogue signal of the frequency-to-voltage converter analyzed for its spectral intensities exactly as in the real time method. The filter output was measured for a period of 10 min, after a 2 min electrical equilibration, at all frequencies. In addition to effectively lowering the center frequency of the filter by a factor of one-fourth, an important advantage of the tape method is that the spectral analysis is performed on the same signal for all frequencies. In view of the long averaging periods required at low frequencies, this considerably reduces the possibility that the spectral analysis will be affected by slow drifts in the magnitude of the voltage fluctuations which might occur during an experiment.

The bandwidth and frequency response of the entire electronic system was calibrated by using a high quality 10^9 ohm resistor (Keithley Instruments, Inc., Cleveland, Ohio, model R20-10^9) as a source of thermal (Johnson) noise. The resistor was well shielded and connected by a very short lead to one of the preamplifier inputs (DC coupled); the other input was grounded. The power spectrum of the noise generated by the resistor, corrected at 5 and 10 Hz for the effect of input stray capacitance, was determined for the various preamplifier bandwidths and for each of the two filter bandwidths. A series of correction factors was then calculated. These are the factors by which an individual reading at a particular setting of the filter center frequency must be multiplied such that the measured noise power of the resistor per unit bandwidth is independent of frequency. In addition to the obvious effect of the band-

width of the preamplifier, the main need for these corrections is the frequency dependence of the gain of the filter and, to a lesser extent, to that of its bandwidth. The latter two correction factors were not very great; the noise spectra obtained without making them does not differ very much from the corrected ones. The calibration of the system was corroborated by using it to determine the power spectrum of an operational amplifier (P85AU) connected so that the effect of its input current noise was preeminent at the output. The spectral intensities, so obtained, were inversely proportional to frequency, as should have been the case.

The system with tape recorder was calibrated with a Hewlett-Packard type 8057A ultra-low frequency precision pseudorandom noise generator (Hewlett-Packard Co., Palo Alto, Calif.). The signal passed through the entire electronic system and was recorded and analyzed in the same manner as for the skin voltages. The spectral intensity of the calibrating noise signal was set to be independent of frequency in the frequency range of interest here. As in the calibration for the real time analysis, a series of correction factors was calculated. These contain an additional component due to frequency response limitations of the FM recording method.

Current was supplied to the skin sample, when required, by a variable Zener diode voltage source (Tektronix, type W plug in unit) in series with a high quality 3.3×10^5 or 10^6 ohm resistor. That the current source was sufficiently stable and free of noise was demonstrated by substituting the combination of two 10 kohm General Radio Co., Concord, Mass., type 500-J resistors in parallel for the chamber and by measuring its noise spectrum with and without the current supplied by the current source flowing through it. When a current equal to the maximum supplied to the skin flowed through the 5 kohm resistance, there was no detectable increase of its noise power at any of the filter center frequencies employed during the measurements on skin. With the chamber filled with Ringer's solution, but containing no skin, there was no detectable increase in noise when even the maximum current flowed. The noise spectrum of the electrodes and chamber, without a skin sample in place but filled with Ringer's solution, was determined frequently to be sure that the electrical fluctuations measured were indeed due to the presence of a skin sample. In a few rare instances, it was necessary to subtract (quadratically) the noise of the electrodes, chamber, and electronic devices from the measurements. This correction never amounted to more than about 25% for the results presented here.

The chamber and electrodes were contained in an electrically shielded box. Skin temperature was controlled by thermostatically adjusting the temperature of either the air surrounding the chamber or a tray of water in which it was immersed. A thermistor probe situated within the fluid near the skin served to monitor the temperature continuously. Unless indicated otherwise, the temperature was 19–21°C. In no case, did it vary by more than 0.5°C during a noise determination sequence. The possible effect of spontaneous temperature fluctuations on the measurements is examined in Results.

At the start of an experiment, a piece of skin was mounted in the holder, the two halves of the chamber were joined together, electrodes inserted, and the chamber filled with Ringer's solution. The DC voltage developed by the skin usually increased slowly, and when it had reached a steady state, measurements of the power spectrum of the fluctuating portion of the potential commenced or a tape recording of the noise was made for subsequent analysis.

The effect of the reactive component of the impedance of the skin was examined in the following manner and found to be negligible. A small constant amplitude sinusoidal current was applied to the skin sample. The resulting sinusoidal voltage which appeared across the skin was measured as a function of frequency. In the range of frequencies covered by the noise measurements, there was no significant dependence of the skin voltage upon the frequency of the current. Thus, the form of the noise spectra is not materially affected by the presence of reactive components of the skin impedance.

The Ringer's solution contained (millimolar): 110 NaCl, 2.5 KCl, 2.0 $CaCl_2$, and 2.0 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Its pH was brought to 7.3 with NaOH.

RESULTS

The power spectrum of the fluctuating component of the voltage developed by frog abdominal skin with Ringer's solution on both sides is shown in Fig. 1 plotted on log-log coordinates. The spectral intensity, the power per unit bandwidth, is proportional to $1/f^a$, where a is a constant and f is frequency; a straight line fitted to the data by the method of least squares yields $a = 1.71$. Such a frequency dependence is characteristic of the high frequency portion of the spectrum of a transport mechanism involving a simple first order relaxation process as a rate limiting step. The inevitable spontaneous deviations around the equilibrium state of the process give rise to fluctuations with a spectral intensity proportional to $(1 + 4\pi^2 f^2 \tau_0^2)^{-1}$ when the process can be described by a single time constant τ_0. In the high frequency region, the frequency dependence is $1/f^2$; deviations occur in the direction

