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RETICULOCYTE LIPOXYGENASE CHANGES THE PASSIVE ELECTRICAL PROPERTIES OF BOVINE HEART SUBMITOCHONDRIAL PARTICLES *

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Purified reticulocyte lipoxygenase oxygenates the polyunsaturated phospholipids of sonified submitochondrial particles from bovine heart as measured by a burst of oxygen uptake. Over the frequency range of 0.5 to 100 MHz, the complex impedance of the submitochondrial particles as a function of the frequency before and after lipoxygenase attack was measured. From these data, the membrane capacity, the conductivity of the membrane and the conductivity inside the particles were calculated. Lipoxygenase action causes a 4-fold increase in the membrane capacity and a 2-fold increase in the membrane conductivity. Using the method of deformation of electric pulses, kinetic measurements were performed. In parallel to the changes of the passive electric properties, a partial inhibition of NADH oxidase and succinate oxidase was caused by the lipoxygenase attack. Oxygen uptake, changes of the passive electric properties and the inhibition of respiratory enzymes were prevented by lipoxygenase inhibitors. Owing to the high oxygen consumption produced by the lipoxygenase reaction, anaerobiosis was reached within the first 30 s in the closed chamber. Therefore, it must be concluded that the changes in passive electric properties and the inhibition of the respiratory enzymes are due to secondary anaerobic processes such as the hydroperoxidase reaction catalyzed by the lipoxygenase or a slow redistribution of peroxidized membrane lipids. The results are discussed in relation to the breakdown of mitochondria during the maturation process of red cells.

Introduction

Peroxidation of membrane-bound lipids has been shown to be a deteriorative process for cells [1,2] and subcellular organelles such as mitochondria [3], microsomes [4] and lysosomes [5]. Loss of membrane integrity and inactivation of membrane-bound enzymes are caused by lipid peroxidation [3,4]. However, the molecular mechanism of the membrane damage has not been well understood.

During the maturation process of red blood cells, which is characterized by the disappearance of mitochondria and ribosomes, there is synthesized a lipoxygenase [6] that is able to oxygenate unsaturated lipids [7]. It has been shown earlier that the attack of this lipoxygenase on isolated mitochondria and submitochondrial particles in vitro leads to damage of their structure and function [8,9]. Moreover, the oxygenated mitochondrial membranes become more susceptible to an ATP-dependent proteolysis system present in the re-

* Dedicated to Professor S.M. Rapoport (Berlin, G.D.R.) on the occasion of his 70th birthday.

ticulocyte [10]. Therefore, the reticulocyte lipoxygenase is closely related to the breakdown of mitochondria during the maturation of red blood cells [11,33].

The drastic effects of the lipoxygenase reaction on mitochondrial membranes should be accompanied by a change in the physicochemical properties of the membranes such as membrane fluidity, surface charge and ion permeability, as has been shown for the lipid peroxidation induced by ferrous ascorbate or ultraviolet irradiation [12]. Until now, nothing has been known about the influence of the lipoxygenase reaction on the passive electric properties of biological membranes. In this report, it is shown that the action of reticulocyte lipoxygenase on submitochondrial particles causes a change in passive electric membrane properties that parallels the inhibition of respiratory enzymes.

Materials and Methods

Reticulocyte lipoxygenase was purified to electrophoretic homogeneity as described elsewhere [14]. Bovine-heart submitochondrial particles were prepared according to Crane et al. [15], with the exception that the disintegration of the mitochondria was performed by sonification (tip-type sonifier Branson B 12; microtip). Lipoxygenase activity was measured photometrically according to Holman [16] as the oxygenation rate with linoleic acid as substrate [11] using a molar extinction coefficient for hydroperoxylinoleic acid of 25000 $M^{-1} \cdot cm^{-1}$ [17].

