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# On the relationship between the nonlinear dielectric properties and respiratory activity of the obligately aerobic bacterium *Micrococcus luteus*

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**Abstract**

We have used our previously-described, four-terminal, nonlinear dielectric spectrometer to study the generation of nonlinear dielectricity by the obligately aerobic, heterotrophic, respiratory bacterium *Micrococcus luteus*. Intact cells of *M. luteus* generate harmonics when excited with single sinusoids of appropriate voltage and frequency. Two frequency windows are observed, respectively below ("low frequencies") and above ("high frequencies") ca 100 Hz. The low-frequency region shows a voltage window of some $\pm 0.4$–$1.6$ V (zero-to-peak, as judged on the outer electrodes). Anaerobic (non-respiring) cells generate odd-numbered harmonics, whilst aerobic (respiring) cells generate even-numbered harmonics. In the high-frequency range, intact cells of *M. luteus* (and also of *Saccharomyces cerevisiae*) generate both odd- and even-numbered harmonics, whether aerobic or anaerobic. In the low-frequency range, the inhibition of respiration by cyanide or by HQNO (2-n-heptyl-4-hydroxyquinoline-*N*-oxide) causes the appearance of odd-numbered harmonics in aerobic cell suspensions. Higher concentrations of cyanide (but not of HQNO) cause the disappearance of all harmonics. These facts are consistent with the known effects of these inhibitors on electron transport in the branched respiratory chain of this organism, and suggest that the major source of nonlinear dielectricity within the respiratory chain is either in the branch to cytochrome $b_{558}$ or proximal to the site at which HQNO binds. Membrane vesicles of *M. luteus* generate harmonics with patterns similar to those found in intact cells. In the low-frequency region, the addition of NADH to the membrane vesicles causes the replacement of odd-numbered harmonics by even-numbered harmonics, providing extremely clear-cut evidence that the type of harmonics observed depends strongly on whether or not the cells are respiring. In the high-frequency region, both odd- and even-numbered harmonics are generated by membrane vesicles, whether or not the membranes are carrying out electron transport. Liposomes consisting of pure phospholipids show similar patterns of harmonics, suggesting that the high-frequency nonlinear dielectricity may be ascribed to the lipid portion of the membranes of cells and vesicles. By contrast, the expression of nonlinear dielectricity at low exciting frequencies is determined by the respiratory system, and whether or not it is active. This phenomenon provides a novel method for the measurement of respiratory activity in situ.

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## INTRODUCTION

We have recently demonstrated the generation of nonlinear dielectricity by yeast cell suspensions [1–3], manifest as harmonics or beat frequencies when cells were excited, within appropriate voltage and frequency windows, by sinusoidally modulated electric fields. In this system, inhibitor studies revealed that the major source of the nonlinear dielectricity observed was the $H^+$-ATPase present in the plasma membrane of this organism. When the exciting field was a single sinusoid of appropriate frequency, the type of harmonics observed depended upon whether the cells were resting or glycolysing: in the former case, odd-numbered harmonics were observed, whilst cells which were actually metabolising glucose and generating ATP via substrate-level phosphorylation generated even-numbered harmonics [1]. This type of behaviour could be accounted for in terms of a simple double potential well model, and the frequency optimum observed, ca 15–20 Hz, was strikingly similar to the $k_{cat}$ of the yeast $H^+$-ATPase [2].

Electrical fields of low frequency, i.e. of a frequency low with respect to the characteristic frequency (ca. 1 MHz) of the $\beta$-dielectric dispersion of cells and tissues (mainly representing the charging of a static membrane capacitance [4–15]) do not effectively penetrate the cell membrane. Thus, and whilst this phenomenon underpins the fact that the nonlinear dielectric method is selective for membranous enzymes in intact cells, there could be no contribution of respiratory chain enzymes, which are located in the mitochondria of these eukaryotic (yeast) cells, to the nonlinear dielectric spectra measured. By contrast, of course, the respiratory chain of prokaryotes *is* found in their cytoplasmic membrane. It was therefore of great interest to extend the nonlinear dielectric spectroscopic approach to a suitable heterotrophic respiratory bacterium, so as to determine whether, in such an organism, there might be a contribution to the nonlinear dielectric spectra from respiratory chain enzymes. The present article describes such experiments using the obligately aerobic soil bacterium *Micrococcus luteus*, at the level of both the intact cell and the membrane vesicle. It was found, for the first time, that the respiratory chain was indeed the major source of nonlinear dielectricity in this organism, and that the type of harmonics observed (odd-numbered vs. even-numbered) depended upon whether or not the respiratory chain was active, a finding complementing that observed for the $H^+$-ATPase in yeast. In addition, we observed the generation of nonlinear dielectricity in the kHz range in cells of both yeast and *M. luteus*; since we also observed this in membrane vesicles and in liposomes consisting purely of phospholipid, it is ascribed to the conformational and configurational dynamics of the lipid portion of the membranes and membrane vesicles studied.

## EXPERIMENTAL

*Biological material. Micrococcus luteus* (syn. *M. lysodeikticus*) was generally obtained as a freeze-dried suspension from the Sigma Chemical Company, Poole,

Dorset. It was rehydrated in a buffer consisting of 50 mM $KH_2PO_4$, 1 mM $MgSO_4$, pH 7.0. In some cases, *Micrococcus luteus* Fleming strain 2665 was used, and was maintained on Difco nutrient agar and grown aerobically as described elsewhere [16]. No substantive differences were observed in the nonlinear dielectric behaviour of the two strains. Membrane vesicles were prepared by osmotic treatment of protoplasts created by treatment of intact cell suspensions with lysozyme. For this, 0.5 g dry wt. of cells were suspended in 10 ml of the buffer described above, to which were added 1.5 mg lysozyme. These were incubated at 20°C for 10 minutes. Then, a crystal of DNAase was added, and the membranes collected by centrifugation and resuspended in 10 ml of the original buffer. Concentrations of membranes are quoted in terms of the equivalent dry weight of cells from which they were prepared.