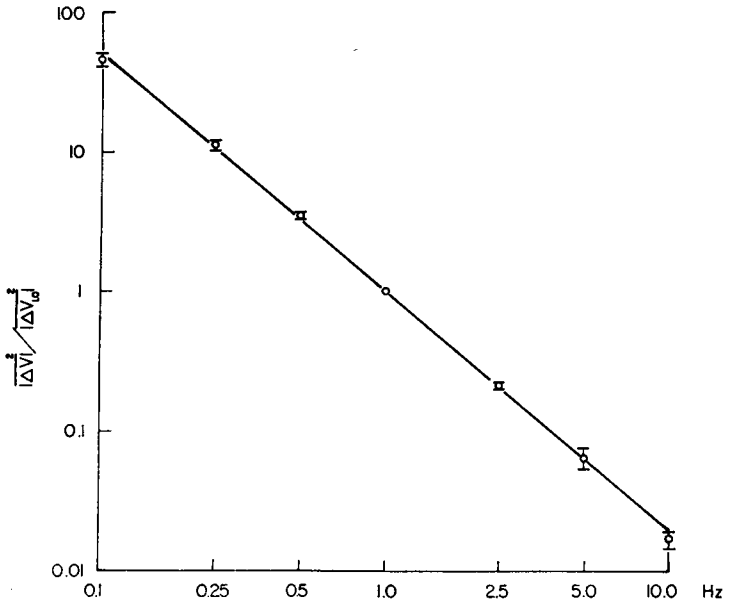

FIGURE 1 Normalized spectral intensity of the voltage fluctuations of frog abdominal skin as a function of frequency with no external current supplied. Each point is the mean obtained from 19 determinations of the spectrum of 12 different samples of skin with Ringer's solution on both sides. The individual power spectra comprising the mean were normalized by dividing the intensities at a given frequency $\overline{|\Delta V|^2}$ by the intensity at 1.0 Hz, $\overline{|\Delta V_{1.0}|^2}$. Bars denote ± 1 SE of the mean. Absolutely, the mean $\pm$SE of the spectral intensity at 1.0 Hz is $1.54 \pm 0.35 \times 10^{-12}$ v² sec. Sample area was 0.32 cm². The straight line, slope -1.71, was fitted to the data by the method of least squares. The resting potential at the time of the determination of the spectra was 57 ± 5 mv, anatomical inside surface positive.

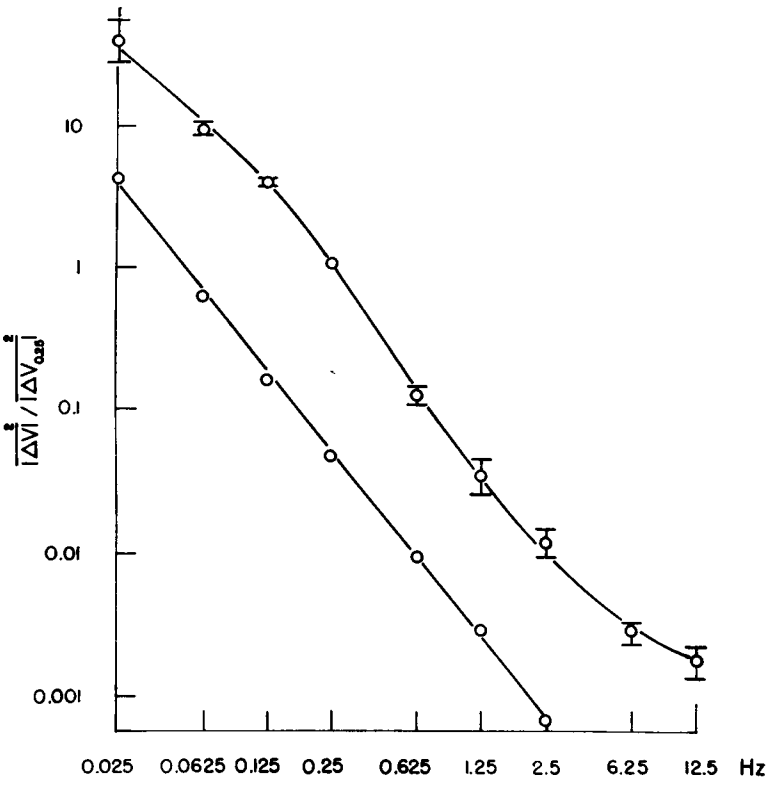

FIGURE 2 Effect of temperature on the ultralow frequency power spectrum of the voltage fluctuations of frog skin. Individual spectra were normalized by dividing the intensity at a given frequency $\overline{|\Delta V|^2}$ by the intensity at 0.25 Hz, $\overline{|\Delta V_{0.25}|^2}$. The upper, high temperature curve is the mean of six normalized spectra determined on five different skin samples; bars denote ±SE. Resting potential at the time of recording the noise sample was 47 ±9 mv; temperature was 31.9 ±0.1°C. The lower curve is the mean of two normalized spectra obtained from two skin samples (different from those employed for the high temperature measurements) with a resting potential of 31 ±4 mv; temperature was 20.4 ±0.1°C. Absolutely, the spectral intensity at 0.25 Hz was 10.5 ±2.9 × 10^{-18} v^2 sec for the 31.9°C spectra and 0.47 ±0.19 × 10^{-18} v^2 sec for the 20.4°C ones. The vertical separation of the two curves accurately reflects their differences in absolute value (cf. Fig. 1).

of $a < 2$ when the process involves a distribution of closely spaced time constants rather than a single one (Ziel, 1959). Possible mechanisms giving rise to these spectra, as well as others, are discussed in detail below.

If the electrical fluctuations of the skin are indeed a manifestation of a relaxation process, then at sufficiently low frequencies the spectral intensity should become constant, independent of frequency. Therefore, with the aid of the time base compression afforded by the tape recording system, a search was made for a low frequency flattening of the power spectrum. The results of these measurements are shown in Fig. 2. There illustrated is the disappointing result that at 20°C, the spectrum remains linear down to 0.025 Hz.

However, if the slope of the power spectrum is less than 2 because of a distribution of relaxation time constants, as has been suggested, then raising the temperature should cause a narrowing of the distribution. In consequence, the low frequency flattening of the power spectrum should occur at a higher frequency, and the maximum slope of the spectrum should more nearly approach 2. This expectation is realized by the graphs of Fig. 2 which show the effect of raising the skin temperature to 32°C. Note the flattening of the curve for low frequencies and the steepening of the slope in the middle range. These curves evidence a relaxation process with a time constant of about 0.6 sec.

