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SPIN-LABEL STUDIES OF MEMBRANE-ASSOCIATED DENATURED HEMOGLOBIN IN NORMAL AND SICKLE CELLS

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Summary

A maleimide spin label (*N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)-maleimide) was reacted with oxyhemoglobin-free cell stromata of normal and sickle cells. The EPR spectrum of spin-labeled red cell membranes showed that the spin labels are attached to at least two different binding sites. There was a major signal, A, which characterized a strongly immobilized environment and a minor signal, B, which characterized a weakly immobilized environment. Quantitative EPR measurements using equal amounts of Hb AA and Hb SS red blood cells demonstrated that Hb SS red cell membranes had an approximately four times higher EPR signal intensity than Hb AA red cell membranes ($(7.98 \pm 1.14) \cdot 10^5$ and $(2.2 \pm 1.2) \cdot 10^5$ spin labels/cell, respectively). Moreover, the ratio of signal intensities A and B are different in these cells. Comparative spectrophotometric studies of membrane-associated denatured hemoglobins of Hb AA and Hb SS red cell membranes suggested that the EPR signal A is derived from spin labels attached to membrane-associated denatured hemoglobin, while signal B is mainly from spin labels attached to membranes. The combination of EPR spectrum of Hb AA membranes pretreated with *N*-ethylmaleimide and that of spin-labeled precipitated hemoglobin further strengthened this conclusion.

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Abbreviations: MalNEt, *N*-ethyl maleimide; SDS, sodium dodecyl sulfate.

Introduction

Despite the accumulation of biochemical and physiological knowledge of hemoglobin and red cell membranes, little is certain about their association in the native red blood cell and in red cell stromata. This may be attributed partly to the variation in amounts of hemoglobin attached to membranes prepared by different methods and partly to the lack of appropriate methods to investigate the nature of the interaction between hemoglobin and membranes.

Recently we have found that there are two types of hemoglobin associated with red cell membrane, soluble oxyhemoglobin and insoluble hemichrome-type denatured hemoglobin [1]. Even for the same blood sample, the amount of oxyhemoglobin associated with membranes varies widely depending on the lysing and washing media employed, while the amount of endogenous denatured hemoglobin on the membrane is constant unless hemoglobin is denatured during measurement. It was also found that the amount of denatured hemoglobin in sickle erythrocytes is approximately 5 times higher than that in normal red cells [1].

In this study, electron paramagnetic resonance (EPR) of the spin label analogue of maleimide, (*N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide, which binds covalently to reactive sulfhydryl groups, was used to investigate the nature of hemoglobin-membrane association.

The spin-label method is a unique physical method to study the conformation of biomolecules [2]. From the EPR signal of the spin label attached to a known site on a molecule, one can monitor the conformation, hydrophobicity, and mobilization of that particular environment. The spin label is also an excellent tool to quantify specific residues of macromolecules since the signal can be quantitatively measured regardless of whether the object is in solution, in a precipitated state, or on intact membranes. Although the spin label maleimide was used to study human and bovine erythrocyte membranes [3—10], the residue to which the spin label was attached is not known. Since the maleimide spin label combines with native and denatured hemoglobin, the EPR spectrum of red cell membranes should be affected by the amount and status of hemoglobin associated with membranes.

The purpose of this study was to investigate quantitatively the spin labels attached to oxyhemoglobin-free red cell membranes prepared from normal and sickle erythrocytes. By comparing the EPR spectrum and the total amount of spin label attached to membranes with the amount of membrane-associated denatured hemoglobin determined by the spectrophotometric method, the proportion of the spin label attached to denatured hemoglobin and membrane proteins can be estimated. It was found that the spin label method can be used for studying the amount as well as the conformation of membrane-associated denatured hemoglobin in normal and abnormal red cell membranes.