*Liposomes* were prepared via the cholate dialysis procedure. 2 g of crude soybean L-$\alpha$-phosphatidylcholine (Sigma type IV-S) plus 0.8 g sodium cholate were added to 20 ml of the phosphate buffer described above, and sonicated in bursts ($3 \times 1$ min cumulative at the full power of an MSE ultrasonicator) until the mixture had dissolved. The cholate phospholipid mixture was dialysed overnight at 4°C vs. phosphate buffer and the liposomes stored at 4°C and used within 3 days.

*Nonlinear dielectric measurements* were performed exactly as described previously [1], save that the oscillator used was a Krohn–Hite model 4400A ultra-low-noise generator [2]. The powers in difference spectra are given to the nearest dB.

*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.

## RESULTS

Figure 1 shows a typical nonlinear dielectric spectrum of a cell suspension (25 mg dry wt $ml^{-1}$) of *M. luteus* (Fig. 1A), a supernatant whose conductivity was adjusted to match that of the cell suspension (Fig. 1B), and their ratio (Fig. 1C). The exciting frequency was 30 Hz and the field strength, as judged by measurements between the outer electrodes was $\pm 0.7$ V $cm^{-1}$ (zero-to-peak). It may be observed that, at this cell concentration, the spectrum contains both odd- and even-numbered harmonics. *M. luteus* is an obligate aerobe, being unable to ferment, and since no attempt was made to oxygenate the cells, the bulk of a cell suspension of this cell density in our reaction vessel would have become anaerobic during the incubation phase prior to taking the spectrum (although the top will have been aerobic). To elaborate this point, and to check that the generation of harmonics was indeed caused by the cells, Fig. 2 shows the dependence on cell concentration of the nonlinear dielectric spectrum of intact cell suspensions of *M. luteus*. It may be observed that, above a concentration of some 25 mg/ml, the magnitude of the odd-numbered (third) harmonic depends monotonically on the cell concentration. However, below 25 mg dry wt. cells/ml, the odd-numbered harmonic disappears and is replaced by an even-numbered one, strongly suggestive that under these conditions the cells become aerobic and generate even-numbered

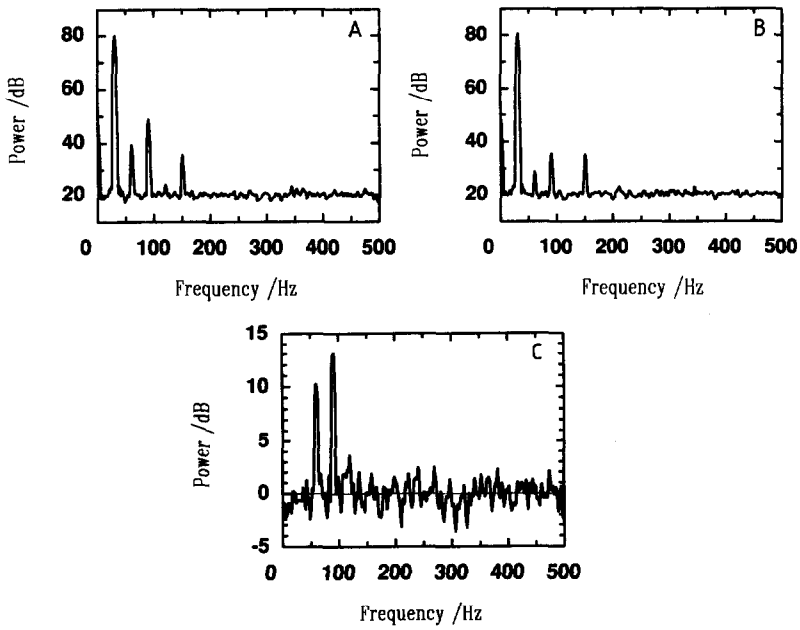


Fig. 1. Nonlinear dielectric properties of *M. luteus*. A suspension of cells (25 mg dry wt. $ml^{-1}$, prepared as described in the Experimental section), was placed in the test cell, and a sample of its supernatant (with its conductivity at 1 kHz adjusted to match that of the cells) was placed in the reference cell. Spectra were obtained using an exciting voltage of $\pm 0.7$ V zero-to-peak (field strength $\pm 1.05$ V $cm^{-1}$ as measured between the outer electrodes, $\pm 130$ mV $cm^{-1}$ as measured between the inner electrodes) at a frequency of 30 Hz, and each spectrum represents the average of 30 blocks. (A) Test cell. (B) Reference cell. (C) Test cell *minus* reference cell.

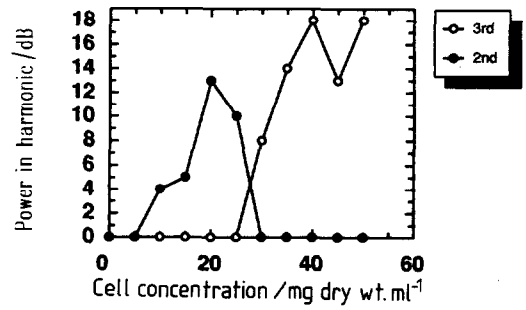


Fig. 2. Effect of cell concentration on the generation of harmonics by suspensions of *M. luteus*. Measurements were made exactly as described in the legend to Fig. 1(C), save that the cell concentration was varied as indicated.

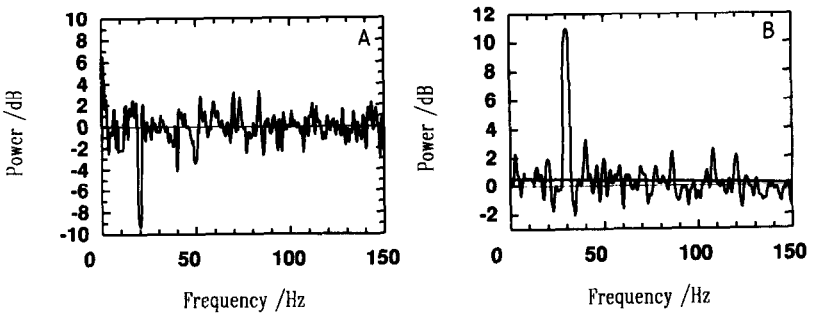


Fig. 3. Effect of respiratory chain activity on the nonlinear dielectric behaviour of intact cells of *M. luteus*. Measurements were made as described in the legend to Fig. 2, at an exciting frequency of 10 Hz and a voltage of $\pm 0.8$ V (zero-to-peak), at a cell concentration of 6 g $l^{-1}$. (A) Aerobic cells. (B) Anaerobic cells.

harmonics via activation of their respiratory chains. This was confirmed by an experiment in which we compared cells at a concentration of 6 g/l under explicitly aerobic and anaerobic conditions (Fig. 3), the latter enforced by filling the reaction vessel completely, closing it to the ingress of atmospheric oxygen and waiting for the cells to use up the residual oxygen. On a day-to-day basis, the concentration at which the cells became anaerobic under routine conditions was rather variable (as may be gleaned, for instance, from Figs. 1 and 2), presumably reflecting variations in the endogenous respiratory rate and temperature of measurement, but was normally in the range 10–25 mg dry wt. $ml^{-1}$. We may mention here that control experiments (lacking cells) indicated that $O_2$ per se had no effect on the generation of nonlinear dielectricity.

