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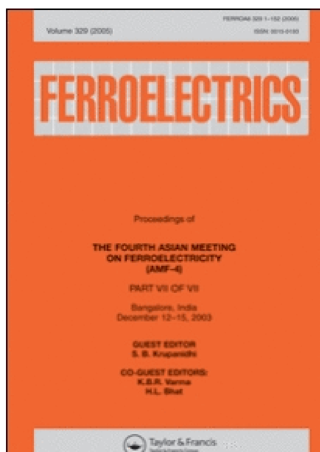

Ferroelectrics
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617887

Mechanisms for the interaction between nonstationary electric fields and biological systems II. Nonlinear dielectric theory and free-energy transduction

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To link to this article: DOI: 10.1080/00150198808227005
URL: http://dx.doi.org/10.1080/00150198808227005

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Ferroelectrics, 1988, Vol. 86, pp. 79–101
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MECHANISMS FOR THE INTERACTION BETWEEN NONSTATIONARY ELECTRIC FIELDS AND BIOLOGICAL SYSTEMS II. NONLINEAR DIELECTRIC THEORY AND FREE-ENERGY TRANSDUCTION

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(Received 14 April 1988)

A discussion of nonlinear dielectric phenomena and their relationship to free-energy transduction in biological systems is given. It transpires that the conditions required for observing the nonlinear dielectric behavior of biological membranes can be expected under easily realizable circumstances, and may potentially form the basis for powerful techniques for studying membrane and other proteins in their native environment.

We develop a nonlinear dielectric theory, which generalizes the dielectric permittivity to include real and imaginary parts of harmonics of the fundamental frequency. An analytical relationship is derived between these permittivities and the kinetic constants of a model protein. Of special relevance is the occurrence of higher harmonics in the dielectric displacement even when the exciting electric field consists of a single sinusoidal frequency. This is manifested by the analytical result that the corresponding higher order permittivities are in general not equal to zero, as well as by the results of a calculation of the Fourier spectrum of the dielectric displacement of a membrane enzyme subjected to a sinusoidal electric field. Interestingly, the Fourier spectrum of the dielectric displacement betrays kinetic characteristics of the enzyme.

In the nonlinear domain discussed here, additional free-energy transduction from the field to the reaction catalyzed by the enzyme is possible. A study of the nonlinear dielectric behavior of biological systems ("2-dimensional dielectric spectroscopy") could serve to account for the fact that free energy may be harvested by enzymes (with a concomitant change in their activities) from energetically rather modest exogenous electrical fields.

1. INTRODUCTION

In the accompanying paper [1], we described some linear, passive electrical properties of biological systems, and argued that it might be fruitful, in order better to understand the relationship between dielectric spectra and the ability of enzymes to transduce free energy from electrical fields, to consider the nonlinear

dielectric behavior of real, biological systems, and exactly how this differed from that of the rather simple model systems usually considered. We started by giving an example of lateral protein motions in spherical shell bilayers.

In this paper we shall try to examine some of the properties to be expected of proteins, and more specifically of enzymes, if one describes their behavior with the more general nonlinear equations. This behavior will include nonlinear dielectric phenomena, such as interfrequency crossing, as well as electrochemical phenomena such as the transduction of free energy from the nonstationary electric field to chemical or transport work. Rather than on reviewing existing literature, we shall focus on principles responsible for these phenomena.

Our ultimate purpose is to formulate an amalgam of dielectric theory, enzyme kinetics and (Mosaic) non equilibrium thermodynamics. We intend this amalgam to be optimal for describing interactions between the catalytic cycles of enzymes and dynamic electric fields. The availability of such a theory should allow us to bridge the existing gap between dielectrics on the one hand and biochemistry on the other. Such a bridge may then enrich biological dielectrics with the spectrum of interesting properties of enzymes and provide (membrane) biochemistry with a tailored tool; a new form of dielectric spectroscopy.

2. NONLINEAR DIELECTRIC BEHAVIOR

2.1. *Where nonlinear dielectric behavior begins in a model protein*

In order better to understand how nonlinear effects arise and under what circumstance they are apt to lead to *measurable* deviations from predictions based on linear response theory, let us specify a simple enzyme model. We imagine a planar bilayer membrane (Figure 1) which is sufficiently large to accommodate many molecules of a particular protein, where we assume that the average distance between proteins is great enough that protein-protein interactions may be ignored. Furthermore, for the sake of simplicity we take it that all protein molecules are oriented in the same direction. Each individual protein can exist in two electrically distinguishable conformational states (which may, as discussed later, be a part of an enzyme catalytic cycle) and makes stochastic transitions between them according to well-defined rate constants which are instantaneous functions of the local electric field, as shown below.

$$e_1 \rightleftharpoons e_2 \quad (1)$$

It is assumed that the various configurations making up each conformation attain internal equilibrium on a time scale much faster than both the inverse frequency

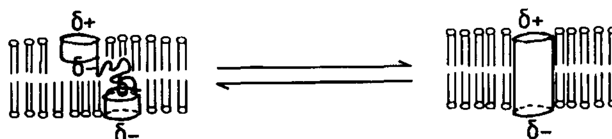

FIGURE 1 A bilayer membrane with an enzyme that can exist in two conformations with different dipole moments.

of any applied field to be considered and the relaxation time of the conformational equilibrium itself [cf., 2].

The imposition of an electric field shifts the chemical potential of a dipolar molecule, g_1 (usually denoted by μ_1, but this would cause confusion with its dipole moment), according to the Gibbs Equation (3), which for isobaric and isothermal conditions is [4–7]:

$$(dg_1^{\phi})_{T,\text{pressure}} = p_1\, dE \tag{2}$$

Here g_1^{ϕ} is the concentration independent part of the chemical potential, and p_1 is the component parallel to the electric field of the partial molar macroscopic polarization. p_i has the dimension (charge · distance) of dipole moment. For a second conformation the analogous equation applies so that:

$$RTd\ln(K_{eq}) = d(\delta g^{\phi})_{T,\text{pressure}} = dE\,\delta p \tag{3}$$

where δp is the difference in partial molar polarizabilities between e_1 and e_2, p_2–p_1. This δp might consist solely of a difference, $\delta\mu$, in permanent dipole moment, which could originate from different orientations of alpha-helical segments between the two conformations [8] or the two conformations of the enzyme may also differ in the position of a charge carried by the protein. If the electric field is homogeneous, the difference in position is δs and the charge is q, then this contributes an extra $q\delta s$ to the difference in polarization. Further, the two conformations of the enzyme may have different polarizabilities α, for instance because the alpha-helices in one conformation can more easily orient relative to the field than in the other conformation. This would contribute a term $E\delta\alpha$ to δp.

Equation 3 gives the change in equilibrium constant for the protein conformational transition with a change in the electric field. Comparing the equilibrium constant in the presence of the electric field, K_E, to that in the absence of the field, K_0, this equation yields:

$$K_E = K_0 \exp[E\delta\mu/(RT) + Eq\delta s/(RT) + \tfrac{1}{2}E^2\delta a/(RT)] \tag{4}$$

Only in the case that $[E\delta\mu + qE\delta s + (\delta a/2)E^2] \ll RT$ can Equation 4 be approximated by the formula [9]

$$(\delta K/K)_{T,\text{pressure}} = (\delta\mu + q\delta s + \tfrac{1}{2}E\delta\alpha)\delta E/(RT) = \delta p\delta E/(RT) \tag{5}$$

For our planar bilayer system it is interesting to analyze the anticipated behavior of the system in response to an alternating field. Assume $\delta\alpha$ and $\delta\mu$ to be positive. For amplitudes such that $E^2\delta\alpha \ll E\delta\mu$, the positive phase of the field will favor the right hand (e_2) state in 2, while in the negative phase, the left hand (e_1) state will be favored. This represents a permanent dipole relaxation mechanism. If, however, $E^2\delta\alpha \gg E\delta\mu$, which would be the case at very large amplitudes of the field, the left hand state (e_1) will be more favored than at zero field during both positive and negative phases of the field. This situation represents an inducible dipole mechanism. The quadratic dependence of the power of the exponential term in Equation 4 on E may explain the "amplitude" window seen in the stimulation of active transport by an A.C. field [7].

