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Fractal Methods to Analyze Ion Channel Kinetics

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We describe the traditional nonfractal and the new fractal methods used to analyze the currents through ion channels in the cell membrane. We discuss the hidden assumptions used in these methods and how those assumptions lead to different interpretations of the same experimental data. The nonfractal methods assumed that channel proteins have a small number of discrete states separated by fixed energy barriers. The goal was to determine the parameters of the kinetic diagram, which are the number of states, the pathways between them, and the kinetic rate constants of those pathways. The discovery that these data have fractal characteristics suggested that fractal approaches might provide more appropriate tools to analyze and interpret these data. The fractal methods determine the characteristics of the data over a broad range of time scales and how those characteristics depend on the time scale at which they are measured. This is done by using a multiscale method to accurately determine the probability density function over many time scales and by determining how the effective kinetic rate constant, the probability of switching states, depends on the effective time scale at which it is measured. These fractal methods have led to new information about the physical properties of channel proteins in terms of the number of conformational substates, the distribution of energy barriers between those states, and how those energy barriers change with time. The new methods developed from the fractal paradigm shifted the analysis of channel data from determining the parameters of a kinetic diagram to determining the physical properties of channel proteins in terms of the distribution of energy barriers and/or their time dependence.

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Science is difficult because it is hard to see what is obvious. Kuhn (1, 2) described how we fall into ways of thinking about scientific problems that he called "paradigms." We analyze and interpret experimental data within the framework of these paradigms. These paradigms give us the tools to understand the world. But when the paradigms do not match the world, then the paradigms prevent us from properly understanding the data. The assumptions of these paradigms are often so deep, and so accepted by mutual consent and repeated use, that we fail to see them. Yet, they have a dramatic effect on how we analyze and interpret experimental data. "We shape our tools, and thereafter our tools shape us" (3). In this article, we describe the traditional nonfractal and the new fractal methods used to analyze the currents through ion channels in the cell membrane. We make clear the hidden assumptions used in these methods and how those assumptions lead to different interpretations of the same experimental data.

PATCH CLAMP

The intracellular electrical voltage controls the internal functions inside the cell and its responses to external stimuli. This voltage is determined by the differences in concentration of ions across the cell membrane and its electrical conductivity. Ions cannot easily cross the lipid bilayer that forms the

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cell membrane. The conductivity of the cell membrane therefore depends on proteins that interact with the ions and assist them in crossing the membrane. One class of such proteins is ion channels. These proteins consist of a linear string of approximately 1000 amino acid residues laced several times through the membrane. Atomic, electric, and hydrophobic forces determine the three-dimensional conformational shape of the channel protein. Heat (thermal fluctuations), or the binding of ligands, or the voltage across the cell membrane supplies enough energy to switch the channel protein from one conformational shape to another. The study of the switching of the channel protein from one conformational shape to another is called ion channel kinetics. Some conformational shapes have a central hole that provides an open conduit for ions to cross the membrane. Other conformational shapes are closed to the passage of ions.

A small *patch* of cell membrane, containing only one ion channel, can be sealed in the mouth of a glass micropipet. The patch can be *clamped* at a constant voltage and the current measured. The membrane seals to the glass and the current through the patch measures the flow of ions passing through the channel protein. During the times when the channel is in a conformational shape open to the flow of ions the small current (picoamperes) through the channel can be resolved. At other times, no current is detected through the channel. Thus, the sequence of durations of times that the channel protein spends in open and closed conformational shapes can be determined (4, 5). This is a remarkable experimental technique because it measures the changes in time of the conformational state of an individual protein molecule. Neher and Sackmann were awarded the Nobel prize in physiology or medicine in 1991 for this accomplishment.

The sequence of times during which the channel protein is open or closed provides information about the structure of the ion channel protein and the dynamics of how it changes from one conformational shape to another. How these open and closed times depend on voltage, ionic concentrations, and ligands also characterizes the ionic selectivity of the channel and its pharmacology. We now describe the methods that have been used to analyze and interpret these data.

NONFRACTAL METHODS USED TO ANALYZE ION CHANNEL DATA

In the 1950s Hodgkin and Huxley measured the electric current through the cell membrane of a giant nerve fiber clamped under different voltage and ionic conditions (6). They stated that "our objective here is to find equations which describe the conductances with reasonable accuracy." They determined the values of adjustable parameters in these equations that best fit their data. "For the sake of illustration we shall try to provide a physical basis for the equations." The physical interpretation of the equations that they proposed was that the conductances depend on the number of discrete activating and inactivating particles occupying discrete locations in the cell membrane. They also added that they "must emphasize that the interpretation given is unlikely to provide a correct picture of the membrane." This was a prescient remark since physical measurements over the next 40 years showed that ion channel proteins were integral units and not the transient assemblies that they had postulated.

Notwithstanding the balanced and cautious remarks of Hodgkin and Huxley, almost all subsequent attempts to fit and model experimental data from ion channels assumed that channel proteins have a few discrete conformational shapes and switch almost instantaneously from one to another. This occurred despite the fact that Fitzhugh (7) and Nagumo *et al.* (8) had shown in the early 1960s that the Hodgkin–Huxley equations could be well approximated by equations having continuous, rather than discrete states. The essential element in the Hodgkin–Huxley equations was not the discrete nature of the channel states, but the nonlinearity of the conductance and voltage. That is, the conductances that determined the voltage across the cell membrane themselves depended on the voltage. Once the voltage started to change, the conductances changed, which changed the voltage even more. This nonlinearity generated and propagated the action potential. Nonetheless, the discrete nature of channel states became the established method to analyze and interpret new types of experimental data that became available over the next four decades.

In the 1980s, the sequence of open and closed times from individual channels was measured by the patch-clamp technique. These times were then used to form histograms of how frequently the channel was open or closed for durations greater than t and

less then $t + \Delta t$. The method used to analyze and interpret these histograms was based on the paradigm that there are a few channel states and that the probability of switching from one state to another depends only the present state of the channel, rather than its history of previous states (4, 5). These mathematical assumptions correspond to a physical model of a discrete set of conformational shapes of the channel protein separated by fixed energy barriers (9, 10). As the channel protein gains or loses energy, it jumps from one conformational shape to another. There is only one pathway between each set of conformational shapes. Since the energies of each conformational shape are constant, the probability of switching between those states is also constant over each increment in time. This picture was consistent with the understanding at that time of chemical and biochemical reactions where the association of $A + B \rightarrow C$ or the dissociation $C \rightarrow A + B$ occurs over a fixed, unique energy barrier. It was also consistent with the Hodgkin–Huxley model where the "discrete activating and inactivating particles" were now interpreted as gating charges, that is, electrical charges in the molecular structure of the channel that clicked into new positions when the channel protein switched into a new conformational shape. The currents due to movement of these gating charges in response to voltage changes had been directly measured.

