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ANALYSIS OF LINEARLY POLARIZED FLUORESCENCE OF CHLOROPLASTS ORIENTED IN POLYACRYLAMIDE GEL

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Linearly polarized fluorescence was measured in mesophyll chloroplasts of maize aligned in polyacrylamide gel. The dependence of fluorescence polarization on the squeezing parameter of the gel sample was studied at room temperature. Polarized fluorescence emission and polarization ratio spectra were recorded at low temperature (−140°C). A theoretical model is proposed for a quantitative description of the polarized fluorescence emission and its dependence on the squeezing parameter. It is shown that chloroplasts are aligned as disc-shaped particles with no detectable deformation of their ultrastructure. The fluorescence emission and the polarization ratio spectra as well as the calculated orientation angles between the transition dipoles and the membrane plane were similar to those obtained with magnetically oriented chloroplasts.

Introduction

One of the basic problems concerning photosynthetic conversion of solar energy deals with the organization of pigment molecules which participate in the energy-transfer and charge-separation processes. A traditional approach used to study the organization of chlorophyll in photosynthetic membranes is associated with polarized light spectroscopy of oriented chloroplasts and subchloroplast preparations. Linear dichroism and polarized fluorescence measurements were applied to determined the orientation of the absorbing and emitting dipoles with respect to the thylakoid membrane plane [1–8].

The alignment of chloroplasts can be achieved by various techniques such as orientation in an external magnetic field [2], spreading and drying of a chloroplast suspension on a glass slide [3] and orientation in flow gradients [9]. The former technique is based upon diamagnetic properties of biomembranes [10,11] while the latter two make use of the asymmetry of the shape of chloroplasts. We used orientation of chloroplasts in polyacrylamide gel which is also based on asymmetry of shape. This technique which was invented in 1978 [12,13] has several advantages: it preserves an aqueous microenvironment for the objects under study and the parameters of squeezing can be controlled and monitored. Thus, it is possible to calculate various distribution functions which are related to statistics of orientation. These statistics are necessary to estimate the angles made by transition dipoles with the membrane plane or with the principal axes of the oriented particles.

Orientation in polyacrylamide gel has been applied in recent studies of reaction centers, pigment-protein complexes and membranes from various photosynthetic organisms [12–16]. Theoretical models have been elaborated to analyse and explain these observations [17–19]. Here we report new results on polarized fluorescence of oriented chloroplasts and propose a theoretical background for them.

Materials and Methods

Mesophyll chloroplasts were isolated from maize leaves as previously described [8] and resuspended in buffer solution containing 0.35 M sucrose, 1 mM $MgCl_2$, 10 mM NaCl, and 1 mM $MnCl_2$. The suspension of chloroplasts was then mixed with the components of the polyacrylamide gel before polymerization (final concentrations: acrylamide, 12%; *N,N'*-methylenebisacrylamide, 0.24%; glycerol, 50%) [12,13]. The samples were polymerized at temperatures not exceeding 40°C in spectrophotometric cuvettes (1 × 1 cm) and squeezed in specially designed cuvettes made of plexiglass. The dimensions of a rectangular sample before squeezing, L_{xo}, L_{yo} and L_{zo}, and those after sqeezing, L_x, L_y and L_z, are related by the equations:

$$L_x = L_{xo}\sqrt{m}\,; \quad L_y = L_{yo}\sqrt{m}\,; \quad L_z = \frac{L_{zo}}{m}\,; \quad m > 1 \qquad (1)$$

where m is the squeezing parameter. We used several cuvettes with different fixed values of m: m = 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 and 2.5. Homogeneity of the squeezed samples was tested visually using the colour pattern observed with the sample placed between two crossed polaroids. With the concentrations mentioned above, the samples were homogenous if m did not exceed 2.0.