In the remainder of this paper, we will consider that $|E^2\delta\alpha/2| \ll |(E\delta\mu + qE\delta s)|$. Then, the field dependent equilibrium coefficient of Equation 4 may be written in terms of a δp that is independent of the field:

$$K_E/K_0 = \phi \equiv \exp[E\delta p/(k_B T)] \tag{6}$$

Thermodynamically consistent field-dependent rate coefficients for reaction 1 may be written:

$$k_f(E) = k_f\phi^f \tag{7}$$

$$k_r(E) = k_r\phi^{f-1} \tag{8}$$

k_f and k_r are the forward and reverse unidirectional rate constants at zero field, respectively. f represents the fraction of the total dipole moment change of the transition expressed between state e_1 and the transition state. The displacement current due to the protein conformational change is given by:

$$(dp/dt)/\delta p = I/\delta p = d(e_1)/dt = -[k_f\phi^f + k_r\phi^{f-1}]e_1 + k_r\phi^{f-1}$$
$$= d(e_1 - e_{1eq})/dt = -[k_f\phi^f + k_r\phi^{f-1}](e_1 - e_{1eq}) + k_f k_r(\phi^{f-1} - \phi^f)/(k_f + k_r) \tag{9}$$

where $e_1 - e_{1eq}$ is the concentration of e_1 minus the concentration of e_1 that would be attained at equilibrium in the absence of the field. The total enzyme concentration $e_1 + e_2$ has been normalized to 1. Without loss of generality also δp and $(e_1 - e_{1eq})$ could be normalized to 1, with p_{eq} being taken to be zero, whilst the rate constants are interpreted as turnover numbers. Let us first consider the case that the equilibrium is perturbed by an oscillating electric field $re(E) = E_0\cos(\Omega t)$, with an amplitude small enough that (by a Taylor expansion of Equation 6)

$$\phi^f \approx 1 + [fE_0\delta p/(RT)]\cos(\Omega t) \tag{10}$$

and the analogous approximation (with $1-f$ replacing f) being valid for ϕ^{f-1}. The above Equation (9), after multiplication by δp, then becomes:

$$dp/dt = -\alpha p + \beta x - \Gamma xp \tag{11}$$

with the dimensionless parameter x representing the electric field dependence

$$x \equiv [E_0\delta p/(RT)]\cos(\Omega t) \equiv x_0\cos(\Omega t) \tag{12}$$

and

$$\alpha \equiv (k_f + k_r) \tag{13}$$

$$\beta \equiv -[k_f k_r \delta p/(k_f + k_r)] \tag{14}$$

$$\Gamma \equiv [k_f f + k_r(f-1)] \tag{15}$$

Note that here, p represents a charge separation defined relative to the planar membrane of Figure 1, and has a definite sign even in the absence of the field. Thus, application of an external field can result in either an increase or decrease in the polarization. This is in contrast to the case of a field applied to a freely and rapidly orientable protein in the previous paper where the polarization always increased due to the field.

In general, Equation 11 does not satisfy the conditions for "linearity" (cf. Equations 2 and 25 of the accompanying article): it forfeits condition iii of Section

2.3 thereof, because the rate coefficient relating dp/dt to p (i.e., $-\alpha - \Gamma x$) depends on the electric field (through x). Since both p and E depend on time, this could give rise to a cross correlation term between p and E (see below). Of course if x were extremely small, then all the terms depending on x in Equation 11 would drop out, but this extreme is not of interest because the field is then completely without any effect.

As discussed in the previous paper [1], the Langevin function (Equation 21 of Reference 1) is approximately linear so long as $x \leq 1$, and this is the realm normally taken to be describable by linear dielectric theory. With this in mind, there are two conditions which, if either are met, allows expressing Equation 11 in a form analogous to Equation 2 of the accompanying paper, i.e.

$$dp/dt \approx O_1 \equiv -\alpha p + \beta x \tag{16}$$

where O_1 stands for the first and lower order terms in x and p. These conditions are $\Gamma x \ll \alpha$ or that $\Gamma p \ll \beta$. Since Γ is typically less than or equal to α, the first condition is trivially met when $x \ll 1$. We thus find that provided that the electric field is small enough (to the extent quite reminiscent of the extent required in Section 2.2 of the accompanying article), the two-state enzyme should be expected to obey linear dielectric theory. However, the above condition need not hold, even under conditions where the Langevin function (Equation 21 of the preceding paper [1]) is given accurately by a linear approximation.

The second condition, $\Gamma p \ll \beta$, holds if $p \ll (\beta/\Gamma)$. The amplitude of p is not only field strength- but also frequency-dependent. For a given field strength, its DC amplitude amounts to:

$$p_{\max} = (k_f k_r x_0 \delta p)/(k_f + k_r)^2 = (\beta/\alpha)x_0 \tag{17}$$

which yields the same condition as before ($x \ll 1$) to insure linear behavior at low frequencies. At high frequencies, the polarization will not come close to its maximum DC value, $p_{\max}$ and the condition for linearity will be less stringent, such that Γp may be much less than β even for $x \approx 1$.

Finally, both of the above conditions to linearize Equation (11) are trivially met if for any value of f

$$\Gamma = fk_f + (f-1)fk_r = 0 \tag{18}$$

and for the most natural choice, in the absence of detailed information, of $f = \frac{1}{2}$, this is equivalent to $k_f = k_r$. This is precisely correct for billiard ball dipoles. For more complex systems, particularly involving activated chemical processes, Equation (18) does not necessarily hold. Then terms from the cross correlation between the dynamic response of the system and the applied perturbation may arise, even in the linear domain of the Langevin function ($E_0 \delta p \leq RT$).

Equation (9) allows us to investigate further when and how "linearity" may break down as x gets closer to 1. We do this by taking along the second order term of the Taylor series for ϕ, in addition to the first order terms shown in Equation (10). We find:

$$dp/dt \approx -\alpha p + \beta x - \Gamma xp + \tfrac{1}{2}\beta(2f-1)x^2 + \tfrac{1}{2}[k_f f^2 + k_r(f-1)^2]x^2 p \tag{19}$$

In order for Equation (19) to be reasonably approximated by the linear Equation (16), the terms including xp, x^2, and x^2p must be negligible. As discussed above, this is immediately fulfilled if $x \ll 1$. If, however, $x < 1$ but $x \approx 1$, a sufficient condition is that $k_f = k_r$ and $f = \frac{1}{2}$, in which case the multipliers of xp and x^2 become identically zero. As $x < 1$, the third order terms containing x^2p also drops. This is precisely the case for spherical (billiard ball) dipoles rotating in a nonsaturating field, but need not in general apply to chemical reactions.

Notice that even under conditions where the terms due to x^2 and x^2p are negligible (e.g., if $f = \frac{1}{2}$ whereby the multiplier of x^2 is zero irrespective of the values of k_f and k_r, and $x^2p < xp$ which is true for $x < 1$), the contribution of Γxp can still be significant (greater than 25% at some time points for $f = \frac{1}{2}$, $k_f = 1000$, and $k_r = 1$).

In the following, we will investigate more formally the phenomenology which may arise as a result of this cross correlation term, Γxp. Our primary motivation is not so much that the dielectric permittivity predicted by linear dielectric theory may not be quite exhibited by the system, but that there will be higher harmonic terms in the polarization, which may be detected with an enhanced signal to noise ratio and may contain much mechanistic information. This, as will be discussed extensively later, may be particularly pertinent for studying the properties of membrane proteins by dielectric spectroscopy.

