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# OXYGEN BINDING TO NITRIC OXIDE MARKED HEMOGLOBIN**

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ABSTRACT. Electron spin resonance spectra of organic phosphate free human hemoglobin marked with nitric oxide at the sixth coordination position of one of the four hemes allow to observe the transition from the tense (T) to the relaxed (R) conformation, as a function of parcial oxygen pressure. The spectra are composites of contributions from $\alpha_T$, $\alpha_R$ and $\beta$ chains spectra, showing the presence of only two conformations: T and R. In the absence of organic phosphates NO binds to $\alpha$ and $\beta$ chains with the same probability, but in the presence of phosphates NO combines preferentially with $\alpha$ chains. The dissociation of NO proceeds at least an order of magnitude faster in T than in R configuration.

RESUMO. Os espectros de ressonância paramagnética eletrônica de hemoglobina humana livre de fosfatos orgânicos, marcada com óxido nítrico na sexta posição de coordenação de uma das quatro hemes, permitiu observar a transição da conformação tensa (T) para a relaxada (R), em função da pressão parcial de oxigênio. Os espectros são somas de contribuições de espectros de cadeias $\alpha_R$, $\alpha_T$ e $\beta$, mostrando a presença de apenas duas conformações: T e R. Na ausência de fosfatos orgânicos o óxido nítrico se liga com a mesma probabilidade a cadeias $\alpha$ e $\beta$, mas na presença de fosfatos orgânicos NO se combina preferencialmente às cadeias $\alpha$. A dissociação do NO é pelo menos uma ordem de grandeza mais rápida na configuração R do que na configuração T.

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In the past several years a considerable interest has developed in the characteristic of nitrosyl hemoglobin. NO binds at the sixth coordination position of the heme and NO-Hb is paramagnetic. NO-Hb is believed to exhibit a very similar behavior to the physiologically important, but diamagnetic, oxy-Hb.

EPR spectra and optical absorption have been able to distinguish between the α chains and β chains (1-23). In the absence of organic phosphates, nitrosyl hemoglobin A is locked in the R configuration at physiological pH, the same as other six coordinated hemoglobins; the organic phosphates (i.e. $InsP_6$) switch it to the T configuration. Extensive use has also been made of mutant $Hb_{Kansas}$ because nitrosyl $Hb_{Kansas}$ persists in the T configuration even in the absence of phosphates (11,16,21,23,25). These experiments have yielded information concerning the relation between the motion of iron in and out of the plane of the porphyrin, the interaction of the paramagnetic electron with the $N_\epsilon$ of his (F8), as well as with the N of NO, and the angle between NO and this plane (23). They have in general helped to clarify the ideas about the mechanism leading to the cooperative effects in hemoglobin (19).

We report in this paper on the use of NO as a marker in hemoglobin, and on the observation of the conformational transition from T to R states during the oxigenation of organic phosphate free hemoglobin.

Our attention is focused on the α chains which are the only ones to show EPR changes during this transition.

## MATERIALS AND METHODS

Hemoglobin A was prepared according to Benesch and Benesch (26), and used at pH 7.0 and 0.05 M phosphate buffer and at concentrations of approximately 10%. The solution was introduced into a tonometer which could be interchangeably adapted to spectrophotometric and EPR measurements. The solution was deoxygenated by washing with $N_2$ and pumping out several times. Controlled volumes of NO gas were intected with a syringe to give approximately a ratio of NO/hemes of about 5%. The solution was shaken for 5 minutes and the excess of NO was pumped out. For the purpose of oxygenation controlled volumes of air were similarly introduced. After each injection of air followed by 5 minutes of shaking at 25°C the solution was transferred (within the tonometer) to an araldite EPR tube and spectrum was obtained at 140°K. The araldite tubes were home made and used because of their resistence to breakage while undergoing abrupt temperature changes.

The oxigenation curve for NO free hemoglobin was obtained, in the condition described above, using the procedure of Benesch at al (27).

EPR spectra were obtained using a Varian V-4500 spectrometer (X-band). Temperature was controlled with the Varian F401 temperature controller. Typical experimental conditions were: 3G modulation, 20 mw power, 5 minute sweep over 500 G range, microwave frequency 8914 MHz. No saturation effects were observed at this power.

Optical measurements were taken with a Beckman DU spectrophotometer using a plastic cuvette of 1.2 mm light path, with a glass spacer reducing the path to 0.2mm and attached directly to the tonometer. This permitted to take optical measurements at Hb concentrations used in EPR runs. $InsP_6$ was purchased from Sigma and added at a ratio of 2 molecules per hemoglobin.