The fluorescence of squeezed samples was studied in the set-up described earlier [7]. The geometry of the measurements is sketched in Fig. 1. Fluorescence was excited by the blue spectral lines of an HBO 200 high-pressure mercury arc lamp; the excitation beam which was filtered by a $CuSO_4$ solution was non-polarized. The fluorescence was passed through a polaroid filter and vertically (V) or horizontally (H) polarized light was transmitted. The intensities I_V and I_H were recorded at each wavelength of emission to obtain the fluorescence polarization ratio (FP) spectra:

$$FP = \frac{I_V}{I_H} \qquad (2)$$

and at each value of m to obtain the dependence of the fluorescence polarization ratio on m. A given sample was first put into a cuvette with m = 1.0, and the polarization ratio was recorded at room temperature to ensure that FP = 1.00. Then the sample was successively squeezed in cuvettes with m = 1.2, 1.4, 1.6, 1.8, 2.0 and 2.5. The dependence of the polarization ratio on the squeezing parameter, m, was determined at room temperature at 685 nm. The fluorescence polarization ratio spectra were determined at − 140°C.

Results

Typical low-temperature fluorescence and fluorescence polarization ratio (FP) spectra are shown in Fig. 1 for the squeezing parameter m = 2.0. The spectra resemble those reported by various authors and the polarization ratio values are as high as those measured in magnetically oriented chloroplasts (e.g., see Refs. 4 and 6). It follows that no appreciable deterioration in the molecular archi-

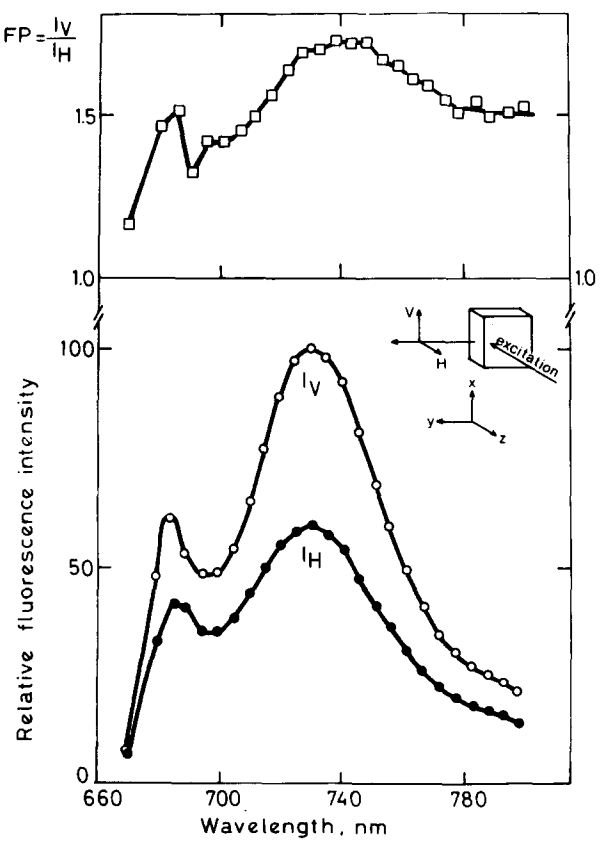

Fig. 1. Polarized fluorescence spectra I_V and I_H and fluorescence polarization ratio (FP) spectra of mesophyll chloroplasts of maize in squeezed gel at − 140°C, m = 2.0. Geometry of measurement is shown in inset.

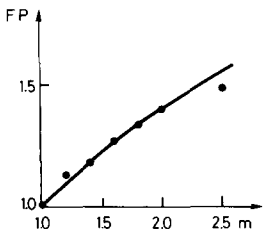

Fig. 2. The fluorescence polarization ratio measured at 685 nm at room temperature as a function of the squeezing parameter, m; ●, measured values; ———, curve obtained from theoretical calculation with $k = 0.245$, $\delta = 70°$.

tecture of chloroplasts in the gel could be detected by fluorescence, and that the chloroplasts were very well oriented by this technique.

The dependence of the fluorescence polarization ratio on the squeezing parameter, FP (m), is depicted in Fig. 2. It shows that FP = 1.0 in non-squeezed samples and that it increases monotonically upon gradual squeezing of the samples.

Theory

Alignment of chloroplasts by squeezing the gel

The chloroplasts can be considered as disc-shaped (oblate) particles, with the axial symmetry of the ellipsoid of revolution possessing the axes b and c (Fig. 3A).

