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On the nonlinear dielectric properties of biological systems

Saccharomyces cerevisiae

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ABSTRACT

The construction of a dual-cell, non-linear dielectric spectrometer is described. The system is applied to the study of resting cell suspensions of *S. cerevisiae*. Substantial harmonics are generated by these cells when stimulated by very modest exciting fields (ca. ± 2 V cm^{-1}, 20 Hz). The generation of these harmonics occurs only in living cells, and in a cell concentration-dependent manner. We studied the 3rd harmonic in detail. The ability to generate this harmonic is observable only within rather narrow voltage and frequency windows. The generation of a third harmonic is strongly inhibited by low concentrations of sodium metavanadate, suggesting that it may be ascribed largely to the H^+-ATPase present in the plasma membranes of these cells. When the enzyme is not at static head, the 3rd harmonic disappears and strong second and 4th harmonics may be observed. Non-linear dielectric spectroscopy constitutes a powerful and convenient means by which to monitor the ability of living cells to transduce exogenous electric field energy. This type of transduction may serve to account for the many reports of the ability of very weak electromagnetic fields to affect biological activity.

INTRODUCTION

The linear, passive audio- and radio-frequency electrical properties of biological systems have been studied since the last century [1–3], and are summarised in several recent reviews [4–15]. In the frequency range below 10 MHz or so, including that of present interest, these properties are conveniently measured as the equivalent parallel conductance and capacitance of an electrochemical cell containing the system under study [16]. Up to these radio-frequencies, most biological systems exhibit two major dispersions, known according to a terminology of Schwan [3] as the α- and β-dispersions. Whilst other sub-dispersions undoubtedly contribute to

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these major dispersions [17,18], and may occasionally be separated from them [7,19–21], it is generally recognised that the β-dispersion of tissues and cell suspensions is caused predominantly by the build-up of charge at the essentially non-conducting plasma membrane surfaces. The α-dispersion, though not always dependent upon the ionic strength of the medium [19], is usually accounted for mainly in terms of the relaxation of counterions tangential to the charged surfaces of the membrane and cell envelope.

In the simplest case of dielectric relaxation, that of the reorientation of a "hard" sphere with a permanent dipole moment μ [22], the statistical mean of the cosine of the angle θ that the dipole makes with the field has a field-dependence that follows the Langevin function $\cos\langle\theta\rangle = \coth x - 1/x$, where $x = \mu E/kT$, A Taylor expansion of this series shows that substantive deviations from linearity do not occur for values of x less than approximately 1, and that to an excellent approximation $\cos\theta = \mu E/3kT$ (see e.g. ref. 6). Thus the dielectric displacement current is proportional to the magnitude of the exciting field, and their ratio, the admittance, independent of it, properties characteristic of a linear system obeying the fluctuation–dissipation theorem [23,24].

Due to the fact that they are suspended or dissolved in conductive aqueous media, biological dielectrics are "lossy". Thus electrochemical reactions, and especially Joule heating, restrict the ac voltages that may be applied to them, and the dielectric properties of biological materials are typically measured using macroscopic electrical fields E of the order 0.1–5 V cm^{-1}. Given the effective dipole moments μ usually encountered, the Langevin factor $\mu E/kT$ is normally miniscule, and, as judged by the independence of the measured admittance from the exciting field, well within the range of linearity. However, it has been pointed out [14,25,26] that this criterion is less than robust; non-linear systems may *appear* linear by this criterion, but may nonetheless generate harmonics (which can in fact give important kinetic information). This lacuna is normally exacerbated since the impedimetric instruments used are designed to filter out currents and voltages at frequencies other than the fundamental [15,27], and thus force the observer to treat the system as linear sensu stricto, whatever the reality.

Indeed, substantial harmonics, in the presence of linear behaviour of the fundamental, have been observed for nerve axons using transmembrane electrodes [28,29] (which do not therefore obey the Nyquist relation [30]), and the generation of a second harmonic by black lipid membranes possessing a difference in transmembrane surface potential has also been known for some time [31,32]. Similar phenomena (of a linear impedance as judged by the fundamental under conditions in which substantial harmonics are generated) have also been observed by Furukawa's group in ferroelectric polymers [33,34]. Finally, the production of harmonics by purely (faradaic) electrochemical systems is well known, and is exploited analytically in the technique of 2nd-harmonic ac voltammetry [35,36]; in a related approach, Yoshikawa and colleagues [37,38] have demonstrated the generation of a variety of harmonics at the surface of chemically modified electrodes, and their modulation by the constitution of the aqueous medium in which they are immersed. However, because

the relevant geometry means that the thickness of the dielectric layer in all of these systems is very small, the relatively modest voltages applied can correspond, in many cases, to substantial transmembrane or trans-interface electrical fields.

When a field of appropriately low frequency is applied to a *suspension* of cells contained between two or more macroscopic electrodes, the charging of the membrane capacitance may also cause a lesser but effective "amplification" of the macroscopic field across the membrane [39,40]. In certain cases in which the membrane of interest contains appropriate enzymes this can cause the performance of useful biological work in a field- and frequency-dependent fashion [41–43]. The general mechanism underlying this effect is that enzymes are not dipolar billiard balls [9,44–47], and can relax between different conformations, some of which may and some of which may not have different vectorial dipole moments from each other; simple model (theoretical) systems of this type can accurately reproduce the data obtained [14,48–55]. An important *corollary* of this which follows from theoretical considerations [14,25,26,52], but one which has yet to be demonstrated experimentally, is that such systems should be expected to display non-linear dielectric properties, in the sense that they should generate harmonics, at relatively modest exciting fields.

There are various means by which one may seek to measure the non-linear dielectric behaviour of a (biological or other) system, for instance by using a high field to excite the system and a low "probing" ac voltage to register the field-dependent dielectric properties [9,56]. In an alternative approach, one may use a pure sinusoidal ac current to excite the system but instead of measuring the ac current *only* at the frequency applied, one studies the entire frequency space of interest by performing a Fourier (or other) transformation to see the extent to which the non-linearities of the system are manifest by the generation of harmonics. By varying the frequency and amplitude of the exciting current one may then build up a 2-dimensional non-linear dielectric spectrum which can then act as a "dielectric fingerprint" of the system under test.

The purposes of the present article are therefore (i) to describe our implementation of the latter type of system for the registration of such non-linear biological dielectric spectra, (ii) to indicate for the first time that they are easily observed in cell suspensions, using intact cells of *Saccharomyces cerevisiae*, and (iii) to provide data which suggest that the H^+-ATPase in the plasma membrane of this organism is the source of the majority of the non-linear dielectricity thus observed.