These assumptions imply that the open- and closed-time histograms have the form of a sum of exponential terms such as $a_1e^{-(b_1t)} + a_2e^{-(b_2t)} + a_3e^{-(b_3t)} + \ldots$ (4, 5). The number of such exponential terms corresponds to the number of discrete channel states. For example, if a channel has one open (O) and two closed (C_1, C_2) states, then the open-time histogram has the form $a_1e^{-(b_1t)}$ and the closed-time histogram has the form $a_2e^{-(b_2t)} + a_3e^{-(b_3t)}$. Typically, the channel cannot switch from any state to every other state. There are only certain pathways between the states. These pathways can be summarized in a kinetic diagram such as $O \leftrightarrow C_1 \leftrightarrow C_2$ or $C_1 \leftrightarrow O \leftrightarrow C_2$.

The method used to analyze and interpret the open- and closed-time histograms was to fit them with the sum of exponential terms and determine the kinetic diagram of the pathways between the discrete states. It is important to understand that the choice of the sum of single-exponential functions was based on the paradigm that there are a few discrete states separated by fixed energy barriers. It is not that different functional forms were tried and that this form was found to best represent the data. Rather, it was assumed that this form was the only meaningful form. If one exponential did not fit the data then the problem must be that there were additional discrete states and thus the sum of two exponential terms was used. If the sum of two exponentials were not sufficient to fit the histograms, then three would be used, etc. Kuhn makes the point that once a paradigm is established, scientific work consists of analyzing and interpreting data within the context established by that paradigm. The difficulties that the paradigm has in matching the data are dealt with by adding additional complexity to the equations of the paradigm, rather than investigating if the paradigm is flawed. A few studies had found evidence of nonlinearities in time (11–13) or the time dependency of the channel structure (14), but these did not influence how the patch data were analyzed.

FRACTAL METHODS USED TO ANALYZE ION CHANNEL DATA

A number of qualitative properties indicated that the patch-clamp data had fractal properties. This suggested new quantitative methods of analyzing the data that have led to a new picture of the physical properties of ion channel proteins.

Three signs indicated that the patch-clamp data had fractal properties:

1. The average rate of openings and closings changed abruptly in time. For a nonfractal process, the change in the average in the data signals a change in the mechanism that is generating the data. Thus, it was suggested that the mechanisms that cause ion channels to open and close were changing erratically in time. However, a mechanism that is fixed in time can also generate fractal data with average properties that change abruptly in time (9, 15–17). For example, a fractal signal in time can consist of a hierarchy of bursts of increased activity. The lowest level of bursts is separated by brief periods of inactivity. These bursts are then clustered into groups separated by longer periods of inactivity. Then, the bursts of bursts are clustered into groups separated by even longer periods of inactivity, etc. The average rate of activity measured will depend on whether it was measured within the bursts or the periods of inactivity, and it will change abruptly when measured at the onset of the next hierarchy of bursts. The pattern of bursts within bursts within bursts was common in the patch-clamp data, which

raised the possibility that the variation in the average rate of openings and closings was due to a constant mechanism that generated a self-similar, fractal pattern.

2. The trace of the current shifting between the open and closed states recorded on a chart recorder looked similar when the chart paper was moving at different speeds. Thus, the pattern of open and closed times was similar when measured at different time resolutions, which is characteristic of a fractal. This is illustrated in Fig. 1.

3. The durations of open and closed times extended over a large range of times from milliseconds to hours. There was no single value that was well representative of the durations of the open or closed times. That is, the open- or closed-time histograms were not a gaussian distribution that could be characterized by an average open- or closed-time duration. Rather, within the limits of the time resolution of the patch-clamp technique, there was an ever larger number of ever smaller open or closed times. The large range of these times, which cannot be represented by one scale, is characteristic of a fractal.

Methods that match the properties of experimental data are likely to be the best approach to analyze the data and interpret the results. We describe here two fractal methods that are useful in analyzing patch-clamp data. These methods would also be useful in analyzing other data that have similar fractal characteristics, such as the rate of respiration (18) or interspike intervals recorded from nerve cells (19).

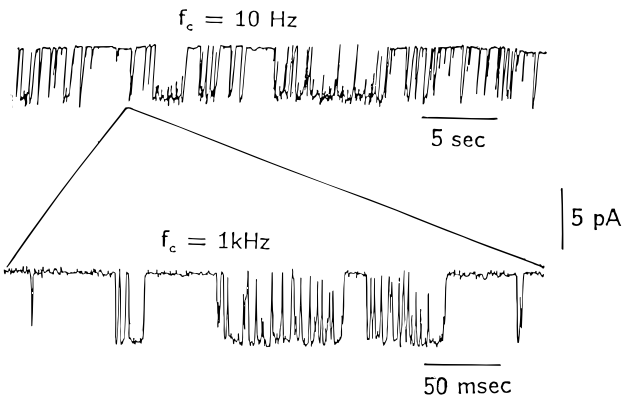

FIG. 1. Using the patch-clamp technique, the current as a function of time was recorded through a single ATP-sensitive potassium channel of rat pancreatic B cells. The channel spontaneously fluctuates between states that conduct and do not conduct the current. This can be seen when the record is displayed at low time resolution (10 Hz). However, when it is displayed at higher time resolution (1000 Hz), each opening or closing can be seen to actually consist of many briefer openings and closings. This is an example of statistically self-similarity in a time signal. The behavior at finer time resolution is similar to itself at coarser time resolution. (This figure by Gillis, Falke, and Misler (38) is reproduced with permission, from the New York Academy of Sciences.)

Multiscale Probability Density Function (PDF)

The nonfractal method constructs histograms of the number of open or closed times, $N(t, t + \Delta t)$, greater than time t and less than time $t + \Delta t$. These histograms are formed from bins of fixed range Δt. The mathematical function that characterizes the data is the probability density function, PDF(t), which is the probability that a time in the data is greater than t and less than $t + \Delta t$. The PDF can be determined from these histograms, namely, $\text{PDF}(t + \Delta t/2) = N(t, t + \Delta t)/(N_T \Delta t)$ where N_T is the total number of open (or closed) times measured.

