

Rotating-Field-Induced Rotation and Measurement of the Membrane Capacitance of Single Mesophyll Cells of *Avena sativa*

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Rotation of single cells (mesophyll protoplasts of *Avena sativa*) induced by a planar, homogeneous rotating field has been observed at a frequency of 20–40 kHz (conductivity of the external mannitol solution $6 \times 10^{-5} \Omega^{-1} \text{cm}^{-1}$). This variation in optimum frequency is largely due to an inverse dependence on cell radius. Rotation direction is opposite to that of the field, and can be reversed at will by reversing the field. The maximum speed of cell spinning was a few cycles per second (and thus always much slower than that of the field) and was proportional to the square of the amplitude of the field.

The rotation of a single cell in a rotating field is expected on the basis of the dipole-dipole theory developed by Holzapfel *et al.*, (J. Membrane Biol. **67**, 1–14 (1982)), for multi-cell rotation. Measurements of the dependence of optimum applied field frequency on medium conductivity indicate that the dipole is generated by interfacial (Maxwell-Wagner) polarization. The required frequency is a linear function of the conductivity of the external solution. This relationship is used to derive a value for the specific membrane capacitance. Further applications of this technique for cell and membrane research are discussed.

Introduction

Rotation of a very small percentage of cells in a linear non-uniform field (frequency range between 100 Hz and 120 MHz) has been described by several authors [1–4].

Zimmermann *et al.* [4] showed that nearly all cells of a given species if in close proximity rotated when exposed to a linear alternating field having a characteristic narrow frequency range. Further work by this group showed that the presence of a minimum of two cells in close proximity is necessary and presented a theory to account for this based on the interaction of two induced dipoles within the field [5]. Implicit in the theory was the local production of a rotating field by the interaction of the two induced dipoles.

Rotation of any *single* cell induced by the same characteristic range of frequencies as observed by Zimmermann *et al.* [4] has now been observed, provided that the field itself rotates rather than alternating along one axis.

Materials and Methods

The experimental chamber consists of four electrodes mounted on a microscope slide as shown in Fig. 1. The essentials of the technique were proven in a chamber with 8 mm electrodes; a smaller chamber with 3 mm electrodes was used when higher field strengths (greater than 37.5 V cm^{-1}) were required.

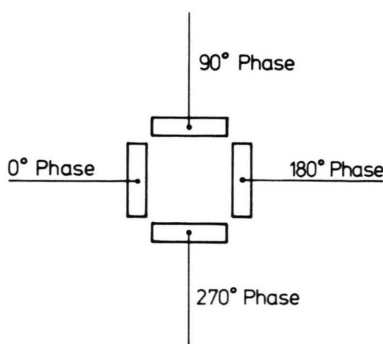


Fig. 1. Schematic diagram of the chamber used to produce the rotating field. Plane electrodes made of platinum foil were bonded to a polymethylacrylate microscope slide; the assembly was sealed with epoxy resin. The electrode spacing was either 3 mm or 8 mm; the height of the electrodes 2 mm or 4 mm, respectively.

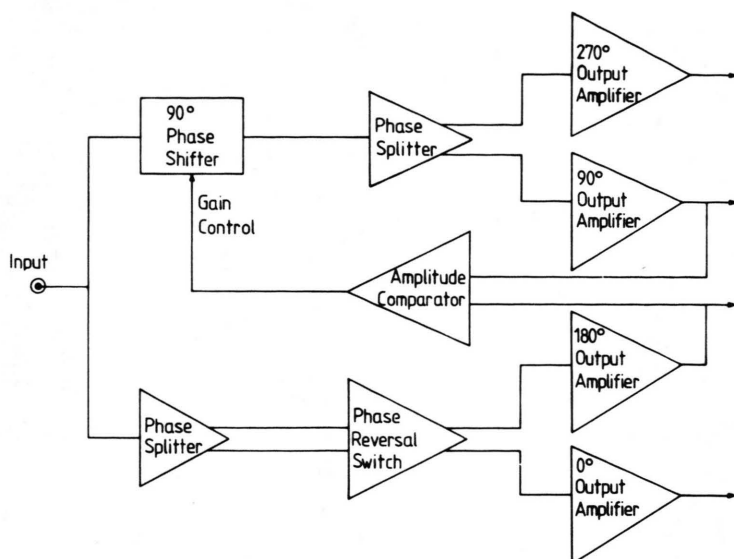


Fig. 2. Block diagram of the four-phase generator. Reversal of the phase switch reverses the direction of rotation of the field in the chamber.

