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NITROSYL HEMOGLOBIN. EPR COMPONENTS AT LOW TEMPERATURES

by

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Abstract - The EPR spectrum of nitrosyl hemoglobin is composed of at least three components (A, B and C) with 1different dependence on temperature and power level. The A component increases at low temperature (7.5K to 30K) and decreases at higher temperature. B component disappears at around 30 K and is replaced by C. Relaxation of A follows Orbach mechanism with energy of $28cm^{-1}$. This behavior can be attributed to phonon induced changes in orientation of NO with respect to the heme plane.

Keywords: Nitrosyl Hemoglobin; EPR Components at low Temperatures.

Introduction

Nitrosyl hemoglobin and nitrosyl myoglobin (HbNO and MbNO) have been fairly thoroughly studied, mainly by the Electron Paramagnetic Resonance (EPR). This is due, in great part, to the fact that NO as a ligand of heme iron has a great deal of similarity in its eletronic structure to the physiologically important oxygen. In both cases iron is ferrous with spin zero. HbNO however is paramagnetic (S=1/2), while oxyhemoglobin (HbO_2) is diamagnetic.

Another reason for the interest in EPR of HbNO (or MbNO) is that the observed complex spectra are sensitive to many factors, such as quaternary and tertiary sructures of the proteins, concentration of NO, pH, degree of hydration, etc. They also differ for NO on α and β chains [1-9].

More recently HbNO and MbNO have been used in the kinetic studies of photodissociation [10,11]. It was shown that EPR is a suitable technique for these studies [6,12,13]. Moreover in meso-Cobalt-Mb it distinguishes the deoxygenated form from the photolyzed one which is not possible in most of the optical measurements [14]. The saturation behavior in a wide temperature range is helpful for a better understanding of the spectra and relevant for defining the adequate conditions for these EPR experiments. Yet despite the large number of EPR experiments with nitrosyl-hemoproteins it is still difficult to unambigously interpret the complex EPR signal of HbNO.

The temperature behavior of MbNO EPR spectra was investigated over a wide temperature range [15-17]. In MbNO it was shown that the spectra result from two and three conformations in thermal equilibrium in solution and crystal respectively [15,16]. The power saturation at low temperatures ($T \leq 4.2K$) and temperature dependence above 80K of HbNO crystals suggest efficient spin-spin and spin-lattice relaxation processes which involve heme-heme magnetic dipolar interaction between both subunits [18]. Temperature and microwave power dependences of EPR spectra of HbNO solutions were not examined yet. In order to better understand HbNO we study the behavior of the EPR signal between 7.5K and 104K, as well as its dependence on microwave power. The saturation method resulted to be a convenient way to separate the overlapping spectra of HbNO. The overlap normally adds to the complexity of the signal. This method allows also to obtain the spin-lattice relaxation time T_1 of HbNO as a function of temperature.

Experimental Methods

Hb solutions were prepared by hemolysis of human blood and were stripped of ions by passage through a Sephadex G-25 (Sigma Co.) column. The solution was diluted to a 0.4mM heme concentration with 0.1M phosphate buffer, pH 6.2. HbNO was obtained as described by Louro et al. [4] by equilibration of the Hb samples with excess nitric oxide gas for at least 2h before freezing.

EPR measurements were performed with a X-band

spectrometer (Varian E-9) in the temperature range from 7.5K to 104K. The temperature of the sample was controlled by a helium flux criostat (Helitran LTD-110) with an APD-E temperature controller (Air Products and Chemical). Temperatures were measured with a Au-FexChromel thermocouple fixed on the tube's wall just above the sample.

Spectra were obtained for different microwave powers at each temperature. All of them can be reproduced by summing different fractions of two of the three components, A, B and C, shown in fig.1. The g values and hyperfine splittings are the values measured from derivatives extrema. Spectrum A was taken at low temperature (7.5K) and low microwave power ($1.2x10^{-4}$mW) and B at low temperature and high microwave power (225mW). Spectrum C was taken at high temperature (104K) and high microwave power. The best composite spectra (figs.2 and 3) for each experimental one were chosen by visual inspection of different trial combinations.

The fractions of the individual spectra (A, B and C), used for the above reproduction, were then used for the saturation and thermal equilibrium studies.

The half saturation power, $P_{1/2}$, as well as estimated values of T_1 and T_2 (spin-lattice and spin-spin relaxation times, respectively) were obtained by continuous wave saturation method as described previously [19].

The linear fittings of figs. 4 and 5b were done using linear regression, minimizing the square root error.

Results and Discussion

Spectra analysis

The EPR signal was obtained as a function of temperature and microwave power. The range covered here lies between 7.5K and 104K and between 225mW and 1.2×10^{-4}mW, respectively.

