NONLINEAR DIELECTRIC SPECTROSCOPY OF BIOLOGICAL SYSTEMS: PRINCIPLES AND APPLICATIONS

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Abstract. Biological cells can be seen, electrically, as consisting of conducting internal and external media separated by a more-or-less non-conducting cell membrane. The classical, linear, β-dielectric dispersion results from the charging up of this nominally 'static' membrane capacitance according to a Maxwell-Wagner type of mechanism, and typically occurs in the radiofrequency range. However, because practically all of the external macroscopic field is dropped across the 5 nm thick cell membrane, there is an effective and substantial *amplification* of the field across this membrane. This is predicted, and is found, to produce substantial nonlinearities when attempts are made to measure harmonics of the single-frequency exciting field. The nature (odd *vs* even) and magnitude of these harmonics changes substantially with cell status and environment, providing opportunities for using the cells themselves as sensing elements to describe their surroundings. Electrode polarisation effects producing nonlinear dielectricity can confound these measurements and must be bypassed or taken into account. Nonlinear dielectric spectroscopy (NLDS) provides a wholly non-invasive approach to cellular characterisation and diagnosis.

1. Introduction

The linear, passive audio- and radio-frequency electrical properties of biological systems have been studied since the end of the 19th century, and have been summarised in a number of reviews [1-9].

For cellular systems, it is conventional to recognise three major dielectric dispersions, in which the permittivity and conductivity change significantly with frequency. These are known as the α-,β- and γ-dispersions, and occur typically in the audio, radio- and microwave-frequency ranges, respectively [1].

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The β-dispersion is the most significant for our initial purposes, as it defines the frequency range below which the cell membrane is charged up by the external exciting electrical field. A typical β-dispersion for microbes is shown in Fig 1. Here the permittivity changes between 'high' values at 'low' frequencies and 'low' values at 'high' frequencies. The frequency at which this is half-completed is known as the characteristic frequency f_c, and is often of the order of 1 MHz or so, although it increases with both internal and external conductivity, and decreases with the cell radius. Such measurements are of historical interest as they led to the recognition that the large membrane capacitance that could be calculated therefrom (approx. $1\mu F.cm^{-2}$) meant that cell membranes must be of molecular thickness (say 5 nm). The dielectric increment depends on cell concentration, and we have therefore also exploited measurements of the β-dispersion to advantage for the on-line, real-time estimation of microbial biomass [10-15].

One consequence of these effects is that at audio frequencies the membrane of biological cell suspensions is effectively fully charged up by the exciting field and thus the cell membrane itself is seen as more or less entirely non-conducting under these circumstances. Specifically, if a cell is exposed to a low-frequency electric field the cell membrane will polarise, amplifying the electric field across the membrane by several orders of magnitude [4; 7; 8; 16; 17]. Consequently, the activity of polar and polarisable membranous enzymes can be modified significantly even by rather weak fields [18]. This strength of amplification means that the fields to which these enzymes are subject can produce nonlinear dielectric effects. In particular, enzymes whose conformational states display different dipole moments are affected kinetically by alternating fields during changes between states [18-24]; correspondingly, one should also expect *on theoretical grounds* [22; 23; 25] that these interactions would be observable via the harmonics produced by their nonlinear dielectric response to a sinusoidal field (Fig 2).

