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Nota Científica 19/80

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Setembro 1980

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September 1980

ABSTRACT. EPR spectra of nitrosil-hemes were used to study the quaternary structure of hemoglobin. Human adult hemoglobin has been titrated with nitric oxide at pH 7.0 and 25°C. After the equilibration of NO among the α and β subunits the samples were frozen for EPR measurements. The spectra were fitted by linear combinations of three standard signals: the first arising from NO - β hemes and the other two arising from NO - α hemes of molecules in the high and low affinity conformations. The fractional amounts of α subunits exhibiting the high affinity spectrum fitted the two-state model (Edelstein, S.J. (1974) Biochemistry 13, 4998-5002) with $L = 7 \times 10^6$, and c^{α}_{NO} and $c^{\beta}_{NO} \sim 0.01$. Hemoglobin has been marked with nitric oxide at one chain using low-saturation amounts of nitric oxide. The EPR spectra were studied as a function of oxygen saturation. Linear combinations of the three standard signals above fitted these spectra. The fractions of molecules exhibiting the high affinity spectrum fitted the two-state model with $L = 7 \times 10^6$, $c_{O_2} = 0.0033$ and $c^{\alpha}_{NO} = 0.08$, instead of $c^{\alpha}_{NO} = 0.01$. Thus, the two state model is not adequate to describe the conformational transition of these hybrids. The results are evidence of the nonequivalence between oxygen and nitric oxide as ligands.

RESUMO. Os espectros de RPE de nitrosil-hemes foram utilizados para estudar a estrutura quaternária da hemoglobina. Hemoglobina humana foi titulada com óxido nítrico em pH 7.0 e 25°C. Estabelecido o equilíbrio da reação do NO com as cadeias α e β as amostras foram congeladas para realizar as medidas de RPE. Os espectros foram reproduzidos a partir de combinações lineares de três sinais padrão: o primeiro devido a NO - β hemes e os outros dois devidos a NO - α hemes de moléculas nas conformações de alta e baixa afinidade. As frações de cadeias α que exibem o espectro de alta afinidade reproduziram os resultados do modelo de dois estados (Edelstein, S.J. (1974) Biochemistry 13, 4998-5002) com parâmetros $L = 7 \times 10^6$ e c^{α}_{NO} e $c^{\beta}_{NO} \sim 0.01$. A hemoglobina foi marcada com óxido nítrico em apenas uma cadeia. Foram estudados os espectros de RPE em função da saturação com oxigênio. Combinações lineares dos três sinais padrão reproduziram esses espectros. As frações de moléculas que exibem o espectro de alta afinidade reproduziram os resultados do modelo de dois estados com parâmetros $L = 7 \times 10^6$, $c_{O_2} = 0.0033$ e $c^{\alpha}_{NO} = 0.08$, em vez de $c^{\alpha}_{NO} = 0.01$. Concluímos portanto que o modelo de dois estados não é adequado para descrever a transição conformacional desses híbridos. Os resultados apresentam uma evidência da inequivalência entre o oxigênio e o óxido nítrico como ligantes.

* Work partially supported by FINEP and CNPq.

INTRODUCTION

In the past several years the study of the properties of nitrosylhemoglobin and nitrosyl hybrids has assumed major importance for various reasons. First, HbNO is expected to be the closest analog to HbO_2 examined to date because nitric oxide and oxygen form similar low-spin complexes with iron, bind nearly isosterically to heme, and carry similar charge distributions[1]. Moreover, binding of NO to hemes produces paramagnetic species with spin 1/2 which can serve as chain specific spin-labels[2] and the EPR spectra of the α chains of HbNO are sensitive to the R and T quaternary structures[3,4,5].

In the absence of organic phosphates nitrosylhemoglobin is essentially in the high affinity quaternary state R. However in contrast to other liganded hemoglobins, inositol hexaphosphate (IHP) switches the allosteric equilibrium towards the deoxy, T structure. Changes in optical absorption, infrared, NMR and EPR spectra detect this transition[6]. The appearence of a well-resolved three-line hyperfine EPR structure has been associated with the α-hemes of hemoglobin in the T structure[5,7,8]. Szabo and Perutz[9] suggested that this signal is due to pentacoordinate NO-hemes and the interpretation of the infrared NO stretching frequencies in nitrosylhemoglobin by Maxwell and Caughey[10] supports this assignment. The EPR spectrum of the NO-hemes of β subunits does not depend on the quaternary structure[8].

Kinetic studies of Cassoly and Gibson[11] indicate a lack of cooperativity and chain nonequivalence in the combination rate constants of NO with hemoglobin. Hille et al.[12] found no evidence for the preferencial binding of NO to either chain in

rapid mixing and freezing EPR experiment. Henry and Cassoly[13] examined the question of chain nonequivalence and concluded that the apparent rate of NO binding to the α chains is four times faster than to the β chains. The difference in affinities of the chains for NO at equilibrium can be explained on the basis of a bigger dissociation rate constant for the β chains relative to the α chains[14]. Experiments using mixtures of nitrosylhemoglobin and deoxyhemoglobin in the presence of IHP confirmed a slow re-equilibration of NO among available heme sites, the equilibrium redistribution beeing attained about 10 min after mixing[14]. The cooperativity in the reaction of NO with hemoglobin arises from differences in the dissociation rate constants. The intrinsic rate constants for the dissociation of the first and fourth molecules of NO differ about 100-fold[15].

In the present work we have measured the dependence of the pentacoordinated α-heme EPR spectrum on the fractional degree of NO saturation at equilibrium and compared the results with the two-state allosteric model[16]. Although the equilibrium binding curve for NO could not be determined, the model provides the dependence of the fraction of the molecules in the R state on the fractional saturation. We have also used the EPR spectra of NO reacted α chains to follow the conformational transition during the oxygenation of hemoglobin marked with NO. This experiment was possible because of the slow rates of dissociation of nitric oxide as compared to oxygen.

MATERIAL AND METHODS

Human hemoglobin was obtained from fresh hemolysates

of erythrocytes washed three times with 0.9% NaCℓ. The stroma was eliminated with toluene. The hemoglobin solution was then passed through a Sephadex G-25 column equilibrated with 0.9% NaCℓ to remove organic phosphates and dialysed overnight in 0.05 M phosphate buffer pH 7.0. The resultant hemoglobin solution was placed in a tonometer, equipped with a septum-fitted injection port, which could be interchangeably adapted to spectrophotometric and EPR measurements. The solution was deoxygenated by repeated evacuation and flushing with nitrogen gas. The final concentration of protein and buffer was about 2 mM and 0.2 M respectivelly.

