the enthalpy associated with the step observed here is quite large, and, furthermore, the optical rotation indicates the loss of most of the α-helical structure which is taken up by 70% of the chain in the native molecule. Hence, a very significant structural change has been shown to occur cooperatively within a narrow limit.

Because of the uncertainty about the conformation of the product of reversible denaturation of myoglobin, it would appear premature to interpret the measured values of the free energy or the enthalpy in terms of the ums of contributions from local interactions, as suggested by various authors.[7,27,28] However, it is in-

(27) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).
(28) J. Hermans, *J. Phys. Chem.*, **70**, 510 (1966).

teresting to notice that the calculated molar specific heat of denaturation of about 1400 cal/mole degree is qualitatively in agreement with the similarly large values which Brandts had to assume in analyzing the denaturation equilibria of chymotrypsinogen and ribonuclease as two-state equilibria.[6,7] Rather than make the assumption of drastic changes in the structure of the denatured molecule with temperature we also would explain this observation by pointing to the observed large specific heat of solution of hydrocarbons in water, which should correspond to a large specific heat in those molecules in which the hydrocarbon side chains are exposed to the aqueous solvent, *i.e.*, the denatured molecules.[27]

Electron-Transfer Characteristics of the Prosthetic Group of Hemoproteins[1,2]

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Abstract: Diffusion-controlled polarograms corresponding to the ferroheme–ferriheme electron transfer were obtained at the dropping mercury electrode, in buffered aqueous solutions in a range of ionic strengths between 0.1 and 0.2. Experimental conditions were judiciously controlled to eliminate spurious inferences due to electric double layer and interfacial adsorption phenomena. Remarkably, the ferroheme–ferriheme electrode reaction proved to be *a two-electron-transfer process, which proceeded with Nernstian reversibility from pH 7 to 13*. The Fe(III) form of the electroreactive species was invariably dimeric. The electroreactive ferroheme species was monomeric at $7 < \text{pH} < 9$ and at $\text{pH} > 12.5$, and dimeric in the intermediate alkalinity domain. Thus the conceptually simplest possible two-electron transfer, *viz.*, Fe(III) dimer $+ 2e \leftrightarrow$ Fe(II) dimer, prevailed when $10^{-5} < [\text{OH}^-] < 3 \times 10^{-2}$. In contradistinction "dimer–monomer cleavage," *viz.*, Fe(III) dimer $+ 2e \leftrightarrow 2\text{Fe(II)}$ monomers, occurred at both higher and lower alkalinities. Unequivocal assignments are offered identifying ligand groups at five octahedral coordination positions of each of the two iron centers in the electroreactive dimers. The two iron–porphyrin building stones of the dimers were linked *via* the remaining coordination sites. Variations in the nature of this "dimerization bond" (by propionate or water bridging) may account for the significantly different Stokes radii of the ferriheme dimers observed at low and high pH.

E mpirical observations have consistently suggested the prevalence of a systematic parallelism between electron-transfer processes in homogeneous solution (isotopic self-exchange: *e.g.*, $*\text{Fe}^{2+} + \text{Fe}^{3+} = \text{Fe}^{2+} + *\text{Fe}^{3+}$; cross reactions: *e.g.*, $\text{Fe}^{2+} + \text{Ce}^{4+} = \text{Fe}^{3+} + \text{Ce}^{3+}$) and electrode reactions occurring at a heterogeneous phase interface. Recent theoretical developments[3] have rationalized these findings in terms of mathematical expressions which correlate the kinetics of self-exchange and cross and electrode reactions. Thus, the electrochemical characteristics of a given redox couple can indeed provide valid clues on electron donor–acceptor behavior in solutions and *in vivo*. On the basis of these considerations, a reinvestigation of the electrochemical behavior of the ferroheme–ferriheme system was undertaken in these laboratories,

under judiciously controlled "model conditions," which were expected to yield insights into the remarkably paradoxical electron-transfer properties of hemoglobin.[4]

The iron–protoporphyrin system has been previously studied in aqueous solutions by prominent investigators[5–7] using classical potentiometric and spectrophotometric methods; their results lead to a significant controversy concerning the involvement of polymeric electron donor–acceptor species. Similar findings were reported by Brdicka and Wiesner[8] on the basis of a brief polarographic investigation. In contradistinction, recent papers on metal porphyrins in nonaqueous solvents revealed straightforward electrochemical be-

(3) R. A. Marcus, *J. Chem. Phys.*, **43**, 679 (1965).

