APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 6, 129-141 (1981)

EPR Characterization of Cellulose Triacetate Fibers Used for Enzyme Immobilization

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Received October 1, 1980; Accepted November 22, 1980

Abstract

EPR studies of a nitroxide spin label and of the nitroxide spin-labeled albumin entrapped in cellulose triacetate fibers were carried out.

The EPR spectra have shown that within the fiber only two phases are present: a liquid one of medium viscosity trapped inside microcavities, and a polymeric one surrounding them.

After entrapment, spin-labeled albumin is distributed mainly in the liquid phase, though a not negligible amount of it remains within the polymeric matrix.

The EPR studies have shown that, after the standard procedure of drying, the albumin is almost completely precipitated, but about 85% of it returns to solution when the fiber is again placed in the solution.

The behavior of the albumin dissolved inside the microcavities toward denaturating agents and pH change, and that of the free albumin in solution is similar; the minor differences noticed indicate a second-order interaction between the fiber and the protein.

Index Entries: EPR characterization of cellulose triacetate; cellulose triacetate, EPR characterization of; fibers, EPR characterization of; albumin, spin-labeled.

Introduction

The use of immobilized enzymes has lately gained widespread interest in the chemical and pharmaceutical industry. In fact, immobilization often results in

0273-2289/81/0600-0129$02.60

an increase of enzyme stability, and the possibility of reuse represents a net advantage in the field of chemical catalysis and automated analysis, allowing an easier handling of enzymes and minimizing the preparation and purification costs.

Several and very diversified methods for enzyme immobilization have been proposed during the last few years (*1–5*) and also in our laboratories an original system of enzyme immobilization has been developed (*6*) that consists in the physical entrapment of enzymes as aqueous solution, inside microcavities of cellulose triacetate fibers, obtained by wet-spinning.

The reactivity and technological problems related to the fiber entrapped enzymes have been sufficiently investigated so far (*7*).

Scanning electron microscopy (SEM) has been used to investigate the structure of cellulose triacetate fibers and the presence within the polymeric matrix of microcavities has been demonstrated. However, more work was felt necessary for better understanding the morphology of the fibers, the distribution of the enzyme within the fiber, the behavior of the enzyme itself. We thought that such information could be obtained by using the spin-label technique, which has lately received widespread attention for detecting structural changes of molecules of biological interest, and for the investigation of the interactions of such molecules with the surrounding (*8, 9*).

In fact, the spin labels are stable free radicals, usually nitroxides, which in solution show a simple EPR spectrum and which can be covalently bound to other molecules quite easily. Since the EPR parameters of a free radical, as the g factor and the nitrogen hyperfine (hf) coupling constant are tensors, the resulting EPR spectrum will depend on the orientation of the radical respect to a space-fixed orientation, such as a static magnetic field. A spin-label EPR spectrum is extremely sensitive to the nature and rate of the motions the label undergoes. If the radical, or a molecule to which the radical is bound, is strongly immobilized, the observed EPR spectrum appears with a typical line-shape, the so-called "amorphous pattern" (*10, 11*), with all spin-labels standing still with random orientations.

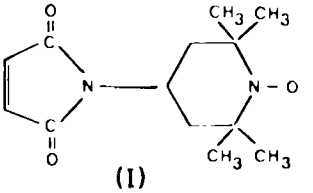
When molecular motion is activated, these anisotropic parameters are averaged and if the motion is sufficiently rapid the observed spectrum becomes "isotropic" as it is when the radical is in a solution of low viscosity. The motion of the spin label is expressed by the correlation time τ_c, which can be quantitatively determined from the observed linewidth $1/T_2$ (*12*). Therefore, it is possible to have informations about the molecular motion of the radical by either the changes of line shape of the EPR spectrum, or by the changes of linewidth, $1/T_2$, of each peak of hf line, thus obtaining indications about the surrounding of the spin label or of the molecule to which it is bound.

In this paper we report the results of our investigations of the morphology of cellulose triacetate fibers carried out by entrapping into them a nitroxide spin-label, and the study of the interaction between the fiber and entrapped bovine serum albumin, labeled with a derivative of the same radical.