MATERIALS AND METHODS

Organism

Sacharomyces cerevisiae was obtained locally as a freeze-dried powder, and resuspended to a concentration of ca. 50 mg dry wt ml^{-1} in a medium consisting of 20 mM KH_2PO_4, 30 mM KCl, 1 mM $MgCl_2$, pH 6.5. All experiments were performed in this medium unless otherwise recorded, within 4 h after the preparation of the stock cell suspension.

Chemicals

All of these were obtained from the Sigma Chemical Company or BDH Ltd., Poole, Dorset. Water was singly-distilled in an all-glass apparatus.

Apparatus

A block diagram of the non-linear dielectric spectrometer which we have devised and implemented in this work is shown in Fig. 1. A description of the design considerations and their implementation follows. The system (Fig. 1A) is built around an IBM-PC-AT-compatible microcomputer (Viglen III, 80386 main processor, Viglen Ltd, Unit 7, Trumpers Way, London W7 2QA). To maximise the performance of the mathematical operations, we incorporated an 80387-compatible coprocessor (Cyrix 83C87). One of the expansion ports of the computer was furnished with a Data Translation model 2823 ADC/DAC board. Because of the

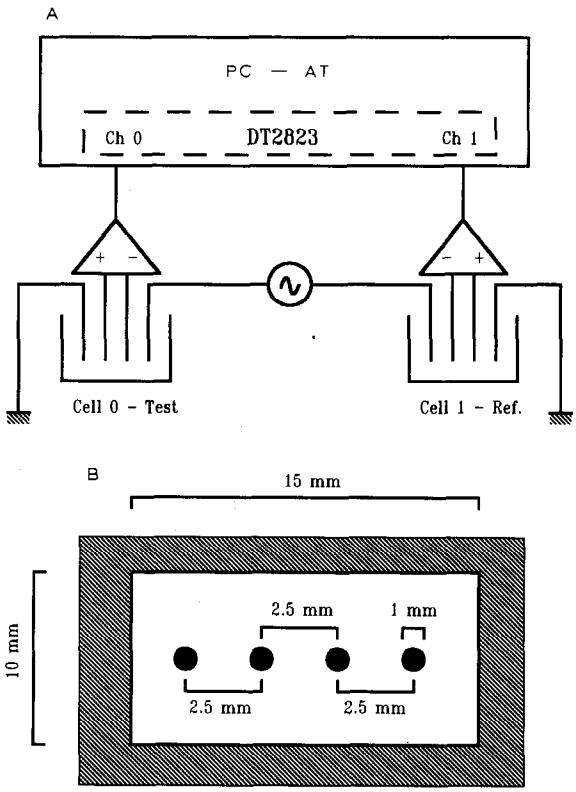

Fig. 1. A block diagram of the non-linear dielectric spectrometer devised for the present work. (A) The overall setup, consisting of matched electrochemical cells connected to a sinusoidal oscillator and, via a Data Translation Analog-to-Digital converter, to an 80386-based microcomputer. (B) Plan of the electrochemical cell, to show the dimensions and geometry. The electrodes were 24-carat gold pins, of length 6.7 mm. The volume of suspension used was 1 ml. For further details, see the text.

expectation that the harmonic signals would have a low magnitude, a 16-bit board was chosen, with 4 differential inputs and a stated upper frequency of 100 kilosamples/s. To minimise the contribution of electrode polarisation phenomena [3,9,15], a 4-electrode system based on gold pin-type electrodes was used (Fig. 1B). Signals were applied to the outer, current electrodes by means of a Thandor TG501 function generator (RS Components Ltd.). The frequency and amplitude of these signals was checked using a Solartron 1200 Signal Processor (Schlumberger Instruments, Farnborough, Hants.) and a Hameg NM 208 Digital Storage Oscilloscope.

The acquisition of the data and its subsequent processing and display was performed using the ILS software (Signal Technology Inc., Goleta, CA), running under control files written in-house. In all cases, spectra were collected as follows: the system of interest was pipetted into the electrode cell, the test waveform applied to the (outer) current electrodes, and the data logged from the voltage electrodes at a sampling frequency (which was typically at 25 times the frequency of the fundamental) and for a time specified by the operator. Time was specified in terms of a number of blocks, each block consisting of 512 samples. At the end of the time specified, the data were Fourier-transformed as follows. Preliminary pre-whitening was carried out [57] by subtracting the mean of the block from the individual samples. Each block was then windowed using a Blackman window [58] and fast-Fourier-transformed using routines within the ILS software to form an ensemble of power spectra. These were then averaged in order to enhance the signal : noise ratio. The method of time- averaging modified periodograms was that due to Welch [59]. The spectral data were stored on the computer's hard disk.

A reference spectrum was acquired using the supernatant, whose conductivity had been adjusted (with distilled water, to compensate for the volume fraction of cells present in the sample; see refs. 60 and 61) to be identical to that of the sample at the frequency of interest. Two different types of control file were used, depending upon whether the reference was to be logged using the same set of electrodes or (as was done in the experiments presented herein) a separate matched cell. In either case, the logging, windowing and Fourier transformation routines were identical, and provided a power spectrum of the "reference" cell, which was also logged on

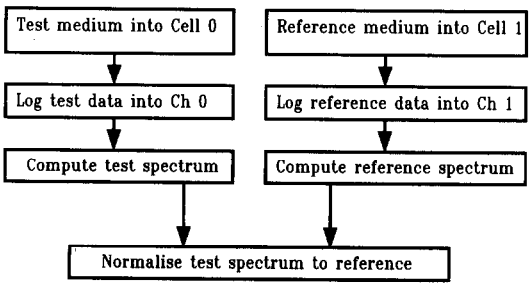

Fig. 2. Block diagram of the method used to obtain non-linear dielectric spectra uncontaminated by artefactual electrochemical phenomena. For further details, see the text.

the computer's hard disk. Finally the "sample" power spectrum so obtained was divided by the "reference" power spectrum, and also stored on the disk. The total time necessary to acquire a difference dielectric spectrum (at 20 Hz, 500 samples per second for 10 blocks) was some 2.5 min. A diagram of the steps involved in the generation of the non-linear dielectric spectra is shown in Fig. 2. It may be stressed that the power of this approach was that it allowed us to deconvolve effects due to nonlinearities within the *electrochemical* system from those due to the biological cells themselves.