(4) J. H. Wang in "Oxygenases," O. Hayaishi, Ed., Academic Press Inc., New York, N. Y., 1962, pp 470–516; J. H. Wang, A. Nakahara, and E. B. Fleischer, *J. Am. Chem. Soc.*, **80**, 1109 (1958); J. H. Wang, *ibid.*, **80**, 3168 (1958).
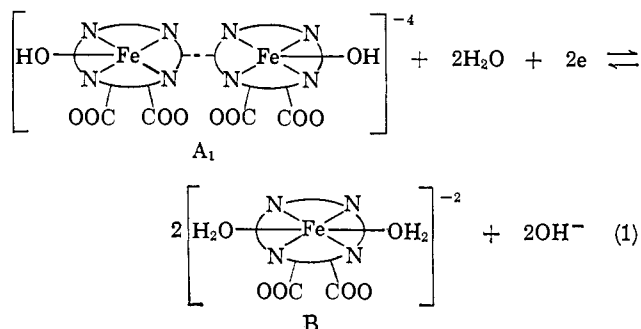(5) J. B. Conant, C. A. Alles, and C. O. Tongberg, *J. Biol. Chem.*, **79**, 89 (1928).
(6) E. S. Barron, *ibid.*, **121**, 285 (1937).
(7) J. Shack and W M. Clark, *ibid.*, **171**, 143 (1947).
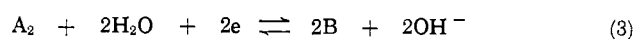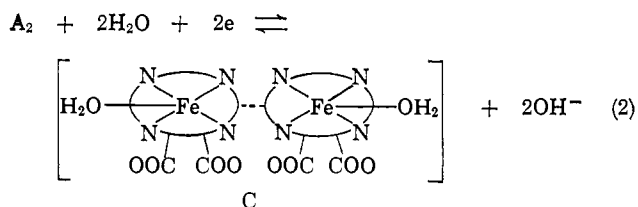(8) R. Brdicka and K. Wiesner, *Collection Czech. Chem. Commun.*, **12**, 39 (1947).

havior patterns.[9-12] Results described in the present article make it evident that the ferroheme–ferriheme electrode reaction can be radically altered by even relatively minor environmental influences (e.g., pH and ionic strength), necessitating careful selection of experimental conditions for the acquisition of meaningful data. The studies reported below were advisedly restricted to an aqueous milieu, on considerations of biological relevance. Extreme care was exercised to arrive at unambiguous conclusions concerning genuinely valid reaction schemes, by taking full account of contemporary knowledge of electric double-layer effects, specific and nonspecific adsorption, etc.[13]

It was reported in a preliminary communication[14] that the electroreduction of ferriheme in aqueous 0.1 M potassium hydroxide proceeded with "Nernstian reversibility" at the dropping mercury electrode (dme), in accordance with the equation

$$\left[HO-\underset{OOC\ \ COO}{(Fe)}\cdots\underset{OOC\ \ COO}{(Fe)}-OH \right]^{-4} + 2H_2O + 2e \rightleftharpoons$$

$$A_1$$

$$2\left[H_2O-\underset{OOC\ \ COO}{(Fe)}-OH_2 \right]^{-2} + 2OH^- \quad (1)$$

$$B$$

where species A_1 denotes a soluble ferriprotoporphyrin dimer, which functioned as a two-electron acceptor yielding the monomeric ferroprotoporphyrin product, B. Equation 1 represents a dimer–monomer reaction which is consistent with the potentiometric results reported by Conant, Alles, and Tongberg,[5] but at variance with findings by Shack and Clark[7] and Barron,[6] who postulated the prevalence of dimer–dimer and monomer–monomer reactions, respectively.

Further investigations have now revealed that under specified experimental conditions reactions 2 and 3 can also take place in aqueous solutions at the dme interface

$$A_2 + 2H_2O + 2e \rightleftharpoons$$

$$\left[H_2O-\underset{OOC\ \ COO}{(Fe)}\cdots\underset{OOC\ \ COO}{(Fe)}-OH_2 \right] + 2OH^- \quad (2)$$

$$C$$

$$A_2 + 2H_2O + 2e \rightleftharpoons 2B + 2OH^- \quad (3)$$

where A_2 denotes a ferriheme dimer, which, however, has a significantly different diffusion coefficient from the electroactive species A_1 involved in eq 1.

The reduction of heme–iron from the trivalent to the divalent state at the dme occurred in a range of po-

tentials between −0.16 and −0.51 v vs. nhe.[15] Well-defined polarographic waves were obtained, whose shape exhibited a remarkable dependence on pH. Dimer–monomer reactions (1 or 3) predominated in two discrete intervals, viz. pH > 12.5 and 7 < pH < 9. In the intermediate range, 9 < pH < 12.5, the dimer–dimer process 2 prevailed.