The voltage fluctuations of frog skin can arise from the active transport system and/or the passive electrical pathways of the skin. To be able to identify the relative contributions of these two sources, it is necessary to consider first the electrical manifestations and consequences of the active transport of sodium.

As indicated above, normally metabolizing frog skin maintains an electrical potential difference across itself when situated between two volumes of chemically identical solution. If this resting potential difference is short circuited to zero through an external circuit, there is a steady net transport of sodium from the solution phase contacting the outside anatomical surface to that against the inside one. The rate of sodium transport is exactly sufficient to account for the short circuit electric current flowing through the skin (Ussing and Zerahn, 1951; Koefoed-Johnson et al., 1952). That is, the electrical transport of species other than sodium is zero. Clearly, under the short circuit conditions, there is no electrochemical gradient across the skin to cause the sodium transport, and the driving force must derive from a chemical reaction. That it is, is demonstrated by the familiar dependence of the short circuit current on the metabolic activity of the skin (Fuhrman, 1952; Zerahn, 1956; Leaf and Renshaw, 1957).

When the potential across the skin is open circuited, i.e. no current supplied to or drawn from an external source, net sodium transport diminishes by about 50%, and anion (chloride) flux through the skin becomes significant (Ussing and Zerahn, 1951; Koefoed-Johnson et al., 1952). The latter flux, necessitated by the physical requirement that each of the two bulk solution phases remains electroneutral, is equal in magnitude and direction to that of sodium. In contrast, under short circuit conditions, this quantitatively exact charge compensation of the sodium transported occurs by complementary electrode reactions at the two current electrodes within the bulk phases; there is no net flux of anion through the skin itself.

If the resting potential across the skin is augmented, by supplying current flowing in the outward anatomical direction, a level can be reached at which net sodium flux through the skin is zero, and all the flow of charge is due to the anions (Ussing and Zerahn, 1951). In this case, as well as in the other two considered, isotopic flux ratio criteria indicate that chloride flux is completely passive (Koefoed-Johnson et al., 1952). There is no direct metabolic contribution to the transport of chloride, the sole driving force being its electrochemical gradient.

Upon the basis of experiments such as these, it is possible to formulate a simple equivalent circuit of the direct current electrical properties of the skin. This is comprised of the combination of a resistance R' in parallel with an element consisting of an emf E in series with a resistance r' where E is due to the active transport of sodium. The potential across the skin V is given by

$$V = \frac{R'}{r' + R'} (I_e r' - E),$$

where I_e is the electrical current supplied to or drawn from an external source. Further evidence for the equivalent circuit is the observation that the steady state current-voltage relationship of the skin is linear for even rather large deviations of V around the resting level (Finkelstein, 1964).

For the purpose of the analysis of the present experiments, a more convenient equivalent circuit is the one consisting of a generator of constant inward current I^*_{Na} the active sodium transport pathway, in parallel with a resistance R. I^*_{Na}, the metabolically linked component of sodium movement, is equal to the current flowing through the skin under short circuit conditions ($V = 0$); it is independent of V. The voltage across the skin V is simply equal to IR, where I, the current flowing through R, is $I_e - I^*$. The component I^* is the passive electrical current flowing through R to compensate for I^*_{Na}; it is equal in magnitude but opposite in direction to I^*_{Na}. The interchangeability of the two equivalent circuits is established by noting that $R \equiv r'R'/(r' + R')$ and $I^*_{Na} \equiv E/(r' + R')$. In both models, the current I_e can include a passive electrical component due to sodium. Indeed, the marked effect on net sodium flux of the potential across the skin is evidence of a significant component of electrical transport of sodium through the skin controlled by this ion's electrochemical gradient and independent of metabolic energy.

In general, the elements of the constant current equivalent circuit of the skin will have fluctuating components of resistance ΔR and of current ΔI. As ΔI_e always can be avoided experimentally (see Methods), ΔI is simply the fluctuation in I^* due to the fluctuation in the rate of active sodium transport. That is, $\Delta I^*_{Na} = \Delta I^* = \Delta I$. ΔR includes, first, the intrinsic variations in the passive electrical resistance of the skin and, second, any effects of fluctuations in the rate of sodium transport which become manifest as resistance changes, as they would for certain transport mechanisms. Since $V = IR$, $V + \Delta V = (I + \Delta I)(R + \Delta R)$ and the fluctuating portion of the voltage across the skin ΔV is given by $\Delta V = I\Delta R + R\Delta I + \Delta R\Delta I$. If ΔR and at least part of ΔI are correlated with correlation coefficient C, then $\Delta I = \Delta I' + C\Delta R$, where the prime denotes the uncorrelated, independent portion of ΔI; when ΔI and ΔR are completely independent, $C = 0$. $R \gg \Delta R$ and $I \gg \Delta I$, so

$$V = (I + CR)\Delta R + R\Delta I'. \tag{1}$$

$\overline{\Delta R} = 0$; $\overline{\Delta I} = 0$, where the bar denotes the mean of the quantity beneath it, so

$$\overline{\Delta V^2} = (I + CR)^2\overline{\Delta R^2} + R^2\overline{\Delta I'^2}.$$

The relative contributions of ΔI and ΔR to ΔV can be ascertained by observing the dependence of ΔV on I, when a constant direct current is supplied from a source external to the skin. Under these conditions, I is diminished or augmented, but, very importantly, ΔI remains unchanged. Since ΔI is a time varying quantity, it is not affected by the direct current from the external source. If ΔR is insignificant, ΔV will not alter when the steady value of I is changed, according to equation 1. In contrast, if ΔI is insignificant, $C\Delta R = 0 = \Delta I'$ and the relationship between ΔV and I is linear, extrapolating to zero. That is, ΔV would disappear when I is reduced to zero by supplying an external current which exactly cancels that caused by the active transport of sodium. Finally, when both ΔR and ΔI are significant, the relationship between ΔV and I is nonlinear and ΔV cannot be reduced to zero by changing I with an external current source. Although C cannot be deduced from the result of experiments in which I is varied, the frequency dependence of the spectrum of ΔV must be independent of I in the important special case of ΔR and ΔI being fully correlated.