Methods

Preparation of red cell membranes. The method of preparation of red cell membranes was essentially the same as that of Asakura et al. [1]. 0.5 ml of packed red blood cells from normal adults and patients with sickle cell anemia

were hemolyzed by adding 30 ml of hemolyzing solution (5 mM phosphate/0.5 mM EDTA, pH 7.5). After the solution was brought to 0.9% NaCl concentration, it was centrifuged at 15 000 × *g*. The supernatant was removed carefully, and the red cell membranes were washed with the hemolyzing solution three more times. The supernatants from the above washings were pooled, and the total hemoglobin concentration was determined by optical spectroscopy with the use of the millimolar extinction coefficient of 15 $mM^{-1} \cdot cm^{-1}$ at 577 nm (heme basis). The hemoglobins associated with the membranes were measured by visible and EPR spectroscopies, respectively, as described below.

Measurements of membrane-associated hemoglobins. After red cell membranes were dissolved in 2.5% sodium dodecyl sulfate (SDS), the amount of hemoglobin associated with membranes was determined by the optical absorption at 404 nm with the millimolar extinction coefficient being 90.9 $cm^{-1} \cdot mM^{-1}$ (heme basis) [1].

EPR measurements of membrane-associated hemoglobin. Red cell membranes prepared from 0.5 ml of packed erythrocytes were suspended in 0.1 M phosphate buffer, pH 7.5, and mixed with 200 ml of 4 mM *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide. The reaction was allowed to proceed for two days at 4° C. Spin-labeled red cell membranes were repeatedly washed with 0.1 M phosphate buffer, pH 7.5, until no free spin labels were detected in the supernatant. The resultant membranes were placed in a EPR capillary tube (inner diameter = 1mm) for EPR measurement. The EPR spectrum was taken at 20° C with a Varian E-9 EPR spectrometer interfaced with a Nicolet Instrument Computer (Model 1074). The EPR spectrum, after being stored in the computer was double-integrated. The quantitation of the spin label in a sample was achieved by comparing the integrated intensity of the EPR spectrum with those of free spin-labeled solutions with various concentrations.

Reaction of membranes with N-ethylmaleimide. Blocking of SH groups was carried out by *N*-ethylmaleimide (MalNEt) prior to spin labeling. 1 ml of 10 mM MalNEt solution in 0.1 M phosphate buffer, pH 7.5, was added to the red cell membranes prepared from 0.5 ml packed cells. The reaction was allowed to proceed overnight. After removal of the unreacted MalNEt by repeated washing, the membranes were spin labeled as mentioned above.

Preparation of spin-labeled native and denatured hemoglobin. Spin-labeled native oxyhemoglobin was prepared by adding a 5-fold excess of the maleimide spin label in 0.1 M phosphate buffer, pH 7.5. After overnight reaction, the unreacted spin labels were removed by passing the mixture through Sephadex G-25 pre-equilibrated with 0.1 M phosphate buffer, pH 7.0. Spin-labeled denatured hemoglobin was obtained by mechanical shaking [11] or by heat denaturation of spin-labeled oxyhemoglobin; both methods yielded the same EPR spectrum.

Results

Spin labeling of red cell ghosts prepared from normal and sickle erythrocytes

In the previous paper [1], we demonstrated that native oxyhemoglobin can be totally removed from red cell ghosts by repeated washings with 5 mM phosphate containing 0.5 mM EDTA, pH 7.5. Incubation of the red cell mem-

branes thus prepared from normal (Hb AA) and sickle (Hb SS) erythrocytes with maleimide spin label resulted in the covalent attachment of the spin label to the membrane; the EPR spectra of the spin-labeled membranes are shown in Fig. 1. It is obvious that the EPR spectra of both spin-labeled Hb AA and Hb SS membranes show mixed spectra consisting of a strongly immobilized signal (signal A) and a weakly immobilized signal (signal B). The existence of two signals indicates that the spin labels are attached to two different types of micro environments on the red cell membranes. Quantitative measurement of the EPR spectra of Hb AA membranes from 12 individuals yields the value $(2.2 \pm 1.2) \cdot 10^5$ spin labels/cell; the same measurement from 12 patients with sickle cell anemia yields the value $(7.98 \pm 1.14) \cdot 10^5$ spin labels/cell. Therefore, Hb SS membranes have about four times as many spin labels attached than do Hb AA membranes.