At considerably more cathodic potentials than reduction of ferriheme, an additional polarographic wave was observed due to the evolution of hydrogen catalyzed by the protoporphyrin ring.

Experimental Procedure

A controlled-potential electronic polarograph described by Kelley,[16] et al., coupled with an X–Y recorder (Moseley Model 7000) was used to determine current–potential curves. A "three-electrode system" consisting of a dme, a saturated calomel reference electrode (sce), and a platinum wire counter electrode was employed throughout this investigation. The rate of polarization was maintained constant at 0.2 v/min. By means of the polarograph's operational amplifier networks the following types of current responses were measured, whenever desired: "instantaneous currents" at a specified time and potential; "maximum currents" corresponding to the maximum size of the dme at a given potential; "average currents" integrated over the lifetime of the drop. Unless otherwise stated, all currents (i) reported in this paper are the maximum currents duly corrected for residual current.

The number of electrons (faradays per mole of electroreduced ferriheme) involved in reactions 1–3 was determined by coulometry at controlled potential. The electrolysis cell consisted of a stirred mercury pool as the cathode and a quiescent mercury pool as the anode, which were separated by porous septa into two discrete compartments. The cathode chamber was also equipped with a dme used to record polarograms while constant potential electrolysis was in progress. The stirred mercury pool cathode was maintained at a constant potential corresponding to the plateau of the relevant polarographic wave. The number of coulombs consumed during the electrolysis was determined by graphical integrations of automatically recorded current–time curves.

The current–potential data on the ferroheme–ferriheme system were obtained in well-buffered aqueous solutions. Because of solubility limitations, the study was limited to alkaline media. The buffer systems employed were[17]

7 < pH < 9	tris(hydroxymethyl)aminomethane (THAM)– hydrochloric acid
9.6 < pH < 11	potassium bicarbonate–potassium hydroxide
9 < pH < 11.5	glycine–potassium hydroxide
pH > 12	potassium hydroxide

Hemin chloride was purchased from Eastman Kodak and Nutritional Biochemical Corp. The commercial material was recrystallized twice from a pyridine–chloroform mixture.[18] Concentrations reported in this paper are consistently expressed in formula weights per liter of solution. Formalities (F) of ferro- and ferriheme species have been reported in terms of monomeric formula weight, regardless of any polymerization which may occur. All other chemicals were reagent grade. Triply distilled water was used for the preparation of solutions.

All experiments were carried out at 25.00 ± 0.05°.

Results

Generally, the electrochemical behavior of the ferro-heme–ferriheme system exhibited two discrete patterns, depending on whether the ionic strength (μ) or the concentration (c^0) of the electroactive species in the

(9) A. Standienda, Naturwiss., 5, 105 (1965).
(10) D. G. Davis and D. J. Orleron, Anal. Chem., 38, 179 (1966); D. G. Davis and R. F. Martin, J. Am. Chem. Soc., 88, 1365 (1966).
(11) D. W. Clack and N. S. Hush, ibid., 87, 4238 (1965).
(12) R. H. Felton and H. Linschitz, ibid., 88, 1113 (1966).
(13) P. Delahay, "Double Layer and Electrode Kinetics," Interscience Division of John Wiley and Sons, Inc., New York, N. Y., 1965.
(14) J. Jordan and T. M. Bednarski, J. Am. Chem. Soc., 86, 5690 (1964).

(15) Throughout this paper, electrode potentials are expressed in accordance with the 1953 Stockholm IUPAC Convention; see J. A. Christiansen, J. Am. Chem. Soc., 82, 5517 (1960).
(16) M. C. Kelley, H. L. Jones, and D. J. Fisher, Anal. Chem., 31, 1475 (1959); 32, 1262 (1960).
(17) R. Bates, "The Determination of pH," John Wiley and Sons, Inc., New York, N. Y., 1964.
(18) H. Fisher, Org. Syn., 21, 53 (1941).

bulk of the solution exceeded 0.2 and 10^{-3} F, respectively. Typical findings corresponding to the two experimental condition domains are summarized below.