In the following we use the degree of polarization, P, which is related to the fluorescence polarization ratio (FP) by:

$$P = \frac{1 - \mathrm{FP}}{1 + \mathrm{FP}} \tag{3}$$

The degree of polarization, as derived in the Appendix, is given by:

$$P(\delta, m) = \frac{(3\cos^2\delta - 1)\,(3\,T(m) - 1)}{3 - \cos^2\delta + T(m)\,(3\cos^2\delta - 1)} \tag{4}$$

where δ is the angle between a transition dipole and the normal to the membrane plane (Fig. 3) and $T(m)$ the distribution function derived by Kuhn and Grün [20]. Its applicability has been analyzed recently [18,19]. The curve $P(\delta)$ is shown in Fig. 4A for $m = 2.0$ where $T(m) = 0.62$. The range of experimental values, $-0.27 \leq P \leq -0.08$,

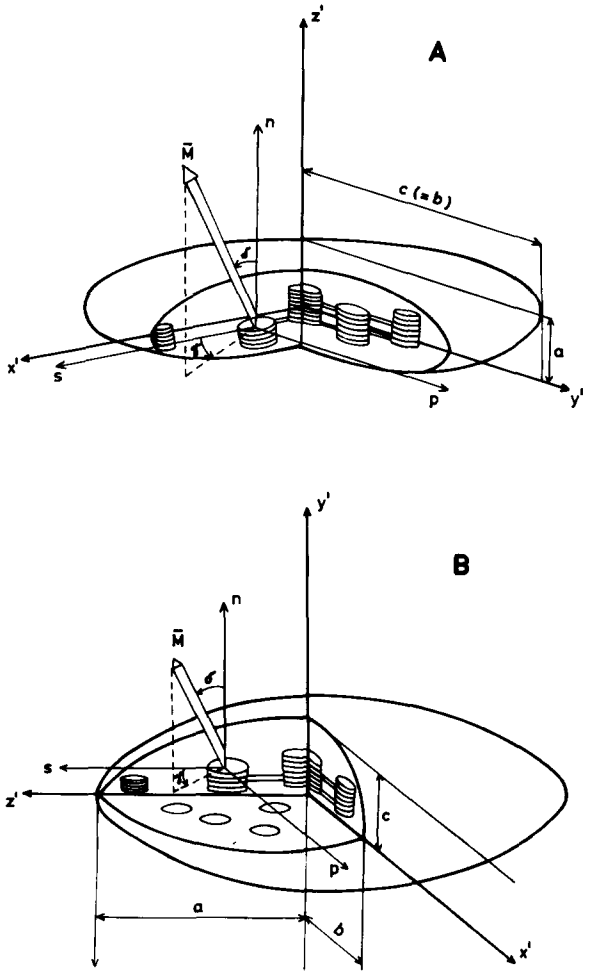

Fig. 3. Disc-shaped (A) and rod-shaped (B) model of chloroplasts with idealized membrane pattern showing the orientation of an $\bar{M}$ transition dipole at the δ angle with respect to the membrane normal.

is completely covered by the range of calculated values, $-0.36 \leq P \leq 0.53$, hence all the experimental results (Fig. 1) can be explained by this orientation model. In order to test our model, calculations were carried out also with the false assumption that the chloroplasts were rod-shaped (oblong) particles with the axial symmetry of ellipsoids of revolution with axes $a > b = c$ (Fig. 3B). The same idealized internal membrane structure was used also in these calculations which were used in the model of disc-shaped chloroplasts. For the model of rod-shaped chloroplasts, P can be

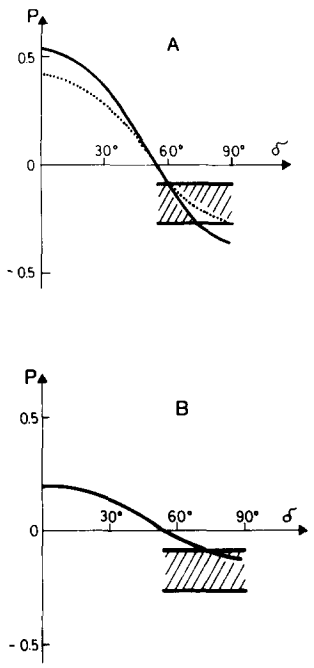

Fig. 4. Degree of polarization $P(\delta, m = 2.0)$ as a function of angle δ between a transition and the normal to the thylakoid membrane plane in disc-shaped (A) and rod-shaped (B) chloroplasts. Dashed area covers P values determined from our experiments. Dotted curve in A represents P_k ($\delta, m = 2.0$, $k = 0.245$).