Since the generation of nonlinear dielectricity by yeast cells showed a rather narrow voltage and frequency window [1–3], we studied whether this might also be true of *M. luteus*. Figures 4 and 5 show, respectively, that within the voltage and frequency range indicated, windows are indeed manifest. These experiments were

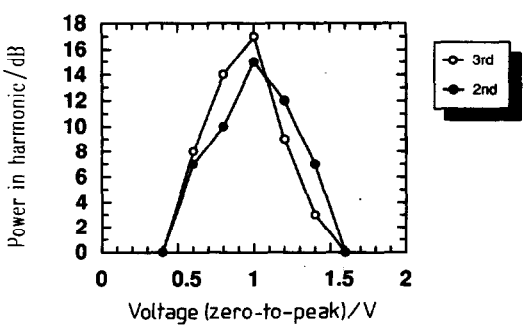


Fig. 4. Effect of voltage on the nonlinear dielectric properties of suspensions of *M. luteus*. Measurements were made exactly as described in the legend to Fig. 2, with an exciting frequency of 30 Hz, a cell concentration of 10 g $l^{-1}$, and the voltage (zero-to-peak) indicated.

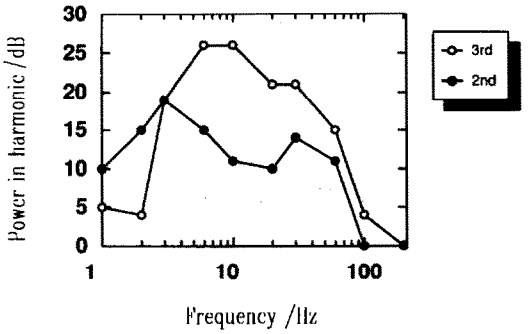


Fig. 5. Effect of frequency on the nonlinear dielectric properties of suspensions of *M. luteus*. Measurements were made exactly as described in the legend to Fig. 2, with an exciting voltage (zero-to-peak) of $\pm 1$ V, a cell concentration of 10 g $l^{-1}$, and the frequency indicated.

carried out at a cell concentration of 10 mg dry wt. $ml^{-1}$, where both odd- and even-numbered harmonics were present; it may be observed that these have rather similar voltage (Fig. 4) and frequency (Fig. 5) windows.

In the experiments with yeast cells [1–3], we did not study in any detail the effects of exciting frequency beyond ca. 100 Hz, since none were observed within the voltage window used at the lower frequencies. However, in the present case, when we extended the exciting frequency to values significantly beyond 100 Hz, a second region of harmonic generation was observed (Fig. 6), with both odd- and even-numbered harmonics being generated. The frequency- and voltage-dependences are shown, respectively, in Figs. 7 and 8, where it may be observed that the field-dependence is rather different (Fig. 8) from that observed in the low-frequency region (Fig. 4). These spectra did not change substantially as a function of whether the cells were aerobic or anaerobic (not shown). However, they did depend upon the presence of cells, as shown in the cell concentration-dependence (Fig. 9).

*M. luteus*, in common with many other bacteria [17,18], possesses a branched respiratory chain [19], cytochromes $b_{558}$ (probably a cytochrome *o*) and $aa_3$ acting as terminal electron acceptors [20–22]. Low concentrations of the respiratory inhibitor 2-n-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) can act partially to inhibit respiration at the *b* cytochrome level (after the branch), whilst cyanide inhibits respiration via both cytochrome $aa_3$ (at "low" concentrations) and cytochrome *o* (at high concentrations); indeed, *low* concentrations of cyanide can actually *stimulate* the overall respiratory rate by diverting the flux of electrons from the well-coupled main chain to the more poorly coupled alternative branch [22]. It was thus of interest to study the effects of these inhibitors on the generation of nonlinear dielectricity by *M. luteus* cells.

Figure 10 shows the effect of HQNO on the generation of harmonics by aerobic cells of *M. luteus*. It may be observed that in the absence of the inhibitor there are very few odd-numbered harmonics, but that increasing concentrations of the inhibitor cause them to appear. That inhibition of the harmonics is not observed

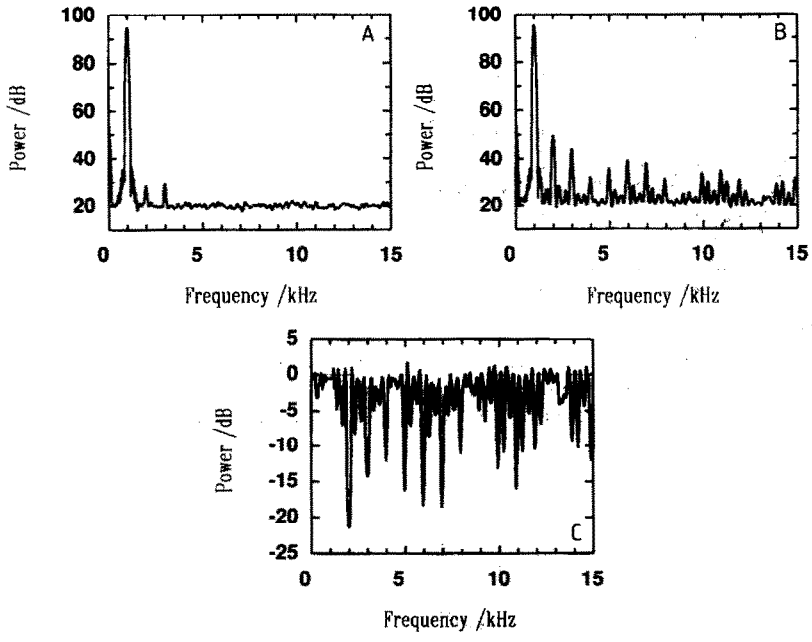


Fig. 6. Nonlinear dielectric properties of intact cells of *M. luteus* at 1 kHz. Measurements were made exactly as described in the legend to Fig. 2, at a cell concentration of 25 g $l^{-1}$, save that the exciting voltage was $\pm 0.8$ V (zero-to-peak) and the frequency of excitation was 1 kHz. (A) Suspension. (B) Supernatant. (C) Suspension *minus* supernatant.