## RESULTS AND DISCUSSION

Oxyhemoglobin and hemoglobin marked with NO (NO/heme = 0.05) exhibit, in the absence of organic phosphates, two vastly different EPR spectra (fig. 1 a,b). These types of spectra have been previously associated with the R and T configurations of hemoglobin (7,11,13, 19,23). Fig. 1 c,d show the corresponding spectra obtained after addition of $InsP_6$. While the Hb spectra with and without organic phosphates are similar (but not identical), the oxyHb spectra differ considerably. This is due to the fact that organic phosphates preserve better the T configuration even in the presence of six coordinated hemes. In the absence of phosphates the T configuration is associated with five coordinated (deoxy), while the R configuration with six coordinated hemoglobin. The observed T and R spectra for the limiting cases of Hb and oxyHb allow one to study in some detail the conformational change from T to R as a function of $pO_2$. The dissociation of NO (see below) is sufficiently slow to be easily taken under account.

After introduction of $O_2$ one obtains a series of EPR spectra as a function of $PO_2$ (Fig. 2). These spectra change in time due to the dissociation of NO. This problem has therefore been studied. In order to distinguish between the dissociation and changes in conformation we have, for each $PO_2$ studied, marked Hb with NO (see Materials and Methods) and obtained an EPR spectrum at 140°K. The amplitudes of some of the EPR lines of this spectrum are shown in Fig. 3a constituting the t = 0 points. The sample was then brought to room temperature and oxygen introduced. After approximately five minutes of shaking the sample was cooled and a spectrum obtained. The sample was then thawed, remaining at room temperature for a time t and was frozen again for another EPR measurement. This procedure was repeated several times yielding data shown in Fig. 3a where t corresponds only to the room temperature portion of the process. Line 1 (Fig. 2a) is known to arise from the $\alpha_T$ hemes (9), while line 3 corresponds to a sum of contributions from $\alpha_R$ and $\beta$ hemes. Changes in the amplitudes of lines between t = 0 and t = 5 minutes are mainly due to the effect of $O_2$ on the conformation of the protein. Line 1 decreases and line 3 increases, because the contribution of $\alpha_T$ decreases and that of $\alpha_R$ increases with increasing $PO_2$. The gradual decrease of the amplitudes of all lines after t = 5 minutes, is due to the dissociation of NO. Fig. 3b shows that the dissociation of NO is equal for $\alpha$ and $\beta$ chains. This is apparent because the ratios of amplitudes of lines representative of $\alpha$ and $\beta$ chains (2 and 3), remain constant with time for all $PO_2$.

The decrease in the ratios of amplitudes with increasing $pO_2$ (Fig. 3b) is due to the conversion of $a_T$ to $a_R$. Ratios of amplitudes of other pairs of lines (not shown) lead to a similar conclusion. We have checked that consecutive freezing and thawing of NO marked Hb did not affect the amplitudes of the EPR lines.

The dissociation of NO has a half time of about 45 minutes at low $PO_2$ (Fig. 3a), and is at least an order of magnitude longer at higher oxygen pressure, indicating that the dissociation is much higher for T than for R configuration.

The T to R transition

In the series of spectra as a function of $pO_2$ and corresponding to t = 5 minutes as shown in figure 2 and at the low concentrations of NO used in the present work, there exist only three groups of Hb molecules; $(\alpha_2\beta_2)$, $(\alpha^{NO},\alpha,\beta_2)$ and $(\alpha_2,\beta^{NO},\beta)$. The first group is diamagnetic, the second and third contribute to the EPR spectra. Fig. 1a shows that these spectra correspond almost entirely to the T configuration (13). Hence the presence of a single NO per molecule preserves most of its deoxy, T character. As $pO_2$ increases, so does the conversion to the relaxed conformation. At highest $pO_2$ one has essentially all the paramagnetic molecules in the $(\alpha^{NO},\alpha^{O_2},\beta_2^{O_2})$ and $(\alpha_2^{O_2},\beta^{NO},\beta^{O_2})$ states, and in R conformation.

The latter spectrum (Fig. 1c) is identical with the nitrosyl-hemoglobin spectrum of Reisberg, taken at the same temperature (16). This shows that NO binds with the same probability to α and β chains and permits us to use Reisberg's α and β chain spectra (Fig. 7 b,c) as characteristic of R configuration.

Since the β spectra do not "feel" the difference between R and T conformation (9,24) and with the assumption that $\alpha_T$ spectrum does not contribute al all at g = 1.985 (H = 3210 G) -which seems reasonable in view of the $\alpha_2^{NO}$ $\beta_2^{deoxi}$ spectra in the presence of $InsP_6$ (24),we were able to subtract the contribution of β chains from all our experimental spectra.

The resulting curves are shown in figure 4 and figure 5. They correspond to the sum of $\alpha_T$ and $\alpha_R$ contribution with varying ratios of the two.

The simulated $\alpha_T + \alpha_R$ spectra are shown in Fig. 6. The comparison of the simulated and experimental β subtracted spectra allows us to obtain the fraction of $\alpha_R$ as a function of $pO_2$ (Fig. 7). The good agreement between simulated and experimental spectra shows that the latter consist at any $pO_2$ of a simple superposition of $\alpha_T$, $\alpha_R$ and β spectra.