Domain A, Diffusion Controlled: $0.1 < \mu < 0.2$, $c^0 < 10^{-3} F$. Sigmoid-shaped cathodic polarograms of ferriheme in the absence of ferroheme in the bulk of the solution were obtained in a pH range between 7 and 13, at voltages consistent with the known equilibrium potentials of the ferroheme–ferriheme couple. However, the analytic geometry of the polarograms exhibited an unusual dependence on pH. At relatively low and relatively high pH, the current–potential curves were of the shape illustrated in a previous paper,[14] i.e., asymmetrical about the inflection point which did not coincide with the half-wave potential; in contradistinction, in an intermediate pH interval, the current–potential curves were symmetrical about the inflection point, which was identical with the half-wave potential. The two types of cathodic polarograms were found to conform to the wave eq 4 and 5 which correspond respectively to dimer–monomer reactions (eq 1 or 3) and a dimer–dimer reaction (eq 2) occurring with Nernstian reversibility and yielding diffusion-controlled

$$E_{\mathrm{dme}} = E^\circ + \frac{\beta RT}{n\mathfrak{F}} \log \frac{f_A D_B}{f_B{}^2 D_A{}^{1/2} K_W{}^2} - \frac{2\beta RT}{n\mathfrak{F}} (\mathrm{pH}) + \frac{\beta RT}{n\mathfrak{F}} \log \frac{(i_d - i)}{i^2} \quad (4)$$

$$E_{\mathrm{dme}} = E^\circ + \frac{\beta RT}{n\mathfrak{F}} \log \frac{f_A{}^2 D_C}{f_C{}^2 D_{A_2} K_W{}^2} - \frac{2\beta RT}{n\mathfrak{F}} (\mathrm{pH}) + \frac{\beta RT}{n\mathfrak{F}} \log \frac{(i_d - i)}{i} \quad (5)$$

currents. In eq 4 and 5, the symbol β ($= 2.303$) is the modulus which converts natural logarithms to their decadic counterparts, E° is the standard potential of the ferro–ferriheme couple, and K_W is the autoprotolysis constant of water. n denotes the number of electrons transferred: $n = 2.0$ if reactions 1–3 indeed prevail. The subscripts refer to the chemical entities identified in eq 1, 2, and 3. The symbol "A" has been used to denote either ferriheme dimer, A_1 or A_2, as applicable. The remaining symbols conform to the notation of Kolthoff and Lingane.[19]

The experimentally observed wave shapes and corresponding pH intervals are summarized in Table I. Significant numerical data are presented in Tables II and III.

Based on the following evidence, it was concluded that the limiting currents obtained under the specified experimental conditions were diffusion controlled.

(a) Diffusion currents, i_d, were proportional to the concentrations, c^0, of ferriheme in the bulk of the solution (Tables II and III, column 2). The quantity i_d/c^0 was constant within $\pm 3\%$.

(b) The ratio of the average to maximum polarographic currents was 0.79 ± 0.01, in satisfactory agreement with the theoretical assignment of 0.81 ± 0.01 for pure diffusion control.

(c) A logarithmic plot of the diffusion current vs.

(19) I. M. Kolthoff and J. J. Lingane, "Polarography," Vol. 1, Interscience Publishers Inc., New York, N. Y., 1952, p 190 ff.

Table I. Synopsis of Polarographic Observations for the Diffusion-Controlled Reduction of Ferriheme in Domain A of Experimental Conditions

pH interval	Analytic geometry	Corresponding electrode process	Applicable wave equation
7–9	Asymmetrical	Fe(III) dimer–Fe(II) monomer	4
9–12.5	Symmetrical	Fe(III) dimer–Fe(II) dimer	5
>12.5	Asymmetrical	Fe(III) dimer–Fe(II) monomer	4

Table II. Numerical Data Obtained under Experimental Conditions where Diffusion-Controlled Dimer–Monomer Electrode Processes[a] Prevailed

c^0, F^b	i_d/c^0, $\mu a/10^{-3}\ F$	No. of electrons involved[c]	Half-wave potential, $E_{1/2}$, v. vs. nhe
THAM–HCl Buffer, pH 8.89, $\mu = 0.1$			
3.083×10^{-4}	1.46	2.2	-0.192
5.582×10^{-4}	1.54	2.1	-0.195
8.000×10^{-4}	1.57	2.1	-0.205
1.156×10^{-3}	1.57	2.0	-0.209
1.499×10^{-3}	1.49	1.9	-0.212
0.1 M KOH buffer, pH 12.86, $\mu = 0.1$			
2.104×10^{-4}	1.71	2.1	-0.473
2.408×10^{-4}	1.82	2.0	-0.476
3.642×10^{-4}	1.82	2.1	-0.480
4.722×10^{-4}	1.70	2.2	-0.483
5.194×10^{-4}	1.73	2.0	-0.488

[a] Reaction 1 or 3, both governed by the wave eq 4. [b] Bulk concentration of electroactive species, expressed in terms of the formula weight of monomeric ferriheme units. [c] Calculated from the slopes of plots of E_{dme} vs. log $i^2/(i_d - i)$ based on eq 4.

