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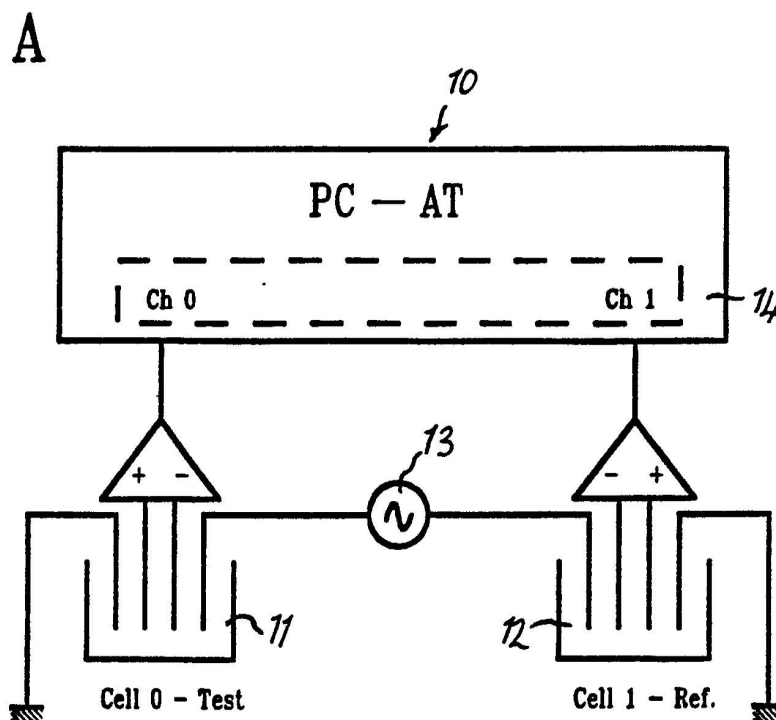
(58) Field of search

UK CL (Edition K) G1N NBMX
INT CL⁵ G01N

(54) Analysis of biological material by a non-linear dielectric spectrum

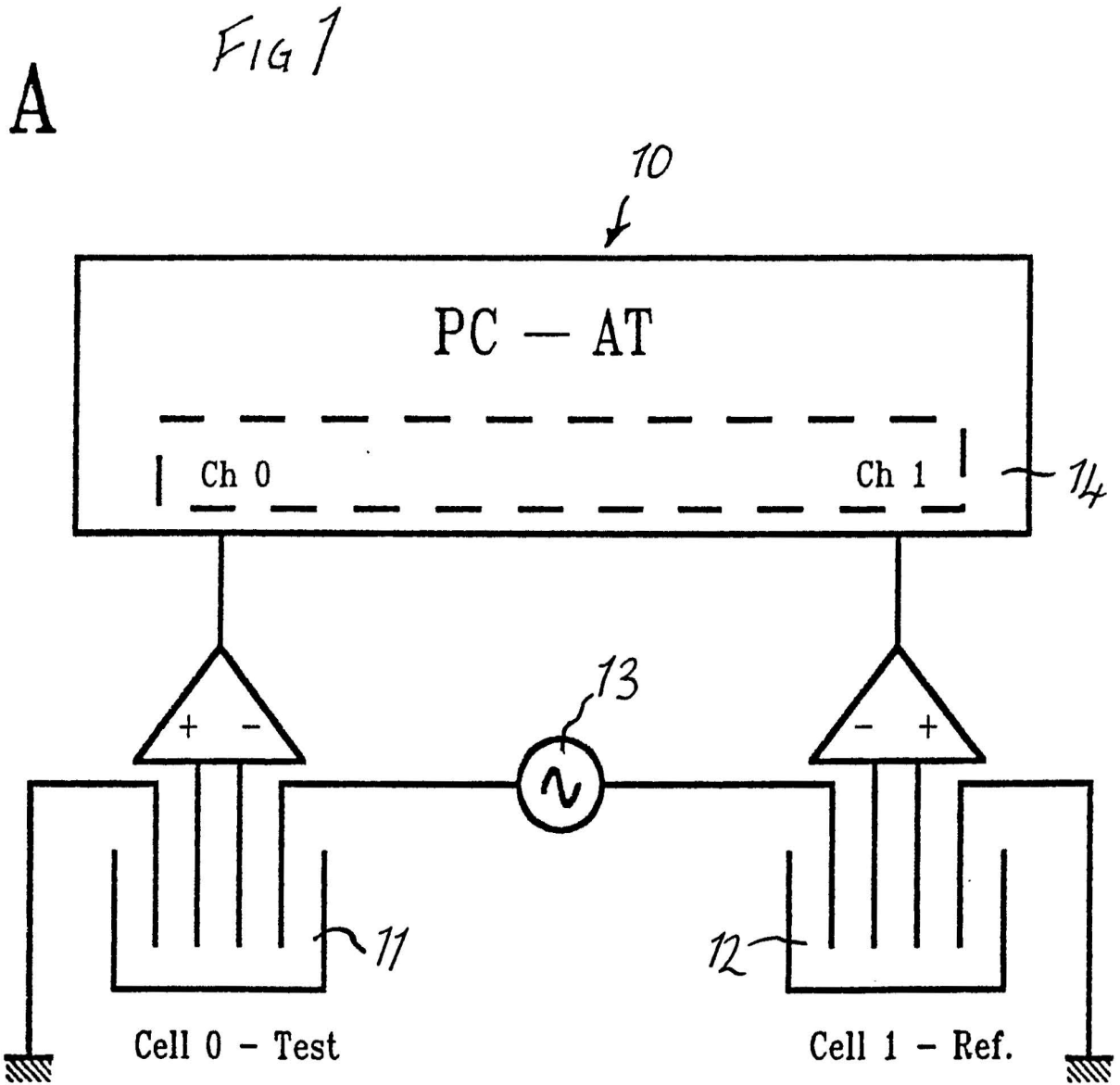
(57) Biological cell material, substrates therefor, or inhibitors of cell metabolism are determined by applying an AC electrical potential across a sample of the biological material so as to produce a non-linear dielectric spectrum, and obtaining a detectable signal from said spectrum. The potential is typically of a first frequency and the measured response is at a second frequency substantially not overlapping with the first frequency. Alternatively, the applied potential may be sinusoidal and the entire frequency of the spectrum may be observed to ascertain the presence of harmonics.

As shown, a sample in test cell O is compared with a reference of the same conductivity in cell 1. AC 13 is applied to both cells and the output of both cells is analysed by microcomputer 14.



At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

GB 2 247 530 A



B

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FIG 2

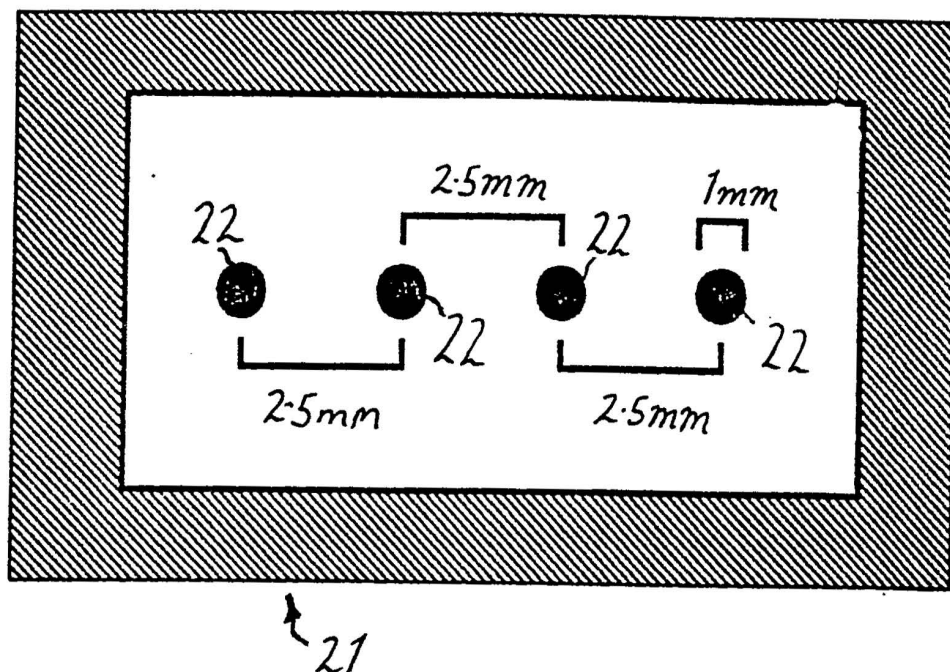
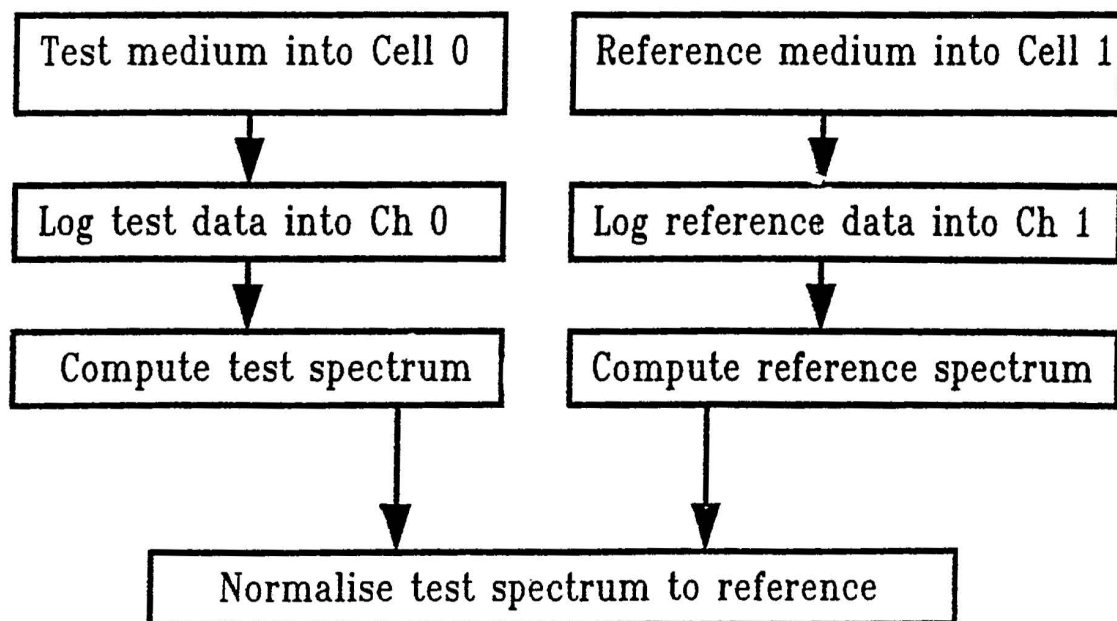


