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EPR EVIDENCE FOR THE EXISTENCE OF A NEW HEMICHROME IN HEAT DENATURED MetHb

by

Odivaldo Cambraia ALVES and Eliane WAJNBERG

Abstract

Electron Paramagnetic Resonance was used to follow the time dependence of heat denaturation of met- and nitrosyl-hemoglobin (metHb and HbNO) at 60° C, 70° C and 80° C. The spectral changes of both complexes indicate that conformational changes in the protein manifest themselves in changes of the equilibrium of hemichromes in metHb and of six- and five-coordinated iron in HbNO. The formation of a hemichrome with $g = 2.45$, 2.27 and 1.85 not described before is observed. A his-Fe-cys complex is proposed for its structure.

Key-words: Denaturation; Hemichrome; EPR; Hemoglobin.

Introduction

Native protein molecules are known to be folded into well defined three-dimensional structures. These structures are usually stable over a fairly wide range of external conditions, but they can be disrupted by sufficiently drastic changes in physical or chemical environments. This process is known as denaturation and it is very important to the understanding of structural and functional aspects of proteins.

During the last sixties the interest on protein denaturation was intense [1–6]. For a period of 15 years it decreased, but the development of news techiniques and theoretical models for the study of proteins have revived this interest [7–13]. There is a large number of theoretical papers in this field, in which the thermodynamic characteristic of the process [7,11] and the role of the adsorbed water [9,12,13] are discussed. Experimental results should establish the applicability of these theoretical models.

Although Electron Paramagnetic Resonance (EPR) is restricted to paramagnetic systems, it has the advantage of not being perturbed by the precipitation of proteins during the denaturation which makes the use of optical techniques difficult. EPR has shown that during heat denaturation of oxyhemoglobin (oxyHb) there occur three events: oxidation to methemoglobin (high spin ferric), the formation of low spin species and under drastic conditions further changes in these species [1].

It was also observed that the high spin form of different types of hemoglobin and

of their α and β subunits is converted, either spontaneously or under the influence of external denaturants such as urea and salicylate, into several low spin forms. These forms are collectively called hemichromes and are brought about through discrete reversible and irreversible changes of protein conformations so that atoms endogenous to the protein become bound as the sixth ligand of the heme iron [5] .

HbNO is a sensitive probe to the heme environment of the protein. It has been reported that the effect of different concentrations of detergent on the EPR signals of nitrosyl hemoglobin (HbNO) is similar to that observed in the transition from the R to the T structure and in the dehydration process of the protein. This result was attributed to the weakening of the fifth bond of the NO–heme complex. Loosening of the protein structure due to the combined effect of eletrostatic and hydrophobic attractions between the detergent molecules and the protein is responsable for this change [14,15].

This work seeks more definite information concerning the denaturation of hemoglobin. The time dependence of heat denaturation of metHb and HbNO at three different temperatures: 60°C, 70°C and 80°C was studied using EPR spectroscopy. The use of different denaturation temperatures allows for a better description of the process. At low temperatures the beginning of the process is clarified while the final state is better characterized at high temperatures. Computational facilities allow for a quantitative analysis of components from the complex spectra. Changes of the equilibrium of EPR components were observed in the denaturation process for both complexes.

Tests to characterize the involvement of the β^{93} SH-group were perfomed by blocking the SH groups in native Hb. To examine further the dehydration effect and the structural changes of heme iron (spin state and symmetry) during the heat treatment of Hb we have investigated the lyophilized metHb and hematin (an iron porphyrin) subjected to the same treatment. Characteristic times of the process were determined.

Materials and Methods

Hemoglobin solution was obtained by hemolysis of human blood. Oxidation to metHb was accomplished by reaction with excess of potassium ferricyanide ($K_3[Fe(CN)_6]$), followed by dialysis in 0.06 M phosphate buffer pH 7.0. The samples were lyophilized in a Labconco 75200 Lyophilizer.

N-ethylmaleimide (NEM) was used to block the reactive sulphydril in hemoglobin. Reaction with NEM was carried out at pH 7.0 with a ratio of 4 NEM : 1 Hb. In this condition only β^{93} sulphydril is blocked [16].

HbNO was prepared in a tonometer by reaction of deoxygenated hemoglobin solution with nitric oxide gas (prepared from the reaction of nitric acid with metallic copper). The addition of NO was made under nitrogen (N_2) atmosphere.

Hemoglobin samples were heated by incubation in a ultrathermostatic water bath (Fanem-111) at the desired temperature T (60°C, 70°C and 80°C with an error of about

1°C). The sample temperature was measured with a Chromel-Constantan termocouple and after a very short time ($\simeq 1min$) equilibrium with the bath was reached. The heating denaturation time t was measured relative to this thermal equilibrium condition taken as $t = 0$. The samples were removed from the bath at different times and cooled by immersion in liquid nitrogen in order to stop the denaturation process.

EPR measurements were perfomed with an (X–band) EPR spectrometer Varian E–9, 100 KHz modulation. Spectra were taken at 200 mW of microwave power and at 80 $\pm$ 2K. Modulation amplitude was limited to avoid broadening of the lines. Temperature was measured by a Chromel-Constantan thermocouple just above the EPR cavity and controlled by a pre-cooled nitrogen flux.

The double numerical integration of the EPR spectra was obtained by the trapezium method. Corrections of spectral baseline were applied. In order to compare the number of molecules with different Fe^{3+} spin states (S=5/2 and S=1/2) corrections were made taking into account the area dependence on the g factor [17], the temperature dependence of Kramer's doublets population due to zero field spliting [18] and the magnetic field range. The scan range correction is more complicated in the case of the high spin met form spectrum because at X band the EPR signal is spread out from 1100 Gauss ($g \simeq 6$) to 3200 Gauss ($g \simeq 2$) and a superposition with hemichrome signals occurs in the high field . However, the high spin low field signal contribution is approximately a factor of 18 higher than contribution of the high field region [19]. For the high spin

component a scan range from 700 to 1700 Gauss was used. The areas of hemichrome signals were obtained with a scan range from 1700 to 3700 Gauss. For the NO complex only the g factor dependence had to be considered.

Results

In figure 1 EPR spectra of human metHb at different denaturation times and temperatures are presented. The spectra show an absorption with $g = 6$ due to component perpendicular to heme symmetry axis of Fe^{3+} (spin state 5/2) [20]. In the high field range the spectra of the denatured samples are characteristic of spin state 1/2 of iron Fe^{3+} with: $g = 2.9$, $g = 2.45$, $g = 2.27$, $g = 1.91$ and $g = 1.85$ [6,21].

At the three heating temperatures studied one observes a decrease of the high spin line intensity without changes in its shape as well as the appearance of the low spin spectrum with $g = 2.27$ and 1.91 predominating. This is followed by the decline of this component, appearance of lines at $g = 2.45$ and $g = 1.85$ and broadening of the $g = 6$ line. Samples with higher degree of denaturation show a $g = 2.01$ line characteristic of dehydrated metHb [22].

In figure 2 the number of high spin, low spin and total Fe^{3+} molecules (in arbitrary units) is shown as a function of the denaturation time at 60°C, 70°C and 80°C. The total number was obtained by summing the high and low spin values taking into account the

corrections described in Materials and Methods.

At 60°C the number of Fe^{3+} in the high spin state increases and then decreases to a constant value with increasing time. At 70°C and 80°C the initial growth can not be observed. The decrease is faster for a higher denaturation temperature. The initial growth is due to the conversion of a residual fraction of oxyHb to metHb as observed in previous work on oxyHb denaturation [1]. At higher temperatures (70°C and 80°C) this step is already concluded at t=0.

The number of molecules in the low spin form grows to a maximum value at a denaturation time that is shorter at higher temperatures. Then there is a decrease to an equilibrium value.

At all temperatures there is an apparent decrease of the numbers of paramagnetic centers with the denaturation process. It could be related to a reduction of Fe^{3+} to Fe^{2+}. This hypothesis is confirmed by the reaction of denatured metHb with NO. NO is known to bind to Fe^{2+} but in excess can reduce Fe^{3+} to Fe^{2+} [23]. NO was therefore added to react only with the estimated amount of Fe^{2+}, that is, 50 % of total quantity of iron for T=80°C and t=0.5 h and 70 % for T=80°C and t=4h. There is no change in the hemichrome spectra but the HbNO spectra are added as can be seen by comparing spectra a to b and c to d in figure 3. Their shape depend on the degree of denaturation of the sample. The parts of the spectra a and c due to HbNO are indicated in b and d respectively.