Table III. Numerical Data Obtained under Experimental Conditions where Diffusion-Controlled Dimer–Dimer Electrode Process[a] Prevailed

c^0, F^b	i_d/c^0, $\mu a/10^{-3}\ F$	No. of electrons involved[c]	Half-wave potential, $E_{1/2}$, v. vs. nhe
KHCO$_3$–KOH Buffer, pH 9.91, $\mu = 0.18$			
1.499×10^{-4}	1.55	2.2	-0.304
2.519×10^{-4}	1.50	2.0	-0.301
3.802×10^{-4}	1.44	1.9	-0.299
5.776×10^{-4}	1.50	1.9	-0.302
7.534×10^{-4}	1.40	2.0	-0.303

[a] Reaction 2, governed by wave eq 5. [b] Bulk concentration of electroactive species, expressed in terms of the formula weight of monomeric ferriheme units. [c] Calculated from the slopes of plots of E_{dme} vs. log $i/(i_d - i)$, based on eq 5.

the mercury pressure was linear and had a slope of 0.48 ± 0.01 compared to an ideal slope of 0.50.

It is evident from eq 4 and 5 that the half-wave potential should vary linearly with pH regardless of whether a dimer–monomer or dimer–dimer electrode process prevails. Theoretically, the derivative $d(E_{1/2})/d(\mathrm{pH})$ should have a numerical value of 0.060 v/pH unit at 25°. This expected dependence was borne out experimentally, the slope of the relevant lines being (0.061 ± 002) v/pH unit. This indicates that, for each electron transferred, the product of the electrode reaction contained one less hydroxyl ion than the reactant.

The experimentally determined half-wave potentials are summarized in Tables II and III, column 4. The following expression for the half-wave potential of a dimer-monomer reaction is easily obtained from eq 4.

$$E_{1/2} = \text{constant} - \beta RT/2\mathfrak{F} \log c^0 \qquad (6)$$

This predicts $dE_{1/2}/d(\log c^0) = -0.030$ v at 25° which compares with experimental findings of -0.029 ± 0.001 v (Table II, column 4). In contradistinction, the half-wave potential should be independent of the bulk concentration of the electroreducible species for a dimer-dimer reaction (eq 5). This invariance is substantiated accordingly by experimental data listed in Table III, column 4.

The last term in eq 4 and 5 is indicative of the type of electron-transfer process occurring at the dme. For reactions 1 or 3 (dimer-monomer) a plot of the quantity $Q_1 = \log [(i_d - i)/i^2]$ vs. E_{dmc} should be a straight line whose slope is equal to $(\beta RT/n\mathfrak{F} = 0.059/n)$ at 25° if Nernstian reversibility prevails and currents are controlled solely by the diffusion of ferriheme from the bulk of the solution to the dme surface. Similarly, a plot of the quantity $Q_2 = \log [(i_d - i)/i]$ vs. E_{dme} should be a straight line whose slope is equal to $0.059/n$ at 25° under conditions where reaction 2 (dimer-dimer) prevailed at the dme.

In the alkalinity intervals 7 < pH < 9 and pH > 12.5, plots of Q_1 vs. E_{dme} were consistent with a reversible two-electron Fe(III) dimer-Fe(II) monomer process. Assignments for n (the number of electrons transferred) calculated from the pertinent slopes on the basis of eq 4 are listed in Table II, column 3, yielding $n = 2.0 \pm 0.1$. Plots of Q_2 vs. E_{dme} in the same pH regions also produced straight lines, with slopes of 0.042 ± 0.002. In principle this would be compatible with a two-electron transfer "rate controlled" by slow steps other than diffusion. However, this possibility was ruled out because it is in disagreement with the observed shift in half-wave potential in accordance with eq 6. Any one-electron-transfer process, whether reversible or irreversible, could be excluded unambiguously because it requires $dQ_2/dE_{dme} > 0.059$. The far-fetched alternative of spurious slopes "simulated" by extraneous effects (e.g., double layer, adsorption, etc.) was carefully considered and ruled out conclusively by the internal consistency of the results.

In the alkalinity interval 9 < pH < 12.5 the "wave analysis" plots, Q_2 vs. E_{dme}, were linear. Values of n calculated from the pertinent slopes on the basis of eq 5 are listed in Table III, column 3, yielding $n = 2.0 \pm 0.1$. This finding rules out, unequivocally, any single electron transfer, as well as any irreversible two-electron transfer. The prevalence of a Fe(III) dimer-Fe(II) dimer process is thus convincingly demonstrated.