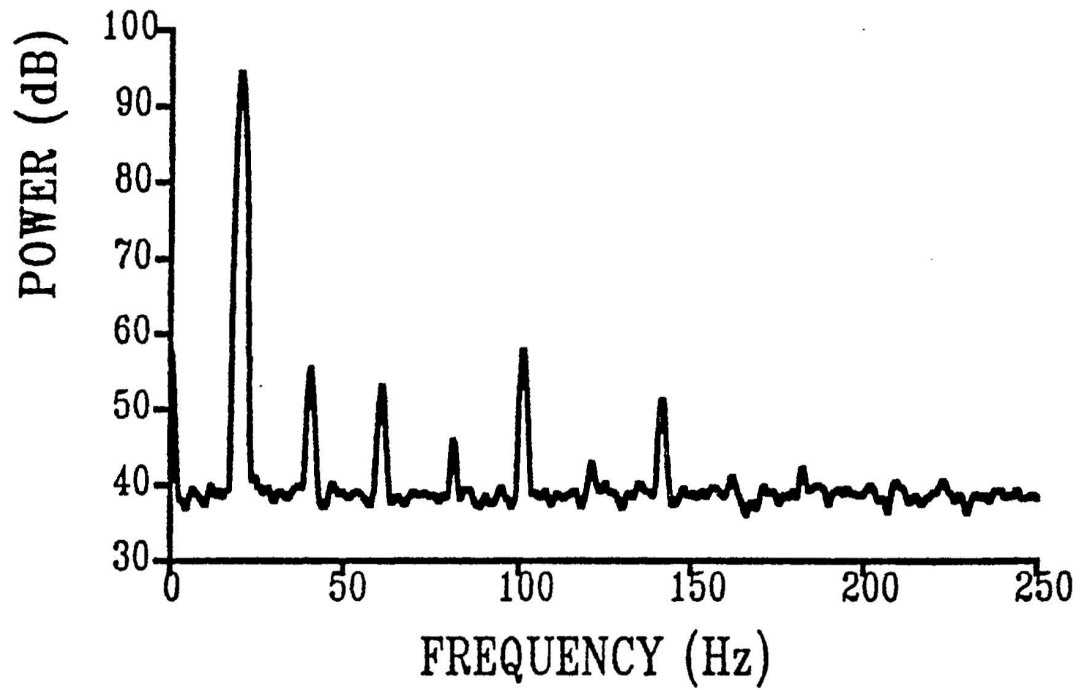
FIG 3



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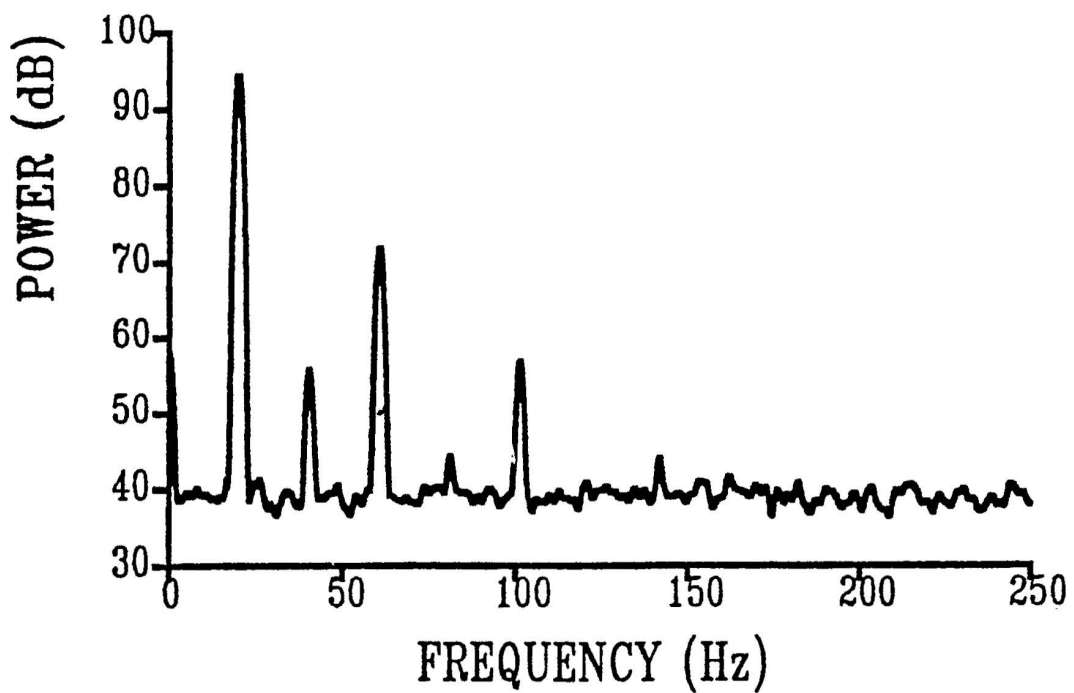
A