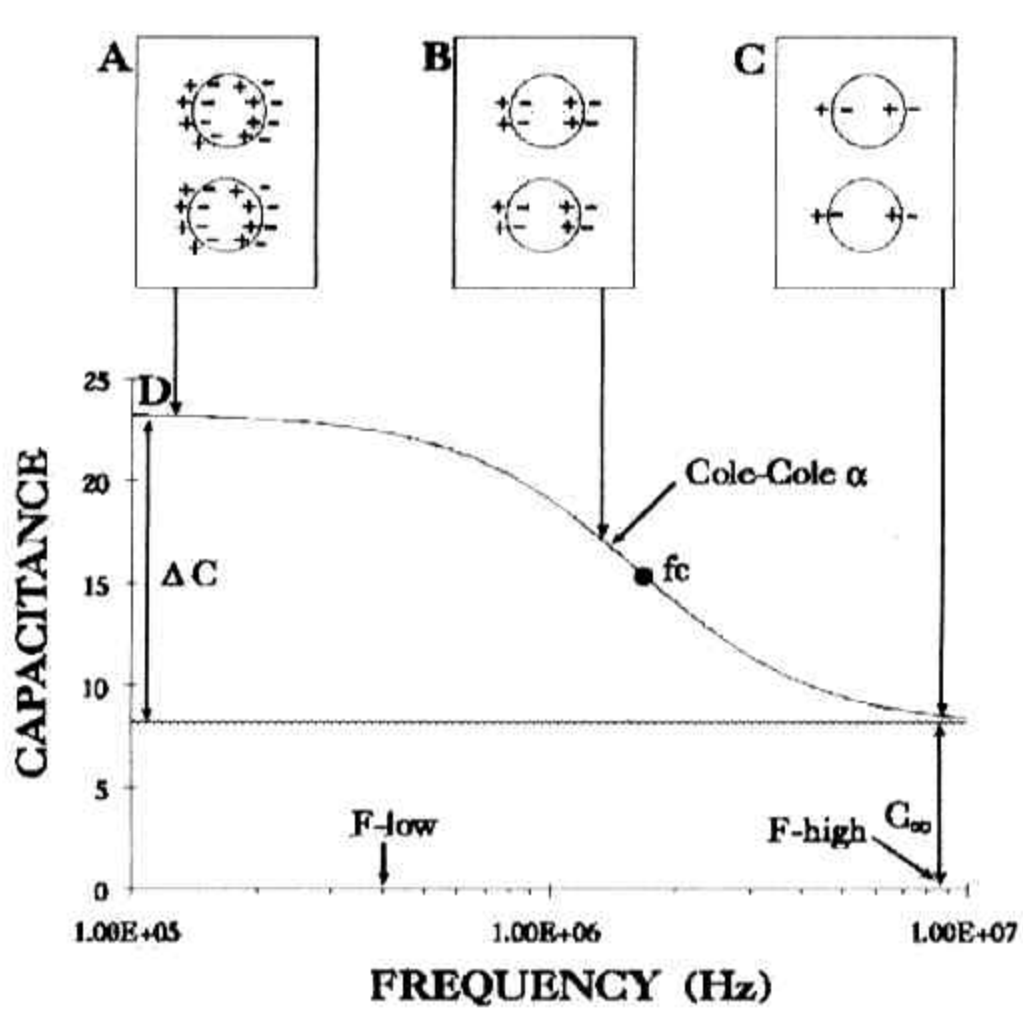

Figure 1. The β-dielectric dispersion of a typical biological tissue or cell suspension. At low frequencies, there is time for intra- and extra-cellular ions to migrate to the cell membrane and charge it up fully (A). This occurs less and less as the frequency is increased (B,C), giving a curve of capacitance against frequency of the form shown in (D). A non-zero value for the Cole-Cole α [26] of the β-dispersion, which characterises its breadth, is usually interpreted (but see [27]) in terms of a heterogeneity of relaxation times caused by heterogeneity in cell size, shape, membrane capacitance and internal conductivity.