For the measurement of the electric parameters, the submitochondrial particles suspended in 0.25 M buffered sucrose (10 mM potassium phosphate, pH 7.4) were placed in a Teflon chamber (chamber constant $z = 10\ m^{-1}$) with one circular platinum electrode on the bottom and another on the top. The distance between the electrodes was 5 mm. The module, $|\Re|$, of the electric impedance and the phase angle φ were measured as a function of the frequency in the region from 0.5 to 100 MHz by means of a Tesla impedance meter BM 508. From the values measured, the electric conductivity of the membranes ($\varkappa_s$), the geometric capacity of the membranes (C_s), the conductivity inside the particles ($\varkappa_i$) and the parameter α as a measure of the size distribution of the particles were calculated according to the theory of Pauli and Schwan [13].

For the kinetic measurements of the changes of passive electric properties, a rapid method was used which is based on the deformation of a rectangular electric pulse [18]. A pulse wave which passes a suspension of particles is deformed and this deformation can be measured as a voltage difference, ΔV. The kinetic measurements were performed as follows. An electric membrane model consisting of capacitors and resistors was constructed in such a way that its passive electrical properties were the same as those of the suspension of submitochondrial particles; consequently, the submitochondrial particle suspension and the electric membrane model deform the pulse wave in the same manner. No voltage difference, ΔV, can be measured. Changes in the passive electrical properties of the submitochondrial particles during the lipoxygenase reaction are indicated by a voltage difference. $\Delta V = f(t)$ reflects the time-course of $\varkappa_0$, $\varkappa_\infty$ and f_0, provided that these parameters change monotonically and proportionally.

All measurements were carried out at 37°C.

Results

From Fig. 1, it can be seen that the incubation of reticulocyte lipoxygenase with mitochondrial membranes causes a burst of oxygen uptake in the absence of any respiratory substrate. Since this oxygen uptake was completely prevented by addition of 100 μM nordihydroguaiaretic acid which also inhibits the reaction of reticulocyte lipoxygenase with linoleic acid or phospholipids as substrate (not shown), the burst of oxygen uptake can be ascribed to an oxygenation of membrane phospholipids catalyzed by the lipoxygenase. As referred to the protein content of the mitochondrial membranes, the rate of oxygen uptake with sonified submitochondrial particles is twice as high as that with frozen-and-thawed mitochondria (Fig. 1). This difference may be due to the higher content of phospholipids per mg protein in the sonified particles owing to the separation of matrix proteins. It has been shown earlier that the action of reticulocyte lipoxygenase on submitochondrial particles causes an irreversible inhibition of the NADH oxidase and succinate oxidase activities at

specific sites [19,20]. In Fig. 2, the respiratory inhibition is shown under the conditions of the measurements of passive electric properties. As

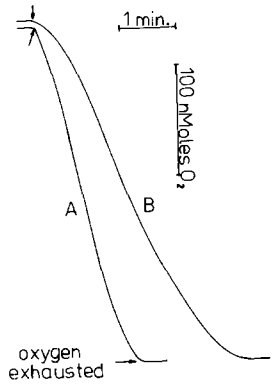

Fig. 1. Oxygen uptake caused by the action of reticulocyte lipoxygenase on beef-heart mitochondria (A) and submitochondrial particles (B). Lipoxygenase (6 nkat) was added as indicated by the arrows to a suspension of frozen and thawed mitochondria (B) (68 mg protein/ml) and sonified submitochondrial (A) particles (70 mg protein/ml). Volume of the reaction chamber was 1.9 ml. Rates of 295 nmol $O_2 \cdot min^{-1}$ (submitochondrial particles) and 150 nmol $O_2 \cdot min^{-1}$ (frozen-and-thawed mitochondria) were calculated.

seen, a partial inhibition of both NADH oxidase and succinate oxidase occurred, with NADH oxidase being more sensitive. Higher amounts of lipoxygenase cause inhibition greater than 90% in both systems (not shown). Prevention of anaerobiosis in the reaction mixture by shaking it frequently led to the same partial inhibition of the respiratory enzymes.