FIG 4



B

FIG 5



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C
Fig 6

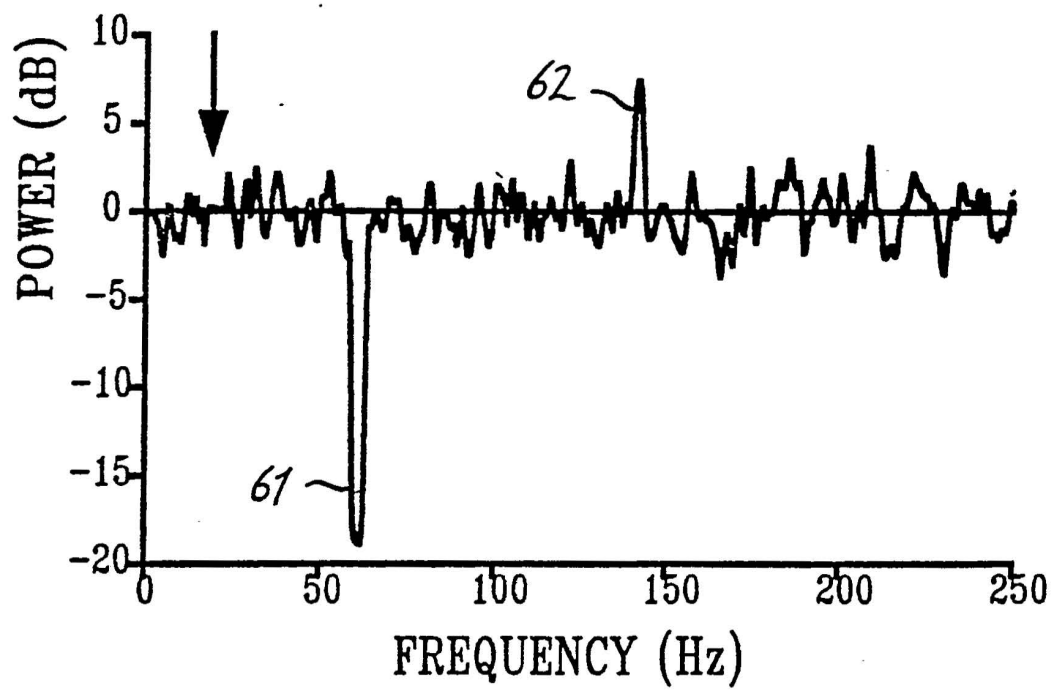
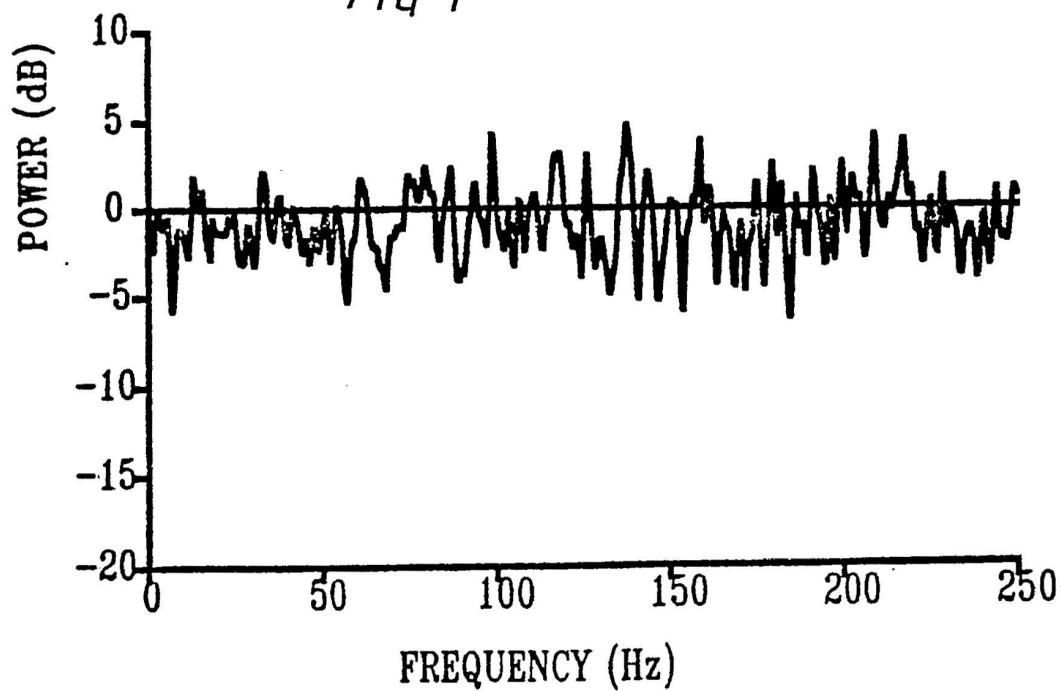
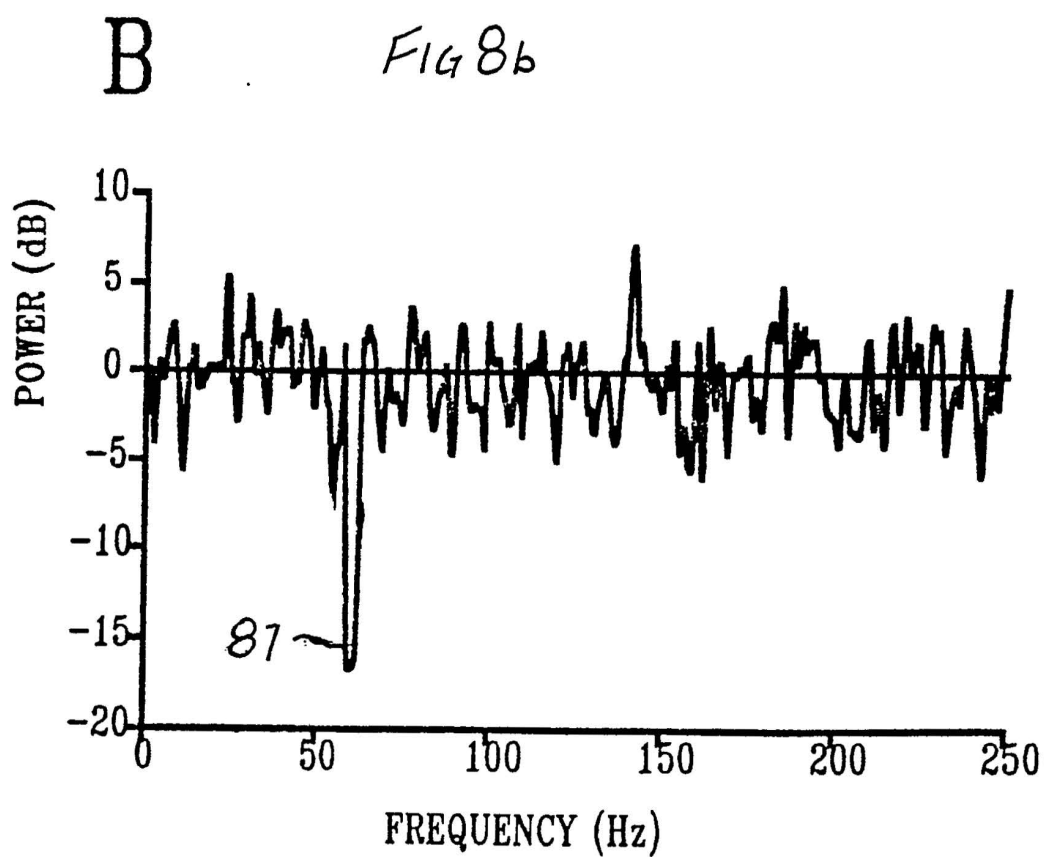
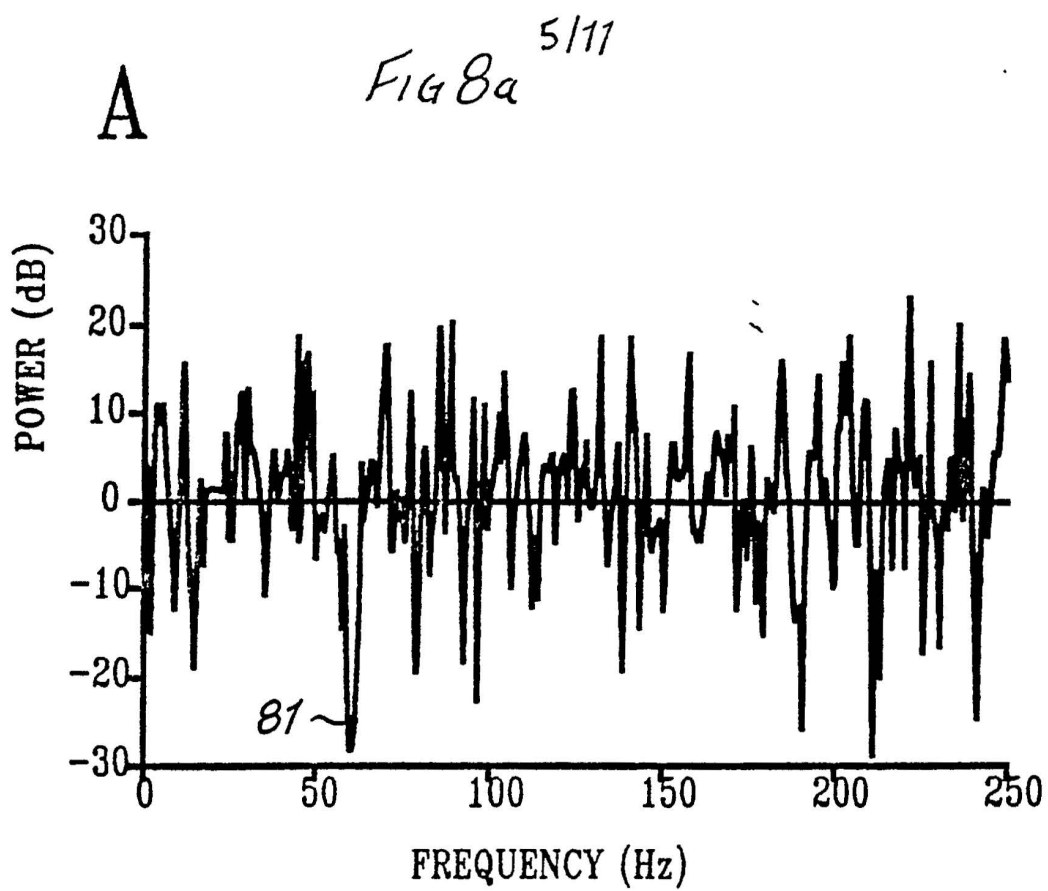