Fig. 3 illustrates the effect of varying I on $\overline{|\Delta V|}$ at a single frequency. Observe, first, that when V is increased by augmenting I from an external source, $\overline{|\Delta V|}$ increases. Therefore, ΔI cannot be the sole source of ΔV. Second, when V is decreased by diminishing I with an external source, $\overline{|\Delta V|}$ decreases and reaches a finite constant value. That is, ΔV does not approach zero as I does. Thus, ΔV does not originate exclusively with ΔR. Both these observations are consistent with equation 1 when neither ΔR or ΔI is insignificant. It follows, therefore, that the voltage fluctuations arise as the combined effect of resistance and current fluctuations within the skin.

If ΔR and ΔI are manifestations of fluctuations in the same single process, as would be the case for certain mechanisms, then ΔR and ΔI are fully correlated and, therefore, must have the same spectrum. The close similarity of the spectra of ΔR and ΔI is evident from the data presented in Fig. 4. There it is seen that the spectrum of ΔV is virtually independent of I when I is varied over a wide enough range so that the relative contributions of the ΔR and ΔI components of ΔV alter considerably. At 20°C, the slope of the spectrum does appear to diminish somewhat for currents which reduce the potential difference across the skin. This may signify that the correlated effect of ΔI is not the sole origin of ΔR or, on the other hand, there is a second order effect of I on the distribution of time constants of the transport process. Although it is not possible to decide this issue for the present, it is clear that the form of the spectra of ΔI and ΔR are not qualitatively different.

The conclusion that ΔV arises as the consequence of the fluctuations ΔI and ΔR implies implicitly that ΔV should disappear when active transport vanishes. In the absence of active transport, $I^* = 0 = \Delta I = \Delta I'$ and, therefore, $\Delta V = 0$, according to equation 1. To determine if ΔV is dependent upon a functioning active transport mechanism, the effect of ouabain, a potent inhibitor of active transport (Koefoed-Johnson, 1958), was examined. In the presence of 0.5 mM ouabain, the potential

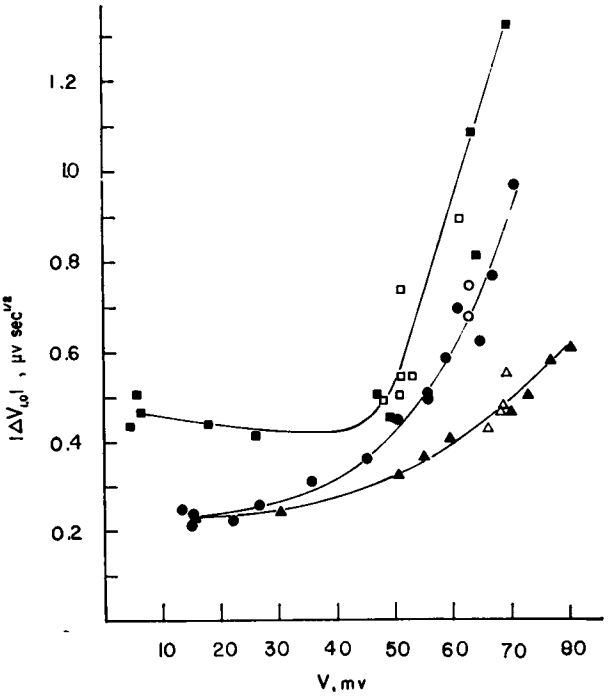

FIGURE 3 Effect of externally supplied current on the voltage fluctuations of frog skin with Ringer's solution on both sides. Results with three different skin samples denoted by circles, squares, and triangles; empty symbols: resting state, no external current; filled symbols: current supplied. The ordinate is the mean of the absolute value of the intensity of ΔV at 1.0 Hz. The abscissa V is the potential developed across the skin, inside positive; displacements from the resting level are due to positive or negative currents supplied. Data points were obtained in an approximately random sequence. Skin resistance was constant over the range of potentials studied and for the samples denoted by circles and triangles was 1.6 kohm cm^2; it was 1.1 kohm cm^2 for the sample denoted by the squares. The calculated current density required to reduce V to zero, the "short circuit" current, was 38 μamp cm^{-2} for the circle and triangle samples and 50 μamp cm^{-2} for the square sample, as obtained by extrapolation to $V = 0$ of the measured current-voltage relationships.

across the skin gradually disappears and concomitantly with it ΔV. The exact quantitative relationship between $\overline{|\Delta V|}$ and V was not determined, but, qualitatively, the graph of $\overline{|\Delta V|}$ at 1.0 Hz appears to be a concave upward monotonically increasing function of V which passes through the origin. There was no evidence of a component of ΔV persisting in the absence of the current generated by active transport. This would not be the case if ΔR and ΔI did not fully account for ΔV.

The resistance fluctuations, however, are not abolished by ouabain. The form of the noise spectrum elicited by passing a constant current through an otherwise electrically silent ouabain-treated skin is indistinguishable from that of the untreated tissue (Fig. 1).

The possibility must be entertained that ΔR and ΔI arise artifactually, rather than