The fractal character, that the open and closed times extend over a large range of time scales, implies that it is important to accurately determine the PDF over a large range of times t. The nonfractal method was not successful at this because the histograms depended on the size, Δt, of the bins used. Small bins include many times when t is small, but few times at large t. Hence, the estimate of the PDF is accurate at small t, but not at large t. Wide bins include more times at large t, but their resolution is poor at small t. Hence, the estimate of the PDF is accurate at large t, but not at small t. Fractals are characterized by events at all time scales. Thus, the fractal method consists of determining the PDF from histograms of different bin sizes and then combining them into a single PDF (20, 21). We use the word *fractal* to describe this method because it was motivated by fractal concepts that suggested the importance of sampling the data at different time scales. However, this multiscale method is capable of accurately characterizing both fractal and nonfractal PDFs.

This fractal method has four advantages over the nonfractal method in determining the PDF: (1) It uses small bins to accurately determine the PDF at small t and large bins to accurately determine the PDF at large t. (2) It uses the PDFs determined from each histogram rather than the histogram itself. These PDFs can then be combined into a single function, while the histograms themselves cannot be combined. (3) It generates more points in the PDF function at time scales that have more data so that functions used to fit the PDF are properly weighted by the relative accuracy of the amount of data at

different time scales. (4) It is efficient at determining the form of the PDF when the number of times is small.

Some aspects of a multiscale approach are present in using histograms with logarithmically increasing bin sizes (22). However, the fractal method described here is considerably simpler and computationally more efficient. Moreover, the histograms determined at different bin sizes in the fractal method can then be used to determine the effective kinetic rate constant (described below).

The fractal method consists of evaluating histograms of different bin sizes, determining the PDF from each histogram, and then combining some of those values to form the completed PDF. The bin size of the first histogram is determined by using trial and error to find the smallest bin $\Delta t_{\min}$ for which there are times in the first four bins. PDF($[k - 1/2]\Delta t$) = $N(k)/(\Delta t\ N_T)$, where N_T is the total number of times and $N(k)$ is the number of times in the range $(k - 1)\Delta t < t \leq k\Delta t$ in the histogram. The value computed from the first bin, $k = 1$, is excluded from the final PDF because it includes all the times unresolved at resolution Δt. The values included in the final PDF are those computed from the second bin, $k = 2$, and higher bins, $k > 2$, stopping at either the first bin, $k = k_{\max}$, which contains no times, or $k = 20$, whichever comes first. The values beyond this range are excluded because the times in these bins are too sparse to give good estimates of the PDF. Then, the next histogram is formed with bin size $2\Delta t_{\min}$ and the values of the PDF are determined using the same procedure. This process is iterated, doubling the bin size of the histogram each time. This is continued until the first time that there are no times in the second, third, or fourth bin. The PDFs determined from the different bin sizes are then combined to form the final PDF. We developed programs in Microsoft QuickBASIC on the Macintosh and C on the Silicon Graphics workstation to implement this method. The C version is presented in the Appendix.

The fractal method is successful is determining PDFs that have single-exponential (Ae^{-at}), multiple-exponential ($Ae^{-at} + Be^{-bt}$), stretched-exponential ($A \exp[-kt^a]$), or power law (At^{-a}) forms. For example, in Fig. 2, the nonfractal fixed bin histogram method and the fractal multiscale method are used to determine the PDF from test data of 1000 times generated from a single exponential PDF and power law PDF. The PDF determined by the fractal method extends over a larger range of times, is more precise (has less scatter with corresponding larger correlation coefficient r^2), and is more accurate (the parameters determined by a least-squares fit are closer to true values of the PDF used to generate the data). The success of this new method illustrates how the fractal paradigm, which emphasizes the importance of analyzing the data over many different time scales, can lead to accurate and efficient new quantitative methods of analyzing experimental data.

Effective Kinetic Rate Constant (k_{eff})

The nonfractal method determined the probability, called the kinetic rate constant, that a channel will switch from open to closed or from closed to open in the next time increment Δt. It was assumed that this probability is constant. The effective kinetic rate constant of the fractal method is a generalization of the kinetic rate constant that does not assume that this probability is a constant.

The self-similarity of a fractal means that an ever larger number of smaller pieces are revealed when the fractal is measured at finer resolution. As these additional pieces are included in the measurement, the value measured for a property (such as length, area, or volume) will change. Thus, the value of property depends on the scale of resolution at which it is measured. The fractal character of the open and closed times therefore suggested that we determine how the probability of a channel switching states depends on the time resolution at which it is measured. That is, the channel must be open or closed long enough for us to detect it in that state. Thus, we determine the conditional probability that it switches states in the next increment of time dt, given that it has already remained open or closed for at least a time that we called the effective time, t_{eff}. The effective time t_{eff} determines the time resolution. We called this conditional probability the effective kinetic rate constant, k_{eff}, because it is the kinetic rate constant measured at a given time resolution (20, 21). The scaling relationship for a fractal characterizes how the value measured depends on the measurement scale. We can therefore analyze the patch-clamp data by determining the scaling relationship of the dependence of k_{eff} on t_{eff}. There will be one scaling relationship $k_{\text{eff}}(t_{\text{eff}})$ determined from the switches from open to closed and another scaling relationship determined from the switches from closed to open.

The same scaling relationship function $k_{\text{eff}}(t_{\text{eff}})$ can be derived from two different approaches. First, in

terms of the fractal scaling, $k_{eff}(t_{eff})$, characterizes how the probability of changing states, k_{eff}, depends on the time resolution, t_{eff}, used to measure that probability. Second, in terms of conditional probabilities, the effective kinetic rate constant k_{eff} characterizes how the probability that the channel switches states depends on the time t_{eff} that it has been in a state. These two interpretations are equivalent because the system must remain in a state a minimum of time t_{eff} to be detected at time resolution t_{eff}.