Fig 7

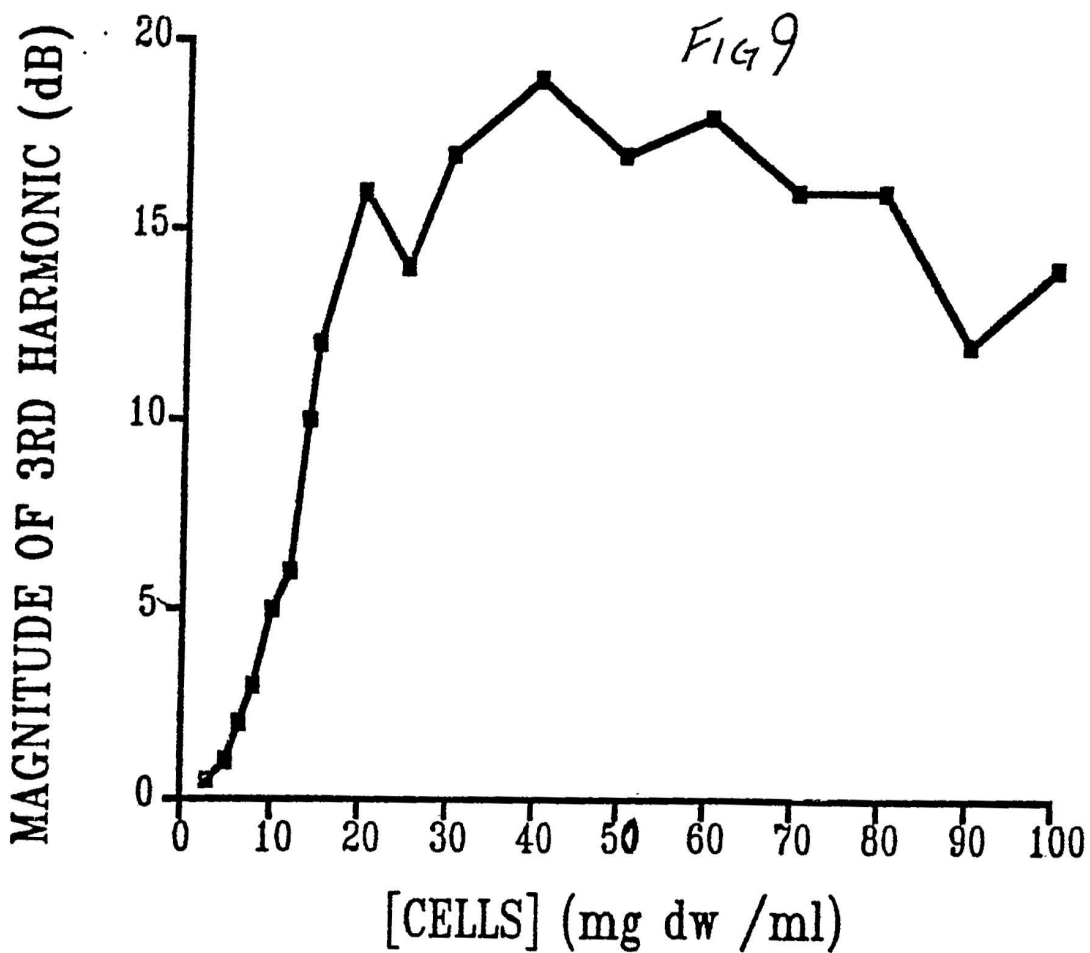
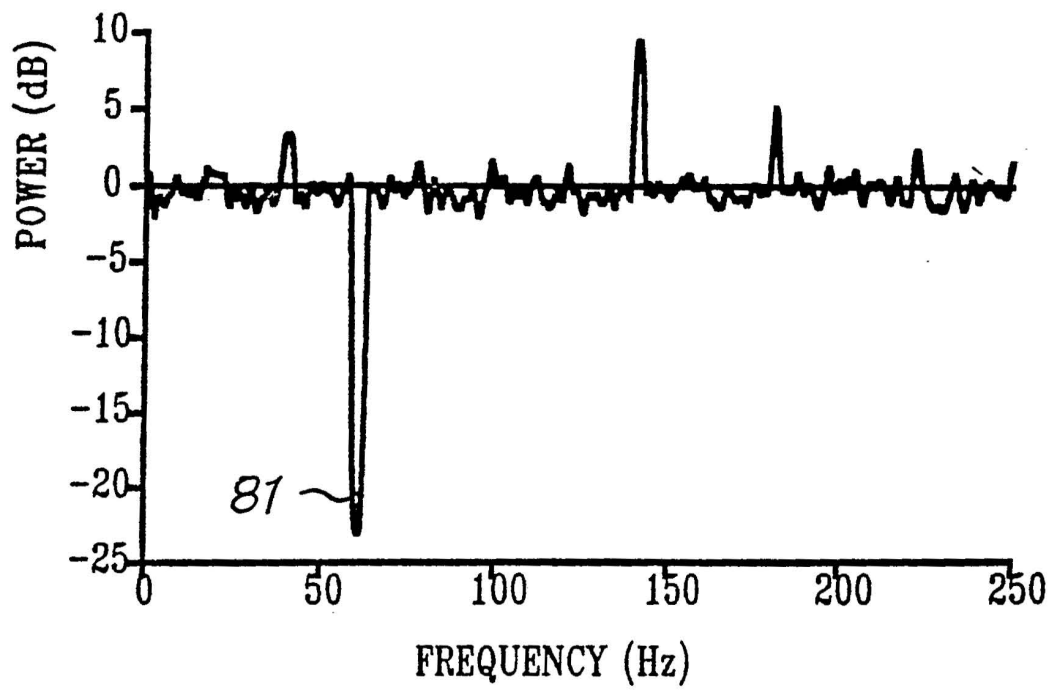




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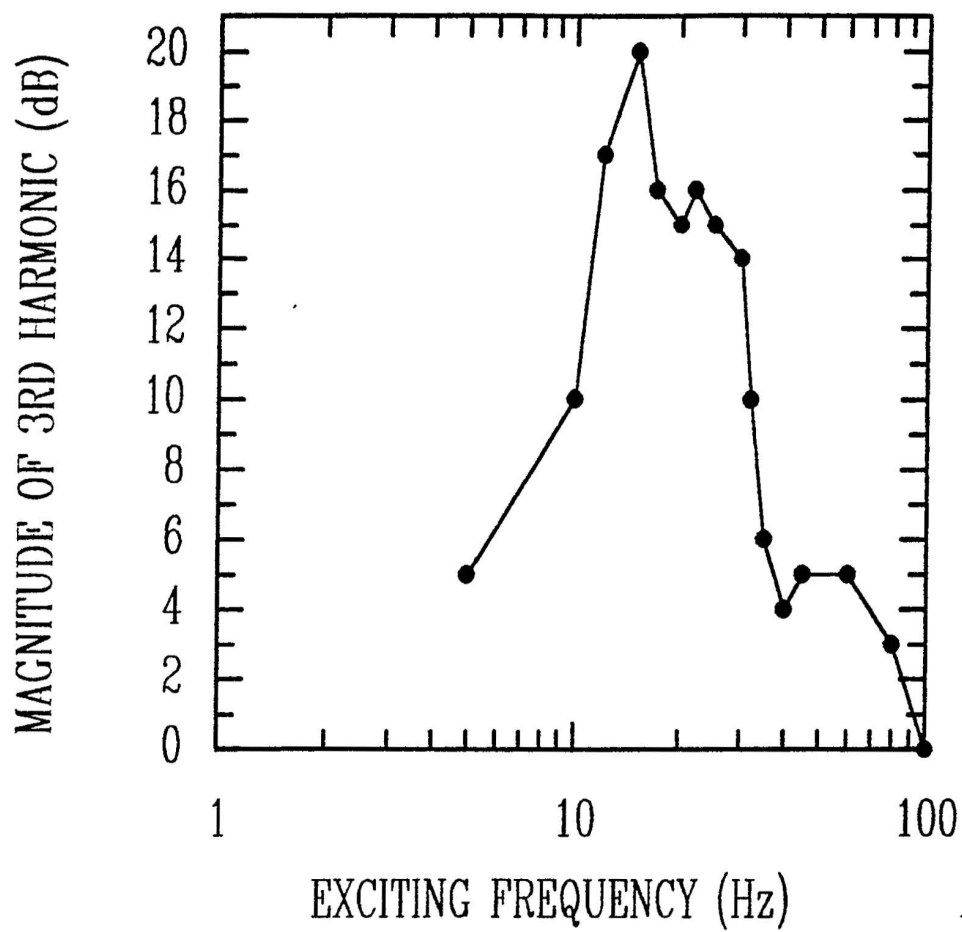
C