The results of the measurements of the passive electric properties are given in Fig. 3. The characteristic frequency, f_0, and the conductivities, $\varkappa_0$ and $\varkappa_\infty$, obtained from the figure and the passive electric parameters calculated from these data according to Eqns. A1–A3 (see Appendix) are summarized in Table I. It should be mentioned here that the volume concentration of the particles in the suspension, which was determined by high-speed centrifugation to be 0.6, is not changed during the lipoxygenase reaction. The radius of the submitochondrial particles was obtained from electron-micrographs, a selection of which is presented in Fig. 4. The membrane thickness was estimated to be 5 nm [21].

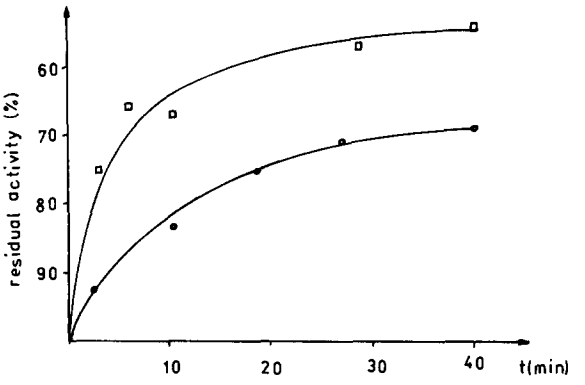

Fig. 2. Inhibition of respiratory enzymes by the action of reticulocyte lipoxygenase on beef-heart submitochondrial particles. 4 ml of the suspension of sonified submitochondrial particles (70 mg protein/ml) was incubated with lipoxygenase (30 nkat/ml) at 37°C. At the times indicated, aliquots were taken off and the activity of respiratory enzymes were measured polarographically in 0.1 M potassium phosphate (pH 7.4) by addition of 3 mM succinate or 0.5 mM NADH. NADH oxidase, □; succinate oxidase, ○.

The capacity of the membranes of sonified submitochondrial particles calculated is in accordance with the value for biological membranes as reported in the literature [22,23]. As can be seen from Table I, the membrane capacity is increased 4-fold during the lipoxygenase reaction. This effect may be explained by an increase in the dielectric

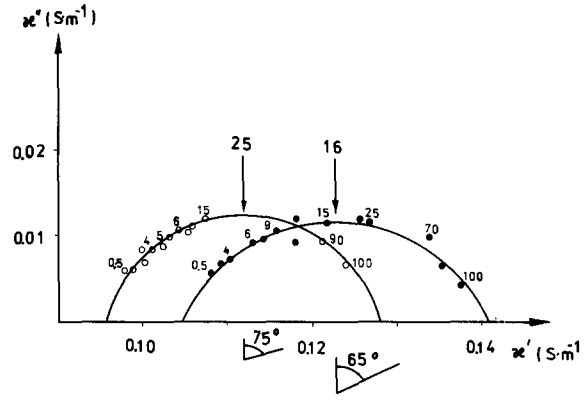

Fig. 3. Dielectric data of sonified submitochondrial particles in the complex admittance plane. 4 ml of a suspension of sonified submitochondrial particles (70 mg protein/ml) were incubated with lipoxygenase (30 nkat/ml) for 45 min at 37°C. Before (○) and after (●) lipoxygenase treatment, the electrical measurements were performed. The numbers on the circle segment show the frequencies at which the measurements were taken. The characteristic frequency is indicated by an arrow.

