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such as the DBBF crosslinked methHb, were reduced at approximately the same rate as methHb. The reduction of methemoglobin could also be accomplished with Sepharose-immobilized FMN, but the reaction rate was considerably slower than with the soluble catalyst.

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**A NEW METHOD FOR THE REDUCTION OF METHEMOGLOBIN
AND METHEMOGLOBIN DERIVATIVES**

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SUMMARY

The chemical reduction of methemoglobin (metHb) with dithionite and other reducing agents has proven unsatisfactory due to undesirable byproducts. We have now developed a method to reduce methemoglobin on a preparative scale, in which metHb is reduced using photoactivated flavin mononucleotide (FMN). The reduction is performed in the absence of oxygen, since oxygen is reduced more rapidly than metHb and leads to the formation of H_2O_2 . DL-methionine was routinely used as the electron donor, although a variety of compounds could act in this capacity. Thus, a 1% metHb solution containing 20 Mm DL-methionine and 20 μ M FMN was reduced by more than 95% in 30 min using a 300-watt light bulb. The reaction rate depended on the methionine concentration as well as on the light intensity. The reaction was independent of pH between pH 6 and 8. Derivatives of metHb, such as the DBBF crosslinked metHb, were reduced at approximately the same rate as metHb. The reduction of methemoglobin could also be accomplished with Sepharose-immobilized FMN, but the reaction rate was considerably slower than with the soluble catalyst.

Key Words: Methemoglobin reduction; Flavin mononucleotide (FMN); Hemoglobin A₀; Photoactivation; Catalytic reductions.

INTRODUCTION

Studies with hemoglobin and myoglobin are often difficult to perform with the pure ferrous proteins because these proteins oxidize spontaneously and relatively rapidly to the corresponding ferric forms. This oxidation makes it difficult to obtain proteins that are 100% in the ferrous form. For the same reason, long-term storage of hemoglobin solutions must be stored for long periods of time at either very low temperatures or in the complete absence of oxygen, a condition that is not always easy to achieve. One is thus often faced with the problem of reducing the methemoglobin that has formed in a hemoglobin solution before valid studies with the hemoglobin can be initiated. The oxidation to methemoglobin can be especially bothersome in storing solutions of precious hemoglobin variants or hemoglobin derivatives.

Various reducing agents have been reported to reverse this oxidative process. Thus, the chemical reduction of the ferric methemoglobin to the ferrous form can be accomplished with a variety of reducing agents, such as dithionite [1], ascorbate [2,3], 5-hydroxyanthranilic acid [4], iron salts and EDTA-metal complexes [5], ferredoxin [6] and NADH in the presence of associated enzymes [7].

Achieving a direct reduction of the ferric iron with a reducing agent is often difficult without either an incomplete reduction of the methemoglobin or the formation of undesirable byproducts. For example, the reduction with dithionite or EDTA results in the formation of SO_3^{--} and formaldehyde, respectively, which react with other parts of the protein and lead to modifications of the protein and deterioration of the pigment [8,9]. The reduction with other reducing agents, such as ascorbate and cysteine, reaches an equilibrium when there are still considerable amounts of methemoglobin present in the solution [see e.g. ref. 10].

Further research into this problem led to the use of catalysts to mediate the transfer of electrons between an electron donor and methemoglobin. Thus, in a preliminary report, Kajita et al. [11] published a method to regenerate oxyhemoglobin using phenazine methosulfate as the catalyst and NADH as the electron donor. The reduction was reported to occur even in the presence of oxygen, but the rate was quite slow, and without indication that a complete reduction could be achieved in this manner.

Several investigations have been reported recently in which small amounts of metHb were reduced electrochemically, using electrodes coated with methylene blue [12,13]. In addition, Durliat and Comtat [14] described the electrochemical reduction

of methemoglobin at a Pt electrode, using flavin mononucleotide (FMN) as the catalyst. Since oxygen is reduced before methemoglobin under those conditions, the reduction was performed under anaerobic conditions. The authors reported that 25 ml of a 1 Mm hemoglobin solution containing 55% methemoglobin could be completely reduced in 25 min, using 0.2 Mm FMN.