Nitric oxide gas was prepared by reacting nitric acid at 30% with metalic copper in the absence of oxygen. The gas was passed through a solution of 1M KOH, collected and stored, at a pressure somewhat higher than 1 atm, in a gas sampling bottle containing distilled watter, to avoid the presence of small amounts of NO_2 in the gas. Calculated volumes of NO gas were injected into the tonometer by gas tight S.G.E. microsyringes. Volumes of air were injected by clinical syringes with vacuum greased piston.

EPR spectra were obtained at -110° C using a Varian V-4500 spectrometer and a Varian F401 temperature controller. Typical experimental conditions were: microwave frequency, 8914 MHz; microwave power 20 mW; field modulation, 3 G; sweep-time, 100 G/min. Optical spectra were recorded in a Beckman-DK-2A spectrophotometer using a plastic cuvette with a glass spacer to provide a 0.2mm path.

For the fractional NO saturation measurements, the

sample was equilibrated during 20 min at 25°C, and frozen, after each NO addition. This time was considered more than sufficient for the NO reaction and redistribution because no change was observed in the EPR spectra afterwards. The time for complete redistribution for samples with IHP is 10 minutes[14]. In the R state, the dissociation rate constant is two orders of magnitude smaller and 10 minutes would be considered insufficient. However, on saturating with NO, the transition to the R state is attained at a high degree of saturation, when the fractions of α and β chains that have reacted with NO are almost equal, and the redistribution is therefore not an important factor.

For the measurements of oxygenation the samples were reacted and equilibrated with NO at a 5% saturation before addition of air. A different sample was used for each experiment to prevent NO_2 formation. Measurements of the decrease of the EPR signal with time at ambient temperature show that only 2 or 3% of NO molecules react with O_2 during the 3 minutes of equilibration with oxygen.

RESULTS

In order to analyse the EPR spectra obtained during the titration of deoxyhemoglobin with NO, we have obtained a set of standard normalized EPR spectra of β chains, and hexacoordinated and pentacoordinated α chains by the following procedure:

1. We have recorded the EPR spectra of isolated NO-β chains, NO-saturated hemoglobin, deoxyhemoglobin reacted with NO at 5%

saturation (Fig.1A) and the latter sample oxygenated and frozen rapidly (Fig.2B).

2. The standard spectrum of pentacoordinated α chains (designated α_T) was obtained by subtracting from Fig. 1A the fraction due to the β spectrum, corresponding to the contribution at $g \leq 2.009$. This procedure was based on three facts. In the first place the addition of IHP does not modify the spectrum of Fig.1A, in times short enough to prevent redistribution among chains. This indicates that the molecules are in the T conformation and only pentacoordinated α subunits are present. Second, the spectrum of pentacoordinated α chains has no contribution in the region of $g \leq 2.009$, as deduced from NO-hybrids[8] and from NO-Hb Milwaukee with IHP[17]. Third, the EPR spectra of NO-β isolated subunits and NO-β reacted hemoglobin are the same[13,18].

3. The hexacoordinated spectrum (designated α_R), normalized to the pentacoordinated spectrum, was obtained by subtracting from Fig.1B the same fraction of the β spectrum.

4. Finally, the β spectrum was normalized to the α_R spectrum using NO saturated hemoglobin to guarantee equal saturation of α and β chains.

Fig.2 shows the normalized α_T, α_R and β EPR spectra, obtained for two different samples, with an error of the order of the noise of the experimental spectra. The α_T spectrum is almost identical to the NO-Hb Milwaukee spectrum[17]. The ratio between NO reacted α and β chains at low saturation was calculated using the normalized α_T and β spectra. Five samples gave a medium value of 3.7, comparable with the value 4, obtained by Henry and Cassoly[13].

Dependence of EPR Spectrum on the Fractional Saturation with Nitric Oxide

EPR spectra were recorded at various degrees of NO saturation (Fig.3). The low dissociation constant of NO permits to obtain the fractional saturation directly from the total amount of NO added. The absence of changes in the shape and intensity of the spectrum as the amount of NO added exceeds the calculated value for complete saturation confirmed the validity of the calculations.

The experimental spectra were fitted by linear combinations of the α_T, α_R and β spectra indicating a two-state transition. In order to compare the experimental results with the two-state model we have used the Edelstein's version [16] which takes into account the nonequivalence of α and β chains. The relative occurence of each state can be expressed in terms of a and b defined as $a = (X)/K_R^\alpha$, and $b = (X)/K_R^\beta$, where K_R^α and K_R^β are the intrinsic R state dissociation constant of the α and β chains for the ligand X, respectively. The saturation expressions for the α and β chains can be obtained separately:

$$\bar{Y}_\alpha = \frac{a(1+a)(1+b)^2 + Lc^\alpha a(1+c^\alpha a)(1+c^\beta b)^2}{(1+a)^2(1+b)^2 + L(1+c^\alpha a)^2(1+c^\beta b)^2} \qquad (1)$$

$$\bar{Y}_\beta = \frac{b(1+b)(1+a)^2 + Lc^\beta b(1+c^\beta b)(1+c^\alpha a)^2}{(1+a)^2(1+b)^2 + L(1+c^\alpha a)^2(1+c^\beta b)^2} \qquad (2)$$

and the total saturation is given by

$$\bar{Y} = \frac{1}{2}(\bar{Y}_\alpha + \bar{Y}_\beta) \qquad (3)$$

where $c^{\alpha} = K_R^{\alpha}/K_T^{\alpha}$ and $c^{\beta} = K_R^{\beta}/K_T^{\beta}$ are the relative affinities of α and β chains, and L is the allosteric equilibrium constant for unliganded conformations.

The fractional amount of chains exhibiting the EPR spectrum characteristic of the R conformation is given by:

$$\bar{I}_{\alpha_R} = \frac{a(1+a)(1+b)^2}{a(1+a)(1+b)^2 + Lc^{\alpha}a(1+c^{\alpha}a)(1+c^{\beta}b)^2} \qquad (4)$$

According to the experimental results $K_T^{\beta}/K_T^{\alpha} \simeq 4$, for NO. The constant c for NO is about 0.01[15,19]. L is independent of the nature of the ligand and values about 10^6 - 10^7 have been quoted[15,21-23].