might be considered to be consistent with the inability of this molecule alone to inhibit fully respiration in this organism [22]. However, the fact that HQNO actually causes a substantial *appearance* of odd-numbered harmonics rather suggests that the part of the respiratory chain being observed is not located at the site

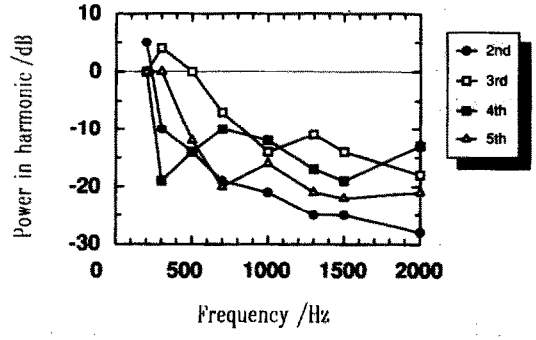


Fig. 7. Effect of frequency on the nonlinear dielectric properties of intact cell suspensions of *M. luteus*. Measurements were made exactly as described in the legend to Fig. 6(C), save that the frequency (above 100 Hz) was varied as indicated.

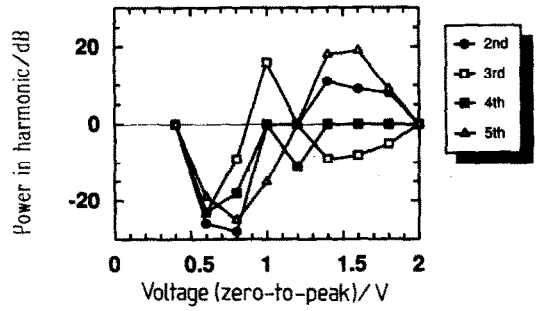


Fig. 8. Effect of voltage on the nonlinear dielectric properties of intact cell suspensions of *M. luteus*. Measurements were made exactly as described in the legends to Figs. 6 and 7, at a frequency of 1 kHz and a cell concentration of 25 g $l^{-1}$, save that the voltage (zero-to-peak) was varied as indicated.

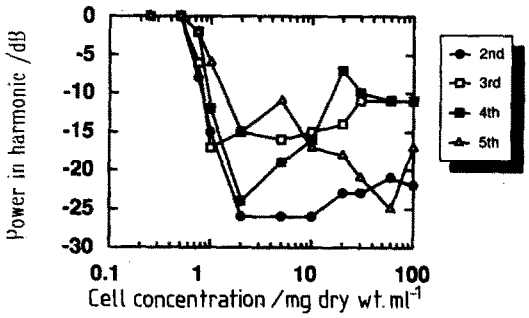


Fig. 9. Effect of cell concentration on the nonlinear dielectric properties of intact cell suspensions of *M. luteus*. Measurements were made exactly as described in the legends to Figs. 6–8, save that the exciting frequency was 1 kHz, the voltage on the outer electrodes (zero-to-peak) was ±0.7 V, and the cell concentration was varied as indicated.

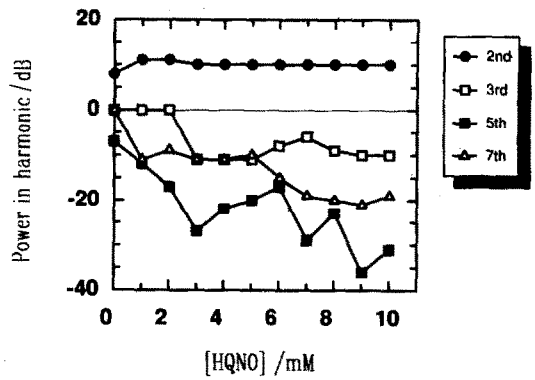


Fig. 10. Effect of HQNO on the nonlinear dielectric properties of aerobic cells of *M. luteus*. Measurements were performed exactly as described in the legend to Fig. 2, at a cell concentration of 3 g $l^{-1}$ an exciting frequency of 30 Hz, and an exciting voltage (zero-to-peak) of ±1 V, save that HQNO was present at the concentrations indicated.

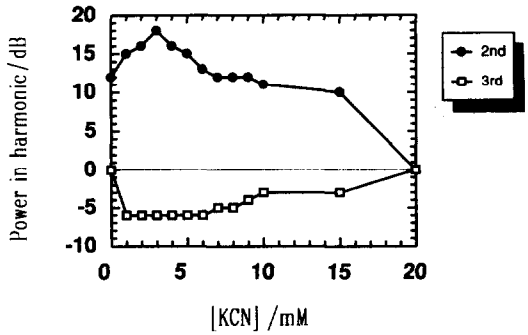


Fig. 11. Effect of cyanide on the nonlinear dielectric properties of aerobic cells of *M. luteus*. Measurements were performed as described in the legend to Fig. 10, at a cell concentration of 12 g $l^{-1}$ an exciting frequency of 30 Hz, and an exciting voltage (zero-to-peak) of $\pm 0.7$ V, save that KCN was present at the concentrations indicated.

of action of the inhibitor, but is being forced towards static head by its presence. In a similar vein, Fig. 11 shows a cyanide titration of harmonic generation by aerobic, intact cells of this organism; low concentrations of cyanide stimulate both second and third harmonics, which are subsequently decreased until at 20 mM cyanide they are no longer visible. These data are exactly consistent with the known influence of cyanide on the respiration of this organism (see above and ref. 22). Figure 12 shows a similar titration of anaerobic cells, where the 3rd harmonic is strongly inhibited during the cyanide titration.