It was observed in this work that with increasing temperature the spectrum of HbNO at low microwave power undergoes a change in the shape which is similar to that observed at low temperature and increasing microwave power. A similar temperature and microwave power evolution was also observed in HbNO crystals and it can not be attributed to the broadening of its components [18]. To check if both spectra dependences are due to an equilibrium between conformations, we analyse each spectrum as a combination of two component spectra, A and B or A and C.

At the lowest temperatures the shape of the signal is strongly dependendent on microwave power. At low power one type of signal prevails (A signal). At higher power this signal saturates and decreases in amplitude. Another signal (B), very weak at low power, increases in amplitude with increasing power and dominates. Hence at 60dB attenuation (1.2×10^{-4}mW) one obtains essentially pure A spectrum and at 0dB (225mW) a pure B spectrum.

For temperatures as high as 30K the intermediate power spectra were well reproduced by summing appropriate fractions of A and B. Such spectra reconstructions at 7.5K are shown as an example in fig.2.

The A spectrum is asymmetric and shows a well resolved

three lines hyperfine structure and another weak structure centered at g=2.009. They are indicative of interactions with the nitrogen of the nitroxide and of the proximal histidine [5,20]. The hyperfine splittings are about 16G and 7G. Two other peaks are seen at g=2.070 and g=1.985. The B spectrum, centered at g=2.013, is quite symmetric and shows no resolved hyperfine structure. Its linewidth is about 70G.

At temperatures between 32K and 45K another spectrum (C) replaces the B spectrum and persists to higher temperatures. C spectrum shows only a slight indication of hyperfine structure (fig.1) at g=2.013, with about 16G splitting. Even at high microwave power we do not observe any contribution of B to the spectra. Its disappearance can not be related to saturation. For temperatures higher than 32K all spectra can only be reproduced by summing appropriate fractions of A and C instead of A and B. The temperature dependence of the spectra at non saturation conditions and their reconstruction using A, B and C are shown in fig. 3.

Since hemoglobin was saturated with NO the three spectra, A, B and C, belong to R quaternary conformation. They resemble the spectra of nitrosyl isolated α or β chains, Hb [2,5,21] or even Mb [15] or erythrocuorin [17] which appear slightly different in the literature depending on pH, temperature and microwave power conditions.

Relaxation

The relaxation behavior of the component spectra, A, B and C, were studied by continuous saturation method. This method

yields experimentally the value of $P_{1/2}$ directly proportional to the relaxation rate T_1^{-1}. A plot of $P_{1/2}$ as a function of temperature (T) was obtained for the A spectrum and is shown in fig.4. The fit indicates that $T_1^{-1} = W\exp(-\Delta/kT)$, where $\Delta = 28cm^{-1}$, $W = 10^{5.1}$ and k is the Boltzman constant. This behavior is typical of the Orbach, two phonons relaxation mechanism with a characteristic energy Δ, observed previously for high spin ferric hemoglobins [22] and for other proteins [23]. Low spin ferric iron in general exhibits a T^n dependence [24].

For the A spectrum T_1 varies from $4.6x10^{-4}s$ at 10K to $1.2x10^{-5}s$ at 100K. As the B and C spectra have high $P_{1/2}$ values, it is not possible to obtain a good fit of their saturation behavior. Nevertheless the range of the values of T_1 is estimated from $6.3x10^{-5}s$ for B spectrum at 7.5K to $6.3x10^{-6}s$ for C at 104K. For invariant linewidths, T_2 is $1.6x10^{-9}s$ at any temperature in all the three spectra.

Saturation measurements by Doetschman and Utterback [18] in HbNO crystals between 1.6K and 4.2K yield an estimative of $2.2x10^{-7}s \leq T_1 \leq 7.2x10^{-6}s$ and $3.1x10^{-8}s \leq T_2 \leq 10^{-6}s$, from linewidths. The T_1 and T_2 values obtained in our work are not in these ranges. It is however known that the linewidths in crystals and in solution are considerably different [25]. On the other hand our T_1 values are of the same magnitude as those measured by Muench and Stapleton [26] in MbNO solutions between 4.2K and 20K. They assumed a linear temperature dependence for T_1^{-1}, with no identification of the relaxation mechanism . We have also checked that these MbNO results fit an exponential temperature dependence

as well as a power one. We believe that, besides the differences between the two proteins, different temperature ranges are responsible for the different results. Care should be taken in analysis of relaxation in spectra which are a combination of two species in nitrosyl-hemoproteins.

The observation of a difference of one order of magnitude between the relaxation rates of spectra A and B is consistent with the observed responses of the spectra to the microwave power at low temperature (fig. 2). The two distinct EPR signals in MbNO solution also present evidence of different saturation behavior and are fairly easily saturated at low temperature ($T<20K$) [15].