In obtaining the experimental spectra as a sum of contributions from α_R, α_T and β, we have noted that the error in the contribution of α_T is small, because this spectrum is quite different from the α_R and β spectra. However the α_R and β spectra are not so different from each other and linear combinations with coefficients differing by 30% produce similar spectra, within the experimental error. To make consistent the comparison of the experimental results with the model, the contribution of the β chains to the spectrum was obtained from the curve $\bar{Y}_{\beta} \times \bar{Y}$ given by the model, for each set of parameters L, c_{α} and c_{β}.

Fig.3 shows the quality of the fit. The contribution of β chains was taken from the curve of Fig.4A and the contributions of α_T and α_R were ajusted to give the best fit to the experimental spectra. It is worth noting that Fig.4A fits quite well the results of Huang[23] for the percentage of

ligation of β subunits calculated from the percentage of decrease of a β NMR peak at pH 7.0. The fractions of the α_R spectrum, calculated from the fits of Fig.3, are plotted against the fractional saturation (Fig.4B). The two-state model fits the results with parameters $L = 7 \times 10^6$, $c^\alpha = c^\beta = 0.01$ considering $K_R^\beta/K_R^\alpha = .4$ or $c^\alpha = 0.02$ and $c^\beta = 0.005$ considering $K_R^\beta/K_R^\alpha = 1$. If the product $c_\alpha c_\beta$ is mantained, i.e., if the ratio of T to R populations for saturated molecules is the same, the curve of $\bar{I}_{\alpha_R}$ versus $\bar{Y}$ is almost insensitive to the ratio K_R^β/K_R^α.

The dependence of the hyperfine EPR signal on fractional saturation is compatible with the two-state allosteric model. Since the parameters L and c's are inside the range of the currently accepted values we can conclude that this signal is a good spectral monitor of the quaternary state T.

Oxygen Binding to NO Marked Hemoglobin

Nitric oxide was used as a marker of the quaternary structure for the oxygenation of hemoglobin. At low fractional NO saturation (~ 5%) the probability of finding molecules with more than one reacted chain is very small. The dissociation constant for NO (10^{-11} - 10^{-12} M) is much smaller than for O (10^{-4} - 10^{-6} M). On oxygenating the marked samples, the NO is fixed at one chain and the changes in the EPR signal reflect the changes in the quaternary structure induced by oxygen binding.

EPR spectra were obtained at various oxygen pressures.

These spectra were fitted by linear combinations of the α_R, α_T and β spectra (Fig.5). The contributions from β chains were calculated from the spectra of the samples before addition of air, when only α_T and β species were present. In these experiments the species that exhibit EPR signal have one subunit locked with NO. Assuming that the two-state allosteric model applies, the allosteric equilibrium constant in the absence of O_2 is $L' = T_1/R_1$, where T_1 and R_1 refers to the quaternary states of molecules with one ligand. Assuming chain equivalence in the reaction of oxygen with hemoglobin, the fraction of molecules in the R state is[24]:

$$\bar{R}(3) = \frac{(1+a)^3}{(1+a)^3 + L'(1+c_{O_2}a)^3} \qquad (5)$$

and the fraction of sites bound by O_2 is:

$$\bar{Y}(3) = \frac{a(1+a)^2 + Lc_{O_2}a(1+c_{O_2}a)^2}{(1+a)^3 + L'(1+c_{O_2}a)^3} \qquad (6)$$

where $a = (O_2)/K_R$, $c_{O_2} = K_R/K_T$ and K_R and K_T are the intrinsic dissociation constants of O_2 for the R and T states.

In the samples used, only the molecules with a NO- reacted α chain exhibit a conformation dependent EPR signal. For these molecules the constant L' is:

$$L' = c_{NO}^{\alpha} L$$

The fraction of sites bound by oxygen, $\bar{Y}(3)$, is not directly accesible because optical absorption detects the oxygenation of both NO-reacted and unreacted molecules. We

have therefore plotted the fractions of α_R calculated from the spectra of Fig.5 versus the fractional saturation $\bar{Y}$, in the absence of NO, obtained at the same experimental conditions of pH, buffer, temperature and concentration. The fractional saturation calculated from the two-state model is:

$$\bar{Y} = \frac{a(1+a)^3 + c_{O_2} a(1+c_{O_2} a)^3}{(1+a)^4 + L(1+c_{O_2} a)^4} \qquad (7)$$

The dependence of the fraction of α_R and the fractional saturation $\bar{Y}$ on the partial oxygen pressure are plotted in Fig.6A. Fig.6B shows the fraction of α_R versus fractional saturation. The theoretical curves calculated from equations (5) and (7) show that the value $c_{NO}^{\alpha} = 0.08$ fits the experimental results. This value is much greater than 0.01, found by Moore and Gibson[15] or 0.02 that fitted the results of saturation with NO. The simple two-state model, therefore does not apply. Oxygen binds to $Hb_4(NO)$ and to $Hb_4(O_2)$ in different ways that cannot be explained simply by the difference between c_{NO} and c_{O_2}.

DISCUSSION

The Two-State Model and the Reaction of Hemoglobin with NO

At low saturation the EPR spectrum of NO reacted hemoglobin is not influenced by the addition of IHP before re-equilibration of NO among the α and β chains. If any hexacoordinated α chain were present in the sample IHP would induce a transition to pentacoordinated species. We then

conclude that in the low affinity conformation of hemoglobin only pentacoordinated α-hemes occur.

Associating the EPR spectrum of pentacoordinated α-hemes to the T conformation, we have concluded that the dependence of the equilibrium ratio of the R and T populations on the fractional saturation with NO satisfies the two-state model. These results are in direct conflict with the results of Hille et al[25], which indicate an equilibrium between hexa- and pentacoordinated NO-α hemes in the low affinity T conformation. From the analysis of the EPR spectra they have concluded that the two-state model is inadequate. In their experiment a rapid mixing device was used for the titration with nitric oxide and the samples were frozen 20 seconds after mixing, before equilibration of NO among the α and β subunits. This process ensures equal populations of reacted α and β chains. However the analysis of the results were based on the assumption of homogeneous mixing. The half-time for the binding of NO at the concentrations used is about 0.2 ms[25]. The 4-jet tangential mixer used ensures the generation of homogeneus solutions within 2-3 ms after mixing. It is clear then, that the samples were not homogeneus at the time of binding. This would explain why, even at low NO saturation, a fraction of hexacoordinated α-NO hemes was present. Inhomogeneus mixing would have produced highly saturated molecules, increasing the population of the R conformation. This invalidates the conclusion that the two-state model is inadequate.