2.2. *The effect of cross correlation between the polarization and the field*

In the accompanying article we reviewed the dielectric behavior expected for systems satisfying the definition of "linearity". Here we shall examine the dielectric behavior to be expected of systems exhibiting significant cross correlation between the field and the polarization, yet still within the linear Langevin domain. First, considering the system of Figure 1, we shall deal with the case in which Equation (11) but not Equation (16) is a fair approximation of the system's behavior. Since $x = x_0 \cos(\Omega t)$ we might start out by assuming that the time-dependent polarization could be approximated by the result of linear dielectrics [cf., 10, 11]:

$$p(t) = p_0 \cos(\Omega t + \theta) \tag{20}$$

then from Equation (11) the displacement current would be written:

$$dp/dt = -(k_f + k_r)p_0 \cos(\Omega t + \Theta) - x_0 \cos(\Omega t) k_f k_r/(k_f + k_r) - [k_f f + k_r(f-1)] x_0 p_0 \tfrac{1}{2}[\cos(\theta) + \cos(2\Omega t + \theta)] \tag{21}$$

The appearance of the term $\cos(2\Omega t + \theta)$ indicates that the dielectric displacement current will contain components oscillating at twice the fundamental frequency. Although this term is multiplied by an assumed small term, $x_0 p_0$, this phenomenon may be observable even at field amplitudes currently in use to make dielectric measurements, by appropriate signal averaging techniques and especially by looking at higher harmonic frequencies.

The above argument is illustrative but not closed: its conclusion is inconsistent with its premise, because the first derivative of Equation (20) does not yield the second harmonics following from Equation (21), i.e., Equation (20) was not a

definitive guess for p. We shall now try to find the true solution for p. To this purpose we acknowledge that (according to the Fourier theorem) the stationary solution of the differential Equation (11) periodic with frequency Ω, can be written as an infinite sum of all the higher harmonics. Bold face refers to complex quantities

$$\mathbf{p}c/\epsilon_0 = \mathbf{P}/\epsilon_0 = (RT/\delta p)\sum_{n=1}^{\infty}[\boldsymbol{\epsilon}_n(\Omega) - 1)]\,\mathbf{x}\exp\{\mathbf{j}(n-1)\Omega t\} \tag{22}$$

where:

$$\mathbf{x} = x_0\exp(\mathbf{j}\Omega t) = [E_0\delta p/(RT)]\exp(\mathbf{j}\Omega t) \tag{23}$$

c in Equation (22) is the concentration of the enzyme per unit volume, linking p (the polarization per mole) to P (the polarization per unit volume). To simplify the algebra, the $\boldsymbol{\epsilon}_n s$ have been formulated in terms of $\mathbf{x}$ rather than $\mathbf{E}$.

Equation (22) generalizes the complex permittivity [1] to a set of complex permittivities, one for each harmonic of the polarization. $\boldsymbol{\epsilon}_n \cdot x_0$ corresponds to the Fourier transform of the dielectric displacement.

Equations such as Equation (11) are in principle defined only for real x and P. Thus, they are valid if the real parts of complex quantities $\mathbf{x}$ and $\mathbf{P}$ are inserted. However, since they must be valid at any time, they must be valid at both $t = T$ and $t = T + \pi/2\Omega$ (i.e., 90° further). If the equation is linear in x and P and if x and P are single harmonics, this implies that the equation must also be valid if the imaginary parts of x and P are inserted and hence for $\mathbf{x}$ and $\mathbf{P}$. This is the basis for the use of complex functions to treat linear dielectrics. However, in the nonlinear equations of nonlinear dielectrics presented here, this simplification is no longer possible. Here the values for x and P to be inserted are the real components of $\mathbf{x}$ and $\mathbf{P}$.

The real component of the complex polarization $\mathbf{P}$ is given by:

$$P/(\epsilon_0 E_0) \equiv \{\mathrm{re}(\mathbf{P})\}/(\epsilon_0 E_0) = (\epsilon_0' - 1) + \sum_{n=1}^{\infty}[(\epsilon_n' - 1)\cos(n\Omega t) + \epsilon_n''\sin(n\Omega t) \tag{24}$$

The term $(\epsilon_0' - 1)$ allows for the possibility (see below) that P oscillates around a value different from its zero-field equilibrium value of zero. Inserting this expression for P and expression 12 for the real component of $\mathbf{x}$ into Equation 11, and realizing that the equality sign must hold individually for every sine and cosine of every harmonic, we find:

$$(\epsilon_0' - 1) = -\tfrac{1}{2}\Gamma x_0(\epsilon_1' - 1)/\alpha \tag{25}$$

$$0 = n\Omega\epsilon_n'' + \alpha(\epsilon_n' - 1) + \tfrac{1}{2}\Gamma x_0[(\epsilon_{n-1}' - 1)(1 + \delta_n^1) + (\epsilon_{n+1}' - 1)] + \beta\delta_n^1 c\delta p/(\epsilon_0 RT) \tag{26}$$

and:

$$0 = n\Omega(\epsilon_n' - 1) - \alpha\epsilon_n'' - \tfrac{1}{2}\Gamma x_0(\epsilon_{n-1}'' + \epsilon_{n+1}'') \tag{27}$$

where δ_n^1 equals 1 if n equals 1, else 0. These equations determine the generalized dielectric permittivities for the first as well as the higher harmonics in the polarization that results from a single component harmonic oscillation in the field.

Equation (11) is only valid for cases where $x_0 \ll 1$. For such values of x_0, higher order permittivities will be much smaller than lower order permittivities. Thus, to

a good approximation, the terms ϵ'_{n+1} and ϵ''_{n+1} may be neglected in Equations (26) and (27). This allows one to obtain closed expressions for the dielectric permittivities:

$$(\epsilon'_1 - 1) \approx -\alpha^2\beta c\delta p/[\epsilon_0 RT(\alpha^2 + \Omega^2 - \tfrac{1}{2}\Gamma^2 x_0^2)] \tag{28}$$

$$\epsilon''_1 \approx \Omega(\epsilon'_1 - 1)/\alpha \tag{29}$$

$$(\epsilon'_n - 1) \approx \tfrac{1}{2}\Gamma x_0(n\Omega\epsilon''_{n-1} - \alpha\cdot\epsilon'_{n-1})/(n^2\Omega^2 + \alpha^2) \tag{30}$$

$$\epsilon''_n \approx -\tfrac{1}{2}\Gamma x_0(n\Omega\epsilon'_{n-1} + \alpha\epsilon''_{n-1})/(n^2\Omega^2 + \alpha^2) \tag{31}$$

The reader may wish to check that the presumption that the magnitudes of the permittivities decrease strongly with n, is retrieved; the harmonics become progressively less important, because the nth harmonic contains the $(x_0)^{n-1}$. The approximations may be improved by reinserting the calculated values for the permittivities for the ϵ_{n+1} terms into Equations (26) and (27) and then repeating the above procedure. The status of the above approximation is that the relative error in any of the calculated permittivities is of the order of x_0.

For $\Gamma = 0$, Equations (28–31) describe a Debye dispersion: all permittivities except ϵ'_1 and ϵ''_1 become equal to zero and ϵ'_1 and ϵ''_1 respectively follow Equations (12) and (13) of the accompanying article. *More importantly, these equations demonstrate that, unless Equation* (18) *applies and* Γ *is* 0, *there will be higher harmonics in the polarization induced by a single sinusoidal component electric field.*

Looking upon $(\boldsymbol{\epsilon}_n - 1)$ as a function of frequency, $\boldsymbol{\epsilon}(n\Omega) = \epsilon'_n - \mathbf{j}\epsilon''_n$, it corresponds to the Fourier spectrum of the polarization that results from an exciting electric field of single frequency Ω. In Section 4.1 (cf., Figure 5) we shall discuss an example of such a Fourier spectrum. Here it is important to note that for a single frequency sinusoidal electric field, the two-state protein model discussed in the present section and represented by Equation (11), gives rise to a Fourier spectrum devoid of frequency components other than the exciting frequency line and its higher harmonics ($n\Omega$ where n is a positive integer). Equations (28–31) demonstrate that ϵ'_n and ϵ''_n and hence the relative amplitudes and phase angles of the higher harmonics vary with (i) the kinetic properties of the protein (such as k_r, k_f, and f), (ii) the difference in dipole moment between the two states, (iii) the frequency of the electric field, and (iv) the amplitude of the electric field.