Knowing the rate of flow of the mercury and the drop time at potentials corresponding to the cathodic diffusion currents of ferriheme, diffusion coefficients of the electroactive species in the various buffered media used in this study were calculated from the applicable expanded form of the Ilkovic equation,[20] yielding

$$D_{A_1} = 1.64 \pm 0.09 \times 10^{-6} \text{ cm}^2/\text{sec in } 0.1 \text{ } M \text{ KOH (pH}$$
$$\approx 13) \text{ and } 25° \qquad (7)$$

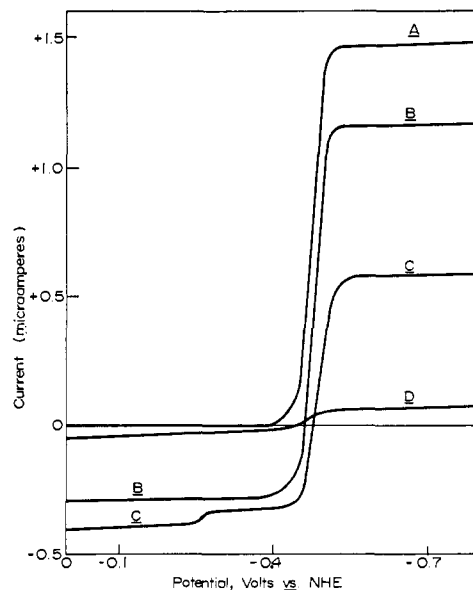(20) I. Kolthoff and K. Izutzu, J. Am. Chem. Soc., 86, 1275 (1964).

Figure 1. Composite current-potential curves in 0.1 M KOH. Polarograms were obtained at various stages during a controlled-potential electrolysis experiment in which ferriheme was converted to ferroheme. Note the normal proportionality of cathodic limiting current to bulk concentration of ferriheme in contrast to the abnormal behavior of the anodic limiting current. For curves A–D, the total electrolysis time (min) and bulk concentrations of ferriheme and ferroheme (milliformal concentrations expressed in terms of the formula weight of monomeric units) are given, respectively: (A) 0, 0.878, 0; (B) 10, 0.673, 0.205; (C) 60, 0.338, 0.440; (D) 120, 0.077, 0.801.

$$D_{A_2} = 1.21 \pm 0.09 \times 10^{-6} \text{ cm}^2/\text{sec at } 7 < \text{pH} < 12.5$$
$$\text{and } 25° \qquad (8)$$

The difference between assignments 7 and 8 is definitely significant. It indicates that two discrete ferriheme dimers were involved in the electrode processes studied in this investigation: A_1 had the smaller Stokes radius and was the electroreducible Fe(III) species in reaction 1; A_2 had the larger Stokes radius and was the electroreducible species in reactions 2 and 3.

However, it was verified by coulometry at controlled potential that 2 faradays was invariably required per mole of ferriheme dimer electroreduced regardless of pH. Extreme care had been taken to ensure the complete absence of oxygen throughout the electrolysis period, because the rapid chemical oxidation of ferroheme by oxygen leads to a regeneration of ferriheme yielding fallacious results.

Considering the "Nernstian reversibility" of the ferroheme-ferriheme electron transfer, anodic polarograms should be obtained in the solutions in which ferroheme has been electrogenerated by controlled-potential electrolysis of ferriheme. A typical family of experimentally determined current-potential curves, which were obtained at various stages during such controlled-potential electrolysis, is shown in Figure 1. These have the general shape of composite waves, involving a cathodic and an anodic segment. The cathodic limiting currents exhibited normal characteristics throughout the controlled-potential electrolysis. They were diffusion controlled and proportional to the concentration of ferriheme remaining in the bulk of the solution. In contradistinction, the anodic seg-
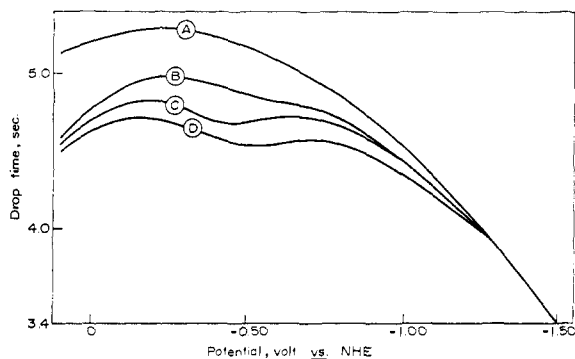
Figure 2. Electrocapillary curves at the dropping mercury electrode, in 0.1 M aqueous potassium hydroxide containing ferriheme. Concentration (F) of ferriheme in bulk of solution: (A) 0, (B) 3.95 $\times$ 10^{-5}, (C) 2.35 $\times$ 10^{-4}, (D) 2.93 $\times$ 10^{-3}.

ment of the current–potential curves exhibited unusual characteristics, viz.:

(a) After short periods of electrolysis ($\sim$10 min, curve B in Figure 1), the anodic limiting current was diffusion controlled.