The results of experiments with NO marked hybrids (8, 9,14,15,18) indicate that α marked chains give two types of spectra, depending on the type of ligands on β chains. Hence, the $\alpha^{NO}$ chains in the tetramer are also expected to yield information about the conformation of the entire molecule. The simple superposition of $\alpha_T$ and $\alpha_R$ indicates that only two global conformations, T and R are important

in the oxygenation process, without having to recurr to additional conformational changes within the individual chains (11).

Without the knowledge of the oxygenation curve for hemoglobin singly marked with NO we cannot use Monod's model (29) to check whether $\bar{\alpha}_R$ is equal to the fraction of molecules in R configuration.

## The effects of $InsP_6$

The difference between the spectra of Hb without and with $InsP_6$ is shown in Fig. 4 together with spectra of pure $\alpha_T$, $\alpha_R$ and β chains (18). This difference is due mainly to contribution from β chains with a 25% contribution of $\alpha_R$. Since all our data indicate that in organic phosphate free Hb NO binds with equal probability to α's and β's, the above result implies that in the presence of $InsP_6$, NO binds preferentially to α chains. A similar result has been recently obtained for binding of $O_2$ to Hb (28).

We also deduce that the contribution of $\alpha_R$ to the organic phosphate free Hb may be as much as 10% of the α's. This contribution is partly responsible for the slight difference between the spectra in Fig. 1a and 1c, and is indicated in Fig. 7. It is due to a single NO bound per molecule. The rest of the difference between Fig. 1a and 1c is due to the preferential binding of NO to α chains in the Hb with $InsP_6$. Comparison of the spectrum in Fig. 1d with the simulations indicates that

$InsP_6$ preserves approximately a 65% T character in NO marked oxyHb.

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FIGURES

Fig. 1 - EPR spectra of NO marked hemoglobin:
a) Hb, b) OxyHb, c) Hb + $InsP_6$, d) OxyHb + $InsP_6$, conditions as in text.

Fig. 2 - EPR spectra of organic phosphate free hemoglogin as a function of $pO_2$: a) 4.5, b) 11.5, c) 19, d) 26.5, e) 38, f) 53.5 mm Hg

Fig. 3 - a) Amplitudes of EPR lines 1 (□, Δ, O) and 3 (■, ●, ⊙) (see Fig. 2a for identification) as a function of $pO_2$, and time after oxigenation:
□ and ■ $pO_2$ = 4.5; Δ and ● $pO_2$ = 19; O and ⊙ $pO_2$ = 38 mm Hg
b) Ratios of EPR lines 2 and 3 (Fig. 2a) as a function of pO, and time after oxigenation:
. - $pO_2$ = 4.5; Δ - $pO_2$ = 11.5; O - $pO_2$ = 19; □ $pO_2$ = 26.5; ▲ - $pO_2$ = 38 mm Hg

Fig. 4 - EPR spectra of: a) $\alpha_T$, b) $\alpha_R$, c) β, d) difference between spectra of organic phosphate free Hb and Hb with $InsP_6$.

Fig. 5 - Experimental, β subtracted EPR spectra (see text).

Fig. 6 - Composites of $\alpha_R$ and $\alpha_T$ spectra (see Fig. 3) in various proportions.

Fig. 7 - Oxygenation curve for NO free hemoglobin, and fraction of $\alpha_R$ as a function of $pO_2$ .