In linear dielectric theory, mechanistic information concerning the process responsible for the observed polarization can only be obtained from the dependence of the single permittivity on the frequency of the exciting electric field. Investigations of nonlinear dielectric properties may yield additional information based on the higher harmonics in the polarization at a given frequency and amplitude of the exciting field. The dependence of the Fourier spectrum of the polarization on the amplitude of the exciting electric field is yet another potential source of information. As the input amplitude is increased, contributions from those *chemical* transitions (remember that simple orientational relaxations *cannot* contribute to the signal at other than the fundamental frequency except through cube and higher order terms in the Taylor expansion of

the electric field factor ϕ) with large δp will show up first in the second harmonic ($n = 2$) spectrum, thus providing for a good selectivity for the method. This contrasts to the case for the fundamental frequency, where contributions from every physical and chemical mechanism with relaxation times shorter than the inverse of the frequency of the field will be seen. The selectivity obtained through nonlinear dielectric spectroscopy is further enhanced if the protein of interest is embedded in a membrane. In such a case, a large fraction of the overall potential drop occurs across the thin ($\approx$5 nm) membrane. In such a case it may be said with confidence that at reasonable (<1 kV/cm) field strengths, all contribution to the higher harmonics of the observed dielectric Fourier spectrum will be due to chemical processes occurring *within* the membrane.

In addition to the emergence of harmonics of the fundamental frequency, at the limit of Langevin linearity (i.e., the region where Equation (11) but not Equation (16) is valid, $E_0 \delta P/(RT) \approx 0.2$) a number of other features appear. One is that the average polarization $\langle P \rangle = \epsilon_0 \epsilon_0' E_0$ of an enzyme exposed to a sinusoidally oscillating exciting field ($\langle E \rangle = 0$) may be different from that pertaining in the absence of the field. Concordantly, the value of $\langle e_1 \rangle$ (i.e., the time average value for the probability for the protein to be in state 1) which satisfies the condition for stationary oscillation $[e_1(t) \equiv e_1(t + 2\pi/\Omega)]$ is not equal to the zero field equilibrium value, but is given by:

$$\langle e_1 \rangle - \langle e_1 \rangle_{\text{zero field}} = \epsilon_0 \epsilon_0' E_0 / (c \delta p) \quad (32)$$

with ϵ_0' given by Equations (25) and (28). Equation (32) demonstrates that an oscillating electric field can drive a protein into a different average conformation. To the extent that proteins in different conformational states may have different catalytic activities, an oscillating electric field may thus affect the catalytic activities.

A second consequence of this phenomenon is that during the initial stages of the applied sinusoidal perturbation, there will be a relaxation of the protein to a new "stationary state", and superimposed on this will be the induced oscillations of the system. During the transient phase, which can last for a significant number of cycles, the time-dependent displacement current will include terms involving $\cos(\Omega t)\exp(-t/\tau)$, where τ is a constant characteristic of the fundamental kinetics of the system and the amplitude of the perturbation. This implies that the Fourier transform will include terms in addition to those arising from the fundamental and harmonic frequencies of the input signal. If the relaxation matrix of a multistate enzyme houses complex eigenvalues, then the new terms are liable to include frequencies of higher harmonics plus and minus the eigenfrequency of the enzyme.

The latter effect may also be met under stationary-state conditions. As we already indicated (in the accompanying article, after deriving Equation (26) for the linear case), resonant behavior in the sense of a local maximum in the frequency dependent permittivity may arise if the relaxation matrix of the enzyme (M) has complex eigenvalues. In the case of linear dielectrics the "damped" resonance would occur if the exciting frequency would equal the eigenfrequency of the enzyme (the resonance would be "damped" because the eigenvalues of the matrix cannot be imaginary; they must have a real component). In nonlinear

dielectrics resonance behavior might be accompanied by Fourier components corresponding to frequencies equal to the fundamental frequency or one of its harmonics plus or minus the eigenfrequency of the enzyme. Indeed, we might suggest that if an autonomous membrane oscillator, such as those frequently found for excitatory [12] or secretory [13] cells, were to be subjected to a sinusoidally oscillating external field, even a stationary response oscillation could contain frequency components relating to sums and differences between the input frequency and the characteristic time constants of the system. An appropriate internal oscillator could consist of a single enzyme driven very far from equilibrium [14], or equally well could be composed of several enzymes working coherently.

The above effects have arisen, even in the linear Langevin domain, due solely to cross correlation between the exciting field and the system response (i.e., due to terms xP). At greater perturbation amplitudes than this, the non-linearity of ϕ must also be explicitly included in the equation for the displacement current

$$\phi = \exp[x_0 \cos(\Omega t)] = \sum_{n=0}^{\infty} I_n^x \cos(n\Omega t) \tag{33}$$

where the I's are the modified Bessel coefficients [15]. Repeating the treatment given above, one sees that also this effect by itself will give rise to higher harmonics in the polarization. Even without cross correlation, energy from the field may appear at frequencies other than the stimulating frequency.

It is important to note that this type of nonlinearity can occur if $E^2\delta\alpha/2 \ll E\delta\mu$ provided that $E\delta\mu > k_B T$. Thus, non-linear dependence does not prove the involvement of changes in polarizability. Even a purely permanent dipole mechanism will display a nonlinear dependence of polarization on E when $E\delta\mu > k_B T$.

In general the input electric field will not be a sinusoidal field of a single frequency. However, the Fourier theorem allows one to treat any periodic input electric field as a sum of a sinusoidal field of the input period plus its higher harmonics. If we write the input field as:

$$\mathbf{E} = \sum_{l=1}^{\infty} |E_l| \exp(\mathbf{j} l \Omega t) \tag{34}$$

the dielectric displacement may be described by:

$$\mathbf{D} = \sum_{n=1}^{\infty} \sum_{l=1}^{\infty} \epsilon_{nl} \epsilon_0 |E_l| \exp(\mathbf{j} l \Omega t) \tag{35}$$

Here ϵ_{nl} is the complex dielectric permittivity in the nth harmonic of the dielectric displacement resulting from the lth component of the input field. $|E_l|$ is the amplitude of the lth harmonic component. Note that, for the dielectric permittivities to be independent of the amplitudes of the components of the input field, the superposition principle would have to hold. This is generally not so: ϵ_{nl} will depend on the Fourier spectrum of the input field. For the single component input field treated above, this was already the case (cf. the x_0 dependencies in Equations (28–31)).

2.3. *Nonlinear dielectrics and the Kronig-Kramers relations*

Above we have dealt with dielectric permittivity for the nonlinear case by defining a series of dielectric permittivities, one for each harmonic in the dielectric displacement. If we would have stuck to the definition of a single dielectric permittivity, then this permittivity would have had to become a function of the input field and even of time:

$$\epsilon = \sum_{n=1}^{\infty} \epsilon_n(\Omega) \exp(\mathbf{j}(n-1)\Omega t) \tag{36}$$

For the same reasons as given below equation (28) in the accompanying article, the polarization and the electric field obey the same symmetry as in the case of linear dielectrics. As a consequence also ϵ defined by Equation (36) and hence equal to the ratio between the polarization and the single frequency electric field, should have the symmetry property defined in Equation (28) of the previous article. One of the main conditions for the derivation of the Kronig-Kramers relationship would hence be fulfilled. However, the dependence of ϵ as defined by Equation (36) on time makes the magnitude of any Kronig-Kramers type of integral undetermined.

For this reason we have defined the higher-order complex permittivities in Equation (22). We shall now investigate whether Kronig–Kramers relations hold for these higher order permittivities. To investigate the symmetry properties of the complex permittivities, we write:

$$\mathrm{conj}(\mathbf{P}(-\Omega)) = \sum_{n=1}^{\infty} \mathrm{conj}[\epsilon_n(-\Omega)]\,\mathrm{conj}\{\exp[-\mathbf{j}(n-1)\Omega t]\}\,\mathrm{conj}[\mathbf{E}(-\Omega)] \tag{37}$$

Using the fact that taking the complex conjugate and inversing the frequency of a periodic wave form retains the same physical process and the fact that Equation (37) must be satisfied for each Fourier component individually, we conclude that, under conditions in which Equation (22) is valid, every higher order complex permittivity must have the same symmetry property as the first order component, which has the symmetry property described by Equation (28) of the previous article [1]. Indeed in the specific example discussed in Section 2.2, the dielectric permittivities did obey this symmetry principle (cf., Equations (28–31)). Thus, the Kronig–Kramers relationships will hold for ϵ even in the case of nonlinear dielectrics. Moreover, they will hold for any of the higher order dielectric permittivities, for as long as the latter are analytic functions and their limit for infinite frequencies goes to zero.