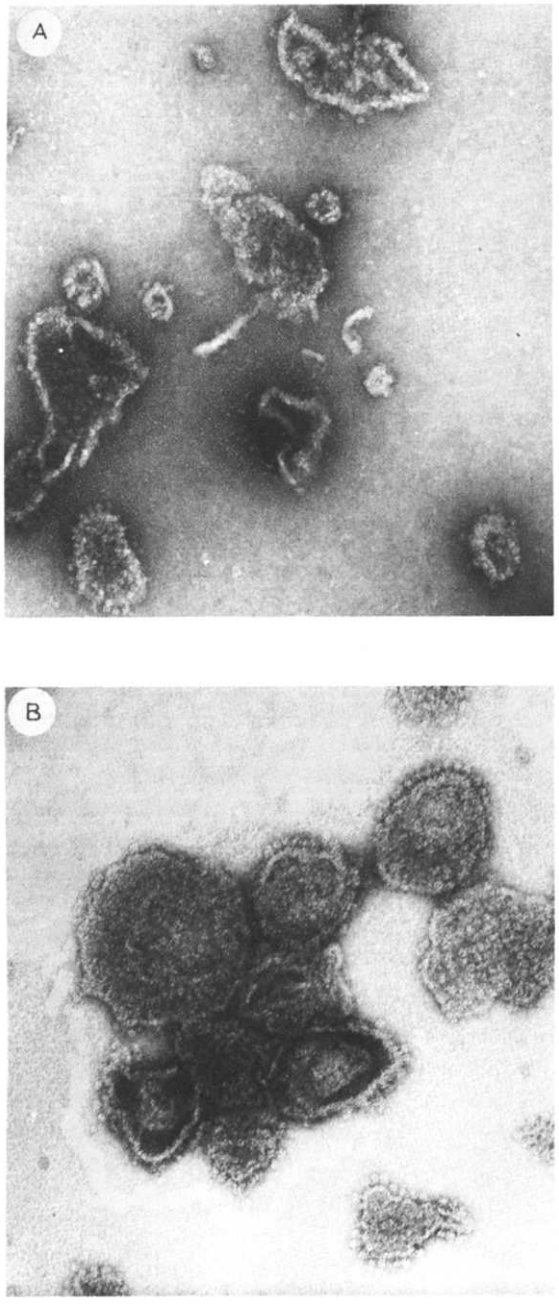

Fig. 4. Electron-micrographs of sonified submitochondrial particles before (A) and after (B) treatment with lipoxygenase. Submitochondrial particles were treated with lipoxygenase as described in the legend to Fig. 2. Before and after treatment, aliquots were submitted to negative staining with phosphotungstenic acid according to Ref. 32. The electron-micrographs were produced by means of an Elmiskop 102 (Siemens AG, Berlin-West; magnification 120000:1) (85200:1 as presented).

TABLE I

PASSIVE ELECTRICAL PARAMETERS OF SONIFIED SUBMITOCHONDRIAL PARTICLES BEFORE AND AFTER LIPOXYGENASE TREATMENT

The experiments were carried out as described in the legend to Fig. 3. f_0, $\varkappa_0$ and $\varkappa_\infty$ were obtained from this figure. The particle diameter, r, was obtained from the electron-micrographs. The conductivity, $\varkappa_a$, of the suspension medium was measured after high-speed centrifugation ($150000 \times g$) as described in Materials and Methods. The passive electric parameters ($\varkappa_s$, $\varkappa_i$, C_s) were calculated from these data according to the Eqns. A1–A3 (Appendix).

Parameter	Before lipoxygenase treatment	After lipoxygenase treatment
r (nm)	60	30
$\varkappa_0$ ($S \cdot m^{-1}$)	0.095	0.105
$\varkappa_\infty$ ($S \cdot m^{-1}$)	0.128	0.140
f_0 (MHz)	25	16
$\varkappa_a$ ($S \cdot m^{-1}$)	0.160	0.167
C_s (10^{-2} $F \cdot m^{-2}$)	2.35	8.57
$\varkappa_s$ (10^{-2} $S \cdot m^{-1}$)	1.03	2.46
$\varkappa_i$ ($S \cdot m^{-1}$)	0.11	0.12
α	0.17	0.28