We shall discuss the validity of associating the EPR spectra of α subunits to the quaternary structures of hemoglobin.

The parameters L, c^{α} and c^{β} of the two-state model that fitted our results are inside the range of the currently accepted values. There is some ambiguity in the values of c^{α} and c^{β} since the model is insensitive to the ratio K_R^{β}/K_R^{α}, provided that $(c^{\alpha}c^{\beta})^{1/2}$ remains equal to 0.01. The ratio K_R^{β}/K_R^{α} cannot be very different from unity, since chain nonequivalence was not noted in experiments on dissociation of nitric oxide from nitrosylhemoglobin in the absence of IHP[15,19].

According to the values of L, c^{α} and c^{β} that fitted our results, the equilibrium constant between the fully liganded T and R conformations is: $T_4/R_4 = L(c^{\alpha}c^{\beta})^2 = 0.07$. This value is about the maximum permitted by the experimental error in the EPR spectra since the spectrum of nitrosyl hemoglobin is caracteristic of the R state, and confirms the observation of a small fraction of the T state in $Hb_4(NO)_4$ molecules[19,26]. Since L must be independent of the ligand, and a value of about 0.01 was found for c_{NO} by kinetic experiments[15], independently of the association of spectroscopic markers to the R or T structures, we have concluded that the EPR spectrum of pentacoordinated α hemes is a good marker for the T quaternary structure of hemoglobin.

On the other hand it is not clear why the NO-β chains are not sensitive to the R→T structural transition. The ratio between the dissociation rate constants of NO from hemoglobin in the T and R states is about 10^2, and has been associated to the formation of pentacoordinated α hemes. However, β-hemes are always hexacoordinated and the dissociation rate constants

vary by a factor of the same magnitude. Therefore, there exist changes in the β subunits, important enough to alter the rate constants, which remain undetected by EPR. The same may be true for the α chains, in which case the two-state model would become only an approximation.

Implications of the Results of Oxigenation of NO-Marked Hemoglobin

The oxygenation of NO-marked hemoglobin shows the R population displaced to higher oxygen pressures, when compared to the results predicted by the two-state model.The value 0.08 for c_{NO}, which fitted these results, is considerably higher than the values obtained by saturation with NO.Considering even the ambiguity in c_α and c_β, $c_\alpha = 0.08$ is highly improbable since it would require $K_R^\alpha/K_R^\beta = 1/16$. This ratio equals 1/2 for isolated chains[15]. It seems, therefore, that the T population of $Hb_4(NO)$ is higher when reacting with O_2 than with NO. Gibson and co-workers[15,27] have already pointed out results which indicated that the R and T states, defined on the basis of their functional behavior with a ligand, depend on the nature of that ligand.

The tertiary structures of the various liganded hemoglobins in the R state differ in details[28]. The tertiary structures of liganded and unliganded T states also differ. Gelin and Karplus concluded that the perturbation on the α-heme caused by the ligand is propagated through the globin along a narrow and well-defined pathway toward the $\alpha_1\beta_2$ interface[29]. These perturbations can propagate through other chains.Although

Perutz maintains that there can be only two quaternary structures R and T[30], Viggiano and Ho proposed that any stretching or weakening of intersubunit linkages would produce a new quaternary structure[31]. Taking this point of view, each quaternary structure (called R or T) could really englobe a set of quaternary structures having minor differences, which can vary according to the nature of the ligand and number of ligands. Our results are consistent with this point of view. This work presents an evidence of the nonequivalence between oxygen and nitric oxide as ligands which could invalidate the use of nitrosylhemoglobin as an analog to oxyhemoglobin.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support of FINEP and CNPq, as well as the cooperation of Dr. Cláudio Siqueira and Eliane Wejnberg.

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FIGURE CAPTIONS

Figure 1 - EPR spectra of NO-hemoglobin at 5% saturation, pH 7.0, T = -110°C: A. Deoxygenated sample. B. Oxygenated sample.

Figure 2 - Normalized EPR spectra, obtained by the procedure described in the text: A. α_T, α chains of hemoglogin in the T conformation. B. α_R, α chains of hemoglobin in the R conformation. C. β chains.

Figure 3 - EPR spectra of NO-hemoglobin at various degrees of NO saturation: • , represent the spectra calculated as linear combinations of the spectra of Fig.2, with coeficients f_{α_T}, f_{α_R}, f_β.

A. $\bar{Y} = 0.20$, $f_\beta = 0.26$, $f_{\alpha_T} = 0.71$, $f_{\alpha_R} = 0.05$

B. $\bar{Y} = 0.29$, $f_\beta = 0.43$ $f_{\alpha_T} = 0.92$, $f_{\alpha_R} = 0.13$

C. $\bar{Y} = 0.39$, $f_\beta = 0.50$, $f_{\alpha_T} = 0.86$, $f_{\alpha_R} = 0.21$

D. $\bar{Y} = 0.48$, $f_\beta = 0.57$, $f_{\alpha_T} = 0.78$, $f_{\alpha_R} = 0.28$

E. $\bar{Y} = 0.65$, $f_\beta = 0.85$, $f_{\alpha_T} = 0.73$, $f_{\alpha_R} = 0.46$

F. $\bar{Y} = 0.80$, $f_\beta = 0.69$, $f_{\alpha_T} = 0.29$, $f_{\alpha_R} = 0.55$

G. $\bar{Y} = 0.90$, $f_\beta = 0.84$, $f_{\alpha_T} = 0.20$, $f_{\alpha_R} = 0.75$

Figure 4 - A. Fractional saturation of β chains, $\bar{Y}_\beta$, versus total fractional saturation, $\bar{Y}$. —— curve calculated using the expressions 2 and 3 of the two-state model with the same parameters as in B.