There is no change of the spectral shape during the heat treatment of dehydrated metHb at any temperature. The g values of the EPR spectra are the same observed in the denaturated metHb solutions, unless for g=1.85 that is not observed. A free radical characterized by a signal at g=2.01 is observed for samples with longer treatament time: 3 hours for T=60° C, 2 hours for T=70° C and 1 hour for T=80° C (spectra not shown). This free radical is characteristic of samples dehydrated metHb with less than 10% of water [22].

The spectra of EPR of hematin under the same heat treatament at 70° C and 80° C are characteristic of the high spin state of Fe^{3+}. There is a very slight change of shape and area of the signal. Both dehydrated hemoglobin and hematin have a constant number of paramagnetic molecules during denaturation.

In figure 4 the EPR spectra of saturated HbNO denatured at 60° C, 70° C and 80° C are shown. The native form has a spectra characteristic of the hexacoordinated iron in the R conformation of HbNO. It is a broadened line with extremes at g=2.038 and g=1.986 [15,24]. Upon heat treatament the low field extreme displaces to g=2.07 and a hyperfine triplet centered at g=2.011 appears. This triplet is due to the hyperfine interation of the electron with the nitrogen of the NO radical as observed in the five coordinated HbNO [15,24,25,26].

The time dependence of the number of HbNO molecules (arbitrary units) is shown in figure 5 for the three denaturation temperatures. The curves show a maximum when

the intensity of signals at g=2.038 and g=1.986 are maximum and a minimum when the hyperfine triplet appears.

There is an increase of the intensity of the line without other changes in the shape superimposed on the transition. This behaviour is characteristic of an increasing number of iron bounded to NO, probably due the nitrosylation of residual metHb, favored by the increase of temperature. It is more evident during the first phase at 60° C and in the second one at 70° C. This EPR spectra changes are not indicative of conformational changes of the protein.

Discussion

From the time dependence of the shape of EPR signals and of the number of Fe^{3+} molecules shown in figures 1 and 2 respectivally it is possible to distinguish two steps in the denaturation process of metHb. The first one is characterized by the conversion of a fraction of the high spin signal into the low spin one. In the second one changes in the low spin signal occur with the decrease of the number of Fe^{3+} molecules. During the last phase high spin signal changes can not be observed. It is important to emphasize that phase is defined by EPR features and can be related to a macroscopic state or a distribuition of microscopic states, and step is defined as transition region between two different phases.

We can define two different transition times (t_1 and t_2) in the denaturation process. t_1 is the time required for a decrease or increase of 50% of the first maximum change in the number of molecules in high spin and low spin state respectively. t_2 is the time required for a decrease of 50% of the second maximum change in the number of low spin molecules. The maximum change of the number of spin molecules in the first phase is quite well determined from the 60° C experiment, while the second low spin maximum change is obtained from the 80° C experiment. For T=60° C the value of t_2 is greater than the time used in the denaturation study (6.5 hours), and thus no low spin spectral changes are evident until this time. The value of t_1 for T=80° C is very small and at t=0 the signal already presents characteristics of the last phase. The t_1 and t_2 values are given in table 1.

T(°C)	t_1 (h)	t_2 (h)
60	0.75 ± 0.25	> 6.5
70	< 0	1.3
80	< 0	0.3

Table 1: Transition times (t_1 and t_2) for metHb denaturation process (see text for definition)

The spectrum of the low spin form of each phase is characteristic of a mixture of

different complexes. The first low spin spectrum, also observed in metHb pH 10.5, is a sum of two components: one with $g_x = 2.9$ and $g_y = 2.27$ and another with $g_x = 2.45$, $g_y = 2.27$ and $g_z = 1.91$. It has been pointed out that because of the strongly distorted symmetry the last complex can not have two identical ligands at the fifth and sixth coordination position of the iron, at least can not have ligands with equal π-binding properties [27]. Because of the similarity of the crystal field data with those of the cytochome P-450 in which one axial ligand is a mercapeptide [28] it is assumed that in this complex a sulphur, probably of a cysteine residue is bound to the fifth site [28,27,29] and an oxygen donor (water or hydroxil) to the sixth site of the heme iron [30,31]. It is called hemichrome P. Figure 6a shows the structure of the β chains in metHb with histidines and cysteines indicated.

The absorption at $g = 2.9$ is characteristic of hemichome H [3]. The other component with $g = 2.27$ overlaps this component of hemichrome P and the line at $g = 1.65$ is unresolved, as occurs in the dehydrated metMb (Neto, L. M. et al unpublished data) or metHb [22]. This hemichrome shows crystal field properties very similar to imidazole and triazole complexes of methemoglobin [27]. Pertubations of tertiary structure of hemoglobin caused by the weakening of hydrogen bonds in the protein matrix move the E helix sufficiently so that the distal histidine is now in a position to bind to the iron [3,21] (figure 6).

The last phase presents a spectra similar to that of dehydrated metHb except for

the shift of the high field line from $g_z = 1.91$ to 1.85. This spectrum is also due to a mixture of hemichrome H ($g_x = 2.9$, $g_y = 2.27$ and $g_z = 1.65$) and another hemichrome with $g_x = 2.45$, $g_y = 2.27$ and $g_z = 1.85$ not observed previously, here called P'.

To verify the binding of cysteine to the fifth site of iron in the second and third phase of denaturation process, we have blocked the β^{93} group with NEM before denaturation. We do not observe either the formation of hemichrome P or P' as shown in figure 7b and d. We conclude that the denaturation process induces displacements and torsion of the F helix, drastically changing the iron coordination at the proximal site in the first step, and additional changes on the ligand at the sixth site in the second one. This additional change characterized by the $g = 1.85$ absorption can be a substituition of water by histidine as suggested for alkaline cytochrome P-450-CAM [32,33]. Figure 6b is the scheme of the heme environment for the proposed structures in the denaturation process.

In the experiment with blocked β^{93} the signal of hemichrome H is isolated in the second and third denaturation phases (spectra b and d in figure 7). The signals of iron bounded to cysteine in P and P', can then be obtained by subtraction of normalized H spectra from those of the second and third phase respectively. The normalized experimental low spin spectra can be reproduced by combining different fractions of H, P and P' spectra (figure 8a). The time dependence of the fractions of H, P and P' spectra for the three denaturation temperatures are shown in figure 8b. We can then

relate the transition time t_1 to the increase of hemichrome P and t_2 to the distortion of hemichrome P into P'.

In the second phase of denaturation process the α subunits (without cysteine (cys) near to the heme) contribute more extensively to the formation of hemichrome H since in the β subunits the hemichome P is formed with the binding of cys β^{93}. Following, in the second step in the β subunits a substituition of hemichrome P into P' is observed. The fractions of H in α subunits (~ 0.3) as well as the sum of the fractions of P and P' in β (~ 0.7) are aproximately constant in the spectral composition during the whole process.

In the second step the number of low spin molecules decreases strongly. This is due the reduction of Fe^{3+} to Fe^{2+} as observed by the nitrosylation of denatured metHb experiment described in the results section. If the reduction of Fe^{3+} to Fe^{2+} was occurring uniformely within metHb chains the maximun values of the fractions of H and P + P' would be 0.5. Our results described above show that the reduction is preferentialy in α chains.

In the spectra of denatured metHb samples where NO was added to bind to Fe^{2+} we observe that the spectrum of the second phase is characteristic of a mixture of R and T conformation (figure 3b, e). In the third phase the NO signal is characteristic of the T conformation (figure 3d, f) [24,26]. Thus the reduction from Fe^{3+} to Fe^{2+} is accompanied by a change similar to that observed in the R–T transition. This change

can only be observed in the contribution of α subunits to EPR spectra (figure 6b) [24,26] . We have no evidence for heme dissociation from globin and dimerization as observed in other denaturation conditions [34], that could be responsable for decrease of the Fe^{3+} signal.

The denaturation process depends on the presence of water. In dehydrated samples the changes observed in EPR spectra are very weak and they are mainly due to the water evaporation. There must be less mobility of the polipeptidic chains in samples with largest degree of dehydration than in hemoglobin solution.

Comparing the EPR spectra of the denatured and dehydrated samples [22] we can note some similarities and differences between these processes. We obverve a strong reduction of high spin signal for both, but in denaturation this reduction is not accompanied by an increase of linewidth as in dehydration. In denaturation broadening ocuurs only after the number of molecules of high spin state reaches its minimun value. The mechanism of formation of hemichromes H and P is problably the same. But there are differences in hemichrome P: the changes observed by denaturation are irreversible [1], while its reversibility depends on the degree of hydration [22]. And finally the denaturation process can go further than dehydration by distorting hemichrome P into P'.