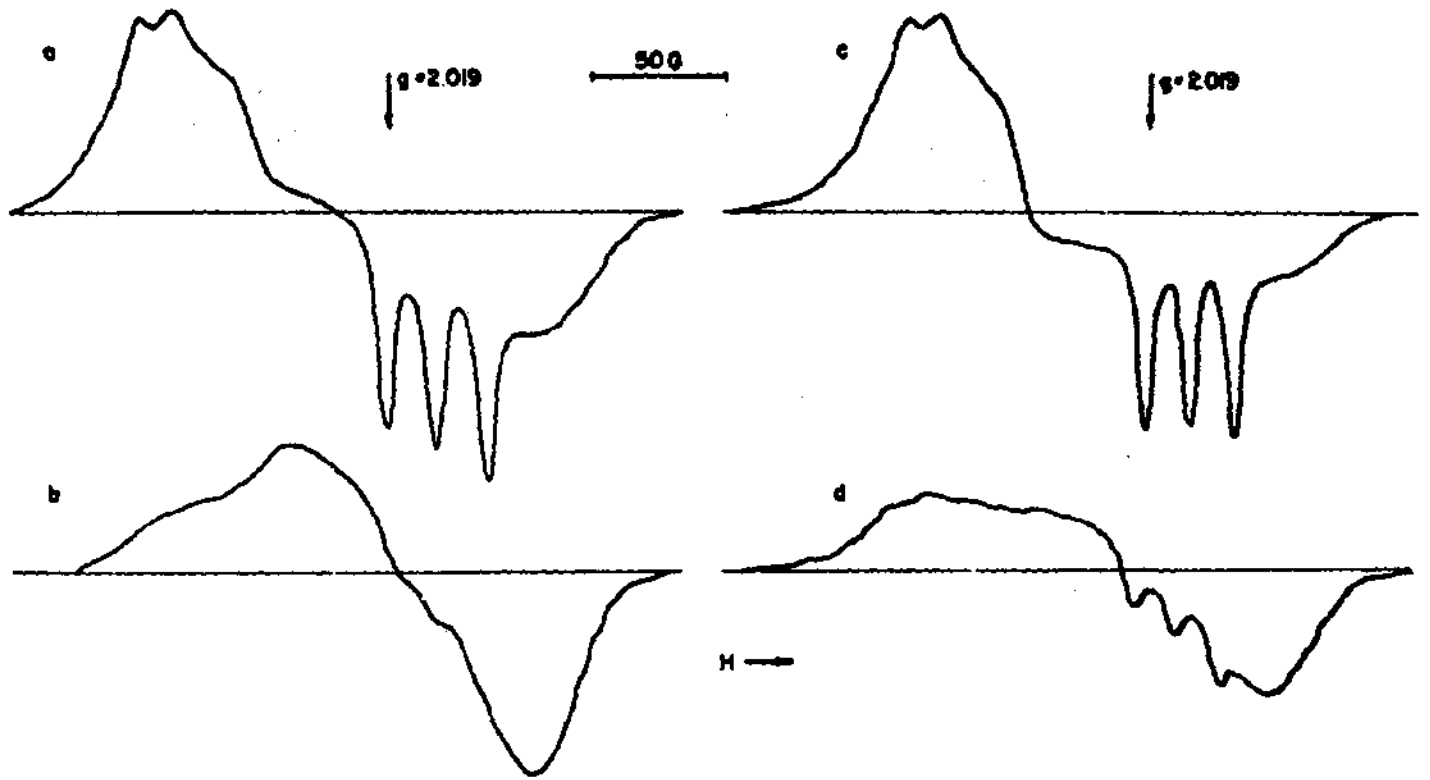
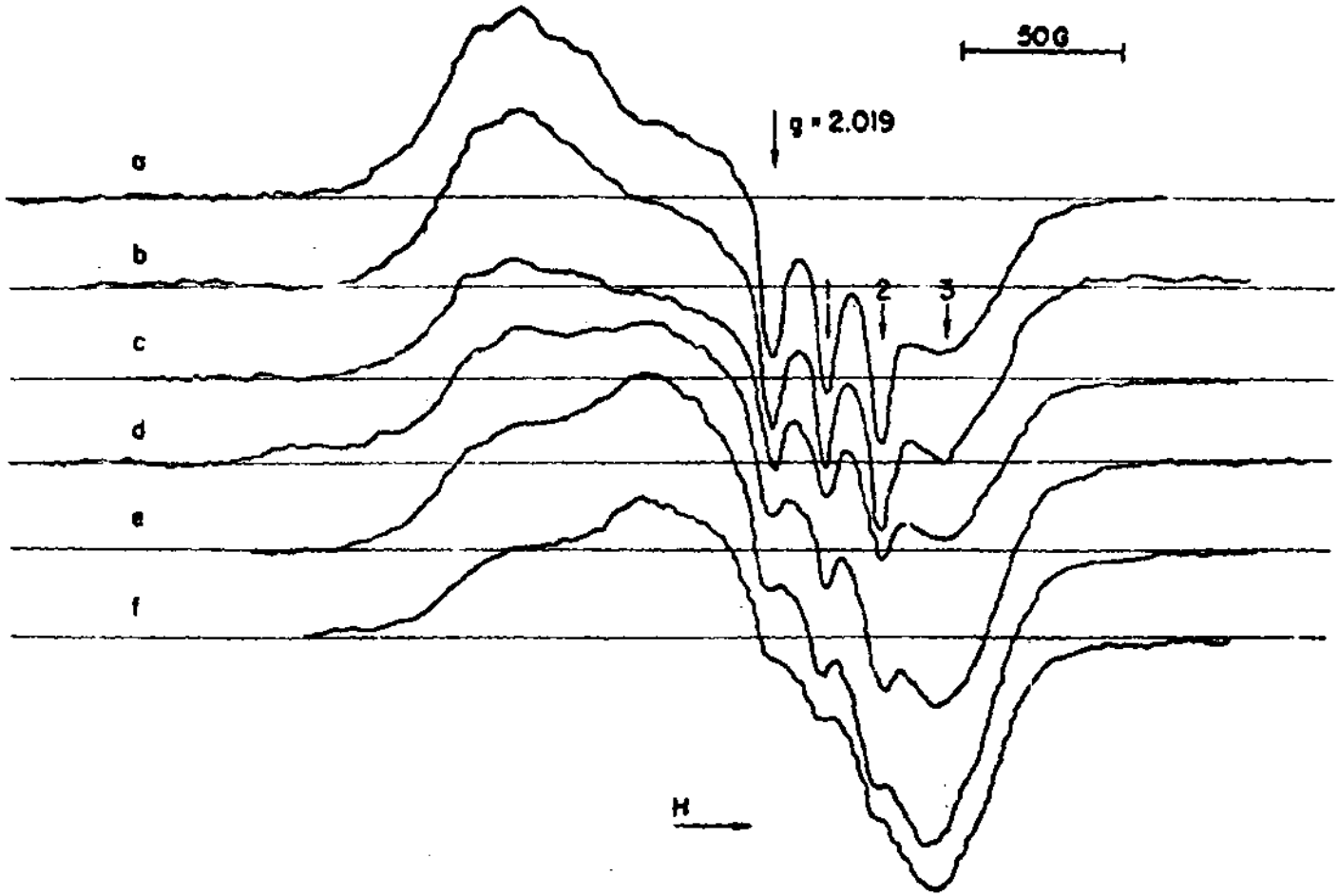