Further examination reveals that Hb AA and Hb SS membranes not only give different EPR absorption intensities, they also have different line shapes. In Fig. 1C the EPR spectra of Hb AA and Hb SS membranes are traced with

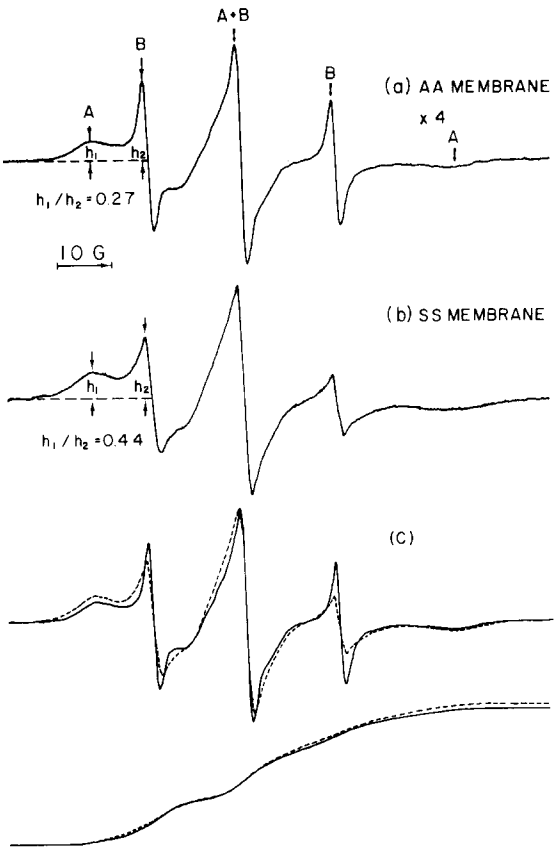

Fig. 1. Comparison of the line shapes of the EPR spectra of spin-labeled Hb AA (a) and Hb SS (b) red cell membranes. Note that the EPR spectrum of spin-labeled Hb AA red cell membranes has increased in intensity by 4 times. In (C) the spectra are traced with the same baseline for comparison. ———, Hb AA membrane; - - - - -, Hb SS membrane. Double-integrated curves at bottom.

the same baseline to demonstrate such differences. It is obvious that the spectral difference is due to a different signal A to signal B ratio; Hb SS membranes has a higher ratio than Hb AA membrane. As a convenient index, we use the ratio of two low field lines, h_1/h_2, to compare the relative population of the spin labels in a strongly immobilized environment to those in a weakly immobilized environment (Fig. 1). For 12 Hb AA membranes, the average value of h_1/h_2 ratio is 0.22 ± 0.09; for 12 Hb SS membranes the average value is 0.54 ± 0.22.

The nature of sites of spin labeling

To establish the origin of these two sites, membranes were treated with MalNEt which mainly reacts with the SH group, prior to spin labeling with *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide. By this treatment, the strongly immobilized signal A, which corresponds to more than 80% of the total EPR absorption, is completely suppressed (Fig. 2a). A similar observation has been reported by Chapman et al. [3].

An interesting finding is that the MalNEt-treated Hb SS stromata show a similar EPR spectrum to those of Hb AA erythrocytes, indicating that the increased number of spin label binding sites for Hb SS membranes can be attributed to the increased reactive sulfhydryl groups in Hb SS membranes. This result leads us to speculate that the major binding sites of red cell membranes may be sulfhydryl groups of denatured hemoglobin.

It was demonstrated in our laboratory that red cell membranes free of oxyhemoglobin contain a considerable amount of denatured hemoglobin, with Hb SS membranes containing about five times more denatured hemoglobin than do Hb AA membranes. To study the possibility that the major binding site of the spin label is the sulfhydryl group of denatured hemoglobin associated with red cell membrane, several experiments and calculations were performed.