It may be mentioned that neither cyanide nor HQNO had any marked effects upon the nonlinear dielectric properties of the cells when the frequency of excitation exceeded 100 Hz, and that the $H^+$-ATP synthase inhibitor venturicidin [23] was also without effect at both low and high frequencies (data not shown).

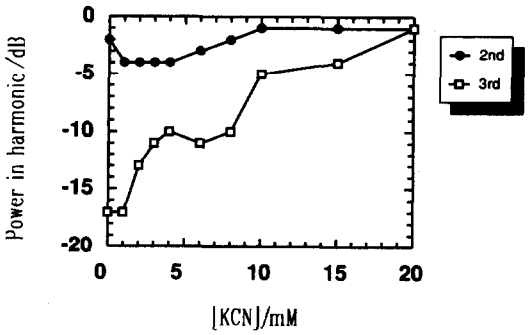


Fig. 12. Effect of cyanide on the nonlinear dielectric properties of anaerobic cells of *M. luteus*. Measurements were performed as described in the legend to Fig. 10, at a cell concentration of 12 g $l^{-1}$ an exciting frequency of 30 Hz, and an exciting voltage (zero-to-peak) of $\pm 0.7$ V, save that KCN was present at the concentrations indicated.

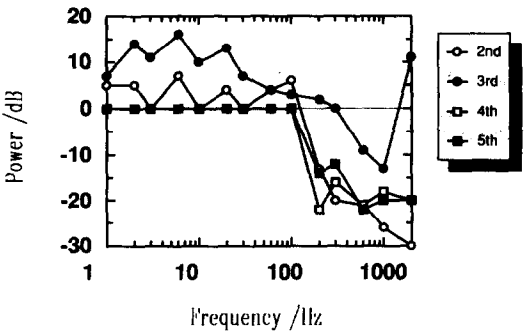


Fig. 13. Effect of frequency on the generation of harmonics by membranes vesicles of *M. luteus*. Measurements were performed as described in the legend to Fig. 7, except that membrane vesicles were present at a concentration of 50 g $l^{-1}$, the voltage (zero-to-peak) across the outer electrodes was $\pm 0.7$ V, and the frequency was varied as indicated.

Each of the above experiments had indicated a clear relationship between the type of harmonics observed (odd vs. even) and the activity of the respiratory chain. However, in intact cells respiration is endogenous, and the proximal electron donor is uncertain. It is therefore much more convenient to study membrane vesicles. Osmotically-prepared cytoplasmic membrane vesicles from respiratory bacteria normally consist of a mixture of so-called right-side-out, inside-out and "scrambled" vesicles [24]; since NADH is membrane-impermeant, only inside-out, and to some extent scrambled, vesicles can respire using exogenous NADH. The addition of NADH (or an NADH-generating system) to membrane vesicles therefore provides a convenient mechanism for turning respiration on and off under otherwise aerobic conditions. We therefore prepared membrane vesicles from *M. luteus* cells and studied their nonlinear dielectric properties.

Figure 13 shows the frequency-dependence of the ability of membrane vesicles of *M. luteus* to generate harmonics. In a manner rather similar to that observed in intact cells (Figs. 5, 7), two regions of frequency-dependence may be observed, respectively below and above ca. 100 Hz. As expected, given the absence of added respiratory substrate, the third harmonic is dominant in the low-frequency region. The voltage window for the low-frequency region is shown in Fig. 14, and, despite the fact that the vesicles are of course smaller than the intact cells from which they are derived, is very similar to that (Fig. 4) of the intact cells (but contains, of course, only a third harmonic, since endogenous respiratory substrates are now absent). Finally, to confirm that the signal is indeed due to the presence of the membrane vesicles, Fig. 15 shows the concentration-dependence of the ability of membrane vesicles to generate a third harmonic.

To confirm the dependence of the type of harmonic on whether or not the membranes were performing electron transport, we compared membranes in the absence of respiratory substrate and those to which NADH had been added. Figures 16A and 16B show their respective nonlinear dielectric properties when excited at 30 Hz. It may be observed (Fig. 16) that the addition of NADH causes

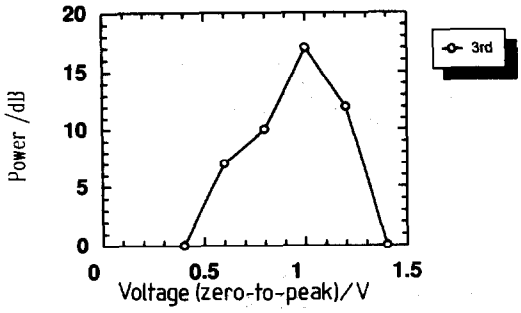


Fig. 14. Effect of voltage on the generation of harmonics by membrane vesicles of *M. luteus*. Measurements were performed as described in the legend to Fig. 13, except that membrane vesicles were present at a concentration of 50 g $l^{-1}$, the frequency of excitation was 30 Hz, and the voltage (zero-to-peak) across the outer electrodes was varied as indicated.

an enormous enhancement of the 2nd and 4th harmonics at the expense of the 3rd harmonic (which had been dominant in the absence of the respiratory electron donor). It may be mentioned again that appropriate control experiments carried out in the absence of membranes showed that neither oxidised nor reduced pyridine nucleotides, nor $O_2$, had any measurable effect in generating nonlinear dielectric responses. Thus, these data provide the clearest possible evidence that the generation of *even*-numbered harmonics by *M. luteus* (in the low-frequency region) depends upon the ongoing activity of the respiratory electron transport chain.