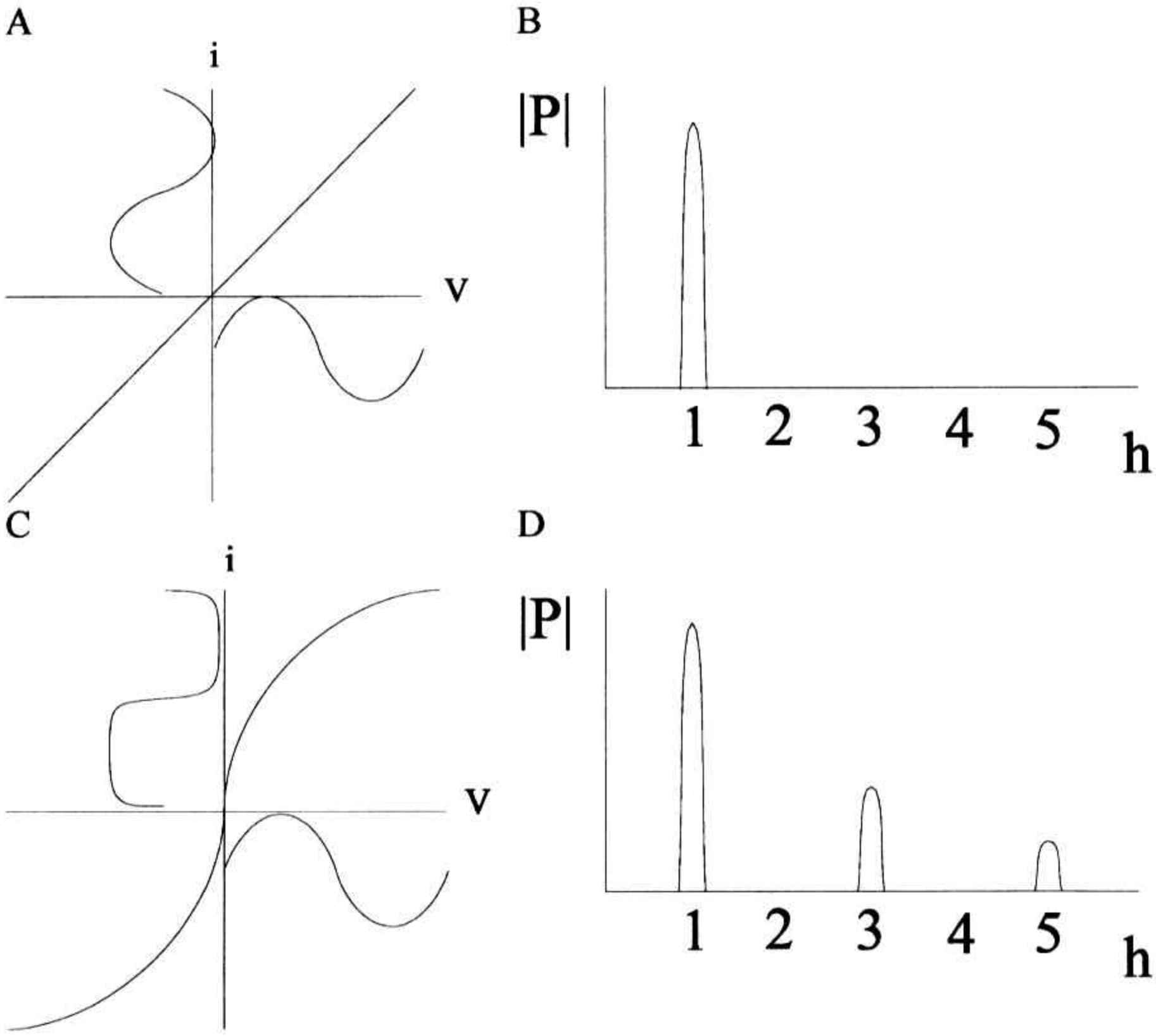

Figure 2. Linear and nonlinear dielectric responses. If the current-voltage characteristic of a system is linear (A) then the result is an output of electrical energy only at the frequency of the exciting voltage (B). Nonlinear systems display a nonlinear current-voltage characteristic and therefore produce harmonics.

Of course nonlinear effects have been at the heart of our understanding of neurotransmission for many years (e.g. [3]), but in those cases the measurements are done with transmembrane electrodes (i.e. at least one cell is intracellular). Our discussion here is confined to those cases in which all the electrodes are extracellular.

The generation of harmonics in response to a purely 'extracellular' sinusoidal electrical field is perhaps most easily understood in terms of the '4-state enzyme' model of a membranous carrier which has a higher affinity for extracellular than intracellular substrate (and conversely a reciprocal kinetic relationship for 'forward' and 'reverse' reactions so as to obey both the Second Law and the Haldane relationship [28]). In addition it has a mobile negative charge in functional linkage to the substrate binding

site. The enzyme can make serial transitions between the 4 states (charge inside/outside, substrate bound/free) and starts with state 4 being the most highly populated (Fig 3A), i.e. with the negative charge facing outwards and the external substrate bound.