First, the number of spin labels attached to red cell stromata is about twice

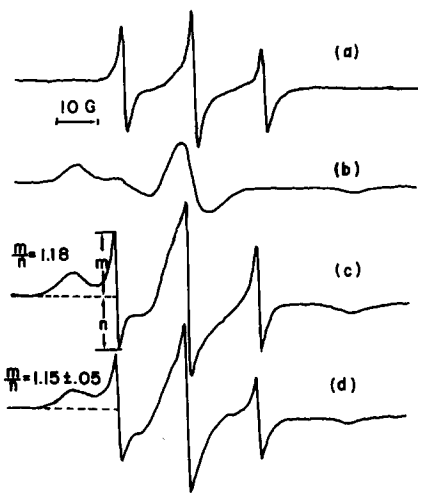

Fig. 2. (a) EPR spectrum of spin-labeled red cell membranes with SH groups previously blocked by MalNEt. (b) EPR spectrum of irreversible hemichrome resulting from mechanical or heat denaturation of spin-labeled hemoglobin. (c) Additive spectrum of spectra (a) and (b) in the intensity ratio 1 : 9. (d) EPR spectrum of spin-labeled red cell membranes (m/n value is the average of four indepdnent measurements).

that of hemoglobin molecules attached. If we assume that each hemoglobin molecule has two reactive sulfhydryl groups, the spin label results agree with results obtained by spectrophotometric measurement of denatured hemoglobin. Second, the complicated EPR spectrum of spin-labeled membrane may be reproduced from the EPR spectra of MalNEt-treated membrane and spin-labeled denatured hemoglobin, as demonstrated further.

Fig. 2a shows the weakly immobilized signal obtained from the spin-labeled membranes with SH groups previously blocked by MalNEt. Fig. 2b shows the denatured hemoglobin (irreversible hemichrome in which the heme iron is oxidized and its fifth and sixth positions are covalently bound by the proximal and distal histidine residues, respectively) obtained from mechanical or heat denaturation of the hemoglobin previously spin labeled with *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide. After adding spectra 2a and 2b in a Nicolet Instrument Computer with the intensity ratio 1 : 9, the resultant spectrum (Fig. 2c) is similar to what one actually obtains from spin-labeled red cell membranes (Fig. 2d).

In order to examine if the spin label method can distinguish oxyhemoglobin which remains in red cell ghosts due to incomplete washing from membrane-associated denatured hemoglobin, we also synthesized a summed spectrum from EPR spectrum of weakly immobilized signal (Fig. 3a) and that of spin-labeled oxyhemoglobin (Fig. 3b). The summed spectrum (Fig. 3c) appears also to be similar to that of spin-labeled red cell membranes (Fig. 2d), but clearly different from it if one compares the m/n ratio of the second peak (Fig. 2c); the ratio of the upper (m) and lower (n) amplitudes with respect to the base line in oxyhemoglobin-containing membrane is 1.45. In contrast the ratio is

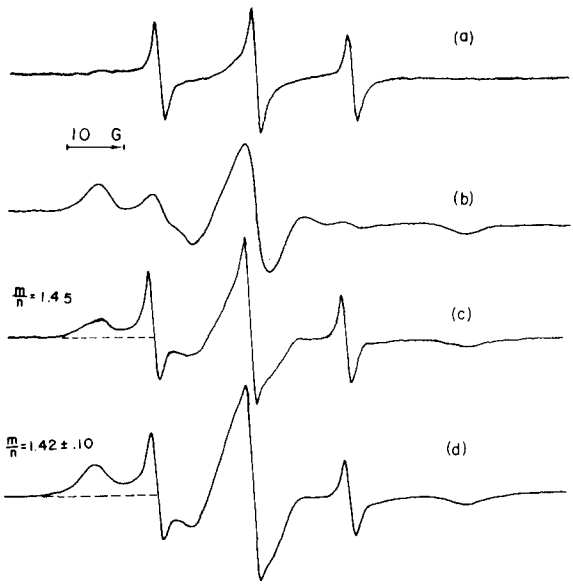

Fig. 3. (a) EPR spectrum of spin-labeled red cell membranes with SH groups previously blocked by MalNEt. (b) EPR spectrum of spin-labeled oxyhemoglobin. (c) Additive spectrum of spectra (a) and (b) in the intensity ratio 1 : 9. (d) EPR spectrum of spin-labeled red cell membranes, resulting from hemolyzing red blood cells by distilled water (m/n value is the average of four independent measurements).

1.15 ± 0.05 for all spin-labeled membrane containing only denatured hemoglobin. The higher m/n ratio of the second EPR peak with respect to the base line is due to the EPR absorption of oxyhemoglobin overlapping the EPR signal B at the same field.