Finally we turned our attention to the high-frequency region (> 100 Hz). As a result of the findings in intact cells of *M. luteus* described above, we again studied cells of baker's yeast at the higher frequencies. We observed (data not shown) that cells of baker's yeast were also capable of generating harmonics almost identical to

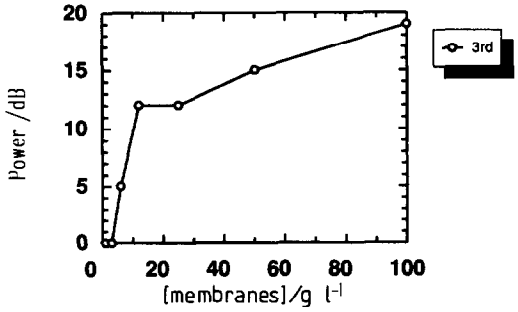


Fig. 15. Effect of vesicle concentration on the generation of harmonics by membrane vesicles of *M. luteus*. Measurements were performed as described in the legend to Fig. 14, with an exciting frequency of 30 Hz, an exciting voltage (zero-to-peak) across the outer electrodes of ±0.8 V, and with the membrane vesicle concentration varied as indicated.

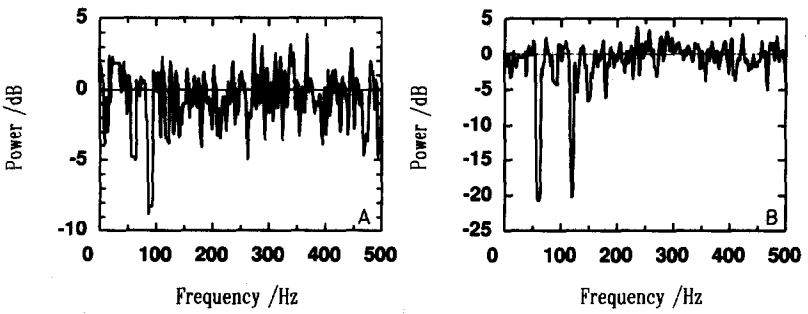


Fig. 16. Effect of NADH on the generation of harmonics by membrane vesicles of *M. luteus*. Measurements were performed as described in the legend to Fig. 13, except that membrane vesicles were present at a concentration of 6 g $l^{-1}$, the frequency of excitation was 30 Hz, and the voltage (zero-to-peak) across the outer electrodes was $\pm 0.8$ V. (A) No NADH present. (B) 10 mM NADH was added 1 min before measurement of the nonlinear dielectric spectra.

those generated by *M. luteus*, when the exciting frequency was in the range 0.6–2 kHz (the latter being the highest exciting frequency at which our equipment could conveniently measure), provided that the voltage (zero-to-peak) on the outer electrodes was in the range $\pm 0.5$–0.9 V. This latter explains why we did not observe these harmonics at high frequencies in our earlier work [1], which was carried out at higher field strengths, outside the voltage window. Since the high-frequency nonlinear dielectricity depended on the presence of cells, both for yeast and for *M. luteus*, we therefore turned our attention to membrane vesicles of *M. luteus*.

Figure 17 shows the generation of nonlinear dielectricity by membrane vesicles of *M. luteus* excited at 1 kHz. The voltage window (Fig. 18) is similar to, though

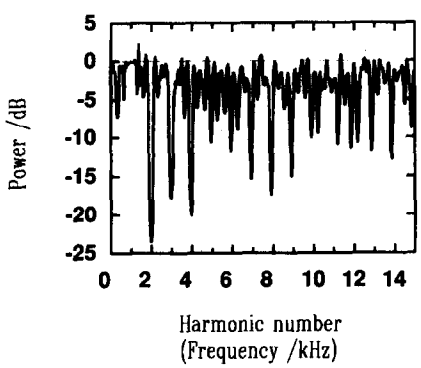


Fig. 17. The generation of nonlinear dielectricity by membrane vesicles of *M. luteus* when excited at 1 kHz. Measurements were performed as described in the legend to Fig. 14, except that membrane vesicles were present at a concentration of 50 g $l^{-1}$, the frequency of excitation was 1 kHz, and the voltage (zero-to-peak) across the outer electrodes was $\pm 0.7$ V.

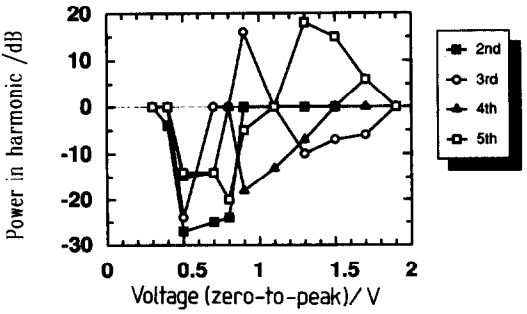


Fig. 18. Effect of voltage on the generation of harmonics by membrane vesicles of *M. luteus* when excited at high frequencies. Measurements were performed as described in the legend to Fig. 17, except that membrane vesicles were present at a concentration of 25 g $l^{-1}$, the frequency of excitation was 1 kHz, and the voltage (zero-to-peak) across the outer electrodes was varied as indicated.

slightly higher than, that of the intact cells (Fig. 4). The concentration-dependence of the harmonics generated by membrane vesicles when excited at 1 kHz is shown in Fig. 19, where it may be observed that it is both monotonic and unselective towards particular harmonics. In this frequency range (above some 100–200 Hz), as in intact cells, the harmonic patterns were practically independent of the presence of NADH (and respiratory or $H^+$-ATP synthase inhibitors; data not shown), i.e. of ongoing respiratory or ATP synthetic activity. Thus, since the vesicles contain only proteins and lipids, one is led to implicate the lipid portion of the vesicle membranes as the source of the nonlinear dielectricity in the high-frequency range. It therefore seemed prudent to check whether pure phospholipid liposomes, lacking protein, might also be able to generate harmonics when excited above 100 Hz.

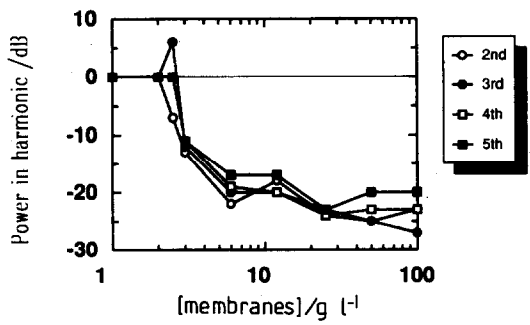


Fig. 19. Effect of vesicle concentration on the generation of harmonics by membrane vesicles of *M. luteus*. Measurements were performed as described in the legend to Fig. 17, with an exciting frequency of 1 kHz, an exciting voltage (zero-to-peak) across the outer electrodes of $\pm 0.8$ V, and with the membrane vesicle concentration indicated.