● , experimental results of Huang [23] at pH 7, without IHP.

B. Fraction of the α_R spectrum, $\bar{I}_{\alpha_R}$, versus total fractional saturation, $\bar{Y}$. —— , curve calculated using the expressions 3 and 4 of the two-state model with parameters $L = 7 \times 10^6$, $c^\alpha = c^\beta = 0.01$ for $K_R^\alpha/K_R^\beta = 0.25$ or $c^\alpha = 0.02$, $c^\beta = 0.005$ for $K_R^\alpha/K_R^\beta = 1.$ + , calculated from the results of Fig.3.

Figure 5 - EPR spectra of NO-hemoglobin at approximately 5% NO saturation, at various oxygen pressures. ● , represent the spectra calculated as linear combinations of the spectra of Fig.2, with coefficients f_{α_T}, f_{α_R}, f_β.

A. pO_2 = 8.5 mm Hg, $f_\beta = 0.21$, $f_{\alpha_T} = 0.73$, $f_{\alpha_R} = 0.05$

B. pO_2 = 17 mm Hg, $f_\beta = 0.25$, $f_{\alpha_T} = 0.81$, $f_{\alpha_R} = 0.22$

C. pO_2 = 25 mm Hg, $f_\beta = 0.41$, $f_{\alpha_T} = 0.67$, $f_{\alpha_R} = 0.41$

D. pO_2 = 33 mm Hg, $f_\beta = 0.38$, $f_{\alpha_T} = 0.52$, $f_{\alpha_R} = 0.73$

E. pO_2 = 38 mm Hg, $f_\beta = 0.39$, $f_{\alpha_T} = 0.45$, $f_{\alpha_R} = 0.85$

Figure 6 - A. ✱ - fractional saturation with oxygen as a function of a (defined in the text) and pO_2: —— , curve obtained using the expression 7 with parameters $L = 7 \times 10^6$ and $c_{O_2} = 0.0033$; ● , experimental points.

1,2,3 and 4 are curves of the fraction of molecules in the R state, $\bar{R}^{(3)}$, calculated using

the expression 5 with the same parameters as ★ , and c^{a}_{NO} = 0.02, 0.05, 0.08, 0.1, respectively; + , experimental points from the results of Fig.5. B. Fraction of molecules in the R state, $\bar{R}^{(3)}$, versus fractional saturation with oxygen $\bar{Y}$, calculated from the expressions 5 and 7, with the same parameters as in A; + , experimental points.