Experimental

Materials and Methods

2,2,6,6-Tetramethyl-4 amino-piperidine-1 oxyl, and 2,2,6,6-tetramethyl-4 hydroxy-piperidine-1 oxyl (4-hydroxy TEMPO) from Aldrich Chemical and bovine serum albumin (BSA) from Sigma Chemicals were used as received. The spin label *N*-(1- oxyl-2,2,6,6-tetramethyl piperidinyl)-maleimide (I) was prepared in this laboratory from 2,2,6,6-tetramethyl-4 amino-piperidine-1 oxyl, following the procedure given by Griffith (*13*) for *N*-(1-oxyl-2,2,5,5-tetramethyl-pyrrolidinyl)-maleimide



(I)

This substance is known to label SH and NH_2 groups of proteins. EPR spectra were obtained at 9500 MHz with a Varian E-4 spectrometer, using the cell at low dielectric loss for liquids.

Reaction of Spin Label (I) with Bovine Serum Albumin

Bovine serum albumin (3g) was dissolved in 50 mL of 0.1*M* sodium phosphate buffer (pH 6.8) at 0°C. Spin label (I) (12 mg) was added to the solution with stirring. The solution was stirred for 24 h, then dialyzed against buffer to remove unbound spin label, and lyophylized.

Entrapment of Spin Label 4-Hydroxy-TEMPO and of Labeled Albumin into Cellulose Triacetate Fibers

The standard method described elsewhere (*6*) was followed: cellulose triacetate was dissolved in methylene chloride and a water/glycerin solution of the spin label or of the labeled albumin was added dropwise with stirring.

Stirring was continued for 30 min in order to obtain a finely dispersed emulsion. The emulsion was extruded through a spinneret and coagulated in toluene. The fibers were then air dried to eliminate the organic solvents.

Results and Discussion

Fibers Morphology

In Fig. 1a is shown the EPR spectrum of the spin label 4-hydroxy-TEMPO entrapped in cellulose triacetate fibers after drying in air for 48 h. Two different signals are clearly identifiable. One, with $A_{max} = 65$ G, is the typical spectrum

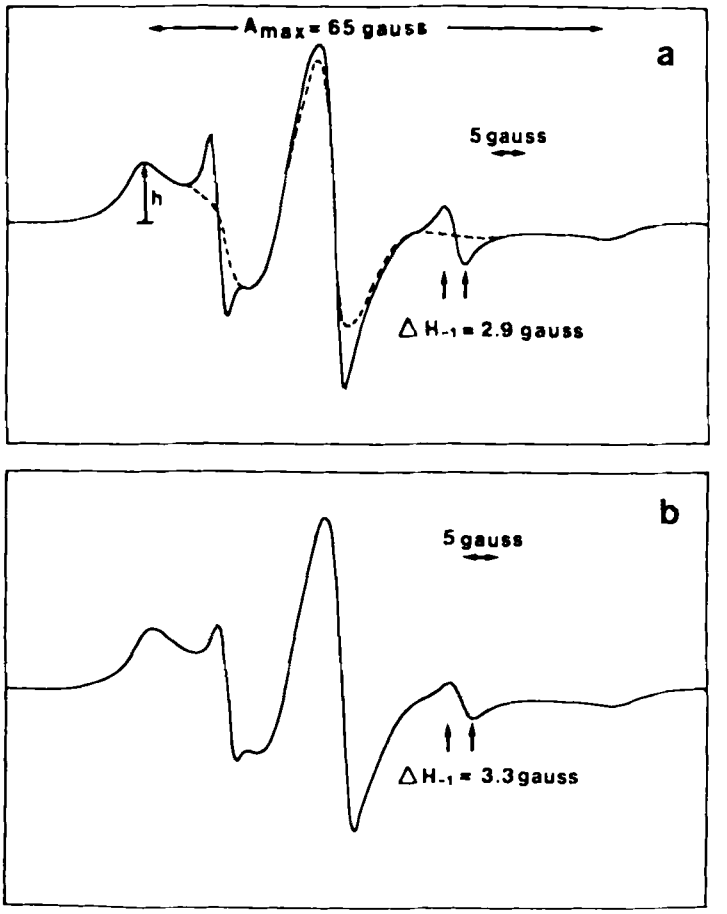


FIG. 1. EPR spectrum of 4-hydroxy-TEMPO entrapped in cellulose triacetate fibers, after drying in air for 48 h (1a), and for 72 h (1b).

of a strongly immobilized radical with a τ_c of the order or higher of 10^{-8} s, while the other, showing a pattern of three rather narrow lines, can be associated with a radical dissolved in a liquid phase of medium viscosity.