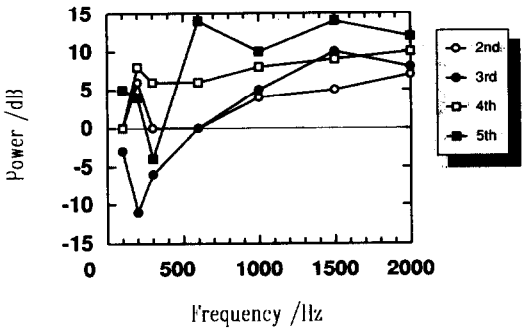


Fig. 20. Effect of frequency on the generation of harmonics by liposomes. Liposomes were prepared as described in the Experimental. Nonlinear dielectric spectra were measured as described in the legend to Fig. 18. The voltage (zero-to-peak) across the outer electrodes was ±7 V and the frequency was varied as indicated.

Figure 20 shows the frequency-dependence of the ability of liposomes to generate harmonics, where it may be observed that data similar to those observable in membranes (i.e. the generation of a plethora of both odd- and even-numbered harmonics) are obtained, although in these very small liposomes significant harmonics appear only when the exciting voltage (zero-to-peak on the outer electrodes) exceeds 4 V (not shown). Thus, it would appear that it is the lipid portion of the cytoplasmic membrane of *M. luteus* (and other organisms) which is predominantly responsible for the generation of harmonics when the cells are excited at frequencies above 100–200 Hz.

## DISCUSSION

We have previously described in detail the construction of a dual-cell, four-terminal, nonlinear dielectric spectrometer, and have demonstrated the generation of nonlinear dielectricity by yeast cell suspensions [1–3]. This was manifest as harmonics when cells were excited, within appropriate voltage and frequency windows, by sinusoidally modulated electric fields of a single frequency. In this system, inhibitor studies revealed that the major source of the nonlinear dielectricity observed was the $H^+$-ATPase present in the plasma membrane of this organism [1–3]. The type of harmonics observed depended upon whether the cells were resting or glycolysing, the former case causing the generation of odd-numbered harmonics whilst cells which were actually metabolising glucose and generating ATP via substrate-level phosphorylation generated even-numbered harmonics [1]. These findings could be explained on the basis of the ability of electric fields to alter the conformational dynamics of (especially) membrane proteins [26–43], whilst the change from odd- to even-numbered harmonics could be accounted for in terms of a simple double-potential-well model [2].

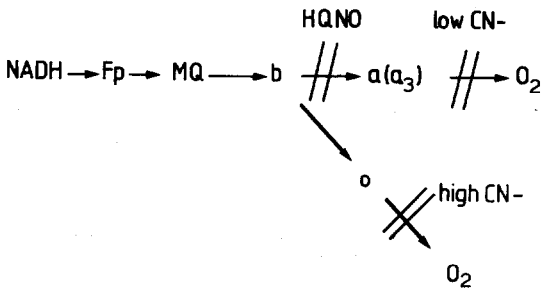


Fig. 21. Simplified diagram of the branched respiratory chain of *Micrococcus luteus*, and of the sites of inhibition of electron transport by cyanide and HQNO [22,46]. The probable involvement of differing redox states of the menaquinone moieties in a so-called Q-cycle is not shown. MQ, menaquinone; *b*, cytochrome(s) *b*; o, cytochrome *o*; a, cytochrome *a*; Fp, flavoproteins.

In the present work, we have used this nonlinear dielectric spectrometer system to study the generation of nonlinear dielectricity by the obligately aerobic, heterotrophic, respiratory bacterium *Micrococcus luteus*. We showed, for the first time in a respiratory system, that intact cells of *M. luteus* generated harmonics when excited with single sinusoids (Figs. 1–5). Two frequency windows were observed, respectively below ("low frequencies") and above ("high frequencies") ca. 100 Hz (Figs. 5, 7). The low-frequency region showed a voltage window of some $\pm 0.4$–1.6 V (0.5–2.1 V $cm^{-1}$, zero-to-peak, as judged on the outer electrodes, 30–400 mV $cm^{-1}$ as judged on the inner electrodes). In the low-frequency region, anaerobic (non-respiring) cells generated odd-numbered harmonics, whilst aerobic (respiring) cells generated even-numbered harmonics (Fig. 3).

Figure 21 shows a simple representation of the branched electron transport chain of *M. luteus* [21], indicating the site of action of appropriate respiratory inhibitors. In the low-frequency region, we found that the inhibition of respiration by HQNO (Fig. 10) or by cyanide (Fig. 11) caused the appearance of odd-numbered harmonics in aerobic cell suspensions. Higher concentrations of cyanide (but not of HQNO) cause the disappearance of all harmonics, in both aerobic (Fig. 11) and anaerobic cells (Fig. 12). These facts are consistent with the known effects of these inhibitors on electron transport via the branched respiratory chain of this organism [21,22], and suggest that the major source of nonlinear dielectricity within the respiratory chain is either in the branch to cytochrome $b_{558}$ or proximal to the site at which HQNO binds (Fig. 21).

Since intact cells of *M. luteus* could respire endogenously, and in order to exclude cytoplasmic processes, it was of interest to study the effects of respiration on the generation of nonlinear dielectricity by this organism at the membrane vesicle level. We found that membrane vesicles of *M. luteus* generated harmonics with patterns (voltage and frequency windows) similar to those found in intact cells (Figs. 13–19). In the absence of an electron donor, the third harmonic was dominant (Figs. 13–16), as expected. However, in the low-frequency region, the

addition of NADH to the membrane vesicles caused the replacement of the odd-numbered harmonics by substantial even-numbered harmonics (Fig. 16), providing extremely clear-cut evidence that the type of harmonics observed depended strongly on whether or not the cells were carrying out respiratory electron transport. It is evident that this phenomenon might be exploited for the measurement of respiratory activity in situ.