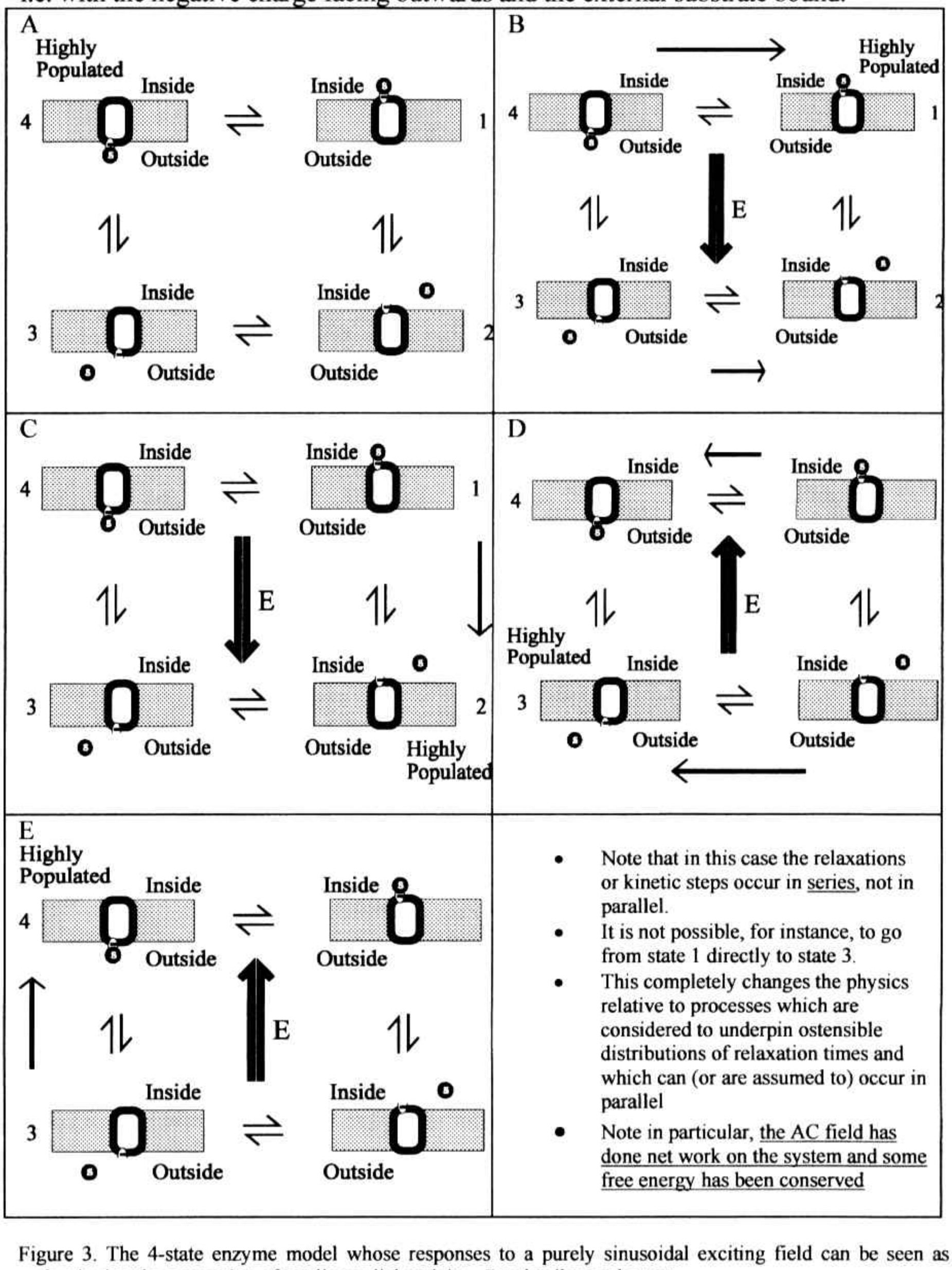

Figure 3. The 4-state enzyme model whose responses to a purely sinusoidal exciting field can be seen as underpinning the generation of nonlinear dielectricity,. For details see the text.