Scanning electron microscopy investigation of cellulose triacetate fibers (*7*) has already indicated the presence inside the fiber of cavities surrounded by porous material. The EPR spectrum confirms this structure since the broad EPR signal can be associated with the spin-label dispersed within the polymeric phase, while the narrower one can be assigned to the radical dissolved in the liquid phase entrapped inside the microcavities.

The shape of the EPR spectrum owing to the spin label dissolved in the liquid phase will depend upon the water/glycerin ratio. In fact, the mobility of the free radical, and thus the width of the EPR lines, is related to the viscosity of the liquid phase. A decrease of the water content will mean higher viscosity and thus wider EPR lines.

In Fig. 1b is shown the EPR spectrum of the fiber of Fig. 1a, after drying at room temperature for further 24 h. As it can be seen, the ΔH_{-1} line is wider, which is clearly related to a decrease of the water content in the liquid phase.

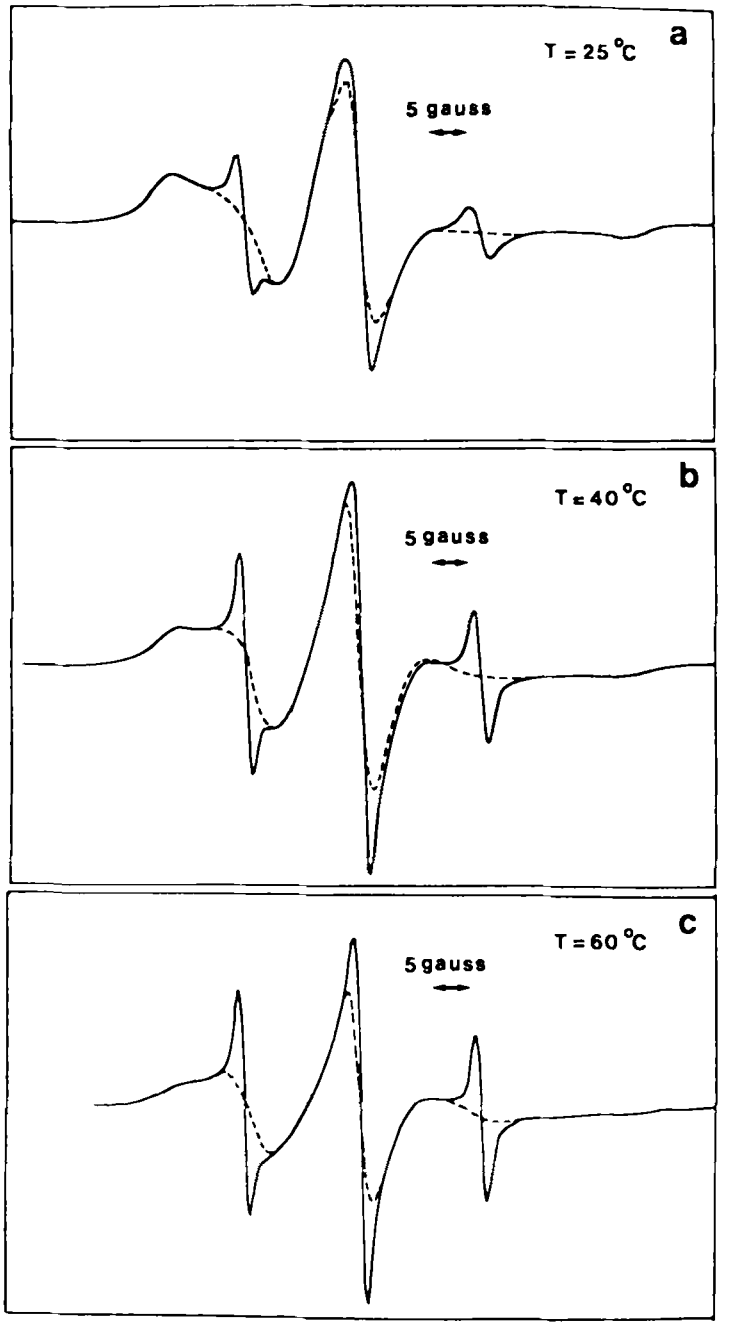


FIG. 2. EPR spectra at different temperatures of 4-hydroxy-TEMPO entrapped in cellulose triacetate fibers.