expressed as:

$$P(\delta,m) = \frac{(1-3L(m))(3\cos^2\delta - 1)}{5 - \cos^2\delta + L(m)(3\cos^2\delta - 1)} \qquad (5)$$

where $L(m)$ is the distribution function characterizing the degree of orientation upon squeezing [18,19]. The curve $P(\delta)$ is depicted in Fig. 4B for $m = 2.0$ where $L(m) = 0.117$. The highest measured FP = 1.72 corresponds to $P = -0.27$. Neither $P(\delta, m = 2.0)$ nor its limiting value $P(\delta, m \rightarrow \infty)$ can explain the observed results. To be specific, no angle corresponds to $P = -0.27$ in this model, since minimal P values are -0.13 at $m = 2.0$ and -0.20 at $m \rightarrow \infty$. Thus, the model of rod-shaped particles, in accordance with the expectations, is unsatisfactory in the case of chloroplasts oriented in polyacrylamide gel. Earlier we have shown that orientation of disc-shaped and rod-shaped macromolecules can be distinguished with the gel-squeezing technique [18,19], and here a similar conclusion is made concerning chloroplasts, cellular organelles possessing a complex membranous structure.

Estimation on the orientation angles of dipoles

Evidently, the thylakoid membranes of chloroplasts do not obey the above idealized arrangement of membranes. Instead of attempting to derive formulae for the $P(m,\delta)$ of disc-shaped chloroplasts with a realistic thylakoid arrangment and geometry let us introduce a parameter, k, characterizing the 'deviation' of the chloroplast ultrastructure from the idealized membrane pattern. Let us assume that $(1-k)100\%$ of the dipoles are bound to ideally ordered membranes and the rest, $k100\%$, are oriented randomly with respect to chloroplast axes. Concerning this arbitrarily introduced parameter characterizing the extent of 'disorder' (e.g., curvature of membranes, marginal parts) of thylakoids the following questions are of interest: (i) what is the highest k value (k_{max}) for the given experimental conditions which is compatible with the results of measurements; (ii) what δ angles can be estimated for a given dipole and a known value of the polarization ratio with different k values between 0 and k_{max}; (iii) is the k value constant under study or does it change upon squeezing?

The degree of polarization of the fluorescence must be corrected for the k disorder parameter. Obviously, $|P_k|$ is lower than $|P|$ for the same orientation angle and squeezing parameter, m (see Appendix). The higher k is, the lower $|P_k|$ should be. Since P_k should sweep all the observed experimental values, the higher boundary for k can be estimated. For the case depicted in Figs. 1 and Fig. 4A, the P_k value is adjusted so that at $m = 2.0$ the extreme $P_k = -0.27$ corresponds to the extreme angle $\delta = 90°$. The k value necessary for this adjustment is $k = 0.245$. It means that if the ultrastructural disorder is accounted for by about 25% of the thylakoids, which are assumed to be aligned at random, not less than 75% of the membranes are perfectly ordered.

The angular dependence of P_k for $k = 0.245$ and $m = 2.0$ is shown in Fig. 4A by the dashed curve. It is clear that the limiting values for angles δ can be found from the two curves $P(\delta,m)$ and

$P_k(\delta,m)$, since for a given fluorescence polarization ratio observed in the experiments, the lowest δ corresponds to $P(\delta,m)$ and the highest δ is found from the $P_k(\delta,m)$ dependence adjusted as described above.

Exact values of k cannot be determined from our measurements. Taking into consideration the electron-microscopic ultrastructure of chloroplasts, however, it is clear that values of the disorder parameter which are closer to 0.25 are more realistic than those close to 0. With $k = 0.245$, 0.2 and 0.15 the orientation angles of the F-735 dipole with respect to the membrane plane can be calculated as 0, 8 and 11° and those of F-685 are obtained as 16, 18 and 19°, respectively, while around 675 nm (FP ≈ 1.2) these angles are about 28°. (These values are not corrected for the overlap of bands of differently oriented emitting dipoles.)

From a comparison of these estimates with earlier analyses yielding similar results in magnetically oriented chloroplasts [8,21], it can be concluded that the above simple calculation using the squeezing and disorder parameters results in good estimates for the different absorbing and emitting dipoles in chloroplast thylakoids.