RESULTS

Figure 3 shows a typical non-linear dielectric spectrum obtained from a suspension of resting cells of *S. cervisiae* using the system described, with an exciting voltage (measured between the outer electrodes) of ± 1.5 V (2.0 V cm^{-1}) at a frequency of 20 Hz, and displays spectra from (A) the sample, (B) the reference and (C) their difference. The following observations may be made: (i) due to imperfections in the generator and the non-linearities inherent in electrochemical systems, the applied waveform is not purely sinusoidal but contains harmonic components which, although very small by comparison with the energy in the fundamental may yet be observed using a measuring system with the present (16-bit) sensitivity and a logarithmic display; (ii) the pattern of harmonics between the "sample" and "reference" cells is markedly different; (iii) upon subtraction of the reference spectrum from the sample spectrum a very strong and apparently negative *3rd* harmonic is obtained. A (positive) 7th harmonic is also reproducibly observed (and on occasion an 11th harmonic), but even harmonics are absent under the stated conditions. We believe this to be the first demonstration of nonlinear dielectricity in a biological cell suspension.

It should be stressed that because the production of power spectra using Fourier transformation results in the loss of phase information [62–67], only the *magnitude* of the 3rd harmonic (or indeed of any other harmonics) is representative of the energy transduced by the cells from the fundamental to the given harmonic. Thus the presence of negative harmonics is due simply to destructive interference between the biologically generated signal at a given harmonic and that from the external signal source. It is not correct to state (and see later) that the cells have 'harvested' energy from the 3rd harmonic present in the exciting signal since no harmonics are observed when the fundamental voltage or frequency of the exciting signal are changed to those of the third harmonic generated by our signal source when the fundamental is set at ± 2 V cm^{-1} and 20 Hz. Thus, variations in the magnitude of a harmonic may be caused by variations in its *absolute* magnitude *and/or* its phase relative to that of the 3rd harmonic present in the exciting signal. Because much of the present study is focussed on the negative 3rd harmonic generated by these cells, we display our data in what follows using the logarithmic (dB) scale, since this serves to accentutate the precision with which alterations in its observable magnitude may be recorded.

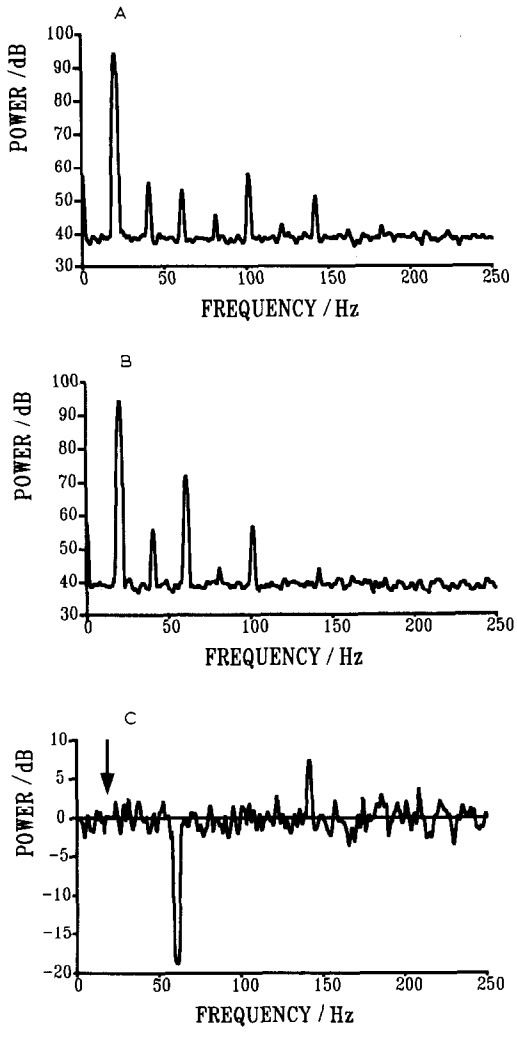

Fig. 3. Non-linear dielectric properties of *S. cerevisiae*. A suspension of cells (50 mg dry wt ml^{-1}, prepared as described in the Methods section), was placed in the test cell, and a sample of its supernatant (with its conductance at 1 kHz adjusted (to 6.5 mS) to match that of the cells) was placed in the reference cell. Spectra were obtained using an exciting voltage of ± 1.5 V (field strength 2.0 V cm^{-1}) at a frequency of 20 Hz, and each spectrum represents the average of 10 blocks. (A) Test cell, (B) reference cell, test cell minus reference cell. In C the arrow shows the exciting frequency.

Whilst there was some day-to-day variation in the exact magnitude of the 3rd harmonic (when viewed on the logarithmic decibel scale), the data displayed were very reproducible within a few dB, particularly for a given batch of cells. Similarly, using identical "samples" (or "reference" supernatants) in both electrochemical cells, no harmonics were observed, (i) indicating that the surface electrochemistry of

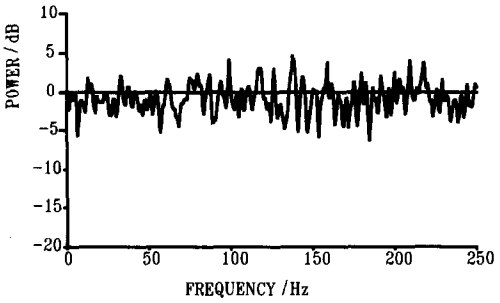

Fig 4. Lack of ability of boiled cells to generate a non-linear dielectric spectrum. The experiment was performed exactly as in Fig 3, except that the cells were placed in a water bath at 100°C for 5 min before carrying out the measurement. The data correspond to those in Fig. 3C, and show the "difference" dielectric spectrum.

their electrodes was well-matched, and (ii) that the yeast cells were indeed the source of the 3rd harmonic observed.

Because of the use of the reference spectrum method, it was clear that the generation of a 3rd harmonic depended upon the presence of yeast cells. The next experiment was designed to establish whether *dead* (boiled) cells could generate a third harmonic, and the data in Fig. 4 show that they did not. This indicated that the presence of potentially active enzymes was prerequisite to the generation of a third harmonic.

To establish whether only harmonics were generated, or whether the power spectra contained non-harmonic components, we varied the number of blocks that were averaged. The data from a representative set of runs are displayed in Fig. 5, where it may be observed that the magnitude of the 3rd harmonic remains essentially constant in the face of a highly variable degree of noise, the variance of the noise decreasing (as expected) in proportion to the number of blocks, such that no true non- harmonic components could be discerned.

The dependence of the magnitude of the 3rd harmonic as a function of the concentration of cells is shown in Fig. 6, where it may be observed that the magnitude of the 3rd harmonic in dB is approximately linear with the concentration of cells up to a cell concentration of some 25 mg dry wt ml^{-1} whereupon a transition to a plateau region may be observed.

The 3rd harmonic generated was usually maximal when the exciting frequency was some 15–20 Hz. Figure 7 displays the magnitude of the 3rd harmonic as a function of the exciting frequency. It may be observed that as the frequency is increased above or decreased below some 15–20 Hz, the magnitude of the 3rd harmonic drops off rather sharply.