The shape of the EPR lines suggests also that inside the fiber are present only these two phases. In fact, EPR spectra recorded at various temperatures (Fig. 2), always show only two signals, which would not be the case if more than two phases were present. The narrowing of the EPR bands is indicative of a decreased viscosity of both phases with increasing temperature.

the more mobile spin labels, bound to the more accessible NH_2 groups of lysine. However, the signal attributed to the strongly immobilized radicals can be associated also to the fraction of labeled albumin precipitated within the fiber, as suggested by the powder spectrum of labeled BSA shown in Fig. 3b.

Therefore, if the amount of precipitated protein increases as a consequence of the treatments that the fiber undergoes, the intensity of the EPR spectrum owing to the strongly immobilized spin label will increase. This will make possible the determination of the amount of protein in solution by simply determining the ratio (h_2/h_1) between the heights of the narrow and the broad signals referred to the same ratio obtained for BSA completely dissolved in solution.

In Table 1 is reported the amount of protein in solution, as a function of the content of water in the fiber, calculated following the aforementioned procedure, while Figs. 4a and 4b show the EPR spectra of labeled BSA in fibers having different water content. Taking into account that after the standard procedure of drying, the fibers contain approximately 40% of water, it is evident from our data that under these conditions the protein trapped within the fiber is mostly precipitated.

When the fiber is again placed in solution, the protein returns to solution as indicated by its EPR spectrum. However, the amount of protein that resolubilizes depends upon the treatment that the fiber undergoes before drying. In fact, although about 85% of the albumin returns to solution if the fiber is treated with a mixture of water/glycerin, a rather lower amount of it is dissolved again if the fiber is treated with a phosphate buffer solution (pH 6.8). Electron microscopy measurements had already anticipated that during the drying process a certain number of the microcavities of the fibers are closed, and that this effect is less evident when the fiber is treated with a mixture of water and glycerin.

The EPR experiments confirm these findings, which can be rationalized by

TABLE 1
Amount of Albumin in Solution as a Function of Fibers Residual Water Content

H_2O content,[a] %	Albumin in solution,[b] %
52	53
43	30
26	0
15	0

[a]Calculated from the dry weight of cellulose triacetate fibers, and expressed as percent of the initial water content (H_2O/glycerin, 70/30).

[b]Calculated from the ratio h_2/h_1 (Fig. 3a), referred to the EPR spectrum of labeled BSA in a solution of H_2O/glycerin 70/30.