Fig 8c



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Fig 10



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FIG 11

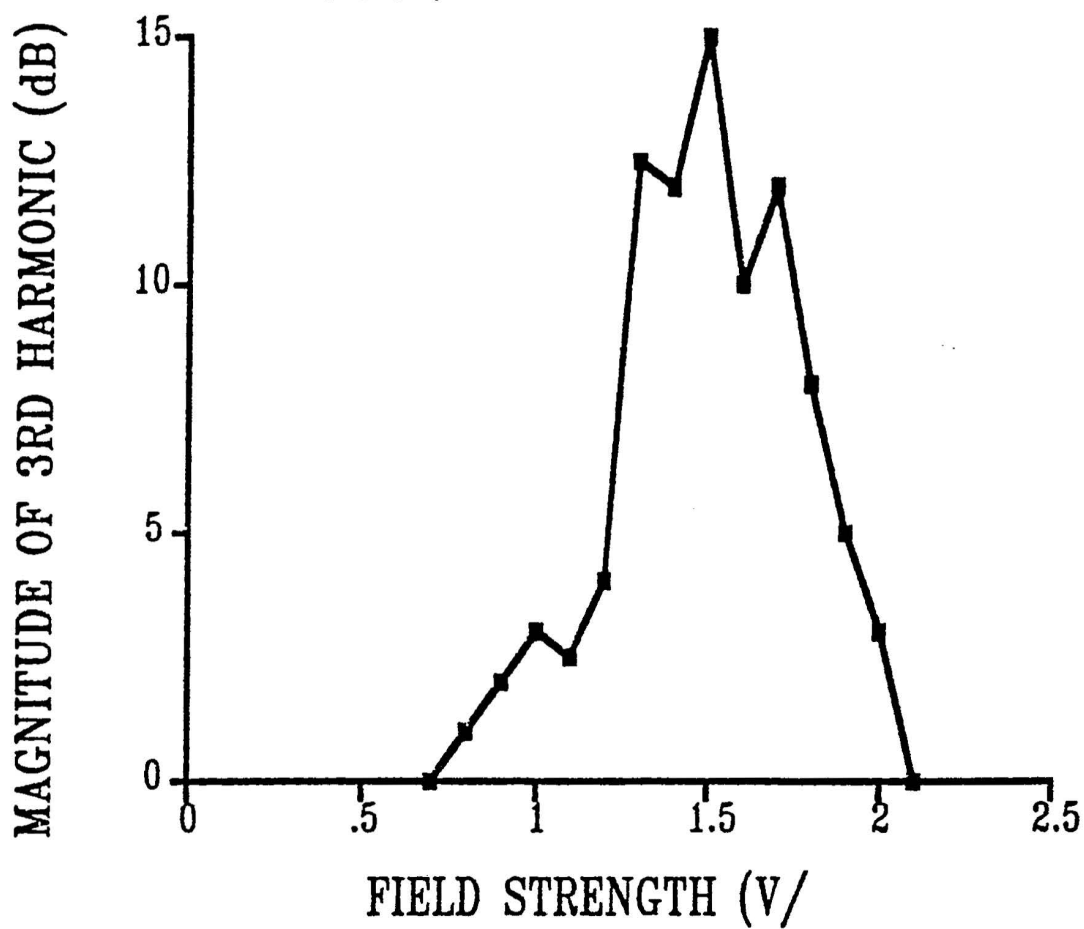
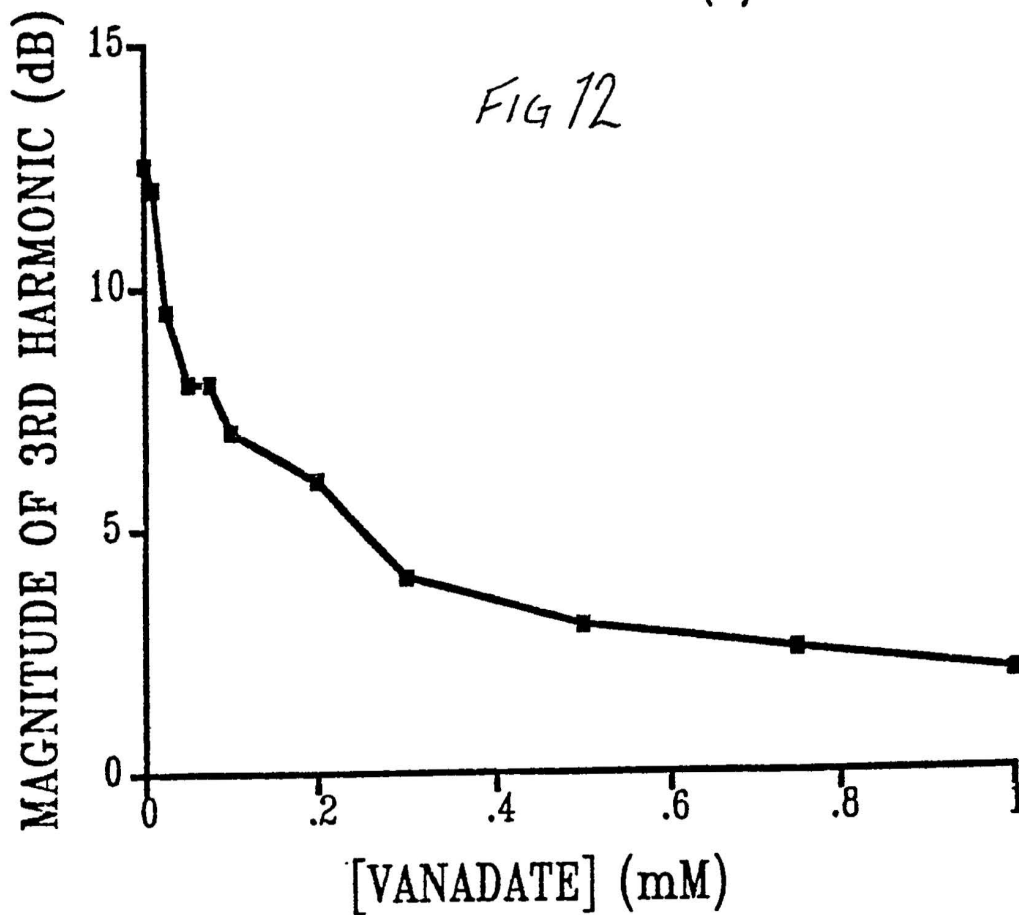


FIG 12



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FIG 13

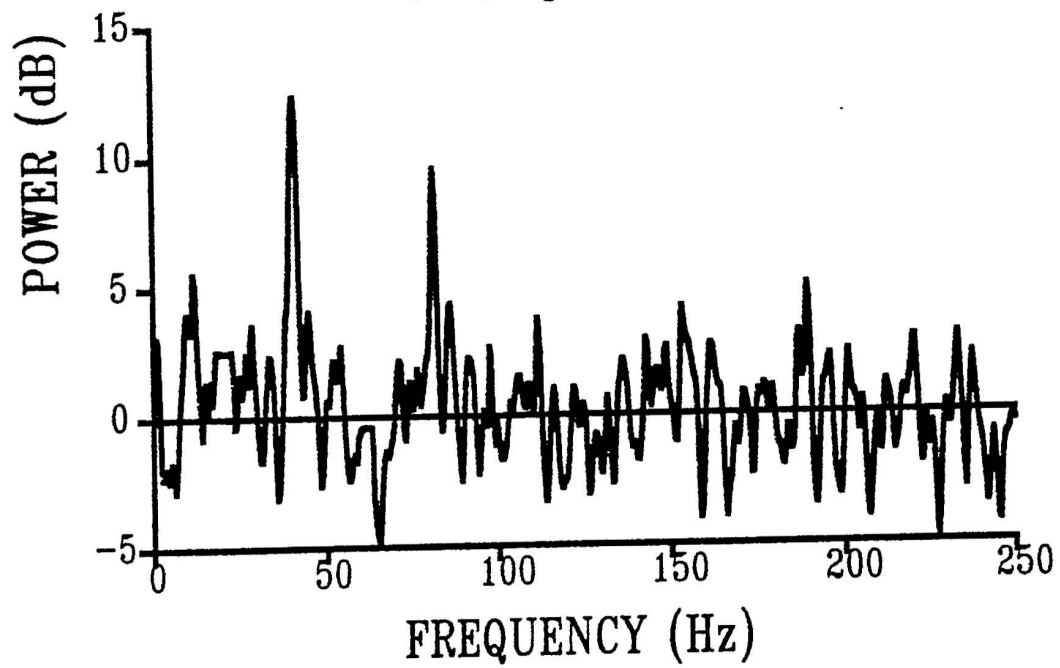
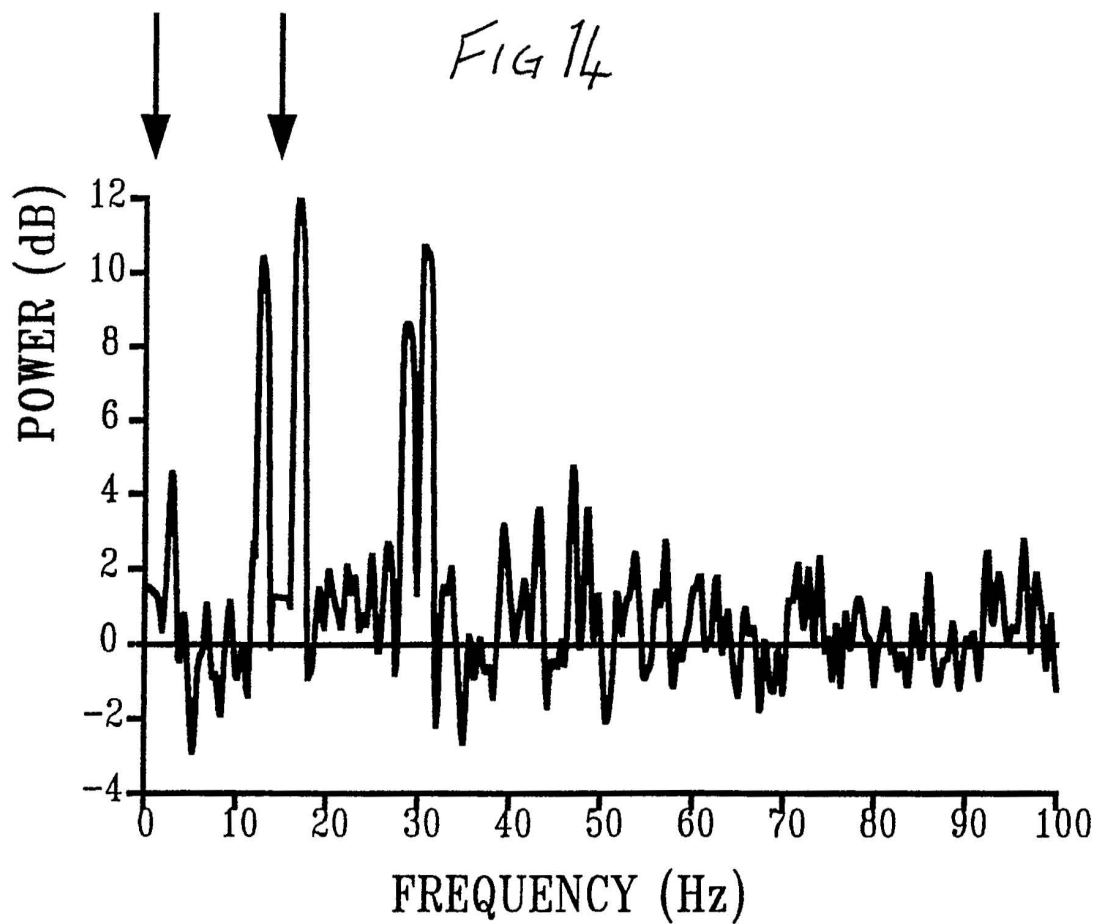