As well as the above frequency window, there was an even sharper voltage or amplitude window within which non-linear dielectric behaviour could be observed. Figure 8 shows that the magnitude of the 3rd harmonic is only significant in a voltage window between ca. 0.6 and 2.1 V (0.8–2.8 V cm^{-1}). Such a sharp

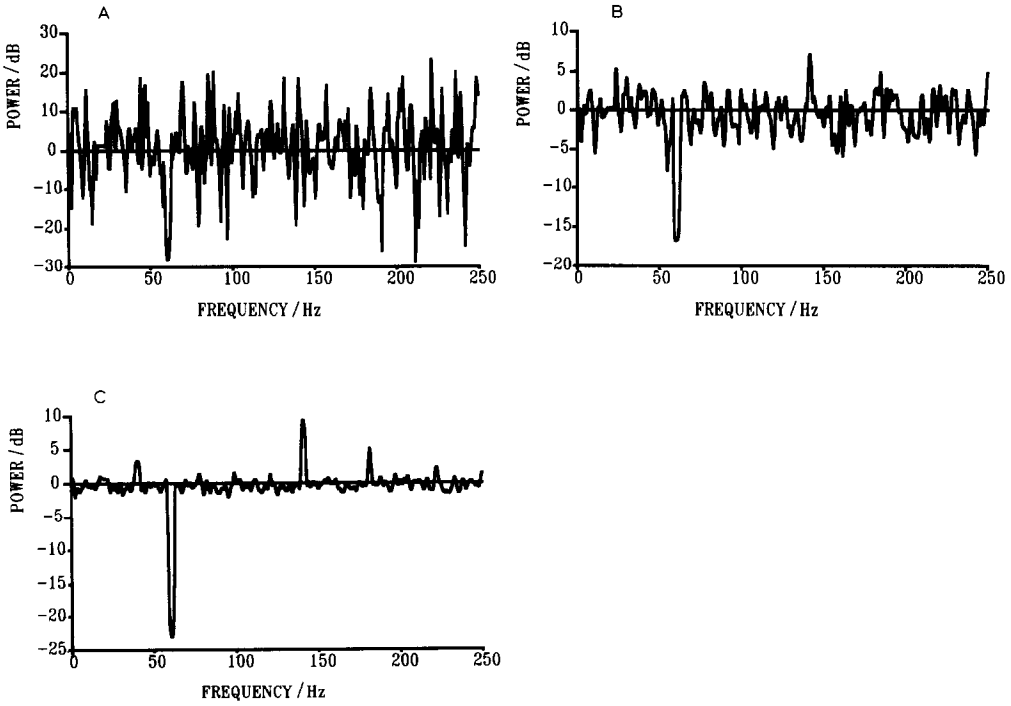

Fig. 5. Effect of averaging on the non-linear dielectric spectrum of *S. cerevisiae*. The experiment was performed as described in the legend to Fig. 3, with an exciting field of ± 2.0 V cm^{-1} at a frequency of 20 Hz, except that the number of blocks averaged to give the difference dielectric spectrum were (A) 1, (B) 10, (C) 100.

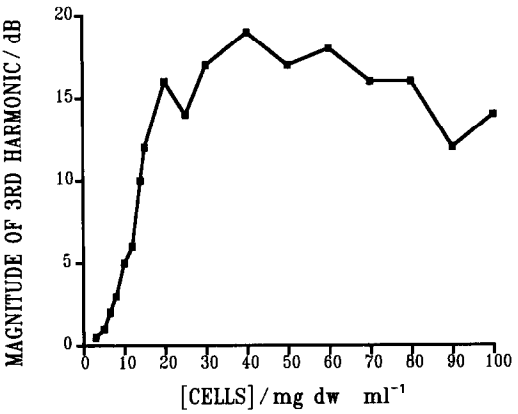

Fig. 6. Effect of cell concentration on the magnitude of the 3rd harmonic in the non-linear dielectric spectrum of *S. cerevisiae*. The experiment was performed as described in the legend to Fig. 3, with an exciting field of ± 2.0 V cm^{-1} at 20 Hz and the cell concentration was varied as indicated.

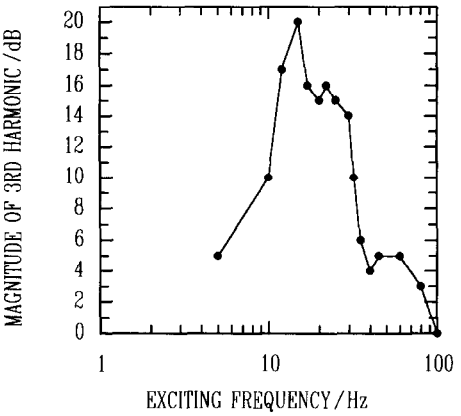

Fig. 7. Effect of exciting frequency on the magnitude of the 3rd harmonic of the non-linear dielectric spectrum of *S. cerevisiae*. The experiment was performed as described in the legend to Fig. 3, except that the exciting frequency was varied as indicated.

amplitude window (albeit at absolute values of the field some 10-fold higher) was also observed by Tsong and colleagues in their studies of the electrostimulation of the erythrocyte Na, K-ATPase [41–43]. When the exciting frequency was varied, the amplitude window observed did not appear to change significantly (data not shown). If the measurement was carried out in the presence of an additional *electrostatic* (dc) field, the magnitude of the 3rd harmonic was strongly decreased, disappearing completely when the dc field exceeded ± 0.4 V cm^{-1} (and the exciting ac field was ± 2.0 V cm^{-1}).

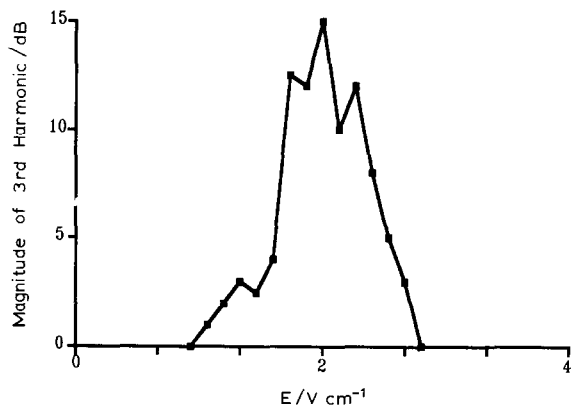

Fig. 8. Effect of the strength of the exciting field on the magnitude of the 3rd harmonic of the non-linear dielectric spectrum of *S. cerevisiae*. The experiment was performed as described in the legend to Fig. 7, at a frequency of 20 Hz, except that the field was varied as indicated.