If the chloroplasts are deformed when aligned by squeezing of the gel one expects k to be a function of m. If they are not deformed one has to be able to fit FP(m) determined experimentally at a definite wavelength (δ = constant) with the assumption that k = constant.

Using the relationships, Eqns. 3, A9, A12 and A13, which can be applied to obtain k values from FP(m) (Fig. 2) and with distinct values of δ we calculated $k(m)$. It was found that with $m \leq 2.0$, k did not depend on the squeezing parameter. (At $m > 2$ homogeneity of the samples could not be attained. Thus, the relative increase in the calculated values of k at $m > 2$, does not necessarily imply an increase in the disorder in chloroplasts.) With $1.4 \leq m \leq 2.0$ with δ orientation angles of F-685 of 72, 70 and 68°, k was obtained in the intervals (0.27,0.32), (0.22,0.25) and (0.12,0.15), respectively. Thus, it can be concluded that no appreciable deformation of the chloroplast ultrastructure occurs upon squeezing the gel sample. An illustration of this conclusion is that the experimental values of FP(m) can be fitted well by the theoretical curve obtained with the assumption that k = constant and δ = constant (The measured FP = 1.40, $m = 2.0$ and $\lambda = 685$ nm, correspond to $\delta = 69.9°$ with $k = 0.245$.)

Conclusions

It was shown that chloroplasts embedded in polyacrylamide gel, upon squeezing of the sample, can be aligned like disc-shaped particles with no appreciable deformation in their ultrastructure. Orientation angles can be estimated from simple calculations by using the squeezing parameter, which can easily be monitored during squeezing, or by introducing an additional parameter characterizing the ultrastructure of chloroplasts. Alignment of chloroplasts in gel can be an efficient and simple tool in revealing the molecular architecture of thylakoids, especially in cases (e.g., in mutants with a poor membrane pattern) where other techniques for alignment of the chloroplasts cannot be efficiently used.

Appendix

In order to calculate fluorescence polarization, it is necessary to find the projections of a given transition dipole vector $\overline{M}$ onto the laboratory-fixed axes x and z (Fig. 1).

The membrane-fixed coordinates $\overline{M}, |\overline{M}| = 1$, can be written as:

$$M_n = \cos\delta$$

$$M_s = \sin\delta\cos\gamma$$

$$M_p = \sin\delta\sin\gamma \qquad \text{(A1)}$$

In rod-shaped and disc-shaped chloroplasts (Fig. 3) the following axes are parallel:

$$P \| x',\ n \| y',\ s \| z' \qquad \text{(A2)}$$

and

$$s \| x',\ p \| y',\ n \| z' \qquad \text{(A3)}$$

respectively.

Orientation of chloroplast-fixed axes x', y' and z' with respect to the laboratory-fixed frame x, y

and z is expressed by Euler's angles φ, ψ, θ (Fig. 5).

The projections in rod-shaped chloroplasts are:

$$M_x = \sin\delta \sin\gamma (\cos\varphi \cos\psi - \sin\varphi \sin\gamma \cos\theta) + \cos\delta (\cos\varphi \sin\psi - \sin\varphi \cos\psi \cos\theta) + \sin\delta \cos\gamma \sin\varphi \sin\theta \quad (A4)$$

$$M_z = \sin\delta \sin\gamma \sin\psi \sin\theta + \cos\delta \cos\psi \sin\theta + \sin\delta \cos\gamma \cos\theta \quad (A5)$$

Corresponding equations for disc-shaped chloroplasts coincide with Eqns. 2 and 3 in the paper of Ganago et al. [18].

Fluorescence emission depends on M^2, and the excitation conditions used here are favourable for effective energy transfer among chlorophylls in a photosynthetic unit. Therefore, we may assume that fluorescence intensities are proportional to $\langle M_x^2 \rangle$ and $\langle M_z^2 \rangle$, where the brackets indicate averaging over all possible values of the angles γ, φ ψ and θ. (We note that the same formulae are valid for absorption of linearly polarized light.) The δ angle shows to what extent the transition dipole is tilted with respect to the membrane normal. The γ angle describes free rotation of a pigment-protein complex within the membrane; the ψ angle describes free rotation of a chloroplast around its axis $a \| z'$ which we proposed to be the axis of symmetry. The term 'rotation' here means that these objects may be found in various positions described by the corresponding angles, and

