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**Ferrocene Mediated Electron Transfer of
Horseradish Peroxidase at Reticulated Vitreous Carbon**

Peter John Bozel

**A Thesis
in
The Department
of
Chemistry and Biochemistry**

**Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada**

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ABSTRACT

Ferrocene Mediated Electron Transfer of Horseradish Peroxidase at Reticulated Vitreous Carbon

Peter John Bozel

Optimization of steady-state colourimetric assays is important for detecting small quantities of horseradish peroxidase (HRP). Investigation of HRP assays using *o*-dianisidine and 2,2'-azino-di[3-ethylbenzthiazoline-6-sulfonate] (ABTS) as colourimetric reducing agents under saturating conditions revealed that *o*-dianisidine gave a more sensitive HRP determination than ABTS at pH 6.0 and 7.5. The effect of 5 mM pyridine and imidazole on the *o*-dianisidine HRP assay was investigated and did not increase the sensitivity of the assay. The minimum concentration of HRP detectable is ~0.1 nM using 120 μ M *o*-dianisidine and 100 μ M H₂O₂ at pH 6.0.

Ferrocene derivatives are among the best mediators of electron transfer between the heme of HRP and carbon electrode surfaces. H₂O₂ may be detected amperometrically using HRP and these mediators. Investigation shows that ferrocenemonocarboxylic acid (FCA) yields a more sensitive determination of H₂O₂ than (dimethylaminoethyl)-ferrocene (DMAF) and potassium ferrocyanide. The minimum concentration of H₂O₂ detectable is ~3 nmol in 2.5 ml electrolyte using 150 μ M FCA and 10 μ M HRP at pH 6.8.

Attempts to modify HRP with FCA and aminomethylferrocene (AMF) show that modified HRP with 100% activity is produced and, after a single G-25 purification the modified HRP can be used to amperometrically detect H_2O_2 . FCA modified HRP is more efficient (ie: higher catalytic current flows upon addition of H_2O_2 per mole of modified protein) for H_2O_2 detection and the minimum concentration detectable is ~ 10 nmol in 2.5 ml, using $15 \mu\text{M}$ of FCA modified HRP. The nature of the modifications is uncertain since dialysis and chromatographic analysis of the FCA and AMF modified HRP failed to identify or separate any derivatives and loss of ferrocene was observed during analysis suggesting that the modifications are reversible.

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To Michael and Steven

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di[3-ethylbenzthiazoline-6-sulfonate]
AMF	aminomethylferrocene
o-di	o-dianisidine
DMAF	(dimethylaminoethyl)-ferrocene
E _{1/2}	half-wave potential
E _{pa}	anodic peak current
E _{pc}	cathodic peak current
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FCA	ferrocenemonocarboxylic acid
FPLC	fast protein liquid chromatography
GOD	glucose oxidase
hepes	[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]
HRP	horseradish peroxidase
i _c	catalytic current
MES	4-morpholineethanesulfonic acid (free acid)
MHRP	ferrocenemonocarboxylic acid modified horseradish peroxidase

M'HRP	aminomethylferrocene modified horseradish peroxidase
Pi	phosphate buffer (from sodium salts)
RVC	reticulated vitreous carbon
sulfo-NHS	n-hydroxysulfosuccinimide

1.0 INTRODUCTION

A chemical sensor is defined as: "...a transducer which provides direct information about the chemical composition of its environment. It consists of a physical transducer and a chemically sensitive layer.¹" A biosensor employs biological materials in the chemically sensitive layer; typically biosensors employ redox proteins in this capacity.

Successful modification of glucose oxidase (GOD) with ferrocenemonocarboxylic acid² (FCA) allowed, for the first time, direct electrical communication between the enzyme and metal electrodes. This research opened the route for the development of biosensors which operate using electrochemical detection of biologically important molecules, such as glucose for the case of GOD. Horseradish peroxidase (HRP) is an enzyme which catalyzes the reduction of H_2O_2 to water. HRP is highly selective for H_2O_2 , commercially available in purified form and well characterized, which makes it a good candidate for biosensor development.

The presented work deals with the study of the electron transfer between reticulated vitreous carbon and HRP as mediated by potassium ferrocyanide, (dimethylaminoethyl)-ferrocene (DMAF) and FCA in the free state as well as showing the results of investigation into the modification of HRP with FCA and aminomethylferrocene.

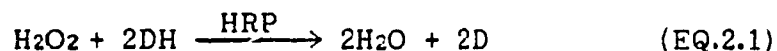
1.1 REFERENCES

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2. Baum, R., (1987) C&EN, March 16, p 24-26

2.0 SPECTROPHOTOMETRIC ASSAYS FOR HORSERADISH
PEROXIDASE: THE STUDY OF O-DIANISIDINE AND
2,2'-AZINO-DI[3-ETHYLBENZTHIAZOLINE-SULFONATE(6)]
AS INDICATOR COMPOUNDS

2.1 INTRODUCTION

Colourimetric enzyme assays have long been employed for peroxidases and other enzymes¹. Equation 2.1 shows a scheme whereby a dye (D, reducing substrate) becomes oxidized as hydrogen peroxide is reduced to water in the presence of HRP:



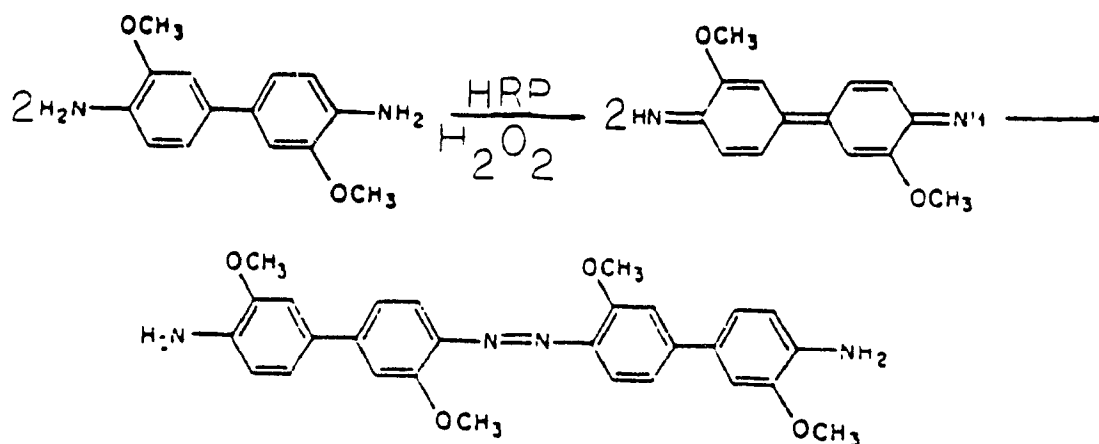
The most important criterion in choosing a dye for this type of assay is that it must be rapidly oxidized in the presence of HRP and H_2O_2 at the pH at which the assay is carried out. The oxidation of the dye must result in a change in absorbance at a known wavelength, which is then monitored spectrophotometrically. The popular dyes used exhibit a change of absorbance in the visible spectrum, therefore assays of this nature are said to be colourimetric². Colourimetric assays are a fast and simple way for determining enzyme activity. This is important for enzyme modification work as any derivatives synthesized may be readily assayed and the activity compared with native protein. This technique may also be used to detect small amounts of immobilized enzyme.

There are several well known dyes for HRP activity determination. Ortho-phenylenediamine, ortho-dianisidine, ferrocytochrome c, guaiacol and 2,2'-azino-di[3-ethylbenzthiazoline-6-sulfonate] (ABTS) are all possible reducing substrates for HRP. Not all of these dyes are suitable for fast and sensitive activity determinations. Ideally the dye should be oxidized by H_2O_2 alone at a rate which is less than 10% the rate of HRP-catalyzed oxidation. Air oxidation of solutions of the dye must also be slow relative to the HRP-catalyzed oxidation. O-phenylenediamine is very light sensitive and oxidation occurs when o-phenylenediamine solutions are exposed to light³; ferrocytochrome c is subject to air oxidation; and although guaiacol has been used as a reducing substrate, some uncertainty exists as to the products of its reaction⁴. For these reasons these compounds were not chosen here as reducing substrates for HRP.

O-dianisidine has proven to be a sensitive substrate for HRP⁵ and studies have revealed a possible mechanism for its oxidation by HRP. Also, nitrogenous compounds such as pyridine and imidazole are known to boost the rate of oxidation of o-dianisidine (turnover) at alkaline pH⁶. In hopes of further increasing the sensitivity of the HRP assay the effects of these compounds on the rate of turnover of o-dianisidine were studied.

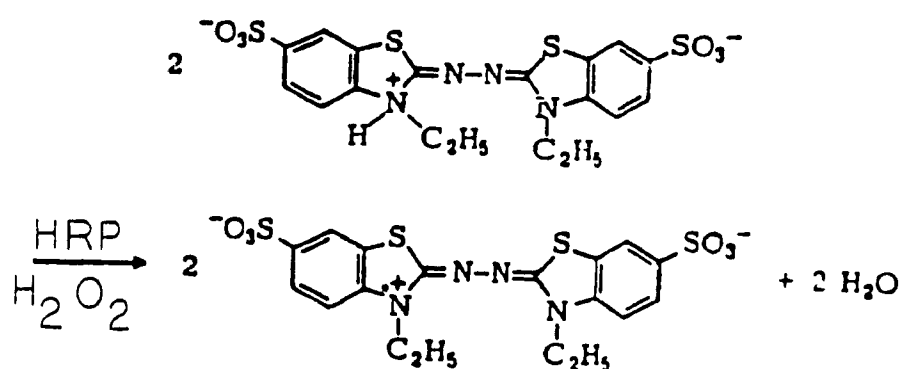
ABTS has also been characterized as a substrate for HRP in enzyme immunoassays⁷ as well as for enzyme free in solution⁴, but no information was found relating the rate of maximum dye turnover to

the concentration of HRP. Hence, the quantitation of HRP colourimetric assays using ABTS and also o-dianisidine as reducing substrates at pH 6.0 and 7.5 are presented in this chapter. In addition the effects of imidazole and pyridine on the rate of oxidation of o-dianisidine at pH 6.0 and 7.5 are reported. Schemes 2.1 and 2.2 show the HRP-catalyzed oxidation of the dyes by H_2O_2 as well as chemical structures of o-dianisidine and ABTS in the reduced and oxidized forms.



Scheme 2.1

Reaction scheme for the oxidation of o-dianisidine by HRP in the presence of H₂O₂. The assay solution becomes orange upon o-dianisidine oxidation.



Scheme 2.2

Reaction scheme for the oxidation of ABTS by HRP in the presence of H₂O₂. The assay solution becomes green upon ABTS oxidation.

2.2 EXPERIMENTAL SECTION

2.2.1 Materials

Horseradish peroxidase (grade 1, lyophilizate) was purchased from Boehringer Mannheim and was used without further purification. The ratio of absorbance of heme (403 nm) to protein (275 nm) is called the purity index and was 3.0 to 3.4 for the HRP used. O-dianisidine dihydrochloride, imidazole and pyridine were obtained from Sigma and 2,2'-azino-di[3-ethylbenzthiazoline-6-sulfonate] (ABTS, registered trademark) was obtained from Boehringer Mannheim as the diammonium salt. All other chemicals were of reagent grade and were used as supplied. Distilled water was further purified by passage through a Barnstead Nanopure water purification system to remove any metal ions which might affect enzyme activity. The specific resistance of water after purification was 18 M Ω -cm.

2.2.2 Methods

Assay Solution Preparation: 0.1 M phosphate buffers were prepared at pH 6.0 and pH 7.5 from the sodium salts. Enzyme solutions were prepared by dissolving a small amount of lyophilized HRP in the appropriate buffer, and concentrations were determined spectrophotometrically using an extinction coefficient of 91 mM⁻¹ cm⁻¹ at 403 nm⁸. The HRP solutions ranged from 0 to 2 μ M. Stock solutions of 120 μ M o-dianisidine were prepared by dissolving 0.038

g in 1 L of buffer and 120 μ M ABTS solutions were prepared by dissolving 0.069 g in 1 L of buffer. The o-dianisidine stock was stable for several weeks at 4 °C wrapped in aluminum foil. The ABTS solutions were always prepared fresh since ABTS solutions were found to be stable only for a short period of time at 4 °C. Stock solutions of hydrogen peroxide were freshly prepared by dissolving 3 μ L of 30% hydrogen peroxide (Anachemia Chemicals) in 10 ml of buffer to yield a solution of 2.7 mM H₂O₂. The stock H₂O₂ was verified as being 30% \pm 2% by permanganate titration⁹. For trials where the effects of imidazole and pyridine were studied the dye solutions were prepared with 5 mM imidazole or pyridine.

HRP Assay Procedure: A Hewlett Packard model 8451A diode-array spectrophotometer was used to obtain assay data. A short computer program was written using Hewlett Packard interface language (HPIL) (see Appendix 7.1) to record absorbance increases at 460 and 436 nm for o-dianisidine and ABTS, respectively.

The rate at which the dyes are oxidized by H₂O₂ alone was determined as follows: 1.81 ml of buffered dye solution was added to a 1 cm cuvette which was placed in the spectrophotometer and stirred using a magnetic stir bar. The cell holder of the spectrophotometer was maintained at 19 \pm 1 °C by a thermostating fluid from a (Lauda) bath. An absorbance reading was made at the appropriate wavelength to serve as the initial (t=0 s) absorbance

reading (A_0). 70 μ L of H_2O_2 solution (final H_2O_2 concentration of 100 μ M) were added, and after a 10 s mixing period the absorbance was recorded every 10 s for a total of 1 min (i.e. total time of reaction was 70 s). The activity of the blank in terms of μ mol dye oxidized per 10 s, was calculated by equation 2.2:

$$\frac{\mu\text{mol dye oxidized}}{10\text{ s}} = \left[\frac{(A_t - A_0)}{n} \right] \times \frac{V \times 10^6}{\Delta\epsilon_{470}} \quad (\text{EQ.2.2})$$

where A_t is the absorbance at a given time t after mixing ($t = 10, 20, 30, 40, 50, 60$ and 70 s), A_0 is the initial absorbance at $t=0$ s, n is the number of 10 s intervals after mixing ($n = 1, 2, 3, \dots, 7$), V is the total volume in the cuvette in L (1.88×10^{-3} L), $\Delta\epsilon_{470}$ is the change in molar absorptivity between the oxidized forms and the reduced forms of the dyes whose spectra are shown in Figures 2.1 and 2.2. The term in square brackets yields the change in absorbance for a given 10 s time interval and multiplication by 10^6 yields the average number μ mol dye oxidized per 10 s interval using the given units of V and $\Delta\epsilon_{470}$. The $\Delta\epsilon_{470}$ used for the calculation of enzyme activities were $18.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for ABTS⁸ at 436 nm and $8.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for o-dianisidine⁸ at 460 nm.

Assay solutions were prepared using 1.80 ml of dye solution plus 10 μ L of enzyme solution. An absorbance reading was made at the appropriate wavelength to serve as the initial ($t=0$ s) absorbance reading (A_0). The enzymatic reaction was initiated by adding 70 μ L of the peroxide stock to the assay solution. After a

10 s mixing period the absorbance of the assay solution was recorded every 10 s as above. Using equation 2.2, the average number of μmol dye oxidized per 10 s, for a given time interval, was calculated. Each trial yielded 7 absorbance change values but only the values obtained for $n=3$ to 6 were used to calculate enzyme activities since mixing caused absorbance changes for $n=1$ and 2 to fluctuate. Saturation kinetics were not always observed for the final interval so the absorbance data for this interval were also ignored. Average activities as well as standard deviations were calculated from the 4 remaining values.

In summary, the time interval 0 to 10 s was for mixing, therefore the first absorbance reading was taken at 10 s, the second reading at 20 s and so on for one minute. Activity of HRP was calculated as the average number of μmol dye oxidized per 10 s, averaged over 4 intervals. Table 2.1 shows the cuvette concentrations of the components of the HRP assays.

Table 2.1
Cuvette Concentrations for HRP Assay*

SUBSTANCE	CUVETTE CONCENTRATION
HRP	0 to 10 nM
o-dianisidine	120 μ M
ABTS	120 μ M
H ₂ O ₂	100 μ M

* Assays performed in 0.1 M phosphate buffer at pH 6.0 and 7.5 at 19 °C with a total volume of 1.88 ml.

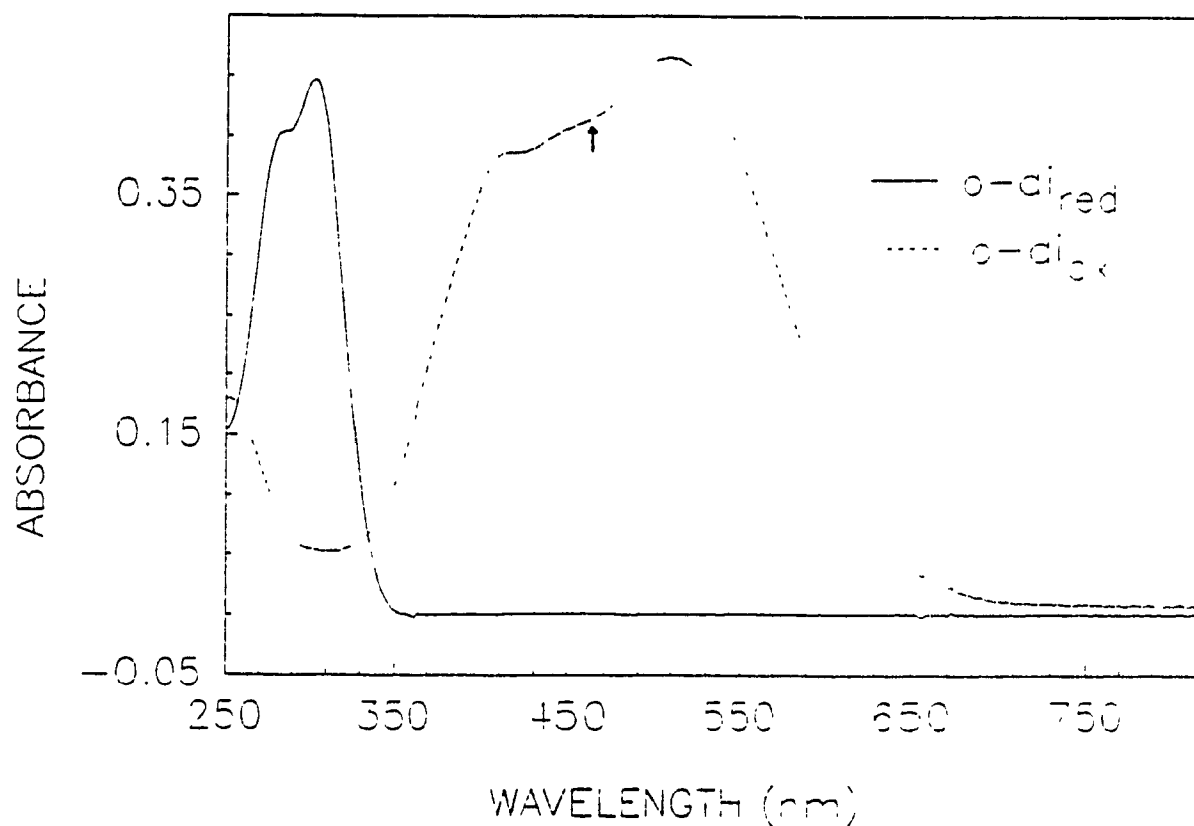


Figure 2.1

Spectra of reduced and oxidized forms of o-dianisidine (50 μ M) in 0.1 M phosphate buffer, pH 6.0, 1 cm pathlength. Dye oxidized using assay procedure with 1 μ M HRP. Monitoring wavelength for the HRP assay using o-dianisidine as reducing substrate is 460 nm.

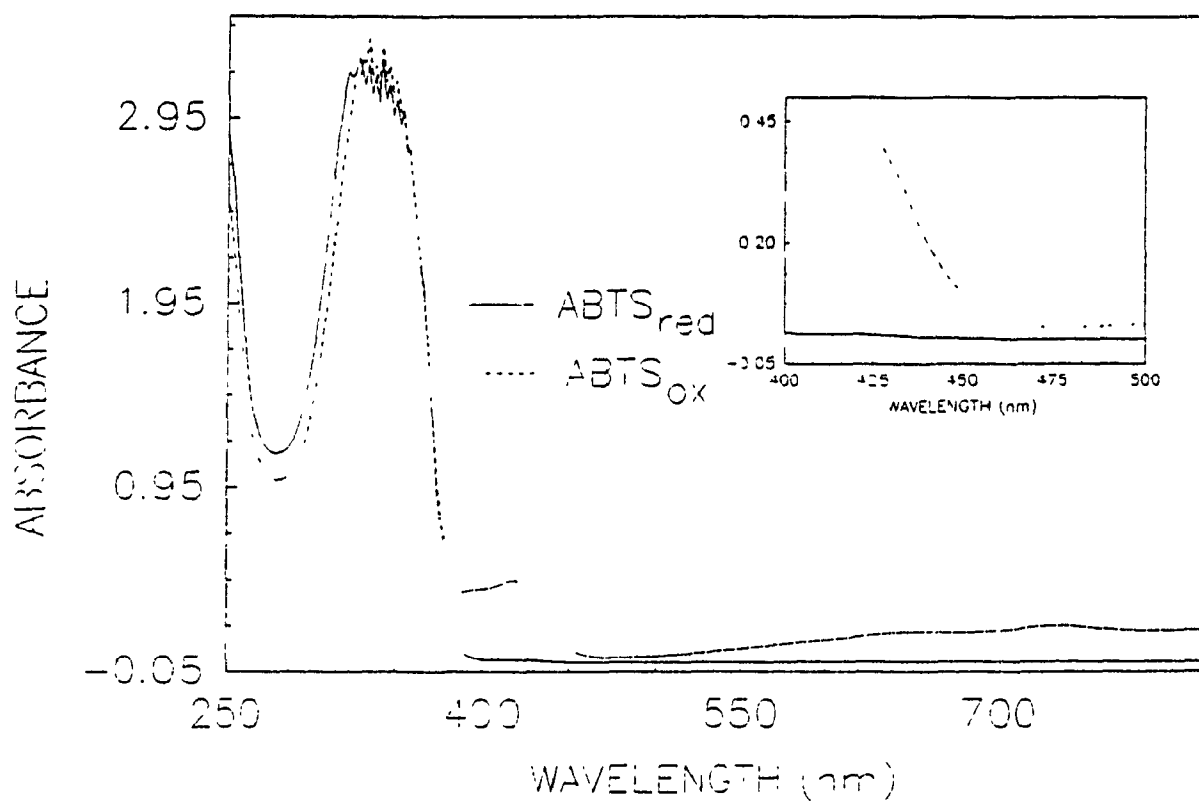


Figure 2.2

Spectra of reduced and oxidized forms of ABTS (15 μ M) in 0.1 M phosphate buffer, pH 6.0, pathlength 1 cm. Dye oxidized using assay procedure with 1 μ M HRP. Monitoring wavelength for the HRP assay using ABTS as reducing substrate is 436 nm.

