KINETIC STUDY OF THE REDUCTION OF METHEMOGLOBIN WITH ASCORBATE USING A COBAS-FARA CENTRIFUGAL ANALYZER

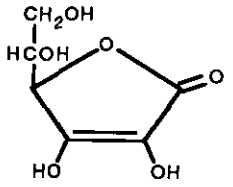
Kilian Dill[1*] and Evelyn L. McGown[2]

[1]Molecular Devices Corporation, 4700 Bohannon Drive, Menlo Park, CA, 94025 and [2]the Blood Research Division, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129. USA

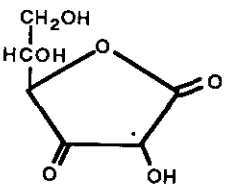
Abstract- The reduction-kinetics of methemoglobin with ascorbic acid were monitored using a COBAS-FARA centrifugal analyzer. The data from the initial rates for the reduction of hemoglobin were monitored as a function of the ascorbate concentration. The results show a first order dependence of the reaction rates on the ascorbate concentration and the rate constant was found to be $1.34 \times 10^{-2}\ s^{-1}M^{-1}$. Ascorbic acid is a one electron donor in this process and consequently forms a stable radical. The presence of oxyhemoglobin interferes with the reduction of methemoglobin by ascorbate.

Iron(III) hemoglobin (methemoglobin) can be reduced by a number of reducing agents,[1-3] but unwanted by-products are usually produced. Recently, we showed that hydrogen gas in the presence of a heterogeneous catalyst imbedded in a conducting organic polymer can be used to regenerate functional hemoglobin and that the by-product (H^+) can be buffered.[3,4]

Ascorbic acid is a biological molecule that has long been recognized as capable of reducing methemoglobin.[5] We demonstrated that erythrocytes can use ascorbate as an electron shuttling-agent to reduce extracellular molecules including methemoglobin.[2] Like other reducing agents, ascorbate produces deleterious by-products, especially at high concentrations and extended exposures.[6-8]



Ascorbic Acid



Stable Radical

Studies of the kinetics of the reduction of methemoglobin have been complicated by by-products (e.g., H_2O_2) which promote reoxidation and damage the protein. To minimize their effects, initial reaction rates must be measured to obtain valid information about the reaction kinetics. The COBAS-FARA centrifugal analyzer (Roche Instruments) is well-suited for studying the kinetics of such reactions because it can mix and monitor multiple reaction mixtures simultaneously. We used the instrument in a somewhat unusual mode to study the kinetics of reaction between ascorbic acid and methemoglobin. Ascorbic acid solutions of differing concentrations were placed in the 'sample cups' and the hemoglobin solution was placed in the 'reagent reservoir'. The instrument was easily programmed to mix the desired aliquots and monitor the reactions at 5 second intervals.

Stroma-free hemoglobin was obtained from the Letterman Army Institute of Research hemoglobin production facility. It was oxidized with a 20% excess of ferricyanide and dialyzed against distilled water to remove hexacyanoferrate ions. The kinetic protocol was as follows: Ascorbic acid stock solutions were freshly prepared (0.075 - 1.2 M) and placed into sample cups. The instrument was programmed to pipet 5 µl of each

ascorbic acid solution into a cuvette with 150 µl of 0.2 M phosphate buffer, pH 7.0. The cuvettes were spun to ensure mixing. A 50 µl aliquot of stock hemoglobin solution was added to each cuvette and the reactions were initiated by spinning the rotor. A_{630} readings were taken at 5 s intervals for 3 minutes. The final concentrations of the components in the cuvettes were 1.8 mM heme, 1.25 - 20 mM ascorbic acid, and 0.1 M phosphate buffer in a volume of 300 µl (including water diluents associated with the automatic pipetting system).

Figure 1 shows a typical plot of the kinetics (initial-rate data) of the reduction of 1.8 mM of 100% methemoglobin by 20 mM ascorbate. As can be seen, a straight line was observed over the short time interval studied. Plots for the lower concentrations of ascorbic acid were similar to Figure 1, except that the data were more scattered. The short reaction time (3 min) was chosen to minimize back reaction (reoxidation) and possible side reactions with oxygen.