constant of the membrane caused by the formation of hydrophilic clusters [24] consisting of lipid hydroperoxides or their decomposition products [25]. The formation of such polar regions within the hydrophobic bilayer may also be the reason for the 2-fold increase in the membrane conductivity. Similar changes of the passive electric membrane properties were also obtained using frozen-and-thawed beef-heart mitochondria instead of sonified submitochondrial particles. A 7-fold increase in the membrane conductivity (change from 8 $S \cdot m^{-1}$ to 55 $S \cdot m^{-1}$) and a 5-fold increase in the membrane capacity (change from $0.8 \cdot 10^{-2}$ $F \cdot m^{-2}$ to $4.3 \cdot 10^{-2}$ $F \cdot m^{-2}$) were measured. It is remarkable that the changes in passive electrical properties with frozen-and-thawed mitochondria are greater than those with sonified submitochondrial particles, despite lower rates of oxygen uptake (Fig. 1). The conductivity of the surrounding medium and the conductivity inside the submitochondrial particles do not differ before and after lipoxygenase treatment. The distribution factor, α, of the submitochondrial particles is low, which means that the particles were very similar in

size. The increase in α during the lipoxygenase reaction indicates that the particles become more heterogeneous in size through the attack by the lipoxygenase, since both shrinkage and fusion of the particles occur. This is also evident from the electron-micrographs (Fig. 4).

The kinetics of the lipoxygenase reaction with submitochondrial particles as measured by the change in the passive electric properties are shown in Fig. 5. The shape of the trace is similar to that of the inhibition of respiratory enzymes shown in Fig. 2, but completely different from that of oxygen uptake. Under conditions identical to those for the measurement of the passive electric properties, the rate of oxygen uptake was very high (in the range of the response time of the oxygen electrode). Even in 10-fold-diluted samples, anaerobiosis in the assay vessel was reached after 1 min. The same was observed when the same concentration of membranes (70 mg/ml) but one-tenth of lipoxygenase concentration was applied. Therefore the oxygenation of membrane-bound lipids catalyzed by the lipoxygenase alone cannot be responsible for the changes in the passive electric properties and the respiratory inhibition (see Discussion). The addition of nordihydroguaiaretic acid (final concentration 100 μM), a well-known inhibitor of lipoxygenases [26], prevented completely the changes in passive electric properties.

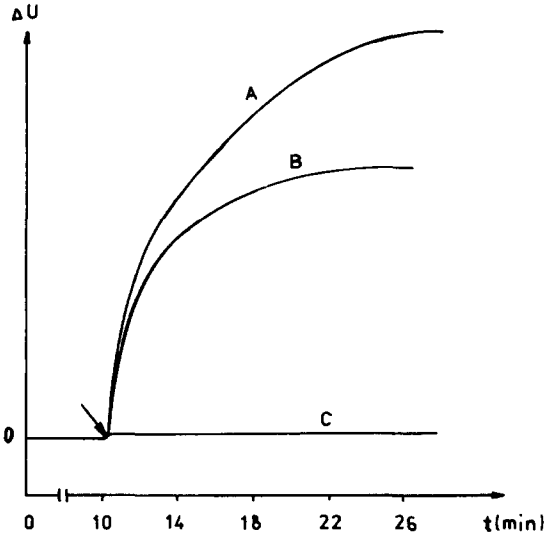

Fig. 5. Kinetics of lipoxygenase reaction with submitochondrial particles measured by means of the method of deformation of rectangular electric pulses. The suspension of submitochondrial particles (70 mg protein/ml) were incubated with lipoxygenase (A, 30 nkat·ml^{-1}; B, 10 nkat·ml^{-1}) at 37°C for the times indicated. The lipoxygenase inhibitor nordihydroguaiaretic acid (100 μM, final concentration, trace C) was added to the particle suspension prior to the addition of lipoxygenase (10 nkat/ml). The start of the reaction is indicated by the arrow.

In separate experiments, we could show that under identical conditions of measurement of the passive electric properties, the lipoxygenase remains active for the investigation period, despite the known tendency of this enzyme to undergo self-inactivation [11,28]; we observed a loss of only a one-third of the activity after 10 min.