2.3 RESULTS

The rate at which the dyes were oxidized by H_2O_2 alone was found to be less than 10% of the rate in the presence of ~ 0.2 nM HRP for o-dianisidine and ~ 2.0 nM HRP for ABTS. Due to this fact the contribution of the oxidation of dye by H_2O_2 was ignored when calculating enzyme activities.

For each concentration of HRP, assays were run in triplicate and the standard deviation calculated for the average activity over the three runs. The concentration of substrate should not be rate-limiting when determining enzyme turnover numbers as maximum turnover of substrate will not be observed. Ideally the absorbance change of the reaction mixture per 10 s interval should not decrease over the total assay time period (1 min). However, Table 2.2 shows that the absorbance change per 10 s interval at the highest HRP concentration (9.8 nM) using ABTS as substrate decreases by 8.5% after the first 4 time periods and then drops by another 13% over the final two intervals to give a total decrease of 21.5%. At an enzyme concentration of 5.3 nM the same trend is observed; the absorbance change per 10 s drops by a total of 18% over 70 s. An average loss of ~ 10 -20% over the course of the assay was observed, even at 1 nM HRP.

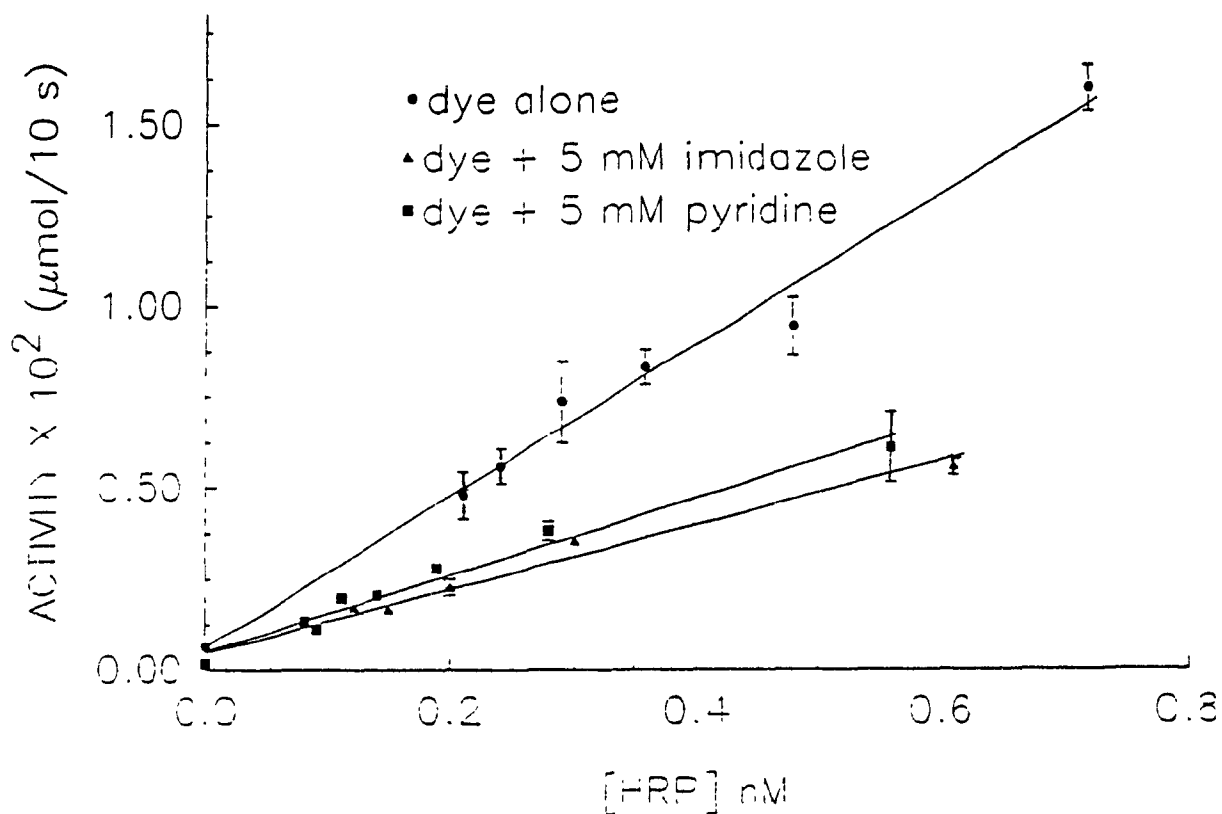


Figure 2.3

Plots of activity (EQ. 2.2) vs. concentration of HRP (nM) at pH 6.0 using o-dianisidine (120 μM) as reducing substrate. The effects of 5 mM imidazole and pyridine on the activity of HRP are also shown.

See Table 2.1 for experimental conditions.

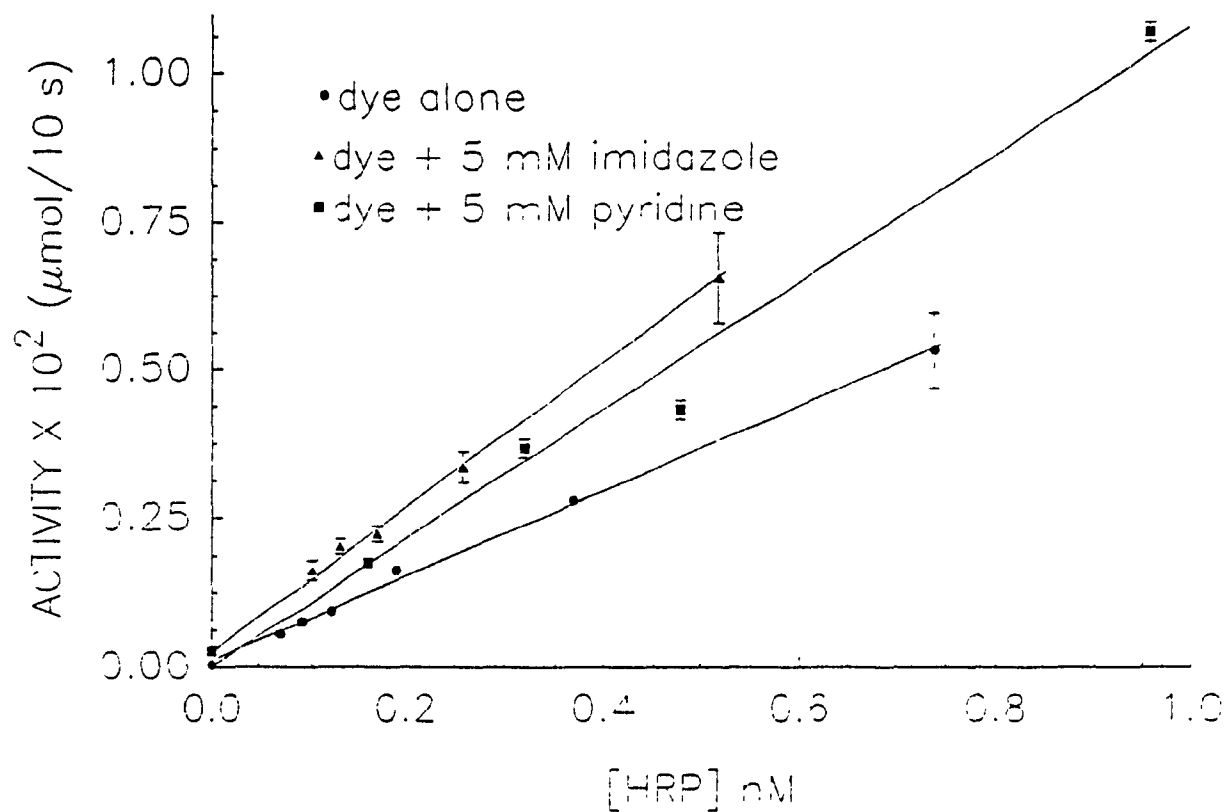


Figure 2.4

Plots of activity (EQ. 2.2) vs. concentration of HRP (nM) at pH 7.5 using o-dianisidine (120 μM) as reducing substrate. The effects of 5 mM imidazole and pyridine on the activity of HRP are also shown.

See Table 2.1 for experimental conditions.

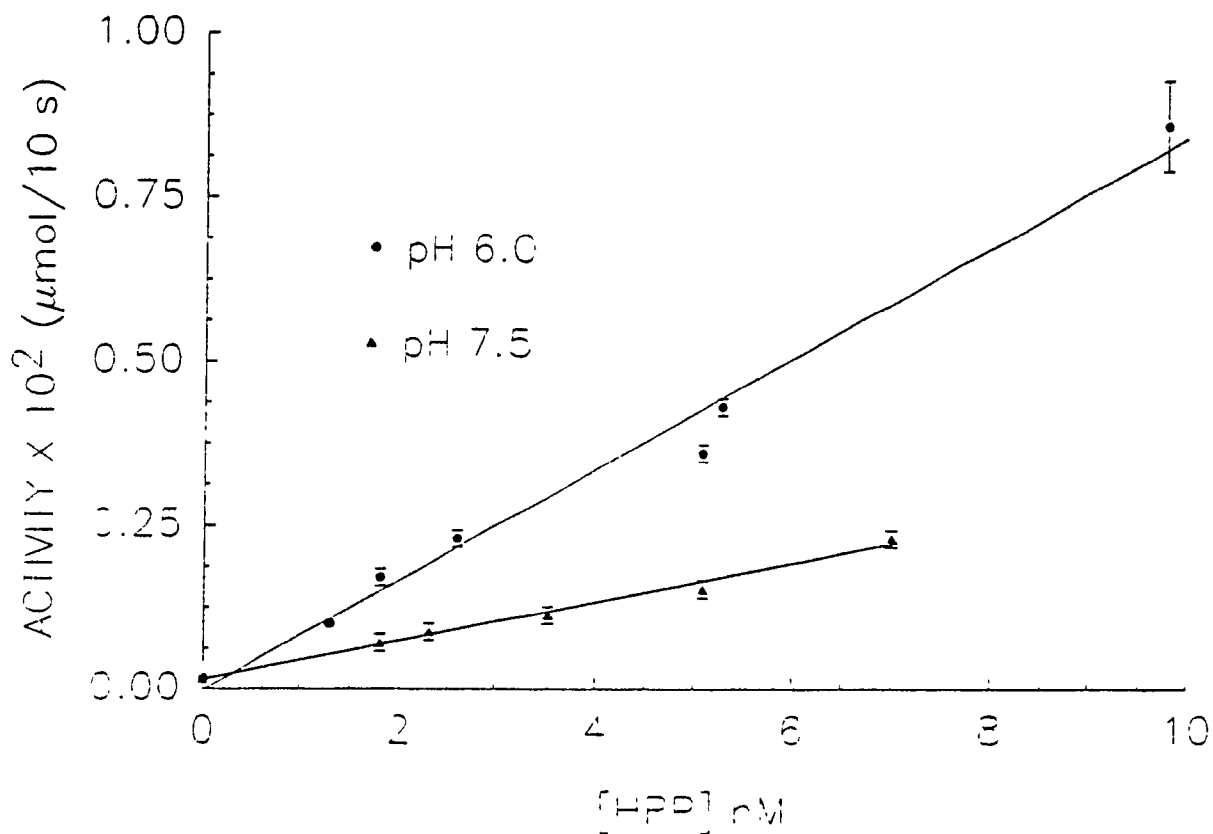


Figure 2.5

Plots of activity (EQ. 2.2) vs. concentration of HRP (nM) at pH 6.0 and 7.5 using ABTS (120 μM) as reducing substrate. See Table 2.1 for experimental conditions.

Table 2.2
Absorbance Changes vs. Time in HRP Assays Using ABTS and
O-Dianisidine as Reducing Substrates^a

	Absorbance Change per 10 s ^c			
	ABTS, 436nm		o-dianisidine, 460 nm	
	9.8 nM HRP	5.3 nM HRP	0.72 nM HRP	0.36 nM HRP
Time interval ^b (s) (n)				
10-20 (2)	0.070	0.045	0.080	0.036
20-30 (3)	0.069	0.044	0.077	0.035
30-40 (4)	0.067	0.042	0.071	0.035
40-50 (5)	0.064	0.041	0.064	0.034
50-60 (6)	0.058	0.040	0.058	0.032
60-70 (7)	0.055	0.037	0.052	0.031

^a0.1 M sodium phosphate pH 6.0, 19 °C, 120 μ M dye and 100 μ M H₂O₂

^bFirst 10 s interval is mixing period.

^cAbsorbance change in 10 s interval due to oxidation of dye at various concentrations of HRP.

Using *o*-dianisidine as a reducing substrate the absorbance change due to oxidation of dye over the course of the assay also decreased by ~20% or less (Table 2.2). However, at the highest concentration of HRP studied (0.72 nM) the absorbance change decreased by 35% indicating that saturating substrate concentrations were not present for the entire reaction period: therefore, the activity of HRP was calculated by using the data from the first four intervals, since the overall decrease in absorbance over these intervals is <20%. A decrease in the absorbance change of <20% over the course of the assay may be attributed to the loss of enzyme activity in the presence of a large excess of H₂O₂ (see Table 2.1). Since the decrease in absorbance change was between 10-20% at each HRP concentration, it did not affect the linearity of the activity vs. concentration of HRP curves shown in Figures 2.3 to 2.5.

Figures 2.3 and 2.4 show plots of μmol *o*-dianisidine oxidized per 10 s vs. concentration HRP (nM) and illustrates the effects of 5 mM imidazole and 5 mM pyridine on the rate of oxidation of *o*-dianisidine by HRP at pH 6.0 and 7.5, respectively. Figure 2.5 contains plots of μmol ABTS oxidized per 10 s vs. nM HRP which show the effect of changing the pH from 6.0 to 7.5 on the rate of oxidation of ABTS by HRP. The slopes of these curves correspond to the turnover of HRP under the various conditions; the calculated turnover numbers are given in Table 2.3.

Table 2.3
Turnover Numbers for HRP Under Various Assay Conditions^a

DYE ^c , pH	TURNOVER NUMBER ^b (s ⁻¹)
o-di, pH 6.0	1170
o-di, pH 6.0 + 5mM imidazole	425
o-di, pH 6.0 + 5mM pyridine	567
o-di, pH 7.5	354
o-di, pH 7.5 + 5mM imidazole	666
o-di, pH 7.5 + 5mM pyridine	525
ABTS, pH 6.0	44
ABTS, pH 7.5	15

^a0.1 M sodium phosphate buffer, 19 °C, 120 μ M dye and 100 μ M H₂O₂.

^bTurnover numbers (number of dye molecules oxidized per molecule of HRP per s) is calculated from the slopes of plots shown in Figures 2.2 to 2.4, divided by 10.

^cAbbreviations used: ABTS, 2,2'-azino-di[3-ethylbenz-thiazoline-6-sulfonate]; o di, o-dianisidine.

2.4 DISCUSSION

Figures 2.3 through 2.5 show that the rate of dye oxidation for *o*-dianisidine and ABTS vs. HRP concentration is linear over the concentration range studied. Pyridine and imidazole (5 mM) do not affect the linearity of this relation, nor do these compounds have any effect on the absorption spectrum of *o*-dianisidine (oxidized or reduced). Table 2.3 shows that at pH 6.0 the turnover of *o*-dianisidine is 64% slower in the presence of 5 mM imidazole and 52% slower in the presence of 5 mM pyridine. Literature values for *o*-dianisidine turnover under these conditions were not found. Figure 2.4 and Table 2.3 show that at pH 7.5 the turnover of *o*-dianisidine is 88% faster in the presence of 5 mM imidazole and 48% faster in the presence of 5 mM pyridine. These results are comparable to those obtained by Claiborne and Fridovich^{5,6} who reported that at pH 7.5, 5 mM imidazole and 5 mM pyridine increased the rate of oxidation of *o*-dianisidine by 68% and 32%, respectively (135 μ M dye, 870 μ M H₂O₂ and 0.1 nM HRP). Only one concentration of HRP was investigated by these workers, and the percent enhancement by these compounds at pH 7.5 are in good agreement with the values presented here.

Figure 2.5 and Table 2.3 show the effect of changing the pH from 6.0 to 7.5 on the HRP assay with ABTS as the reducing substrate. The turnover of ABTS by HRP is three times as rapid at pH 6.0 than at pH 7.5. A value for the turnover of ABTS was calculated from data obtained by Putter et al.⁴ (1.7 mM ABTS, 0.83 mM

H₂O₂ and nM range HRP at pH 6.0) and is ~100. These workers also determined that the minimum concentration of HRP detectable using ABTS as reducing substrate is ~1 nM. The present study is in agreement with these results. The effects of nitrogenous compounds on turnover of ABTS were not investigated since these compounds did not increase the turnover of o-dianisidine beyond the value observed at pH 6.0 with no nitrogenous compounds.

Table 2.3 shows that the turnover of o-dianisidine is much greater than ABTS under the present conditions. The study of HRP immunoassays by Porstmann et al.³ also compared the efficiency of ABTS and o-dianisidine as chromogens for the determination of HRP. The conditions of this study were different (0.7 mM H₂O₂, 0.32 mM dye at pH 5.0 for o-dianisidine and pH 4.2 for ABTS) and the results indicate that the sensitivity of the enzyme immunoassays is better using ABTS. The study by Porstmann et al. was conducted on IgG-coupled HRP which may alter the reactivity of HRP with o-dianisidine and ABTS.

The current study shows that o-dianisidine gives the most sensitive HRP determination with no booster compound present at pH 6.0. Under the conditions outlined, the lowest value of enzyme concentration which can be distinguished from the blank is about 0.1 nM. Under identical conditions using ABTS, the lowest value of enzyme concentration which may be distinguished from the blank is approximately 1.0 nM. Although the booster compounds enhance

o-dianisidine turnover at pH 7.5, this is still not large enough to make the assay more sensitive at pH 7.5 compared to pH 6.0 with no booster compounds. In Chapters 4 and 5 activities of chemically-modified HRP will be expressed as the percent activity of the native protein using o-dianisidine as reducing substrate at pH 6.0, and the conditions outlined in Table 2.1.

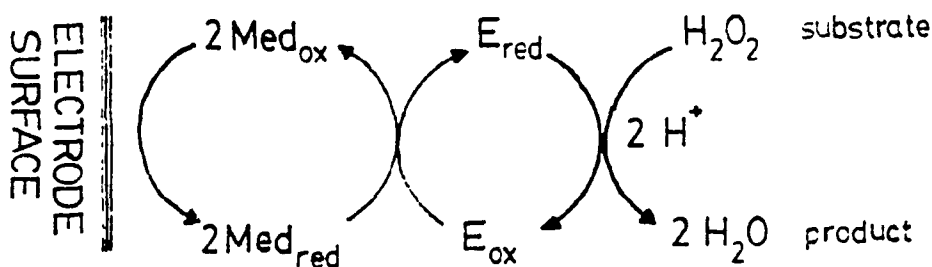
2.5 REFERENCES

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3.0 STUDIES OF ELECTRON TRANSFER BETWEEN
RETICULATED VITREOUS CARBON AND HORSERADISH
PEROXIDASE AS MEDIATED BY POTASSIUM FERROCYANIDE,
FERROCENEMONOCARBOXYLIC ACID AND
(DIMETHYLAMINOETHYL)-FERROCENE

3.1 INTRODUCTION

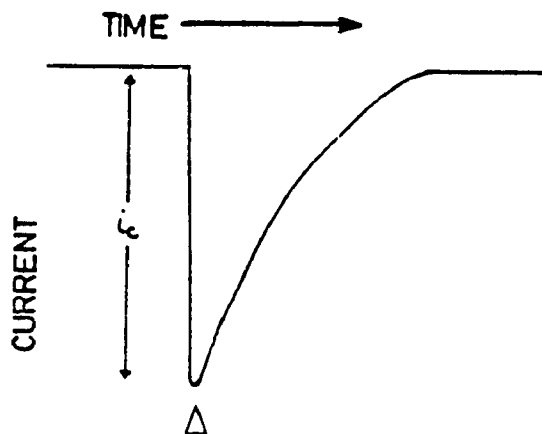
Direct electron transfer between redox proteins and traditional electrode materials (such as gold, platinum and carbon) is not normally observed¹. This difficulty has been overcome through the use of mediator compounds which readily transfer electrons between the protein and the electrode surface². These compounds are usually metal based complexes whose metal center can exist in at least two oxidation states. Using redox mediators, it is possible to electrochemically detect substrates for enzymes which undergo oxidation or reduction steps in their catalytic cycle. For HRP an outline of an electrochemical assay for the detection of H₂O₂ is shown in Scheme 3.1:



SCHEME 3.1

where E is HRP, Med is the mediator and the arrows show the transfer of electrons in the reduction of H_2O_2 to H_2O by HRP.