3. NONLINEAR EFFECTS OF DYNAMIC ELECTRIC FIELDS ON ENZYMES

3.1. *Structural and catalytic effects of nonstationary electric fields*

One of the main purposes of the above discussion was to serve as an introduction to another, though cognate, type of nonlinear behavior which is of strong

relevance to the problem of how an exogenous electrical field (where the average energy of interaction μE may be less than $k_B T$) can affect the kinetics of an enzyme [7, 9, 16–18; see also 19–21]. The following considerations hinge on the fact that a chemical system, when exposed to an electric field, can minimize its free energy by two mechanisms. The most commonly considered involves motion such as rotation of dipoles or electrophoresis of dipolar proteins within a spherical shell. The second involves chemical relaxation, in which chemical transformation of molecules to states of lower free energy in the field is stimulated. This second possibility has been discussed in the context of homogeneous systems by Schwarz [22]. Crucially, the return to the starting state in the absence of the field may, be either a back reaction or a forward reaction completing a catalytic cycle. As discussed for instance by Kell and Harris [19], electric fields can modulate existing interaction between enzymes and hence the coupling of chemical reactions.

While dipole rotation in the homogeneous case minimizes the chemical effect, movement of proteinaceous dipoles in the "spherical" bilayer in cell suspensions *could maximise* the chemical effect of the field (once steady state has been reached) by localizing the dipolar molecules in regions of the cell surface where the differential interaction energy between two conformational states of a protein is the greatest, thus increasing the interaction between the enzymes.

As discussed above, electric fields can also directly affect the rate constants for transitions in an enzymes catalytic cycle, this especially if the catalytic transition involves a conformational change of the protein in which its dipole moment changes. Thus an electric field can modulate the catalytic effectiveness of an enzyme by affecting the rate of one of its rate controlling steps. Alternatively an electric field can cause a redistribution of an enzyme over a conformation in which it is an active catalyst and a conformation in which it is much less so. Catalytic effects such as these can be caused by both stationary and nonstationary electric fields, as discussed in more detail elsewhere [17, 18].

3.2. *Dynamic Field Driven Enzyme Cycling: free-energy transduction*

When the effect of the electric field is said to be catalytic, it is meant that the electric field cannot drive the reaction catalyzed by the enzyme away from equilibrium, and merely speeds up or slows down an enzyme-catalysed process. For transduction of free energy from a dynamic electric field to chemical or transport reactions to occur [7, 16, 23], the latter reactions must be driven away from their zero field equilibrium. Free energy absorbed from the field can then be stored in a metabolically useful form rather than being dissipated. The efficiency and rate with which energy is converted is a function of the matching between the frequency of the input field and fundamental kinetic coefficients of the system [18] and therefore represents a potentially useful means for determining the dynamic properties of membrane enzymes. A simple physical picture of the field induced cycling of an enzyme is presented in Figure 2 [17]. The basic idea is that during the positive phase of the field, the overall relaxation from state 4 to 2, dictated thermodynamically by the field, proceeds initially more rapidly via state 1 than by state 3, while during the negative phase of the field, the net flux from state 2 to 4 occurs mainly via state 3. Thus net clockwise flux may be driven by an oscillating

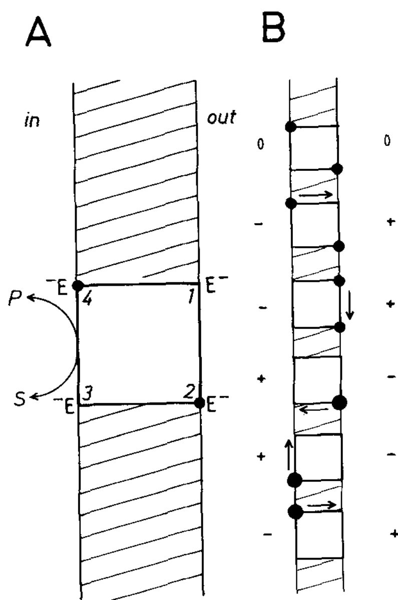

FIGURE 2 A four-state enzyme in a membrane that can catalyze free-energy transduction from an oscillating electric field into a chemical reaction. (A) The enzyme. In states 1 and 2 the negative end of its dipole moment points to the OUTside of the membrane, in states 3 and 4 it points to the INside. The transition from state 3 to state 4 is coupled to the conversion of substrate S to product P. (B) The effect of an oscillating electric field on the enzyme. The polarity of the field is indicated by the 0, −, and + on the two sides of the membrane, Time runs from top to bottom. The enzyme is in the states indicated by ●. The arrow indicates the prevalent transition of the enzyme under the ambient field conditions. In the absence of an electric field it is in states 2 and 4 (these are the most stable conformations). As a result of the oscillating electric field the enzyme will move clockwise through its catalytic cycle [16, 17] and thus convert S to P. In case the chemical potential of P exceeds that of S, free-energy transduction from the oscillating electric field to the free-energy difference between P and S may occur.

field, even against an unfavorable free energy difference between P and S, *given certain characteristics of the enzyme.* One important factor seems to be that the majority of the field dependence reside in the net rate coefficient for the forward process for the overall reaction step $4 \rightarrow 1 \rightarrow 2$, but in the rate coefficient for the reverse process for the step $4 \rightarrow 3 \rightarrow 2$. This may be achieved either by having state 4 exhibit a much greater affinity for substrate on the outside than state 1 for substrate on the inside [16], or by an *a priori* apportionment factor $f > \frac{1}{2}$ for the process $4 \Leftrightarrow 1$, and $< \frac{1}{2}$ for $3 \Leftrightarrow 2$ [24, 25].

These concepts have also allowed [16–18, 23, 24, 26] discussion of a number of points in regard to energy coupling by enzymes in membranous system, in terms of Coulombic interactions.

3.3. *Free-energy transmission in nonlinear dielectrics*

3.3.1. *General principles*

Because of the emergence of higher harmonics in nonlinear dielectrics, the composition of a proper energy balance sheet requires that both the fundamental

and harmonic frequencies and the possible exchange of free energy between them be taken into account. We shall limit the discussion to the stationary state, and first discuss the case of a single frequency sinusoidal input electric field. Using Equation (34) of the previous paper for the definition of the input power (W_{in}) and Equation (22) for the dielectric displacement, we find:

$$W_{in} = \sum_{n=1}^{\infty} \mathrm{re}(\mathbf{E})\, \mathrm{re}[\epsilon_0 \boldsymbol{\epsilon}_n \mathbf{j} n\Omega E_0 \exp(\mathbf{j} n\Omega t)]$$

$$= \epsilon_0 \sum_{n=1}^{\infty} n\Omega (E_0)^2 \cos(\Omega t)\{\epsilon_n' \sin(n\Omega t) + \epsilon_n'' \cos(n\Omega t)\} \quad (38)$$

Again (cf. Section 2.6 of the previous paper [1]) one may distinguish two fractions in W_{in}, i.e., W_{r1} and W_2:

$$W_{r1} = \epsilon_0 (E_0)^2 \cos(\Omega t)\Omega \sum_{n=1}^{\infty} n\epsilon_n' \sin(n\Omega t) \quad (39)$$

$$W_2 = \epsilon_0 (E_0)^2 \cos(\Omega t)\Omega \sum_{n=1}^{\infty} n\epsilon_n'' \cos(n\Omega t) \quad (40)$$

If, as in the case of linear dielectrics, only the fundamental frequency occurs in the dielectric displacement (i.e., $\boldsymbol{\epsilon}_n = 0$ for $n > 1$), then Eqs. 35 and 36 of the previous article are retrieved.

As in linear dielectrics, W_{r1} is temporarily stored power in the sense that its time integral over an entire cycle is zero:

$$G_{r1} = \epsilon_0 (E_0)^2 \sum_{n=1}^{\infty} n\epsilon_n' \int_{-\pi}^{\pi} \cos(\alpha) \sin(n\alpha)\, d\alpha = 0 \quad (41)$$

W_2 consists of the same W_a as in the linear case plus W_{r2}, i.e., a sum of components corresponding to harmonics of the fundamental frequency. Time integrals of W_{r2} over the entire field cycle reduce to zero: this is also temporarily-stored free energy. The higher harmonics in the dielectric displacements do not absorb free energy out of the single component exciting electric field.