As discussed in detail later, the electrical fields involved in these experiments are (in terms of the Langevin factor) quite miniscule, and the system would normally be expected to lie well within the domain of linearity. Indeed, observations of the fundamental, and linear impedance measurements using a Hewlett-Packard 4192A Impedance Analyser (see ref. 60), indicated that the *linear* impedance was independent (within experimental error) of the magnitude of the exciting voltage in the range studied. Thus, as intimated in the introduction, it is quite feasible to have a system in which the impedance *appears* linear but which is in fact non-linear. (We recognise that the potential-dependent *capacitance* (thickness) of a bilayer membrane can exhibit a quadratic dependence upon the potential difference across it [69–72]. However, at the potentials used here the quadratic term is negligible.)

As outlined by Tsong [73], membrane proteins are particularly powerful candidates for interacting with electrical fields for a variety of reasons, including the following: (i) the membrane protein cannot rotate from one side of the membrane to the other and dissipate electrical energy by simple Debye-like rotation of this type; (ii) as described above, the membrane can "amplify" the exciting signal; (iii) membrane proteins have substantial dipole moments [74]. In addition, of course, in common with all proteins, they can effect transitions between different conformational states possessing different dipole moments. Thus in seeking a mechanistic basis for the remarkable generation of non-linear dielectricity that we have observed one is led to consider the membrane proteins of this organism.

A washed cell suspension of *S. cerevisiae* with no added carbon substrate, slowly metabolising endogenous stores under the presumably essentially anaerobic conditions pertaining in the relatively concentrated suspensions normally used, may be expected to generate ATP by substrate-level phosphorylation. As a major potential mechanism of "slip" or "overflow metabolism" [75], this ATP may be expected to be utilised by the H^+-ATPase present in this organism [76–80] to drive the uptake of cations such as K^+ from the external medium until a state of "static head" (see ref. 81) is attained, in which the free energy of hydrolysis of ATP is balanced against the free energy stored in the K^+ gradient (and indeed those of other ions]. The distribution of enzymic conformational macrostates would then be approximately determined by their basic free energies [82] and the net turnover (rate of entropy production) of the enzyme would be minimal. In this sense the energetic macrostates of the enzyme may then be regarded as a symmetrical potential well. This would then explain the generation of odd but not even harmonics. Our working hypothesis, then, was that the H^+-ATPase in the plasma membrane of this organism, as the main enzyme potentially active under the physiological conditions used, was in fact the major source of the nonlinear dielectricity observed.

It is well known that the catalytic cycle of enzymes of this type (the so-called E_1E_2 enzymes] involves an enzyme-bound phosphate intermediate, and that their activity can be inhibited by low concentrations of pentavalent vanadium compounds [83] whose trigonal bipyramidal structure is thought to mimic the transition state of the phosphate during its hydrolysis, trapping the enzyme in its E_2 conforma-

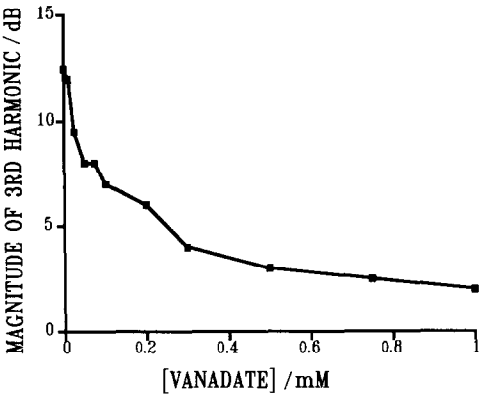

Fig. 9. Effect of sodium metavanadate on the magnitude of the 3rd harmonic of the non-linear dielectric spectrum of *S. cerevisiae*. The experiment was performed as decribed in the legend to Fig. 3, with an exciting field of ± 2.0 V cm^{-1} at 20 Hz, in the presence of the concentrations of sodium metavanadate indicated.

tion [84,85]. Thus it was of interest to study the effect of vanadate on the ability of resting cell suspensions of *S. cerevisiae* to generate a 3rd harmonic.

Figure 9 shows the effect of quite modest concentrations of vanadate on the magnitude of the 3rd harmonic, where it may be observed that the generation of this harmonic is essentially completely abolished by 1 mM sodium metavanadate, and with a K_i^{app} (when the ordinate is plotted using a dB scale) of approximately 0.15 mM. This suggests strongly that the H^+-ATPase in the plasma membrane of these cells is the main source of the non-linear dielectricity, and further serves to exclude phenomena such as dielectrophoresis [6,68] as the source of the non-linearities observed.

As described above, a 3rd harmonic was reproducibly observed with resting cell suspensions of *S. cerevisiae*, could be ascribed to the presence of the H^+-ATPase in this organism, and should be expected to reflect a situation in which the enzyme was at static head. The question then arose as to how this behaviour might be modified when the enzyme was driven away from static head and was able (or expected) to do work. To this end, the following experiment was performed. Resting cells were taken, their (usual) non-linear dielectric spectrum recorded, and a metabolisable carbon source (D-glucose) added. Alter a short lag period of some 20 minutes, the spectrum displayed in Fig. 10 was recorded. Remarkably enough, the 3rd harmonic had disappeared and was replaced by substantial 2nd and 4th harmonics. These even harmonics were also vanadate-sensitive (data not shown). When static head was again attained, the spectrum returned to its starting shape, with a substantial 3rd but no even harmonics (data not shown). This behaviour is consistent with the view that *when carrying out net work (energy transduction)*, the enzyme represents an *asymmetric* potential well with the rectification necessary [52] for the absorption of exogenous electric field energy. A parallel measurement of the dielectric permiitivity

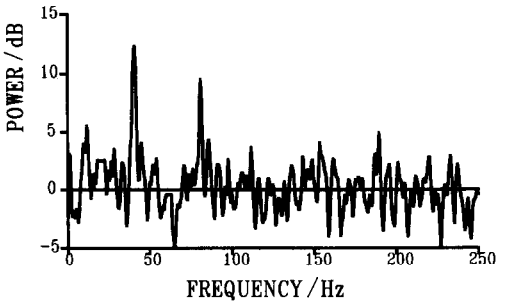

Fig. 10. Effect of glucose on the non-linear dielectric spectrum of *S. cerevisiae*. The experiment was performed as described in the legend to Fig. 3, except that the cells were preincubated with 100 mM D-glucose for 20 min.

at 0.3 MHz, a monitor of intact cellular biomass [86], did not show any observable changes during this experiment (not displayed). Thus non-linear dielectric spectroscopy provides a sensitive means of distinguishing the *metabolic* states of living cells.