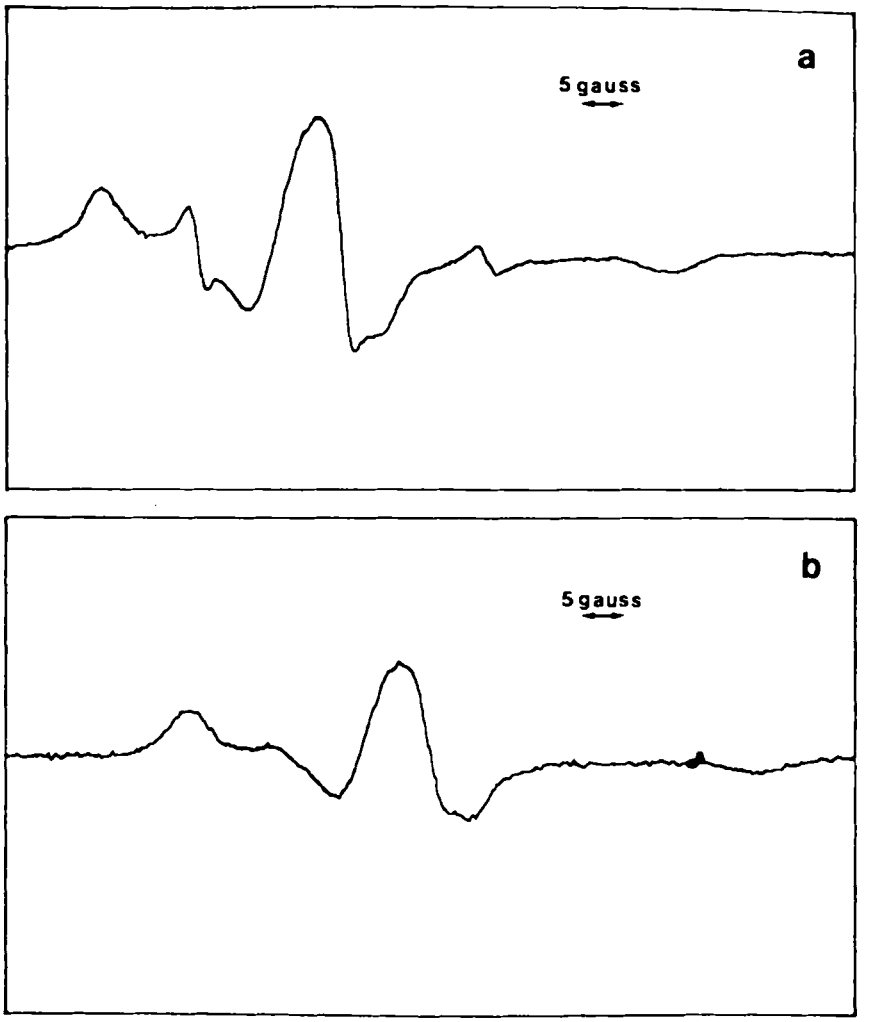


FIG. 4. EPR spectrum of labeled BSA, entrapped in cellulose triacetate fibers having different, water content: 4a, 50% of the initial water content; 4b, 30% of the initial water content.

assuming that glycerin stabilizes the structure of the fiber and that a certain amount of protein remains trapped within the polymeric phase as a consequence of the closing of some cavities during the drying process.

The presence of less accessible albumin, i.e., that dispersed within the polymeric phase, has been confirmed by treating with ascorbic acid fibers containing labeled BSA. In fact, nitroxide radicals are effective electron acceptors and therefore readily reduced by ascorbic acid, with consequent disappearance of the EPR signal. The different rate of reduction of spin labels located in different regions of biological membranes has been exploited to detect the presence of regions of different hydrophilicity (*14, 14a*).

Thus, the presence of differently accessible albumin within the fiber can be pointed out by the different reduction rate of the nitroxide radical bound to BSA.