This same conditional probability function is widely used in other disciplines. In renewal theory

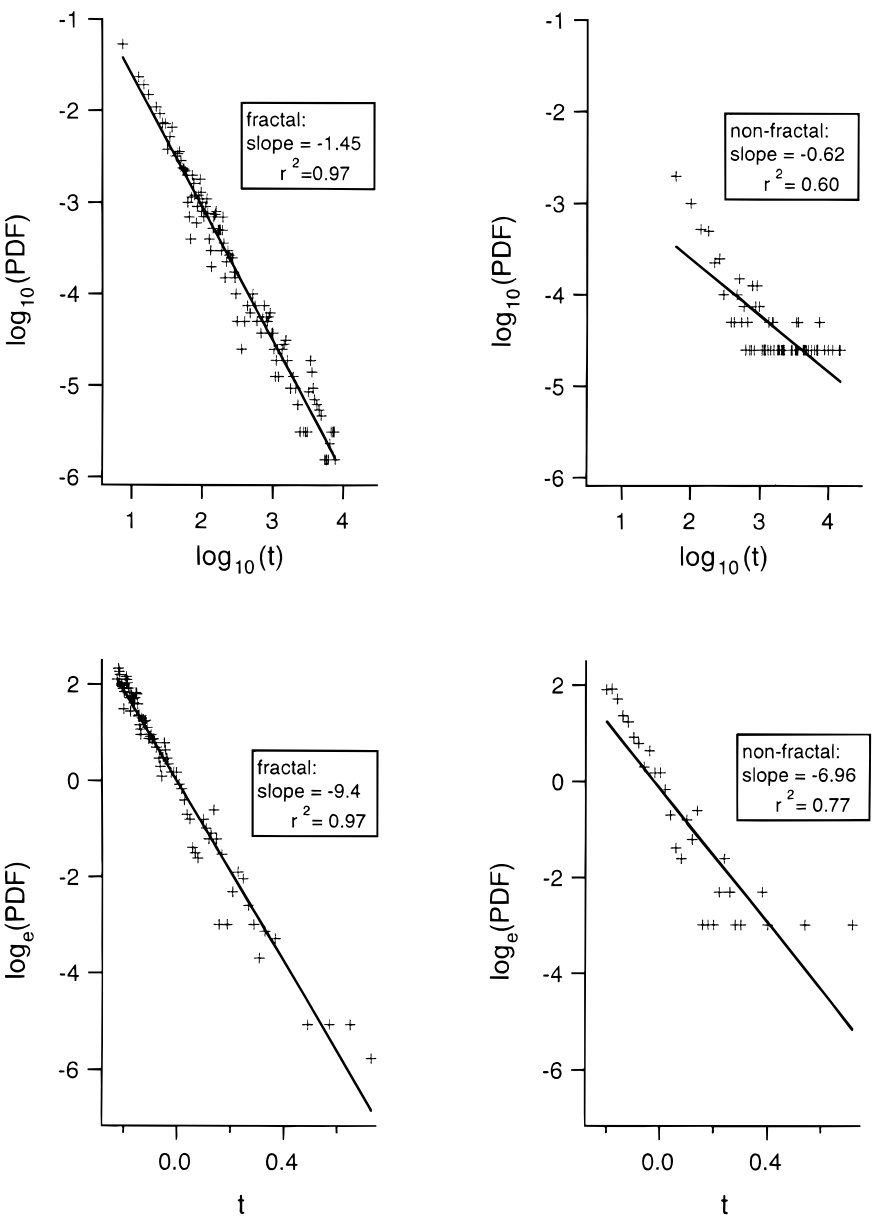

FIG. 2. This figure illustrates that methods of evaluating the probability density function (PDF) motivated by fractal concepts are both more precise and more accurate than nonfractal methods whether the PDF has fractal, power law or nonfractal, single-exponential form. One thousand closed times were simulated from PDFs of the form $t^{-1.5}$ (top) and $\exp^{-10t}$ (bottom). The PDFs determined by the fractal method (left) are more precise (less scatter, larger correlation coefficient r) and more accurate (the parameter values found by least-squares fit are closer to the correct values of -1.5 and -10) than those obtained by the nonfractal method (right).

(which studies the failure rates of components) it is called the age-specific failure rate, and in life insurance and epidemiology it is called the survival rate (23, 24). These conditional probabilities all measure the probability of changing states in the next time increment Δt, given that the system has already survived a time t.

A number of different methods can be used to determine $k_{eff}(t_{eff})$ from the patch-clamp data. The simplest and best method is to use the histograms of different bin size Δt used to determine the PDF described above. For each histogram, $t_{eff} = \Delta t$, where Δt is the bin size. A single exponential, Ae^{-kt}, is then fit to the second, third, or fourth bins of the PDF. This can be done by using least squares to fit a straight line to log[PDF(t)] versus t. The slope of this line is then given by $k = k_{eff}$. The procedure is repeated for each histogram to evaluate the function $k_{eff}(t_{eff})$. The C program in the Appendix to compute the PDF also includes the computation of $k_{eff}(t_{eff})$. The accuracy of this method is illustrated in Fig. 3, where the $k_{eff}(t_{eff})$ function computed in this way closely matches that computed analytically from test data of different models of channel kinetics (20).

There are also additional methods to compute $k_{eff}(t_{eff})$ (9): (1) The maximum likelihood estimator of the kinetic rate constant can be evaluated by using only open or closed times within a given time range, which determines the effective kinetic rate constant at the effective time set by that range of times (25).