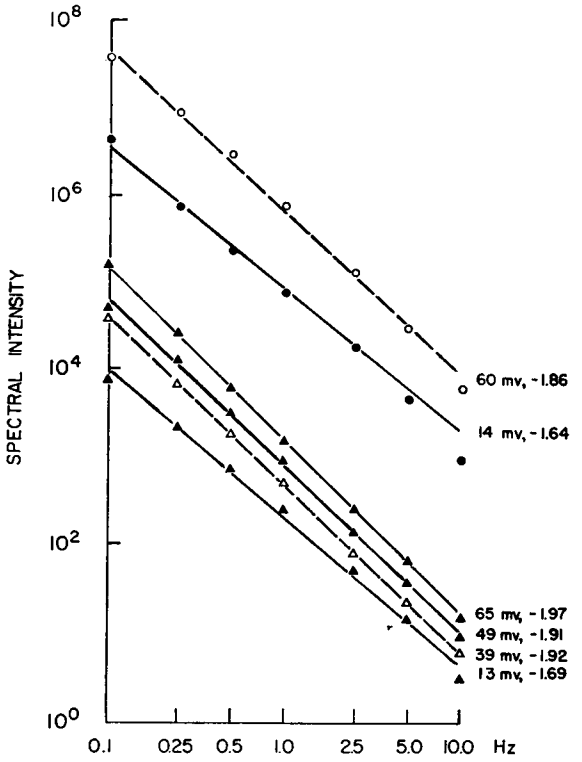

FIGURE 4 Effect of externally supplied current on the spectral intensity of the voltage fluctuations of frog skin with Ringer's solution on both sides. Two different skin samples denoted by circles and by triangles. Empty symbols and broken lines: no external current; filled symbols and solid lines: current supplied. For the sake of clarity, the two sets of data are separated arbitrarily along the vertical axis with respect to each other; the relative positions of the data points within each set are preserved. Absolutely, the spectral intensities at 1.0 Hz are 3.74×10^{-14} v^2 sec for the sample denoted by the circles and 1.26×10^{-14} v^2 sec for the triangle sample in the resting state with no added current. The straight lines drawn through the data points were calculated according to the method of least squares. The numbers near these lines denote the mean potential difference across the skin, inside positive, during the determination of the spectrum and the slope of the line, respectively. The skin sample denoted by circles is the same as that similarly identified in Fig. 3. The empty triangle data points are the means of three separate spectra determined before, during, and at the end of the measurements with current flow. The "13 mv" spectrum is the mean of two consecutively determined spectra.

as a naturally occurring manifestation of the active transport mechanism. After all, the mounted skin sample is compressed between two perforated disks, a procedure which undoubtedly damages the skin somewhat. It is conceivable that this damage at the edges of the area sampled electrically appears as random electrical fluctuations of sufficient intensity to mask the normal ones associated with transport. This problem was approached experimentally by determining the effect of mounting the skin in a holder designed to increase the relative amount of damaged

TABLE I

EFFECT OF EDGES ON ΔV

	Four-hole holder‡	One-hole holder§
I^*, μamp	11.6 ±0.5‖	13.0 ±1.0
R, kohm	5.4 ±0.2	5.9 ±0.3
$\overline{\lvert\Delta V\rvert}/I^*$	0.70 ±0.06	0.97 ±0.08
$\overline{\lvert\Delta V\rvert}/V$	0.129 ±0.011	0.148 ±0.009

The units of $\overline{\lvert\Delta V\rvert}$ are arbitrary but identical for all measurements; sample area for both holders: 0.32 cm².

‡ Nine measurements on five different samples.

§ 14 measurements on five different samples.

‖ Mean ±SE.

sample edge while maintaining constant the area of skin under study. Thus, if edge effects are significant, then when the ratio (sample edge)/(sample area) is increased, the fluctuations should rise even though the area is constant. On the other hand, if edge phenomena do not make significant contributions to the fluctuations, then their magnitude should be unaltered between the two situations.

A comparison was made of the spectral intensities of ΔV with the skin mounted in the usual singly perforated holder (see Methods) and when in a holder identical with it except for being pierced by four holes. The holes were each 1.6 mm in diameter and lay within a 1 cm diameter circle. The area of skin exposed to electrical measurement is the same in both cases, 0.32 cm², but with the four-hole holder, the circumference of the portion of the skin sample available for measurement is twice as great. Thus, the contribution of any edge effect should be twice as great with the four-hole holder.

The spectral intensity of ΔV at 1.0 Hz (±18% nominal bandwidth) was measured (10 min sample) for a different set of skin samples for each of the two holders. The results of these measurements are summarized in Table I. In addition to determining the spectral intensity, the resistance of the sample was measured by observing the voltage displacement caused by small direct currents of both polarities. From the resistance so obtained and the potential across the skin in the absence of externally supplied current, a calculation was made of the current I^* flowing through the skin responsible for V; the spectral intensities are expressed as fractions of I^* and V. Both such "noise figures," Table I, are lower for the four-hole holder, although the differences are not very significant. More importantly, the fluctuations in V do not increase when the skin holder ought to cause proportionately greater edge damage, warranting the attribution of the fluctuations to the main, experimentally unperturbed, body of the skin sample.

Thus far, it has been tacitly assumed that the voltages analyzed are random signals; it is important to know if, indeed, this is actually so. The signals always appeared upon casual observation to be random, but it must be shown quantitatively

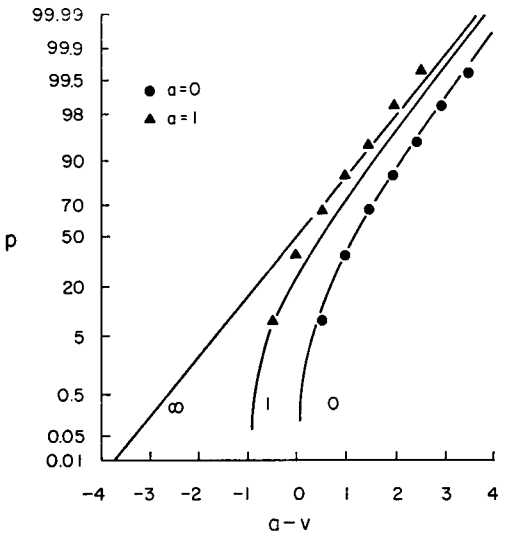

FIGURE 5 Probability distribution function of the envelope of a signal consisting of a sine wave plus gaussian random signal passed through a narrow bandwidth filter. The ordinate p is 100 times the probability that the envelope is less than v, where v = amplitude of signal envelope per rms random signal; a = amplitude of sine wave per rms random signal. The continuous curves are the theoretical distributions for various values of a, denoted by the numbers near the curves. The data points, denoted by circles and triangles, were obtained from the analysis of a 40 min sample of the 0.1 Hz signal obtained with the ±6% filter bandwidth setting from frog skin with Ringer's solution on both sides. No external current supplied; potential across skin was 80 mv. The circles are the data plotted with the assumption that $a = 0$; the triangles correspond to the same data with the assumption that $a = 1$. The circles coincide closely with the $a = 0$, zero sine wave content theoretical curve. Therefore, $a = 0$ for the experimental data.

that they contained no steady periodic component, in fact. The problem of the probability density function of the envelope of a signal consisting of a sine wave plus narrow band gaussian noise has been treated (Rice, 1954), and its solution is illustrated in Fig. 5. Also plotted there is a probability distribution obtained from frog skin voltage fluctuations at 0.1 Hz. The experimental points coincide closely with the curve corresponding to zero sine wave content, indicating that the signals measured and analyzed here are of a gaussian random process.