The four electrodes are driven with equal voltages of four phases spaced by 90° , which are produced by the unit shown in Fig. 2. The production of a rotating field in this manner is discussed in Appendix A. The unit, using sine wave drive from a Toellner TE7702 function generator, produces up to 15 volts peak per electrode from 5 Hz to 500 kHz. The function generator was calibrated against a frequency counter (Tektronix DC 503). Mesophyll cell protoplasts of *Avena sativa* were prepared as by Hampp and Ziegler [6].

Observations were confined to the horizontal centre of the chamber; protoplasts were held at the vertical mid-line by layered isotonic solutions of equal volume (lower layer: 0.5 M sucrose; upper layer: 0.5 M mannitol).

Such solutions made with glass-distilled water had conductivities (measured at 1 kHz) of $6 \times 10^{-6} \Omega^{-1} \text{cm}^{-1}$ approximately; higher and better-defined conductivities were produced by the inclusion of KCl or NaCl without change of total osmolarity.

Observations were made using a Leitz "Orthoplan" microscope (fitted with 6 mm working distance objectives) with the addition of a Grundig video system for rotation speed measurements.

For characteristic frequency measurements the protoplast diameter was measured against an eyepiece graticule, and the field frequency varied for maximum rotation speed as assessed by eye. 30 V cm^{-1} field strength was sufficient and repeatability for any given protoplast better than $\pm 20\%$.

Experimental Results

Single cell rotation was observed in all protoplasts. In media of conductivity $6 \times 10^{-5} \Omega^{-1} \text{cm}^{-1}$, the characteristic frequency (i.e. that giving maximum rotation speed) was between 20 and 40 kHz. At higher conductivities the characteristic frequency increased linearly (Fig. 3).

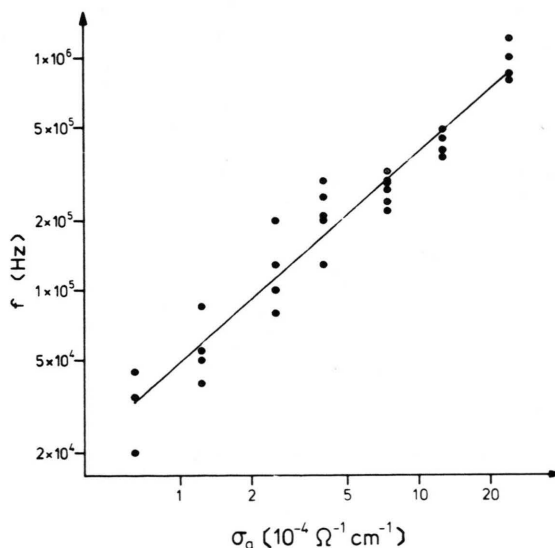


Fig. 3. The dependence of characteristic frequency on external solution conductivity. Both axes are logarithmic, the line is the least-squares fit to logarithmic data. (37 experimental results from 37 protoplasts.)

This is consistent with the results of Holzapfel *et al.* [5] for multi-cell rotation in which method the close presence of other cells and increased significance of ion leakage at high suspension densities will have increased the effective medium conductivity above the published value of $10^{-5} \Omega^{-1} \text{cm}^{-1}$.

Much of the scatter of the data in Fig. 3 is due to the heterogenous nature of the protoplast population. For example, at a given conductivity cells of larger radii exhibited generally lower characteristic frequencies; by plotting the product of frequency and radius ($f \cdot R$ product) for each cell against the conductivity (Fig. 4) the scatter is reduced. (The cell radii ranged from 0.8 to $2.2 \times 10^{-3} \text{cm}$.) This has a basis in the theory presented in the Discussion.

The conductivity was generally adjusted with the aid of KCl; use of NaCl gave indistinguishable results.

The rotation speed was found to vary as the square of the field strength (Fig. 5). This is as reported by Holzapfel *et al.* [5] for multi-cell rotation, but a rotating field of 14 V cm^{-1} is sufficient for 1 radian s^{-1} rotation speed as against 90 V cm^{-1} linear field strength in the earlier method. This higher sensitivity enabled those observations in the high conductivity range (Figs. 3 and 4) to be made without the excessive heating and turbulence which would have resulted from linear field methods.