HRP has been studied in such a scheme at graphite disk electrodes³, and the results reveal that ferrocene derivatives are good mediators for electron transfer between HRP and glassy carbon. This work led to the development of a hydrogen peroxide assay system based on amperometric quantitation. Several different ferrocene derivatives were examined as possible mediators. At a constant reducing potential for the mediator, upon introduction of H_2O_2 electrons flow into the system, reducing H_2O_2 to H_2O . This results in an increase of the cathodic current of the system and an idealized current response is shown below, where the triangle indicates the addition of H_2O_2 :



The initial increase in cathodic current is known as the catalytic current (i_c), which is directly proportional to the amount of H_2O_2 in the system.

In the present work a reticulated vitreous carbon (RVC) working electrode was employed in a typical three electrode system. The

reason for employing this material was the possibility of increased sensitivity for the detection of hydrogen peroxide due to greater surface area of the RVC compared to traditional materials such as glassy carbon. By increasing the available electrode surface area more enzyme will reside at the electrode surface, which should increase i_c for a given amount of H_2O_2 .

This chapter presents the results obtained at RVC for three mediators: potassium ferrocyanide, ferrocenemonocarboxylic acid (FCA) and (dimethylaminoethyl)-ferrocene (DMAF).

3.2 EXPERIMENTAL SECTION

3.2.1 Materials

All materials as per Section 2.2.1 with the following additions: ferrocenemonocarboxylic acid and (dimethylaminoethyl)-ferrocene were obtained from Aldrich. Potassium ferrocyanide and H₂O₂ (30% solution) were purchased from Anachemia chemicals. The 10 μ L gas tight syringe was purchased from Hamilton, the Ag/AgCl reference electrode used for all experiments was purchased from Aldrich, and RVC (100 pores per inch) was obtained from High Performance Materials, Inglewood, California.

3.2.2 Methods

Cell Construction: The all glass cell, shown in Figure 3.1, was constructed as described by Yocom⁴. The cell was equipped with two compartments separated by a fine glass frit. A side arm was included to hold the counter electrode (a platinum wire) and to act as a port for injection of H₂O₂. The working electrode was a rectangular piece of RVC of dimensions 2 cm x 1 mm x 5 mm. Electrical contact with the RVC was established by attaching an alligator clip to one end of the electrode which was wrapped with aluminum foil. The RVC electrode was partly coated with clear nail varnish to ensure that only half (10 x 1 x 5 mm) was in contact with the sample solution.

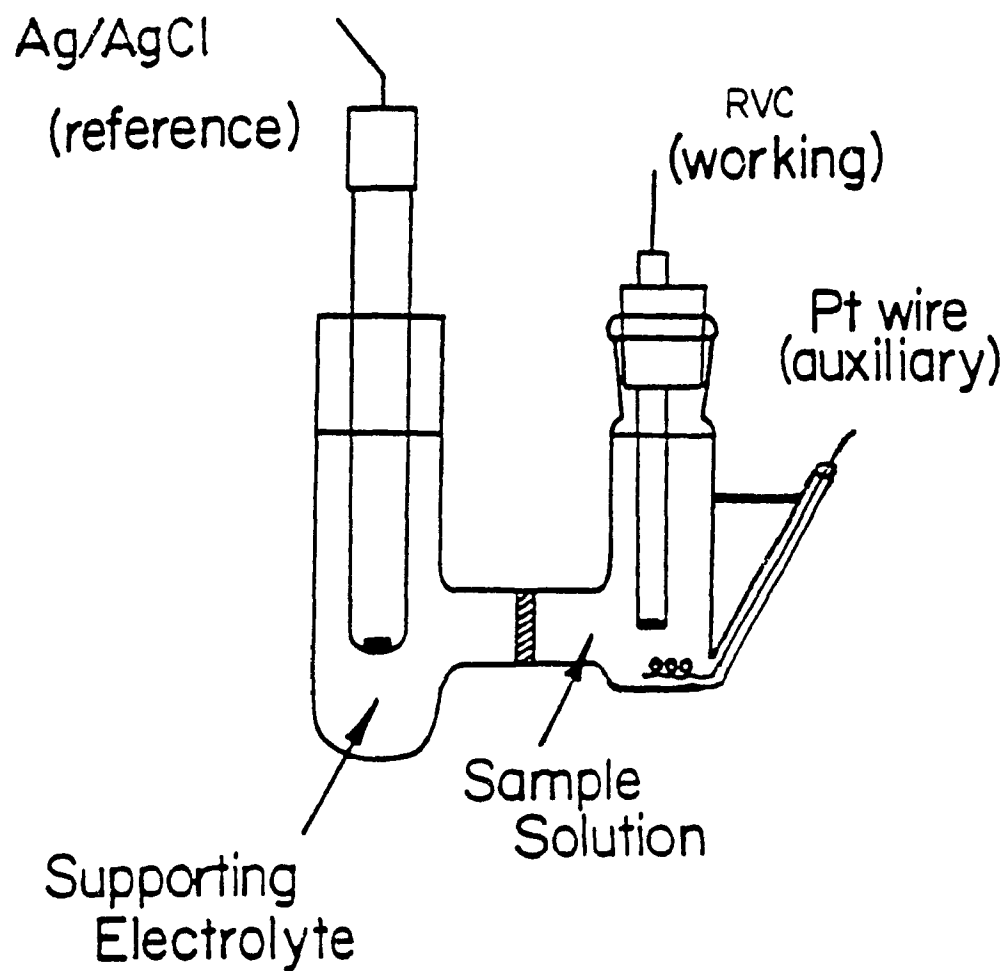


Figure 3.1. Electrochemical cell used for voltammetry and amperometric experiments.

Electrochemical Procedures:

Cyclic Voltammetry: The supporting electrolyte used for all electrochemical experiments was 75 mM KCl buffered with 25 mM phosphate (sodium salt) at pH 6.8. Standard linear sweep (cyclic) voltammetry was performed on each mediator using a PAR 362 potentiostat and voltammograms were recorded using a Philips PM 8143 X-Y recorder. The working compartment of the electrochemical cell was filled with 2.5 ml of 150 μ M mediator solutions (prepared in supporting electrolyte) and the reference compartment was filled with enough supporting electrolyte to cover the reference electrode. Scans (2 mV/s) were initiated from +0.6 V to +0.1 V and back. Half wave potentials were calculated using equation 3.1:

$$E_{1/2} = (E_{pc} + E_{pa})/2 \quad (EQ.3.1)$$

where E_{pc} and E_{pa} are the potentials corresponding to the cathodic and anodic peak current maxima, respectively.

Electrochemical HRP Assays: Solutions for measurements as per Scheme 3.1 of i_c vs. nmol H_2O_2 were prepared by dissolving lyophilized HRP in the electrochemical buffer which also contained 150 μ M mediator. The concentration of HRP was determined spectrophotometrically using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ at 403 nm⁵. Using a 10 μ L gas-tight syringe, 4 μ L of an H_2O_2 solution of known concentration were injected at the electrode surface to initiate the enzymatic cycle of HRP. The stock solution of H_2O_2 was verified as being 30% by permanganate titration⁶ and

samples of H_2O_2 were prepared by dilution. All solutions were deaerated by bubbling with argon prior to use. The potential of the system was kept constant at 0.0 V vs. Ag/AgCl using a PAR 362 potentiostat. Current responses were recorded with an RTI-800 analogue-to-digital converter board which was programmed in compiled basic to store the digitized current-time data (see Appendix 7.2) for information on the RTI-800 board and the computer program written to acquire and store current vs. time data). Data acquisition was initiated 20 s before injection of H_2O_2 and the duration of data acquisition was 150 s (see Figure 3.3 for typical data). All experiments were performed at room temperature in unstirred solutions.

3.3 RESULTS

For the electrochemical assay outlined in Scheme 3.1 to work, the system must be poised at a potential which is sufficiently negative to reduce any mediator molecule coming in contact with the electrode. Therefore, the half-wave potential ($E_{1/2}$) was calculated for each mediator (using equation 3.1) from the cyclic voltammetry data in the presence and absence of HRP. All mediators showed reversible behaviour at RVC. In the presence of HRP the shape of the voltammograms did not change but the $E_{1/2}$ values of the mediators were shifted slightly anodically. Figure 3.2 shows the voltammograms obtained for the various mediators and for 10 μ M HRP alone. Table 3.1 lists the observed $E_{1/2}$ values and peak separations (peak separation = $E_{pc} - E_{pa}$) for the mediators tested as well as literature values for $E_{1/2}$.

Current vs. time profiles for the reduction of H_2O_2 by HRP were obtained for the three mediators by poisoning the working electrode at a constant potential of 0.0 V with respect to Ag/AgCl. 4 μ L injections of a given amount of H_2O_2 were performed in triplicate for a given enzyme solution. Solutions were freshly prepared and used until the activity of the HRP decreased and gave non-reproducible results. The maximum number of injections an enzyme solution could endure was 20-30. After each injection the solution was stirred magnetically for 1-2 min to reduce any leftover H_2O_2 and after stirring had been stopped the current was allowed to

Figure 3.2. DC cyclic voltammetry of HRP and mediators in 25 mM phosphate buffer, pH 6.8 and 75 mM KCl. In all cases the working electrode was RVC, the counter electrode was platinum wire, and reference electrode was Ag/AgCl. Scan rate 2 mV/s. (a) 10 μ M HRP, no mediator present, (b) 150 μ M (dimethylaminoethyl)-ferrocene, (c) 150 μ M ferrocenemonocarboxylic acid and (d) 2 mM potassium ferrocyanide.

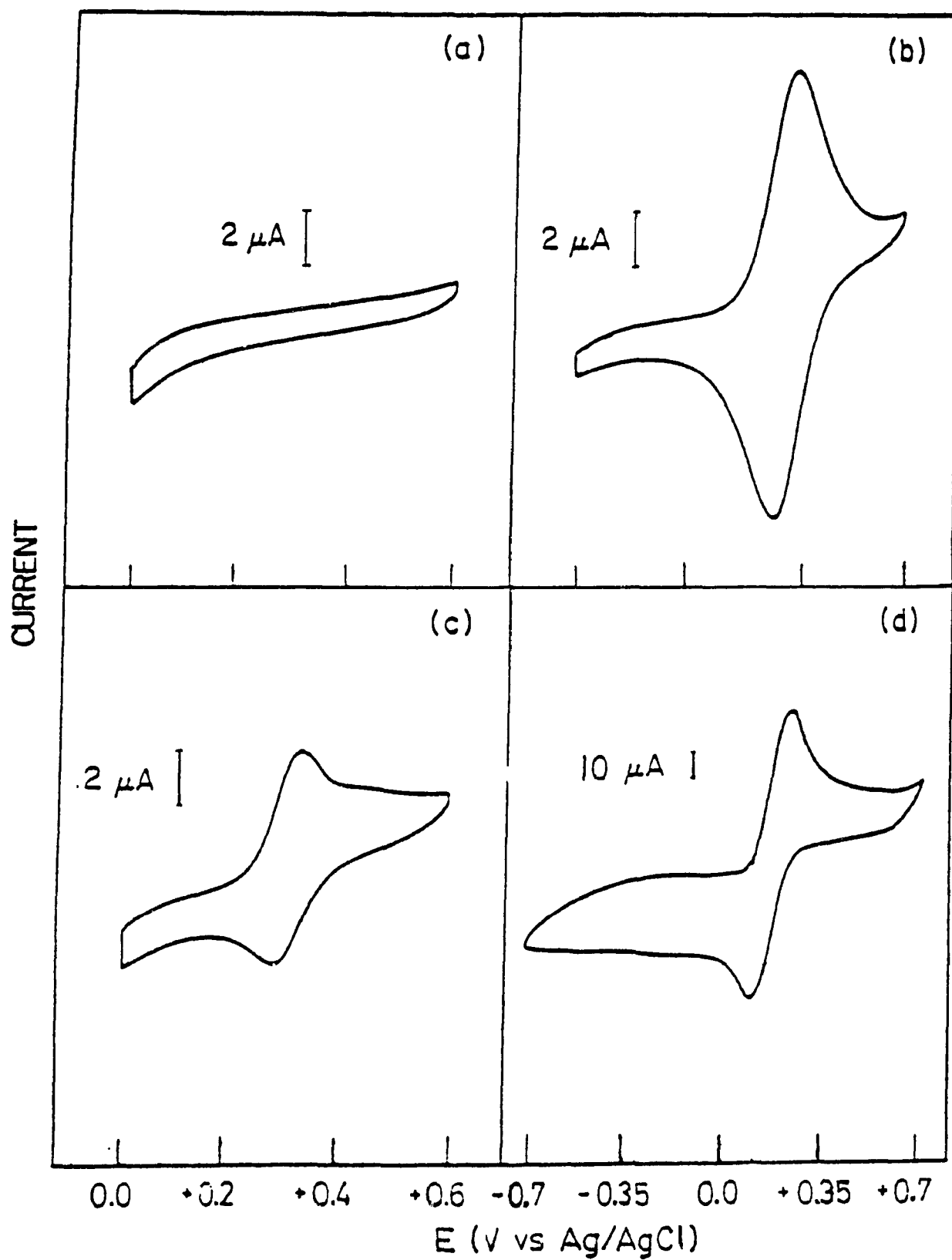


Table 3.1
Half-Wave Potentials and
Peak Separations For Mediators^a

MEDIATOR ^e	E _{1/2} Lit value ^d (mV)	Without HRP		With HRP ^b	
		E _{1/2} ^c (mV)	Peak Separation ^c (mV)	E _{1/2} ^c (mV)	Peak Separation ^c (mV)
Fe(CN) ₆ ⁴⁻	256 ⁷	265	105	280	100
FCA	231 ³	307	60	315	60
DMAF	356 ³	376	58	385	57

^a Electrolyte was 2.5 ml of 25 mM sodium phosphate, pH 6.8 and 75 mM KCl. Electrochemical cell used is shown in Figure 3.1.

^b Concentration of HRP was 10 μ M.

^c Maximum deviation for all E_{1/2} values and peak separations is ± 5 mV.

^d Literature values of E_{1/2} are at glassy carbon vs. Ag/AgCl for ferrocenes and at RVC for ferrocyanide. Numerical superscripts indicate the reference numbers given in Section 3.5.

^e Concentration of potassium ferrocyanide was 2 mM and concentration of FCA and DMAF was 150 μ M.

equilibrate to a constant value before performing the next injection. Figure 3.3 exhibits a typical plot of current vs. time after an injection of H_2O_2 , using FCA as mediator. The observed i_c was plotted vs. nmol of H_2O_2 added for each mediator. Figures 3.4 and 3.5 show i_c vs. nmol H_2O_2 plots for the mediators. Linearity of the plot is good for ferrocyanide (Figure 3.4) over the range of H_2O_2 studied, but the corresponding plots for the ferrocene mediators (Figure 3.5) show more scatter in the data than would be required for the electrochemical assay to be a good quantitative tool. The plot for FCA shows saturation behaviour beginning at ~ 10 nmol H_2O_2 . A blank was performed to verify that H_2O_2 is not directly reduced by mediator in the electrochemical assay. The assay was run under identical conditions as above, with no HRP present in the assay mixture. Injections of H_2O_2 revealed that no increase in cathodic current flowing in the cell was observed indicating that H_2O_2 is not directly reduced by the mediator in the assay.

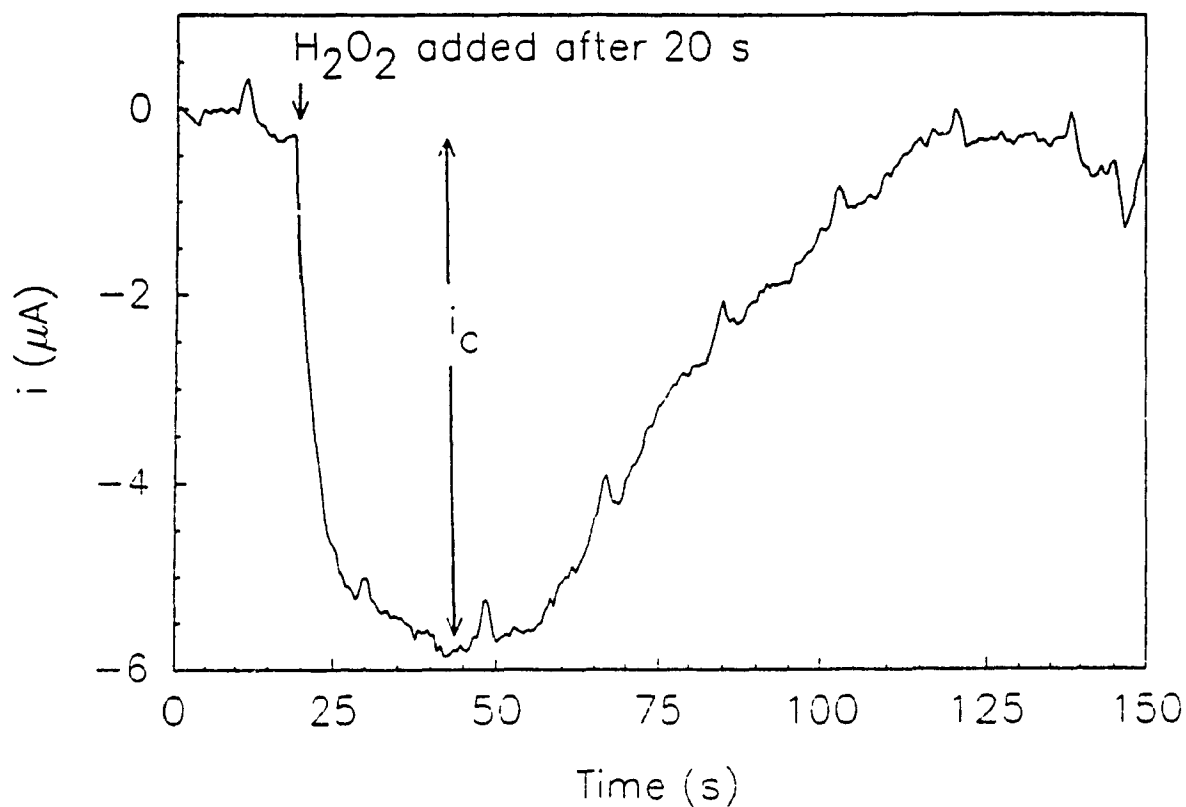


Figure 3.3

Plot of observed current i (μA) flowing in the HRP electrochemical assay system vs. time (s) following injection of 3.6 nmol of H_2O_2 at the electrode surface employing 150 μM FCA as mediator. Electrolyte was 2.5 ml of 25 mM sodium phosphate, pH 6.8 and 75 mM KCl. Cell used is shown in Figure 3.1. The concentration of HRP was 10 μM .