The imposition of the first (positive-going) phase of the sinusoidal electric field causes the negative charge to move across the membrane (Fig 3B) and the enzyme to adopt state 1. Since the affinity for the substrate when facing inwards is much lower, the enzyme relaxes in an electrically silent manner to state 2 (Fig 3C). Now the polarity of the sinusoidal field reverses, and the negative charge moves back to face outwards (state 3, Fig 3D), after which it again binds fresh substrate (state 4, Fig 3E). The result of this is that although the enzyme and the field are both back where they started, the field has caused the enzyme to do chemical work by moving a molecule of substrate across the membrane. Thus the field affects the enzyme [18-20] and some 15 years ago we pointed out that the enzyme must therefore affect the field, specifically by giving a nonlinear response in a dielectric measurement [22; 23; 25]. It was therefore predicted that Fourier analysis of the voltage induced across the inner electrodes in a conventional 4-terminal set-up of the type we and others had used in linear dielectrics [4; 8; 29-32] would demonstrate the transduction of single-frequency excitations into harmonics.

2. Results

Starting in the early 1990s, we showed that these predictions were indeed borne out [33-38]. The basic schematic of the apparatus is given below.

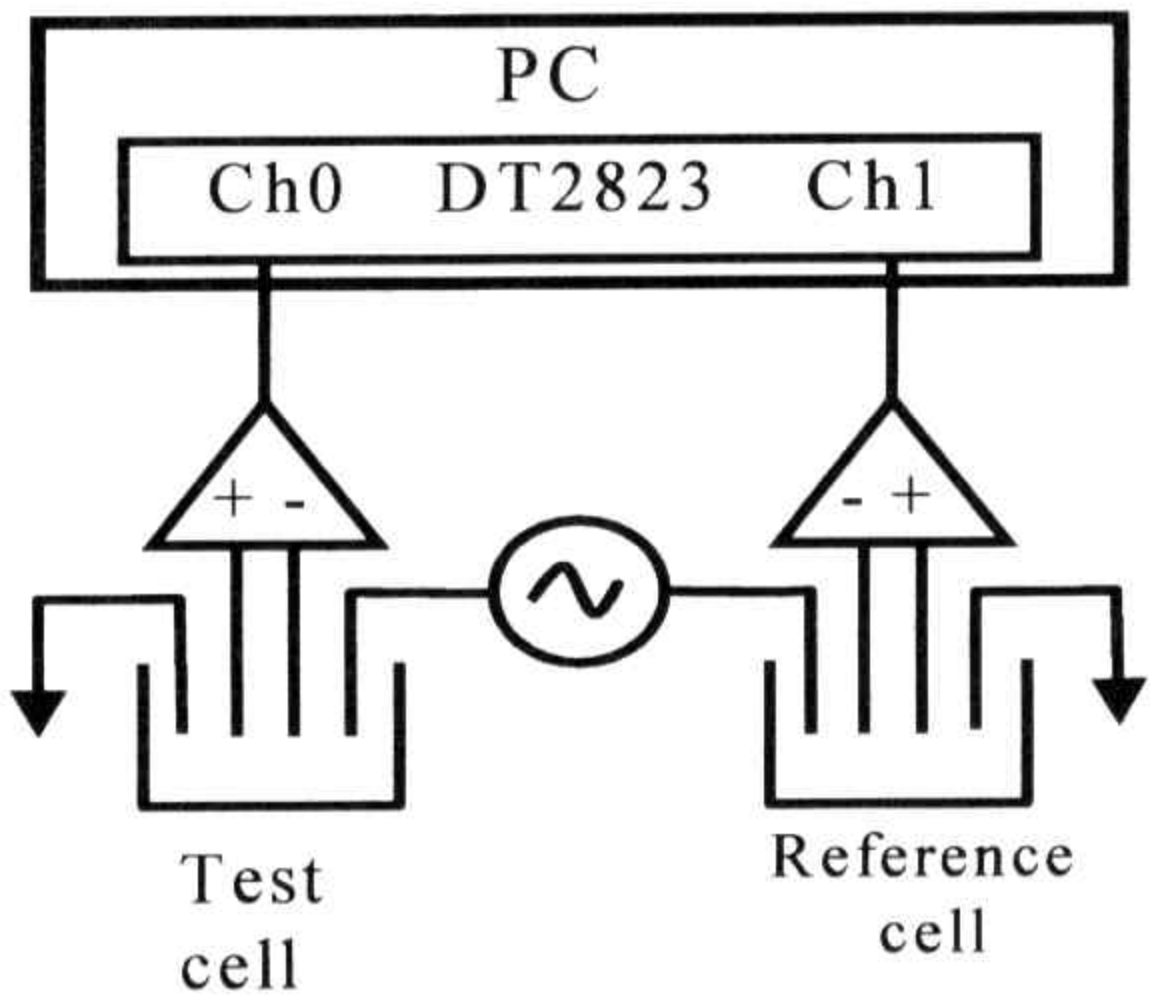

Figure4. A set-up for nonlinear dielectric spectroscopy (redrawn after [37]). AC (usually in the form of a pure sinusoid, but other waveforms are possible – e.g. [36]) is applied to the outer electrodes of a 4-terminal system and the resulting waveform captured across the inner electrodes using an analogue-to-digital converter connected to an input amplifier of high input impedance. Fourier transformation (following Blackman-Harris windowing) permits the determination of harmonic or other frequencies not present in the exciting waveform. To remove the contribution of electrode-derived nonlinearities the signal from the reference cell, containing only conductivity-matched supernatant, is removed from the signals obtained in the test cell. In the original work gold pin electrodes were used. A survey of other electrode materials is given in [39].