The reduction of methemoglobin using photochemically reduced FMN as a catalyst was first reported by Yubisui et al. [15]. These authors showed that the reduction of methemoglobin by reduced FMN is extremely fast compared to the rates obtained with other agents, and is also quite specific. The photoactivated FMN was reduced using EDTA as the electron donor. McCormick et al. [16] published a list of electron donors that readily react with photoactivated FMN, most of which reduce photoactivated FMN at a rate similar to that of EDTA. In a similar study, Frisell et al. [9] showed, however, that the oxidation of EDTA (and several other compounds) by photoactivated FMN results in the formation of an almost equimolar amount of formaldehyde. Formaldehyde reacts rapidly with amino groups in proteins, a characteristic which makes EDTA considerably less attractive as an electron donor to reduce compounds such as methemoglobin.

In all cases cited above [with the exception of ref. 14] the experiments on the reduction of methemoglobin were carried out more or less on an analytical scale and only small quantities of

methemoglobin were reduced. It was the goal of the present study to develop a method for the reduction of methemoglobin for use on a preparative scale.

MATERIALS AND METHODS

Riboflavin mononucleotide, riboflavin, DL-methionine, and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. FMN immobilized on Sepharose was also obtained from Sigma; this product contained 0.68 μ M FMN per ml of packed resin. Hemoglobin A₀ and DBBF-hemoglobin were prepared in our facility as previously described [17-19].

Preparation of Methemoglobins - A small molar excess (10 to 20%) of potassium ferricyanide was weighed out and dissolved in a minimum amount of water. The hemoglobin solution was cooled in an ice bath and the ferricyanide solution was then added, slowly, under stirring, and in the dark. Stirring was continued in the cold and in the dark for about 30 min. The oxidized hemoglobin solution was then allowed to warm up to room temperature and was passed through a mixed bed resin column to remove the reaction products. If, after passage through the column, the conductivity of the solution was more than 10 mhos, the solution was again passed through a freshly mixed bed resin column. Alternatively, the reaction products may be removed by extensive dialysis against cold distilled water.

Reduction of Methemoglobins - Twenty ml of a 0.1% methemoglobin solution in 10 mM tris buffer, pH 7.2, were placed in a 20-ml

Coulter counting vial. The appropriate amount of reductant was added in solid form to yield a final concentration of 20 mM. The vial was closed with a rubber stopper, equipped with an oxygen electrode and with two 18G1 and one 20G3 hypodermic needles. The long needle was used to add reagents to the reaction mixture and to remove samples for analysis. The solution was continuously stirred with a small stirring bar. The solution was deoxygenated by continuously flushing pure nitrogen gas over the solution through one of the 18G needles, with the other needle serving as a vent. Two hundred microliters of a 0.01% solution of FMN was added to the hemoglobin solution in the dark (final concentration: 2 μ M) and nitrogen flushing was continued until the oxygen pressure was less than 2 torr. The reduction was started by turning on the light source. At specified time intervals 200- μ l samples were removed and the remaining methemoglobin concentration was determined spectrophotometrically. Fig. 1 shows a diagram of the reaction vessel.

RESULTS

Effect of Oxygen on the Reduction of Methemoglobin

The oxidation-reduction potential for the reduction of molecular oxygen to hydrogen peroxide is 0.295 V at pH 7.0 [20], whereas the redox potential for the reduction of the ferric to the ferrous form of hemoglobin is 0.16 V under the same

conditions [12,21]. Acting on a methemoglobin solution at neutral pH, reduced FMN will therefore first reduce any oxygen present before it will reduce the methemoglobin. It is well known that hydrogen peroxide reacts rapidly with ferrous hemoglobin to form methemoglobin. Therefore, to achieve a complete reduction of the methemoglobin, it is imperative that the reduction with reduced riboflavin be done in the absolute absence of oxygen.

Rate Profile of Methemoglobin Reduction

Fig. 2 shows a typical set of data obtained when 0.1% methemoglobin was reduced using 20 mM DL-methionine as the electron donor. The reduction was rapid and virtually linear, but slowed down when the reaction was near completion. The results of two independent experiments are shown, illustrating the slight variation in rate that may be observed between individual experiments. The figure also illustrates the increase in deoxyhemoglobin in the reaction mixture, which closely parallels the reduction of the methemoglobin.

Effects of Various Catalysts

In a first set of experiments we compared the effectiveness of FMN with that of methylene blue and phenazine methosulfate as a catalyst in the reduction of methemoglobin. We found the latter two to be quite inferior to FMN. The results obtained with phenazine methosulfate as a catalyst for the reduction of

oxygen as well as methemoglobin are shown in Fig. 3. In the absence of light the reduction was very slow, just a few percent per hour, as was illustrated by Kajita et al. [11]. Illumination of the reaction mixture resulted in a large increase in the rate and yielded the results shown in Fig. 3. Nevertheless, the rate of reduction is much slower than with an equivalent amount of FMN. Furthermore, the reaction does not appear to go beyond the reduction of about 50% of the methemoglobin. Adding more phenazine methosulfate did not result in any further reduction. The results obtained with methylene blue were similarly inferior to those obtained with FMN.