DISCUSSION

A variety of recent theoretical and experimental work has focussed on the possibility that enzymes, particularly those in membranes, can capture and transduce the energy in a non-stationary electrical field for the production of useful (bio)chemical work [14,25,26,41–55,87]. We and others have pointed out that such systems should be expected to display non-linear dielectric properties, in the sense that they should generate harmonics, at the rather modest exciting fields normally employed in linear dielectric measurements [9,15,25,26,52,55].

In the present work, we have subjected this proposal to a first experimental test, by constructing a non-linear dielectric spectrometer which can apply sinusoids of a given frequency and, by means of Fourier transforms, can analyse the response of the biological system at *a range of* frequencies. This approach, in contrast to the simplest forms of white noise application [88], does not require us to *know* which if any non-linearities occur in the system. Using resting cell suspensions of *Saccharomyces cerevisiae*, we demonstrated that in the presence of a very modest exciting field of only ± 1–2.5 V cm^{-1} at 15–20 Hz, sizeable 3rd and 7th harmonics could be observed under conditions in which the linear impedance was (as expected) voltage-independent.

Further experiments showed (i) that the signal required living cells, (ii) that it depended on their concentration, (iii) that it had a very sharp voltage (field) window, (iv) that only harmonics were generated, and (v) that the optimal frequency of excitation was some 15–20 Hz. Its sensitivity to low concentrations of the inhibitor sodium vanadate indicated that the source of the 3rd harmonic was overwhelmingly due to the H^+-ATPase in the plasma membrane of this organism

[76–80], which, due to its structural and mechanistic similarity to the Na,K-ATPase [85] may be expected to transduce exogenous electrical energy in a similar manner to that demonstrated by Tsong and colleagues for the latter [41–43].

Since the characteristic frequency of the charging time for the cell membrane under the conditions of conductivity used is many times greater than the frequencies applied here [60, 86], the electric potential V_m generated across the (spherical) yeast plasma membrane is given by $V_m = 1.5\ rE \cos\theta$ where E is the macroscopic field, r the cell radius and θ the angle between the field and the membrane normal [10,39,40]. For the present cell radius of 3 μm and a field of 2 V cm^{-1}, we obtain a *maximum* (field-dependent change in) membrane potential of 0.9 mV. For a membrane thickness of 5 nm, the maximum oscillatory *transmembrane* field E_m is 1800 V cm^{-1}. Typical membrane proteins have *permanent* dipole moments m of 100–1000 Debye units [10,74]. For convenience we will assume that relevant *changes* in dipole moment due to field-dependent conformational changes of target enzymes are 500 D (this is very charitable; they are likely to be much less since 500 D equates to the displacement of 10 full charges across the membrane [20]!). The Langevin factor $\mu E_m/kT$ is then equal to some 0.075, i.e. *substantially* less than 1. Thus despite the application of a field which would normally be regarded as very modest [89], we have observed the generation of a substantial third harmonic by the *S. cerevisiae* cells.

Astumian and Robertson [52] recently gave the equations for describing the behaviour of a 2-state enzyme in a black lipid membrane when excited with a sinusoidally modulated eletric field, and showed that all harmonics could be generated under appropriate conditions. They indicated that electroconformational coupling caused a rectification of the exciting signal, together with a dc shift and that the 2nd harmonic, but *not* the odd ones, was very sensitive to the concentration of the substrate of the (theoretical) model enzyme.

The same authors [52] also considered suspensions of spherical cells, similar to those used in the present work, and found that the first and 3rd harmonics disappeared when there was a spherical geometry. The present results are at first sight in contradiction to this analysis, since we regularly observed substantial 3rd (and other odd) harmonics in resting cell suspensions. However, the "spherical symmetry" required in the Astumian and Robertson analysis extends to the molecular level, and assumes, for instance [52], that the H^+-ATPase enzymes are randomly arrayed in the plane of the membrane. Not only is this moderately unlikely in principle, but it has been shown that low-frequency fields can cause the lateral movement of proteins in the plane of the membrane; dc fields will tend to cause the accumulation of appropriately charged proteins at a single pole [90] whilst ac fields cause their (statistical) accumulation at both poles facing the electrodes (see ref. 7, 19–21, 91). That this occurs in yeast cells is strengthened by recent studies on the temperature-dependence of their β-dielectric dispersion [18]. Thus our observation of a substantial 3rd harmonic is not in contradiction to the analysis of Astumian and Robertson [52].

The cells which we studied were resting, in the sense that they had no added

carbon substrate, and it is to be assumed that the H^+-ATPase had reached a state of static head. Under these circumstances, Astumian and Robertson [52] showed that even harmonics are not to be expected, and indeed we did not find any. Inverting this argument, it *is* to be expected that if the enzyme is made to do work then even harmonics should appear. When the cells were given glucose and allowed to glycolyse, this behaviour was indeed observed; the 3rd harmonic disappeared and substantial even harmonics were observed. Although the nature and magnitude of these harmonics is expected to reflect the kinetics of the enzyme as it acts in situ, the present type of Fourier analysis does not permit us to extract the required information on the turnover number of the enzymes.

Notwithstanding, the present experiments have shown for the first time in cell suspensions that the conformational flexibility of enzymes can manifest in the generation of non-linear dielectric spectra at very modest values of the exciting field, in which the Langevin factor is extremely small and in which purely linear behaviour would formerly be expected. The present approach and analysis may serve to account in part for the many (and increasing) number of reports in which very weak electromagnetic fields have been shown to elicit biological or biochemical responses [92–114].

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REFERENCES

1 G.N. Stewart, J. Physiol., 24 (1899) 356.
2 R. Hübner, Arch. Gesamt. Physiol. Mensch., 133 (1910) 237.
3 H.P Schwan, Adv. Biol. Med. Phys., 5 (1957) 147.
4 E.H. Grant, R.J. Sheppard and G.P. South, Dielectric Properties of Biological Molecules in Solution, Oxford University Press, Oxford, 1978.
5 O. Schanne and E.R.P Ceretti, Impedance Measurements in Biological Cells., Wiley, Chichester, 1978.
6 R. Pethig, Dielectric and Electronic Properties of Biological Materials, Wiley, Chichester, 1979.
7 D.B. Kell and C.M. Harris, J. Bioelectricity, 4 (1985) 317.
8 K.R. Foster and H.P Schwan in C. Polk and E. Postow (Eds.), CRC Handbook of Biological Effects of Electromagnetic Fields, CRC Press, Boca Raton, 1986, p. 27.
9 D.B. Kell in A.P.F. Turner, I. Karube and G.S. Wilson (Eds.), Biosensors; Fundamentals and Applications, Oxford University Press, Oxford, 1987, p. 428.
10 R. Pethig and D.B. Kell, Phys. Med. Biol., 32 (1987) 933.
11 K.R. Foster and H.P Schwan, CRC Crit. Rev. Biomed. Eng., 17 (1989) 25.
12 C.L. Davey and D.B. Kell in R. Paris (Ed.), Electric Field Phenomena in Biological Systems, IOP Short Meetings Series Vol. 21, Institute of Physics, London, 1989, p. 51.
13 S. Takashima, Electrical Properties Properties of Biopolymers and Membranes, Adam Hilger, Bristol, 1989.

