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The Nonlinear Dielectric Properties of Biological Systems: A Set of Methods for Assessing the Behaviour of Enzymes *in situ*

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The frequency-dependent linear, passive electrical properties of biological systems have been widely studied, usually by measuring their macroscopic capacitance and conductance as the in- and out-of-phase components of the AC admittance of the system (see Grant *et al.*, 1978; Pethig, 1979; Kell, 1987; Pethig and Kell, 1987; Kell and Davey, 1990). Since the admittance is by definition linear, the electronics of the instrumentation generally used, are designed (using suitable filters) to remove currents and voltages at other than the exciting frequency. In essence, this amounts to the use of a type of rose-coloured spectacles, *forcing* the experimenter to treat the system as if it were linear even if it is not. Since a variety of recent theoretical work (Kell *et al.*, 1988; Westerhoff *et al.*, 1988; Astumian and Robertson, 1989; Davey and Kell, 1990) has indicated that enzymatic systems might be expected to show *nonlinear* dielectric behaviour, even at very modest exciting fields, we have designed and constructed a dual-cell, nonlinear dielectric spectrometer, in which sinusoids of a single frequency are applied to the outer, current electrodes of a 4-terminal electrochemical cell but in which the voltage waveform induced across the inner electrodes is analysed, using Fourier techniques (Kell, 1987), to give its frequency content at *a range of frequencies* (Woodward and Kell, 1990). The use of a 2-cell configuration (cell suspension-*minus*-supernatant) permits the registration of the biological behaviour uncontaminated by the well-known ability (Bard and Faulkner, 1980) of purely electrochemical systems to generate harmonics when excited with pure sinusoidally modulated fields.

Using this system, we have studied resting cell suspensions of the yeast *S. cerevisiae* (Woodward and Kell, 1990). Substantial, odd-numbered harmonics were generated by these cells when stimulated by very modest exciting fields (ca 2 $V.cm^{-1}$, 20 Hz). The generation of these harmonics occurred only in living cells,

Biothermokinetics
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and in a cell concentration- dependent manner. We initially studied the third harmonic in detail. The ability to generate this harmonic is observable only within rather narrow voltage and frequency windows (±1–2.5 V.cm $^{-1}$, 1–100 Hz). The generation of a third harmonic was strongly inhibited by low concentrations of sodium meta- vanadate and by very low concentrations of dibenzhydryl carbodiimide, a more potent analogue (Beechey and Knight, 1978) of the better-known dicyclohexyl carbodiimide, suggesting that the production of this harmonic could be ascribed largely to the H^+-ATPase present in the plasma membranes of these cells. It is worth pointing out that the optimal exciting frequency, 20 Hz, is just that of the k_{cat} for the H^+-ATPase of *S. cerevisiae* (e.g. Wach *et al.*, 1990), providing, in principle, a novel and convenient means for estimating this parameter *in vivo*.

When the cells were permitted to glycolyse by the addition of glucose to the cell suspension, the enzyme passed from a state approximating static head to one in which it was forced to turn over. Under these conditions, the third harmonic disappeared and strong second and fourth harmonics could be observed (Woodward and Kell, 1990).

Since the system studied was demonstrably non-linear, it was to be expected that the application of *more than one* sinusoidal frequency to the outer electrodes might lead to the generation of *non-harmonic* frequencies across the inner electrodes. This was indeed shown to be the case (Woodward and Kell, 1991); if the frequencies applied to resting cell suspensions were f_1 and f_2, waveforms at frequencies of $f_1 \pm 2f_2$ and $f_2 \pm 2f_1$ could be observed, and the voltage windows were such that it had to be assumed that both exciting frequencies cooperated in their generation.

We also addressed the question of whether the substrate for the H^+-ATPase of *S. cerevisiae* was a free pool of ATP, as follows. If glycolysing cells were inhibited by a(ny) glycolytic inhibitor, such that the glycolytic flux is reduced by, say, 50%, the turnover number of all the relevant enzymes in systems exhibiting "pool" behaviour would be reduced by 50%. By contrast, if the system exhibits (perfect) "microcompartmentation" (see, e.g., Welch, 1977; Welch and Clegg, 1986; Masters *et al.*, 1987; Keleti *et al.*, 1989), to reduce the overall flux by 50% would mean that half the enzymes were turning over at their original rate whilst half were not turning over at all. The present method allows us, for the first time, to distinguish these, since the former generate even harmonics whilst the latter generate odd ones. We therefore carried out a titration with iodoacetamide, a rather specific inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase (Webb, 1966), which may be used to decrease the ATP-generating glycolytic flux. With a glycolysing control system (in the absence of iodoacetamide) that generated only even harmonics, we found that the addition of low concentrations (up to 0.4 nmol.mg^{-1} dry wt) caused a diminution in the even harmonics *that was almost exactly mirrored by an increase in the third harmonic*. This heterogeneity in the response therefore indicates strongly that the H^+-ATPase was connected to the ATP-generating reactions of glycolysis according to the "microcompartmentation" model, a finding consistent with other data on the membrane ATPase in this (Brindle *et al.*, 1990) and related eukaryotic systems (Fossel and Solomon, 1976; Solomon, 1978).

Nonlinear dielectric spectroscopy constitutes a powerful and convenient means by which to monitor the ability of living cells to transduce exogenous electric field

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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 (e.g. Lin, 1989). We also anticipate that further analysis of this type of behaviour may allow one to devise an on- line probe for the *kinetics* of appropriate enzymes *in situ*.

Acknowledgments

We thank the Wolfson Foundation for financial support of this work, and Drs Dean Astumian, Baldwin Robertson, Bob Todd and Hans Westerhoff for many stimulating discussions.

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