(b) After longer periods of electrolysis ($\sim$1 hr, curve C in Figure 1), the anodic limiting current did not increase and was not diffusion controlled. This is contrary to normal expectations. A splitting of the wave also occurred.

(c) Finally, approaching exhaustive electrolysis (curve D in Figure 1), the anodic branch of the composite wave decreased and disappeared completely, becoming indistinguishable from the residual current.

This abnormal pattern of behavior of the composite waves exemplified in Figure 1 for a medium containing 0.1 M potassium hydroxide was similarly observed in the entire acidity region studied in this investigation regardless whether the prevailing electrode process was reaction 1, 2, or 3. This parallels the observations noted by Brdicka and Wiesner,[8] who were unable to obtain anodic polarographic waves when a ferriheme solution was chemically reduced to ferroheme with hydrosulfite.

If the normally shaped composite wave (curve B in Figure 1) corresponded to the reversible dimer–monomer electrode reaction (eq 1), which has been substantiated earlier in this study under comparable experimental conditions in the absence of ferroheme, it should conform to a mathematical expression which is the applicable analog of eq 1 at 25°

$$E_{dme} = E° + 0.0295 \log \frac{f_{A_1}D_B}{f_B{}^2 D_{A_1}{}^{1/2}K_W{}^2} - 0.059pH +$$

$$0.0295 \log \frac{(i_{dc} - i)}{(i - i_{da})^2} \quad (9)$$

where i_{dc} and i_{da} refer to the cathodic and anodic diffusion currents, respectively. A wave analysis plot of $Q_3 = \log [(i_{dc} - i)/(i - i_{da})^2]$ vs. E_{dme} constructed from the data obtained from curve B in Figure 1 was linear with a slope of 0.031 v (which compares with a theoretical assignment of $\beta RT/2\mathcal{F} = 0.0295$ v), verifying that the relevant composite wave indeed corresponded to the postulated dimer–monomer electrode process. In contradistinction, similar wave analysis plots for curve C in Figure 1 were nonlinear, indicating that the

electrode process was no longer solely diffusion controlled.

Electrocapillary curves determined in this investigation suggest that the nature of the mercury–solution interface underwent significant changes in the presence of ferro- and ferriheme. Figure 2 demonstrates this behavior in 0.1 M potassium hydroxide in which the bulk concentration of ferriheme was varied, while the bulk concentration of ferroheme was zero. Similar effects were noted under all experimental conditions described previously. A characteristic dip appeared in the electrocapillary curves presented in Figure 2 at approximately -0.47 v vs. nhe. This coincides with the potential where appreciable reduction of ferri- to ferroheme occurred in this medium. Thus the dip is evidently related to a change in the mercury–solution interface caused by the electrogeneration of ferroheme. However, when ferroheme was added to the bulk of the solution in varying concentration (by controlled-potential electrolysis of ferriheme), no further change in the electrocapillary curve was observed.

Domain B, Nondiffusion Controlled: $\mu > 0.2$, $c^0 > 10^{-3}$ F. Under these conditions the polarographic waves of ferriheme contained appreciable kinetic contributions reflecting rate-determining effects of chemical processes preceding the electrode reaction proper. The prevalence of this situation was ascertained from the following observations: (a) the ratio between limiting currents and the concentration of ferriheme in the bulk of the solution was no longer invariant, but decreased with increasing c^0; (b) with increasing ferriheme concentration or ionic strength, the ratio of the average to maximum polarographic currents decreased from the "purely diffusion-controlled value" of 0.80 to the "purely kinetically controlled value" of 0.60; (c) although the limiting current increased with the height of the mercury column, it did so less rapidly than the square-root dependence required by the diffusion equation. These trends were observed in all buffer systems employed in this study, as well as upon enhancement of ionic strength by addition of the following strong electrolytes: potassium chloride, potassium nitrate, sodium fluoride, potassium fluoride, and sodium perchlorate.