14 C.L. Davey and D.B. Kell in M. O'Connor, R.H.C. Bentall and J.S. Monahan (Eds.), Emerging Electromagnetic Medicine, Springer, Berlin, 1990, p. 19.
15 D.B. Kell and C.L. Davey in A.E.G. Cass (Ed.), Biosensors: a Practical Approach, Oxford University Press, Oxford, 1990, p. 125.
16 H.P. Schwan in W.L. Nastuk (Ed.), Physical Techniques in Biological Research, Vol. 6B. Academic Press, New York, 1963, p. 323.
17 C.L. Davey, G.H. Markx and D.B. Kell, Eur. Biophys. J., 18 (1990) 265.
18 L.E. Ferris, C.L. Davey and D.B. Kell, Eur. Biophys. J., 18 (1990) 276.
19 D.B. Kell, Bioelectrochem. Bioenerg., 15 (1983) 405.
20 D.B. Kell and C.M. Harris, Eur. Biophys. J., 12 (1985) 181.
21 C.M. Harris and D.B. Kell, Eur. Biophys. J., 13 (1985) 11.
22 P. Debye, Polar Molecules, Dover Press, New York, 1929.
23 R. Kubo, J. Phys. Soc. Jpn., 12 (1957) 570.
24 G. Williams, Adv. Mol. Relax. Proc., 1 (1970) 409.
25 D.B. Kell, R.D. Astumian and H.V. Westerhoff, Ferroelectrics, 86 (1988) 59.
26 H.V. Westerhoff, R.D. Astumian and D.B. Kell, Ferroelectrics, 86 (1988) 79.
27 J.R. Macdonald, Impedance Spectroscopy, Wiley, New York, 1987.
28 L.J. DeFelice, W.J. Adelman Jr., D.E. Clapham and A. Mauro in W.J. Adelman Jr. and D.E. Goldman (Eds.), The Biophysical Approach to Excitable Membranes, Plenum, New York, 1981, p. 37.
29 S. Miyamoto and H.M. Fishman, IEEE Trans. Biomed. Eng., BME-33 (1986) 644.
30 H.M. Fishman, H.R. Leuchtag and L.E. Moore, Biophys. J., 43 (1983) 293.
31 W. Carius, J. Coll. Interface Sci., 57 (1976) 301.
32 V.S. Sokolov and V.G. Kuz'min, Biofizika, 25 (1980) 170.
33 T. Furukawa, K. Nakajima, T. Koizumi and M. Date, Jpn. J. Appl. Phys., 26 (1987) 1039.
34 T. Furukawa, M. Tada, K. Nakajima and I. Seo, Jpn. J. Appl. Phys., 27 (1988) 200.
35 A.J. Bard and L.R. Faulkner, Electrochemical Methods, Wiley, Chichester, 1980.
36 E.R. Brown and J.R. Sandifer in B.W. Rossiter and I.F. Hamilton (Eds.), Physical Methods of Chemistry, Vol. 2, 2nd ed., Electrochemical Methods., Wiley, New York, 1986, pp. 273–432.
37 K. Yoshikawa, M. Shoji, S. Nakata and S. Maeda, Langmuir, 4 (1988) 759.
38 S. Nakata, K. Yoshikawa, M. Shoji, H. Kawakami and T. Ishii, Biophys. Chem., 34 (1989) 201.
39 U. Zimmermann, Biochim. Biophys. Acta, 694 (1982) 227.
40 T.Y. Tsong and R.D. Astumian, Bioelectrochem. Bioenerg., 15 (1986) 457.
41 E.H. Sepersu and T.Y. Tsong, J. Membr. Biol., 74 (1983) 191.
42 E.H. Sepersu and T.Y. Tsong, J. Biol. Chem., 259 (1984) 7155.
43 D.-S. Liu, R.D. Astumian and T.Y. Tsong, J. Biol. Chem., 265 (1990) 7260.
44 G.R. Welch and D.B. Kell in G.R. Welch (Ed.), The Fluctuating Enzyme, Wiley, New York, 1986, p. 451.
45 D.B. Kell, Univ. Wales Rev. Sci. Technol., 1 (1988) 64.
46 D.B. Kell, ISI Atlas Sci.: Biochemistry, 1 (1988) 25.
47 D.B. Kell in H. Fröhlich (Ed.), Biological Coherence and Response to External Stimuli. Springer, Heidelberg, 1988, p. 233.
48 H.V Westerhoff, T.Y. Tsong, P.B. Chock, Y. Chen and R.D. Astumian, Proc. Natl. Acad. Sci. USA, 83 (1986) 4734.
49 R.D. Astumian, P.B. Chock, T.Y. Tsong, Y. Chen and H.V. Westerhoff, Proc. Natl. Acad. Sci. USA, 84 (1987) 434.
50 T.Y. Tsong and R.D. Astumian, Progr. Biophys. Mol. Biol., 50 (1987) 1.
51 T.Y. Tsong and R.D. Astumian, Annu. Rev. Physiol., 50 (1988) 273.
52 R.D. Astumian and B. Robertson, J. Chem. Phys., 91 (1989) 4891.
53 R.D. Astumian, P.B. Chock, T.Y. Tsong and H.V. Westerhoff, Phys. Rev. A, 39 (1989) 6416.
54 T.Y. Tsong, D.-S. Liu, F. Chauvin, A. Gaigalas and R.D. Astumian, Bioelectrochem. Bioenerg., 21 (1989) 319.