Using this apparatus, it was established that a variety of biological cell suspensions produced harmonics when excited by single-frequency electrical fields, odd-numbered harmonics were favoured when cells were resting, with the balance shifting towards even-numbered ones when they were metabolically active. These harmonics were not produced by boiled cells, could be found only in rather narrow voltage and frequency windows, the latter typically in the decade 10-100 Hz, and could be related to the kinetics of the 'target' enzymes. Inhibitor and mutant studies showed that identifiable membranous enzymes were the main source of the nonlinear dielectricity. In the eukaryotic baker's yeast (*Saccharomyces cerevisiae*) the enzyme was the membrane-located H^+-ATPase [35; 37], while in the bacteria *Micrococcus luteus* [34] and *Rhodobacter capsulatus* [33] the membrane-located respiratory chains were involved. The optimal excitation frequency for observing these effects, which could also be elicited by excitation at 2 frequencies, neither of which alone could serve, was considered to equate to the turnover number of the main target enzyme [36]. Other nonlinear effects, believed to reflect other motions in membranes, could be observed at higher excitation frequencies [34]. The use of multivariate and machine learning methods, including statistical, neural and evolutionary computing [39; 40], allowed quantitative models to be formed which could relate (a) the pattern of harmonics generated in response to varying voltages and frequencies to (b) the concentration of molecules such as glucose. Of course the non-invasive measurement of glucose is of massive significance in the management and prognosis of diseases such as diabetes [41]. Some data from glucose biotransformations by *S. cerevisiae* are given in Fig 5.