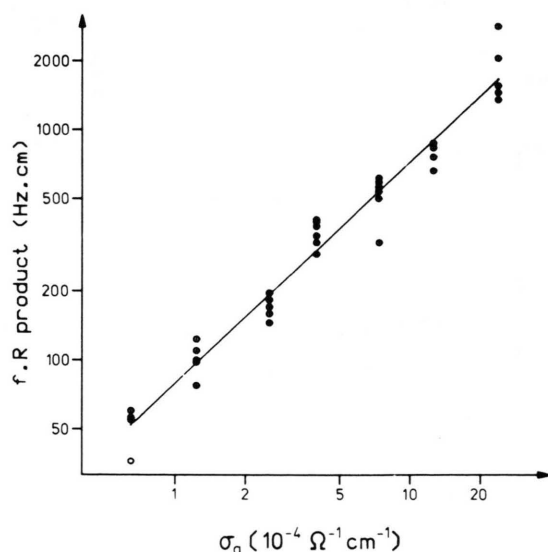


Fig. 4. The dependence of the product of characteristic frequency and cell radius on external solution conductivity. Both axes are logarithmic, the line is the least-squares fit to logarithmic data. (Same experimental results as in Fig. 3.)

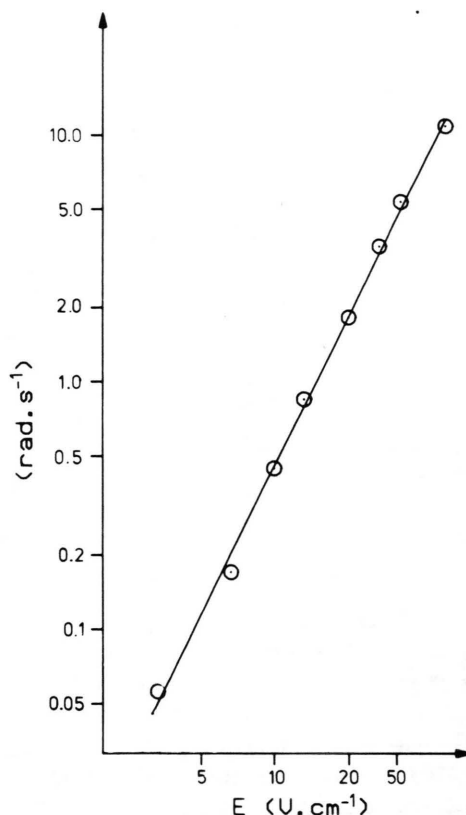


Fig. 5 Dependence of protoplast rotation speed on applied field strength. Both axes logarithmic, the line is the least-squares fit to logarithmic data. Gradient of line is 2.00 ± 0.04 (\pm standard deviation). The rotation speed varies as the square of the field strength. (Cell radius, $1.6 \times 10^{-3} \text{cm}$, medium conductivity $6.4 \times 10^{-5} \Omega^{-1} \text{cm}^{-1}$, characteristic frequency 28 kHz . Other cells give the same gradient, even though the rotation speed may be different.)

Fig. 5 shows no indication of a threshold effect down to the lowest voltages used (giving 1 revolution in 2 min approximately). Studies of slower rotation were not attempted due to lateral drift of the cell under observation during the 10 min or more required for such a measurement. Lateral drift is to be avoided because only the chamber's central field can be considered homogeneous, and will inevitably change as the electrodes are approached. With constant applied voltage, field-induced lateral motion (dielectrophoresis, indicating an inhomogeneous field) was appreciable near the electrodes, but the rotation rate was less than in the centre of the chamber. This is because the proximity of the large flat electrode shorts out that component of the rotating field parallel to it and thus reduces the

rotation rate. This is equivalent to electrically converting the field to a linear one, which was found to stop rotation.

For demonstration purposes, rotation of a single protoplast in the entire volume of the 8 mm chamber was observed. The low probability of such a single protoplast being in the central field made it more practical to use about 10 protoplasts in the 3 mm chamber. In order to avoid interactions and field inhomogeneities all data were taken using protoplasts in the central volume and distant from the influence of other cells.

At the strengths of uniform field available in these large chambers, no pearl chains were observed in rotating or linear fields. High suspension densities gave rise (in the rotating field) to planar clusters of cells, the whole cluster rotating in the same direction as but more slowly than the component cells.

The measurements presented here are based on protoplasts studied within 5 h of preparation. Protoplasts stored at 4 °C for 24 h usually show lysis of some of the population, but the remaining whole cells and those few vacuoles liberated intact still rotate. Lysis debris did not show rotation.