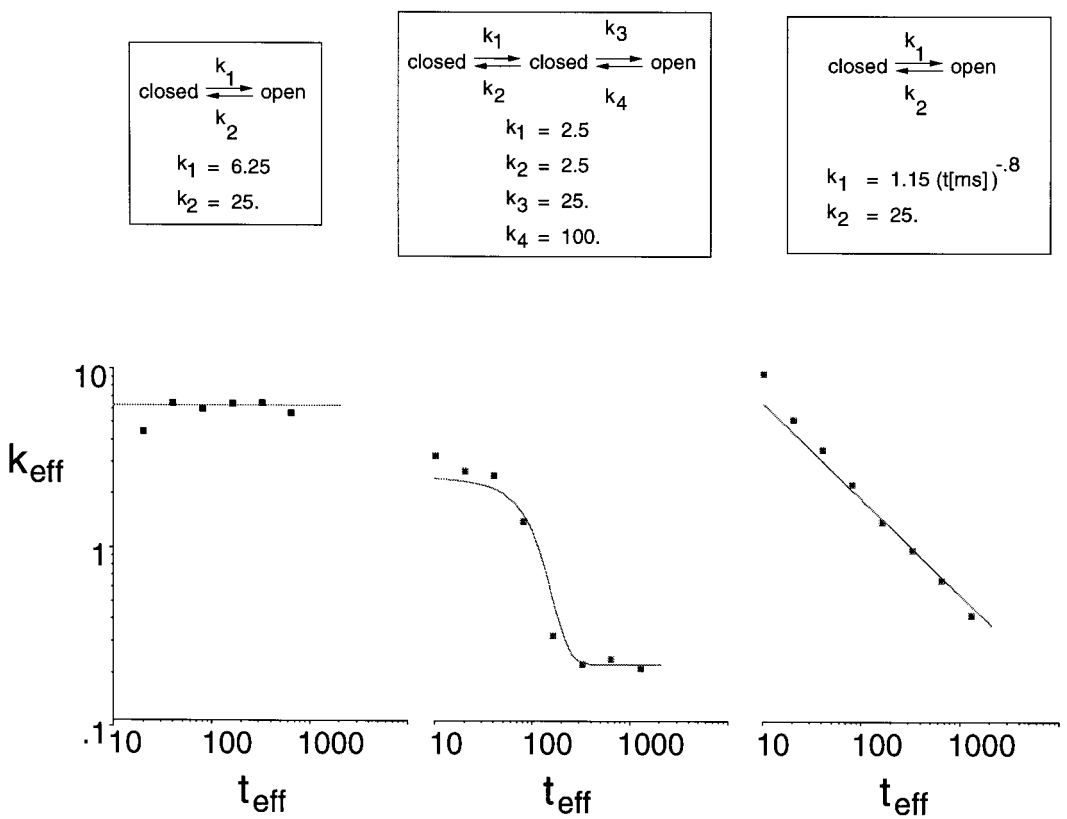

FIG. 3. The fractal method analyzes the statistical properties of the open and closed times by determining how the probability of switching between these states depends on the time resolution at which it is measured. The effective kinetic rate constant, k_{eff}, is the probability of switching from the closed to open or from the open to closed states. The fractal scaling relationship is characterized by the dependence of k_{eff} on the effective time scale t_{eff} at which it is measured. This relationship can be determined from histograms of different bin size, where the bin size $\Delta t = t_{eff}$. These plots show that this procedure is an accurate method of determining k_{eff} from the open or closed times. Two thousand three hundred forty-one closed times were computed from each of three kinetic diagrams that have PDFs of single-exponential (left), multiple-exponential (middle), and power law (right) forms. Histograms of different bin size were then used to determine the k_{eff} to switch from the closed to open state from these closed times. For these kinetic diagrams, the scaling function $k_{eff}(t_{eff})$ can be evaluated analytically. Considering the relatively small number of closed times, the values determined by using the fractal method of histograms of different bin size (points) are a good match to the analytic result (lines).

(2) As described below, the PDF is directly related to the k_{eff}, and thus $k_{eff}(t_{eff})$ can be determined by numerical differentiation of the PDF. (3) The effective time scale can be set by resampling the current data at different analog-to-digital conversion rates and the effective kinetic rate constant can be determined from single-exponential fits of the histograms evaluated at each sampling rate (20). (4) Correlation functions can be used to determine the kinetic rate constant directly from the current data without the need to first identify and measure the duration of open and closed times (26). The effective kinetic rate constant can be determined at different delay times in these correlation functions, which correspond to different effective time scales.

Relationship between PDF and k_{eff}

The PDF and k_{eff} fractal methods of analyzing the patch-clamp data are related to each other. For example, the best method in practice to determine k_{eff} uses the histograms of the open and closed times evaluated at different bin sizes that are needed to determine the PDF. Formally, k_{eff} is a transformation of the PDF (20, 21). That is,

$$k_{eff}(t_{eff}) = -\{d[\ln P(t)]/dt\} \quad \text{evaluated at } t = t_{eff} \tag{1}$$

where

$$P(t) = \int_{t'=t,\infty} \mathrm{PDF}(t')\, dt' \tag{2}$$

defines the cumulative probability that the channel is open or closed for a time t. This quantitative relationship can be understood in the following qualitative way. The PDF characterizes the frequency of short and long times. The k_{eff} characterizes how many additional short times are found when the data are examined at finer temporal resolution. Thus, evaluating an appropriate function of the PDF, at an effective time scale, makes it possible to determine how many additional short times will be found when the data are examined at finer temporal resolution.

Although mathematically equivalent, in practice, some functional forms are easier to recognize when the data are plotted using one method or the other. Both methods are good at identifying PDFs with single-exponential (Ae^{-at}) or power law (At^{-a}) forms. The PDF method is better at identifying well-separated segments of these forms. The k_{eff} method is better at identifying PDFs with a stretched-exponential ($A \exp[-kt^a]$) form.

Interpretation of the Results of the Fractal Methods of Analysis

The nonfractal method assumed that channel proteins have a small number of discrete conformational states separated by fixed energy barriers and that they switch along unique pathways from one state to another. The fractal method of analyzing the data did not make these assumptions. How can we then interpret the results of the fractal PDF and k_{eff} methods? Two different interpretations have been proposed (9, 10). The *structural* approach interprets the PDF and k_{eff} plots in terms of a distribution of energy barriers that separate a set of open and closed conformational substates. The *dynamical* approach interprets the PDF and k_{eff} plots in terms of how rapidly the energy barriers that separate the open and closed states change in time. Other interpretations may also be developed in the future. The central point is to interpret the results of the fractal method in terms of physical properties of channel proteins so that we can use the data to understand the structure and function of the channel, or of pieces of the channel, under different experimental conditions.

The number and size of energy barriers that separate the states or their time dependency can be directly determined from the PDF and k_{eff} plots. In the structural interpretation, the difference in energy between two states, ΔE, is proportional to $\log(k)$, where k is the kinetic rate constant of switching between these states. The distribution $g(\Delta E)$, the relative number of energy barriers of energy difference ΔE, is related to the distribution $g(k)$, the relative number of the rate constants across those energy barriers. The relationship is that $g(k)\, dk = g(\Delta E)\, d(\Delta E)$. The distribution $g(k)$ depends on the cumulative probability $P(t)$, which is the integral of the PDF:

$$P(t) = \int_{k=0,\infty} g(k)\, e^{-kt}\, dk\,. \tag{3}$$

The distribution $g(k)$ can be determined as the inverse Laplace transform of $P(t)$. In the dynamical interpretation, the time dependence of $k(t)$ is determined from the relationship of the effective kinetic rate constant, namely, $k(t) = k_{eff}(t_{eff})$, where $t = t_{eff}$.

Thus, the PDF and k_{eff} can be used to determine the physical properties of the channel protein from the patch-clamp data. The nonfractal method had made very restrictive assumptions on the nature of the physical properties of channel proteins. Namely, it was assumed that channel proteins have a small number of discrete states separated by fixed energy barriers. The goal of the nonfractal analysis was to determine the parameters of these states that best fit the data. The nonfractal analysis did not address the question of whether channel proteins have these assumed properties. The interpretation of the fractal method uses a much less restrictive set of assumptions. The goal of the fractal analysis is to determine the number of channel states and the distribution of energy barriers between them, or the variation in time of the energy barriers between those states. Thus, the fractal analysis can reveal if a channel has a small number of discrete states separated by fixed energy barriers, as assumed by the nonfractal method. In addition, it can also reveal if a channel has a large number of states and time-dependent energy barriers.