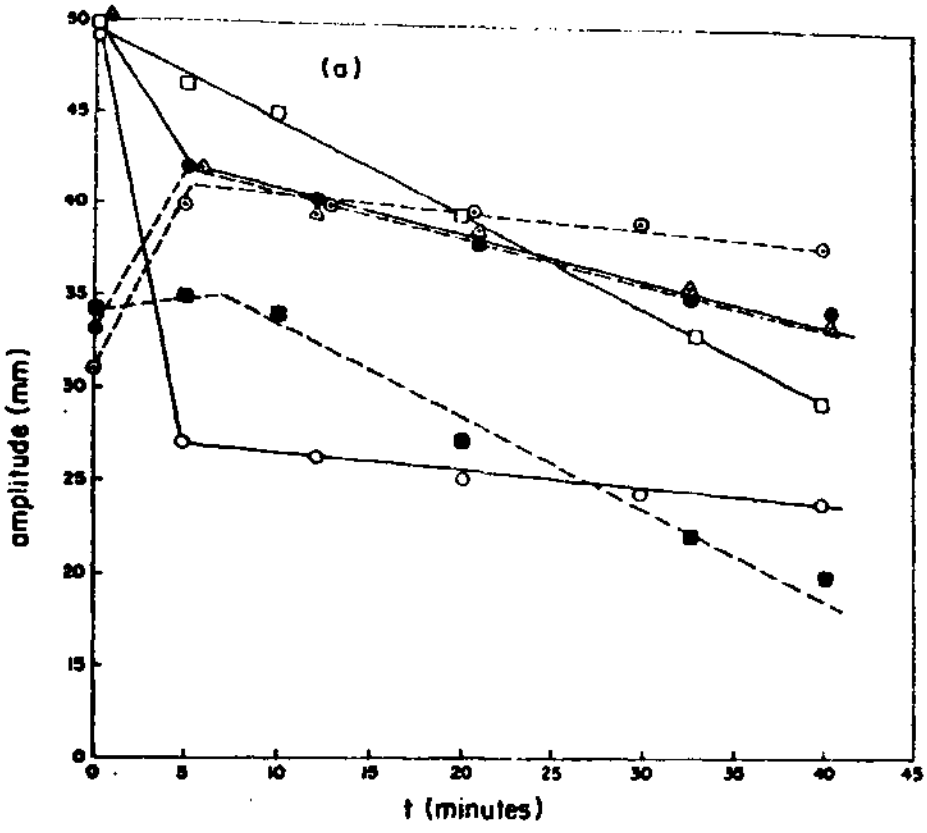