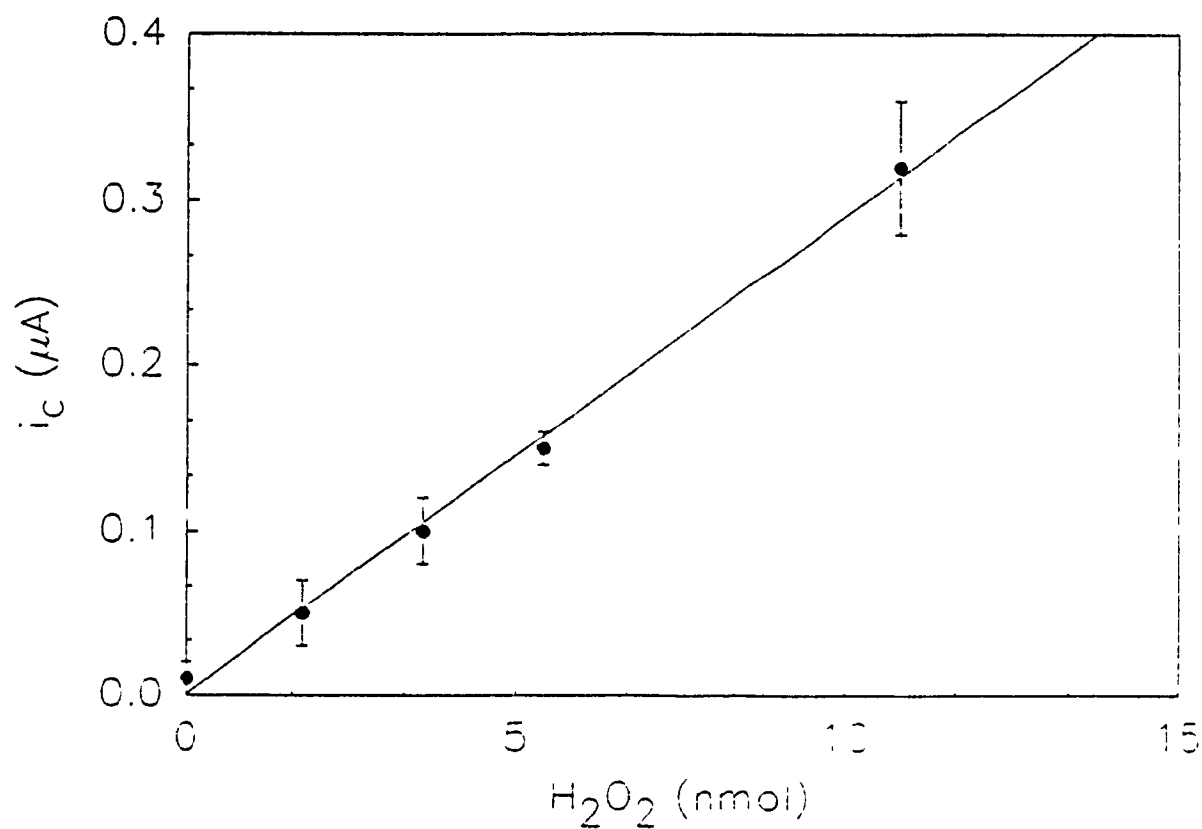


Figure 3.4

Plot of i_c (μA) vs. nmol H_2O_2 injected at electrode surface using $150\ \mu\text{M}$ potassium ferrocyanide as mediator in HRP electrochemical assay (see Figure 3.3 for experimental conditions)

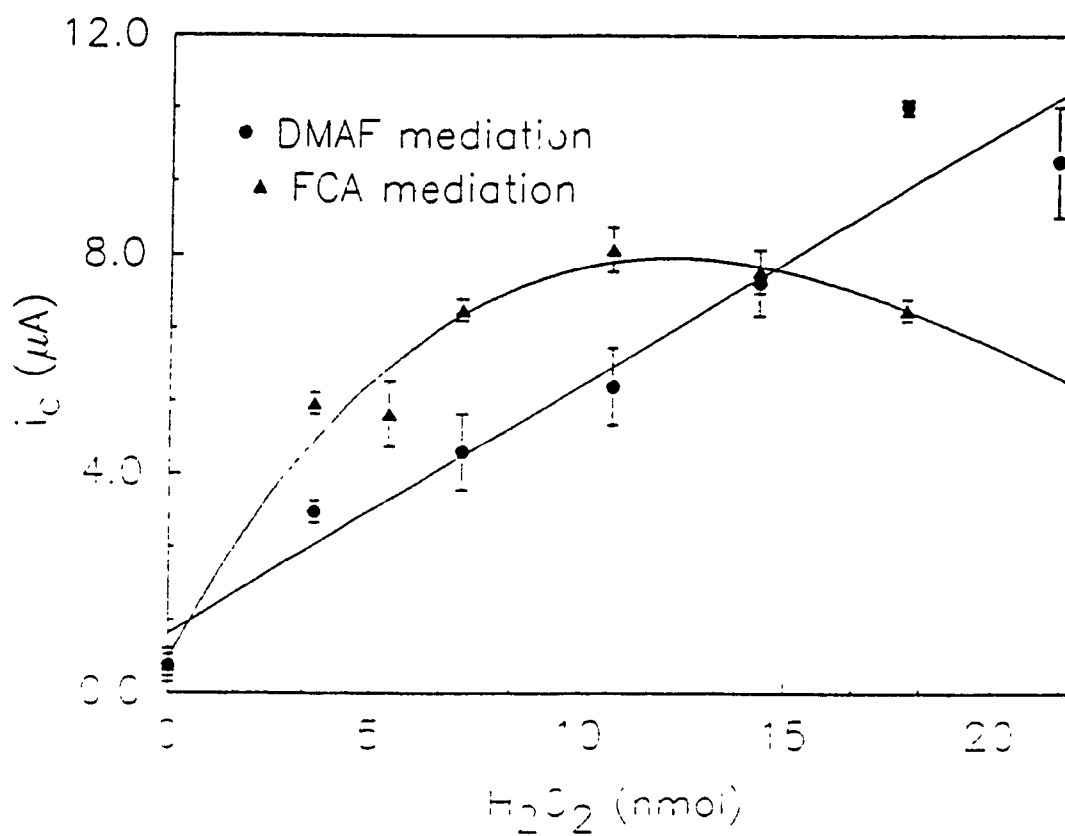


Figure 3.5

Plot of i_c (μA) vs. nmol H_2O_2 injected at electrode surface using 150 μM FCA and DMAF as mediators (see Figure 3.3 for experimental conditions)

3.4 DISCUSSION

Cyclic voltammetry of the ferrocene mediators shows reversibility (peak separations of 60 mV) and ferrocyanide also exhibited quasi reversible behaviour at RVC with a peak separation of 150 mV. The half-wave potential for ferrocyanide is 265 mV at RVC, which is in good agreement with the literature value at RVC of 256 mV. $E_{1/2}$ values for the ferrocene mediators are 307 and 376 mV at RVC for FCA and DMAF, which is similar to the $E_{1/2}$ value in the literature for DMAF at glassy carbon (Table 3.1), but a difference in $E_{1/2}$ of 76 mV is observed. This indicates that FCA does not behave at RVC as it does at glassy carbon. No comparable data at RVC was found in the literature for the ferrocenes. An arbitrary potential of 0.0 V was chosen for the operation of the HRP electrochemical assay (Scheme 3.1) since this value ensured complete reduction of any oxidized mediator at the electrode surface, facilitating the operation of the assay.

The performance of the HRP assay may be evaluated using the calibration curves shown in Figures 3.4 and 3.5. These figures reveal the range over which i_c vs. nmol H_2O_2 is linear. Figure 3.4 shows that the relation between i_c and nmol H_2O_2 is linear up to 10 nmol H_2O_2 injected using potassium ferrocyanide as mediator. The linearity of this curve and the reproducibility of i_c from injection to injection is good evidence that the HRP electrochemical assay works as predicted in Scheme 3.1. The number of coulombs passed during a run should

also be proportional to the amount of H_2O_2 injected. However, tailing of the current vs. time responses makes this type of measurement difficult, and often the current did not return to baseline. The non-homogeneity of sample solutions following H_2O_2 injection could account for this fact. Although the bulk of H_2O_2 was delivered to the electrode surface, the extent of diffusion of H_2O_2 around and away from the electrode would depend on many variables, such as the speed of injection etc. Reduced mediator could also become trapped in the pores of the RVC electrode⁸, limiting the rate of H_2O_2 reduction and affecting the tail of the current vs. time response of the system.

Figure 3.5 shows that the relation between i_c and nmol H_2O_2 injected is linear up to 22 nmol H_2O_2 for DMAF and to -10 nmol H_2O_2 for FCA. Also, the scatter in these plots result in large errors in the calculated slopes. Table 3.2 gives estimates of the slopes of the linear portion of the calibration curves which indicates the sensitivity of the assay using a particular mediator.

Table 3.2
Evaluation of Mediators for the
HRP Electrochemical Assay for H₂O₂

MEDIATOR	SLOPE OF CALIBRATION CURVE ^a (μA/nmol H ₂ O ₂)
$\text{Fe}(\text{CN})_6^{4-}$	0.025
FCA	0.6
DMAF	0.4

^a Slopes were calculated from data points shown in Figures 3.6 and 3.7. For FCA the slope was estimated from the data points up to 10.5 nmol H₂O₂.

The data in Table 3.2 clearly indicate that the ferrocene mediators yield the highest ic per nmol of H₂O₂. From Figure 3.5 it is also clear that the current response using FCA mediation levels off after 10 nmol H₂O₂. Hill et al.³ obtained a calibration curve for FCA at graphite which exhibited good linearity up to 0.5 μM H₂O₂ with a slope of 0.2 μA/μM H₂O₂. It is difficult to compare the results obtained by these workers with those obtained here. Their working volume was 0.7 ml as opposed to 2.5 ml, the volume of H₂O₂ solution introduced was 5 μL, the concentration of HRP was 30 μM and the solution was stirred prior to data collection. However, assuming a homogeneous

H₂O₂ solution in the present work, the linear portion of the FCA calibration curve would yield a slope of 1.5 $\mu\text{A}/\mu\text{M}$ H₂O₂. This suggests that the sensitivity of the HRP electrochemical assay is increased by the injection method at RVC. The reasons for the non-linearity of the FCA calibration curve in the present work are unclear, but it may be caused by interactions with the electrode surface due to the carboxyl side-group as well as the inhomogeneity of the solution.

The non-homogeneous injection method used here was employed for lack of a better alternative. Attempts at making the assay solution homogeneous failed: magnetic stirring after the introduction of H₂O₂ to the cell caused fluctuations in the current vs. time data of 10 μA or more. Potential jump, i.e. changing from an oxidizing to a reducing potential for the mediator after stirring in a sample of H₂O₂, resulted in an initial decrease in current of $\sim 100 \mu\text{A}$, which made the reading of i_c impossible. From the observed i_c vs. nmol H₂O₂ curves it was decided that the injection method is sufficient as a qualitative tool for evaluating the efficiency of the mediators at RVC.

In summary, the data presented show that RVC supports electron transfer to HRP via the mediators studied for the electrochemical assay of H₂O₂ under the conditions of this work. The results obtained for current response following injection of H₂O₂, suggest that the ferrocenes are more efficient at mediating electrons between HRP and RVC than ferrocyanide. A study on the oxidation of ferrocene and some substituted ferrocenes in the presence of HRP⁹ revealed that the electron-exchange-rate between ferrocene derivatives and HRP is $\sim 10^4$

times greater than that for ferrocyanide. The data presented are in agreement with this result. This study also indicates that although higher sensitivity for H_2O_2 at RVC as opposed to glassy carbon is achieved, the results are more qualitative than those obtained at glassy carbon since higher scattering of data points is observed for plots of i_c vs. concentration of H_2O_2 . This may be due to the nature of the working electrode and/or the inhomogeneity of H_2O_2 in the assay solutions.

3.5 REFERENCES

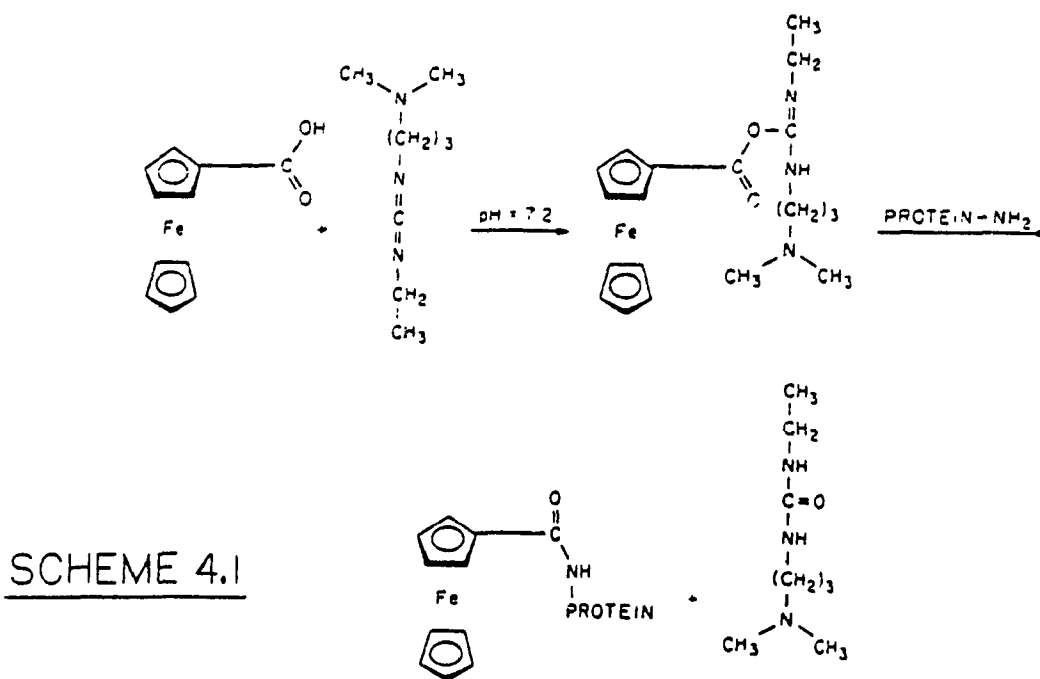
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4.0 ATTEMPTED COVALENT MODIFICATION
OF HORSERADISH PEROXIDASE
WITH FERROCENEMONOCARBOXYLIC ACID

4.1 INTRODUCTION

In chapter 3 it was shown that ferrocene derivatives can efficiently mediate electron transfer between HRP and RVC. The development of HRP electrochemical assays (Scheme 3.1) is evidence that a biosensor for H_2O_2 could be constructed using electrochemical detection. Such a biosensor could also be used to detect metabolites such as glucose and urea¹, which produce H_2O_2 during their enzymatic oxidation. To create a traditional dipstick type of biosensor for the detection of H_2O_2 employing Scheme 3.1, the device would have to contain immobilized HRP and mediator. Immobilization of HRP with free mediator presents several problems. Physical immobilization (e.g. dialysis membrane) offers very short lived enzyme activity¹ as well as the possibility of mediator diffusion through the membrane. Gel entrapment (e.g. polyacrylamide) increases the lifespan of HRP¹, but hinders diffusion of mediator to the protein and mediator can also leach out of the gel. Covalent attachment of HRP to a solid support would require free mediator be present in the test solution. To overcome these potential problems, we sought to covalently bind a ferrocene derivative to HRP. The modified HRP could then be immobilized and its ability to function as a biosensor for H_2O_2 evaluated.

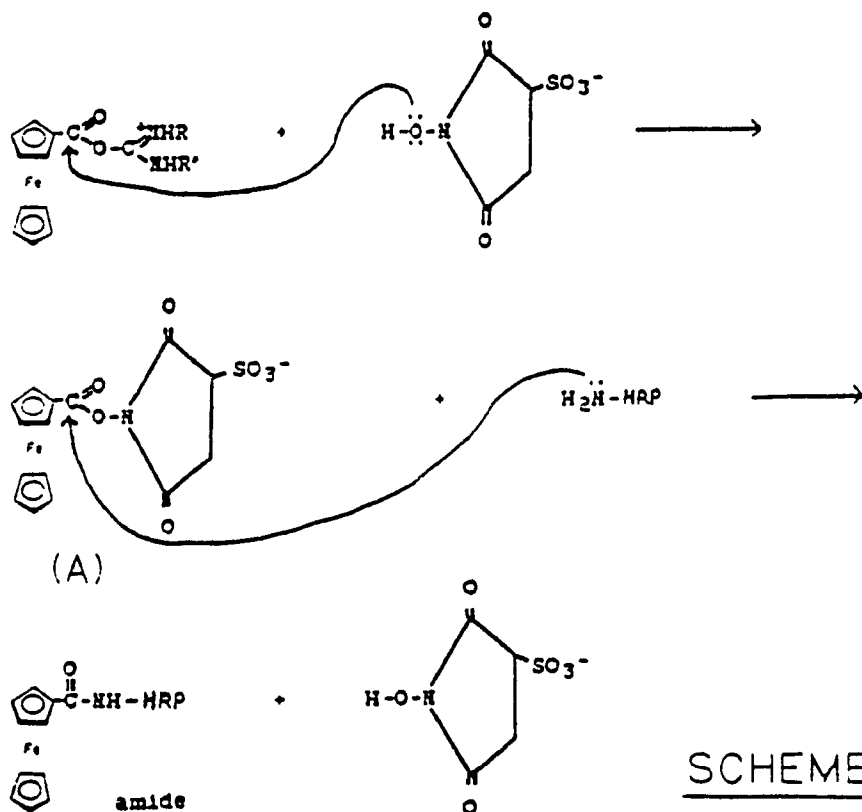
For the ferrocene-modified protein to function in the desired capacity, the covalently bound ferrocenes would have to mediate electron transfer between the heme of HRP and the electrode surface in a manner similar to free mediator (Scheme 3.1). Carbodiimides have been shown to be effective agents for the formation of amide bonds between primary amines and carboxylic acids². Degani and Heller^{3,4} used 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to promote covalent bond formation between FCA and lysine groups of glucose oxidase (GOD). The mechanism for the modification is outlined in Scheme 4.1:



SCHEME 4.1

It was found that 12 \pm 1 molecules of FCA could be attached to GOD in this manner. The modified GOD has good enzyme activity and also exhibits redox behaviour at a glassy carbon electrode.

Because of the success that Degani and Heller had with their FCA derivative of GOD, modification of HRP with FCA was attempted. Carbodiimide-promoted bond formation between the 6 lysines of HRP (see Appendix 7.3 for amino acid content of HRP) and FCA was the goal of this work. The experimental conditions used by Degani and Heller were followed with one major difference: N-hydroxysulfosuccinimide (sulfo-NHS)⁵ was used to promote amide bond formation as shown below (Scheme 4.2):



Sulfo-NHS forms a more hydrolysis resistant ester (A) than the carbodiimide, which then undergoes nucleophilic attack by a primary amine, forming the desired amide bond. Sulfo-NHS has been shown to greatly enhance carbodiimide-promoted covalent bond formation between tris(4,4'-dicarboxy-2,2'-bipyridine)ruthenium(II) and the lysines of horse heart cytochrome c⁶. Thus, it seemed likely that one could covalently link FCA to HRP and the results of the attempted modification are presented in this chapter.

4.2 EXPERIMENTAL SECTION

4.2.1 Materials

All materials as per Sections 2.2.1 and 3.2.1 with the following additions: [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES) was obtained from Aldrich. 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC) was purchased from Sigma and N-hydroxysulfosuccinimide from Pierce (sulfo-NHS). 0.2 μ m-pore single-use filters were purchased from Gelman Sciences. All gel-filtration and ion-exchange resins as well as fraction collector (RediFrac) and peristaltic pump (MicroPerpex S) were obtained from Pharmacia. YM 30 (30,000 MW cut off) ultrafiltration membranes and ultrafiltration cells were obtained from Amicon. Dialysis tubing, MW cut off 25,000, was obtained from Spectrum Medical Industries Inc.

4.2.2 Methods

HRP Modification: 0.15 M HEPES, pH 7.3 \pm 0.1 was used to buffer the reaction. 80 mg of FCA were dissolved in 4 ml of buffer; the pH was monitored during dissolution and adjusted to 7.3 \pm 0.1 with 5 N NaOH. 100 mg of EDC were then added, followed by 50 mg of sulfo-NHS and the pH adjusted if needed. Attempted coupling reactions were initiated by adding 10 mg of lyophilized HRP to give a final concentration of 87 mM FCA, 130 mM EDC, 57 mM sulfo-NHS and 57 μ M HRP. The solution was placed in a glass vial, sealed and left immersed in an ice bath for 17 h. After this time the solution

was centrifuged and the supernatant was filtered using a 0.2 μ m -pore filter. The enzyme was then separated from unreacted FCA, EDC, sulfo-NHS and other small reaction products by gel filtration on a 2x20 cm column of Sephadex G-25 equilibrated with 25 mM phosphate (sodium salts), pH 6.8, and 75 mM KCl. This buffer was also used as the eluent and the modified HRP (MHRP) was eluted first followed by a bright orange band containing unreacted FCA and the other small molecules.

Electrochemistry: Cyclic voltammograms and current response data were obtained using the procedures outlined in Section 3.2.2.

Anion Exchange Chromatography: Samples of native HRP and MHRP were subjected to anion-exchange chromatography on a 1x15 cm column of DEAE-Sepharose equilibrated with 5 mM sodium acetate, pH 4.4 at 4 °C. MHRP was transferred into 5 mM acetate, pH 4.4, by several cycles of dilution and ultrafiltration using a YM 30 membrane. Native HRP was dissolved in 2-3 ml of 5 mM acetate, pH 4.4, and loaded onto the column. After the solution drained into the bed, 2 ml of starting buffer were applied and the elution was begun. A linear gradient from 5 mM to 500 mM acetate, pH 4.4, was employed at a flow rate of 7.5 ml/h. Fractions were collected and absorbance readings at 403 nm were made for each fraction using a Hewlett Packard 8451A diode array spectrophotometer. Elution profiles of A₄₀₃ vs. fraction number were plotted.

Dialysis Experiments: Dialysis of MHRP was performed after purification on G-25. Samples were dialyzed against 75 mM KCl buffered with 25 mM phosphate, pH 6.8. The ratio of buffer outside the bag to sample inside was 100:1. Samples were kept at 4 °C with no stirring. Voltammograms were performed at different times on the sample to check for FCA peaks.