The PDFs of the open or closed times found in the patch-clamp data are either entirely of one functional form or of segments of these forms. These forms are either single exponentials (Ae^{-at}), stretched exponentials ($A \exp[-kt^a]$), or power laws (At^{-a}). These forms represent a continuum of different physical properties of the channel protein. In the structural interpretation, as the form of the PDF changes from a single exponential, to a stretched exponential, to a power law, the number of conformational substates increases and the width of the distribution of energy barriers widens. In the dynamical interpretation, the energy barrier to exit a state changes in time. For all channels, the energy barrier is either constant or increases with time, as if the dynamics of the channel protein cause it to settle into an ever more stable configuration. In the dynamical interpretation, as the form of the PDF changes from a single exponential, to a stretched exponential, to a power law, the energy barrier increases more rapidly in time. Thus, it is possible to interpret how the changes in the PDF caused by voltage, ionic composition, ligands, and temperature are altering the physical properties of ion channel proteins.

Figure 4 illustrates how the fractal method of analyzing the patch-clamp data can be used to yield information about the physical properties of channel proteins. The closed times from a potassium channel in neuroblastoma × glioma cells (27) are a straight line on plots of log(PDF) versus log(t) and log(k_{eff}) versus log(t_{eff}), indicating that they have a power law (At^{-a}) form. Thus, this channel protein has a large number of closed conformational shapes, many different ways that its shape can change to reach the open state, a wide distribution of energy barriers out of the closed state, and/or an energy barrier to exit the closed state that increases rapidly with the time already spent in the closed state. On the other hand, the open times from a potassium channel in hippocampal neurons have a single-exponential PDF and a constant k_{eff} (21). Thus, this channel protein has only one open conformational shape, only one way that it changes its shape to close, a narrow distribution of energy barriers out of the closed state, and/or an energy barrier to exit the open state that remains constant in time. The closed times of a chloride channel from skeletal muscle (28) have a PDF and k_{eff} with segments of different forms at different time scales. At intermediate times, the PDF is approximately a stretched exponential ($A \exp[-kt^a]$) and the k_{eff} is approximately a power law (At^{-a}), indicating that the dominant process that operates at this time scale corresponds to one with an intermediate number of closed conformational shapes, an intermediate number of ways its shape can change out of these states, a distribution of energy barriers of modest width, and/or an energy barrier to exit the closed state that rises slowly in time.

NONFRACTAL VERSUS FRACTAL PARADIGMS

The nonfractal and fractal methods interpret the same data in different ways. The nonfractal method assumes certain restricted characteristics for the physical properties of the ion channel protein, namely, it has a small number of discrete conformational shapes, separated by fixed energy barriers. This can be represented as a kinetic diagram with a small number of states connected by pathways characterized by kinetic rate constants equal to the probability per second that the channel switches from one state to another. The goal of the nonfractal method is to determine the parameters of this kinetic diagram, that is, the number of states, how they are connected, and the values of the kinetic rate constants connecting them.

The fractal method assumes a more general class of possible characteristics for the physical properties

of the channel protein, such as a distributions of energy barriers (in the structural interpretation) or their time dependency (in the dynamical interpretation). This more general class of characteristics also includes, as one special case, the more restricted characteristics assumed by the nonfractal method. The goal of the fractal method is to determine the physical properties of the channel protein.

There is divided opinion as to the relative merits of the nonfractal and fractal methods of analyzing the data and their interpretations of the results. Since each method has a different goal, some arguments talk past, rather than address each other. The goal of the nonfractal method is to determine the parameters of the kinetic diagram that best match the data. The goal of the fractal method is to deter-