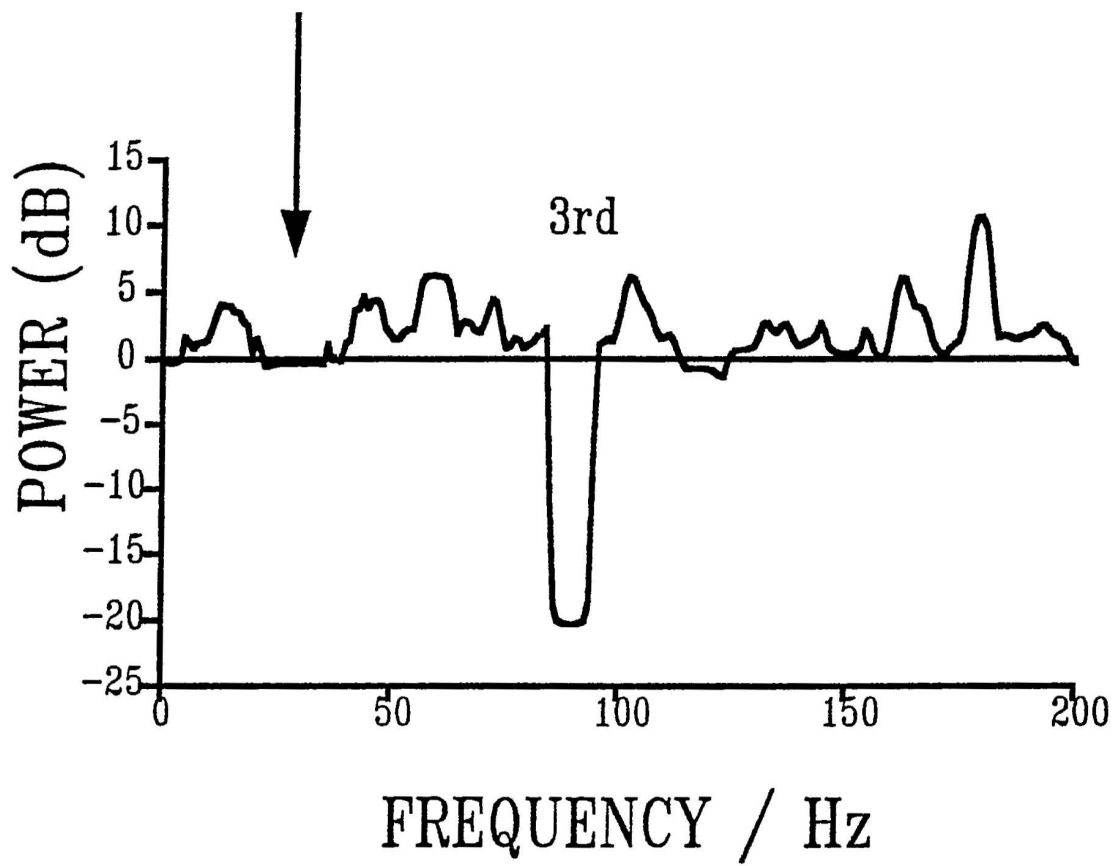
FIG 14



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FIG 15

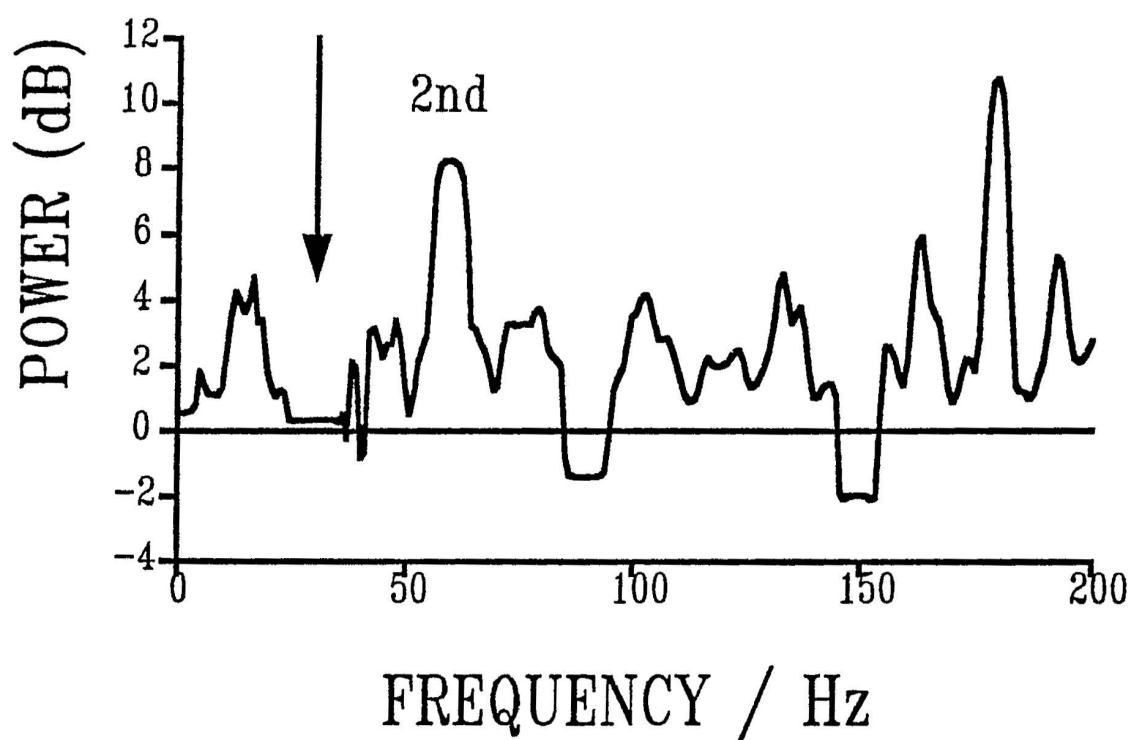
Micrococcus luteus



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FIG 16

Micrococcus luteus



Analytical Method

This invention relates to a method of analysis of biological cell materials and particularly, but not exclusively to analysis of materials comprising enzymes in membranes, as well as analysis of substrates for such cell materials and enzymes.