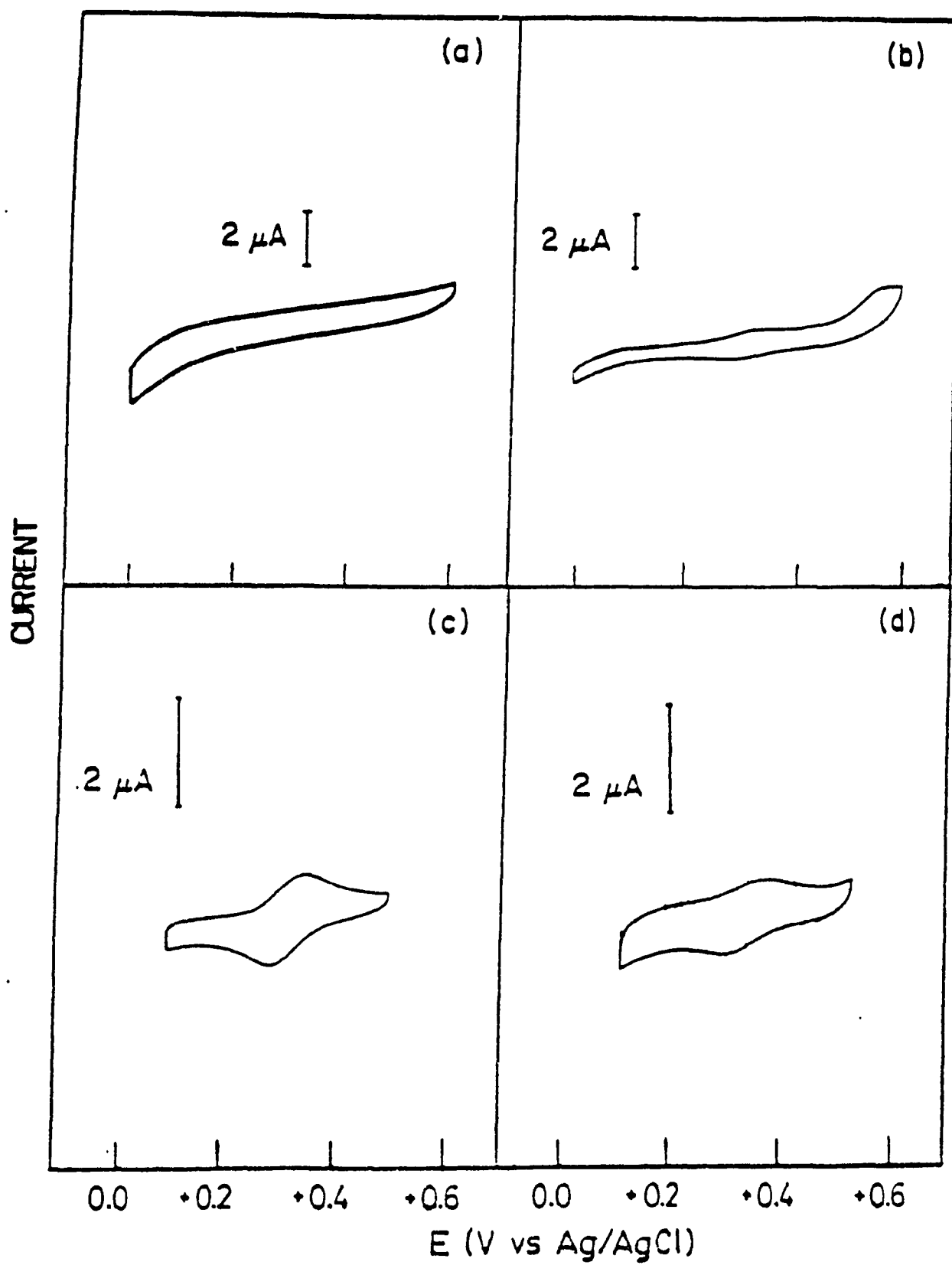
4.3 RESULTS

Cyclic voltammograms of MHRP following G-25 purification were obtained to determine if FCA was present in the sample. Figure 4.1 shows the voltammograms obtained for native HRP and MHRP. Peaks due to FCA are not observed for 15 μ M native HRP, Figure 4.1 (a), whereas peaks due to FCA are observed for 15 μ M MHRP after a single G-25 purification, Figure 4.1 (b). Figure 4.1 (c) shows FCA peaks for the sample of MHRP from 4.1 (b) which was used in the HRP electrochemical assay, and Figure 4.1 (d) MHRP after a second G-25 purification. A 15 μ M sample of MHRP, which had undergone a single G-25 purification, was used to see if a current response could be observed from the sample in presence of H_2O_2 . Current response data was acquired using the protocol given in Section 3.2.2 but without the addition of free mediator. Figure 4.2 shows the plot of i_c vs. nmol H_2O_2 for 15 μ M MHRP.

To investigate the nature of the modification, anion exchange chromatography was performed on samples of native HRP and MHRP. Figure 4.3 shows chromatograms for native HRP and MHRP on DEAE-Sepharose. The chromatograms indicate that the cationic nature of HRP has been altered by the modification procedure since some MHRP binds to the resin. Samples of 15 μ M MHRP (main peak) showed no reduction or oxidation peaks for FCA after exposure to

DEAE-sepharose. Dialysis of MHRP was performed as described in Section 4.2.2 and Figures 4.4 (a) through (d) show voltammograms obtained for MHRP before and after dialysis.

Figure 4.1. DC cyclic voltammetry of native HRP and MHRP. Experimental conditions as per Figure 3.2. (a) 15 μ M native HRP, (b) 15 μ M MHRP after one G-25 purification, (c) MHRP from (b) after use in the HRP electrochemical assay, (d) MHRP from (c) after a second purification on G-25.



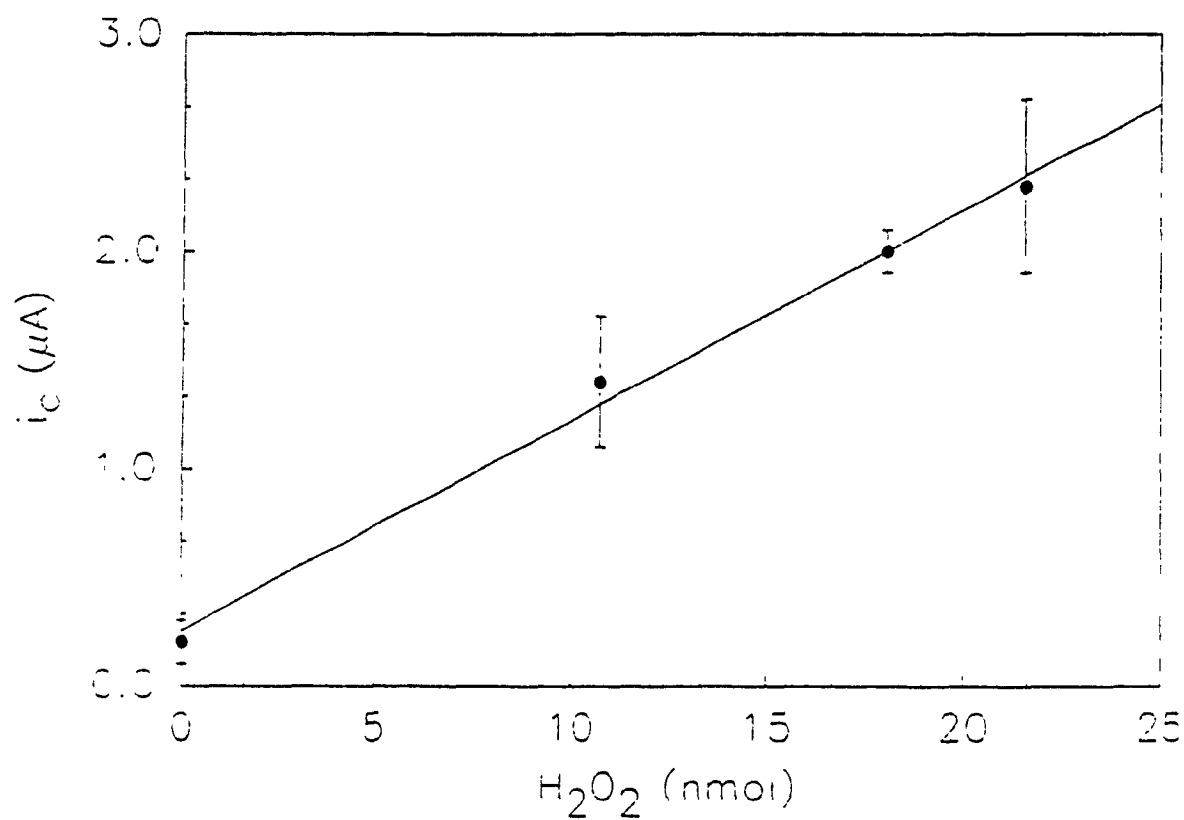


Figure 4.2

Plot of i_c vs. $\text{nmol H}_2\text{O}_2$ injected at electrode surface employing MHRP. Experimental conditions as per Figure 3.3 except that $10 \mu\text{M}$ MHRP was used without free mediator.

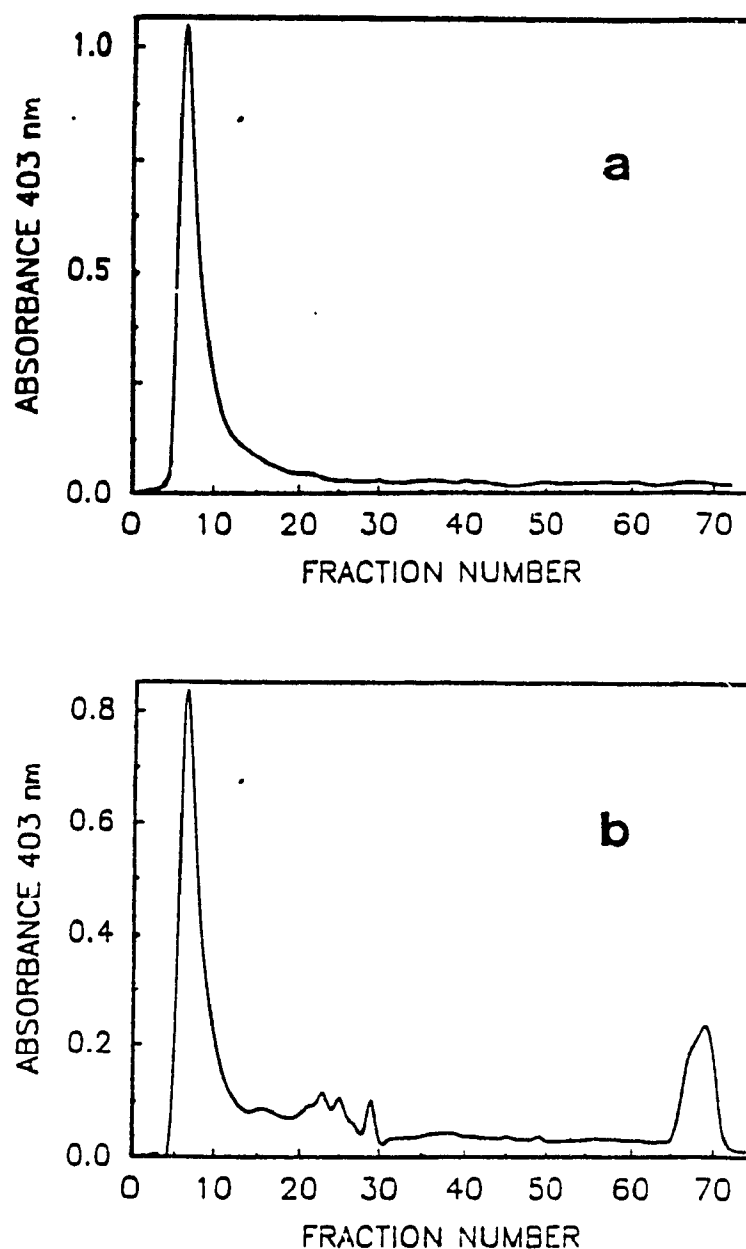
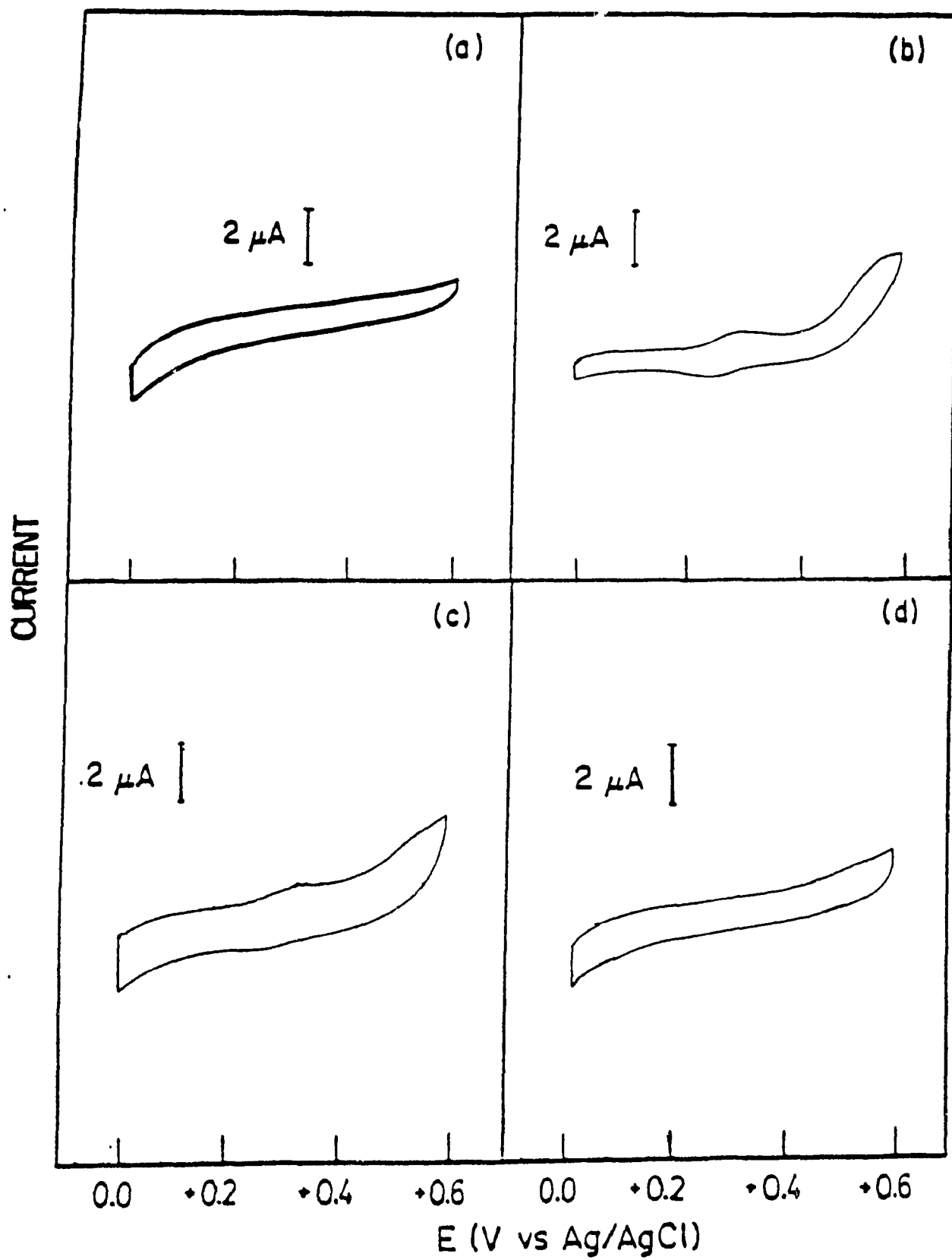


Figure 4.3

Elution profiles of (a) native HRP and (b) MHRP on DEAE-Sepharose. Column: 1x15 cm. Eluent: linear gradient from 5 to 500 mM acetate, pH 4.4. Flow rate 7.5 ml/h. Fraction size 1 ml. 10 mg of protein in ~3 ml loaded in both cases with a recovery of 95%.

Figure 4.4. DC cyclic voltammetry of MHRP which had undergone dialysis against 25 mM phosphate, pH 6.8, and 75mM KCl. Experimental conditions as per Figure 3.2. (a) 10 μ M native HRP, (b) 20 μ M MHRP before dialysis, (c) 20 μ M MHRP after 4 h of dialysis, (d) MHRP after 24 h of dialysis.



4.4 DISCUSSION

Figure 4.1 shows cyclic voltammetry of native HRP and of MHRP. Figure 4.1 (a) shows that native HRP exhibits no oxidation or reduction peaks when cycled between 0.0 V and +0.6 V. The voltammogram in Figure 4.1 (b) clearly shows that there is reduction and oxidation due to FCA taking place at the electrode surface. The calculated $E_{1/2}$ value from this voltammogram is 310 mV, which is in agreement with the $E_{1/2}$ value of 315 mV obtained for free FCA in the presence of HRP (see Table 3.1). This result is similar to that obtained by Degani and Heller for GOD who observed redox activity for FCA-modified GOD at glassy carbon^{3,4} whereas no redox activity for untreated enzyme was observed. MHRP was assayed using the o-dianisidine assay (see Chapter 2) and was found to have 100% activity relative to native HRP. These results indicated that a current response to H_2O_2 might be obtained using the protocol of Chapter 3. Figure 4.2 shows that with 10 μ M MHRP present, a linear response to H_2O_2 is observed with a slope of 0.1 μ A/nmol H_2O_2 . This value is six times smaller than the slope of the linear portion of the calibration curve employing free 150 μ M FCA as mediator (Table 3.2).

Figures 4.1 (c) and (d) show that although MHRP still shows FCA peaks at RVC after being used in the electrochemical assay, a second G-25 treatment of the day-old MHRP results in diminished oxidation and reduction peaks, suggesting that FCA may not be covalently bound to HRP. This result prompted further investigation to

determine the nature of the modification by ion exchange chromatography. Anion exchange was the method of choice since any non-covalently bound FCA should be removed because of its anionic carboxylate side group.

Figure 4.3 (a) shows that native HRP elutes from the anion exchange resin in the void volume (~5 ml) of the column. This result is expected since the pI of HRP (isoenzyme C) is 8.0-8.9⁷. Approximately 55% of MHRP was eluted in the void volume (Figure 4.3 (b)), less than 10% eluted in fractions 10-30, ~15% eluted in fractions 65-70 (~180 mM acetate) and the remaining HRP could only be removed with 1 M acetate. Similar chromatograms were obtained following three different modification attempts. FCA peaks were not observed for voltammograms for solutions containing 15 μ M MHRP which had undergone anion exchange. None of the fractions from the DEAE column showed redox activity at RVC, suggesting that FCA attachment was reversible under anion exchange. MHRP which is retained by the resin (fractions 65-70 and tightly-bound MHRP) is thought to be modified with sulfo-NHS and not FCA because of the interaction of MHRP with DEAE.

Figures 4.4 (b)-(d) show the effect of dialyzing MHRP against 75 mM KCl buffered with 25 mM phosphate, pH 6.8. These voltammograms clearly show that dialysis reduces the magnitude of the oxidation and reduction peaks observed for 15 μ M MHRP, and after 24 h dialysis, the sample of MHRP shows no discernable redox peaks.

Heller used 3 M urea to reversibly denature glucose oxidase during the modification in order to expose as many lysine residues as possible. The present work did not employ urea and this may account for the apparent lack of covalent modification since the lysines of HRP may not be exposed. The use of urea in the modification would have to be investigated to clarify this point.

In summary, the modification procedure produced a modified HRP which has a 100% activity. After a single G-25 purification, a linear plot of i_c vs. nmol H_2O_2 was obtained for MHRP similar to those in Chapter 3. This indicates that MHRP may be of use in developing a biosensor for H_2O_2 . The nature of the modification, however, is still uncertain since FCA is removed from MHRP by dialysis and anion exchange chromatography. The extent of FCA loss by these treatments is not clear since cyclic voltammograms were performed at relatively low concentrations (10-15 μM) of HRP. It is possible that by increasing the concentration of MHRP after dialysis or anion exchange that FCA may be detected by cyclic voltammetry. Further study is necessary to determine if this is true. Also the current response of MHRP to H_2O_2 after dialysis and anion exchange needs to be investigated at various MHRP concentrations to determine if MHRP could be useful in a biosensor for H_2O_2 .

4.5 REFERENCES

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5.0 ATTEMPTED COVALENT MODIFICATION OF HORSERADISH PEROXIDASE WITH AMINOMETHYLFERROCENE

5.1 INTRODUCTION

The results of Chapter 4 show that FCA-treated HRP responds to H_2O_2 in the desired manner even though the nature of the modification is unsure. Examination of the amino acid composition of HRP (Appendix 7.3) reveals that there are 48 aspartic acid and 20 glutamic acid residues in HRP (isoenzyme C). This makes a total of 68 free carboxyl groups in HRP as opposed to the 6 primary amine groups on lysine residues. Thus the possibility of binding a ferrocene derivative to the carboxyls of HRP is very attractive.

The reasons that FCA was used in the first modification attempt are its commercial availability and the reported FCA modification of GOD by Degani and Heller¹. A ferrocene with a primary amine side-group is required if the sulfo-NHS-carbodiimide cross linking chemistry outlined in Chapter 4 is to be employed for carboxylate modification; thus, aminomethylferrocene (AMF) was synthesized to act as modifier for HRP.

In an attempt to covalently link AMF to HRP, the reaction conditions were varied and cyclic voltammetry was used to investigate the presence of AMF. Ion exchange chromatography at ambient pressure and high pressure (FPLC), as well as dialysis were used to

probe the nature of the modification. This chapter presents the results of the attempted HRP modification using carbodiimide-promoted amide bond formation between AMF and HRP.

5.2 EXPERIMENTAL SECTION

5.2.1 Materials

All materials as per Sections 2.2.1, 3.2.1 and 4.2.1 with the following additions: aminomethylferrocene (AMF), which was synthesized in our laboratory from ferrocene carboxaldehyde (Aldrich), was identified by mass spectroscopy. Cacodylic acid (free acid) was obtained from Sigma and 4-morpholineethanesulfonic acid (MES, free acid) from J.T. Baker. FPLC equipment was purchased from Pharmacia and consisted of an LCC-500 controller, 2 P-500 pumps, an MV-7 valve for sample loading, Mono-S column and a UV-monitor with a 280-nm filter. FPLC chromatograms were recorded using a Kipp and Zonen BD40 chart recorder.

5.2.2 Methods

AMF Molar Absorptivity Determination: 3-6 mg of AMF were dissolved in 50 ml of anhydrous ether and a molecular weight of 215 ($\text{FeC}_{11}\text{H}_{14}\text{N}$) was used to calculate the molarity of the solutions. Spectra were taken using an HP 8451A diode array spectrophotometer and Figure 5.1 shows the spectra of AMF in anhydrous ether and in 0.1 M MES, pH 3.6. Figure 5.2. shows a plot of absorbance at 440 nm (visible maximum) vs. concentration of AMF in anhydrous ether from which an extinction coefficient of $0.14 \text{ mM}^{-1} \text{ cm}^{-1}$ was estimated.