Only one step is distinguishable by EPR in the heat denaturation of HbNO. The initial phase is characterized by a R conformation spectra and the second one is char-

acterized by a well resolved hyperfine triplet. We define the transition time (t_x^{NO}) as the time necessary for the number of HbNO molecules to decrease to one half of its maximum change. The values of t_x^{NO} are given in table 2.

We have taken the spectrum of the native sample (A) and the sample with higher degree of denaturation (80° C, t=6.5 h) as representative of the denaturated one (B). The normalized experimental spectra can be reproduced by combining different fractions of A and B (figure 4). The transition time t_x^{NO} can be also defined from the behaviour of the fractions of these components (figure 9) as the time where crossover at 0.5 occurs. These values agree quite well with those obtained using the number of HbNO molecules (see table 2).

T(°C)	t_x^{NO} $(h)^a$	t_x^{NO} $(h)^b$
60	> 5.3	> 5.3
70	0.3	0.5
80	< 0	0

Table 2: Transition time t_x^{NO} for HbNO heat denaturation (see text for definition). [a] Using numbers of NO molecules from figure 5. [b] Using fractions of spectra A and B from figure 9

Similar spectra to B were obtained using other denaturing agents (pH, detergents)

and explained as being due to diverse changes in the heme environment [35,36]. A scheme of the iron binding for this change is shown in figure 6b.

We observe that denatured metHb with NO shows a spectrum of a fivecoordinated T conformation (fig. 3f) different from that of denatured HbNO R conformation (B, fig. 5). The T conformation is characterized by a hiperfine triplet centered at $g = 2.014$ and a shoulder at $g = 2.11$, while in the denatured R conformation the hyperfine triplet is centered at $g = 2.011$ and the shoulder is not observed (figure 10). Despite this difference, it was shown that any hyperfine triplet of HbNO is due to the weakening or rupture of the bond between iron and proximal histidine in the α subunits [26].

The transition times of both complexes depend on the denaturation temperature. The larger value of t for metHb as compared to HbNO indicates greater stability of the former to heat denaturation. EPR evidences two transition times for metHb while only one for HbNO. t_1 is associated to the substituition of the proximal histidine by cysteine at the fifth position of iron in β subunits and substituition of water by histidine at the sixth position in α subunits. t_2 is associated to the substituition of water molecule probably by a histidine at the sixth position in β chains and reduction of Fe^{3+} to Fe^{2+} (fivecoordinated) mainly in the α chains. The t_x^{NO} is the time required to the transition from six to five coordinated systems. t_2 and t_x^{NO} are related to similar conformational change in the α subunits figure 6b. Although the results indicate paralelism between the break of histidine bond in α chains for both complexes, in metHb it is the second step

of denaturation and their resulting structures are different as revealed by EPR spectra (figure 10).

For metHb extended heat treatment resulted in further spectral changes, not observed before with a 4 min heat treatment [1]), while for HbNO we could not observe the EPR spectra with features of iron with axial symmetry of other denaturation conditions [14]. It is shown here that in metHb the reduction of Fe^{3+} molecules to Fe^{2+} occurs preferentially in the α subunits.

The intermediate and experimentally separable species studied in the heat treatment in this work opens the possibility of studing their functional and structural properties separately.

This work is an extension of previous ones on denaturation of hemoglobin. This comparative study using different Hb complexes and structural states contributes to the understanding of protein stability by showing the relevance of hydrophobic interactions and of the solvatation water for altering the protein native structure.

The process can be described as a continous change of the heme environment and iron coordination.

Acknoledgements

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Figure captions

Figure 1 – EPR spectra of human metHb for different denaturation times and temperatures.

Figure 2 – Time dependence of the number of Fe^{3+} molecules of denatured metHb. Δ high spin state, $\diamond$ low spin state and $\square$ total. Denaturation temperature: (a) T=60°C; (b) T=70°C and (c) T=80°C.

Figure 3 – EPR spectra of denatured metHb before and after the addition of a controlled volume of NO gas. (a) T=80°C, t=0.5h; (b) Same as (a) with NO; (c) T=80°C, t=4h; (d) Same as (c) with NO; (e) and (f) Same as (b) and (d) showing the HbNO region.

Figure 4 – EPR spectra of HbNO with different denaturation times and temperatures. (- - -) fit with different fractions of native (A) and denatured (B) spectra. From the top to bottom composite spectra are given by 0.6 A + 0.4 B and 0.88 A + 0.12 B.

Figure 5 – Time dependence of the numbers of HbNO molecules under heat treatament

Figure 6 – (a) The β subunits in metHb with histidines and cysteynes indicated. (b) The heme environment structural changes in the denaturation process of methHb and HbNO.

Figure 7 – EPR spectra of metHb (a) denatured at T=70°C, t=0.5h (b) same as (a) treated with NEM before denaturation, (c) at T=80°C, t=4h and (d) same as (c) with NEM. High spin spectra region not shown.

Figure 8 – (a) Low spin metHb EPR spectra. (—) Experimental and (- - -) fit with different fractions of hemichrome H, P and P ′ samples. From the top to bottom the spectra are reproduced by 0.25 H + 0.75 P, 0.25 H + 0.6 P + 0.1 P′, 0.3 H + 0.45 P + 0.3 P ′, 0.3 H + 0.15 P + 0.5 P′, 0.4 H + 0.15 P + 0.5 P′ and 0.375 H + 0.6 P′. (b)Time dependence of H(□), P(△) and P′(◇) fractions in the composite spectra.

Figure 9 – Time dependence of fractions of normalized A (△) and B(□) spectra of HbNO under heat treatament

Figure 10 – Comparison between EPR spectrum of (a) denatured R conformation of HbNO and (b) NO added to denatured metHb.

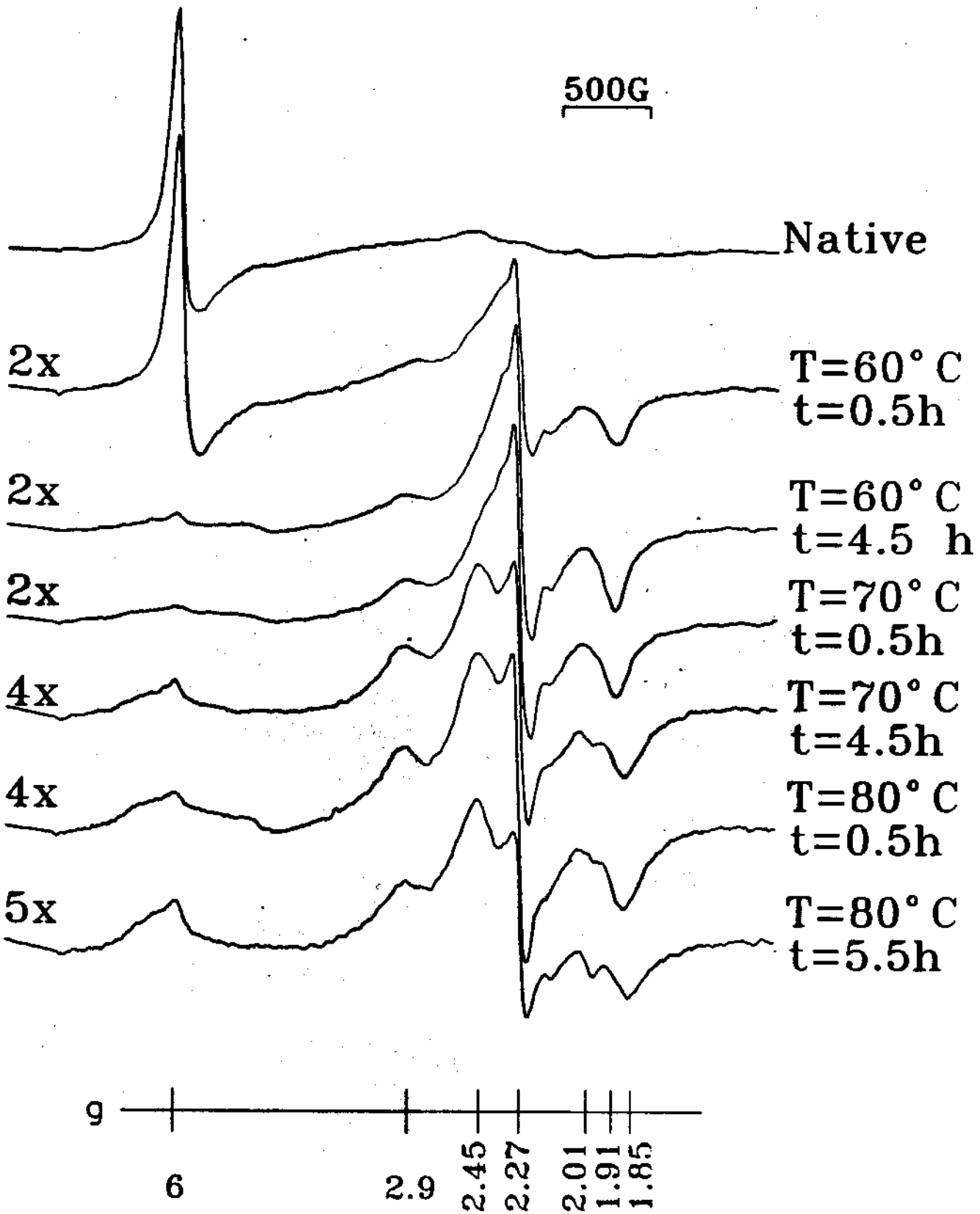

500G
Native
2x
T=60° C
t=0.5h
2x
T=60° C
t=4.5 h
2x
T=70° C
t=0.5h
4x
T=70° C
t=4.5h
4x
T=80° C
t=0.5h
5x
T=80° C
t=5.5h
g
6
2.9
2.45
2.27
2.01
1.91
1.85