Discussion

In this communication, it is shown that the reaction of reticulocyte lipoxygenase with mitochondrial membranes causes marked changes in the passive electric properties of the membranes. Similar changes in the electric parameters were observed using sonified submitochondrial particles and frozen-and-thawed mitochondria as substrate for the lipoxygenase reaction. As shown under the conditions of our experiments, the oxygen in the chamber was exhausted within the first 30 s. A calculation from the concentration of the oxygen and membrane protein and from the data on the phospholipid content of heart mitochondrial membranes [27] shows that only about 0.01 mol of hydroperoxyphospholipids per mol phospholipid were formed. Furthermore, the changes in the passive electric properties of the membranes continue under conditions of anaerobiosis. From these two circumstances, it follows that the changes in passive electrical properties and the inhibitions of respiratory enzymes are caused by anaerobic processes subsequent to the lipoxygenase-catalyzed oxygenation of membrane-bound lipids. We propose that the changes studied here were brought about by the lipohydroperoxidase activity of the reticulocyte lipoxygenase. This enzyme exerts an anaerobic hydroperoxidase activity with 13-hydroperoxyoctadecadienoic acid in the presence of 9,12-all-*cis*-octadecadienoic acid which amounts to one-fifth of the aerobic oxygenase activity with 9,12-all-*cis*-octadecadienoic acid as substrate [28]. The analogous

activity of the lipoxygenase with hydroperoxyphospholipids as substrate may be responsible for the changes observed here. Another possibility to explain the kinetics is the slow redistribution of the hydroperoxylipids within the membrane leading to covalent interactions with integral membrane proteins which may severely damage the membrane structure and function.

The study of the interaction of the reticulocyte lipoxygenase with mitochondrial membranes is of interest from two aspects. Firstly, this lipoxygenase may be a useful tool for the study of general processes of peroxidative membrane damage supplementing the frequently used artificial catalysts such as ferrous ascorbate. Secondly, this system may serve as model for the events taking place during the maturation process of reticulocytes. A main event is the degradation of mitochondria, which is initiated by the action of the lipoxygenase [11]. The changes in the passive electrical properties demonstrated here may explain the following observations related to the maturational breakdown of mitochondria:

(1) It has been shown in experiments on the maturation of reticulocytes in vitro that there is an uncoupling of oxidative phosphorylation in the intact cell [29]. The increase in the conductivity of mitochondrial membranes caused by the reticulocyte lipoxygenase action as demonstrated here may be one of the reasons for the enhanced proton permeability, i.e., for uncoupling.

(2) Rapoport et al. [30] have shown that the breakdown of mitochondrial proteins is caused by an ATP-dependent proteolytic system in reticulocytes, the action of which is preceded by the lipoxygenase attack. The marked changes in passive electrical properties may reflect also a change in lipid-protein interaction [31] leading to an exposure of integral membrane proteins to extramitochondrial degradation systems.

Appendix

The conductivity, $\varkappa$, of a suspension of particles surrounded by a membrane can be characterized by a complex number consisting of a real part, $\varkappa'$, and an imaginary part, $\varkappa''$. $\varkappa$ may be represented by a vector in the complex admittance plane. The ends of these vectors for various frequencies are situated on a semi-circle or a segment. The frequency, f_0, at which the imaginary part, $\varkappa''$, reaches its maximum is the characteristic frequency (Fig. 1). For frequencies $f \ll f_0$ and $f \gg f_0$, the imaginary component of $\varkappa$ disappears and the real part is $\varkappa_0$ and $\varkappa_\infty$. From the values f_0, $\varkappa_0$ and $\varkappa_\infty$, which can be determined experimentally, the conductivity of the membrane, $\varkappa_s$, the conductivity inside the particles, $\varkappa_i$, and the geometric capacity of the membrane, C_s, can be calculated according to the Eqns. A1–A3:

$$\varkappa_i = \frac{2\varkappa_a(1-p) - \varkappa_\infty(2+p)}{\frac{\varkappa_\infty}{\varkappa_a}(1-p) - 1 - 2p} \tag{A1}$$

$$C_s = \frac{1}{2\pi f_0}\left(\frac{1}{r\left(\frac{1}{\varkappa_i} + \frac{1-p}{\left(1+\frac{p}{2}\right)2\varkappa_a}\right)} + \frac{\varkappa_s}{d}\right) \tag{A2}$$