Discussion

Holzapfel *et al.* [5] showed that rotation can be predicted for two or more cells in a linear field by interaction between the field and a field-induced dipole the direction of which varies due to a symmetrical interaction with a near-by cell. (This effect only operates in the region of a dispersion in the dipole inducing mechanism.) In the case of a rotating field with such a frequency, the phase lag of the induced dipole is the direct cause of a continuous torque. The induced dipole vector and the field vector must be multiplied together to give the torque, so rotation speed can be expected to vary with the square of the field, as indeed found (Fig. 5). This was also found in the multi-cell system of Holzapfel *et al.* [5], but contradicts the report by Mischel and Lamprecht [7], and the analysis of the ambipolar charging model by Pohl [8], both of which suggest a linear dependence. The quantitative treatment of single cell rotation is presented in Appendix B.

The direction of rotation of the cell will depend on that of the field, whilst in the multi-cell theory

the direction depends on the orientation (with respect to the field) of the line connecting the cell centres.

The rotation of protoplasts and vacuoles, but not of lysis fragments with broken membranes, indicates that a closed membrane is required, and that the dielectric properties of the materials alone are not sufficient for such rotation.

A closed (*i.e.* insulating) membrane system in relatively conductive media will become charged in an electric field, depending on the time constants of the system as discussed below.

The quantitative considerations of such an interfacial (Maxwell-Wagner) polarization will be shown to agree with the experimental data. It is therefore likely that charging of the membrane is the only mechanism operating and that other dielectric effects in biological materials [8–10] are not significant in the frequency range used here.

The electrical time constant of the charging process of the membrane of a spherical cell of radius R (cm) having specific membrane capacitance C_m (F cm⁻²) is given by [9, 11]:

$$\tau = RC_m(\varrho_i + \frac{1}{2}\varrho_a) \quad (1)$$

where τ is the time constant and ϱ_i , ϱ_a the internal and external resistivities, respectively (in Ω cm). The membrane resistance is assumed to be very high.

If only solutions of resistivity high compared to the cell interior are used, then the internal term ϱ_i can be neglected. That is:

$$\tau = \frac{1}{2} RC_m \varrho_a \quad (2)$$

Replacing τ by $1/2\pi f$ and ϱ_a by $1/\sigma_a$, where σ_a is the external conductivity (Ω^{-1} cm⁻¹) then:

$$f = \sigma_a / \pi RC_m \quad (3)$$

i.e. the Maxwell-Wagner dispersion frequency will be directly proportional to the medium conductivity.

The plot of f against σ_a (Fig. 3) shows considerable scatter, partly due to geometric variations between individual cells. When the radius of each cell is included by plotting the $f \cdot R$ product against σ_a the scatter is much reduced (Fig. 4).

Re-arrangement of Eqn. (3) gives:

$$f \cdot R = \sigma_a / \pi C_m \quad (4)$$

showing that the gradient of such a plot yields the membrane capacitance.

Figs. (3) and (4) have logarithmic axes in order to better display the wide range over which results were taken. The appropriate slope was taken from a linear plot of the same data as Fig. 4 (not shown). This was a least-squares fit to the data and yielded a mean value of:

$$C_m = 0.48 \pm 0.07 \mu\text{F cm}^{-2}.$$

This value includes the standard deviation of the slope of the fitted line, and allowance for systematic errors in the calibrated frequency generator ($\pm 0.1\%$), the radius measurement ($\pm 5\%$) and the conductivity measurement ($\pm 5\%$).

If the plasmalemma and tonoplast are assumed to have similar individual capacitances and be approximately series-connected (the vacuole occupies at least 70% of the protoplast) the value for each membrane becomes $1 \mu\text{F cm}^{-2}$ approximately.

This is the value accepted as typical for most biological membranes [9, 10, 12].

The linear dependence on conductivity and close agreement with values calculated from other data provide very strong evidence that the characteristic frequencies necessary for rotation result from the electrical charging of the membrane through the solution resistance. The total scatter ($\pm 40\%$) of the $f \cdot R$ product for any given conductivity is greater than can arise from known sources of error, the greatest of which is in finding the characteristic frequency ($\pm 20\%$). It is believed that the remainder results from intrinsic variability in the protoplasts, possibly caused by membrane structure, cell morphology etc.

Eqn. (1) predicts that the membrane time constant should also depend on the internal conductivity, which cannot easily be changed in protoplasts. However, recent results, using a linear alternating field on vesicles in which the lipids had been polymerized [13] show that increase in the internal conductivity leads to an increase in characteristic frequency as expected.