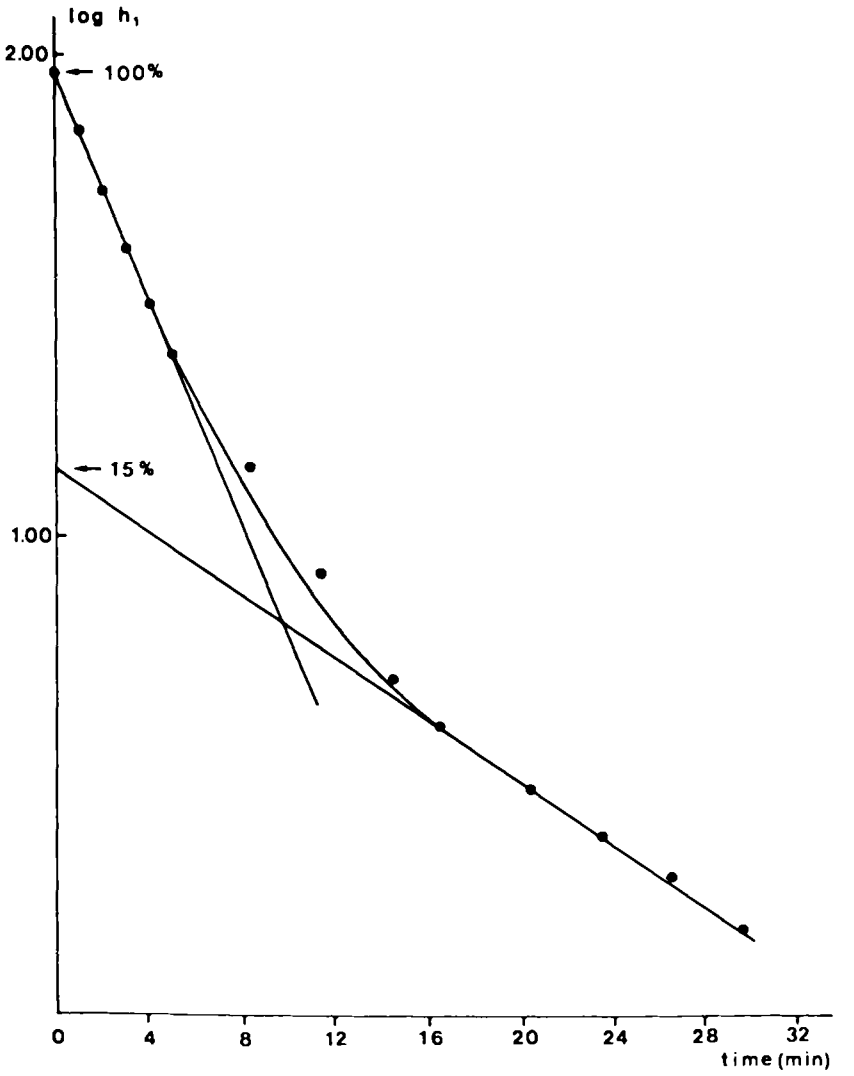


FIG. 5. Kinetics of reduction of labeled BSA with ascorbic acid; h_1 represents the intensity of the EPR line associated with the less mobile spin-label.

Figure 5 reports the kinetics of reduction of labeled BSA, monitored by the decrease in intensity of the EPR lines associated with the less-mobile spin label when the fiber is treated with ascorbic acid.

As can be observed, the curve shows two different slopes, which can be taken as an indication of the presence of differently accessible albumin. From the intercept with the ordinate one can evaluate the amount of the less accessible protein most likely dispersed within the porous phase, and this turns out to be around 15%.

The presence of two signals owing to the labeling of albumin at two different positions can be exploited also to detect the existence of an interaction between the fiber and the entrapped albumin. In fact, the ratio $h_1/(h_1 + h_2)$ (see Fig. 3a) can be related to the changes of mobility of the spin label and thus to the modifications of the secondary and tertiary structure of labeled albumin.

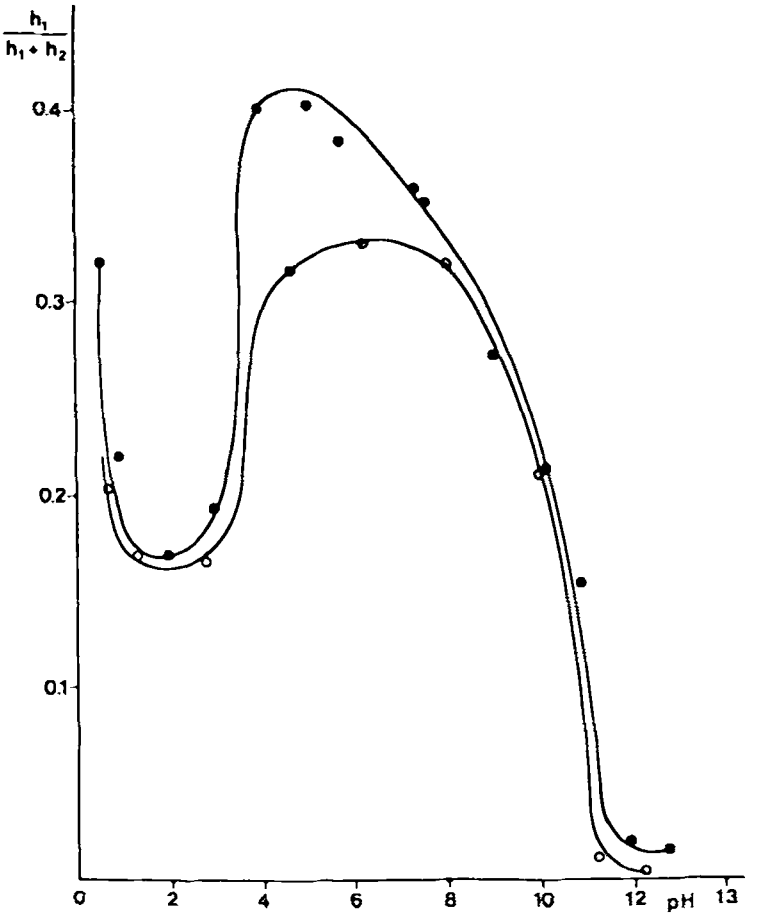


FIG. 6. Variation, as a function of pH, of the ratio between the strongly immobilized and more mobile spin label bound to BSA: ○ labeled BSA in 0.1 *M* KCl solution; ● labeled BSA entrapped in cellulose triacetate fibers. h_1 and h_2 are related to the concentration of the strongly immobilized and more mobile spin label, respectively (see Fig. 3a).