In case the exciting electric field consists of more than one frequency, our analysis requires the use of Equation (35) instead of Equation (22). Here, we will make this generalization. The free energy exchanged with the external electric field consists of two components, G_{r1} and G_2, which as before are the time integrals of the two components of the input power.

$$W_{r1} = \epsilon_0 \sum_{nlm} |E_l|\, |E_m|\, \epsilon_{nl}' \sin(n\Omega t) \cos(m\Omega t) \quad (42)$$

Note that $(\Sigma_l E_l)(\Sigma_l E_l)$ may be written $(\Sigma_m \Sigma_l E_l E_m)$ which is why the double sum over E in the above equation appears. As usual, integration of this part of the interaction power over complete field cycles yields zero.

$$W_2 = \epsilon_0 \sum_{nlm} |E_l|\, |E_m|\, \epsilon_{nl}'' \cos(n\Omega t) \cos(m\Omega t) \quad (43)$$

Integration of W_2 over complete cycles recovers only the terms resulting from coupling between input and output harmonics of the same frequency:

$$G_a = \int_0^{2\pi} W_2 d(\Omega t) = \sum_{lm} |E_l|\,|E_m|\,\epsilon''_{ml} \cdot \pi \tag{44}$$

Terms in W_2 containing $\cos(n\Omega t)\cos(m\Omega t)$ with $n \neq m$, merely function as temporary storage of free-energy. Thus, in the nonlinear dielectric case, both ϵ'_{ml} (for $m \neq l$) and ϵ''_{ml} $(m \neq l)$ correspond to reversible free-energy exchange between the electric field and the enzyme. Only ϵ'_{ml} with $m = l$ gives rise to free-energy absorption from the field.

As discussed above (Section 3.2), certain enzymes when placed in a nonstationary electric field can harvest free energy from it and transduce that to chemical work or transport work. For such a case, the relationship between free-energy dissipation, output work, and free energy absorbed from the field per cycle is:

$$j_c \Delta G_{\text{out}} = \sum_{lm} |E_l|\,|E_m|\,\epsilon''_{ml}\pi - \Phi \tag{45}$$

Here j_c is the number of turnovers of the output reaction per field cycle [cf., 16]. The net heat exported from such a system is (per field cycle) [3]:

$$j_q = \sum_{lm} |E_l|\,|E_m|\,\epsilon''_{ml}\pi - j\Delta H_{\text{out}} \tag{46}$$

If we convert Equation (45) to units of $(\text{time})^{-1}$ by multiplication by $2\pi\Omega$, we obtain for the dissipation function Φ:

$$\Phi_{\text{conv}} = -\Phi_{\text{abs}} + \Phi \tag{47}$$

where Φ_{conv} is $-J\Delta G_{\text{out}}$ and Φ_{abs} is the free energy irreversibly absorbed per unit time.

We wish to note that the situation is somewhat paradoxical: harvest of free-energy from an oscillating electric field (even one at a well-defined single frequency [7, 16]) by an enzyme requires nonlinear behavior (though, as we shall discuss, not nonlinear behavior with respect to the polarization of the enzyme). Nonlinear dielectric behavior (i.e., with respect to the polarization) gives rise to higher harmonics, which do not absorb free energy from a single component input field. The inescapable conclusion is that the free-energy converter must direct free energy absorbed from the exciting field at the fundamental frequency to the higher harmonics, as well as/or to the thermodynamically uphill chemical or transport process. We shall next examine if, as in the case of enzymes following linear equations (cf. Section 2.6 of ref. 1), this absorbed free-energy is merely dissipated, or if it may be transduced to chemical or transport work.

3.3.2. *A special case: free-energy transduction in an enzyme with linear dielectric behavior*

In Section 2.6 of the previous article, we demonstrated that in systems described with linear equations only, no free-energy transduction from the field to some output process could occur. In this demonstration, we used the assumption that

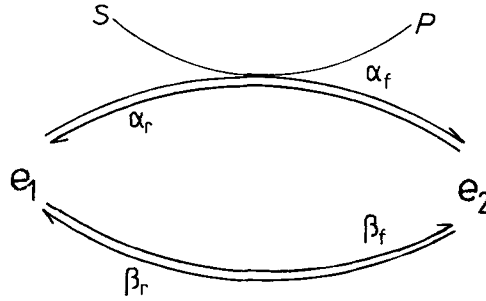

FIGURE 3 Diagram of a two-state enzyme. Note that there are two pathways between the two states e_1 and e_2, such that there is a catalytic cycle. The upper, or α-branch, may be coupled to the conversion of S to P. Its forward (i.e., from state 1 to state 2) rate constant is α_f, its reverse rate constant α_r. The forward and reverse rate constants of the lower (β-) branch are β_f and β_r respectively. For the enzyme to act as a catalyst it must cycle, i.e., move from state 1 to state 2 through the α-branch and back through the β-branch, or *vice versa*. If states e_1 and e_2 differ in effective dipole moment, then the four rate constants must depend on the electric field, as further described in the text.

the flux along any single pathway leading from one enzyme state to another could individually be expressed by a *linear* equation of the form of Equation (38) of the previous paper. Here, we will show that for a simple, but more realistic, two state model for an enzyme, net cyclic flux may not obey a linear equation even if the polarization can be treated as entirely linear. We consider the schematic two state enzyme diagram shown in Figure 3, with the rate coefficients being given by

$$\alpha_f = \alpha_{f0}[1 + fx] \tag{48}$$

$$\alpha_r = \alpha_{r0}[1 + (f - 1)x] \tag{49}$$

$$\beta_f = \beta_{f0}[1 + hx] \tag{50}$$

$$\beta_r = \beta_{r0}[1 + (h - 1)x] \tag{51}$$

and we let $\alpha_{f0} = \beta_{r0}$ and $f = 1$ and $h = 0$. In this case, the change in the probability of enzyme state e_1 (i.e., the polarization) with time is given by

$$de_1/dt = J_\alpha + J_\beta = -(2\alpha_{f0} + \alpha_{r0} + \beta_{f0})e_1 + [\alpha_{r0} + \beta_{r0}(1 - x)] \tag{52}$$

which is a strictly linear relationship according to our previous definitions since all of the cross correlation terms cancel. However, the cyclic (catalytic) flux is

$$2J \equiv J_\alpha - J_\beta = (\alpha_{r0} - \beta_{f0})e_1 + (\alpha_{f0} + \beta_{f0})xe_1 - [\alpha_{r0} + \beta_{r0}(x - 1)] \tag{53}$$

This includes potentially non-zero cross correlation terms (xe_1), and shows that a dynamic field (given by x) may induce net cyclic flux in such a system.

If also $\alpha_{r0} = \beta_{f0}$ the reaction catalyzed by the enzyme is at equilibrium, and for that case this demonstration does not prove free-energy transduction from the field to the output reaction. When we however considered that substrate is in ten fold excess over product and calculated $\langle\Phi\rangle$, $\langle\Phi_{\text{abs}}\rangle$, and then from Equation (47)) $\langle\Phi_{\text{conv}}\rangle$ with $x = x_0\cos(\Omega t)$, we obtained Figure 4 (Astumian *et al.*, 1988), which illustrates that a portion of the free energy irreversibly absorbed by an enzyme from the field may be transduced to the output reaction of that enzyme. The amount of free energy transduced is frequency dependent.

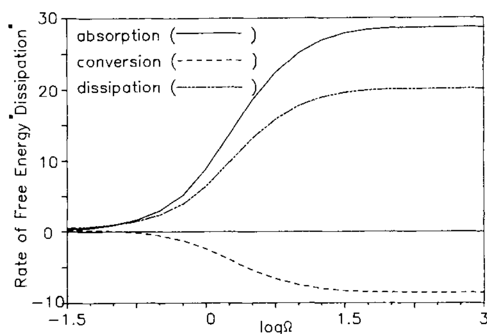

FIGURE 4 Free-energy absorption, dissipation and conversion calculated for the two state model enzyme of Figure 3 exposed to an oscillating field [$x = 0.8\cos(\Omega t)$] versus frequency, Ω. All zero field rate coefficients were taken to be unity, with field dependencies according to Equations 48–51 of the text. The apportionment factors were taken to be $f = 1$, and $h = 0$.