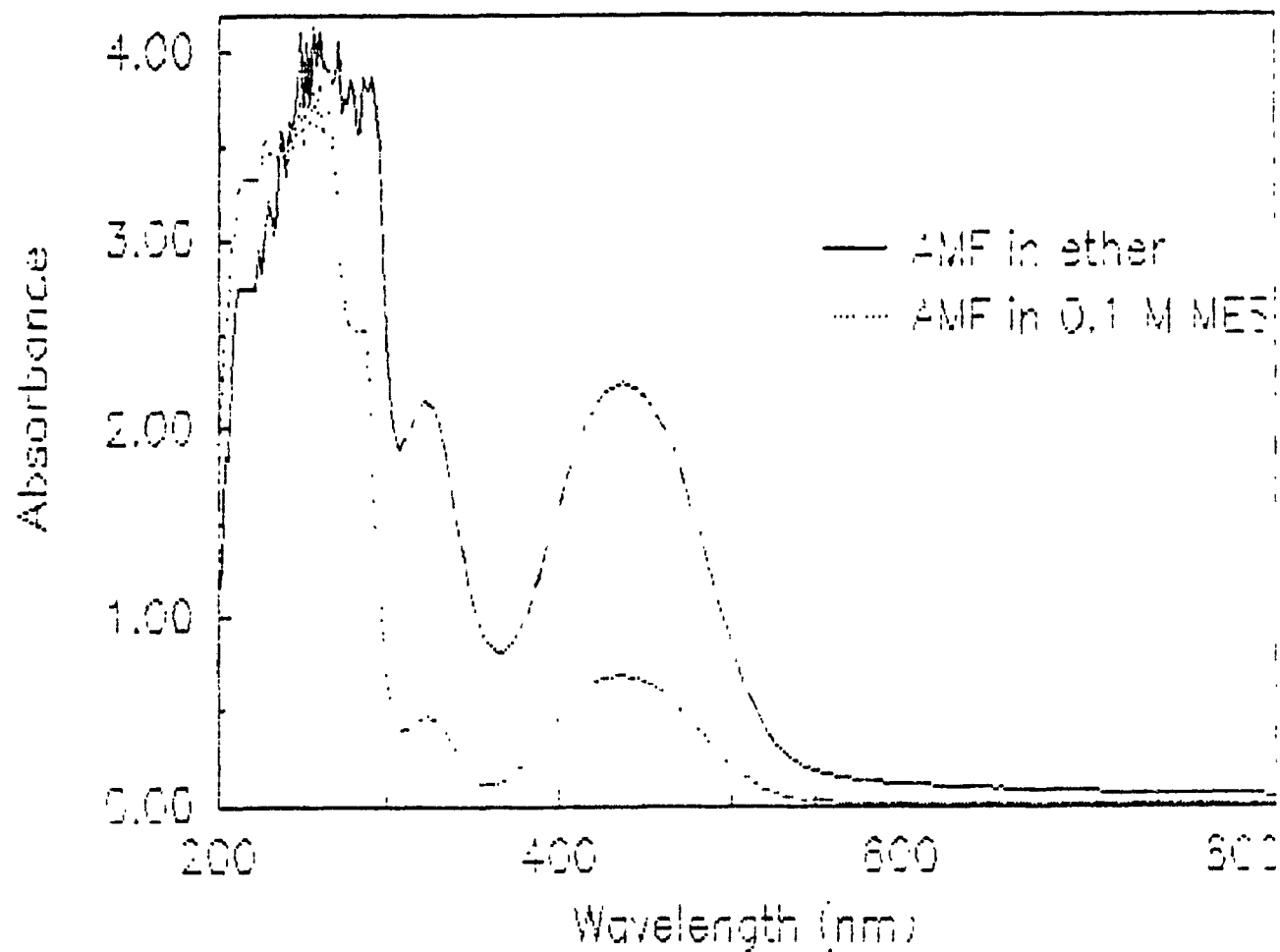


Figure 5.1

Spectra of 14 mM aminomethylferrocene in anhydrous ether and 4 mM aminomethylferrocene in 0.1 M MES, pH 3.6.

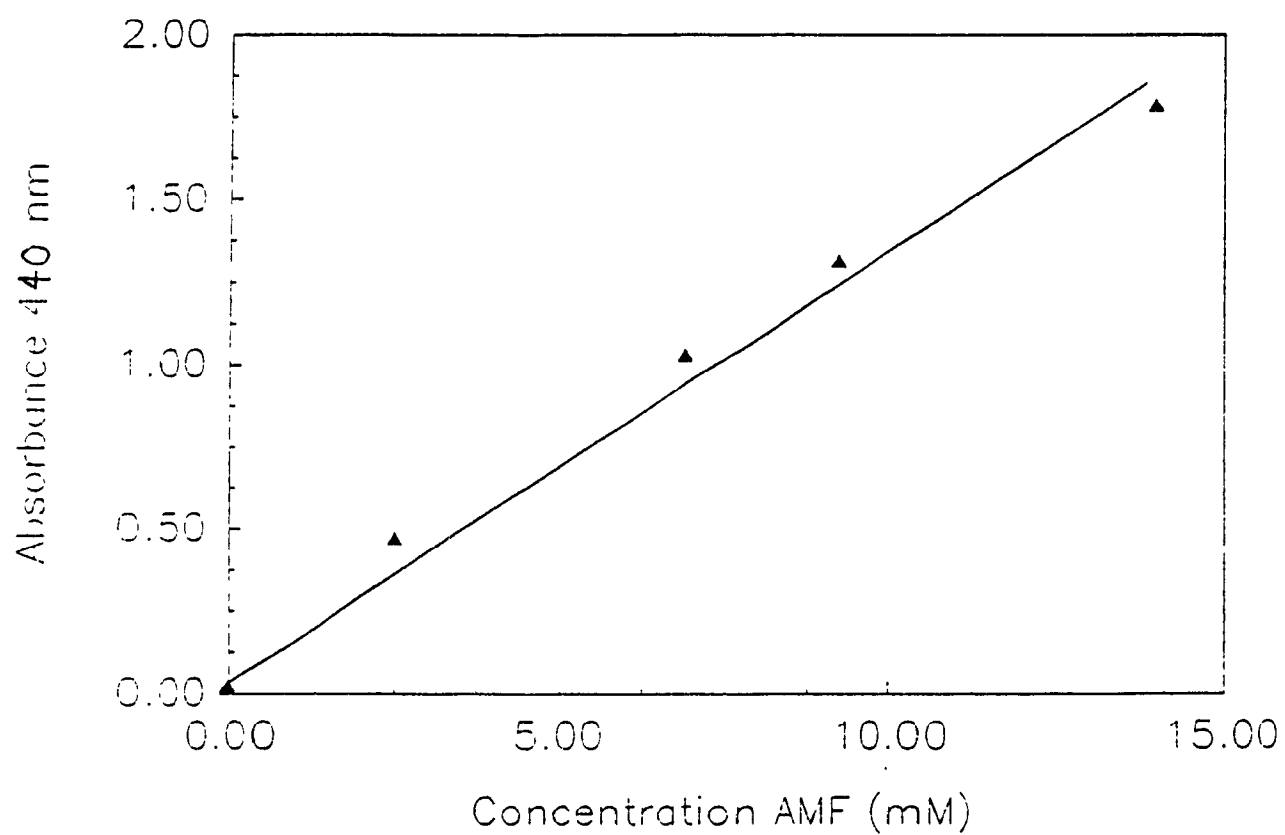


Figure 5.2

Plot of absorbance at 440 nm vs. concentration AMF (mM) for
extinction coefficient determination.

Dissolution of AMF in Aqueous Solution: AMF is virtually insoluble in neutral aqueous solutions but is highly soluble in diethyl ether. By dissolving AMF in ether and extracting it into water at pH <1, it was found that the aqueous layer (containing AMF) could be brought to the desired pH, with little precipitation of dissolved AMF. This technique was used for trials 2 and 3 (see Table 5.1) where concentration of AMF in the reaction mixture was ~1 mM.

Several attempts at solvating AMF using this extraction technique did not work as AMF precipitated from the ether layer upon addition of aqueous solutions. It was subsequently found that the extraction did not work with ether which contained (unknown) impurities. Using a freshly opened bottle of Fisher anhydrous ether, up to 5 mM ($A_{440} \sim 0.7$) AMF can be extracted into 0.1 M MES, pH 3.6, using the following method: dissolve AMF in ~3 ml anhydrous ether until the absorbance at 440 nm is ~2. Add 3 ml of 0.1 M MES to the AMF solution and shake gently for several minutes until the absorbance at 440 nm of the aqueous layer is 0.6-0.7. Adjust the pH of the aqueous layer, which is ~4.3 after the extraction, to 5.2 ± 0.1 with 5 N NaOH. This buffered solution can then be used to dissolve the reagents for the modification.

HRP Modification: AMF solutions were prepared as outlined above, the reagents for the modification were added and the pH

Table 5.1
Conditions Employed for Modification
of HRP by Aminomethylferrocene^a

Trial	pH ^b	Conc'n of EDC (mM)	Conc'n of Sulfo-NHS (mM)	Conc'n of HRP (μ M)	Conc'n of AMF (mM)
1	8.0	50	20	200	0.07
2	6.0	50	20	50	1.1
3	5.2	50	20	50	1.1
4	5.2	10	5	75	4.2
5	5.2	10	5	75	4.2
6	5.2	10	5	75	4.2
7 ^c	5.2	10	5	75	4.2

^a After preparing AMF solutions as described in Section 5.2.2, samples were treated as outlined in Section 5.2.2.

^b Trials 1 and 2, 0.1 M phosphate buffer; trial 3, 0.1 M cacodylate buffer; trials 4-7, 0.1 M MES buffer.

^c Trial 7 was carried out in triplicate.

adjusted to give the reaction mixtures listed in Table 5.1. The various reaction mixtures were further treated as in the HRP modification procedure of Section 4.2.2.

Methods of Analysis: Gel filtration of M'HRP was performed as in Section 4.2.2. Cyclic voltammograms and current response data were obtained using the procedure outlined in Chapter 3, Section 3.2.2. Anion exchange chromatography at ambient pressure and dialysis experiments on modified HRP (M'HRP) were performed as outlined in Chapter 4, Section 4.2.2. Cation exchange chromatography of M'HRP was performed on a 1x15 cm column of CM-Sephacrose equilibrated with 5 mM sodium acetate, pH 4.4 at 4 °C. M'HRP was transferred into 5 mM acetate, pH 4.4, by several cycles of dilution and ultrafiltration using a YM 30 membrane. Native HRP samples were dissolved in 5 mM acetate pH 4.4 and 2-3 ml of sample were carefully loaded onto the column. After the solution drained into the bed, 2 ml of starting buffer were applied and the elution was begun. A linear gradient from 5 mM to 500 mM acetate, pH 4.4, was employed at a flow rate of 10 ml/h. Fractions were collected and absorbance readings at 403 nm were made for each fraction using a Hewlett Packard 8451A diode array spectrophotometer. Elution profiles of A₄₀₃ vs. fraction number were plotted.

Fast Protein Liquid Chromatography: Samples of native HRP and M'HRP (Trials 4 through 7) were subjected to FPLC using a Pharmacia Mono-S column (cation exchanger) equilibrated with 5 mM

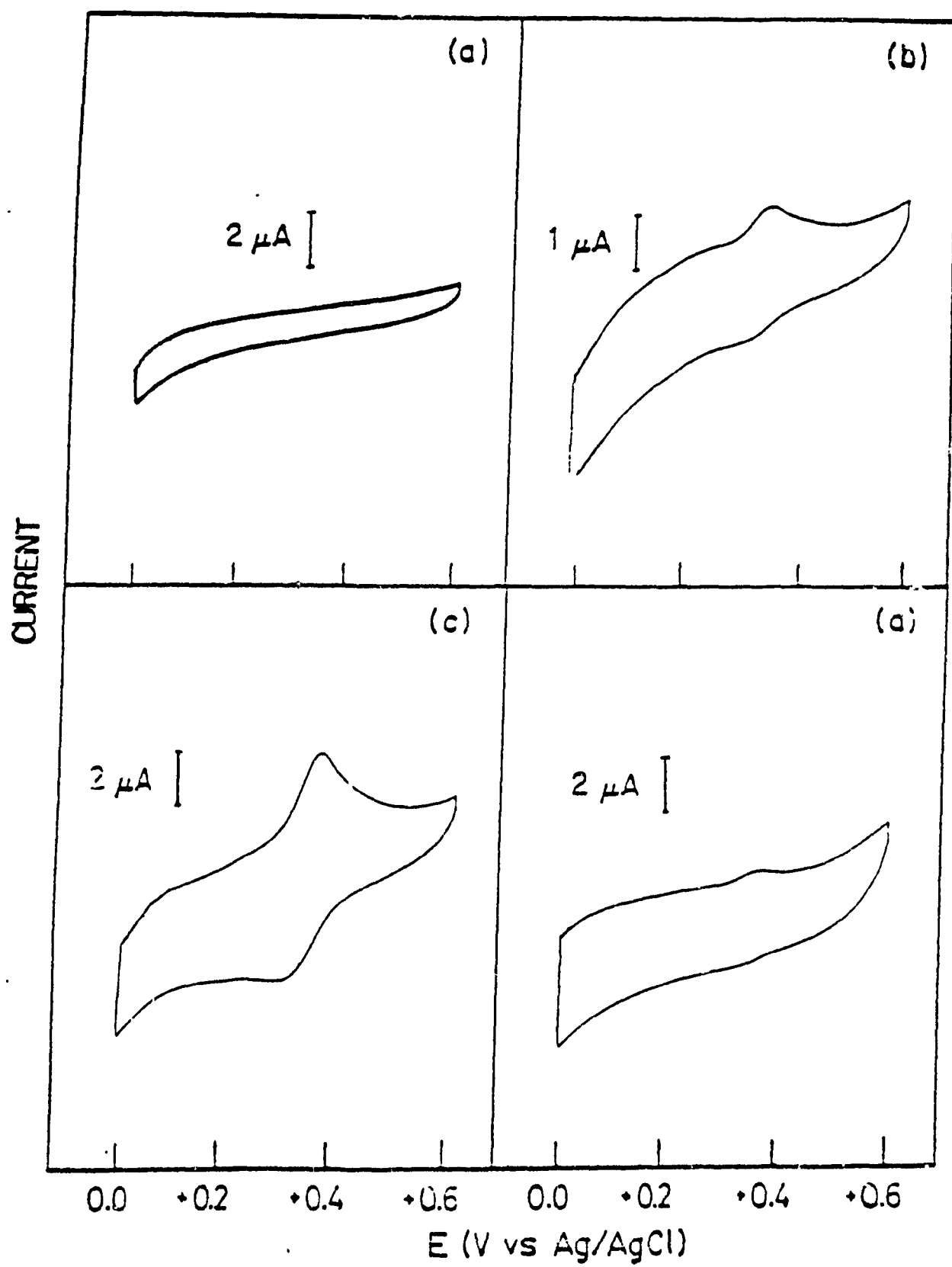
acetate buffer (pH 4.4). 100 μ L samples of native HRP and M'HRP were applied to the column using a manual injection port on the MV-7 valve, and a short computer program was written using LCC-500 controller language to control sample loading and to generate a linear gradient of 5 to 100 mM acetate with a 300 mM acetate step gradient after 20 ml of elution. The flow rate was 1 ml/min and fractions were not collected due to the small size of sample loaded and the computer program is presented with explanation in Appendix 7.4.

5.3 RESULTS

All samples of M'HRP underwent purification on a G-25 column as in Section 4.2.2. Voltammograms of M'HRP at different stages after modification and G-25 purification were recorded to establish the extent of AMF modification and its stability to ion exchange chromatography and dialysis. Figure 5.3 (a) shows the voltammogram obtained for 10 μ M native HRP and Figure 5.3 (b) shows the voltammogram obtained for Trial 1. The concentration of AMF for Trial 1 (Table 5.1) was ~ 0.07 mM and the M'HRP showed reversible behaviour at RVC with $E_{1/2}$ of 332 mV, compared to 265 mV obtained for the hydrochloride salt of AMF at glassy carbon². The sample was transferred into 5 mM acetate buffer, pH 4.4, by several cycles of dilution and ultrafiltration and purified on DEAE-Sephacrose. The protein eluted in the void volume of the column as in Figure 4.3, and no peaks due to AMF were observed for this sample after the above treatment.

Trials 2 and 3 were performed using the ether extraction method to dissolve AMF. The pH was lowered as carbodiimide-promoted amide bond formation between carboxyl groups of proteins and primary substituted amines reportedly works best between pH 4.5 and 5.0³. Due to the fact that AMF peaks were observed for trial 1, samples 2 and 3 were dialyzed as in the case for FCA (Section 4.2.2), and Figures 5.3 (c), (d) and 5.4 (a), (b) show the

Figure 5.3. DC cyclic voltammetry of native HRP and M'HRP performed in 25 mM phosphate buffer, pH 6.8 and 75 mM KCl. In all cases the working, counter and reference electrode were RVC, platinum wire and saturated Ag/AgCl, respectively. Scan rate 5 mV/s. (a) 10 μ M native HRP, (b) 14.8 μ M M'HRP from Trial 1, after modification procedure, (c) 40 μ M M'HRP from Trial 2, after 2 h c² dialysis, (d) 40 μ M M'HRP from Trial 2, after 21 h of dialysis.

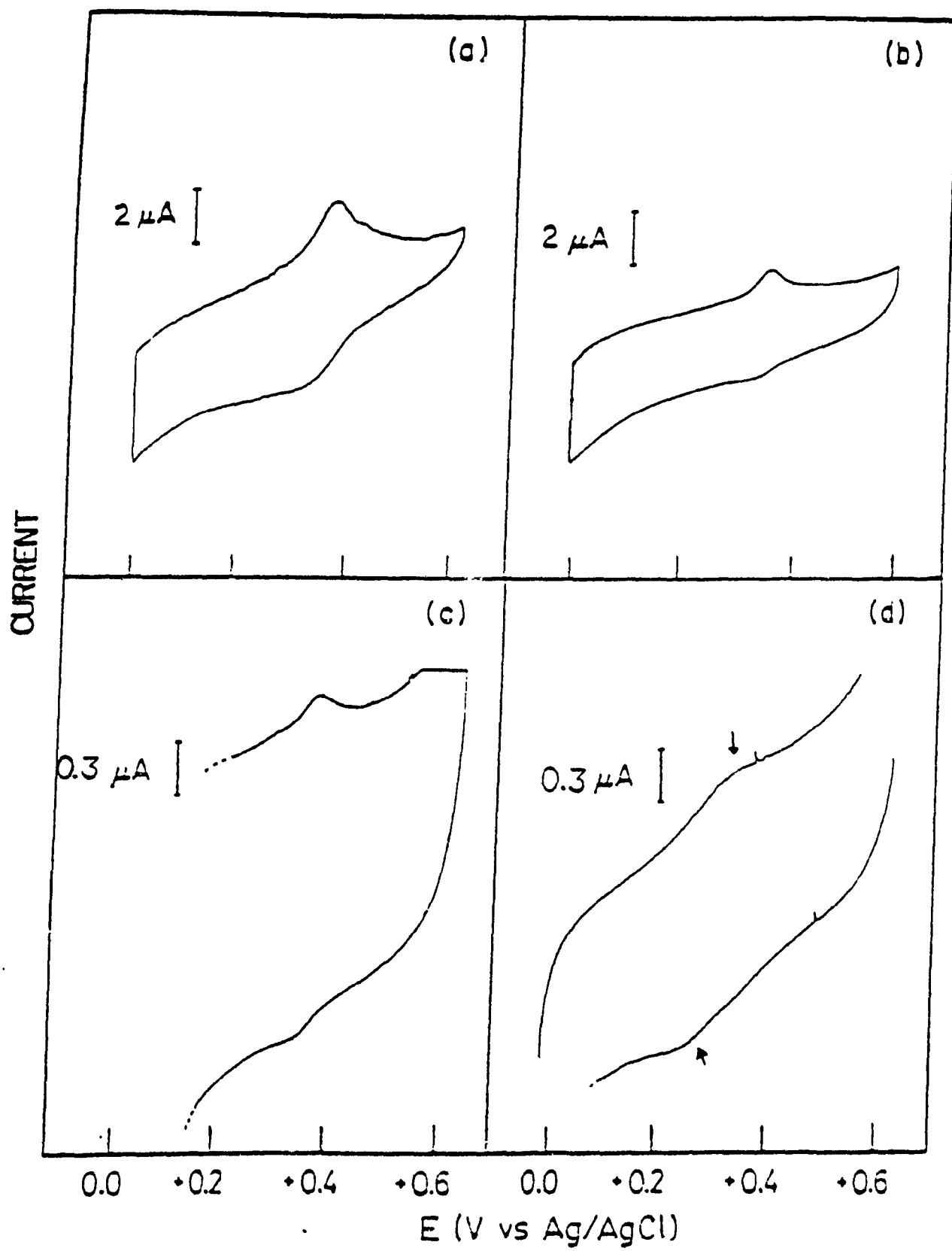


voltammograms obtained for 40 μ M M'HRP after dialysis. The voltammograms show that AMF peaks decreased for both samples of M'HRP after 21 h dialysis, but the decrease was not as marked for the sample of Trial 3 (pH 5.2). This result suggests that lowering the pH yields M'HRP which is more resistant to dialysis; therefore, all subsequent modifications were performed at pH 5.2. After 48 h of dialysis the samples from both Trial 2 and Trial 3 showed voltammograms which were void of oxidation and reduction peaks due to AMF.

Work by Staros et al.⁴ showed that sulfo-NHS-carbodiimide coupling of glycine to hemocyanin worked best using 5 mM of sulfo-NHS. These workers showed that higher concentrations of sulfo-NHS actually decreases the yield of cross-linked product, therefore, the concentration of sulfo-NHS was lowered to 5 mM for Trials 4-7.

To test the stability of the modification to anion exchange chromatography, M'HRP from Trials 4 and 5 were subjected to DEAE-sepharose as in Chapter 4, and Figure 5.5 shows the elution profile of M'HRP from Trial 5. All M'HRP eluted in the void volume (like native HRP) and no protein was retained by the column, which differs from the elution profile of "FCA-modified" HRP (MHRP) shown in Figure 4.4 (b). This suggests that M'HRP is less negatively

Figure 5.4. DC cyclic voltammetry of M'HRP: (a) 40 μ M M'HRP from Trial 3, after 2 h of dialysis, (b) 40 μ M M'HRP from Trial 3, after 21 h of dialysis, (c) 40 μ M M'HRP from Trial 4, after DEAE-Sephadex chromatography. Scan rate 10 mV/s, (d) 40 μ M M'HRP from Trial 5, after DEAE-Sephadex chromatography. Scan rate 20 mV/s. Experimental conditions as per Figure 5.3.