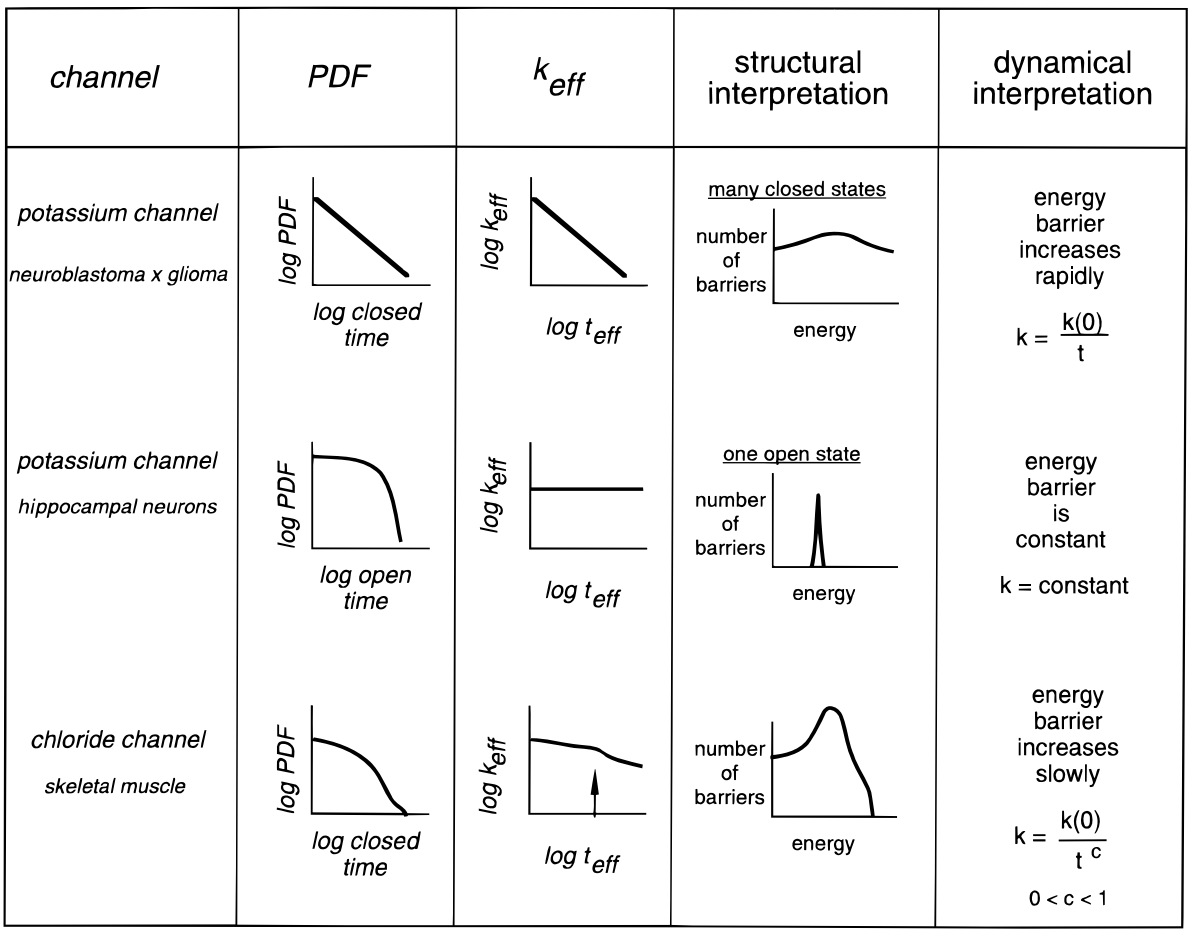

FIG. 4. The fractal method uses the multiscale probability density function (PDF) and the effective kinetic rate constant (k_{eff}) to analyze the open and closed times. The results can then be interpreted in terms of the energy structure of the channel (structural interpretation) or the time dependency of the energy barriers (dynamical interpretation). *Top*: The closed times of a potassium channel in neuroblastoma × glioma cells measure by McGee *et al.* (27) are a straight line on plots of log(PDF) versus log(t) and log(k_{eff}) versus log(t_{eff}). This indicates that this channel protein has a large number of closed states and therefore a wide distribution of energy barriers, and/or an energy barrier to exit the closed state that increases rapidly with the time. *Middle*: The open times from a potassium channel in hippocampal neurons measure by Liebovitch and Sullivan (21) have a single-exponential PDF and a constant k_{eff}. This indicates that this channel protein has only one open state and therefore a narrow distribution of energy barriers, and/or an energy barrier to exit the open state that remains constant in time. *Bottom*: The closed times of a chloride channel from skeletal muscle measured by Blatz and Magleby (28) have a PDF and k_{eff} with segments of different forms at different time scales. At intermediate times, the PDF is approximately a stretched exponential ($A \exp[-kt^a]$) and the k_{eff} is approximately a power law (At^{-a}). This indicates that the dominant process that operates at this time scale has an intermediate number of closed states and therefore a distribution of energy barriers of modest width, and/or an energy barrier to exit the closed state that rises slowly in time.

mine the physical properties of the ion channel protein. The fractal approach has questioned whether the assumptions used in the nonfractal analysis are a valid representation of the physical properties of ion channel proteins.

A number of arguments have been presented in support of the nonfractal approach (for details, see Refs. 29–33): (1) The existence of proteins in distinct conformational forms, such as the forms of myoglobin either bound or unbound to oxygen, supports the idea that channel proteins have a small number of distinct states. (2) The time course of chemical reactions has been successfully characterized by changes between a small number of fixed energy levels. (3) The brief duration of the gating currents, the shifts in electrical charges when the channel protein changes shape, indicates a sudden change from one discrete conformational shape to another. (4) Although biophysical measurements have found that proteins, such as myoglobin, have a large number of conformational substates and time-dependent changes in structure, the conditions under which those measurements were done (typically low temperatures and fast picosecond time scales) are not representative of conditions in the cell membrane (body temperature at millisecond time scales). The properties of channel proteins may be different from those of other proteins. (5) By adding additional states to the kinetic diagrams and adjusting the additional parameters, the PDFs of the nonfractal method can fit the data to high accuracy. (6) The parameters determined from the kinetic diagrams provide numbers to characterize experimental data. The dependence of these numbers on different experimental conditions can then be determined. The fractal method does not provide a simple set of such parameters.

In our view, the arguments in support of the fractal method and its interpretation are more convincing (for details, see Refs. 9, 10, 34–37): (1) Extensive theoretical and experimental work over the last 20 years, using many different computational and experimental techniques, has demonstrated that globular proteins have large numbers of conformational substates, wide distributions of energy barriers between these states, and energy barriers that vary in time (9, 10). (2) The dynamics of motions in proteins is now known to be an essential element in how they function in chemical reactions. For example, the motions within myoglobin play an important role in how pieces of the protein fluctuate out of the way so that oxygen can reach its binding site in the interior of the protein. Static models, with a few fixed energy barriers, do not predict the correct rates of oxygen binding in myoglobin. (3) In systems of many interacting pieces nonlinear interactions often lead to coherent global effects. The existence of simple gating currents does not necessarily imply that there are only simple, discrete changes in protein structure. (4) Many different proteins have been found to have large numbers of conformational substates and time-dependent changes in structure over a wide range of temporal and temperature scales (9, 10). These measurements have not yet been done on channel proteins in the cell membrane. There is no evidence to suggest that channel proteins have properties that are manifestly different from those of all other proteins. (5) Additional states are added to the kinetic diagrams of the nonfractal method until those models fit the data. Thus, a high goodness of fit is achieved, not necessarily because they are valid, but because of the additional number of adjustable parameters (34, 35). (6) Some scientists desire a standard formula to determine the parameters that characterize their data. They have relied on the rate constants of the kinetic diagrams of the nonfractal method to provide these parameters. However, since channel proteins do not have the properties assumed by the nonfractal method, those parameters do not provide a meaningful way to understand the structure and function of channel proteins. The difficult and exciting part of science is to discover the underlying physical mechanisms that are responsible for the observed characteristics of the data. There is no simple mechanical or automated analysis that can accomplish this task.

CONCLUSION

To analyze the open and closed times of ion channel proteins recorded by the patch-clamp technique, the nonfractal method assumed that channel proteins have only a few discrete states separated by fixed energy barriers. The goal was to determine the parameters that characterize the kinetic diagram. Additional states were added to kinetic diagrams so that these models can fit the data, without testing the validity of the assumptions. In fact, extensive theoretical and experimental evidence demonstrated that proteins have many conformational substates, broad distributions of time-dependent energy barriers, contradicting the assumptions of the nonfractal method.

Fractal methods provide new ways to analyze and interpret these data. The fractal methods more closely match the multiscale and self-similar properties of the data. Thus, the results of the fractal analysis can better characterize the properties of the data. There are different ways to interpret the results of the fractal analysis. The structural and dynamical interpretations make less restrictive assumptions about the physical properties of channel proteins than the assumptions made by the nonfractal methods. These interpretations of the fractal method are more consistent with the known biophysical properties of proteins. This makes it possible to use the data to determine the physical properties of channel proteins and their dependence on voltage, ionic composition, ligands, and temperature. The goal of the fractal approach is to use the data to reveal the physical properties of channel proteins, rather than to determine the parameters of a kinetic diagram. This change in paradigm may lead us to a better understanding of the physical properties of ion channels, their energy structure, and the role of time-dependent motions in the function of channel proteins.