To keep the mathematics simple, we have thus far limited the discussion to enzymes that are taken to be describable by a two state diagram (although by implication in such a diagram as shown in Figure 3, each conformational state consists of a multitude of configurational substates which are assumed to be in rapid equilibrium). In general, however, enzymes tend to have many states [2, 27], and importantly for the catalysis of biological free-energy transduction [3], transitions between states occur in an ordered fashion [note that indeed, the two state model with $f = 1$ and $h = 0$ is obtained by reduction of the four state model of Figure 2 treating states 1 and 3 as steady state intermediates [11]]. Referring now to Figure 2, an enzyme in state 4 can get to state 2 by either passing through state 1 or state 3. Depending on the instantaneous magnitude of the field, one of these paths may be more kinetically favorable than the other. What we see illustrated in the field induced cycling of Equation (53) is a case of the oscillating field causing an alternation of the relative favorability of the two paths. This may lead to a tendency to undergo net clockwise (or counterclockwise, depending on the characteristics of the enzyme) cycling. An output force will also cause a tendency to cycle in one direction or the other, and if these two tendency are counterposed free energy transduction from the oscillating electric field to a chemical reaction will occur if the "tendency" due to the field is greater than that due to the output reaction.

3.3.3. Free-energy transduction and nonlinear dielectric behavior

In Section 2.6 of the previous article, we demonstrated that in linear dielectric systems no free-energy transduction from the field to some output process could occur. We shall now show why in the case of nonlinear dielectrics such a demonstration is no longer possible. In the case of nonlinear dielectrics, k_c and k_{-c}, as well as k_E in Equation (38) of the previous article [1] may depend on E. Taking the average of this equation yields terms like $\langle k_c e_1 \rangle$, which, because both k_c and e_1 now vary with time, in a correlated fashion, no longer yields $\langle k_c \rangle \langle e_1 \rangle$. Also, as shown by Equation (32), in the nonlinear case $\langle e_1 - e_{1eq} \rangle$ may not equal zero. Consequently, in the case of nonlinear dielectrics, it cannot be excluded

that the flux across a conformational transition of an enzyme can become nonzero due to a pushing effect of the oscillating electric field. However, for a two-state enzyme with only one reaction path connecting the two conformations, the average reaction rate along the single pathway must equal zero; for catalysis to occur an enzyme must be able to undergo a cycle [3, 27].

The simplest cycle consists of two distinguishable transitions between two states (cf. Figure 3). We shall assume that only one of these transitions is coupled to the interconversion of S and P. In order to simplify the algebra, we shall take a special case: pathway 1, which interconverts S to P is characterized by Equation (11) and the rate constants $\frac{1}{2}\alpha$, $\frac{1}{2}\beta$ and k_f (note that its $f = 1$), whereas pathway 2 is characterized by Equation (11) and rate constants $\frac{1}{2}\alpha$, $\frac{1}{2}\beta$, and $-k_r$ (its $f = 0$). As a consequence of these choices, Equation (11) applies to the sum of the transitions along the two pathways (α and β) (with $\Gamma = k_f - k_r$): If $k_f \neq k_r$ the enzyme exhibits nonlinear *dielectric* behavior in the sense specified in Section 2.2.

Subjecting the enzyme to the field described by Equation (12), we obtain for the average flux along either path:

$$\begin{aligned} c\langle v_1\rangle \delta p &= -\tfrac{1}{2}\alpha\langle P\rangle - \tfrac{1}{2}k_f\langle Px\rangle \\ &= -\tfrac{1}{2}\alpha\epsilon_0(\epsilon_0' - 1)E_0 - \tfrac{1}{2}k_f\epsilon_0(\epsilon_1' - 1)E_0 \\ &= -\tfrac{1}{4}x_0(\epsilon_1' - 1)(k_f - k_r)\epsilon_0 E_0 \end{aligned} \tag{54}$$

Thus, the conversion of P to S can be driven by the oscillating electric field, provided that $k_f \neq k_r$. The rate at which this occurs depends on the square of the electric field (through x_0E_0). The phenomenon arises because of the cross correlation between P and x ($\langle Px\rangle \neq 0$) and the nonlinear dependence of the dielectric displacement current on the electric field.

3.4. *Dynamic electric fields as intermediates between enzymes*

Above we have shown that enzymes of which the effective dipole moment varies as they proceed through their catalytic cycle, can be driven around that cycle by nonstationary electric fields in their environment. Conversely, as such an enzyme turns over, it will generate a nonstationary electric field. Only if many such enzymes turn over in synchrony, will a macroscopic fluctuating electric field become detectable (such a phenomenon might occur upon excitation of a membrane with light-driven proton or electron pumps with a train of intense light flashes). In the absence of such a synchrony, the fluctuating electric field generated by turnover of an enzyme will only be significant in the immediate vicinity of that enzyme. We have estimated that less than 2 nm away that field would be comparable in magnitude to the macroscopic transmembrane electric fields reported across biological membranes and implicated in biological free-energy transduction [18, 26]. Putting 1 and 2 together and being aware of the fact that in some cases of biological free-energy transduction part of the free-energy required to drive observed processes is missing [3, 35], we proposed that two enzymes juxtaposed in a biological membrane may exchange free energy through

dynamic interactions [17, 18, 24, 26, 36]. The one enzyme would generate locally a fluctuating electric field, the other would be driven by it in much (though not quite [24]) the same way as enzymes can be driven by an externally imposed oscillating electric field (cf., the previous sections). We recently established that this is consistent with thermodynamics [24, 26, 11].

The exciting possibility is that this kind of energy coupling between membrane enzymes constitutes an important aspect of biological free-energy transduction. In separate publications we have been reviewing preliminary evidence for this [18, 37]. This line of thought suggests that experimental methods inspecting the interactions between dynamic electric fields and macromolecules (such as dielectric spectroscopy), offer the potential of significantly increasing our insight in biological free-energy transduction. What is needed however is a substantial improvement of the signal to noise ratio of many such methods. Below we shall discuss what observations one may expect and how one may adapt dielectric spectroscopy so as to improve the signal to noise ratio.

4. EXPECTED NONLINEAR DIELECTRIC OBSERVATIONS IN ENZYME STUDIES

4.1. Calculated nonlinear dielectric spectrum for a membrane enzyme

For the enzyme depicted in Figure 2 we showed previously that in theory free-energy transduction may occur from a single component electric field to transport work [16]. For one of the calculated conditions with a single frequency sinusoidal field as the input, (■) in Figure 5 shows the Fourier spectrum (in terms of the norm of the Fourier components) of the calculated dielectric polarization. In line with the theoretical considerations given above, higher harmonics appear in the polarization.

The x's in Figure 5 indicate that the Fourier spectrum of the dielectric displacement depends on the magnitude of the output free-energy of the system. Rather strikingly, if the output free-energy is lowered to zero, all even harmonics disappear from the dielectric displacement. This probably results from the occurrence of symmetry in the kinetics of this particular model enzyme under such level flow conditions: it is probably not a general phenomenon at level flow. Further evidence for the dependence of the Fourier spectrum of the dielectric displacement on the kinetics of the enzyme, stems from Δ in Figure 5: 10-fold increase of all the kinetic constants of the enzyme (or a tenfold reduction in the frequency of the field) alters the Fourier spectrum.

We also performed a calculation at very low field strength ($x = 0.01$) and found that the dielectric displacement then only contained the fundamental frequency (0). At extremely high electric fields (not shown), the dielectric displacement itself as a function of time began to look like a square wave and its Fourier spectrum like the well defined Fourier spectrum of a square wave [15].

From these calculations we conclude that especially the relative amplitudes of the higher harmonics may contain kinetic information about the enzymes present in a biological specimen.