Although these signals are random, it is possible that they arise indirectly, and uninterestingly, from the effect of unavoidable temperature fluctuations. The magnitude of the temperature fluctuations required to account for the voltage fluctuations can be inferred from knowledge of the temperature coefficients of the skin resistance and potential. These were obtained by varying the temperature of the chamber and observing the effect on V and on R. V varies by a mean of 2.6%/°C (six determinations on three different skin samples with a mean open circuit resting potential of 42 mv) while R varies by a mean of −2.4%/°C (five determinations

on three samples). The voltage fluctuations of frog skin when between volumes of Ringer's solution amount to about 2.4 μv rms in the 0.1–10 Hz measurement band (when, as here, the area under investigation is 0.32 cm^2), as obtained by integrating the curve of Fig. 1. This is 0.0044% root mean square (rms) of the average skin potential, Fig. 1, and could be accounted for, therefore, by a temperature variation of 0.0017°C rms in the 0.1–10 Hz band. The maximum resistance fluctuation accounting for the data of Fig. 1 is that calculated by assuming $\Delta I = 0$. Then, according to the analysis leading to equation 1, $\Delta V/V = \Delta R/R$, which is 0.0044% rms and requires a temperature deviation of 0.0018°C rms.

There are two possible sources of temperature fluctuation: random heat exchange between the skin and the solutions surrounding it, and random temperature variations of the solutions themselves. In the first instance, the spectral intensity of the mean square temperature fluctuation is $4kbT^2/(b^2 + 4\pi^2f^2c^2)$, where k is Boltzmann's constant, T is absolute temperature, b is the coefficient of heat transfer between the skin and the surrounding solutions, and c is the heat capacity of the skin (Ziel, 1954). Thus, the power spectrum has nearly the same qualitative form as that of frog skin. The following calculation, however, shows that, quantitatively, spontaneous random heat exchange between the skin and it surround is an extremely unlikely source of the fluctuations in electrical potential observed.

The total theoretical rms temperature fluctuation of the skin is $(kT^2/c)^{1/2}$. If the fluctuations in V are to be accounted for fully by an rms deviation of skin temperature of 0.0017°C, as calculated above, then c must be 9.9×10^{-14} cal degree^{-1}. An estimate of the size of the structure within which the temperature fluctation would have to occur can be made as follows. If the heat capacity of the structure is 1.0 cal degree^{-1}, then it would weigh 9.9×10^{-14} g; with a density of 1.0, its volume would be 9.9×10^{-14} cm^3. The area of skin under investigation here is 0.32 cm^2 so the thickness of the active transport structure, if it were a flat sheet, would be 3.1×10^{-13} cm. This thickness is completely implausible for a biological layer having the electrical resistance of frog skin and within which active transport occurs.

The possible contributions of solution and chamber temperature fluctuations must be considered then. This question was approached empirically as follows. A thin, glass disk perforated by 10 μ diameter straight wall pores, was positioned in the usual location of the skin, instead of the skin. The chamber was filled with Ringer's solution which was then diluted until the resistance measured between the voltage electrodes was about the same as when a skin sample was present. A current of 11 μamp, about the maximum employed with skin samples in place, was passed through the chamber. Since the temperature coefficient of the resistance of electrolyte solutions is about -2%/°C, close to that of the skin, any significant temperature fluctuations of the solutions would be manifest as voltage fluctuations dependent upon the magnitude of the current flowing. With the identical instrumentation employed for the frog skin measurements, no such noise was detectable over the entire 0.1–10 Hz frequency range. The electrical noise with the glass disk in place

was independent of the presence or absence of current flow and was about equal to the theoretical Johnson noise of the measured electrolyte resistance. Thus, if significant temperature fluctuations do occur, they do so with spectral intensities outside the frequency range of the present experiments. There is, therefore, little reason to attribute ΔV or ΔR of frog skin to spontaneous temperature fluctuations.

This experiment also demonstrates that there are no relevant fluctuations generated at either the voltage or current electrodes of the chamber when current is supplied to the system.

DISCUSSION

Before considering particular mechanisms which might generate the voltage fluctuations associated with active transport, it is necessary to examine theoretically some purely physical sources of noise which might be expected to be evident in an electrochemical system such as frog skin. Green and Yafuso (1968) in their study of the electrical noise of inert ion-exchanger membranes examined two mechanisms of its production which should be considered here. The first of these, which they found under some experimental conditions, is current dependent noise arising from concentration fluctuations occurring across a narrow high resistance layer. In frog skin, such layers might exist at membrane-solution interfaces or within the cell membranes themselves. The power spectrum of the concentration fluctuations, however, is of the form $1/f^{1/2}$, which eliminates it therefore as an explanation of the present results. Nevertheless, at frequencies lower than explored here, or under special experimental conditions, such noise, if present, might appear. It would be worthwhile to search for it as its analysis could provide important clues to the nature of the boundary layer processes of biological membranes.

More relevant in the present context, perhaps, are the concentration fluctuations wnich arise during the diffusion and drift of ions in a constant electric field. Green and Yafuso have shown that the spectral intensity of these fluctuations is proportional to $1/f^2$ when $f/D \ll (zF\nabla\varphi/RT)^2$ and proportional to $1/f^{3/2}$ when $f/D \gg (zF\nabla\varphi/RT)^2$, where D is the diffusion constant of the ion, z its charge, F the Faraday, $\nabla\varphi$ the field strength, R the gas constant, and T absolute temperature. For a univalent ion at 293°K, the $1/f^2$ dependence would obtain below 10 Hz if D is the order of the free solution value for univalent ions, about 10^{-5} cm^2 sec^{-1}, and $\nabla\varphi \gg 60$ v cm^{-1}. The field strength condition can be met easily by biological membranes, and it is possible, therefore, that the power spectrum of Fig. 1, with its slope lying midway between 2 and $\frac{3}{2}$, is due to the passive movement of ions, i.e., it is unrelated to active transport. This interpretation is further supported by the observation that the power spectrum obtained when current flows through ouabain-treated, active transport inhibited skin is identical to that of the tissue when actively transporting sodium. Thus, it can be argued that in the inhibited skin the required $\nabla\varphi$ is provided by the externally supplied current flowing through the skin resistance, and in the transporting one it arises from the potential created by active transport.