Temperature Dependence

From the saturation measurements of A, B and C spectra it can be seen that at attenuations higher than 40dB (one tenth of the smallest $P_{1/2}$) none of the three spectra is saturated. Fig. 5a shows that at 50dB ($1.3x10^{-3}$mW) the intensity of the A signal decreases with increasing temperature. The intensity of B is less sensitive to temperature below 30K and vanishes at this temperature when C appears. C signal intensity is almost constant up to 104K.

Above 30K the A signal decreases rapidly. In consequence at liquid nitrogen temperature, at which most spectra have been reported, we observe almost pure C spectrum, which is almost identical to those observed by Henry and Banerjee [5] and Sanches [9].

At 50dB attenuation, the ratios of amplitudes (F) of B/A up to 30K and C/A below 30K are shown in fig. 5b. The B/A ratio below 30K is almost constant. The absolute value of this ratio depends on saturation and normalization conditions. Above 30K there exists a thermal equilibrium between species A and C with an enthalpy $\Delta H = 1.1 kJ/mol$ and entropy $\Delta S = 22.5 x 10^{-3}$ kJ/mol.K.

We believe that the changes observed at 30K correspond to change of the bond angle of the ligand (Fe-N-O). X-ray diffraction of HbNO [27] crystals gives about 150° for the Fe-N-O angle at room temperature while EPR measurements at 77K give about 110° [28]. Changes of the EPR signal of MbNO solutions [15] and crystals [16] demonstrate that the conformation of the bonding of NO is drastically altered upon freezing. Different conformations have also been deduced from infrared measurements of stretching bands in carboxy myoglobin (MbCO). Three different absorption peaks have been associated to three different CO angles with respect to the heme with differences of the order of $20 cm^{-1}$ in energy [29].

Conclusions

We believe that the low temperature EPR studies of HbNO help in elucidating this complex system. It became gradually apparent that the condition, under which the EPR spectra are taken, is of essential importance. The level of the klystron power affecting the relaxation processes can change completely the features of the spectra which, as we show here, are composed of

two spectra at all temperatures studied (7.5K to 104K). Further experiments should be performed to elucidate the difference of magnitude of the relaxation rates of the species.

The relaxation results evidence a low lying energy level of $28cm^{-1}$ for the A component. Since one does not expect in the proposed energy level diagram such small difference in energy between the ground and excited electronic states [30], we propose that the $28cm^{-1}$ refers to a difference between two different geometries (conformations) of liganded heme, produced by different Fe-N-O angles.

The postulated reorientation of NO at 28 cm^{-1} can be induced by low frequency phonon modes of the exterior medium in the 10-100 cm^{-1} range [31]. The decrease of the amplitude of A spectrum above 30K is consistent with this interpretation, but it occurs faster than that (100 cm^{-1}, given by the slope of fig. 5a at temperatures above 30K). Other effects however may also contribute to this decrease as observed for MbCO by Ansari et al. [29]. Since the spins which disappear in the A spectrum do not appear in a new spectrum (C), we believe that NO conformation changes to a new one which is spin silent.

The low temperature B species reorients to a C species at about the same temperature. This reorientation changes the aspect of the spectrum, but both B and C remain paramagnetic with approximately the same number of spins.

It is also interesting to observe that the inelastic neutron scattering spectra of myoglobin show a maximun in the density of vibrational states at $25cm^{-1}$ [32].

The observed effects do not allow for a straightforward relationship between A, B and C components and α and β chains. Although there is a strong similarity between A and α, and B and C and β, the spectra can also be associated to the different EPR species of MbNO [15] and erythrocuorin [17] which have only one polypeptidic chain. The EPR spectra of HbNO have a complex temperature and power dependence which may include the contribution of the spectra of α and β chains.

It became apparent, particularly in the optical work of the Illinois group [29], that the ligands in the case of MbCO do not form an unique angle with the heme plane. The different groups of ligands exhibit different dissociation kinetics. We observe in HbNO, by a different technique, phenomena which we attribute also to ligand orientation. It remains of course to be seen whether these effects play any role under the physiological conditions.

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Figures Captions

Fig.1- EPR spectra of HbNO (0.4mM, pH 6.2) A, B and C components. A at 7.5K and $1.2x10^{-4}$mW, B at 7.5K and 225mW and C at 104K and 225mW.

Fig.2- Microwave power dependence of EPR spectra of HbNO (0.4mM of heme, pH 6.2) at 7.5K. ——— experimental spectra; ——— composite spectra. From top to bottom, ratios of fractions of B/A: 14.4, 4.8, 0.91, 0.17 and 0.1.

Fig.3- Temperature dependence of EPR spectra of HbNO (0.4mM of heme, pH 6.2) at $1.3x10^{-3}$mW. ——— experimental spectra; ——— composite spectra. From top to bottom, ratios of fractions of: B/A - 0.1, 0.29 and 0.25; C/A - 0.94, 1.9 and 5.