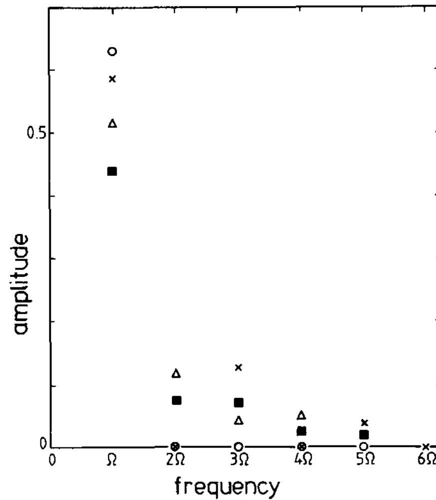

FIGURE 5 Fourier components in the dielectric displacement of a model membrane enzyme subject to an oscillating electric field. The four-state enzyme described in Reference 16 was subjected to a sinusoidal oscillating electric field (frequency Ω). Its polarization as a function of time was calculated through numerical integration of the appropriate rate equations, combining flow through the upper and the lower branches (cf. Figure 2). A Fourier analysis was then performed using the REALDFT operator of MLAB on three periods of the polarization after discarding the first (nonstationary) half-period. Standard case (■): field amplitude 71 mV (across the membrane), $\rho = 1$ ($\equiv$case 3 of Reference 16). (×): $\rho = 1$ ("level flow") ($\equiv$case 1 of Reference 16). (O): as (■) except that the field amplitude was reduced to 2.4 mV (amplitude was multiplied by 100). (△) at tenfold lower frequency of the exciting electric field, otherwise as (■). At frequencies other than the harmonics, no polarization was found.

4.2. Experimental feasibility of Non-Linear Dielectric Spectroscopy of biological systems

Let us estimate how membrane protein conformational changes may lead to observable results along the lines of Schwarz's discussion of a chemical contribution to the "linear" dielectric increment except here specified for the case of membrane embedded proteins. If the protein of interest is present at a concentration of 10 mg/ml and has a molecular mass of 500 000 (values typical for the ATP hydrolase in the chromatophore experiments of Kell and Harris [28, 29]); if the relevant difference in permanent dipole moments between the two states of interest is 500 D ($1.67 * 10^{-27}$ Cm, equivalent to 2–3 charges moving across the membrane); and if we assume (incorrectly) that Kirkwood's g parameter attains only its minimum value of unity, we have from Equation (23) of the previous article [1; see also 30] at 25°C, $\epsilon_1' = 30$ permittivity units, which is easily measurable. Given that other enzymes are certainly present and that Kirkwood's g factor is doubtless >1, it is not impossible that intramolecular conformational changes contributed to the novel low-frequency dielectric dispersion (μ-dispersion) observed previously [28, 29], particularly since it is to be expected that increasing the viscosity of the aqueous phase will affect enzymatic (inter-conformational) rate constants [31].

That simple non-linear systems may have the effect of "dumping" electrical

energy into frequencies different from the exciting one, or converting it into electrically silent work, opens up an entirely new arena for the dielectric investigation of membrane and other processes [32]. What are the possibilities for generating a strong enough oscillating field in real life? In homogeneous solution, even for dipoles, or dipolar transitions involving changes of 1000 D, the field strength required to go beyond the linear Langevin regime would be 1 MV/m. Such an AC field is probably near the limit of those attainable in aqueous solutions without dielectric breakdown. In a cell suspension, however, because of the basic geometry of a spheroidal closed nonconducting membrane immersed in a relatively highly conducting medium, the application of lower, (say <10 kV/m) fields can result in changes in the membrane potential of ≈ 50 mV, an intramembrane field strength of ≈ 10 MV/m, at the poles of the cells [7]. Any transmembrane proteins in this region will therefore experience an extremely intense electric field, which if oscillatory will certainly lead to significant non-linearity. Thus, we conclude that measurement of the nonlinear dielectric properties of membrane proteins at easily attainable external field strength is in principle accomplishable (although we note that, given the well-known tendency of simple electrochemical systems to generate harmonics [33], a 4-terminal approach would prove technically prudent), and the characterization of the frequency response of the system may well present a useful method for determining both structural and functional properties of membrane proteins.

Of particular relevance is the promise of specificity of the higher harmonics for membrane enzyme-linked processes. From Equations (28) and (30), we may estimate for the case that $\delta = 1$ and $k_f = k_r$ that the amplitude of the second harmonic is $x_0/2$ times the amplitude of the fundamental frequency, in the low-frequency limit. In other words, where the amplitude of the fundamental frequency in the dielectric displacement is proportional to $E\delta\mu$, the amplitude of the second harmonic is proportional to $(E\delta\mu)^2$, and that of the third harmonic to $(E\delta\mu)^3$. We shall compare bulk water with dipole moment of 1.85 D [34] in a field of 10 kV/m to a membrane protein with dipole moment difference between two conformations of 185 D in a field of 10 MV/m. Consequently the x-factor for the protein is about 100 000 times greater than that for water. We use a protein concentration of 1 mg/ml, and a protein molecular mass of 0.5 Md. This leads to a concentration ratio of protein to bulk water of $4/10^8$ (mole/mole). Thus, at the fundamental frequency, the dispersion by the water will swamp out that by the protein by a factor of 250. However, even at the second harmonic, the dispersion by the protein will exceed that by the bulk water, by a factor of 400. At the third harmonic the dispersion by the membrane protein will exceed that by the bulk water by a factor of 40 million. It is to be recalled here that we are imposing an oscillating sinusoidal electric field of but a single frequency.

We conclude that the rather general properties of typical proteins, i.e. their possession of conformational flexibility between states of different dipole moments, leads to their expression of extremely interesting nonlinear dielectric behavior, and that this may indeed be exploited in understanding their dynamics and ergo in biosensing [32]. Further enhancement of specificity may be obtained by specifically looking at higher harmonics at zero phase shift, which is indicative of saturation behavior.

CONCLUDING REMARKS

In these twin papers we have been searching through the theories of dielectrics, enzyme kinetics and non equilibrium thermodynamics for the best combination for describing the interactions between enzymes and dynamic electric fields. Although linear dielectrics does describe many interesting phenomena connected with the interaction of oscillating electric fields and proteins or membranes, it is not fit to address interactions that affect catalysis.

More specifically, three phenomena necessitated the exit from the linear domain. First, the interaction between thermodynamic parameters (such as electric fields) and rate constants tends to follow exponential dependences. Such dependences are linear only at low magnitudes of the applied field. As soon as the applied field is large enough to be of energetic significance (i.e., to exceed RT), its effect on the system can no longer be approximated by a linear dependence. Second, because of the conservation of the total enzyme concentration, enzyme kinetics its intrinsically nonlinear. Finally, oscillations in the electric field may entrain fluctuations in the state probabilities of the enzyme. The correlations between the oscillations and the fluctuations also affect the average rates of processes [11]. We wish to point out that in our studies, nonlinearity arises already from the interaction of a single enzyme molecule with its nonstationary environment. That is, we do not require ferroelectric properties such as cooperative interaction between many enzyme molecules or many lipid molecules. Nonlinear theories developed by various authors for such ferroelectric systems [e.g., 38, 39, see also reviews in 40, 41] are not fit to address the interaction between dynamic electric fields and the catalytic cycling of enzymes. Of course, our emphasis on the role of fluctuations in catalysis and free-energy transduction is not new (cf., 40, 41 and references therein).

In the second paper we have elaborated a description of the nonlinear interactions between nonstationary electric fields an enzymes. This required us to cross the limits of linear relations imposed by linear dielectric theory. The rewards are ample. First, the resulting nonlinear theory provides a smooth transition between dielectric theory, enzyme kinetics and non equilibrium thermodynamics. Second, it proved that when transgressing the bounds of the linear domain, one comes to expect that enzymes may transduce free energy from nonstationary electric fields. This opens up a wealth of concepts that may be important for bioenergetics and biochemistry more in general; hitherto elusive energetic "intermediates" in biological free-energy transducing systems may turn out to amount to nonstationary electric fields. Third, it suggest a new approach to dielectric spectroscopy, which may strongly increase the "signal" to "noise" ratio, where the "signal" is the properties of enzymes that are involved in their interactions with dynamic electric fields *in vivo*.

ACKNOWLEDGEMENTS

We thank Rick Welch for discussion and suggestions to improve the manuscript.

This study was supported in part by the Netherlands organization for scientific research (N.W.O.) and the Biotechnology Directorate of the Science and Engineering Research Council, U.K.

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