We also tested immobilized FMN as the catalyst using a commercial preparation in which FMN was immobilized onto Sepharose via the ribose moiety. One ml of packed resin was suspended into the reaction mixture, which also contained 0.1% methemoglobin and 20 mM methionine. The observed rate of methemoglobin reduction was 1.56% per minute, which is about 13.5% of the rate that we obtained in a parallel experiment with an equivalent amount of soluble FMN.

Comparison of Various Electron Donors

Table I tabulates the initial rates of methemoglobin reduction, obtained with various electron donors that were selected from the list of donors published by Frisell et al. [9]. The results clearly show that the reduction is fastest in the

presence of ascorbate, followed by EDTA and methionine. Unfortunately, oxidation of EDTA by photoactivated FMN leads to the formation of formaldehyde [9], which is detrimental to proteins. Several other electron donors also form formaldehyde or acetaldehyde, including glycine, dimethylglycine and sarcosine [9]. We further found that reductions using ascorbate as the electron donor did result in the formation of turbidity toward the end of the reaction; for this reason we did not use ascorbate in the bulk of our experiments.

Effect of Electron Donor Concentration

The dependence of the reaction rate on the methionine concentration is shown in Table II. The rate increases rapidly with increasing methionine concentration up to 20 mM, where it seems to begin to level off. This could indicate that the oxidation of methionine may be the rate-limiting step in the series of reactions leading to the reduction of methemoglobin.

pH Dependence of the Rate of Reduction

We subsequently investigated the pH dependence of the reaction. As shown in Table III, the rate is independent of pH between 6 and 8. Comparable results were obtained using EDTA as the electron donor (data not shown).

Effect of the Optical Density of the Solution

The reduction of methemoglobin proceeds much faster in more dilute solutions of methemoglobin, as shown in Table IV. This at first may suggest that the rate of reduction is also dependent on the methemoglobin concentration. However, Table IV also shows the optical densities at 450 nm of the reaction mixtures containing various methemoglobin concentrations. The photoactivation of FMN occurs maximally at 450 nm [22]. Since the total light path of our reaction vessel was 20 mm, it is clear that at a concentration of 0.1% methemoglobin, less than 10% of the light completely penetrates the reaction mixture, and at 0.2% methemoglobin this value is less than 1%. Hence, the high absorbencies at 450 nm of the higher methemoglobin concentrations prevented the light from completely penetrating the reaction mixture, and consequently limited the photoactivation of FMN. Therefore, the decrease in rate with increasing methemoglobin concentration may primarily reflect a decrease in photoactivation of the FMN, due to absorbance of the activating light by the reagents. If this interpretation is correct, then using a reaction vessel with a smaller light path would lead to an increase in the rate under otherwise identical conditions. This was indeed found to be the case (data not shown).

DISCUSSION

The reduction of methHb by reduced FMN proceeds with a rate constant of $3.3 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$, which is very fast compared to the rate with other reducing agents [15]. Moreover, the redox potential ($E^{\circ'}$) of FMN/FMNH₂ is -0.219V, whereas that of Fe⁺⁺⁺/Fe⁺⁺ in hemoglobin is 0.144V [21, 23, 24]. These values indicate an equilibrium constant of about 10^{12} for the reduction of methHb by FMNH₂ at pH 7.0. The reduction of methemoglobin is thus not only rapid, but also goes to completion even in the presence of catalytic amounts of FMNH₂, as long as the FMNH₂ is regenerated. The use of FMN as the catalyst thus provides a convenient way to reduce small amounts of methHb that may be present in hemoglobin solutions.

The regeneration of FMNH₂ can be accomplished with a variety of electron donors following activation of the FMN by light. The photoactivation of FMN is also a very fast process. Therefore, the rate-limiting step in the overall reaction scheme is most likely the reduction of the photoactivated FMN by the electron donor. Our finding that the overall reaction rate is dependent on the methionine concentration would be consistent with this conclusion. However, since the redox potentials of most of these electron donors and their oxidation products are unknown, the theoretical overall reaction equilibria of the FMN-catalyzed

reduction of metHb with the various electron donors cannot be calculated. Our experimental results nevertheless show that a virtually complete reduction of methemoglobin can be obtained with a number of different electron donors.