FIG. 1

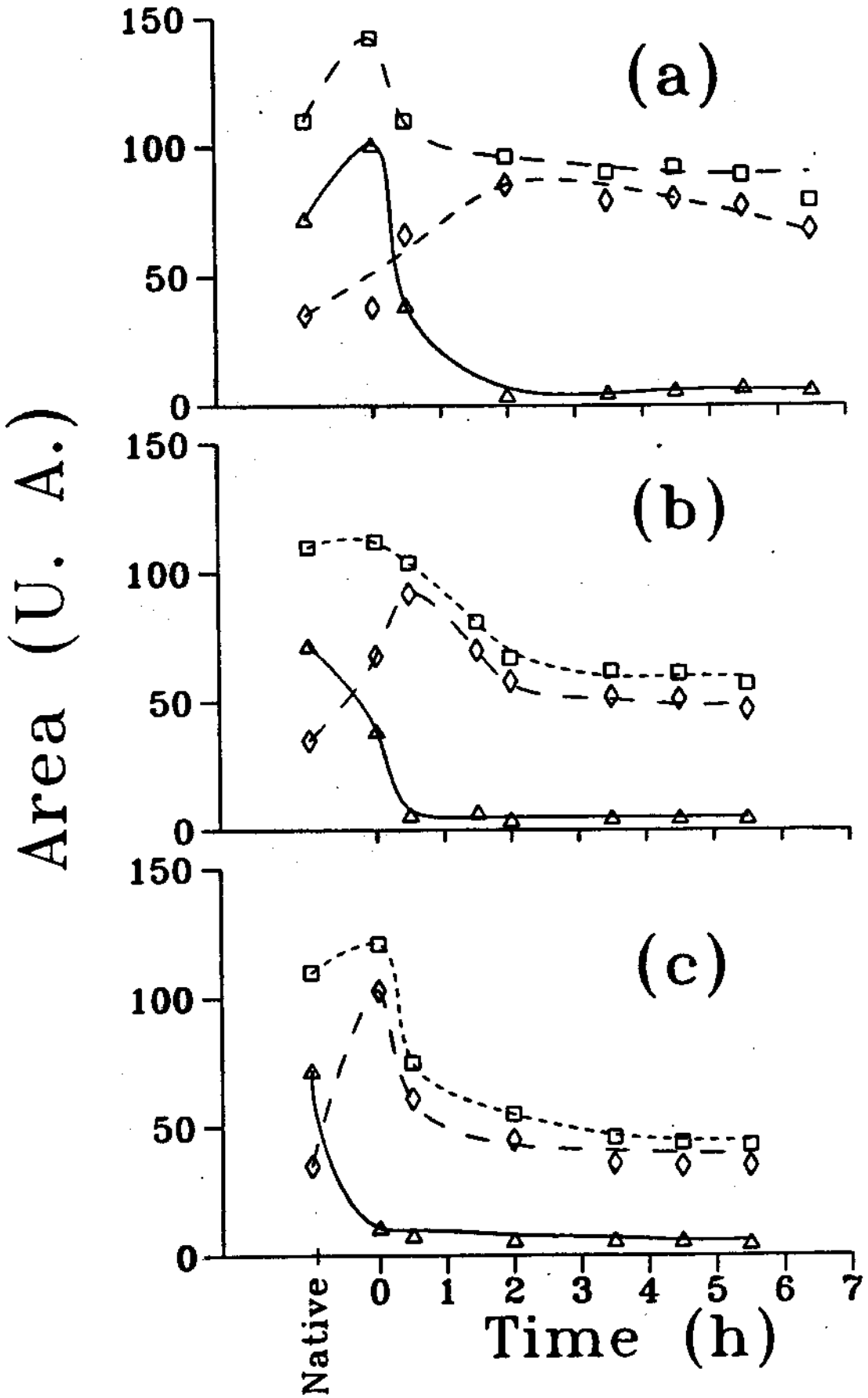


FIG. 2

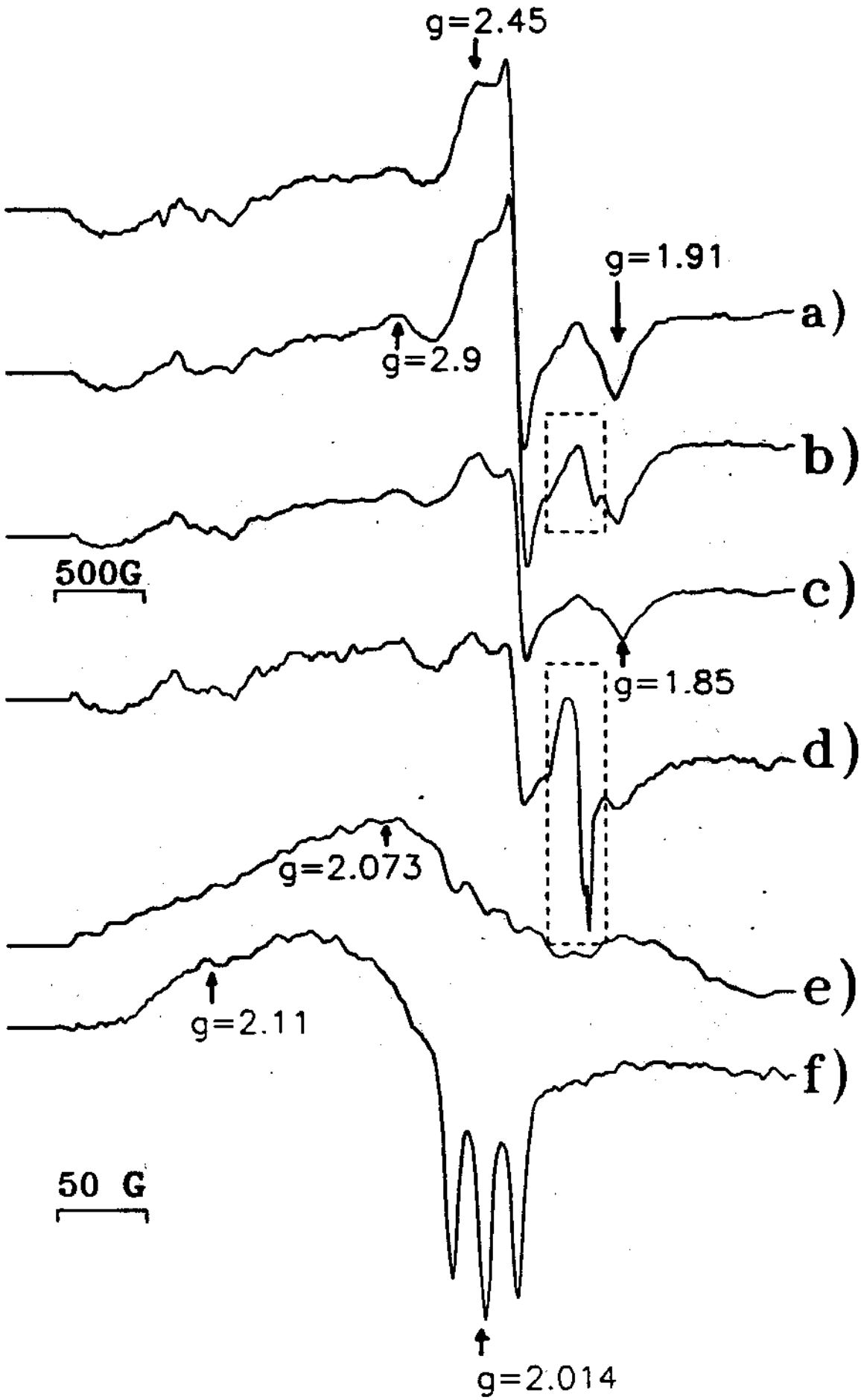


FIG. 3

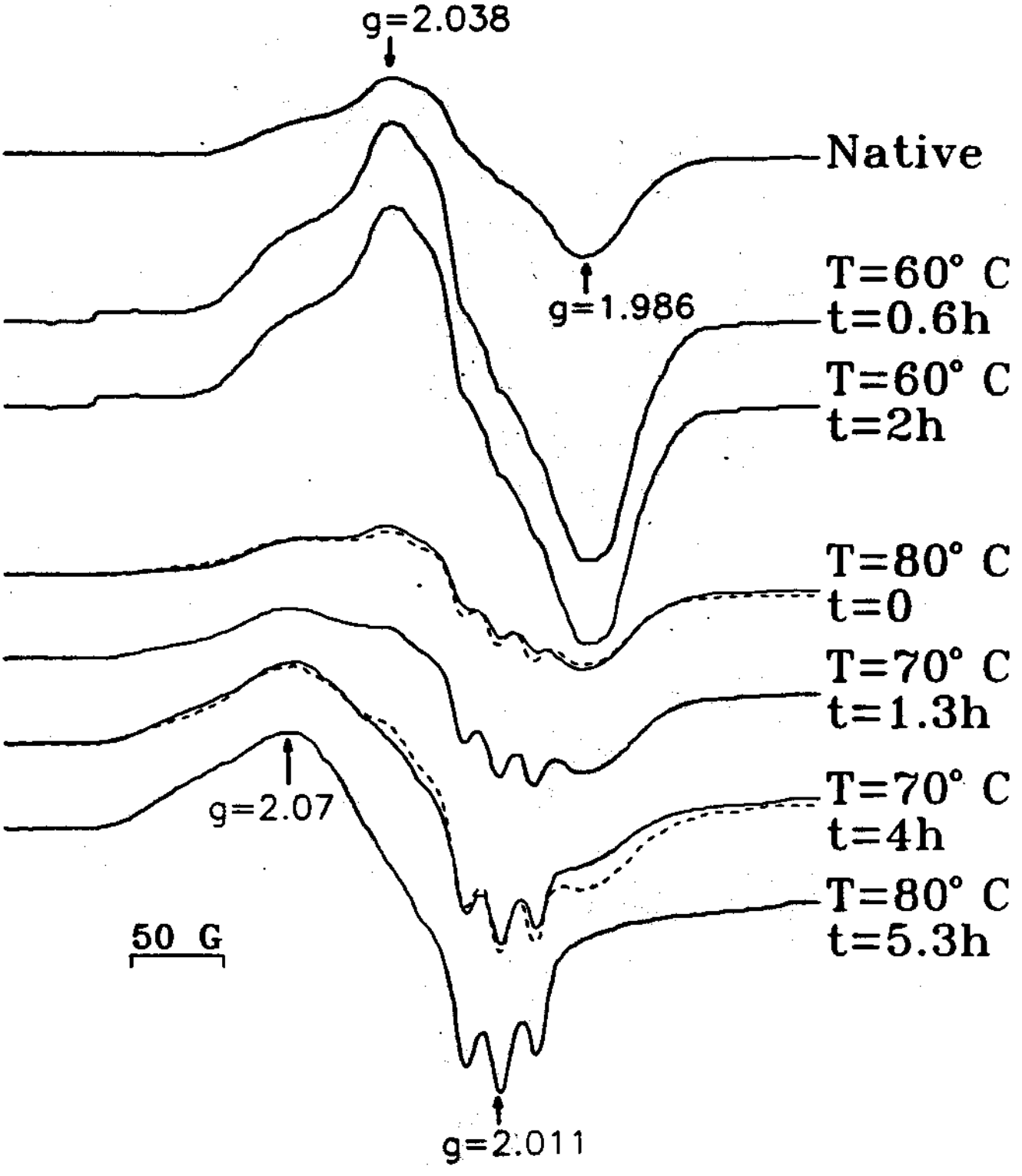

g=2.038
Native
g=1.986
T=60° C