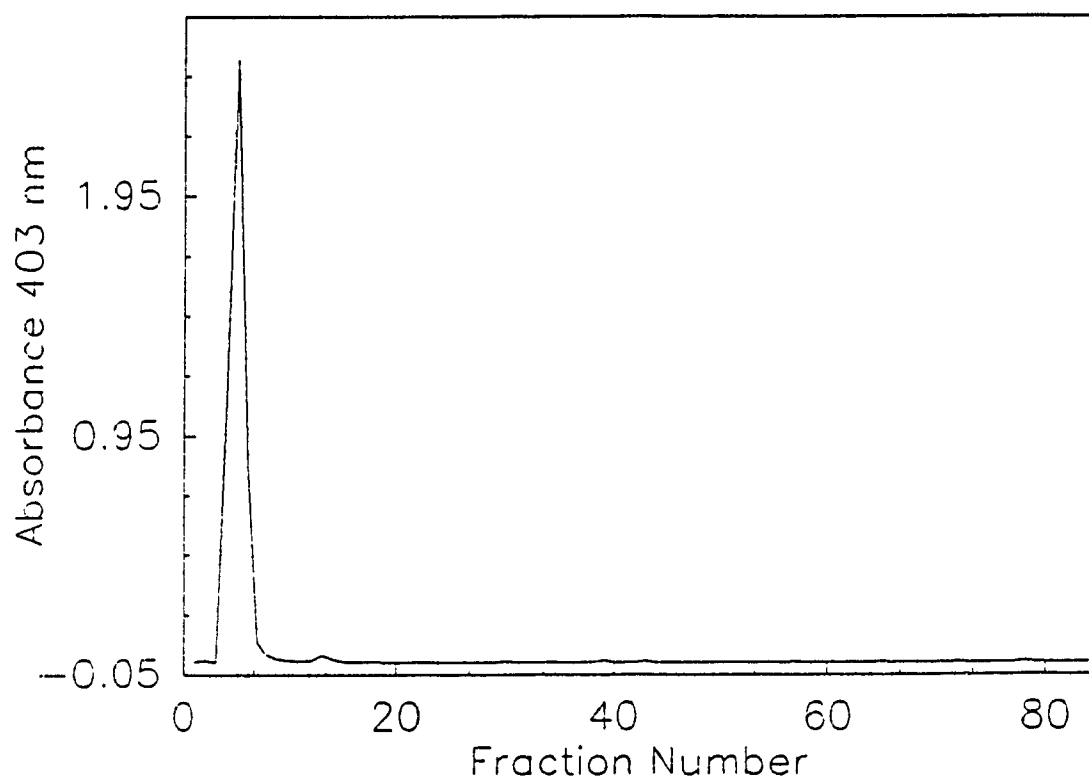


Figure 5.5

Elution profile of M'HRP on DEAE-Sepharose. Column: 1x15 cm. Eluent: linear gradient from 5-500 mM acetate, pH 4.4. Flow rate: 7.5 ml/h. Fraction size: 1 ml. ~3 ml of 100 μ M M'HRP loaded with a 95% recovery.

charged than MHRP, possibly because no sulfo-NHS derivatives of HRP were formed due to the lower concentration of sulfo-NHS in the reaction mixture (5 mM vs. 57 mM).

Figures 5.4 (c) and (d) show the resulting voltammograms for samples of Trials 4 and 5 after exposure to DEAE-sepharose (10 mV/s and 20 mV/s respectively). AMF peaks are observed which indicates that M'HRP retained some of the redox activity due to AMF even after exposure to DEAE-sepharose. The same result as above was obtained for the M'HRP from Trial 6.

Due to the observation of AMF peaks in voltammograms for M'HRP which had undergone DEAE-sepharose purification, cation exchange FPLC (see Appendix 7.4 for information on the resin) was performed on M'HRP in an attempt to further characterize the nature of the modification. Figure 5.6 (a) shows the chromatogram obtained for a sample of native HRP from Mono-s with 280 nm monitoring. Figure 5.6 (b) and (c) shows the chromatograms obtained for samples of M'HRP from Trials 4 and 5, respectively. Figure 5.6 (d) shows the FPLC elution profile for a sample of M'HRP obtained after concentration from ~10 ml to ~2 ml of M'HRP, dilution to 10 ml with 5 mM acetate buffer, pH 4.4, and concentration to ~2 ml by ultrafiltration of pooled M'HRP from Trials 5 and 6. Figure 5.7 compares FPLC chromatograms for native HRP, the same sample shown in Figure 5.6 (d) and M'HRP from Trial 7, at twice the sensitivity of Figure 5.6.

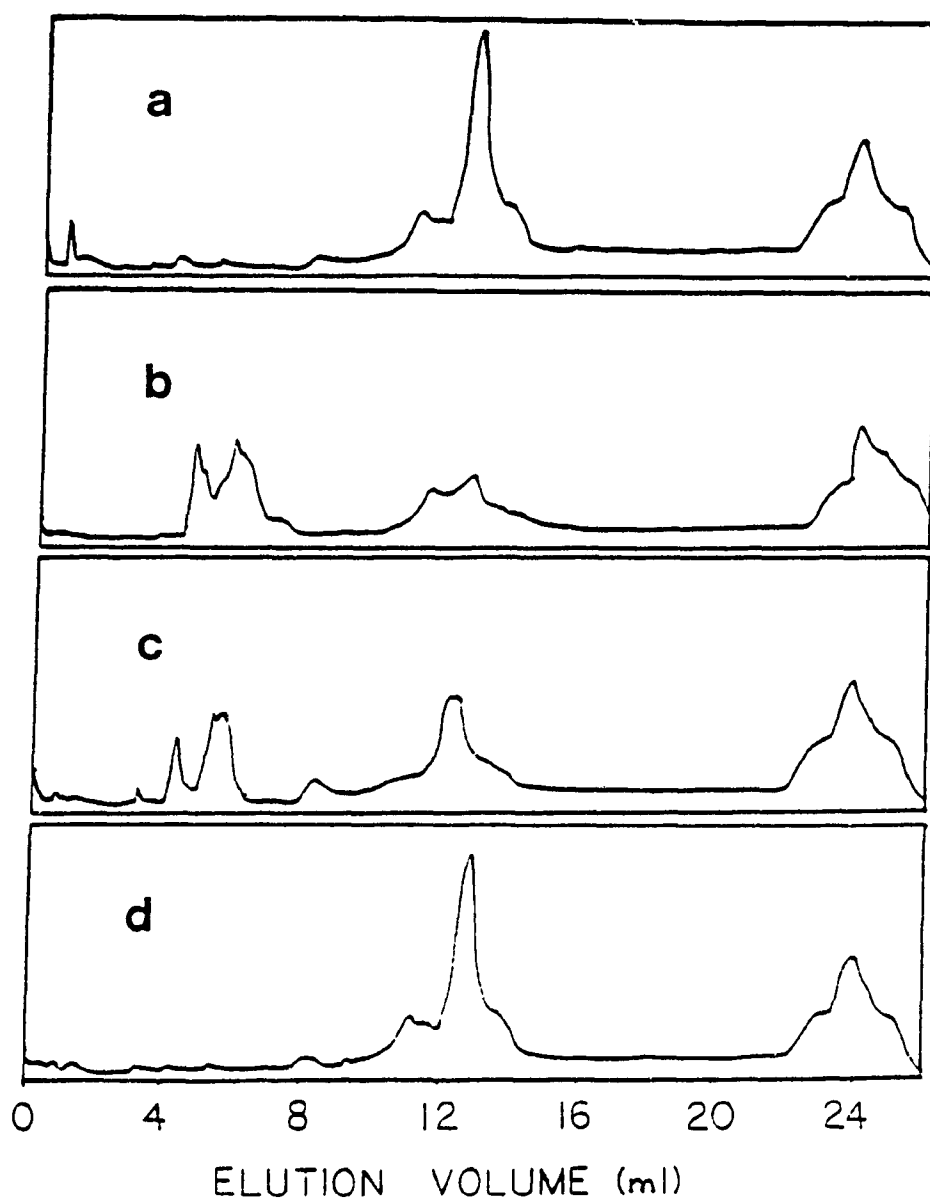


Figure 5.6

FPLC chromatograms of (a) native HRP, (b) M'HRP from Trial 4, (c) M'HRP from Trial 5, (d) pooled samples of M'HRP from Trials 4 and 5 after ultrafiltration. Column: Pharmacia Mono-S. Monitoring wavelength: 280 nm. Eluent: linear gradient, 5-100 mM sodium acetate, pH 4.4. A step-gradient of 300 mM acetate was applied at 20 ml to remove any remaining protein. Flow rate: 1 ml/min, monitor sensitivity of 0.02 (see Appendix 7.4).

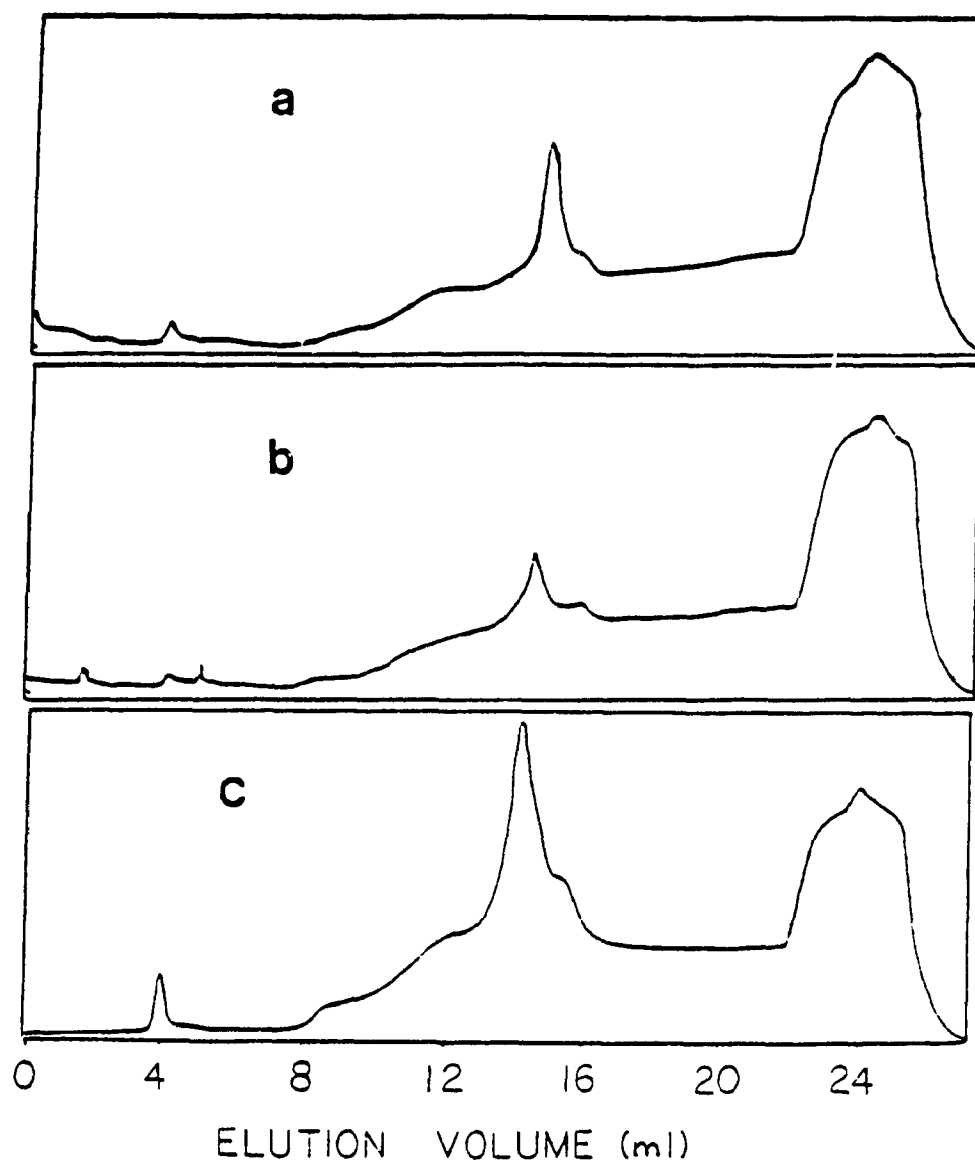


Figure 5.7

FPLC chromatograms of (a) native HRP, (b) pooled samples of M'HRP from Trials 4 and 5 after ultrafiltration, (c) M'HRP from Trial 7. Experimental conditions as per Figure 5.6 except monitor sensitivity was changed to 0.01 (see Appendix 7.4).

The peak which appears in all chromatograms at 24 ml was verified as being a solvent peak resulting from stepping up the gradient from 100 to 300 mM acetate. Figures 5.6 (b) and (d) show the chromatograms obtained for M'HRP from Trials 4 and 5 which had undergone DEAE-sepharose purification. The chromatograms obtained for these samples showed small native peaks at 12 ml and two other peaks at 4 and 6 ml. Initially, it was thought that these peaks were modified HRP, but these peaks were not seen for Trials 6 or 7. Also, pooled M'HRP from Trials 4 and 5 after 2 days storage at 4 °C, gave the chromatogram in Figure 5.6 (d) which looks identical to that obtained for native HRP, Figure 5.6 (a), suggesting that the sample had changed over the storage period. Subsequent FPLC on native HRP, M'HRP from Figure 5.6 (d), and M'HRP from Trial 7 (Figure 5.7 (a), (b) and (c), respectively) showed little difference between native HRP and M'HRP.

In a further effort to detect any chromatographic difference between native HRP and M'HRP, cation exchange on CM-sepharose was run. Figure 5.8 shows the elution profile obtained for native HRP on CM-sepharose. This result shows that the HRP obtained from Boehringer Mannheim (lot no. 11389224-62) is not 100% isoenzyme C, since there is a small amount of some other isoenzyme present. The main band for native HRP eluted at 160 mM. Figure 5.9 shows the elution profile obtained for M'HRP which had been previously run on

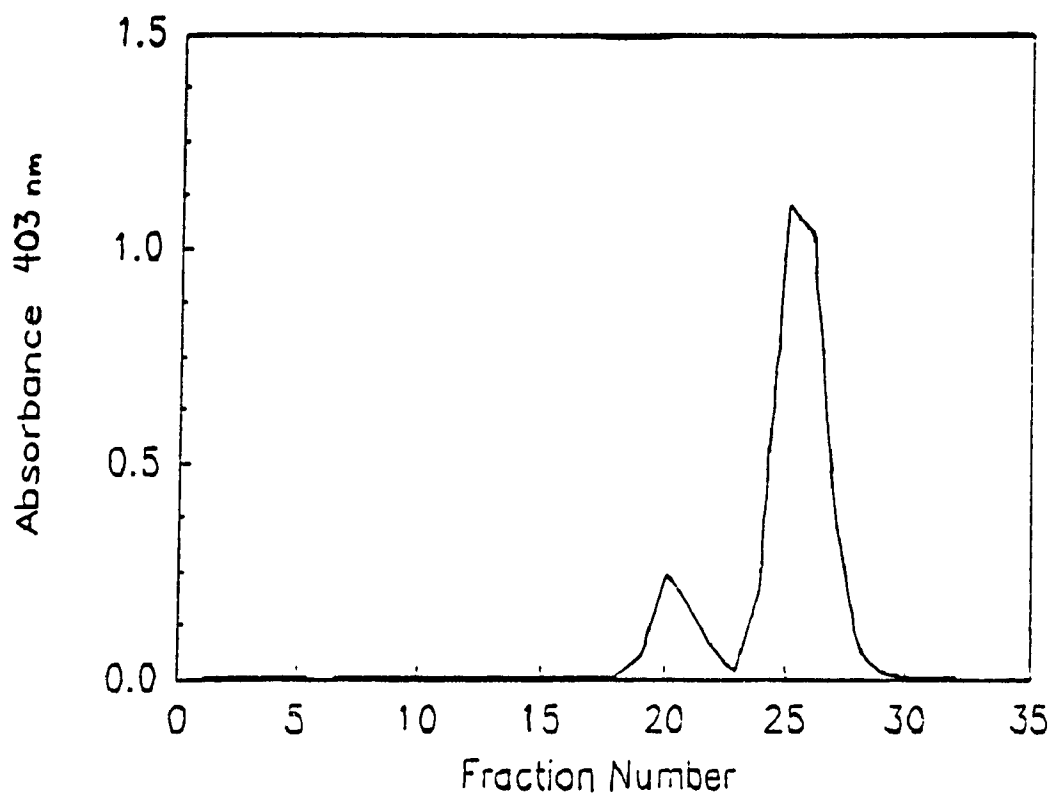


Figure 5.8

Elution profile of native HRP on CM-Sepharose. Column: 1x15 cm.

Eluent: linear gradient, 5-500 mM acetate, pH 4.4. Flow rate: 7.5 ml/h.

Fraction size: 2.5 ml. 10 mg of HRP in ~3 ml loaded with 95% recovery.

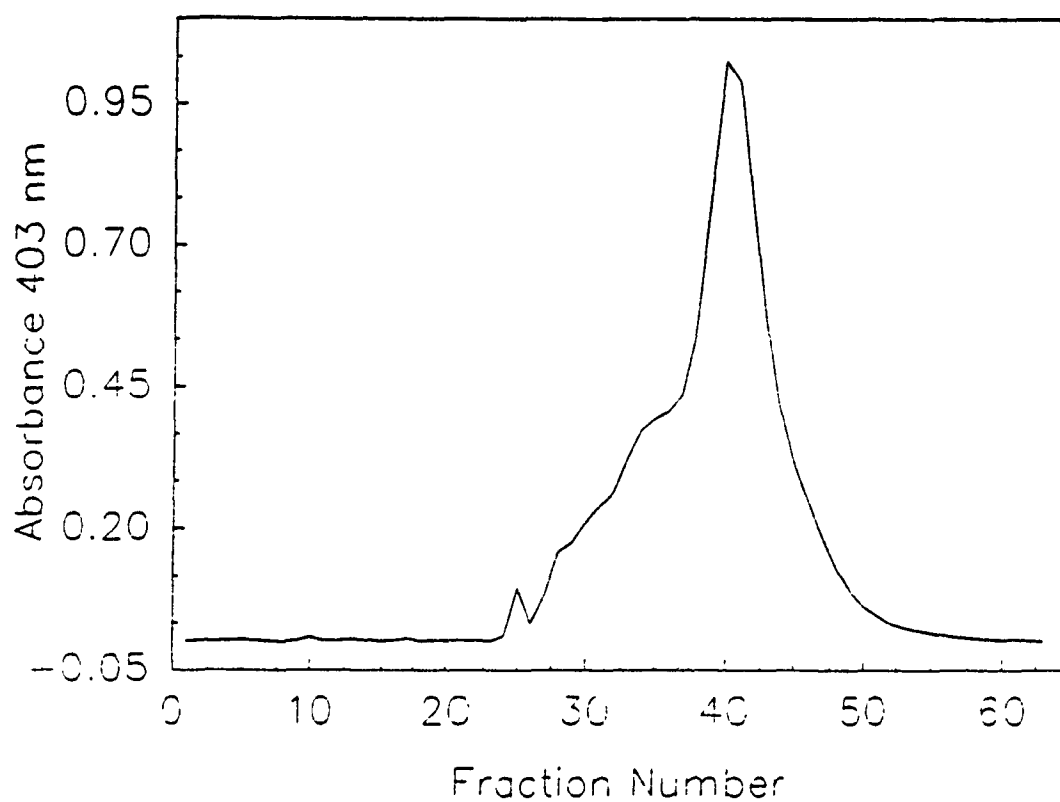


Figure 5.9

Elution profile of M'HRP (pooled from Trials 4-6) on CM-Sepharose.

Column: 1x15 cm. Eluent: linear gradient, 5-500 mM acetate, pH 4.4.

Flow rate: 7.5 ml/h. Fraction size: 1.5 ml. -3 ml of 100 μ M M'HRP loaded
with a 95% recovery.

DEAE-sepharose (pooled from Trials 4-6). There are no longer 2 distinct peaks as in native, and the main peak eluted at -150 mM with a large shoulder which may be AMF-modified HRP. Attempts to observe AMF peaks in voltammograms on 40 μ M M'HRP after anion exchange on DEAE-sepharose and subsequent cation exchange on CM-sepharose (all fractions) failed.

M'HRP was assayed using the o-dianisidine assay (see Chapter 2) and was found to have 100% activity with respect to native. In order to obtain an amperometric response for M'HRP, as in Chapter 4 for MHRP, M'HRP (pooled from the triplicate modification of Trial 7 and which had been subjected to G-25 filtration only) was subjected to injections of H_2O_2 using the protocol of Chapter 3. Figure 5.10 shows that 40 μ M M'HRP, from Trial 7, yielded a linear response to H_2O_2 and the slope of the response curve is $-0.1 \mu\text{A/nmol H}_2\text{O}_2$, which is the same as the slope observed for 10 μ M MHRP shown in Figure 4.2. This result indicates that M'HRP is not as efficient in the electrochemical assay for H_2O_2 as MHRP. Table 5.2 summarizes the M'HRP analyses.