Two observations, however, contradict this view of the origin of ΔV. First, the data of Figs. 3 and 4 demonstrating that ΔV cannot be assigned fully to the effect of ΔR are inconsistent with the constant field drift-diffusion mechanism. The fluctuations generated during diffusion and drift of an ion in a constant field $\nabla\varphi$ are concentration fluctuations and are evident, therefore, as resistance fluctuations. If these were the only significant fluctuations, ΔV would vanish when the passive movement of ions attendant to active transport is eliminated by an external current source, as when the skin is short circuited. Thus, the finding that ΔV reaches a minimum, finite asymptotic value as the potential across the skin approaches zero (Fig. 3) indicates that sources of fluctuations in addition to ΔR must be sought.

Second, the shape of the ΔV noise spectrum which emerges upon raising the temperature of the skin cannot be explained by the drift-diffusion fluctuations, as is evident from the asymptotic spectra of Green and Yafuso given above. According to their analysis, the spectrum of the drift-diffusion fluctuations shifts from a $1/f^{3/2}$ to a $1/f^2$ dependence as frequency decreases. The high temperature data of Fig. 2 does show such a transformation at about 1 Hz, but it is reversed at 0.25 Hz. The restriction of the $1/f^2$ dependence to a narrow middle frequency band, rather than as a low frequency asymptote, is clearly inexplicable as a manifestation of drift-diffusion fluctuations. As has been stated already, the high temperature spectrum of Fig. 2 is more completely explained as evidence of a relaxation process.

Consider then the following plausible mechanisms of active transport which might underlie the electrical fluctuations of frog skin measured and analyzed here. The first of these is the familiar one of active transport mediated by carrier molecules specific for the species transported. As applied to the particular case of frog skin, the hypothesis of carrier-mediated metabolically dependent ionic transport postulates a mechanism of the following general category. At the outer region of the skin, n Na^+'s combine with each carrier molecule C^m, where m is its ionic valence, to form $Na_n^+C^m$ which then moves across the skin to the inner region where it dissociates to form free Na^+ and C^m. Na^+ enters the solution phase in contact with the inner anatomical surface and C^m diffuses back across the skin. For the present considerations, it is unimportant whether or not C^m and/or $Na_n^+C^m$ is charged, C^m is mobile or is one of a chain of fixed sites. Nor does it matter whether metabolic energy enters the scheme as a driving force to transport $Na_n^+C^m$ or simply to form and/or dissociate $Na_n^+C^m$. The important matter is that a rate limiting step in the over-all transport process be a reaction of the form

$$nNa^+ + C^m \rightleftharpoons Na_n^+C^m. \tag{2}$$

Provided this is so, then ΔI, the fluctuations in the electrical current due to active transport, can arise from the inevitable spontaneous fluctuations in the concentration of $Na_n^+C^m$. Furthermore, if a very likely mobility condition obtains, then the noise spectrum of ΔR is similarly accounted for.

Each spontaneous dissociation of $Na_n^+C^m$ which occurs within the skin before its arrival at the inner anatomical border of the skin would have two effects: first, one less Na^+ is successfully transported by the skin, thereby diminishing the compensatory current I^* and, second, the concentration of free Na^+ and of C^m within the skin increases. Except for special circumstances (see equation 4, below), the latter causes an alteration in the electrical resistance of the skin. Thus, in this scheme, ΔI and ΔR are fully correlated in time as they are caused by the same event, the dissociation of the $Na_n^+C^m$.

The question of the form of the spectrum of the fluctuations in $Na_n^+C^m$ is analogous to that regarding the generation-recombination noise of the current carriers in solid state materials. Detailed treatments of this problem abound (Ziel, 1954, 1959; Vliet and Fassett, 1965) and are readily applied to the present situation. If ΔI is the consequence of the spontaneous dissociation of $Na_n^+C^m$, then the spectral intensity of ΔI^2, $S_{I^*}(f)$, is

$$S_{I^*}(f) = 4I^{*2}\frac{\overline{\Delta N^2}}{N_0^2}\frac{\tau_0}{1 + 4\pi^2 f^2 \tau_0^2}, \tag{3}$$

where N_0 is the equilibrium number of $Na_n^+C^m$, $\overline{\Delta N^2}$ its mean square fluctuation, and τ_0 is the average lifetime of a $Na_n^+C^m$ generated by the reaction between Na^+ and C^m. The power spectrum of the component of ΔV^2 due to ΔI^2 is simply $S_{I^*}(f)R^2$ where R is the average equilibrium value of the skin resistance.

The fluctuation in skin conductance arising from the concentration fluctuations of $Na_n^+C^m$ is calculated as follows. The fluctuation in total skin conductance ΔG is the sum of three components,

$$G = \Delta G_{Na} + \Delta G_{C^m} + \Delta G_{Na_n^+C^m},$$

where G_{Na} etc. denote the conductance due to Na^+ etc. The conductance G_i, due to the ith ion, is

$$G_i = Fz_i\mu_i c_i A/L,$$

where F is the Faraday, z_i is the magnitude of the ion's charge, μ_i is its velocity under unit potential gradient, c_i is its molar concentration, A is the area of the sample under study, and L is its thickness. Thus, the fluctuation in G_i due to the fluctuation in the number of i within the sample is given by $\Delta G_i = Fz_i\mu_i\Delta N_i/PL^2$, where N_i is the total number of i in the sample volume and P is Avogadro's number. If the fluctuations in N_{Na^+}, N_{C^m}, and $N_{Na_n^+C^m}$ are solely due to fluctuations in $Na_n^+C^m$ arising from the dissociation of the complex in accord with equation 2, then

$$n\Delta N_{Na^+} = \Delta N_{C^m} = -\Delta N_{Na_n^+C^m} \equiv \Delta N,$$

and

$$\Delta G = \frac{F\Delta N}{PL^2}\left(\frac{1}{n}\mu_{Na} + m\mu_{C^m} - (n-m)\mu_{Na_n^+C^m}\right).$$