t=0.6h
T=60° C
t=2h
T=80° C
t=0
T=70° C
t=1.3h
g=2.07
T=70° C
t=4h
T=80° C
t=5.3h
50 G
g=2.011

FIG. 4

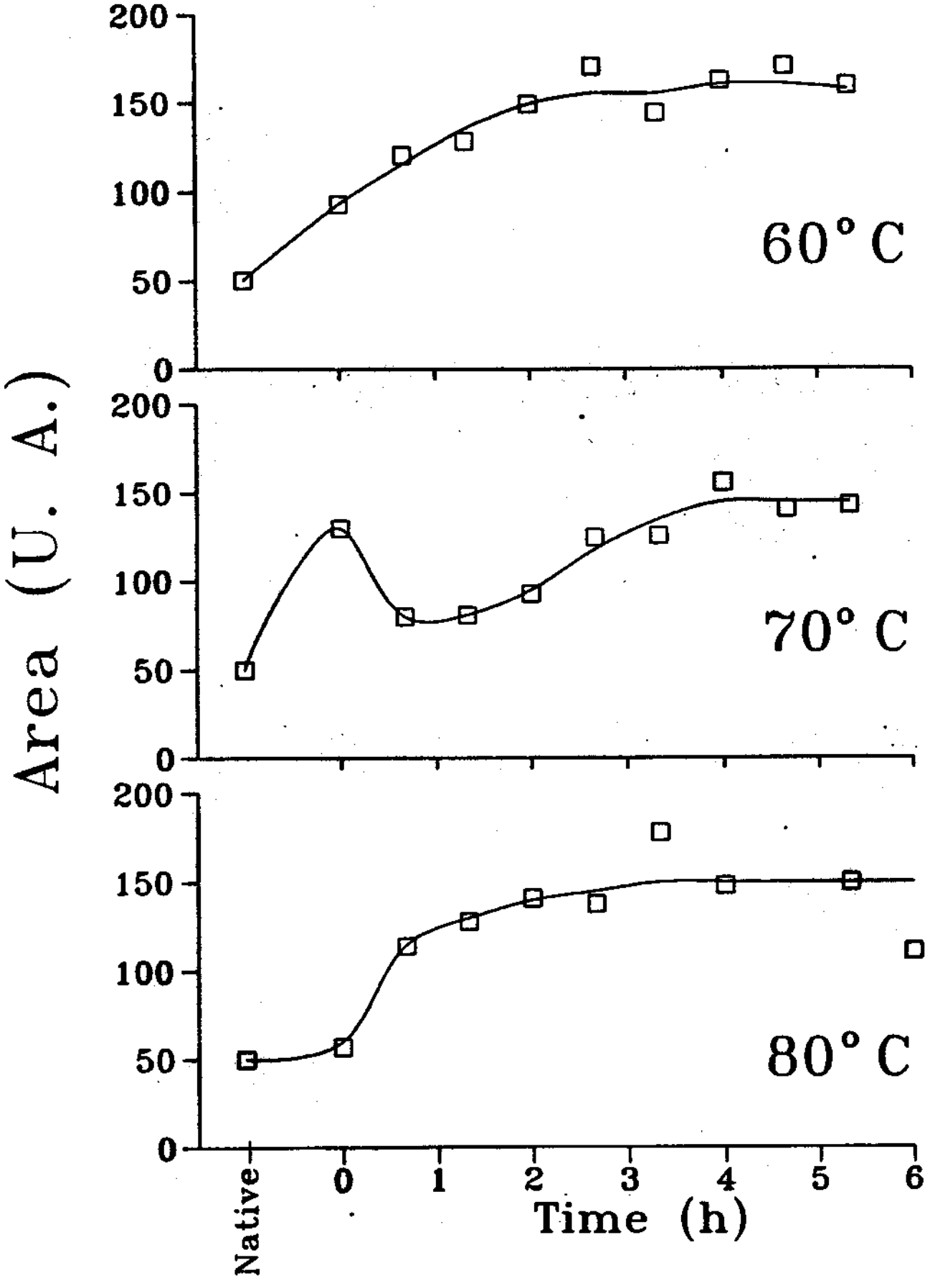
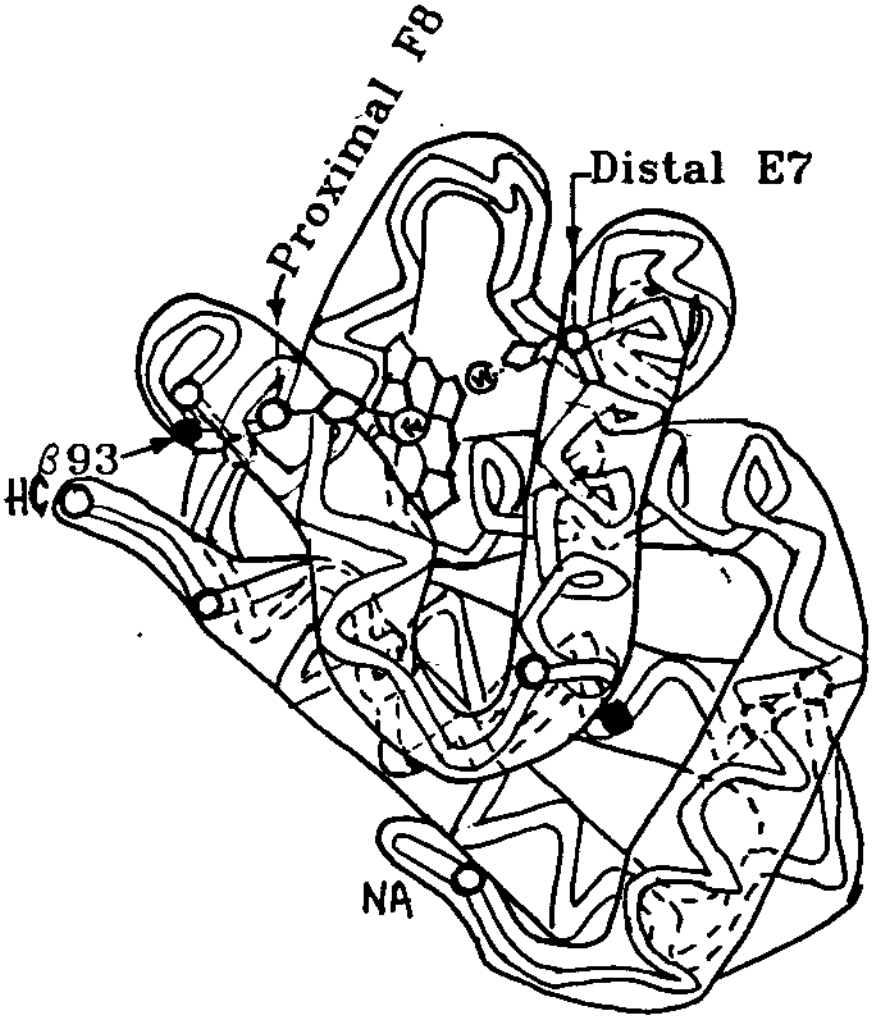