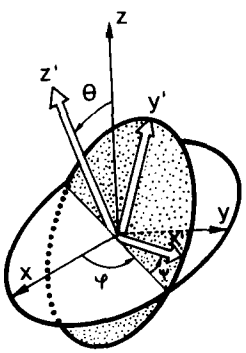

Fig. 5. Relation between chloroplast-fixed axes x', y' and z' and the axes x, y and z of a laboratory-fixed frame as given by Euler's angles θ, φ and ψ.

no values of these angles are preferred or can be fixed in the experimental conditions. Then, φ is related to different orientations of chloroplasts within a gel sample with respect to its z-axis. We have proposed (see Eqn. 1) that z is the axis of symmetry of a sample. The averaging must be done over the angles γ, ψ and φ. The θ angle shows how the symmetry axes of chloroplasts, z', are tilted with respect to the symmetry axis of the sample, z, and how this tilt is changed due to squeezing of the gel. It has recently been shown [18,19] that under the deformation used here, the integration over θ yields substitutions of $\cos^2\theta$ with distribution functions $L(m)$ and $T(m)$ for rod-shaped and disc-shaped particles, respectively. Explicitly, these functions are given by:

$$L(m) = \frac{1}{m^3 - 1}\left\{-1 + \sqrt{\frac{m^3}{m^3 - 1}}\,\log\left(\sqrt{m^3} + \sqrt{m^3 - 1}\right)\right\} \quad (A6)$$

$$T(m) = \frac{m^3}{m^3 - 1}\left(1 - \frac{\arctan\sqrt{m^3 - 1}}{\sqrt{m^3 - 1}}\right) \quad (A7)$$

$L(m)$ equals $1/3$ at $m = 1$ and decreases to zero if $m \to \infty$. $T(m)$ equals $1/3$ at $m = 1$ and increases to 1.00 when $m \to \infty$.

Combination of Eqns. A5 and A6 leads to:

$$\langle M_z^2 \rangle = \tfrac{1}{4}\{1 + \cos^2\delta + L(m)(1 - 3\cos^2\delta)\} \quad (A8)$$

for rod-shaped chloroplasts, and the corresponding calculations for disc-shaped chloroplasts yield:

$$\langle M_z^2 \rangle = \tfrac{1}{2}\{1 - \cos^2\delta - T(m)(1 - 3\cos^2\delta)\} \quad (A9)$$

In both cases, $\langle M_x^2 \rangle$ can be found from the relation

$$\langle M_z^2 \rangle + 2\langle M_x^2 \rangle = 1 \quad (A10)$$

which holds due to symmetry of squeezing (Eqn. 1).

The degree of polarization is given by:

$$P = \frac{\langle M_z^2 \rangle - \langle M_x^2 \rangle}{\langle M_z^2 \rangle + \langle M_x^2 \rangle} \quad (A11)$$

if the spectral bands do not overlap. Eqns. A9 and A11 yield Eqn. 4, while Eqns. A8 and A11 lead to Eqn. 5. From Eqns. A8 and A9 it is evident that the results obtained for disc-shaped and rod-shaped chloroplasts differ not only due to distribution functions $T(m)$ and $L(m)$ but also due to a difference in symmetry. Thylakoid membranes are imagined to be parallel to the axis of symmetry in rod-shaped chloroplasts and perpendicular to the axis of symmetry in disc-shaped chloroplasts. The difference is significant, since the normal to the membrane plane is believed to be the axis of symmetry for the membrane. On the other hand, if in rod-shaped chloroplasts the membrane planes are parallel to their a-axis but not parallel to each other, the projections $\langle M_z^2 \rangle$ and $\langle M_x^2 \rangle$ are still given by Eqns. A8 and A10, since the same symmetry is preserved on average.

The ultrastructure of the chloroplasts was taken into consideration with the k disorder parameter. Since the averaged projections of the dipoles at random are equal to $1/3$, it can be written that:

$$\langle M_z^2 \rangle_k = \langle M_z^2 \rangle (1-k) + \tfrac{1}{3}k \qquad \text{(A12)}$$

and

$$P_k = \frac{\langle M_z^2 \rangle - \langle M_x^2 \rangle}{\langle M_z^2 \rangle + \langle M_x^2 \rangle - \frac{2}{3}\frac{k}{1-k}} \qquad \text{(A13)}$$

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