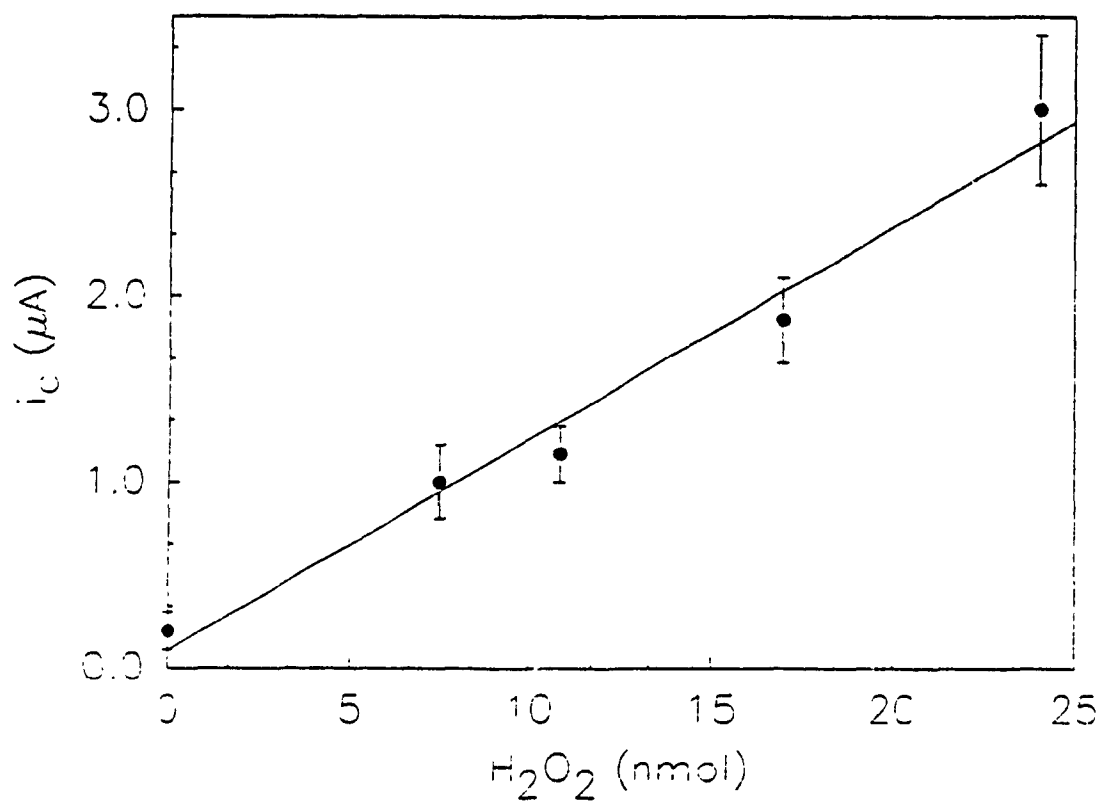


Figure 5.10

Plot of i_c vs. nmol H₂O₂ injected at electrode surface employing 40 μ M M'HRP from Trial 7. See Figure 3. for experimental conditions.

Table 5.2
Summary of M'HRP Analysis

Trial^a	Filt^b	dialysis^c	DEAE^d	CM^d	CV^e
1	x	x	✓	x	-
2	x	✓	x	x	+
3	x	✓	x	x	+
4	✓	x	✓	x	+
5	✓	x	✓	✓	-
6	✓	x	✓	x	+
7	x	x	x	x	+

^a All samples of M'HRP initially purified on G-25 as in Section 4.2.2.

^b Filt; ultrafiltration, performed as described in Section 5.3.

^c Dialysis of M'HRP carried out as described in Section 4.2.2.

^d Ion exchange chromatography of M'HRP on DEAE-sepharose and CM-sepharose as described in Sections 4.2.2 and 5.2.2, respectively.

^e Cyclic voltammetry was performed on samples of M'HRP after the different treatments indicated in columns 2-6. +, - signifies whether AMF peaks were observed.

5.4 DISCUSSION

The attempted modification of HRP with AMF produced M'HRP which, after a single G-25 purification, is active and mediates electron transfer between the heme of M'HRP and RVC. A linear calibration curve is obtained similar to that for MHRP in Chapter 4, but it takes ~4 times more M'HRP to achieve the same degree of efficiency in the electrochemical assay as MHRP. This observation is difficult to explain since there are 11 times more carboxyl groups (aspartic acid plus glutamic acid residues) in HRP than primary amines (lysine residues). Assuming that the desired residues are being modified in MHRP and M'HRP, a possible reason for the lower efficiency of M'HRP in the electrochemical assay may be that the carboxyl groups modified in M'HRP are further from the heme than the corresponding lysine residues in MHRP. Unfortunately, the crystal structure of HRP is unknown, and this claim cannot be substantiated. Another possible explanation for the lower efficiency of M'HRP could be that AMF and FCA interact differently with the carbohydrate moiety (HRP is 18% carbohydrate⁵) under the modification conditions. MHRP may have more ferrocene associated with it than M'HRP through this interaction, accounting for the discrepancy in efficiency of the derivatives in the electrochemical assay. The iron content of the HRP derivatives could be determined using atomic absorption spectroscopy, and the results would reveal which contains more iron.

The nature of the modification is uncertain. However, M'HRP seems to be more resistant to dialysis than MHRP since AMF peaks were observed in voltammograms after 21 h dialysis of M'HRP but no FCA peaks were observed after 24 h dialysis of MHRP. This conclusion may not be valid, since solutions of 20 μ M of MHRP vs. 40 μ M M'HRP were used for voltammetry after dialysis. A repeat of the MHRP work at 40 μ M would be necessary to draw a more substantiated conclusion.

Table 5.2 shows that M'HRP, after further purification on DEAE-sepharose, retains some of the AMF that had originally been associated with it, since AMF peaks are observed (Figure 5.4). After purification of M'HRP on CM-sepharose following on DEAE-sepharose, solutions of 40 μ M M'HRP did not show redox activity due to AMF (Table 5.2). It is possible that AMF may have been removed from M'HRP on the cation exchange resin due to the cationic nature of its primary amine side-group. Assuming amide bond formation between AMF and HRP, it is possible that purification on DEAE-sepharose and then on CM-sepharose hydrolyzes the amide bond formed and results in the loss of AMF from M'HRP.

In summary, preliminary investigation shows that M'HRP does not work as efficiently as MHRP in the electrochemical assay for H_2O_2 , and characterization of M'HRP by anion and cation exchange chromatography at ambient pressure and FPLC does not give conclusive evidence for covalent derivatization of HRP.

5.5 REFERENCES

1. Degani, Y., Heller, A., (1987) J. Phys. Chem., 91, 1285-1289.
2. Frew, J., Harmer, M., Hill, A., (1986) J. Electroanal. Chem., 201, 1-10.
3. "Chemistry and Biochemistry of amino acids, peptides and proteins",
(1971) Dekker, New York, chapter 7.
4. Staros, J., Wright, R., Swingle, D., (1986) Anal. Biochem., 156, 220-222.
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6.0 SUMMARY

6.1 Contributions to Knowledge

1- O-Dianisidine yields more sensitive results for detection of small quantities of HRP than ABTS.

2- Mediation of electron transfer by free FCA yields more sensitive results for detection of H_2O_2 in the HRP electrochemical assay than DMAF and potassium ferrocyanide.

3- HRP may be modified with FCA and AMF using sulfo-NHS enhanced carbodiimide-promoted amide bond formation. The resulting modified protein is functional in the HRP electrochemical assay and FCA modified HRP is more efficient for detecting H_2O_2 than AMF modified HRP. The nature of these modifications is uncertain as not all FCA initially associated with HRP after modification remains after purification on DEAE-sepharose in the case of FCA modified HRP. Similarly, not all AMF initially associated with HRP after modification remains after purification on DEAE-sepharose and CM-sepharose.

6.2 Further Experimentation

Repetition of the MHRP purification and subsequent voltammetry at a concentration at least 40 μ M is necessary to verify that all FCA is lost by MHRP by this treatment.

Purification of M'HRP on CM-sepharose immediately after modification would show if the loss of AMF from M'HRP after DEAE-sepharose and CM-sepharose purification is due to the interaction of the cationic side-group of AMF with the cation exchange resin.

Immobilization of MHRP and M'HRP immediately after modification and testing the immobilized derivatives for loss of ferrocene, loss of activity and efficiency in the HRP electrochemical assay would reveal whether or not MHRP and M'HRP would be practical for use in an amperometric biosensor for H_2O_2 .

7 APPENDICES

7.1 Hewlett Packard Interface Language (HPIL) Computer Program Used To Record Assay Data On The HP 8451A Spectrophotometer

The following program was written by Eddy Cheung and modified by Peter Bozel to record absorbance data for the ABTS assay every 10 s for a total of 1 min using HPIL¹. Line 30 sets the wavelength to be monitored (nm) and lines 50 to 80 record the absorbance data. Lines 90 to 110 calculate and print activity as defined by EQ. 2.2. To modify the program for the o-dianisidine assay line 30 becomes LAMBDA 460 and in line 100 the value 8.3 is substituted for 18.6 ($\Delta\epsilon_{\text{dye}}$). Line 81 requests the operator to input a blank, which is the absorbance of the assay solution before addition of H_2O_2 (A_0 in EQ.2.2).

```

5 ! HIT [RUN] AFTER 10SEC OF MIXING REAGENTS
10 ERASE STATUS
20 PRINTER 1
30 LAMBDA 436
40 Y-SCALE
50 MEASURE .1,10,0,60
60 FOR X=0 TO 6
70 IF NMEAS=X THEN 70
80 NEXT X
81 PRINT "INPUT BLANK?"
82 INPUT B
90 FOR I=0 TO 60 STEP 10
100 A=[(VALUE(I)-B)]x[(1.88)(10e6)/18.6]
110 PRINT "ACT(";I;")= ";A
120 NEXT I
130 END

```

7.2 Analogue to Digital Conversion of Current (μ A)
vs. Time (s) Data for the HRP Electrochemical Assay System

The following computer program was written by Peter Bozel. It was written to acquire digitized current vs. time data from a PAR 362 potentiostat. The program stores the digitized data in a two column (current vs. time) ascii file. The program has the ability to plot acquired data before saving. It is written in basic and has been compiled with the Microsoft QuickBASIC compiler. The executable file is called chramp.exe. The program is menu driven and makes use of drivers² for the analogue to digital converter board (RTI-800) which were purchased from Analog Devices. Parameters for the board's operation are user defined in the parameter definition segment (line 510). These parameters control the duration and frequency of data acquisition, as well as other hardware parameters. The direct memory access (DMA) option³ was used to facilitate data acquisition and the CALL COL800 (line 1040) command controlled the board's operation.

```
1  REM          c PETER J. BOZEL          November 1988
100 DEFINT A-Z
105 DIM SCANARR!(1000)
110 DIM REALARR!(1000)
111 DIM Y1!(1000)
112 DIM X2!(1000)
120 DIM LIN(8)
125 LCHAN=15
130 BOARD=1
135 PCHAN=1
140 RANGE=100
142 SCALE=1
145 MULT=3
150 COUNT=1
151 GAIN=1
152 ERSTAT=0
155 TEXT1$=""
160 TEXT2$ = ""
165 KEY OFF
170 SCREEN 1
175 CLS
180 LOCATE 10,2
185 PRINT "DATA ACQUISITION AND PLOTTING PACKAGE"
190 LOCATE 14,6
195 PRINT "FOR CHRONOAMPEROMETRY STUDIES"
200 LOCATE 24,8
205 PRINT "PRESS 'C' TO CONTINUE."
210 A$ = INKEY$
215 IF A$ <> "C" AND A$ <> "c" THEN 210
220 SCREEN 2
225 SCREEN 0
230 CLS
235 LOCATE 10,25
240 PRINT "FUNCTION SELECTION MENU"
```

```

245 PRINT ""
250 PRINT "          Function option codes:"
255 PRINT "          A - Acquire chronoamperometric data."
265 PRINT "          D - Define data acquisition parameters."
270 PRINT "          E - Terminate session."
280 PRINT "          P - Plot chronoamperometric data."
290 PRINT "          S - Store chronoamperometric data on disk."
295 LOCATE 24,1
300 PRINT "          Enter option code:"
305 Q$ = INKEY$
310 IF Q$ = "" GOTO 305
315 Q$ = CHR$(ASC(Q$) AND 95)
320 IF Q$ = "D" THEN GOSUB 500
325 IF Q$ = "A" THEN GOSUB 1000
330 IF Q$ = "P" THEN GOSUB 3000
335 IF Q$ = "S" THEN GOSUB 4000
355 IF Q$ = "E" THEN GOTO 365
360 GOTO 230
365 SCREEN 0
370 CLS
375 END
500 CLS
505 LOCATE 6,26
510 PRINT "PARAMETER DEFINITION SEGMENT"
515 LOCATE 8,1
516 INPUT "          Enter an integer denoting the logical channel for the COLLECT op-
eration. Values may range from 0 to 255:          ",LCHAN
525 INPUT "          Enter the number of the physical analog input channel from which
data will be taken. Values may range from 1 to 8:          ",PCHAN
530 INPUT "          Enter gain. This amplifies low level signals. Values are 1, 10,
100, or 500:          ",GAIN
535 INPUT "          Enter range. Remember that range x mult is the time between succ-
essive conversions. Values are 1, 10, 100, 1000 or 10000:          ",RANGE
540 INPUT "          Enter mult. Legal values are between 3 and 32767:          ",MULT
545 INPUT "          Enter count. This is an integer which specifies the number of in-
dividual readings to be taken. Valid values are between 3 and 32767:          ",COUNT
550 INPUT "          Enter scale of potentiostat in uA. Values are 1, 10, 100, 1000,
or 10000:          ",SCALE
595 CLS
597 LOCATE 18,18
600 PRINT "          Enter two lines of descriptive text:"
610 LINE INPUT "",TEXT1$
615 LINE INPUT "",TEXT2$
620 LOCATE 24,1
625 PRINT "Change parameter definitions [ y/n ]?"
630 A$ = INKEY$
635 IF A$ = "" THEN 630
640 A$ = CHR$(ASC(A$) AND 95)
645 IF A$ = "Y" THEN 500
650 RETURN
1000 SCREEN 0
1005 CLS
1010 LOCATE 10,28
1015 PRINT "Data Acquisition Segment"
1020 CALL INITIALIZE(ERSTAT)
1025 IF ERSTAT=0 GOTO 1040
1030 PRINT "Initialize error at 1020: ",ERSTAT
1035 STOP
1040 CALL COL800(LCHAN,BOARD,PCHAN,RANGE,MULT,COUNT,GAIN,ERSTAT)

```

```

1045 IF ERSTAT=0 GOTO 1060
1050 PRINT "collect setup error at 1040:",ERSTAT
1055 STOP
1060 PRINT "no error on collect setup"
1065 CALL COLLECT(LCHAN,SCANARR!(0),ERSTAT)
1070 IF ERSTAT=0 GOTO 1085
1075 PRINT "collect error at 1065:",ERSTAT
1080 STOP
1085 PRINT "no error on collect"
1090 CALL CHECK(LCHAN,ERSTAT)
1095 IF ERSTAT=0 GOTO 1090
1100 IF ERSTAT=117 GOTO 1115
1105 PRINT "collect error during check at line 1090:",ERSTAT
1110 STOP
1115 PRINT "collect complete"
1120 GOSUB 2000
1125 RETURN
2000 N=0
2005 FOR LOOP=0 TO COUNT-1
2010 SCANARR!(LOOP)=(SCANARR!(LOOP)/(409.6*GAIN))-(5/GAIN)
2015 REALARR!(LOOP)=(SCANARR!(LOOP)*SCALE)
2017 N=N+1
2020 NEXT LOOP
2025 RETURN
3000 CLS
3005 IF N=0 GOTO 3045
3010 LOCATE 10,22
3015 PRINT "ERROR: NO DATA AVAILABLE FOR PLOTTING"
3020 LOCATE 24,1
3025 PRINT "Strike any key to return to main menu."
3030 AS = INKEY$
3035 IF AS = "" THEN 3030
3040 RETURN
3045 LOCATE 10,30
3050 PRINT "DATA PLOTTING SEGMENT"
3055 LOCATE 1,1
3060 NSTEP = 1
3065 NPLOT = 0
3070 FOR LOOP = 0 TO N - 1 STEP NSTEP
3075   Y1!(NPLOT)=REALARR!(LOOP)
3080   NPLOT = NPLOT + 1
3085 NEXT LOOP
3090 YMIN1! = Y1!(0)
3095 YMAX1! = Y1!(0)
3100 FOR LOOP = 0 TO NPLOT - 1
3105   IF Y1!(LOOP) < YMIN1! THEN YMIN1! = Y1!(LOOP)
3110   IF Y1!(LOOP) > YMAX1! THEN YMAX1! = Y1!(LOOP)
3115 NEXT LOOP
3120 YSCALE!=180/(YMAX1!-YMIN1!)
3125 XSCALE!=600/(NPLOT-1)
3130 SCREEN 2
3135 LOCATE 10,1
3140 PRINT "C";CHR$(10);"U";CHR$(10);"R";CHR$(10);"R";CHR$(10);"E";CHR$(10);"N";
CHR$(10);"T"
3145 LOCATE 25,76
3150 PRINT "TIME"
3155 LOCATE 1,1
3160 PSET (20,195)

```

```

3165 DRAW "c1 nu195 nr575"
3170 FOR LOOP = 0 TO NPL0T - 1
3175   PSET ((LOOP * XSCALE!) + 20, (YMAX1! - Y1!(LOOP)) * YSCALE! + 5)
3180 NEXT LOOP
3185 A$ = INKEY$
3190 IF A$ = "" THEN 3185
3195 SCREEN 0
3200 RETURN
4000 CLS
4005 IF N : 0 GOTO 4045
4010 LOCATE 10,22
4015 PRINT "ERROR: NO DATA AVAILABLE FOR STORAGE"
4020 LOCATE 24,1
4025 PRINT "Strike any key to return to main menu."
4030 A$ = INKEY$
4035 IF A$ = "" THEN 4030
4040 RETURN
4045 LOCATE 10,30
4050 PRINT "DATA STORAGE SEGMENT"
4055 LOCATE 12,1
4060 PRINT "Current data file directory:"
4065 LOCATE 14,1
4070 ON ERROR GOTO 4195
4075 FILES "b:*.dat"
4080 ON ERROR GOTO 0
4085 NAM$ = ""
4090 LOCATE 23,1
4095 INPUT "Enter file storage name [ 1-8 characters ]:",NAM$
4100 IF LEN(NAM$) <= 1 AND LEN(NAM$) >= 8 THEN 4150
4105 CLS
4110 LOCATE 10,16
4115 PRINT "ERROR: FILE NAME < 1 CHARACTER OR > 8 CHARACTERS"
4120 LOCATE 24,1
4125 PRINT "Strike any key to continue."
4130 A$ = INKEY$
4135 IF A$ = "" THEN 4130
4140 CLS
4145 GOTO 4045
4150 FILE$="b:"+NAM$+".dat"
4155 X2!(0)=0
4156 FOR LOOP=1 TO COUNT
4157 X2!(LOOP)=X2!(LOOP)+(COUNT*MULT)
4158 NEXT LOOP
4159 OPEN FILE$ FOR OUTPUT AS #1
4160 PRINT #1, CHR$(34)+FILE$+CHR$(34)
4165 FOR I=0 TO N-1
4170 PRINT #1, X2!(I);",",REALARR!(I)
4175 NEXT I
4180 CLOSE #1
4185 RETURN
4195 PRINT "no existing data files":RESUME NEXT

```

7.3 The Amino Acid Content of Isoenzyme C of HRP (Aibra et al.⁴).

Chemical composition of isoenzyme C of horseradish peroxidase			
Residue	number	Residue	number
Lysine	6	Isoleucine	13
Histidine	3	Proline	17
Arginine	21	Serine	25
Aspartic Acid	48	Threonine	25
Glutamic Acid	20	Cystine	8
Glycine	17	Methionine	4
Alanine	23	Phenylalanine	20
Valine	17	Tyrosine	5
Leucine	35	Tryptophan	1

7.4 Program Used to Control Gradient and Sample Loading Using the
LCC-500 Controller and Information on Mono-S Resin.

The following program was written by Peter Bozel in LCC-500 controller language⁵ to load a 100 μ M of native or modified HRP on a mono-s column. It runs 3 ml of 5 mM acetate through the column (1 ml/min) at which time the sample is loaded. After loading, 2 ml of starting buffer are pumped through and then a linear gradient from 5 to 100 mM acetate is run over 15 ml. A total of 20 ml of buffer has run through the column at this point and by trial and it was discovered that almost all HRP eluted from the column by this time. The gradient is then stepped up to 300 mM acetate for 3 ml and then stepped down to 5 mM acetate to prepare for other runs. An excerpt from instructions supplied with the Mono-S column states: "Mono-S is a strong cation exchanger based on a beaded hydrophylic resin with a particle size of 10 μ m. The charged group on the gel is -CH₂-SO₃⁻."

0.00	ML/MIN	1.00
0.00	CM/ML	1.00
0.00	CONC %B	0.0
3.00	VALVE.POS	1.2
3.00	CONC %B	0.0
5.00	VALVE.POS	1.1
5.00	CONC %B	0.0
20.00	CONC %B	35.0
20.00	CONC %B	100
23.00	CONC %B	100
23.00	CONC %B	0.0
26.00	CONC %B	0.0

REFERENCES

- 1 HP 8451A Diode Array Spectrophotometer, Operators Manual, p 8-4, Hewlett Packard Company.
- 2 RTI-800/815 Users Manual, Analog Devices, 1987.
- 3 RTI-800, 802, 815, 817, 820, Software Manual, Analog Devices, 1987.
- 4 Aibara, S., Kobayashi, T., Morita, Y., (1981) J. Biochem., 90, 489-496
- 5 Pharmacia Liquid Chromatography Controller LCC-500 PLUS, Instruction Manual, Chapter 4.