Fig. 1


50G
g = 2.019
a
b
c
d
e
f
1
2
3
H



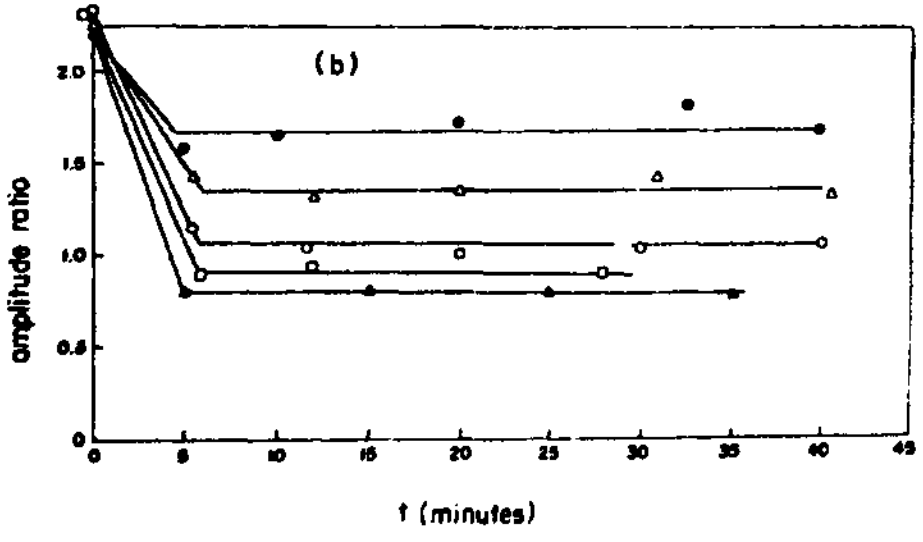




Fig. 3

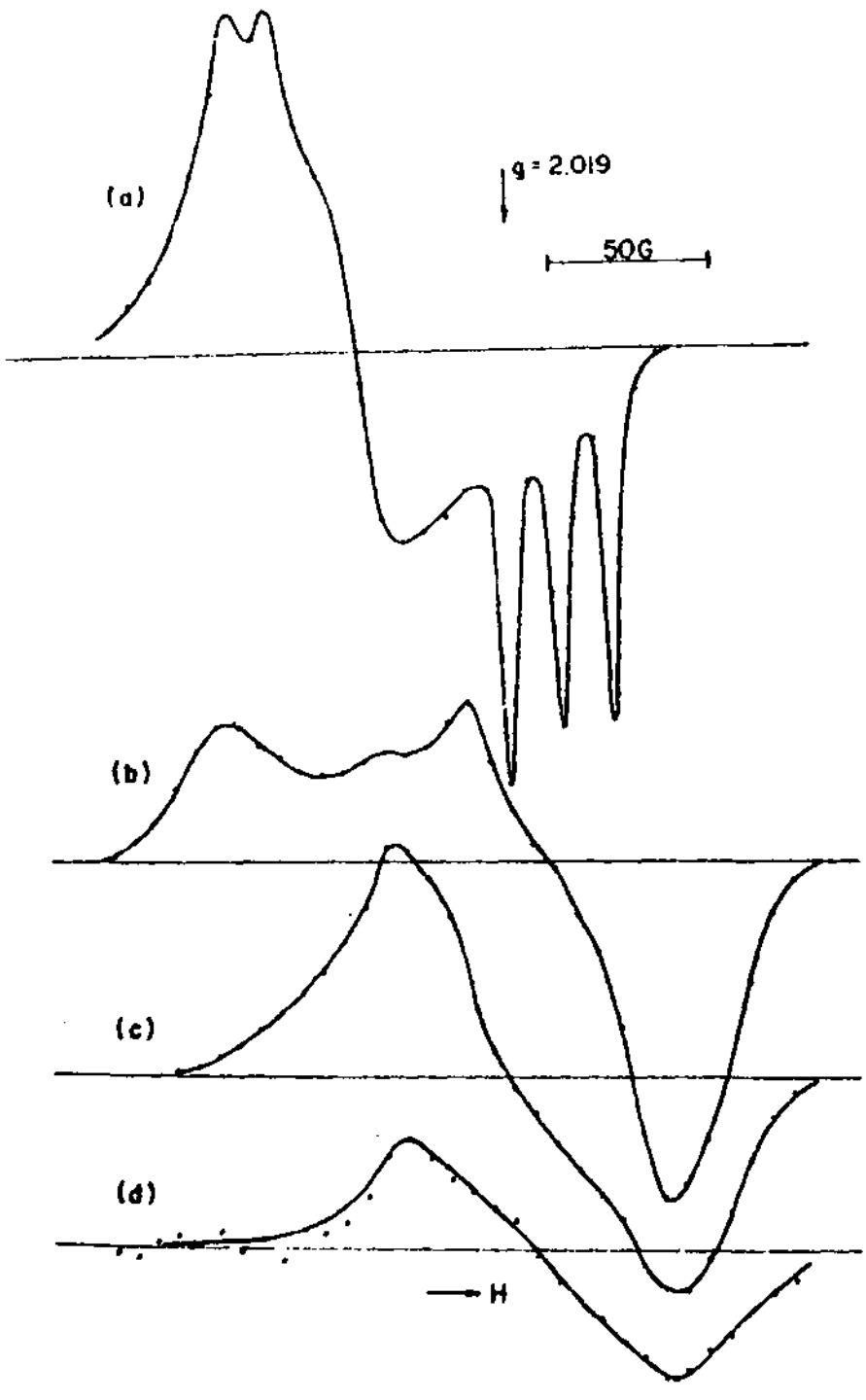