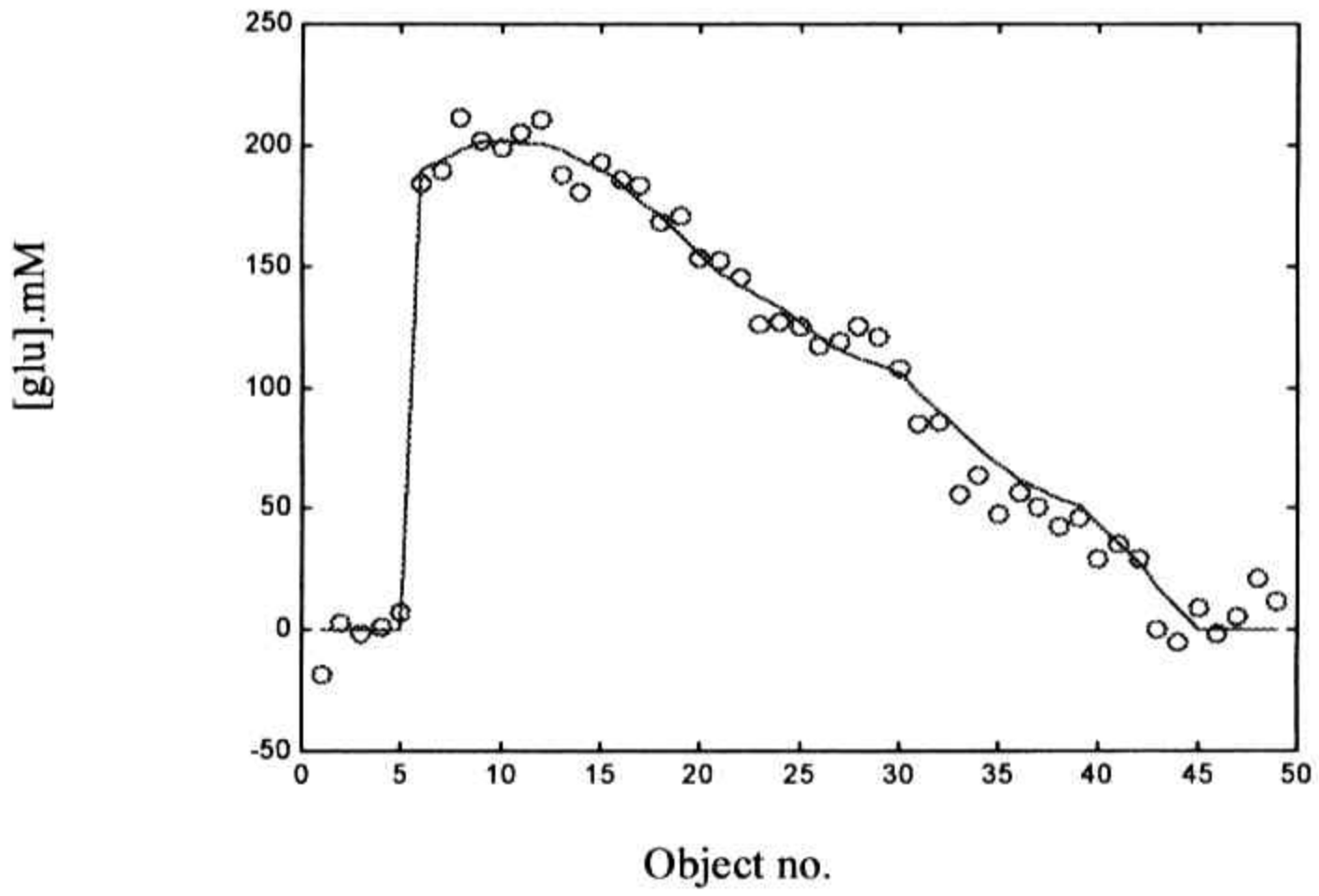

Figure 5. Predicted glucose in a fermentation run using a model produced using genetic programming (see e.g. [42-49]) from an entirely separate run [40]

A problem with all of these measurements, however, is that the nonlinear current-voltage relationships characteristic of the electrode-electrolyte interface [39; 50-52] also generate harmonics. These must be compensated in either hardware or software to

determine the 'purely' cellular responses, they can vary 'stochastically' in response to changes in electrode surface conditions, especially fouling by proteins, and seem to be largest when using electrodes (such as Pt) normally considered best for *linear* dielectric measurements in lossy media [39]. Although this is adequate for laboratory measurements, and some electrodes such as heavily chlorided Ag/AgCl may be essentially satisfactory [39] and can be greatly improved by chemical protection of the electrode surfaces [53], electrode polarisation interferences present the greatest impediment to the uptake of NLDS for the purposes of non-invasive sensing. Present work is aimed at eliminating them completely.

3. Conclusions and Prospects

Nonlinear dielectric spectroscopy of biological systems is an important (if recondite) technique with which one can interrogate cells directly so as to establish their responses to a variety of substances, which thereby become determinands. The essential basis of the effect is understood in qualitative terms, and there have been a number of successes in exploiting it for diagnostics. The elimination or bypassing of electrode polarisaton interferences remains the most important obstacle to be overcome; this would open up the field completely. To date we have not measured the nonlinear phase angle, and this is an attractive possibility. Technical advances mean that higher frequencies are more easily accessible than they were before. We also need to improve our understanding of, and ability to attack, the 'inverse problem' of NLDS (and see [54] for metabolic systems), i.e. given the nonlinear dielectric responses, produce a parameterised nonlinear dynamic model which can explain them. Finally, the availability of systematic gene knockout [55; 56] and other strains, whose metabolic changes can be determined straightforwardly [57], provides an obvious avenue for the further and more detailed dissection of those gene products contributing to the generation of nonlinear dielectricity in biological systems.

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