The linear passive audio and radio frequency electrical properties of biological cell materials are well known. In the frequency range below about 10 MHz, these properties are conveniently measured as the equivalent parallel conductance and capacitance of an electrochemical cell containing the system under study. Up to these radio-frequencies, most biological cell materials exhibit two major dispersions, known as the alpha and beta dispersions. Whilst other sub-dispersions contribute to these major dispersions, and may occasionally be separated from them, the beta dispersion of tissues and cell suspensions is caused predominantly by the build-up of charge at the essentially non-conducting plasma membrane surfaces. The alpha dispersion, though not always dependent upon the ionic strength of the medium, is usually accounted for mainly in terms of the relaxation of counter-ions 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, the statistical mean of the cosine of the angle which the dipole makes with the field, has a field-dependence following the Langevin function:-

$$\cos \langle \theta \rangle = \coth x - 1/x,$$

where $x = E/kT$. A Taylor expansion of this series shows that substantial deviations from linearity do not occur for values of x less than approximately 1, and that to an excellent approximation $\cos \langle \theta \rangle = E/3kT$. Thus the dielectric displacement current is proportional to the magnitude of the exciting field, and their ratio, the admittance, independent of it. These properties are characteristic of a linear system obeying the fluctuation-dissipation theorem.

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 cell materials are typically measured using macroscopic electrical fields E of the order $0.1 - 5 \text{ V.cm}^{-1}$. Given the effective dipole moments usually encountered, the Langevin factor $\mu E/kT$ is normally minuscule, and, as judged by the independence of the measured admittance from the exciting field, well within the range of linearity.

It is known to measure the linear properties of biological materials using impedimetric instruments designed to filter out currents and voltages at frequencies other than the fundamental. This results in materials which appear to have linear characteristics but are in fact non-linear dielectrics.

We have surprisingly found that, by applying a pure sinusoidal AC current to excite a biological cell material, if instead of observing the AC current only at the frequency applied, the entire frequency range of interest is observed, a non-linear dielectric spectrum may be produced from a biological material at voltage levels at which hitherto, the material has been expected to exhibit linear properties only.

The entire frequency range of interest may be studied by performing a transformation on the signal, for example a Fourier transform.

According to a first aspect of the invention there is provided a method of analysing biological cell material, substrates therefor or inhibitors of cell metabolism for such cell material, which method comprises causing an AC electrical potential across a sample of the biological material so as to produce a non-linear dielectric spectrum, and obtaining a detectable signal corresponding to the spectrum obtained.

According to a second aspect of the invention, there is provided a method of analysing biological cell material, substrates therefor, or inhibitors of cell metabolism for such cell material, which comprises causing an electrical potential of one or more initial frequencies to fall across the biological material, and measuring the response of the material at at least one response frequency, wherein the at least one response frequency is substantially not overlapping with the initial frequency or frequencies.

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 cause a lesser but effective "amplification" of the macroscopic field across the membrane. In certain cases in which the membrane of interest contains appropriate enzymes this can cause performance of useful biological work in a field and frequency-dependent fashion. A general mechanism underlying this effect is that enzymes are not dipolar "billiard balls" and can relax between different conformations, some of which may and some of which may not have different vectorial dipole moments from each other.

The electrical potential applied to the sample of the biological material may comprise a relatively high field applied to excite the material and a relatively low probing AC voltage to register the field-dependent dielectric properties of the material.

Preferably, however, a sinusoidal AC current is used to excite the material and the entire frequency range of interest is observed by performing a transformation to see the extent to which the non-linearities of the sample are manifest by the generation of harmonics. By varying the frequency and amplitude of the exciting current, a two-dimensional non-linear dielectric spectrum can be built up which can act as a dielectric fingerprint of the sample under test.

Advantageously a reference non-linear dielectric spectrum is produced using the supernatant of a biological material, the conductivity of which is adjusted to be substantially identical to that of the sample of the biological material. The spectrum from the sample is then divided by the reference spectrum, resulting in the deconvolution of the effects due to non linearities within the electrochemical system from those due to the biological cells themselves.

The third harmonic of the non-linear dielectric spectrum is of particular interest as its magnitude is indicative of the concentration of cells in the biological sample. The invention thus provides a method of observing the third harmonic in a non-linear dielectric spectrum obtained from a biological

sample. It also provides a method of determining the concentration of cells in a cell suspension, since the magnitude of the third harmonic is indicative of the concentration of cells in such a suspension. The invention also provides a method of monitoring the ability of living cells to transduce exogenous electric field energy. The inventors have discovered that the particular harmonics present in the non-linear dielectric spectrum obtained from a biological sample are indicative of the metabolic state of living cells in a biological sample.

There is further provided by the present invention apparatus for carrying out a method as hereinbefore described, which apparatus comprises:-

- a) retaining means for retaining a sample of living cell material;
- b) means for applying an AC electrical potential across said sample so as to produce a non-linear dielectric spectrum; and
- c) means for obtaining a detectable signal corresponding to said spectrum.

The production of power spectra using Fourier transformation results in the loss of phase information, and therefore only the magnitude of the 3rd harmonic (or indeed of any other harmonics) is used to display the energy transduced by the cells from the fundamental to the given harmonic in the present system. 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. The cells do not "harvest" 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 generated by our signal source when the fundamental is set as 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. The data are presented in logarithmic (dB) scale, since this serves to accentuate the precision with which alterations in the observable magnitude of harmonics may be recorded.

Whilst there is some day-to-day variation in the exact magnitude of the 3rd harmonic (when viewed on the logarithmic decibel scale), the data obtained are 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 are observed, (i) indicating that the surface electrochemistry of their electrodes is well-matched, and (ii) that living cells are indeed the source of the 3rd harmonic observed.

The electrical fields involved in the following experiments are (in terms of the Langevin factor) quite minuscule, 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 indicated that the linear impedance was independent (within experimental error) of the magnitude of the exciting voltage in the range studied. Thus, it is quite feasible to have a system in which the impedance appears linear but which is in fact nonlinear. The potential-dependant capacitance (thickness) of a bilayer membrane can exhibit a quadratic dependence upon the potential difference across it. However, at the potentials used here the quadratic term is negligible.

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. 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 nonlinear dielectricity that we have observed one is led to consider the membrane properties 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", this ATP may be expected to be utilised by the H^+ -ATPase present in this organism to drive the uptake of cations such as K^+ from the external medium until a state of "static head" 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 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. We have determined that the H^+ -ATPase in the plasma membrane of this organism, as the main enzyme potentially active under the physiological conditions used, is the major source of the nonlinear dielectric response observed.

The inventors have shown for the first time in cell suspensions that the conformational flexibility of enzymes can manifest in the generation of nonlinear 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 invention will now be described by way of example only, with reference to the accompanying drawings, in which:

Figure 1 is a schematic representation of a non-linear spectrometer for carrying out the method according to the invention;

Figure 2 is a schematic representation of the electrochemical cell used in the spectrometer of Figure 1;

Figure 3 is a block diagram of a particular method used to obtain non-linear dielectric spectra uncontaminated by artefactual electrochemical phenomena;

Figure 4 is a representation of a non-linear dielectric spectrum of Saccharomyces cerevisiae (S. cerevisiae);

Figure 5 shows a non-linear dielectric spectrum obtained from a reference cell;

Figure 6 shows a spectrum obtained by subtracting the spectrum of Figure 4 from the spectrum of Figure 3;

Figure 7 is a non-linear dielectric spectrum obtained from a suspension of boiled cells;

Figure 8 (a) - (c) show the effect of averaging on the non linear dielectric spectrum of S. cerevisiae;

Figure 9 shows the effect of cell concentration on the magnitude of the third harmonic in the non-linear dielectric spectrum of S. cerevisiae;

Figure 10 shows the effect of exciting frequency on the magnitude of the third harmonic of the nonlinear dielectric spectrum of S. cerevisiae;

Figure 11 shows the effect of the strength of the exciting field on the magnitude of the 3rd harmonic of the non-linear dielectric of S. cerevisiae;

Figure 12 shows the effect of sodium metavanadate on the magnitude of the third harmonic of the non-linear dielectric spectrum of S. cerevisiae;

Figure 13 shows the effect of glucose on the non-linear dielectric spectrum of S. cerevisiae;

Figure 14 shows another embodiment of a non-linear dielectric spectrum of S. cerevisiae cells;

Figure 15 shows a non-linear dielectric spectrum of an anaerobic suspension of Micrococcus luteus; and

Figure 16 shows a non-linear dielectric spectrum of an aerobic suspension Micrococcus luteus.

Referring to Figure 1, a nonlinear dielectric spectrometer for use in carrying out the method according to the invention is designated generally by the reference numeral 10. The spectrometer 10 comprises two electrochemical cells 11,12. Cell 11 is a test cell containing a sample of the biological system whose nonlinear dielectric properties are being studied. In this example the biological system comprises a system comprising a suspension of S. Cerevisiae. The concentration of

(the cells was approximately 50mg dry wt. ml⁻¹ in a medium of 20 mM KH₂PO₄, 30 mM KCl, 1mM MgCl₂ pH 6.5. The tests were carried out within four hours of preparing the suspension. Cell 12 is a reference cell comprising the supernatant of the test suspension, the conductivity of which had been adjusted with distilled water, to compensate for the volume fraction of cells present in the sample to be identical to that of the sample at the frequency of interest. The cells 11,12 are connected to a sinusoidal oscillator 13, via a Data Translation Analog - to Digital converter, to an 80386 - based microcomputer 14.