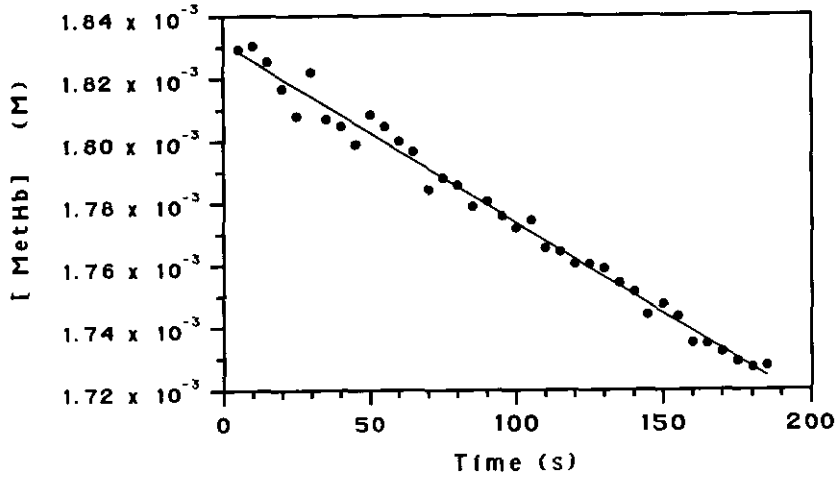


Figure 1. Kinetics of the reduction of methemoglobin (100% Met at a concentration of ~1.8 mM heme) with ascorbate (at a concentration of 20 mM). Optical readings were taken every 5 seconds. Each data point represents the average of two data sets.

Figure 2 is a plot of the initial rate of methemoglobin reduction vs. ascorbate

concentration. The plot is a straight line indicating that the reaction is first order in ascorbate. The reaction can thus be defined by $d[MetHb]/dt = k[Heme]^1[Ascorbate]^1$, with the slope of the line equal to $k[Heme]$.[1] A value of 1.34×10^{-2} $s^{-1}M^{-1}$ for the rate constant was obtained from the plot. These results can only be explained as a reduction of heme via a concomitant one-electron oxidation of the ascorbate to the relatively stable ascorbate radical (see structure), which apparently does not afford further reduction.[9]

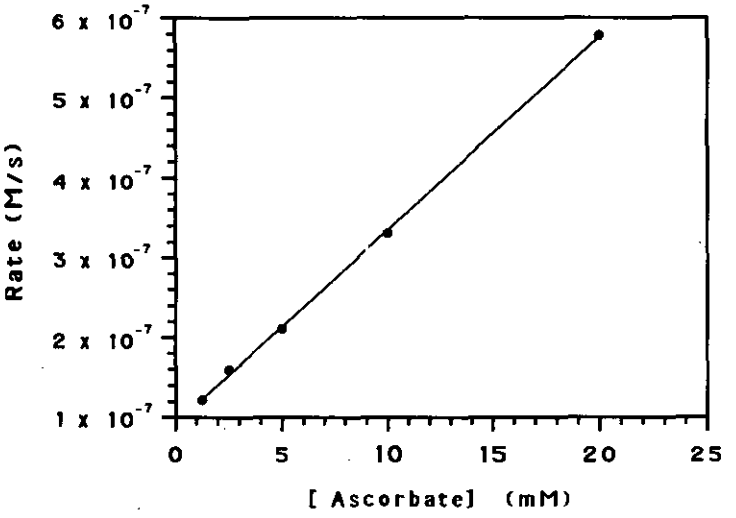


Figure 2. Plot of the rate of Methemoglobin reduction as a function of ascorbate. Data were taken from 100% MetHb data and the negative rates (for the reduction of MetHb) are redefined as positive values.

Presumably our results represent a reduction of the β-chains of hemoglobin because they are preferentially reduced by ascorbic acid.[6,7] We also investigated the reduction of hemoglobin/methemoglobin mixtures. This system was complicated by the presence of oxyhemoglobin that produced side reactions.[5,8,10] We found that the rates of reduction of 42% methemoglobin samples (concentration of 1.8 mM total heme) were always about 10-fold lower than those observed for the corresponding 100% methemoglobin samples. Nevertheless, we observed linear initial rates for the

reductions with a direct first order dependence on ascorbate concentration. By-products such as hydroxyl radicals (from the reaction of oxyhemoglobin with ascorbate) appear to reduce the reaction rate significantly.[8] The most pronounced effects on our initial rates were observed at the lowest ascorbate concentrations.
The kinetic data clearly show that methemoglobin can be reduced with ascorbate via a one electron transfer. The COBAS-FARA was found to be ideal for studying biological reaction and determining the rates of the reactions.

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