FIG. 5



Fe – Fe

O – His

● – Cys

W – H_2O

FIG. 6a

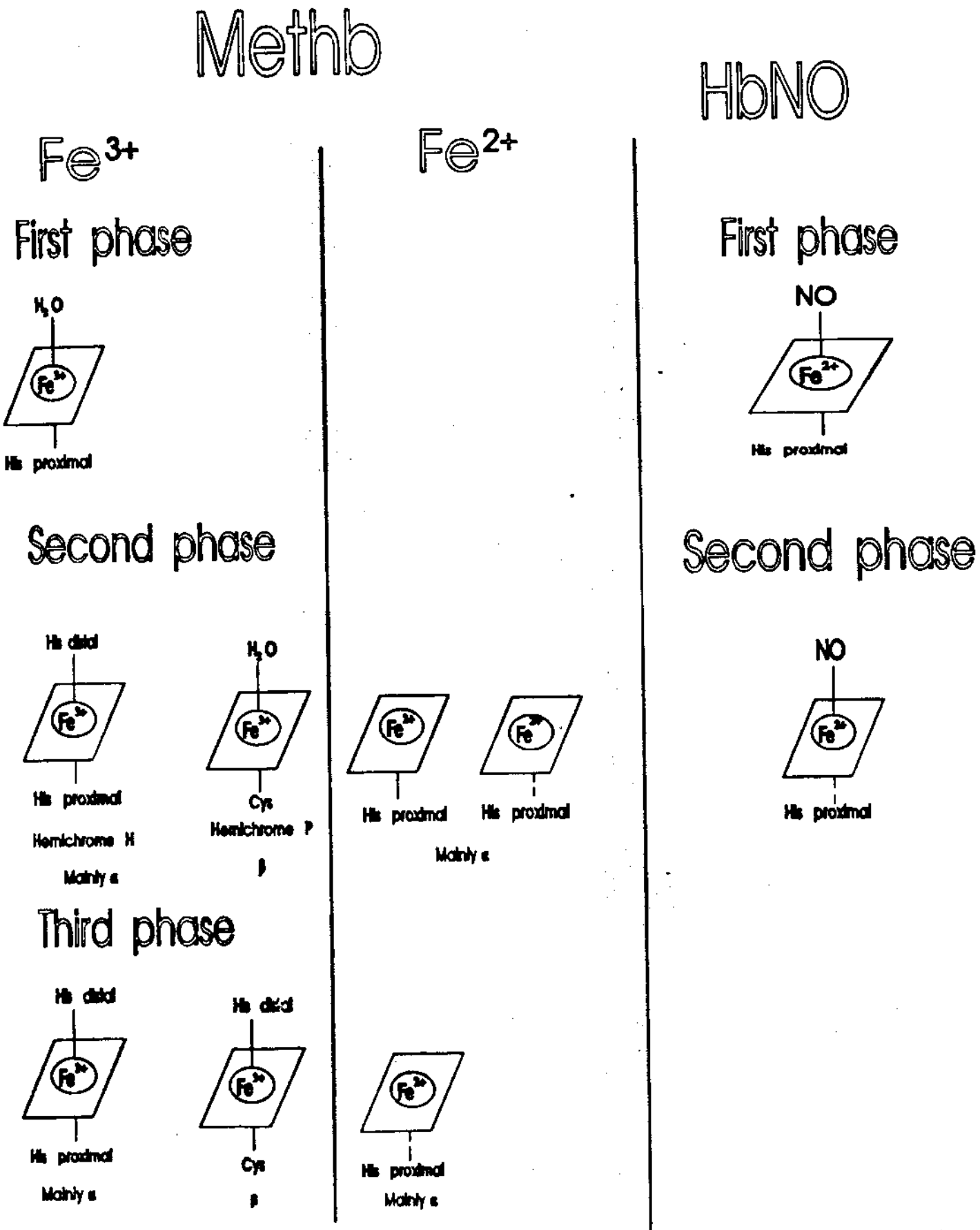

Methb
HbNO
Fe3+
Fe2+
First phase
First phase
H2O
NO
Fe3+
Fe2+
His proximal
His proximal
Second phase
Second phase
His distal
H2O
NO
Fe3+
Fe3+
Fe3+
Fe3+
His proximal
Cys
His proximal
His proximal
Hemichrome H
Hemichrome P
Mainly α
β
Mainly α
Third phase
His distal
His distal
Fe3+
Fe3+
Fe3+
His proximal
Cys
His proximal
Mainly α
β
Mainly α