The sequence of the chemical reactions during the reduction of methemoglobin thus appears to be the following: FMN is photoactivated by light of 450 nm, producing the photoactivated FMN*. This is reduced by methionine to FMNH₂ in what appears to be the rate-limiting step. The reduction is a two-electron reduction, presumably yielding methionine sulfoxide as the initial product. The FMNH₂ reacts very rapidly with one mole of methemoglobin, yielding deoxyhemoglobin and the semiquinone form of FMN, followed by a regeneration of the catalyst by the reduction of a second mole of methemoglobin.

Of the various electron donors tested, we found ascorbic acid to be the most effective, followed by EDTA. It was somewhat surprising to find that sulfhydryl-containing compounds such as cysteine and dithioerythritol, which are well known reducing agents, were less effective than the thioether methionine in reducing photoactivated FMN.

We also did a few experiments using immobilized FMN as the catalyst, because immobilized FMN can be readily separated from the reaction products by filtration. This is important, because

as soon as oxygen is readmitted to the reaction mixture, the FMN will reduce the oxygen to hydrogen peroxide, which in turn will quantitatively reoxidize hemoglobin back to methemoglobin. It is thus essential that the catalyst be removed before the anaerobic conditions are terminated, unless the mixture is kept in complete darkness. As expected, we found that the immobilized FMN is less effective than soluble FMN under comparable conditions. However, the activity of the immobilized FMN appears to be sufficiently high to reduce large amounts of methemoglobin by packing the immobilized FMN in a small diameter column, over which a methemoglobin solution would be filtered while the column was illuminated with an appropriate light source. Experiments along these lines are presently in progress.

The data presented in Table IV indicate another potential problem that may be encountered when large amounts of methemoglobin are to be reduced with this method. The wavelength at which FMN is optimally activated is about 450 nm. Hemoglobin, however, has a considerably high absorbance at this wavelength. Therefore, FMN present in a solution containing a high concentration of methemoglobin, such as 0.2% and higher, will only be activated at the surface, but little light will penetrate the solution deeply enough to activate the remaining FMN. This difficulty can be overcome by leading concentrated methemoglobin solutions through a small diameter glass column which is surrounded by light sources. The ideal diameter of such a column

can readily be calculated from the absorbance at 450 nm of the solution that is to be reduced.

SIMPLIFIED DESCRIPTION OF THE SYSTEM

Methemoglobin can be effectively and completely reduced to deoxyhemoglobin with several reducing agents when photoactivated flavin mononucleotide (FMN) is used as a catalyst. Reducing agents include ascorbate, EDTA, methionine, cysteine, and others. The reduction occurs readily using a white light source, but it must be done in the absence of oxygen. Methemoglobin solutions of 0.1% (0.06 mM) are reduced within minutes in the presence of 2 μ M FMN and 20 mM of one of the reducing agents.

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TABLE I
Reduction of Methemoglobin With
Various Electron Donors^a

Electron Donor (20 mM)	Rate of Reduction (%/min)		
	Exp. 1	Exp. 2	Average
Ascorbate	6.71	6.16	6.435
EDTA	5.88	6.05	5.965
dl-Methionine	4.01	4.20	4.105
Nicotine	4.75	3.13	3.940
Dithioerythritol	2.77	2.98	2.875
l-Cysteine	1.49	1.65	1.570

^a MetHb: 0.075%

TABLE II

Dependence of the Photochemical Reduction of Methemoglobin on the Methionine Concentration^a

Methionine (mM)	Rate of Reduction (% per min)
0	0.43
5	4.65
10	8.16
20	11.92

^a MetHb: 0.075%

TABLE III

Rate of Reduction of Methemoglobin
at Various pH Values^a

pH	Rate of Reduction (% per min)
5.96	2.482
6.04	2.107
6.95	2.469
7.06	2.013
7.96	2.335
8.01	2.338

^a MetHb: 0.10%; methionine: 20 mM

TABLE IV

Rate of Reduction As A Function Of
Methemoglobin Concentration^a

Methemoglobin (%)	Reduction Rate (% per min)	OD ₄₅₀ in 20 mm light path
0.050	9.690	0.592
0.075	3.846	0.885
0.100	2.235	1.172
0.125	0.999	1.468
0.150	0.740	1.770
0.200	0.281	2,303

^a Methionine: 20 mM.