$$\varkappa_s = \frac{d}{r} \cdot \frac{\frac{\varkappa_0}{\varkappa_a} \cdot \frac{1-\frac{p}{2}}{1-p} - 1}{\frac{1}{\varkappa_i} + \frac{1+2p}{(1-p)2\varkappa_a} - \frac{\varkappa_0}{\varkappa_a} \cdot \frac{1+\frac{p}{2}}{1-p}\left(\frac{1}{\varkappa_i} + \frac{1-p}{(1+2p)2\varkappa_a}\right)} \tag{A3}$$

where p is the volume concentration of the particles, $\varkappa_a$ the conductivity of the surrounding medium and d the thickness of the membrane. These equations are valid provided that there are no interactions among the particles, that the shape of the particles is spherical, with radius r, and that the conductivity of the membrane, $\varkappa_s$, is much lower than that of the medium inside the particles, $\varkappa_i$, and the conductivity of the surrounding medium, $\varkappa_a$.

For the determination of the passive electric properties ($\varkappa_s$, $\varkappa_i$, C_s) the module of the electric impedance $|\Re|$ and the phase angle, φ, are measured as a function of the frequency (see Material and Methods). The influence of the capacity of the empty chamber, C_∞, the parasitic capacity of the wires, C_p, and the wire inductivity, L_p, on the values measured were eliminated by the following equations (A4, A5) to obtain the real part, Y', and

the imaginary part, Y'', of the admittance of the particles under investigation.

$$Y' = \frac{\frac{\cos\varphi}{|\Re|}}{\left[1+\omega L_p\left(-\frac{\sin\varphi}{|\Re|}-\omega C_p\right)\right]^2+\omega^2 L_p^2\left(\frac{\cos\varphi}{|\Re|}\right)^2} \quad \text{(A4)}$$

$$Y'' = \frac{-\frac{\sin\varphi}{|\Re|}-\omega C_p+\omega L_p\left[\left(\frac{\sin\varphi}{|\Re|}+\omega C_p\right)^2+\left(\frac{\cos\varphi}{|\Re|}\right)^2\right]}{\left[1-\omega L_p\left(\frac{\sin\varphi}{|\Re|}+\omega C_p\right)\right]^2+\omega^2 L_p^2\cdot\frac{\cos^2\varphi}{|\Re|^2}}-\omega C_\infty \quad \text{(A5)}$$

The conductivity, $\varkappa = \varkappa' + j\varkappa''$, results from the admittance, $Y = Y' + jY''$, by multiplication with the chamber constant, z. From the plot of $\varkappa'$ vs. $\varkappa''$ in the complex admittance plane, the parameters $\varkappa_0$, $\varkappa_\infty$ and f_0 were obtained as shown in Fig. 6. The distribution parameter, α $(0 \leqslant \alpha < 1)$, is a measure of the size distribution of the particles under investigation. If $\alpha = 0$, all particles are uniform in size and there are no interactions between them. In this case, the plot of $\varkappa'$ vs. $\varkappa''$ turns out to be a semi-circle. A high heterogeneity in size of the particles is characterized by an α value approximating 1. In this case, the plot of $\varkappa'$ vs. $\varkappa''$ is a circle segment.

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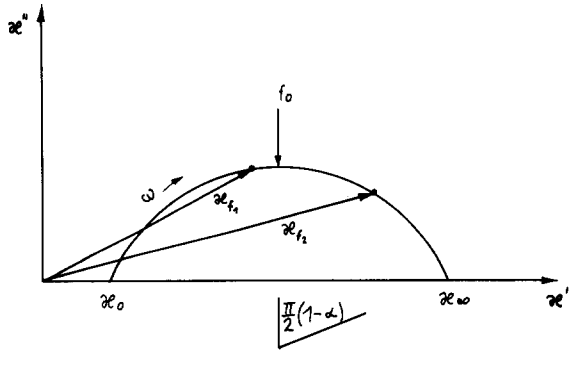

Fig. 6. Dielectric data for a suspension of particles plotted in the complex admittance plane (Scheme).

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