APPENDIX

The following program in C computes the multiscale probability density function (PDF) of the open or closed times and the effective kinetic rate constant (k_{eff}) by the fractal methods described in the text. This version requires 8 MB of RAM to execute. If necessary, this size can be reduced by reducing the size of the input data array. The DATA input into the program are a floating point file of up to n__max = 2,000,000 open or closed times. The output of the program is the PDF generated from histograms HYST of different bin size j*dt, where dt is the minimal bin size chosen by the user. The first output file consists of sets of two floating point numbers: the time, t = hlp1, and the probability density function at that time, PDF(t) = hlp. The second output file consists of sets of three floating point numbers: the effective time scale, t_{eff} = j*dt, the slope of a semilogarithmic fit to the second to fourth bins of the histogram, which is the effective kinetic rate constant k_{eff} = B, and the correlation r = corr of the least-squares fit used to determine k_{eff}. To operate the program, a name such as PDF should be specified when it is compiled. Then execute the command line "PDF FileIN FileOUT1 FileOUT2" where FileIN is the name of the input data file, FileOUT1 the name of the output file for the PDF, and FileOUT2 the name of the output file for the k_{eff}.

```
#include<stdlib.h>

#include<stdio.h>

#include<math.h>

#include<time.h>

#include<string.h>

#include<curses.h>

#define n_max 2000000

#define bin_max 20

#define rate_max 3
```

```
void memall(void);
void inputdata(void);
void allocerr(void);
void getinfo(void);
void calc(void);
int hystog(float);
void rate(float);

long int  n;
float     dt, mini;
char      dane[25], output[25], rateout[25];
float     *DATA, *HYST;
FILE      *pli, *plj;

void main(int ile, char *co[])
{
  strcpy(dane, co[1]);
  strcpy(output, co[2]);
  strcpy(rateout, co[3]);
  memall();
  inputdata();
  getinfo();
  calc();
}
```

```
void memall(void)
{
  DATA = (float*) calloc (n_max, sizeof(float));
  if (DATA == NULL)
    allocerr();
  HYST = (float*) calloc (bin_max, sizeof(float));
  if (HYST == NULL)
    allocerr();
}

void getinfo(void)
{
  printf("Enter dt:  ");
  scanf("%f", &dt);
}

/*********************
Czyta dane          *
*********************/
void inputdata()
{
 long int      ii;
 float         help, hpp = 0.0;
 FILE          *ple;
```

```
  if ((ple=fopen(dane, "rt")) == NULL)
   {
    for(;;)
    {
     if ((ple=fopen(dane, "r")) != NULL) break;
     printf("\n \n FILE CAN'T BE OPENED!! try again:  ");
     scanf("%s", &dane);
    };
   };
   mini = 0.00;
   for (ii = 0; feof(ple) == 0; ii++)
   {
     fscanf(ple, "%f \n", &help);
     DATA[ii] = 3.3 * help;
      hpp += DATA[ii];
      if (help <= mini)
        mini = help;
   };
   n = ii + 1;
  printf(" \n %i  points were stored \n", n);
  printf("The sum is  %f  \n ", hpp);
  fclose(ple);

}
```

```
void calc(void)
{
 int     i, check;
 float   j;

 if ((pli = fopen(output, "wt")) == NULL)
  {
    printf("Sorry can not open output file... \n");
    exit(1);
  };
if ((plj = fopen(rateout, "wt")) == NULL)
  {
    printf("Sorry can not open rate output file... \n");
    exit(1);
  };
for (j = 1.000; ; j *= 2.000)
{
  for (i = 1; i < bin_max; i++)
    HYST[i] = 0.00;
  check = hystog(j);
  if (check >=3)
    break;
  rate(j);
 };
```

```
 fclose(pli);
 fclose(plj);
}

int hystog(float j)
{
  int         i, st;
  long int    nn;
  long int    k;
  float       hlp, hlp1;

   st = 0;
   nn = 0;
   for (k = 0; k < n; k++)
    for (i = 0 ; i < bin_max; i++)
    {
     hlp = mini+dt*j*i;
     hlp1 = mini+dt*j*(i+1);
  if((DATA[k] >= hlp) && (DATA[k] < hlp1))
     HYST[i] += 1.0;
    };
   for (i = 1; i < bin_max; i++)
   {
    if (HYST[i] == 0.00)
     break;
     hlp1 = mini + ((float)i + 0.50) * j * dt;
     hlp = HYST[i] / ((float) n * j * dt);
         fprintf(pli, "%f      %f\n", hlp1, hlp);
    };
   for (i = 1; i < 5; i++)
    if (HYST[i] == 0)
      st++;
   return(st);
}

void rate(float j)
{
 int    i;
 float  LOGHYS[rate_max], XAXIS[rate_max];
 float  s, sx, sy, sxx, sxy, A, B, delta, stt, siga, sigb, corr;
 float  cov, chi;

s= sx= sy= sxx= sxy= A= B= delta= stt= siga= sigb= corr= 0.00;
cov = chi = 0.00;
for (i = 1; i < rate_max + 1; i++)
{
 if (HYST[i] == 0.0)
 {
  printf("\n\n Rate can't be calculated for %f -- zeroes encoutered \n", j);
  return;
```

```
    };
    LOGHYS[i - 1] = log(HYST[i]);
    XAXIS[i - 1] = mini + ((float)i + 0.50) * j * dt;
   };
   s = rate_max;
   for (i = 0; i < rate_max; i++)
   {
     sx += XAXIS[i];
     sy += LOGHYS[i];
     sxx += (XAXIS[i] * XAXIS[i]);
     sxy += (XAXIS[i] * LOGHYS[i]);
   };
   for (i = 0; i < rate_max; i++)
     stt += ((XAXIS[i] - (sx/s)) * (XAXIS[i] - (sx/s)));
   delta = s * sxx - (sx * sx);
   A = ((sxx * sy) - (sx * sxy)) / delta;
   B = ((s * sxy) - (sx * sy)) / delta;
   siga = (1.0 + (sx * sx / (s * stt))) / s;
   sigb = 1.0 / stt;
   cov = -1.0 * sx / (s * stt);
   corr = cov / (sqrt(siga * sigb));
   fprintf(plj, "%f      %f      %f \n ", j * dt, B, corr);
}

void allocerr(void)
{
  printf("\n\n ALLOCATION ERROR!!!!\n");
  printf(" \n\n   PRESS A KEY to finish \n");
 /* getch();*/
  exit(1);
}
```

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