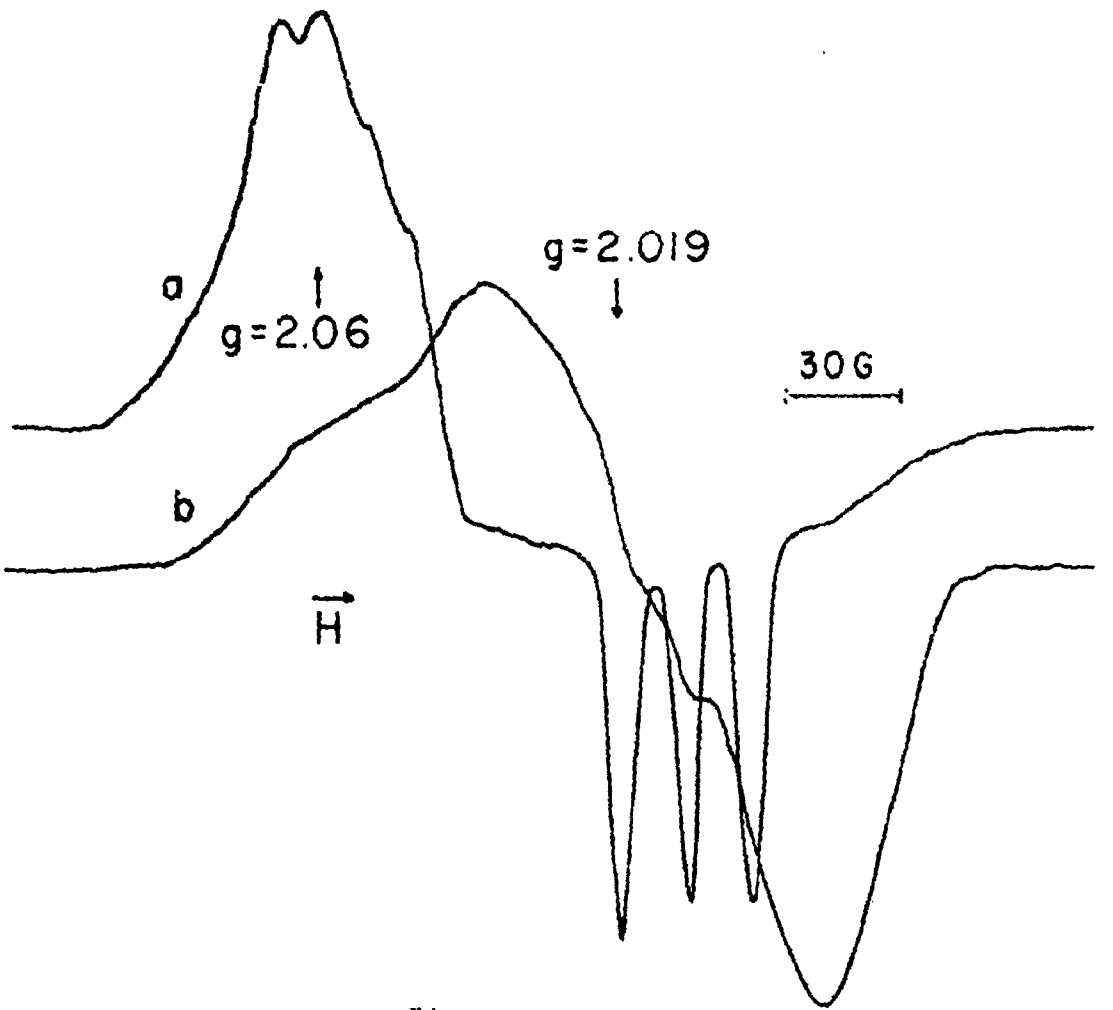

Figure 1

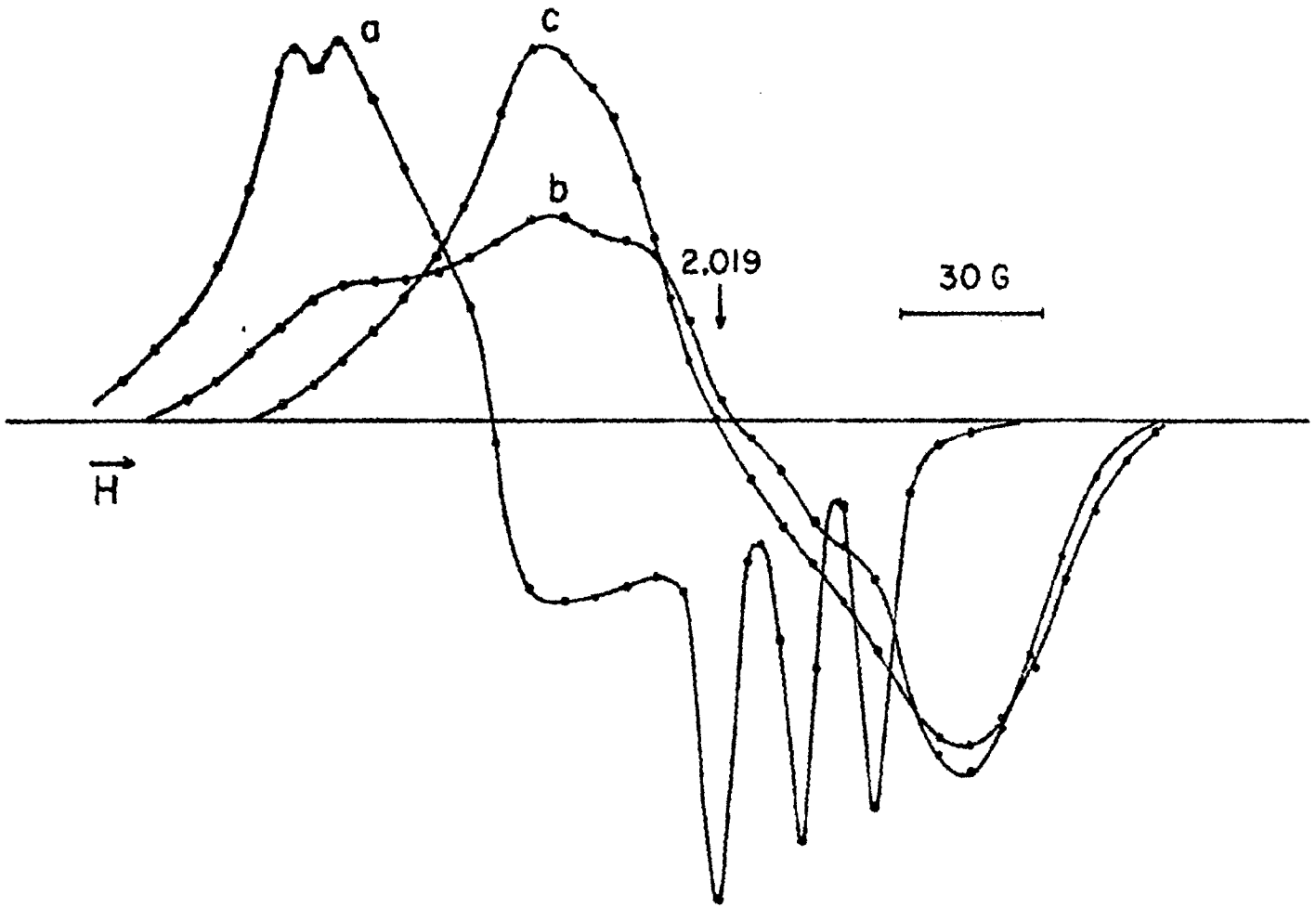

Figure 2

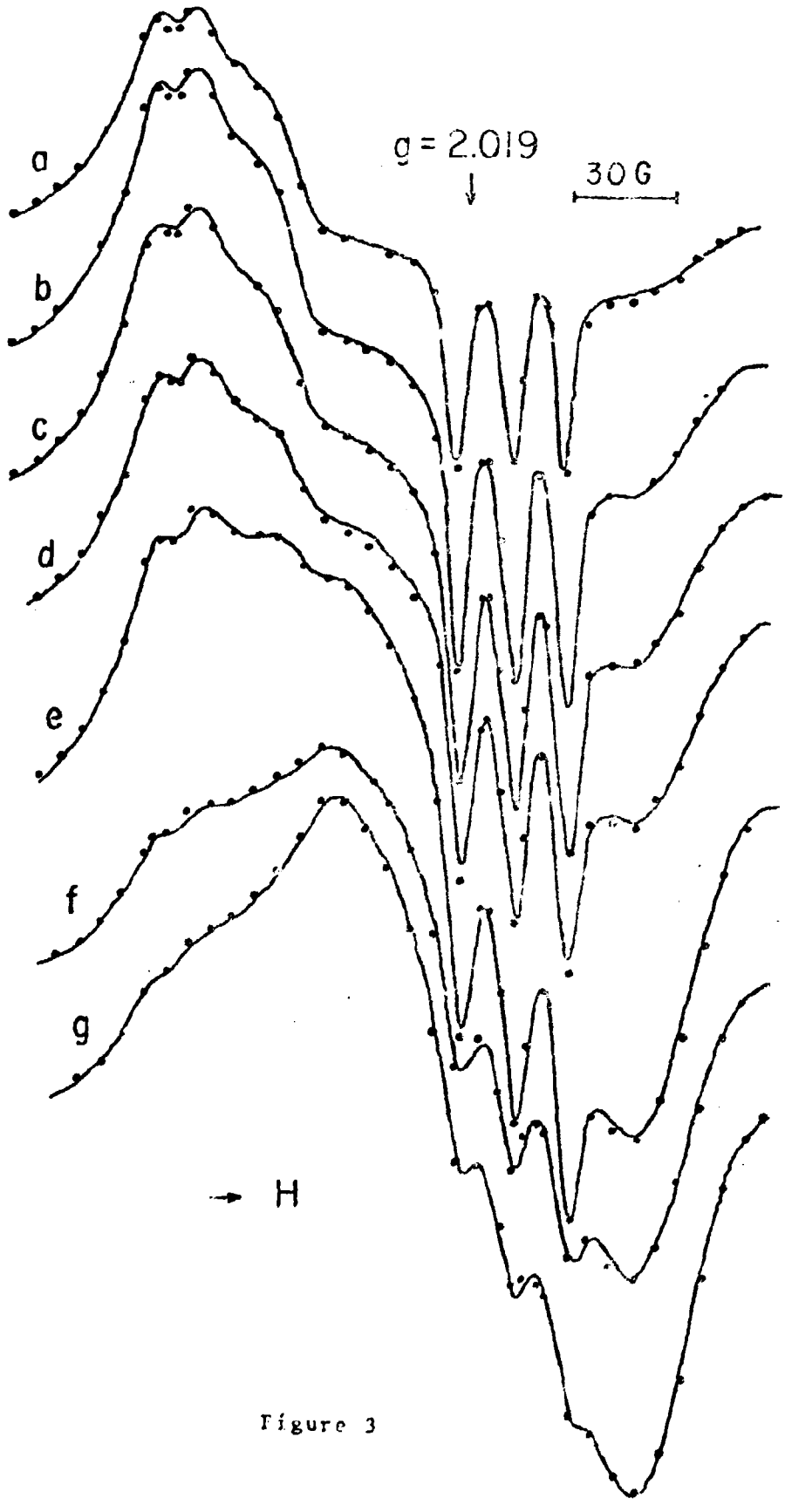

Figure 3