FIG. 6b

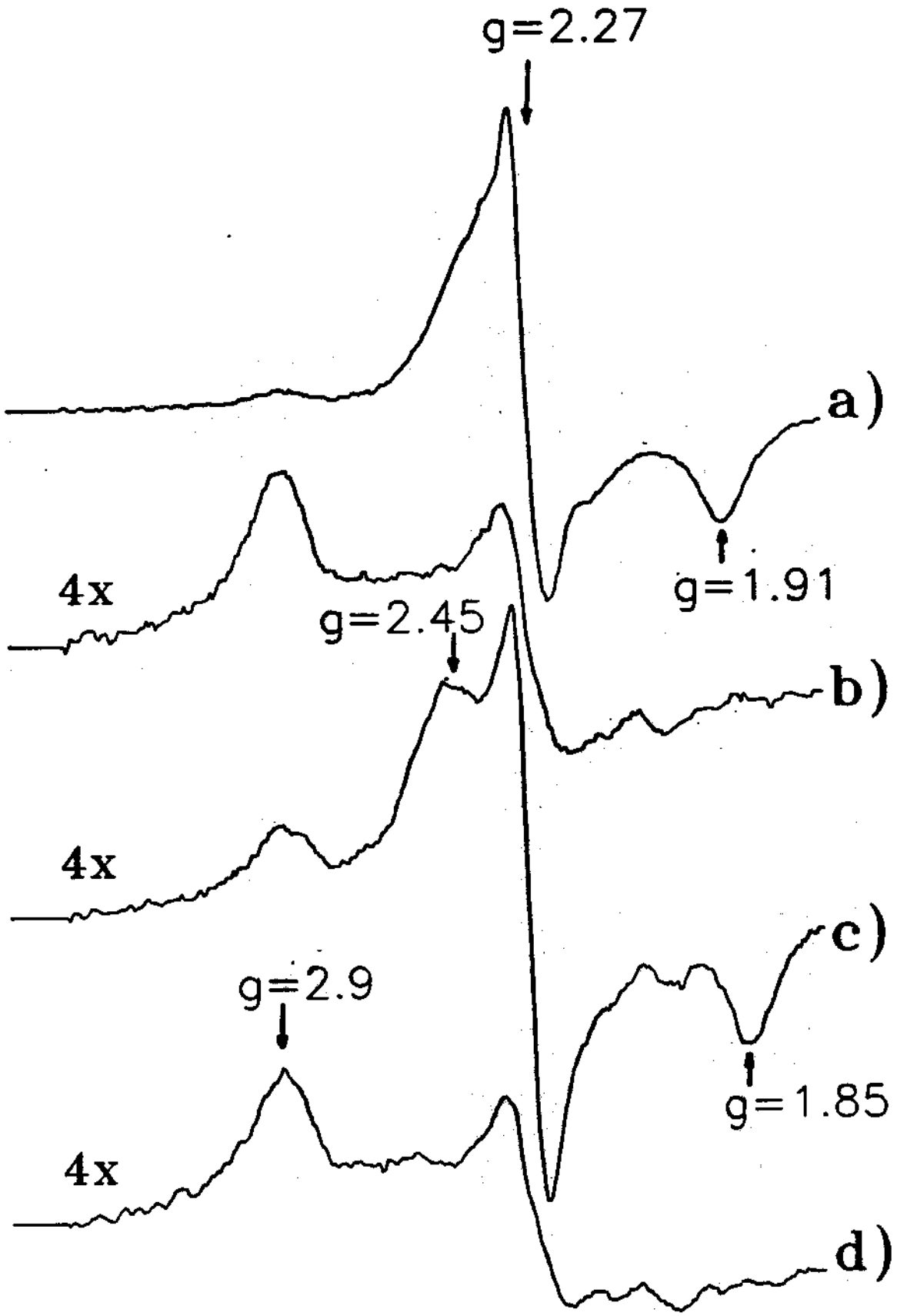


FIG. 7

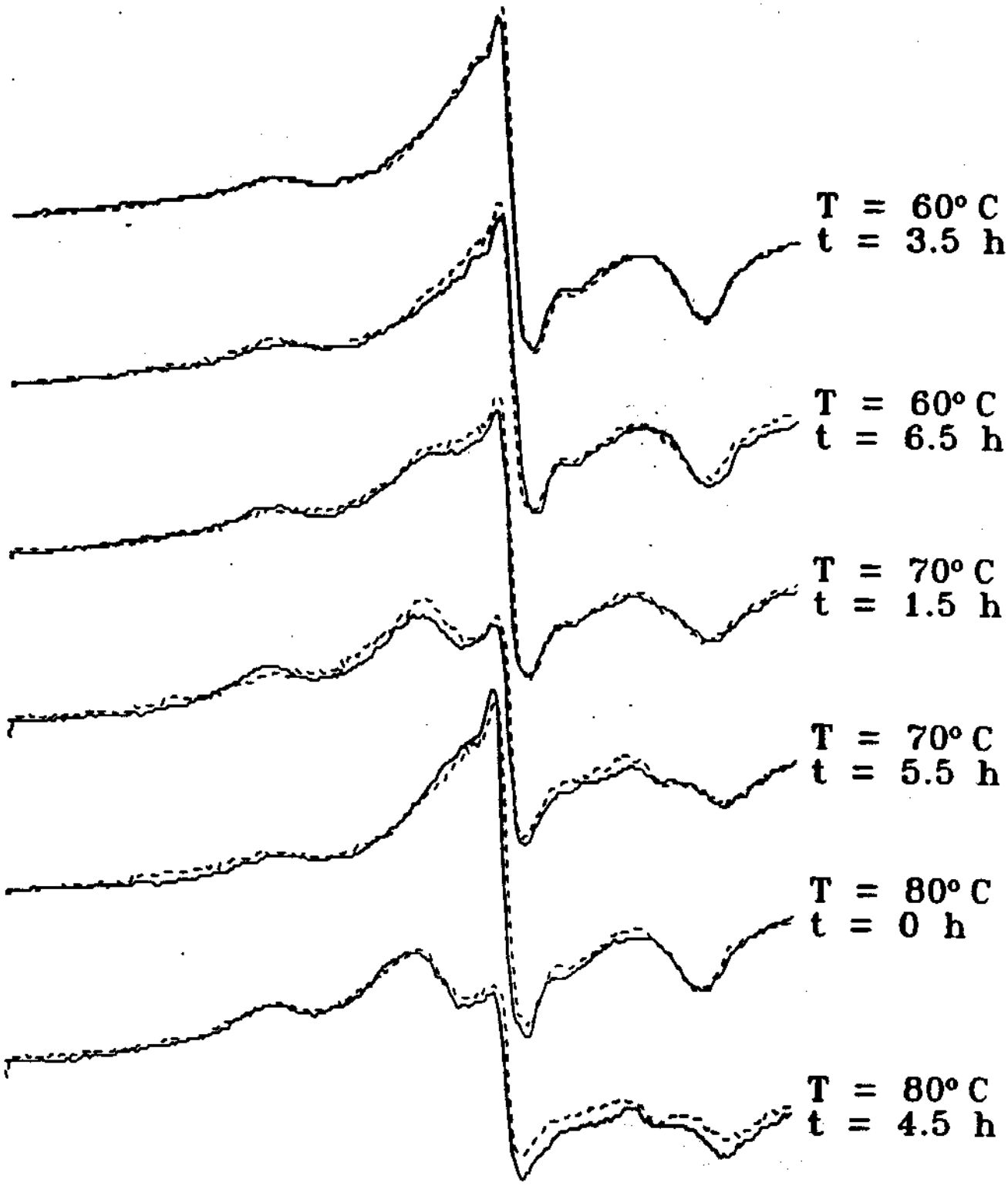


FIG. 8a

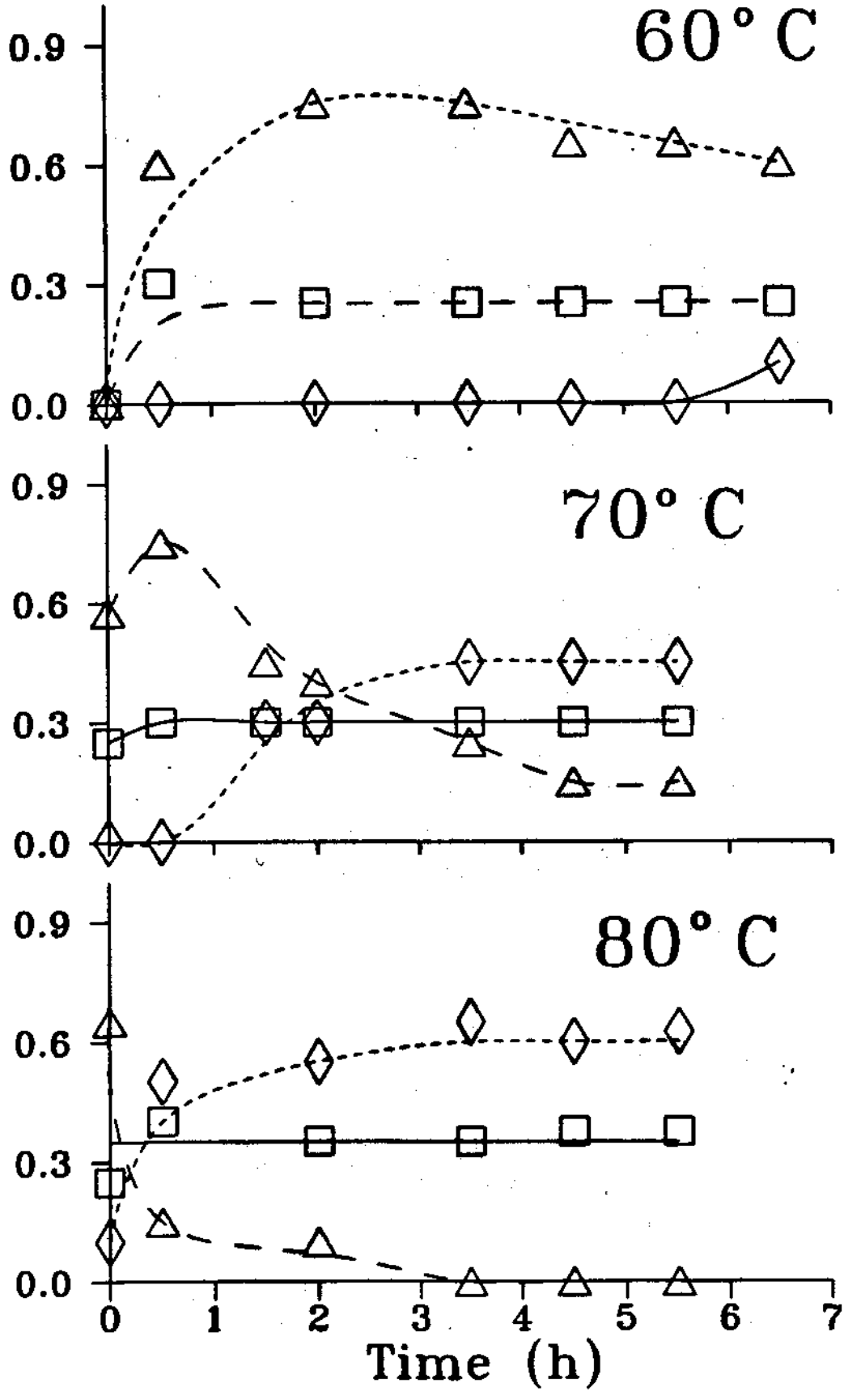


FIG. 8b