Actually the type of EPR spectrum shown in Fig. 3c is observed in some spin-labeled red cell membranes containing oxyhemoglobin. Anderson and Turner [12] have reported that red cell membranes prepared by washing with distilled water contained a constant amount (3%) of hemoglobin associated with membranes. Spin labeling of such oxyhemoglobin-containing membranes gives an EPR spectrum (Fig. 3d) with m/n ratio being 1.42 ± 1.0, similar to the spectrum shown in Fig. 3c. In practice, this consideration is not important because all red cell ghosts prepared by the phosphate/EDTA buffer do not contain oxyhemoglobin as confirmed spectrophotometrically by using a double-beam spectrophotometer.

Discussion

EPR spectroscopy has been employed to study the structure of red cell membranes [3—10] using *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide as a spin label probe. The spectrum consists of two kinds of signals (signals A and B), reflecting the existence of two different types of binding sites for the spin label. Signal A, a strongly immobilized signal, comprises more than 80% of total EPR absorption. Neither the binding site nor the nature of the binding of the spin label on red cell membranes has been described.

Quantitative comparison of EPR spectra of spin-labeled red cell membranes from normal adults and patients with sickle cell anemia affords an insight into this problem.

There are several different units in use and described in the literature to quantify membrane-associated hemoglobin; namely, its percentage relative to the total hemoglobin content in the red blood cells, mg of membrane-associated hemoglobin in $1 \cdot 10^9$ ghosts, and the number of hemoglobin molecules associated with the membrane in one red blood cell. It is possible to convert one value to another. For instance, Sears [13] reported that membrane-associated hemoglobin is 0.01 mg/$1 \cdot 10^9$ cells for Hb AA membranes, which corresponds to $(0.01 \cdot 10^{-3} \times 6.0 \cdot 10^{23})/64\,000 \cdot 10^9) = 0.9 \cdot 10^5$ hemoglobin molecules/cell (where $6.0 \cdot 10^{23}$ and 64 000 are the Avagadro's number and the molecular weight of hemoglobin, respectively), which is also equivalent to 0.01 pg/cell = 0.01/30 = 0.033% (one red blood cell contains 30 pg of hemoglobin [14]). The latter value is in agreement with that obtained by Asakura et al. [1] for Hb AA membranes.

We have observed that the number of spin labels attached to one cell is $(2.2 \pm 1.2) \cdot 10^5$ and $(7.98 \pm 1.14) \cdot 10^5$ for Hb AA and Hb SS red cell membranes, respectively. The spin labels are mainly attached to the β-93 sulfhydryl groups of denatured hemoglobin associated with red cell membrane; therefore, one can calculate the number of molecules of denatured hemoglobin associated with membrane and the percentage of membrane-associated denatured hemoglobin in relationship to the total hemoglobin in a red blood cell. The values for Hb AA and Hb SS membranes so calculated are tabulated in Table I.

TABLE I

AMOUNT OF MEMBRANE-ASSOCIATED DENATURED HEMOGLOBIN FROM Hb AA AND Hb SS RED CELL MEMBRANES MEASURED BY OPTICAL AND EPR METHODS

	Membrane-associated denatured Hb (% ± S.D.)	Number of molecules of denatured Hb/cell ($\times 10^5$ ± S.D.)	Number of spin labels/cell ($\times 10^5$ ± S.D.)
Hb AA membrane			
Optical method	0.042 ± 0.011 (n = 19)	1.2 ± 0.3 (n = 19)	—
EPR method	0.039 ± 0.020 (n = 12)	1.1 ± 0.6 (n = 12) *	2.2 ± 1.2 (n = 12)
Hb SS method			
Optical method	0.210 ± 0.100 (n = 10)	6.0 ± 2.9 (n = 10)	—
EPR method	0.140 ± 0.020 (n = 12)	3.99 ± 0.57 (n = 12) *	7.98 ± 1.14 (n = 12)

* Values calculated based on the assumption that two spin labels attached to one hemoglobin molecule.