If there exist a strong interaction between the fiber and the entrapped protein that may hinder its mobility, the behavior of the entrapped protein should be different from that observed in solution.

In Fig. 6 is reported the variation of the above mentioned ratio, as a function of pH, for labeled BSA in fiber and in solution.

In both cases a transition is evident between pH 2 and 4, which can be associated with the large variation on volume of BSA in this range of pH, already detected by other experimental techniques (*15, 16*), and owing to the unfolding of the protein molecule.

Also the denaturation of the protein at basic pH, or with denaturing agents such as urea (Fig. 7), can be monitored by the spin-label technique that indicates that the process takes place in practically the same fashion both in fiber and in solution.

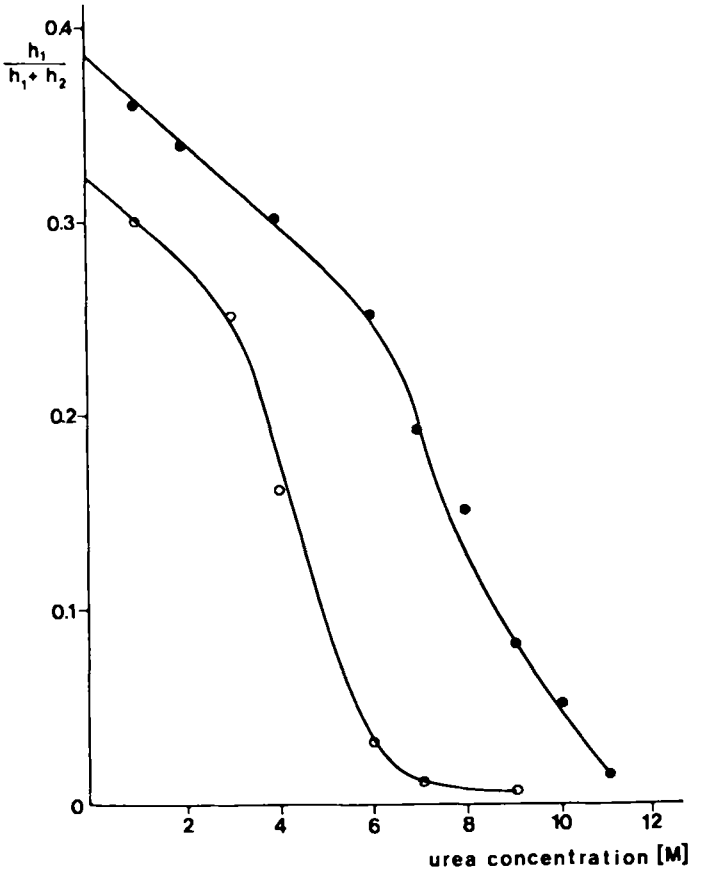


FIG. 7. Variation, as a function of urea concentration, of the ratio between the strongly immobilized and more mobile spin label bound to BSA: ○ labeled BSA in 0.1 *M* KCl solution; ● labeled BSA entrapped in cellulose triacetate fibers. h_1 and h_2 are related to the concentration of the strongly immobilized and more mobile spin label, respectively (see Fig. 3a).

From these results it is evident that the entrapped protein must not interact very much with the fiber if it can undergo large conformational modifications as those taking place as function of pH, when the structure goes from globular ($[\eta] = 2.7$ cm^3/g) to random coil ($[\eta] = 22$ cm^3/g). Therefore it is not too far fetched to affirm that the behavior of the protein is the same in fiber and in solution. Certainly, some interaction between the fiber and BSA takes place, as indicated by the variation of ΔH_{-1} linewidth as a function of pH and urea concentration (Fig. 8). As it can be seen, only for limit values of pH and high urea concentration, the linewidth is similar both in fiber and in solution. This fact is indicative of a second-order interaction, not yet identified, which, howver, seems to have little influence on the basic behavior of albumin.