In the high-frequency range, intact cells of *M. luteus* (and also of *Saccharomyces cerevisiae*) generated both odd- and even-numbered harmonics (Figs. 6–9), whether the cells were aerobic or anaerobic. Similarly, when excited in the high-frequency region, both odd- and even-numbered harmonics were generated by membrane vesicles, whether or not the membranes were carrying out electron transport (Figs. 13, 17–19). We also found that liposomes consisting of pure phospholipids showed very similar patterns of harmonics (Fig. 20), strongly suggesting that the high-frequency nonlinear dielectricity may be ascribed to the dynamic properties of the lipid portion of the membranes of cells and vesicles (and see also refs. 44,45).

It is concluded, on the basis of the present work, and of that described previously [1–3], that both respiratory and fermentative microorganisms can generate nonlinear dielectricity when excited within the appropriate voltage and frequency windows, and that this nonlinear dielectricity is caused, in these circumstances overwhelmingly, by the electric field-induced conformational dynamics of their *membrane-located* enzymes. Future work will be directed towards a more molecular characterisation of these effects and to the more rapid acquisition of nonlinear dielectric spectra.

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REFERENCES

1 A.M. Woodward and D.B. Kell, Bioelectrochem. Bioenerg., 24 (1990) 83.
2 A.M. Woodward and D.B. Kell, Bioelectrochem. Bioenerg., 25 (1991) 395.
3 D.B. Kell and A.M. Woodward, in H.V. Westerhoff (Ed.), Biothermokinetics, Intercept Press, London, in the press.
4 H.P. Schwan, Adv. Biol. Med. Phys. 5 (1957) 147.
5 E.H. Grant, G.P. South and R.J. Sheppard, Dielectric Properties of Biological Molecules in Solution, Oxford University Press, Oxford, 1978.
6 O. Schanne and E.R.P. Ceretti, Impedance Measurements in Biological Cells, John Wiley, Chichester, 1978.
7 R. Pethig, Dielectric and Electronic Properties of Biological Materials, John Wiley, Chichester, 1979.
8 D.B. Kell and C.M. Harris, J. Bioelectricity, 4 (1985) 317.

9 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, FL, 1986, p. 27.
10 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.
11 R. Pethig and D.B. Kell, Phys. Med. Biol., 32 (1987) 933.
12 K.R. Foster and H.P. Schwan, CRC Crit. Rev. Biomed. Eng., 17 (1989) 25.
13 C.L. Davey and D.B. Kell, in R. Paris (Ed.), Electric Field Phenomena in Biological Systems. IOP Short Meetings Series 21. Institute of Physics, London, 1989, p. 51.
14 S. Takashima, Electrical Properties of Biopolymers and Membranes. Adam Hilger, Bristol, 1989.
15 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.
16 A.S. Kaprelyants and D.B. Kell, J. Appl. Bacteriol., in the press.
17 B.A. Haddock and C.W. Jones, Bacteriol. Rev., 41 (1977) 47.
18 W.J. Ingledew and R.K. Poole, Microbiol. Rev., 48 (1984) 222.
19 M. Fujita, S. Ishikawa and N. Shimazono, J. Biochem., 59 (1966) 104.
20 G.V. Tikhonova, V.Yu. Artzatbanov, T.V. Sheiko and D.N. Ostrovsky, Dokl. Acad. Nauk USSR, 260 (1981) 497.
21 V.Yu. Artzatbanov and D.N. Ostrovsky, Biochem. J., 266 (1990) 481.
22 V.Yu. Artzatbanov, T.V. Sheiko, M.A. Lukoyanova and A.S. Kaprelyants, J. Gen. Microbiol. 137 (1991) 1485.
23 S.J. Ferguson and P. John, Biochem. Soc. Trans., 5 (1977) 1525.
24 S.J. Ferguson, D.B. Kell and P. John, P. FEMS Microbiol. Lett., 3 (1978) 151.
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 T.Y. Tsong and R.D. Astumian, Bioelectrochem. Bioenerg., 15 (1986) 457.
28 E.H. Sepersu and T.Y. Tsong, J. Membr. Biol., 74 (1983) 191.
29 E.H. Sepersu and T.Y. Tsong, J. Biol. Chem., 259 (1984) 7155.
30 D.-S. Liu, R.D. Astumian and T.Y. Tsong, J. Biol. Chem., 265 (1990) 7260.
31 G.R. Welch and D.B. Kell, in G.R. Welch (Ed.), The Fluctuating Enzyme, Wiley, New York, 1986, p. 451.
32 D.B. Kell, Univ. Wales Rev. Sci. Technol., 1 (1988) 64.
33 D.B. Kell, ISI Atlas Sci.: Biochemistry, 1 (1988) 25.
34 D.B. Kell, in H. Fröhlich (Ed.) Biological Coherence and Response to External Stimuli. Springer, Heidelberg, 1988, p. 233.
35 H.V. Westerhoff, T.Y. Tsong, P.B. Chock, Y. Chen and R.D. Astumian, Proc. Natl. Acad. Sci., 83 (1986) 4734.
36 R.D. Astumian, P.B. Chock, T.Y. Tsong, Y. Chen and H.V. Westerhoff, Proc. Natl. Acad. Sci., 84 (1987) 434.
37 T.Y. Tsong and R.D. Astumian, Progr. Biophys. Mol. Biol., 50 (1987) 1.
38 T.Y. Tsong and R.D. Astumian, Ann. Rev. Physiol., 50 (1988) 273.
39 R.D. Astumian and B. Robertson, J. Chem. Phys., 91 (1989) 4891.
40 R.D. Astumian, P.B. Chock, T.Y. Tsong and H.V. Westerhoff, Phys. Rev., A 39 (1989) 6416.
41 T.Y. Tsong, D.-S. Liu, F. Chauvin, A. Gaigalas and R.D. Astumian, Bioelectrochem. Bioenerg. 21 (1989) 319.
42 B. Robertson and R.D. Astumian, Biophys. J., 57 (1990) 689.
43 B. Robertson and R.D. Astumian, J. Chem. Phys., 94 (1991) 7414.
44 W. Carius, J. Coll. Interf. Sci., 57 (1976) 301.
45 V.S. Sokolov and V.G. Kuz'min Biofizika, 25 (1980) 170.
46 T.V. Sheiko, M.A. Lutoyanova, A.S. Kaprelyants, V.Yu. Atrzatbanov and D.N. Ostrovsky, Biokhimiya, 55 (1990) 263.