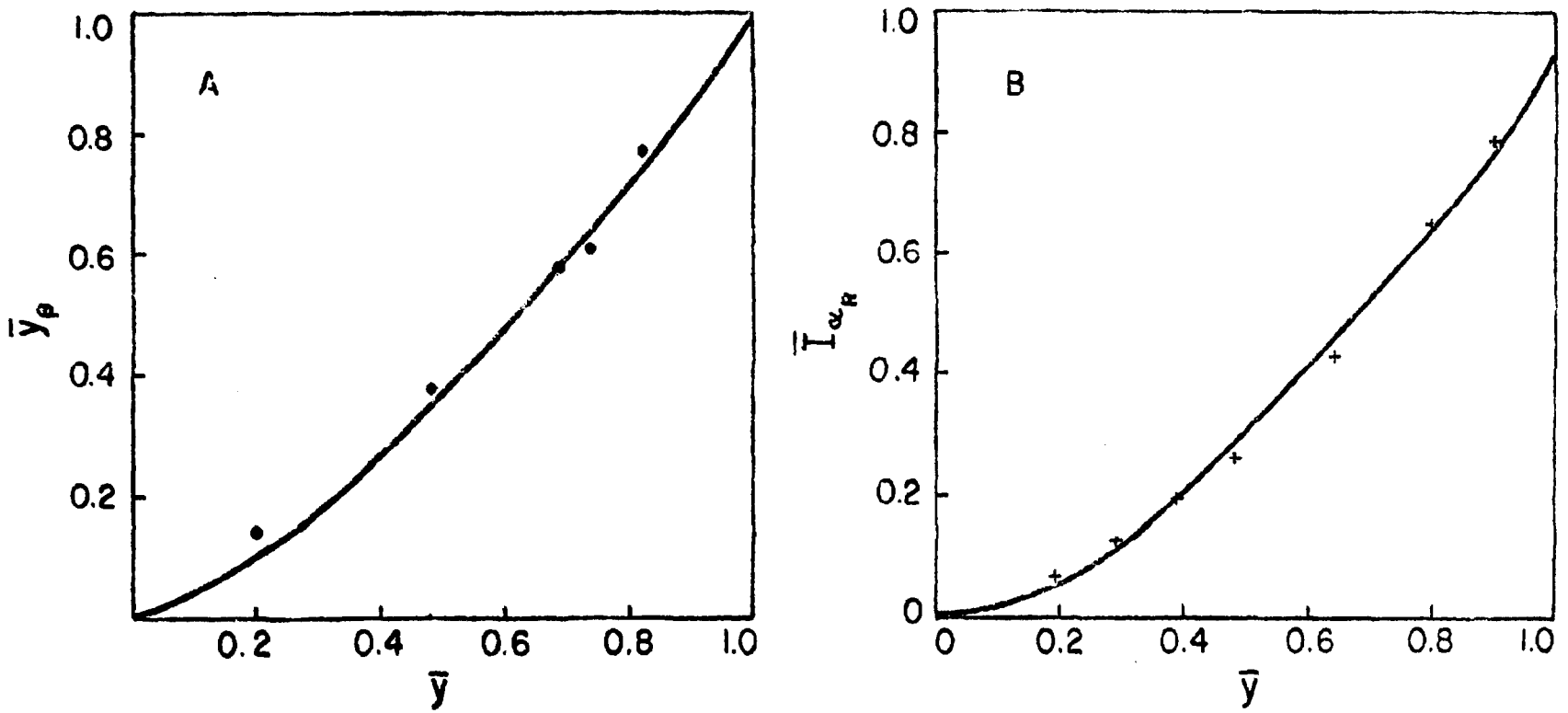

Figure 4

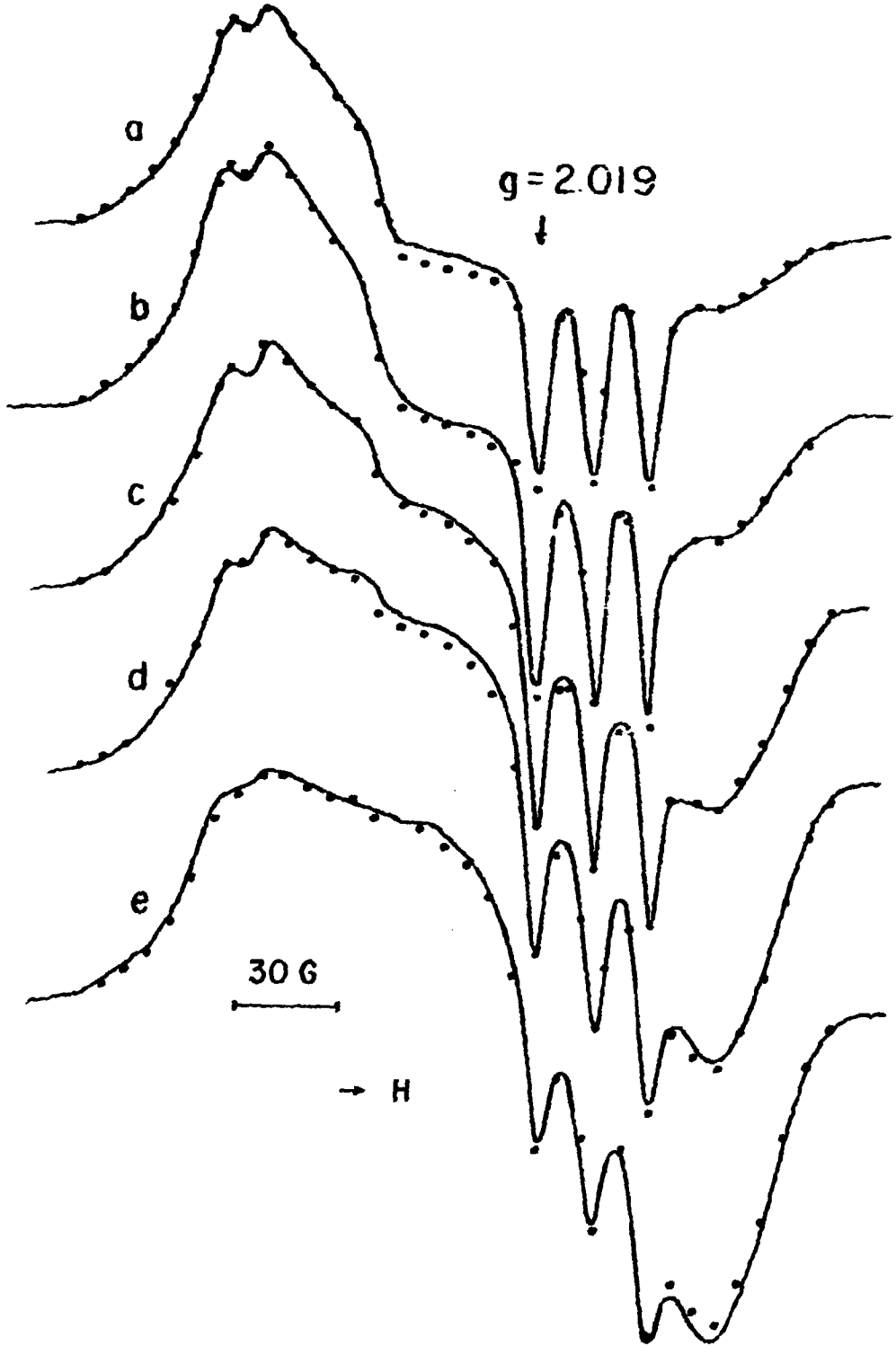

Figure 5

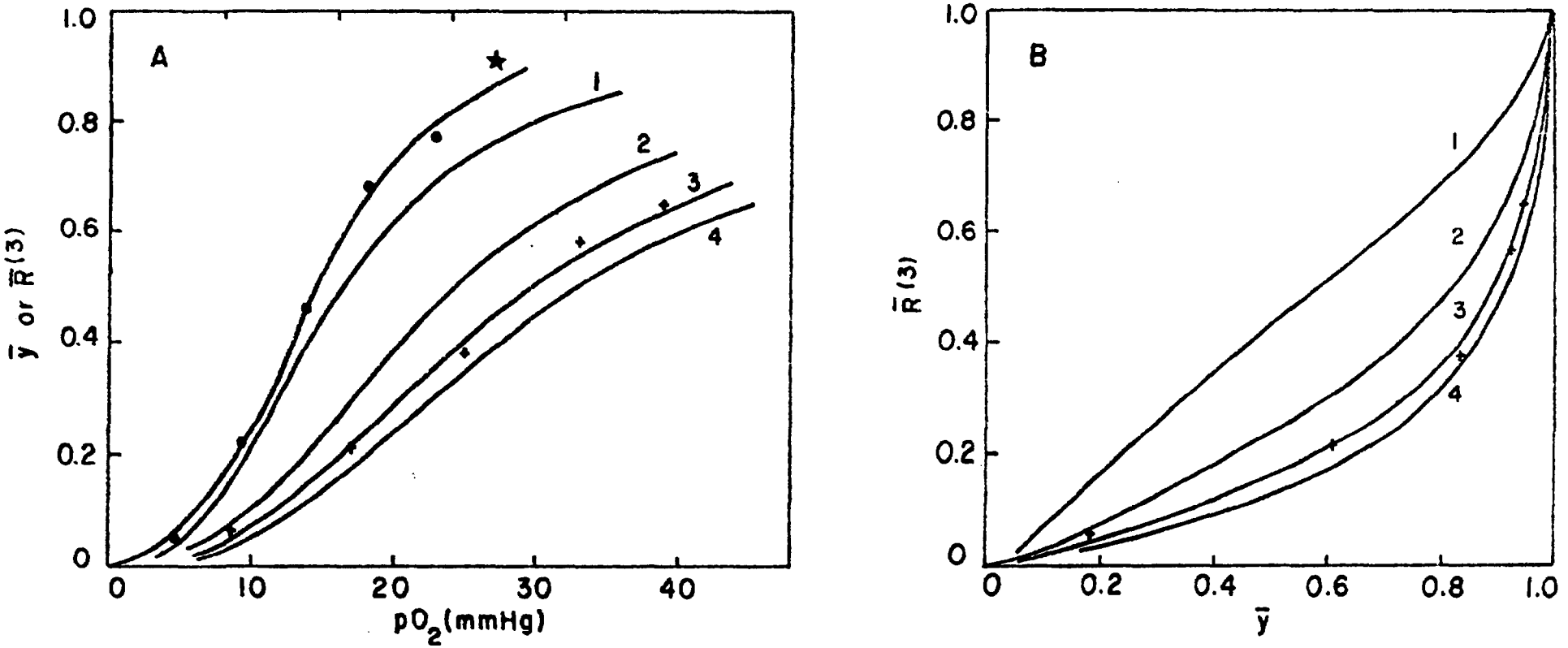
A
B
1
2
3
4
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
$\bar{y}$ or $\bar{R}(3)$
pO_2(mmHg)
$\bar{R}(3)$
$\bar{y}$
0.2
0.4
0.6
0.8
1.0

Figure 6