Fig.4- Temperature dependence of $P_{1/2}$ (proportional to T_1^{-1}) of component A.

Fig.5a-Temperature dependence of the intensity of components A (□), B(○) and C(◊) at $1.3x10^{-3}$mW.

Fig.5b-Temperature dependence of the ratios of intensity (F) of B(○) and C(◊) relative to A, at $1.3x10^{-3}$mW.

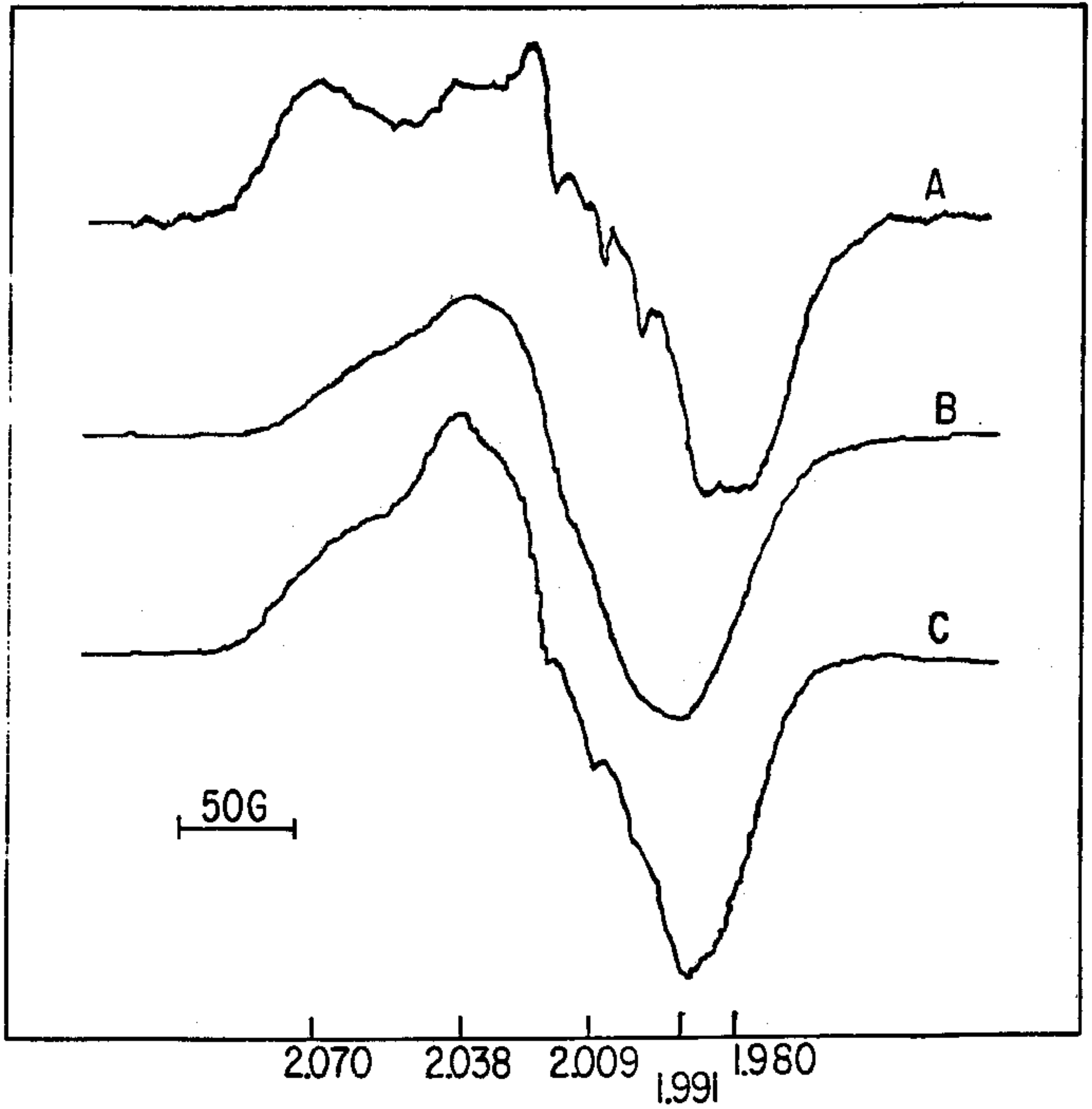

Fig. 1

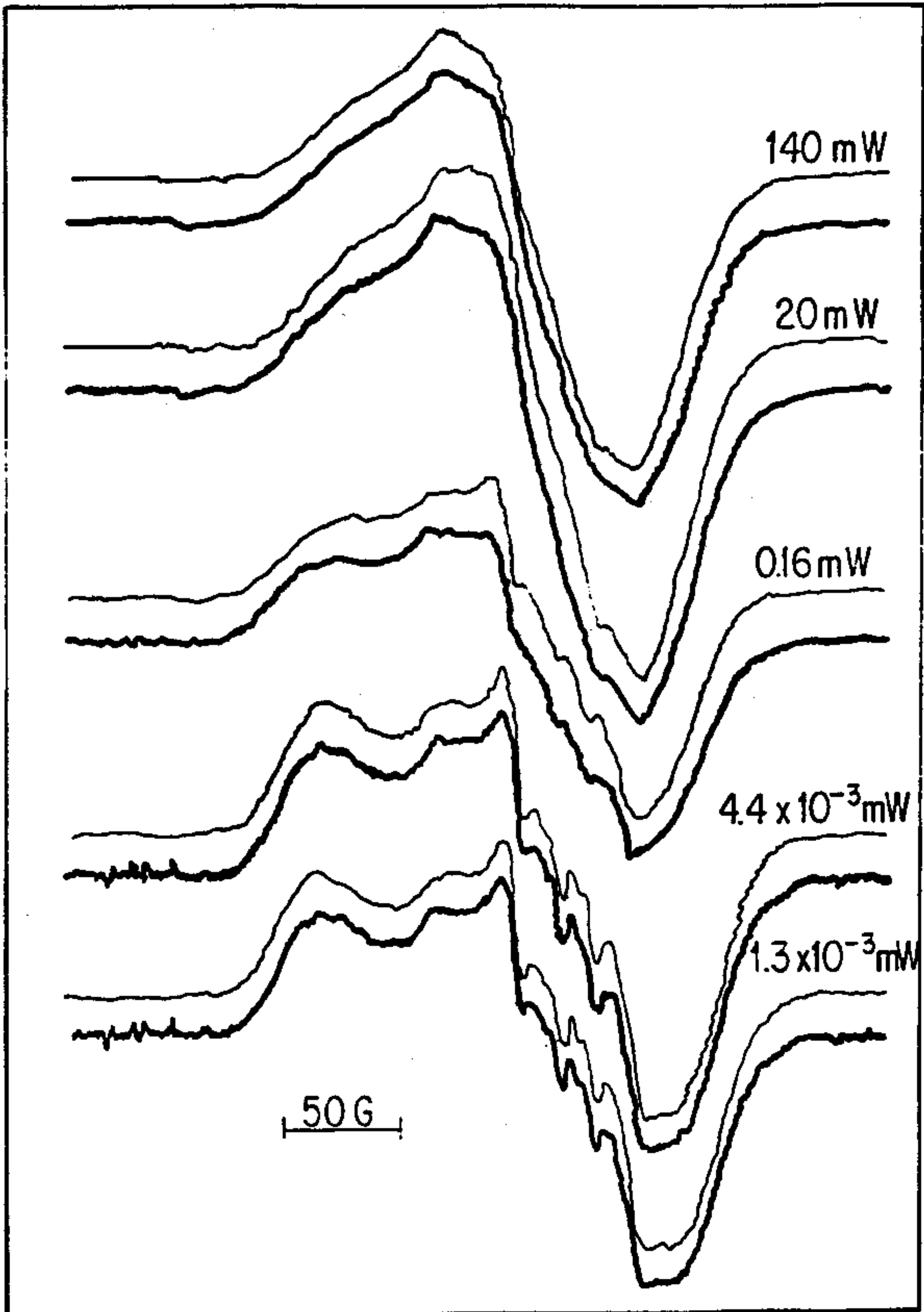