Concluding, the following can be derived from our EPR investigations:

1. Apparently, only two phases are present within cellulose triacetate fibers prepared with our procedure.

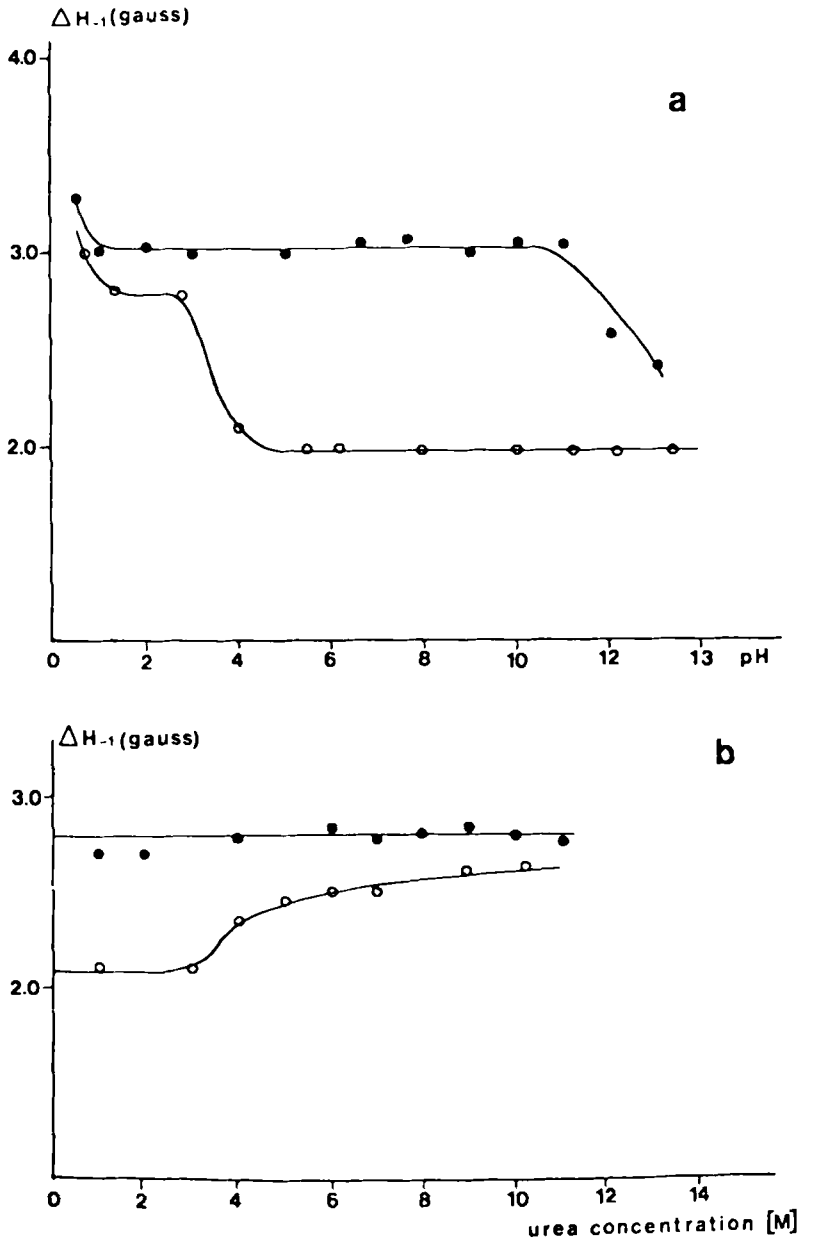


FIG. 8. Variation (ΔH_{-1}), as a function of pH and urea concentration, of the width of the low field EPR line (see Fig. 3a) of labeled BSA: ○ labeled BSA in 0.1 *M* KCl solution; ● labeled BSA entrapped in cellulose triacetate fibers.

2. When trapped within the fiber, albumin is mostly dispersed in the liquid phase.
3. After the standard drying procedure albumin is almost completely precipitated.
4. When the fiber is placed again in solution, the protein is redissolved, its quantity depending upon the procedure of drying.

5. Inside the fiber the protein behaves as in solution. Interactions with the polymeric matrix are of second order.

These conclusions are, at least in part, likely extendable also to those cases in which an enzyme, instead of albumin, is immobilized into cellulose triacetate fibers.

Of course, definite conclusions in this respect could be drawn only after repeating our investigation with a labeled enzyme. For this purpose we have started an investigation on penicillin acylase that we found to retain its activity after labeling with the spin label I. The results of this investigation will be the subject of a following paper.

Acknowledgments

The authors wish to thank M. Barbini and A. Ludovici, for their excellent technical assistance.

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