The occurrence of rotation of isolated cells as much as 4 mm from parallel plane electrodes indicates that uniform field effects are responsible. Although Zimmermann and coworkers [4, 5] observed rotation everywhere between the electrodes in the chamber they used, the adjacent cell or cells necessary for rotation will have made the local field non-uniform. This applies even in the centre, where the field is relatively uniform, and such intra-cell

effects also make it difficult to calculate the effective field strength in multi-cell rotation.

The field strength required for single-cell rotation is approximately six times less than that for multi-cell rotation. If allowance is made for the non-uniformity due to other cells this factor is somewhat higher still. The reason for this higher sensitivity is not known in detail, although the single cell rotation results entirely from a direct interaction between induced dipole and field, whereas the multi-cell rotation also depends on the interaction between two such induced dipoles. It is also possible that the effect of a rotating field on the membrane is different seen at the molecular level.

It is known that steady d.c. fields can lead to aggregation of membrane proteins [14, 15]. Alternating fields may also lead to an aggregation of proteins and formation of particle-free lipid domains [16, 17], which conclusion was reached following the observation of "field stability". This is the resistance of cells (initially exposed to alternating fields for long times) to field pulses which would normally be of damagingly high intensity or long duration [16, 17]. Such resistant cells show no apparent deterioration after pulses that would have caused irreversible breakdown in other cells.

A rotating field has an effect averaged over a much wider region around the perimeter of the cell and so should not give aggregation of intramembranous particles. Cell fusion research [16, 17] can therefore benefit from the use of this technique.

Conclusion

The rotation reported here can be explained in terms of an induced dipole. This supports the theory of Holzapfel *et al.* [5] and makes it unnecessary to call upon hypothetical new effects such as radio frequency generation by the cell itself, such as postulated by Pohl [18, 19].

The dependence of the phenomenon upon a polarisation dispersion will enable the technique to be used to quantify the underlying physical parameters (*e.g.* membrane capacitance, ionic shell and double layer relaxation times, dielectric dispersions etc.). The effect of membrane probes can also be investigated.

The use of the technique described here will simplify and improve the accuracy of cellular spin experiments in several ways. For example, the use

of a uniform field removes the complication of field strength calculations necessary for the non-uniform geometries analysed by Lafon and Pohl [20]. Secondly, a cell in the centre of these large chambers is far removed from the electrical or mechanical disturbance created by proximity to an electrode.

Finally we note that in multi-cell rotation the rotation velocity depends on the orientation of the two cells relative to the field, the distance between them (and possibly on their contact), and on both their radii. In single cell rotation, there is only one radius to consider.

The lack of complicating factors will make this method useful for determining the effects of variables such as viscosity and temperature on rotation speed.

It is also conceivable that cell rotation can be used for detecting interactions between the membrane and very low concentrations of ecotoxicological substances.

Acknowledgement

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Appendix A

Description of a rotating field

When two sinusoidally varying linear electrical fields differing in phase by 90° are superimposed, a field, E , can be considered to result (Fig. 6.)

If both fields have peak amplitude E_0 (V cm^{-1}) and frequency f (Hz), then

$$E_y = E_0 \cos \omega t$$

and

$$E_x = E_0 \cos(\omega t - 90^\circ) = E_0 \sin \omega t$$

where

$$\omega = 2\pi f.$$

The amplitude of the resultant is given by:

$$E^2 = E_0^2 (\cos^2 \omega t + \sin^2 \omega t).$$

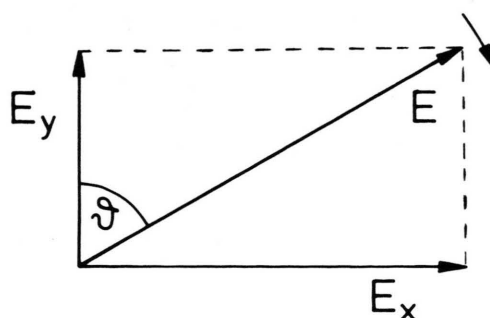


Fig. 6. The superposition of two orthogonal linear field vectors and the resultant field, E , at a given instant in time. θ is the angle of E with respect to the y -vector.

Therefore

$$E = E_0 \quad (\text{A1})$$

(independent of t).

The angle θ in Fig. 6 is given by:

$$\tan \theta = \frac{E_0 \sin \omega t}{E_0 \cos \omega t} = \tan \omega t.$$

Therefore

$$\theta = \omega t. \quad (\text{A2})$$

The resultant field rotates f times per second but has constant instantaneous amplitude E_0 , which is the peak value of the component fields. The increase in average amplitude is due to the addition of two fields.