Fig. 4

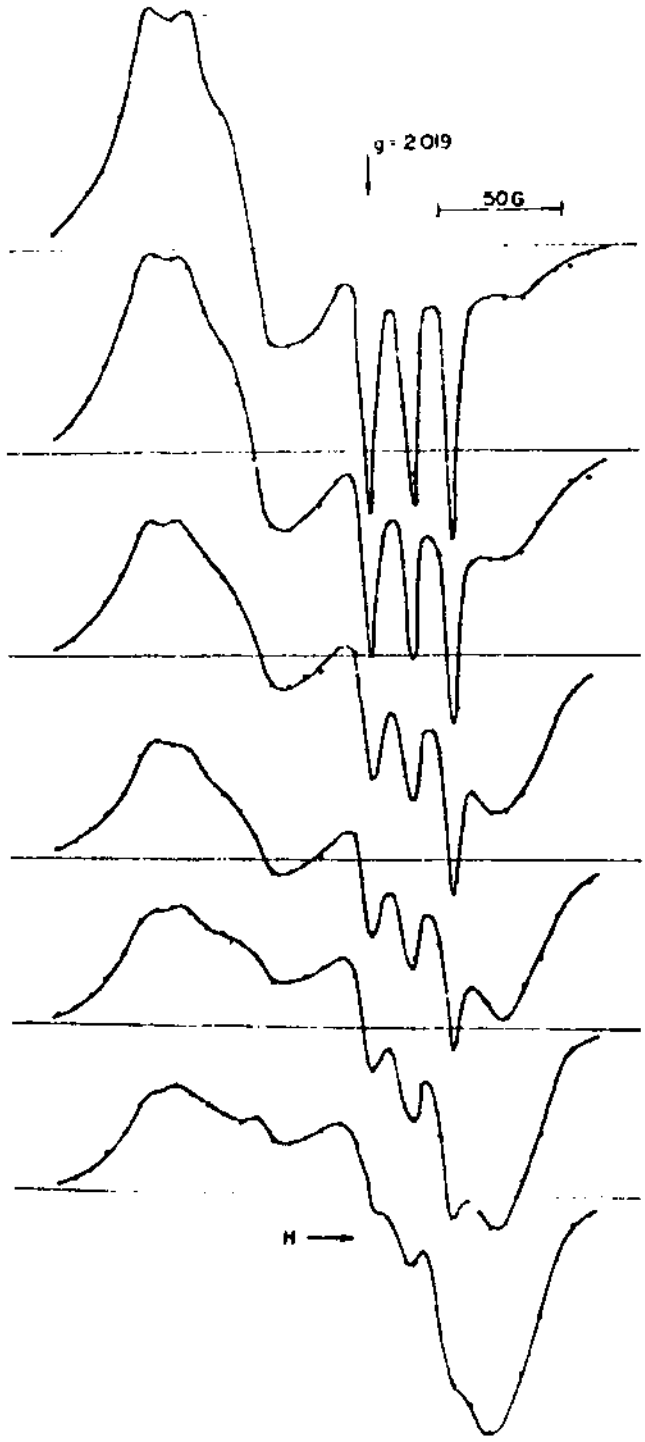



Fig. 5

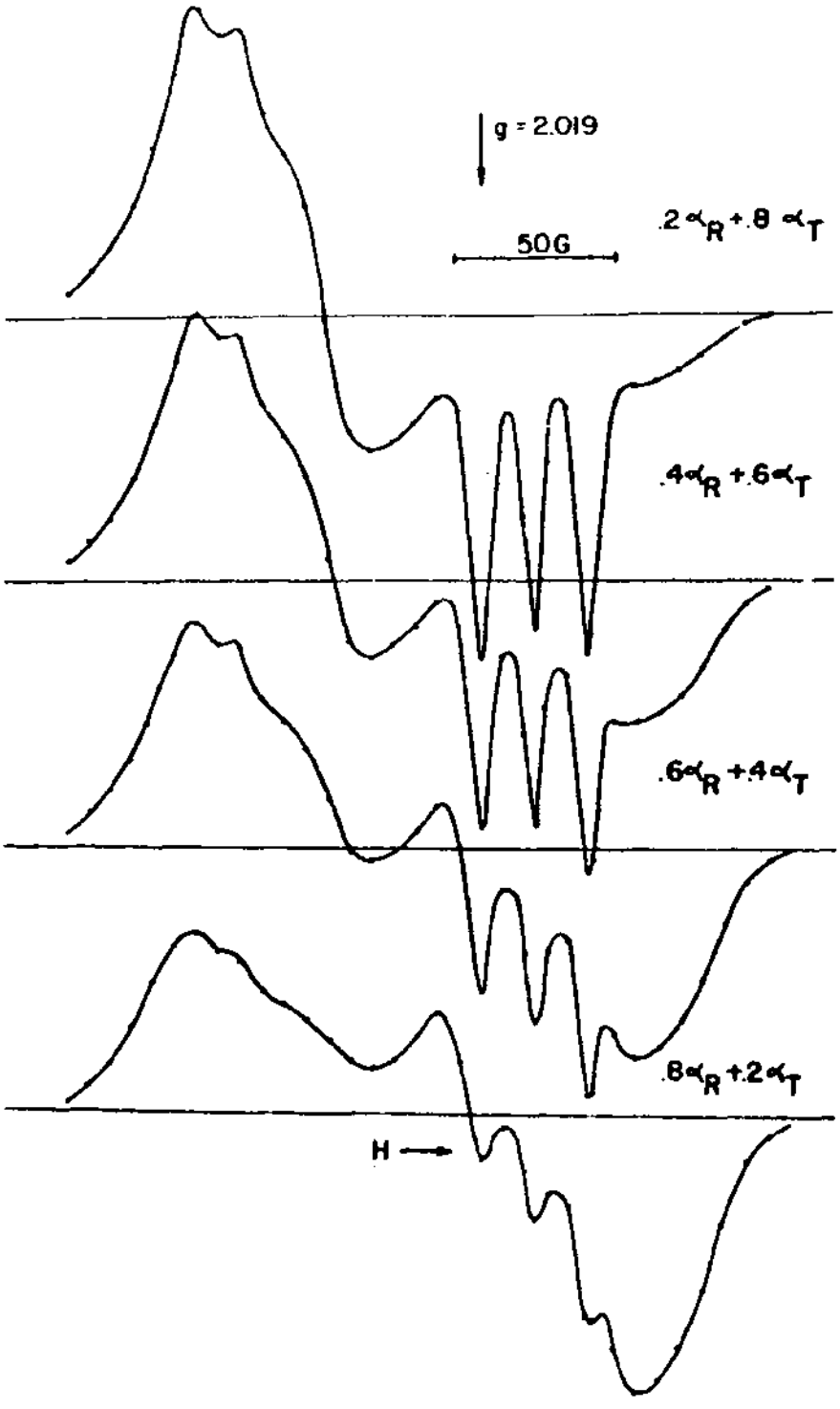



Fig. 6