FIGURE LEGENDS

- Fig. 1. The reaction vessel used in these experiments.
- Fig. 2. Time course of the reduction of methemoglobin with photoactivated FMN and of the formation of deoxy-hemoglobin. The results of two independent experiments are shown.
Methemoglobin: 0.1%; FMN: 2 μ M; dl-methionine: 20 mM; buffer: 10 mM tris-HCl, pH 7.2. Total volume: 20 ml.
- Fig. 3. Time course of the reduction of methemoglobin using photoactivated phenazine methosulfate. Conditions as in Fig. 2, except that FMN was replaced with 33 μ M phenazine methosulfate.

Fig 1

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Fig. 2

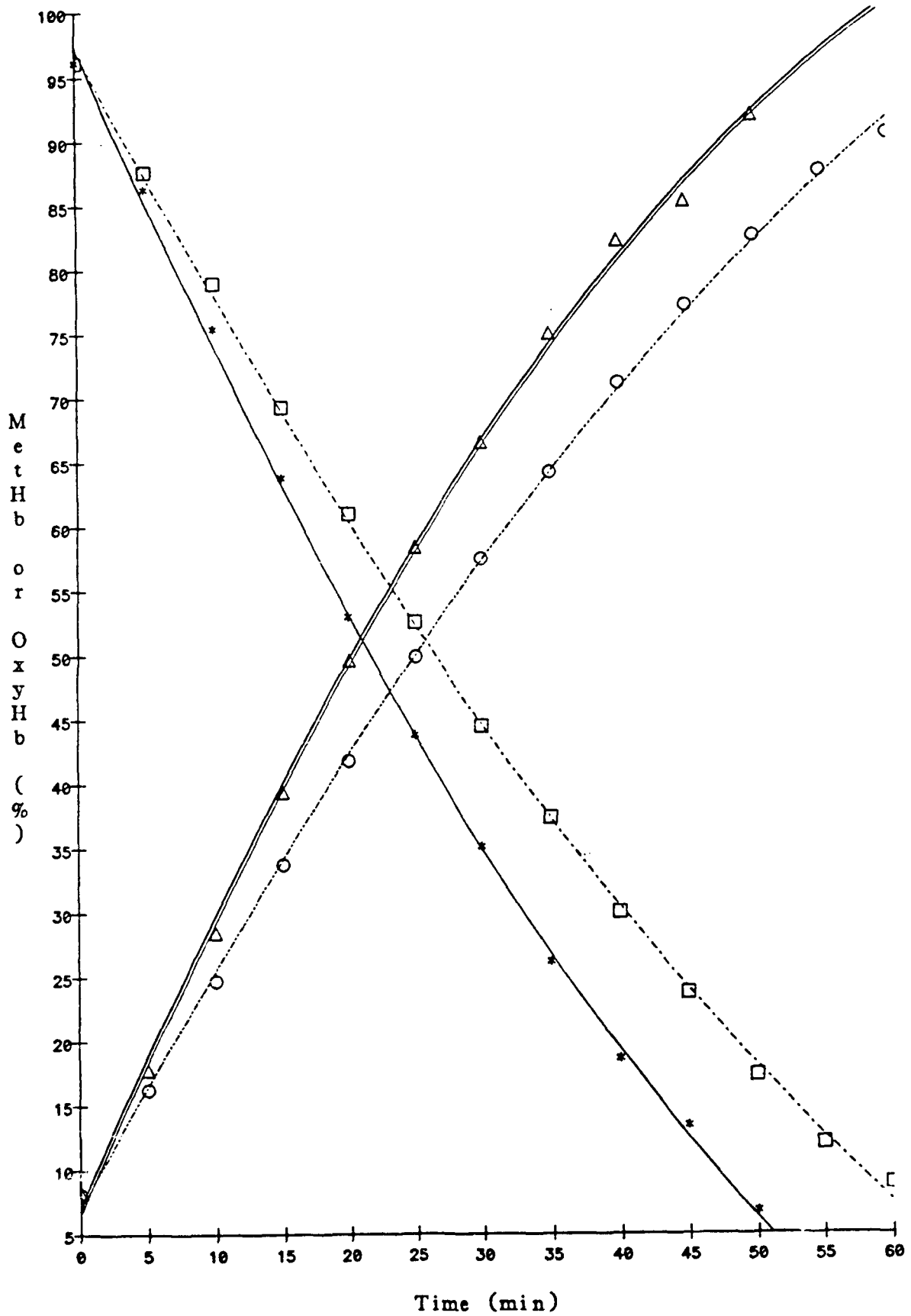


Fig. 3