The superimposed fields can be produced in a chamber such as shown in Fig. 1 driven by four voltages (two electrodes per field in a balanced system) with progressive 90° phase differences.

In theory three, five, or more electrodes can be used; the production of the necessary phase shifts over a wide frequency range would require greater electronic complexity.

Appendix B

The frequency dependence of the torque

Eqn. (C 7) of Holzapfel *et al.* [5] states that a linear alternating field:

$$E_y = E_0 \cos \omega t$$

will induce a linear dipole:

$$M_y = \frac{G}{1 + (\omega \tau)^2} (\cos \omega t + \omega \tau \sin \omega t) \quad (\text{B1})$$

where $G = -2\pi\epsilon_0 a^3 E_0$ and the original vector notation has been adapted to the axes in Fig. 6.

The rotating field results from superimposing a second linear field at right angles. This field E_x is already defined as phase delayed by 90° , and so induces a dipole

$$M_x = \frac{G}{1 + (\omega\tau)^2} (\sin \omega t - \omega\tau \cos \omega t). \quad (B2)$$

The amplitude of the resultant is given by:

$$\begin{aligned} M &= (M_y^2 + M_x^2)^{1/2} \\ &= \frac{G}{1 + (\omega\tau)^2} [\sin^2 \omega t + \cos^2 \omega t \\ &\quad + (\omega\tau)^2 (\sin^2 \omega t + \cos^2 \omega t)]^{1/2}. \end{aligned}$$

Therefore

$$M = G [1 + (\omega\tau)^2]^{-1/2}. \quad (B3)$$

To calculate the torque, it is necessary to know the angle φ this instantaneous dipole makes with the instantaneous field (Fig. 7).

If the angle α describes the instantaneous position of the dipole vector with respect to the axes previously used, then:

$$\begin{aligned} \sin \varphi &= \sin (\vartheta - \alpha) \\ &= \sin \vartheta \cos \alpha - \cos \vartheta \sin \alpha \\ &= \frac{M_y}{M} \sin \vartheta - \frac{M_x}{M} \cos \vartheta. \end{aligned}$$

Use of Eqns. (B1), (B2), (B3) gives:

$$\begin{aligned} \sin \varphi &= [1 + (\omega\tau)^2]^{-1/2} (\omega\tau \sin^2 \vartheta + \omega\tau \cos^2 \vartheta) \\ &= \omega\tau [1 + (\omega\tau)^2]^{-1/2}. \end{aligned} \quad (B4)$$

From (B3) and (B4) we conclude that, for constant ω , the induced dipole is of constant amplitude and rotates with a constant phase difference behind the field.

The torque exerted on the dipole M by the field at angle φ is

$$N = E \cdot M \sin \varphi.$$

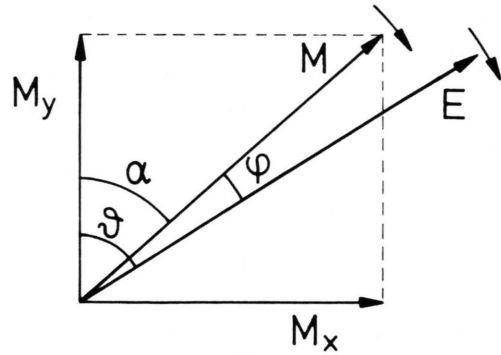


Fig. 7. The superposition of two orthogonal linear dipoles such as result from dielectric polarization involving the component fields of Fig. 6. The phase lag (φ) of the resultant dipole (M) with respect to the resultant field (E) is also shown.

Substitution of Eqns. (B3) and (B4) gives

$$N = E \cdot G \frac{\omega\tau}{1 + (\omega\tau)^2}. \quad (B5)$$

Hence, by differentiation, maximum torque occurs at

$$\omega\tau = 1.$$

Also, if $\omega\tau$ is very large or small, the torque vanishes.

The term G contains a factor in E , so the torque is proportional to E squared.

These last three findings are in common with the analysis by Holzapfel *et al.* [5] of the multi-cell rotation.

The form of (B5) shows identical dependence on $\omega\tau$ with the Debye-equation for the magnitude of the loss factor due to a dielectric dispersion [10].

This gives insight into the source of energy that may maintain rotation and justifies the use of the term "characteristic frequency" for the field frequency giving maximum rotation. (This term is normally used for the central frequency, where the loss is maximised, of a dielectric dispersion).

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