The spectral intensity of ΔN^2 is $S_{I^2}(f)N_0^2/I^{*2}$ (Ziel, 1959) and, therefore, that of ΔG^2, $S_G(f)$, is

$$S_G(f) = \frac{4F^2}{P^2L^4}\left[\frac{1}{n}\mu_{Na^+} + m\mu_{C^m} - (n-m)\mu_{Na_n^+C^m}\right]^2 \frac{\overline{\Delta N^2}\,\tau_0}{1 + 4\pi^2 f^2 \tau_0^2}. \quad (4)$$

Note that $S_G(f) > 0$ if $\frac{1}{n}\mu_{Na^+} + m\mu_{C^m} \neq (n-m)\mu_{Na_n^+C^m}$. In the special case of the inequality not obtaining, the conduction fluctuations vanish.

It is evident from equations 3 and 4 that the frequency dependence of the spectral intensities of ΔG^2 and ΔI^2 are identical. Consequently, the frequency dependence of the spectrum of ΔV^2 is of the form $(1 + 4\pi^2 f^2 \tau_0^2)^{-1}$. For $4\pi_0^2 f^2 \tau_0^2 \gg 1$, the power spectrum varies as $1/f^2$; for $4\pi^2 f^2 \tau_0^2 \ll 1$, it is a constant, independent of f.

Thus, the spectra presented in Results, particularly those at 32°C, Fig. 2, correspond closely to that of a carrier-mediated transport mechanism.[1] At 20°C, the deviations from the $1/f^2$ dependence can be attributed to τ_0 having a distribution of values rather than a single one, as suggested above. These data, together with the additional observation that the spectra of ΔI^2 and ΔR^2 are nearly identical (Fig. 4), constitute good evidence that the active transport of sodium involves its interaction with a molecule specific to it.

It would be highly desirable to measure directly the instantaneous correlation between ΔR and ΔI. After all, it is quite conceivable that they are due to two independent mechanisms with the same spectrum. There are difficult technical problems to be surmounted in making such measurements, but the effort is well justified as a direct determination of the correlation in time of ΔR and ΔI would be convincing further proof that they arise from the dissociation of a sodium-carrier complex.

It is of interest to compare the value of τ_0 inferred from the noise spectra with the time it takes an actively transported sodium ion to cross the frog skin. The transit time for a sodium ion is several minutes, as can be deduced from the kinetics of

[1] The shape of the high temperature spectrum of Fig. 2 requires further comment. The curve has an inflection point, at about 0.4 Hz, centered within a region where the frequency dependence of the spectral intensity is about $1/f^2$. At higher and lower frequencies, the curve flattens. The high frequency flattening is not a characteristic of the generation-recombination mechanism, which suggests that at 32°C an additional source of noise becomes significant at high frequencies. Apparently, raising the temperature has two effects. First, the intensity of all noise sources is increased (cf. the absolute values of Figs. 1 and 2). Second, the shift of the main noise component to a simple relaxation spectrum characterized by a single time constant means that, because of the $1/f^2$ dependence, it vanishes within the bandwidth of the measuring system. Consequently, at high frequencies, the additional noise source is revealed. The data is too sparse to speculate about either its spectrum or origin.

tracer flux data (Curran et al., 1963). As the τ_0 of the 32°C spectrum is about 0.6 sec, there would be, therefore more than ample time for significant dissociation (and recombination) of $Na_n^+C^m$ to occur within the skin. This conclusion is significant because the theoretical spectra of equations 3 and 4 do not obtain when τ_0 is greater than the transit time (Ziel, 1954, 1959). The value of τ_0 which emerges from the present measurements is thus internally self-consistent with both the analysis presented and the known kinetics of the active sodium transport of frog skin.

There is at least one other interpretation of the frog skin noise spectra which can and should be entertained at this stage. In a more general sense, the electrical fluctuations of frog skin are simply evidence of a first order relaxation process. As a specific example, the rate limiting step in active transport need not be the reaction of equation 2 but rather one which provides the energy for the over-all transport process. The inevitable spontaneous fluctuations around the equilibrium point of the energy yielding reaction would lead to fluctuations in the amount of energy available for transport, and, as a final consequence, I^* would fluctuate. The frequency dependence of the spectral intensity of the energy fluctuations would be identical to that of equations 3 and 4. Hence, the spectrum of ΔI would be the same as for the generation-recombination mechanism. Unexplained, however, is why the energy fluctuation mechanism would lead to resistance fluctuations ΔR with the same spectrum as that of ΔI.

The plausibility of this mechanism as the source of ΔI can be tested by determining the rate constants of the metabolic pathways underlying active transport. This can be achieved by perturbing macroscopically the skin's temperature or oxygen supply and observing spectrophotometrically the kinetics of ensuing metabolic relaxations. The time constant of the rate limiting step, so determined, can then be compared with or contrasted to that deduced from the electrical fluctuations.

Stochastic variations in the electrical properties of nerve have been detected and analyzed. A comparison of these spectra to the present ones is of interest because, unlike frog skin, active transport makes a negligible direct contribution to the resting potential of nerve. In contrast to frog skin, both the node of Ranvier of frog nerve (Derksen and Verveen, 1966) and the giant axon of lobster (Poussart, 1969, 1971) display a noise power spectral intensity which varies as $1/f$. In the node, the measurements were of ΔV^2 with constant membrane current; in the lobster axon, they were of ΔI^2 under voltage clamp. Under the conditions of the nerve studies, there is some evidence that the fluctuations are associated with the passive flux of potassium across the membrane. The $1/f$ noise spectra of nerve is evidence, therefore, that the mechanism of such ionic movements is quite different from that of metabolically dependent ionic transport. This conclusion is neither surprising nor novel, but it does enhance the credibility of the present interpretation of the electrical fluctuations of frog skin.

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