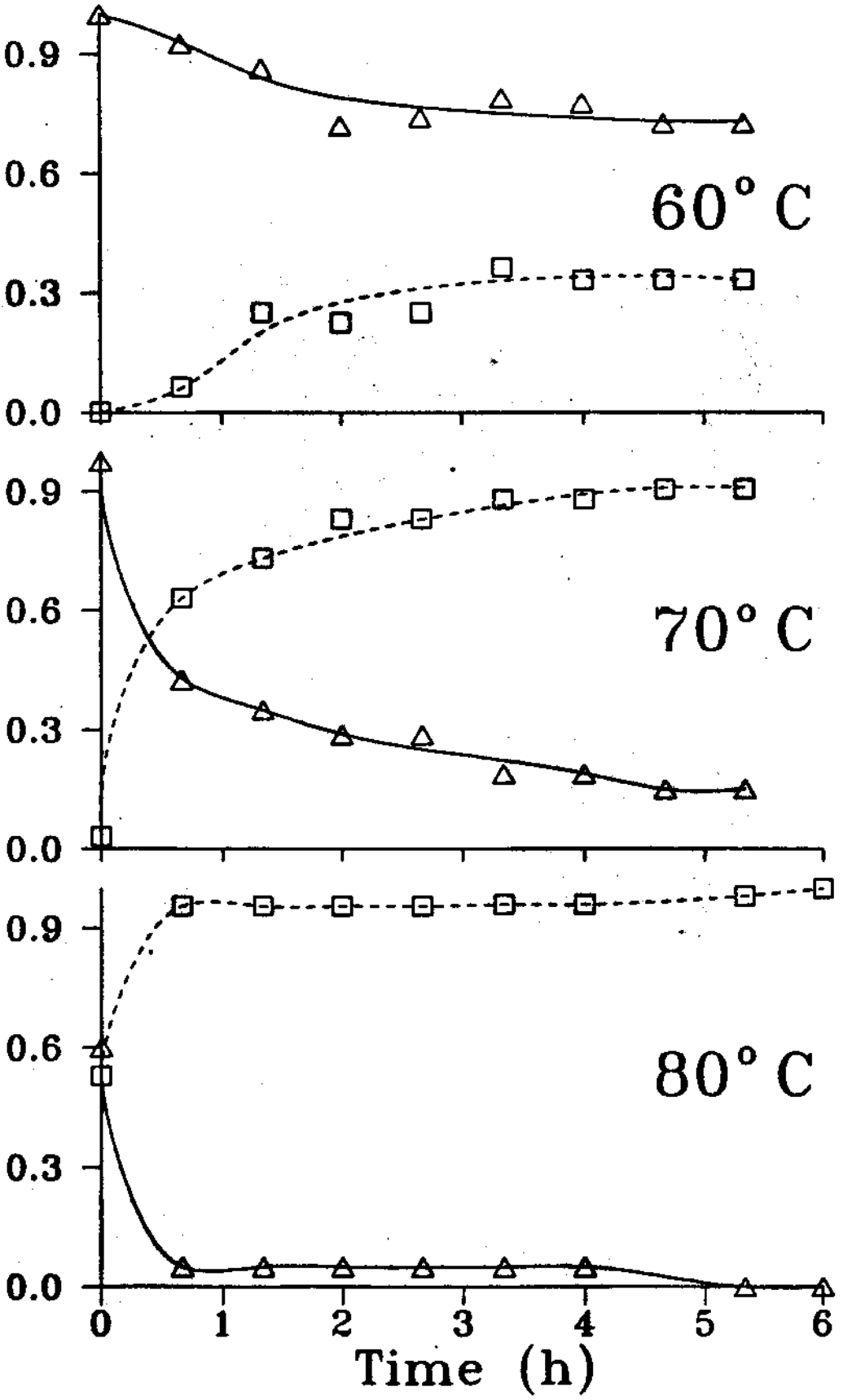


FIG. 9

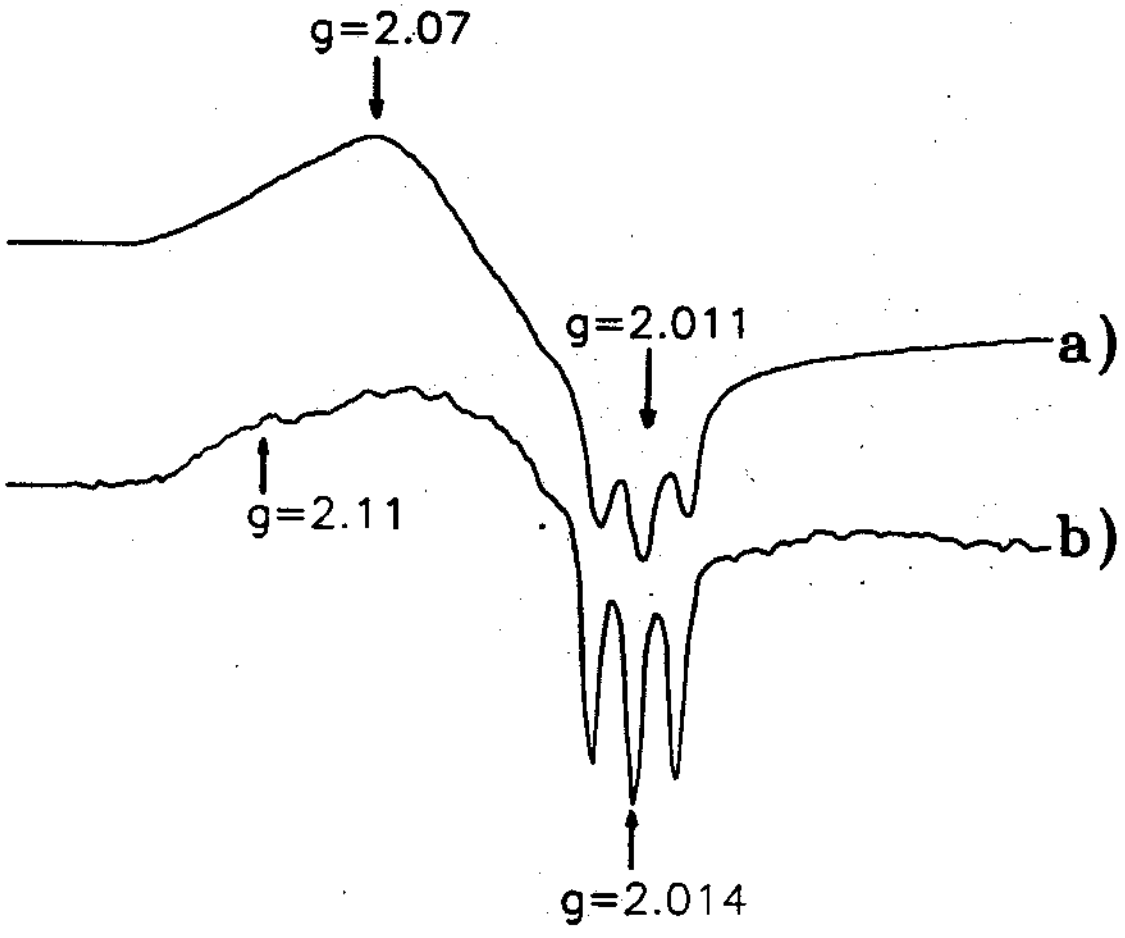


FIG. 10

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