Fig. 2

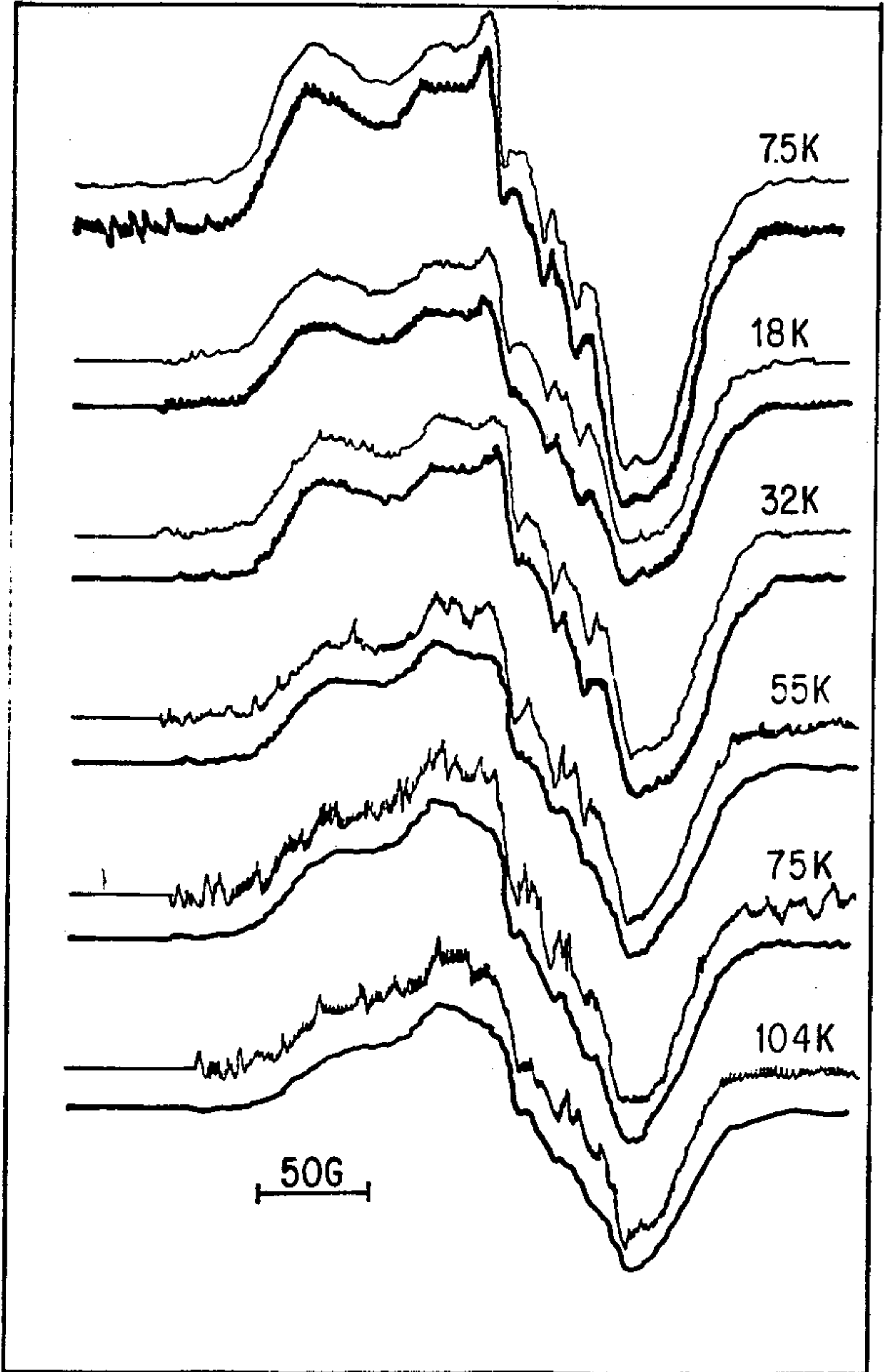

Fig. 3

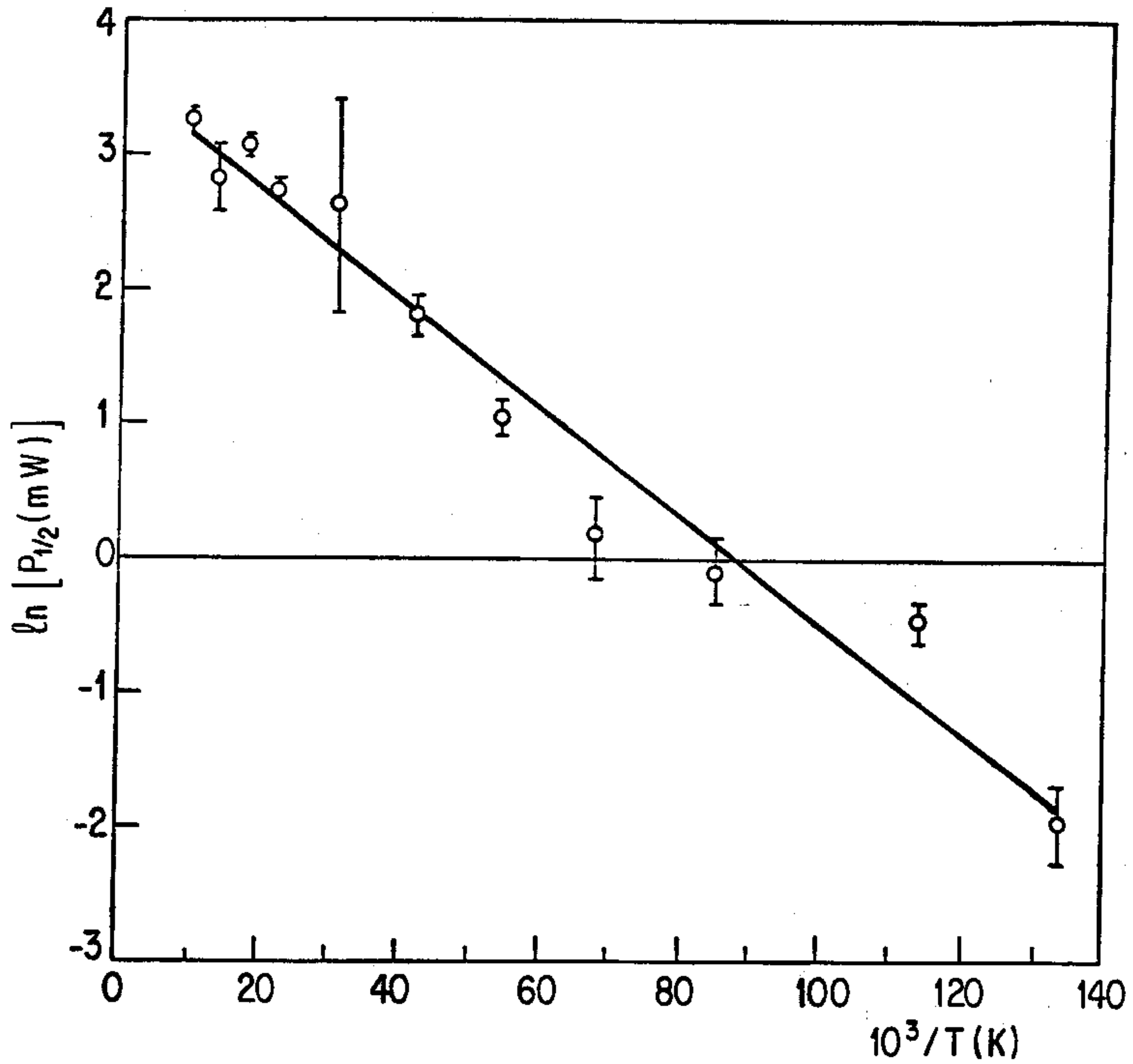

Fig. 4

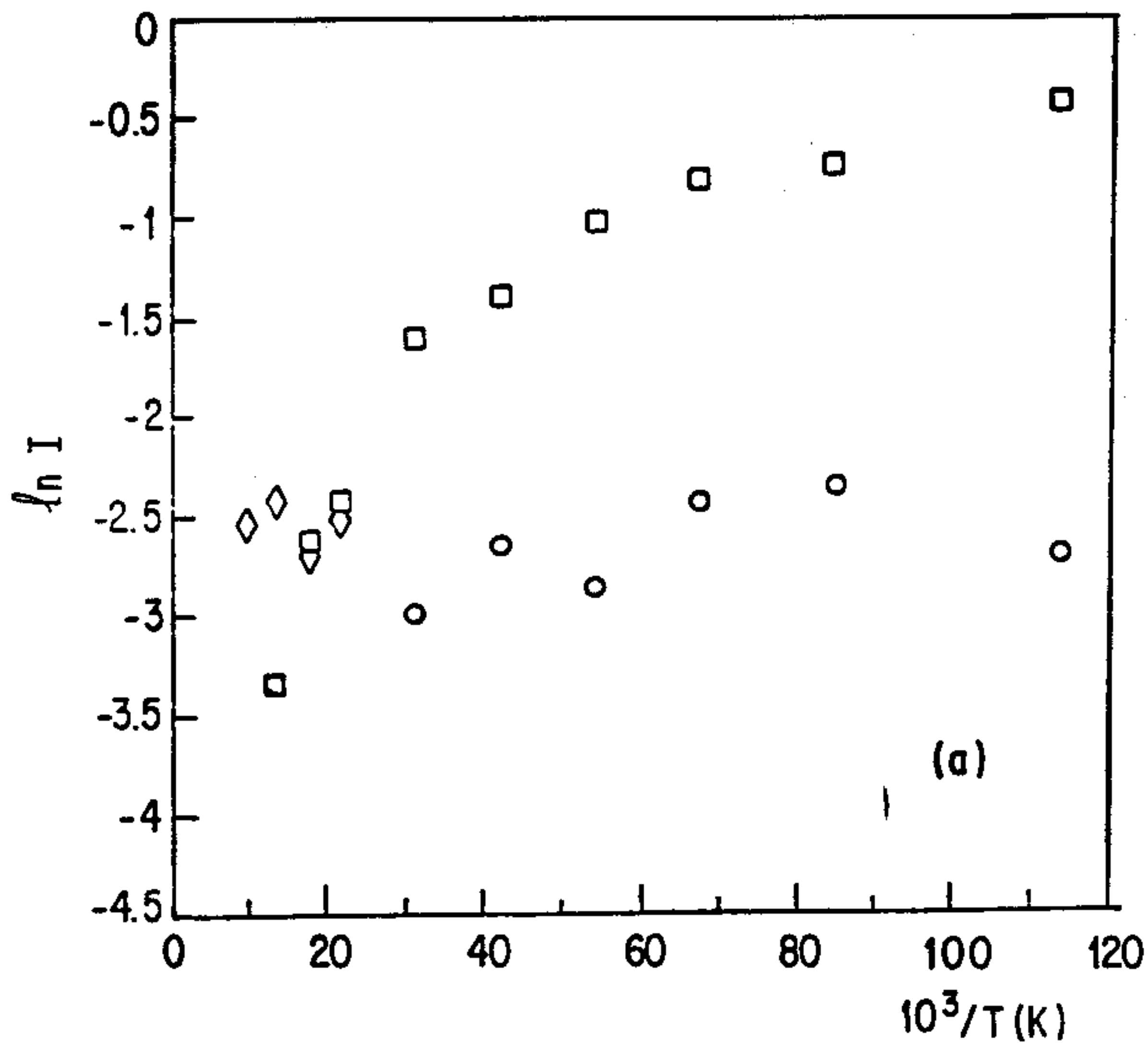