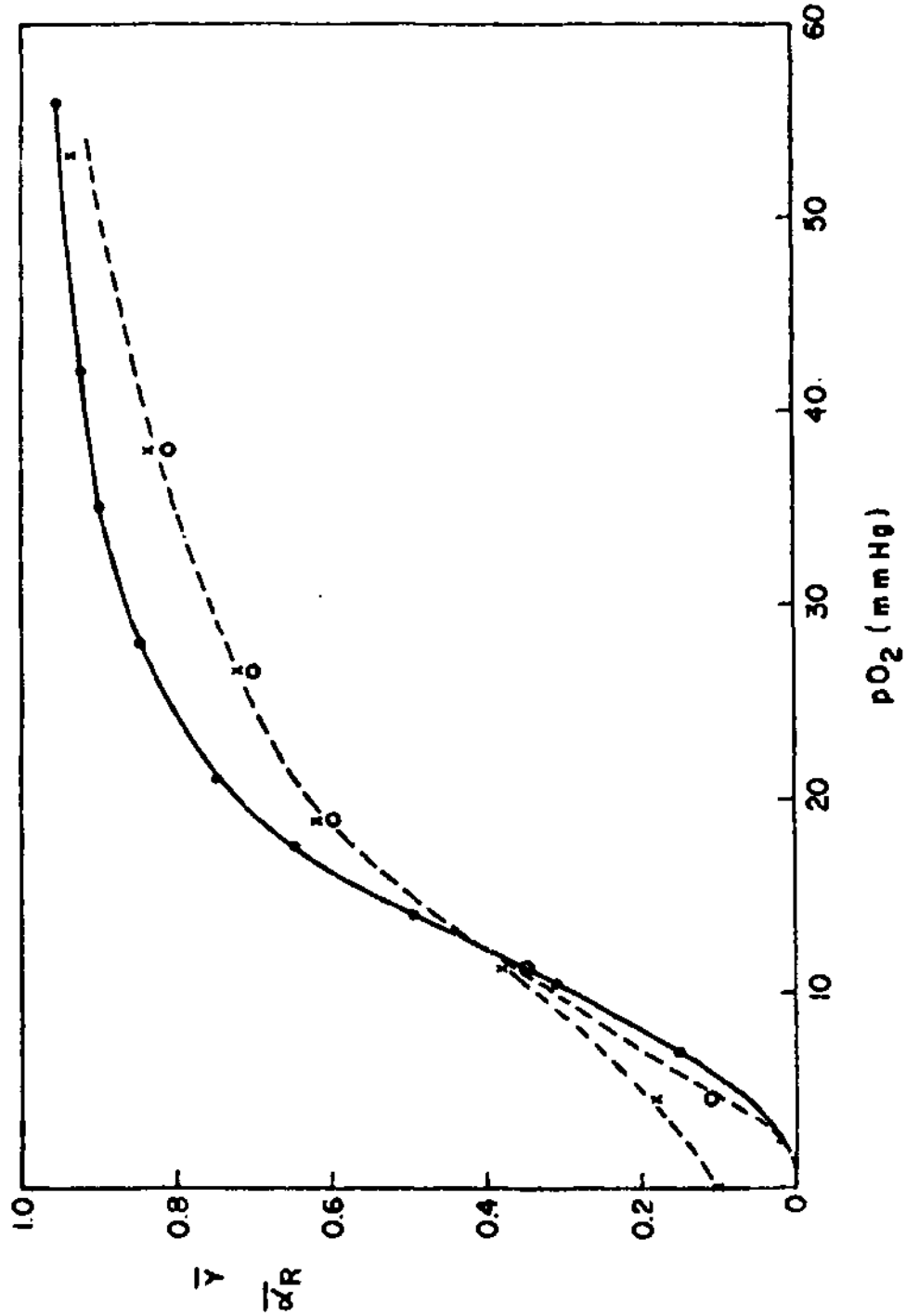

1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
$\overline{Y}$ $\overline{\alpha_R}$
$pO_2$ (mmHg)

Fig. 7