The system is built around an IBM-PC-AT compatible microcomputer (Viglen III, 80386 main processor from Viglen Ltd, LONDON U.K.). To maximise the performance of the mathematical operations, an 80387 compatible co-processor (Cyrix 83C87) was used. One of the expansion ports of the computer was furnished with a Data Translation model 2823 ADC/DAC board. Because of the 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/second.

Referring to Figure 2, an electrochemical cell 21 is shown. To minimise the contribution of electrode polarisation phenomena a 4-electrode system based on gold pin-type electrodes 22 was used. 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 were checked using a Solartron 1200 Signal Processor (Schlumberger Instruments, Farnborough, Hants) and a Hameg HM 208 Digital Storage Oscilloscope.

The acquisition of the data and its subsequent processing and display was performed using the ILS software (Signal Technology Inc, Geoleta, California) running under control files.

The spectra were collected using the following method:

the sample cell suspension was pipetted into the electrode cell 11 (Fig 1), the test waveform applied to the (outer) current electrodes, and the data logged from the (inner) 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 by subtracting the mean of the block from the individual samples. Each block was then windowed using a Blackman window 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 spectral data were stored on the computer's hard disk.

A reference spectrum was acquired using the supernatant in cell 12 (Fig 1), whose conductivity had been adjusted (with distilled water), to compensate for the volume fraction of cells present in the sample 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 described 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 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 minutes. A diagram of the steps involved in the generation of the non-linear dielectric spectra is shown in Fig 3. The advantage of this approach was that it allowed the effects due to non-linearities within the electrochemical system from these due to the biological cells themselves to be deconvolved.

The spectrometer 10 may be used for the registration of non-linear biological dielectric spectra, to indicate for the first time that they are easily observed in cell suspensions, and to show that the H^+ - ATPase in the plasma membrane of Saccharomyces cerevisiae is the source of the majority of the non-linear dielectric response thus observed.

Figure 4 shows a typical non-linear dielectric spectrum obtained from a suspension of resting cells of S. cerevisiae using the spectrometer of Figure 1, with an exciting voltage (measured between the outer electrodes) of 1.5V (2.0 V.cm⁻¹) at a frequency of 20 Hz, and displays spectra from the sample (Figure 4) the reference (Figure 5) and their difference (Figure 6). Due to imperfections in the generator and the nonlinearities 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. The pattern of harmonics between the "sample" and "reference" cells is markedly different, and upon subtraction of the reference spectrum from the sample spectrum a very strong and apparently negative third harmonic 61 (Fig 6) is obtained. A (positive) 7th harmonic 62 is also reproducibly observed (and on occasion an 11th harmonic), but even harmonics are absent under the stated conditions.

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. Figure 7 shows that no third harmonic is generated using dead (boiled) cells. This indicated that the presence of potentially active enzymes was a prerequisite to the generation of a third harmonic.

To establish whether only harmonics were generated, or whether the power spectra contained non-harmonic components, the number of blocks that were averaged was varied. The data from a representative set of runs are displayed in Figs 8a to 8c where it may be observed that the magnitude of the 3rd harmonic 81 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 9 where it may be observed that the magnitude of the 3rd harmonic in dB is substantially 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. Fig 10 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 nonlinear dielectric behaviour could be observed. Fig 11 shows that the magnitude of the 3rd harmonic is only significant in a voltage window between about 0.6 and 2.1 V (0.8 - 2.8 V.cm⁻¹). 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).

It is well known that the catalytic cycle of enzymes of this type (the so-called E₁E₂ enzymes) involves an enzyme-bound phosphate intermediate, and that their activity can be inhibited by low concentrations of pentavalent vanadium compounds whose trigonal bipyramidal structure is thought to mimic the transition state of the phosphate during its hydrolysis, trapping the enzyme in its E₂ conformation.

Figure 12 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 again suggests strongly that the H⁺-ATPase in the plasma membrane of these cells is the main source of the non-linear dielectric response, and further serves to leave phenomena such as dielectrophoresis as the source of the non-linearities observed. The harmonics were also completely

abolished by the H^+ -ATPase inhibitor dibenzhydryl carbodiimide at a concentration of 0.2 pmol/mg dry wt of cells.

As described above, a 3rd harmonic was reproducibly observed with resting cell suspensions of S. cerevisiae. This 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. An experiment to determine how this behaviour might be modified when the enzyme was driven away from static head and was able (or expected) to do work was carried out. Resting cells were taken, their (usual) non-linear dielectric spectrum recorded, and a metabolisable carbon source (D-glucose) added. After a short lag period of some 20 minutes, the spectrum displayed in Fig 13 was recorded. Remarkably, the 3rd harmonic had disappeared and was replaced by substantial 2nd and 4th harmonics. These even harmonics were also vanadate-sensitive. When static head was again attained, the spectrum, returned to its starting shape, with a substantial 3rd but no even harmonics. 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 for the adsorption of exogenous electric field energy. A parallel measurement of the dielectric permittivity at 0.3 Mhz, a monitor of intact cellular biomass, did not show any observable changes during this experiment. Thus non-linear dielectric spectroscopy provides a sensitive means of distinguishing the metabolic states of living cells.

Non-linear dielectric properties may also be manifested as the frequency mixing of a plurality of fundamentals. Fig 14 shows the non-linear dielectric spectrum recorded when a suspension of S. cerevisiae was excited with fields (0.9V/cm each) at frequencies of 1Hz and 15 Hz. The frequency mixing to produce signals at sums and differences of integral multiples of the fundamental is clear.

The electric potential V_m generated across the (spherical) yeast plasma membrane is given by $V_m = 1.5 Re \cos$ where E is the microscopic field, r the cell radius and the angle between the field and the membrane normal.

For the present cell radius of 3 microns and a

field of 2 V.cm^{-1} a maximum (field-dependent change in) membrane potential of 0.9 mV was obtained. 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. For convenience we will assume that relevant changes in dipole moment due to field-dependent conformational changes of target enzymes are 500D (they are however likely to be much less since 500D equates to the displacement of 10 full charges across the membrane). The Langevin factor $\mu E/kT$ 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, we have observed the generation of a substantial third harmonic by the S. cerevisiae cells.

The invention as hereinbefore described has been carried out using suspensions of S. cerevisiae, with or without glucose; however the method is of general applicability and is not limited to the use of the former substrates. Figure 15 shows a spectrum illustrating the nonlinear dielectric behaviour of an anaerobic suspension of Micrococcus luteus. Since this organism cannot ferment, such cells are resting, and display an odd-numbered (third) harmonic. Figure 16 shows an aerobic suspension of Micrococcus luteus where the organism can respire and which causes the generation of an even-numbered (second) harmonic.

Substrates which can be determined by the method according to the invention include oxygen, glucose, lactic acid or lactate, or the like. Similarly, inhibitors of cell metabolism can be determined by the method according to the invention; examples of such inhibitors include vanadates, as described above with reference to Figure 12.

Claims:

1. A method of analysing biological cell material, substrates therefor, or inhibitors of cell metabolism for said cell material, said method comprising the steps of:
causing an AC electrical potential to fall across a sample of the biological material so as to produce a non-linear dielectric spectrum, and obtaining a detectable signal corresponding to said spectrum.
2. A method of analysing biological cell material or substrates therefor or inhibitors of cell metabolism for said cell material, which method comprises causing an electrical potential of one or more initial frequencies to fall across the biological material, and measuring the response of the material at at least one response frequency, characterised in that at least one response frequency is substantially not overlapping with the initial frequency or frequencies.
3. A method according to claim 1 or 2, wherein the electrical potential comprises a relatively high field applied to excite the system, and a relatively low probing AC voltage to register the field-dependent dielectric properties of the biological material.
4. A method according to any of claims 1 to 3, wherein said potential is sinusoidal, and the entire frequency range of interest is observed by performing a translation to ascertain the extent to which the non-linearities of the material are manifest by the generation of harmonics.
5. A method according to any of claims 1 to 4, wherein said material comprises a tissue or a cell suspension.
6. A method according to claim 5, wherein a further electric potential is applied to a supernatant of said dispersion or suspension, the conductivity of said supernatant being adjusted to be substantially identical to that of said biological material.

7. A method according to any of claims 1 to 6, which method further comprises observing the third harmonic of said non-linear dielectric spectrum.
8. A method according to any of claims 1 to 7, which method comprises observing at which method even-numbered harmonics of said non-linear dielectric spectrum.
9. Apparatus for analysing biological cell material substrates therefor, or inhibitors of cell metabolism for said cell material, which method comprises:
 - a) retaining means for retaining a sample of said biological material;
 - b) means for causing an AC electrical potential to fall across said sample so as to produce a non-linear dielectric spectrum; and
 - c) means for obtaining a detectable signal corresponding to said spectrum.