Fig. 5a

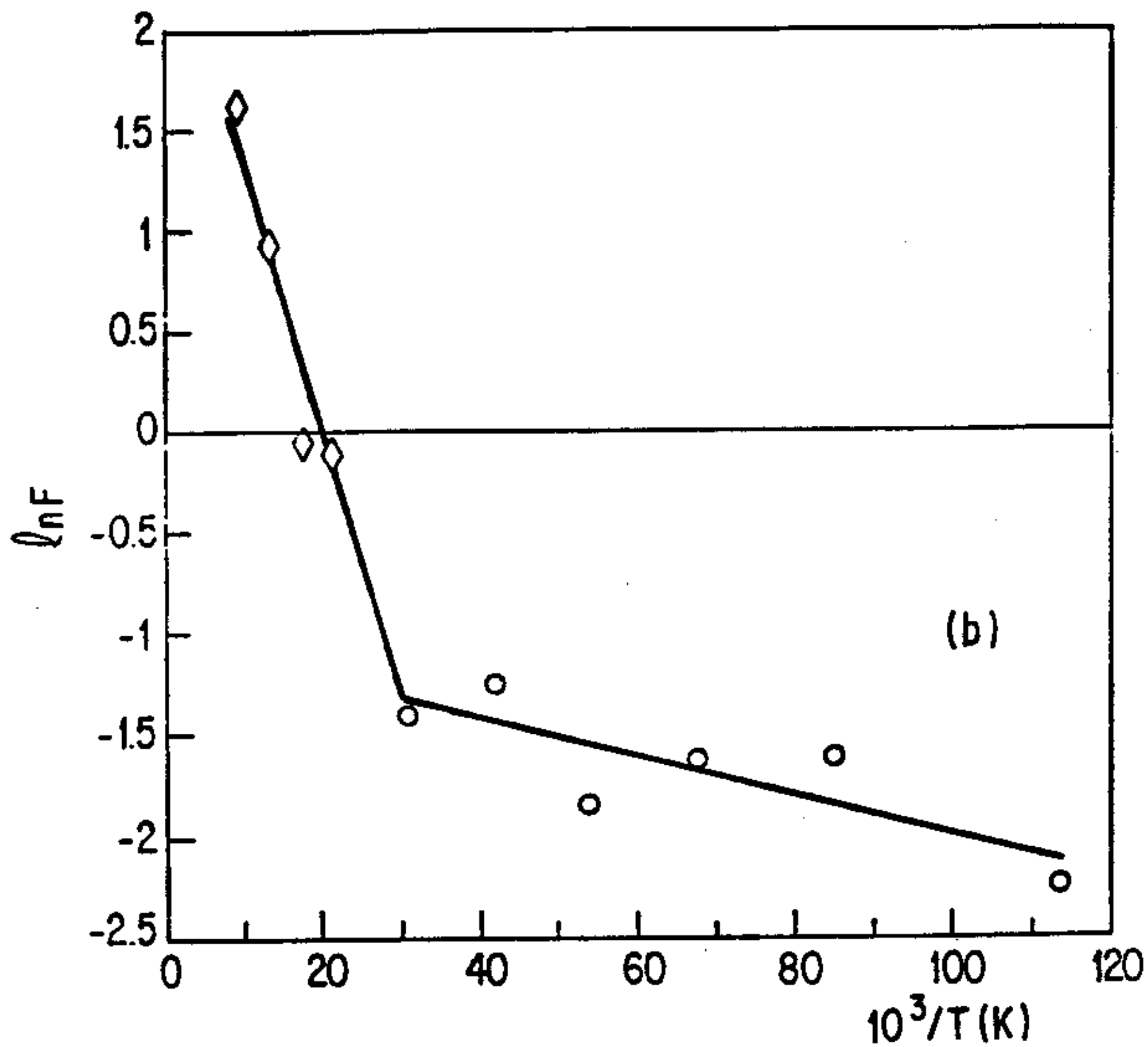

Fig. 5b

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