55 B. Robertson and R.D. Astumian, Biophys. J., 57 (1990) 689.
56 A. Persoons and L. Hellemans, Biophys. J., 24 (1978) 119.
57 P.I. Richards, IEEE Spectrum, January (1967) 83.
58 F.J. Harris, Proc. IEEE, 66 (1978) 51.
59 P.D. Welch, IEEE Trans. Audio Electroacoust., AU-15 (1967) 70.
60 C.M. Harris and D.B. Kell, Bioelectrochem. Bioenerg., 15 (1983) 11.
61 R.W. Lovitt, R.P. Walter, J.G. Morris and D.B. Kell, Appl. Microbiol. Biotechnol., 23 (1986) 168.
62 J.S. Bendat and A.G. Piersol, Random Data; Analysis and Measurement Wiley-Interscience, New York, 1971.
63 D.C. Champeney, Fourier Transforms and their Physical Applications, Academic Press, New York, 1973.
64 G.E.P Box and G.M. Jenkins, Time Series Analysis; Forecasting and Control, revised ed., Holden-Day, Oakland, CA, 1976.
65 P. Bloomfield, Fourier Analysis of Time Series; an Introduction, Wiley, New York, 1976.
66 R.N. Bracewell, The Fourier Transform and Its Applications, 2nd ed., McGraw-Hill, Kogakusha, Tokyo, 1978.
67 C. Chatfield, The Analysis of Time Series; an Introduction, 3rd ed., Chapman and Hall, London, 1984.
68 H.A. Pohl, Dielectrophoresis, Cambridge University Press, New York, 1978.
69 W. Almers, Rev. Physiol. Biochem. Pharmacol., 82 (1978) 96.
70 O. Alvarez and R. Latorre, Biophys. J., 21 (1978) 1.
71 S.H. White and W. Chang, Biophys. J., 36 (1981) 449.
72 P. Schoch, D.F. Sargent and R. Schwyzer, J. Membr. Biol., 46 (1979) 71.
73 T.Y. Tsong in F.T. Hong (Ed.), Molecular Electronics, Plenum Press, New York, 1990, p.83.
74 E. Neumann, Progr. Biophys. Mol. Biol., 47 (1986) 197.
75 O.M. Neijssel and D.W Tempest, Symp. Soc. Gen. Microbiol., 29 (1979) 53.
76 A. Goffeau and C.W. Slayman, Biochim. Biophys. Acta, 639 (1981) 197.
77 F. Malpartida and R. Serrano, Eur. J. Biochem., 116 (1981) 413.
78 J.H. McCusker, D.S. Perlin and J.E. Haber, Mol. Cell. Biochem., 7 (1987) 4082.
79 D.S. Perlin, C.L. Brown and J.E. Haber, J. Biol. Chem., 263 (1988) 18118.
80 R. Serrano, Biochim. Biophys. Acta, 947 (1988) 1.
81 H.V. Westerhoff and K. van Dam, Thermodynamics and Control of Biological Free-Energy Transduction, Elsevier, Amsterdam, 1987.
82 T. Hill, Free Energy Transduction in Biology, Academic Press, New York, 1977.
83 B.J. Bowman and C.W. Slayman, J. Biol. Chem., 254 (1979) 2928.
84 R.L. Smith, K. Zinn and L.C. Cantley, J. Biol. Chem., 255 (1980) 9852.
85 R.K. Nakamoto and C.W. Slayman, J. Bioenerg. Biomembr., 21 (1989) 621.
86 C.M. Harris, R.W. Todd, R.W. Lovitt, S.J. Bungard, J.G. Morris and D.B. Kell, Enz. Microbial Technol., 9 (1987) 181.
87 A.A. Pilla, P. Sechaud and B.R. McLeod, J. Biol. Phys., 11 (1983) 51.
88 P.Z. Marmarelis and V.Z. Marmarelis: Analysis of Physiological Systems: the White-Noise Approach, Plenum Press, New York, 1978.
89 J.C. Weaver and R.D. Astumian, Science, 247 (1990) 459.
90 A.E. Sowers and C.R. Hackenbrock, Proc. Natl. Acad. Sci. USA, 78 (1981) 6246.
91 U. Zimmermann and J. Vienken, J. Membr. Biol., 67 (1982) 165.
92 A.S. Presman, Electromagnetic Fields and Life, Plenum Press, New York, 1970.
93 H.S. Burr, Blueprint for Immortality, Neville Spearman, London, 1977.
94 A.R. Sheppard and M. Eisenbud (Eds.), Biological Effects of Electric and Magnetic Fields of Extremely Low Frequency, New York University Press, New York, 1977.
95 H. Fröhlich, Adv. Electron. Electron Phys., 53 (1980) 85.
96 W.R. Adey, Physiol. Rev., 61 (1981) 435.
97 K.H. Illinger (Ed.), Biological Effects of Non-Ionising Radiation, American Chemical Society, Washington, 1981.

98 H.L. Konig, A.P. Krueger, S. Lane and W. Sonnig, Biological Effects of Environmental Electromagnetism, Springer, Heidelberg, 1981.
99 R.O. Becker and A.A. Marino, Electromagnetism and Life, State University of New York Press, Buffalo, 1982.
100 H. Fröhlich and F. Kremer (Eds.), Coherent Excitations in Biological Systems, Springer, Heidelberg, 1983.
101 M. Grandolfo, S.M. Michaelson and A. Rindi (Eds.), Biological Effects and Dosimetry of Nonionizing Radiation, Plenum Press, New York, 1983.
102 M.J. Allen and P.N.R. Usherwood (Eds.), Charge and Field Effects in Biosystems, Abacus Press, Tunbridge Wells, 1984.
103 W.R. Adey and A.F. Lawrence (Eds.), Nonlinear Electrodynamics of Biological Systems, Plenum Press, New York, 1984.
104 A. Chiabrera, C. Nicolini and H.P. Schwan (Eds.), Interactions between Electromagnetic Fields and Cells, Plenum Press, New York, 1985.
105 D.B. Kell and H.V. Westerhoff in G.R. Welch (Ed.), Organised Multienzyme Systems: Catalytic Properties, Plenum Press, New York, 1985, p. 63.
106 A.A. Marino and J. Ray, The Electric Wilderness, University of San Francisco Press, San Francisco, 1986.
107 R. Nuccitelli (Ed.), Ionic Currents in Development, Alan R. Liss, New York, 1986.
108 C. Polk and E. Postow (Eds.), CRC Handbook of Biological Effects of Electromagnetic Fields, CRC Press, Boca Raton, 1986.
109 J. Black, Electrical Stimulation; its Role in Growth, Repair and Remodeling of the Musculoskeletal System, Paeger, New York, 1987.
110 M. Blank and A. Findl (Eds.), Mechanistic Approaches to Interactions of Electric and Magnetic Fields with Living Systems, Plenum Press, New York, 1987.
111 H. Fröhlich (Ed.), Biological Coherence and Response to External Stimuli, Springer, Heidelberg, 1988.
112 A.A. Marino, Modern Bioelectricity, Plenum Press, New York, 1988.
113 J.C. Lin (Ed.), Electromagnetic Interaction with Biological Systems, Plenum Press, New York, 1989.
114 C.W. Smith and S. Best, Electromagnetic Man, Dent, London, 1990.