As a comparison, we also measured the amount of membrane-associated denatured hemoglobin by optical spectroscopy. These values are also listed in Table I. Similar values achieved from EPR and optical methods for both Hb AA and Hb SS membranes strengthen the previous suggestion that the spin labels are mainly attached to the β-93 sulfhydryl groups of the denatured hemoglobin associated with membrane. Furthermore, if the spin labels mainly react with membrane proteins, as generally assumed in previous studies, one would expect similar intensities of EPR spectra for Hb AA and Hb SS cells, since the membrane proteins for these cells are similar by quantitative SDS-polyacrylamide gel electrophoresis [15,16]. In contrast, the binding sites of the maleimide spin label on Hb SS membranes are found to be 4—5 times as numerous than those on Hb AA membranes, a result with agrees with the increased accumulation of denatured hemoglobin in Hb SS cells by SDS-polyacrylamide gel electrophoresis [16] and by spectrophotometric measurement.

As to the binding site of the spin label, it is difficult to judge whether previous studies and our present study refer to the same labeling sites because the results of previous studies were not quantitative. The previous studies also did not state the amount and ratio of denatured hemoglobin remaining in their membrane preparation.

It was demonstrated that membrane-associated denatured hemoglobin is irreversible hemichrome in nature [1]. It is now clear that the higher values of membrane-associated hemoglobin obtained in some previous studies were due to incomplete removal of oxyhemoglobin from membranes. The fact that oxyhemoglobin can be completely removed by changing the pH and ionic strength of the washing medium indicates that its binding is an ionic one. The mode of association between the hemichromes and the membranes is not clear. Jacob proposed that their association is by disulfide bonding [17]. Using SDS-polyacrylamide gel electrophoresis, Winterbourn and Carrell [18] concluded that covalent bonding, in particular disulfide bonding, was not involved in the association. After eliminating this possibility, they suggested hydrophobic bonding as the likely alternative. It is worthy to point out that from the line shape of the EPR spectrum one can distinguish denatured hemoglobin from oxyhemoglobin. As shown in Fig. 2, the EPR spectrum of spin-labeled red cell membranes can be generated by the addition of spin-labeled membrane free from

hemoglobin (signal B) and that of spin-labeled denatured hemoglobin. Since substitution of the latter by spin-labeled oxyhemoglobin gives a distinctly different EPR spectrum, EPR spectroscopy may afford a way to probe the state of the hemoglobin associated in the membranes.

As to the weakly immobilized signal (signal B), the binding site of the spin label is not known at present. By blocking SH groups with MalNEt prior to spin labeling (*para*-chloromercuribenzoate cannot be used because it has been shown to extract membrane proteins from red blood cells [19]), it can be shown that signal B is the only signal remaining and that it accounts for 5—20% of the total spin labels. In addition, the number of spin labels attached to MalNEt-treated membranes prepared from Hb AA and Hb SS cells are similar. We have shown that the ratio of the spin labels in these two different environments, represented by h_1/h_2, are different for Hb AA and Hb SS membranes; furthermore, the difference is reproducible. Since signal B can be obtained by the method mentioned above, one can separate the EPR spectrum of red cell membrane into pure signal A and pure signal B by computer manipulation. Therefore, the percentage of the intensity of signal A (or signal B) as the total can be estimated after double-integration. The mean of the fraction of the intensity of signal B as the total intensity is calculated to be 18 ± 5% for five Hb AA membranes and 7 ± 3% for five Hb SS membranes. These values are in agreement with the higher h_1/h_2 value for Hb SS membrane and with the conclusion we arrived at previously that Hb SS membrane contains a greater amount denatured hemoglobin than does Hb AA membrane.

It has been reported that unlike MalNEt, its spin label analogue, *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide, is less specific to SH groups and also reacts with the ϵ-NH_2 group of lysine [20]. These results suggest that the weakly immobilized EPR spectrum originated from the spin label bound to the ϵ-NH_2 group of lysines of the membrane protein. It is worthwhile to point out that MalNEt reacts mainly with the sulfhydryl groups. Nevertheless, it can react with the NH_2 groups of lysine at pH 7 with reactivity 1000 times less than that for the sulfhydryl group [21]. The reactivity with lysine becomes more significant as the pH increases and the time increases [21]. Our conclusions may not be absolute and may need modification accordingly.

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