Catalytic Hydrogen Wave. In addition to the wave corresponding to the electroreduction of heme–iron from the trivalent to the divalent state (occurring in the potential range -0.16 to -0.51 v vs. nhr), ill-defined cathodic polarograms were invariably observed in the presence of ferriheme in a range of highly negative potentials, viz., -1.4 to -1.5 v vs. nhe. The corresponding wave heights were approximately eight times larger than the ferroheme–ferriheme wave in the same solution. Qualitatively, the anomalous wave height, I, increased with increasing concentration, c^0, of ferriheme in the bulk of the solution. However, the ratio I/c^0 was not constant, but decreased under the following circumstances: (a) increase of c^0; (b) increase of μ, (c) addition of surfactants such as Triton-X and polyacrylamide. Such trends are generally characteristic of catalytic hydrogen waves.[21]

Discussion

In domain A of experimental conditions (i.e., in buffered aqueous solution of ionic strength between

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0.1 and 0.2 and at submillimolar concentration levels of the electroreactive species), the ferroheme-ferriheme electron transfer was diffusion controlled and occurred with Nernstian reversibility at the dropping mercury electrode. This was the case even though both the divalent and trivalent forms of heme-iron were adsorbed at the electrode surface. This is accounted for by the prevalence of comparable adsorption coefficients of, and surface coverages by, the relevant Fe(II) and Fe(III) species. Situations of this type are known to yield ideal polarograms which conform to classical reversible wave equations.[22]

The present polarographic investigation has conclusively confirmed that the ferroheme-ferriheme electrode reaction involved a two-electron transfer in the entire accessible alkalinity range of pH 7 to 13. Under the specified experimental conditions the electroreducible ferriheme species was invariably a dimeric unit with two electron-acceptor centers. Dimeric ferriheme species are known to coordinate two hydroxyl groups.[23] Since the tetradentate porphyrin ligand forms a Fe(III) chelate which is thermodynamically very stable (stability constant $> 10^{29}$), the two building stones of the dimer must necessarily be linked through octahedral positions which are perpendicular to the virtually planar tetrapyrrole network.[24] According to Lemberg and Legge[25] dimerization may occur *via* a double propionate linkage (from one ferriheme unit to the iron center of the other and *vice versa*), while Walter[26] contends that the coupling is through a water bridge between the two iron centers. We postulate that the difference in the Stokes radii (r) of the two discrete dimeric electroreducible ferriheme species identified in the present investigation is accounted for as follows: A_1 (involved in reaction 1, $r = 14$ A) is dimerized *via* the double-propionate-type linkage; A_2 (involved in reactions 2 and 3, $r = 19$ A) is water bridged. We consider that, as an alternative to water, hydroxyl bridging in A_2 is unlikely, because (a) eq 2 suggests quite definitely that the process A_2 + 2e $\leftrightarrow$ C occurred without severance of the dimeric linkage and (b) ferroheme is generally known to coordinate water in preference to hydroxyl;[25,27] such tendency is substantiated by the assignment of water molecules to complete the coordination shells of the iron(II) in species B and C in eq 1 and 2; these assignments are unequivocal in view of the observed pH dependence of the relevant half-wave potentials.

The reduced (ferrous) form of the ferroheme-ferriheme couple in equilibrium with the dme was found to be dimeric in a narrow pH region, *viz.*, 9 < pH < 12.5, and monomeric when 7 < pH < 9 as well as when pH > 12.5. This situation predicates a discontinuous pattern in the nature of the ferroheme-ferriheme elec-

trode process. A dimer-dimer reaction (eq 2) prevailed between pH 9 and 12.5, while dimer-monomer-type cleavages (eq 1 and 3) occurred both at lower and higher alkalinities. This remarkable discontinuity cannot be attributed to a specific "anion effect," because identical results were obtained in different buffer systems of comparable pH. Under no circumstances did a single-electron-transfer process occur. This is perhaps reasonable in view of the fact that the ferriheme dimers have relatively large cross sections (on the order of 100 to 400 A²), which is likely to favor the simultaneous transfer of two electrons. It is interesting to note that the dimer-dimer reaction (eq 2) represents conceptually the simplest and most efficient conceivable two-electron transfer.

The anomalous behavior of the composite current-voltage curves in Figure 1 is accounted for by the aggregation of ferroheme into polymeric species during the controlled-potential electroreduction of ferriheme. These polymers having a relatively smaller diffusion coefficient gave rise to abnormally small diffusion currents. As the bulk concentration of ferroheme was increased in the course of the electrolysis, the aggregation of ferroheme was enhanced to such an extent that the corresponding diffusion current became immeasurably small. The observed Nernstian reversibility of process 1 indicates that the polymerization was not instantaneous. The ferroheme formed by the electroreduction of the ferriheme dimer A_1 must have existed at the mercury-solution interface for an appreciable time as the monomeric unit B before polymerizing. Evidence for the formation of polymeric ferriheme and ferroheme aggregates in solutions slightly different from those studied in the present investigation has been reported in the literature.[7,28]

Our findings in domain B of experimental conditions illustrate the extreme sensitivity of the ferroheme-ferriheme electron transfer to environmental conditions. This was further substantiated by such phenomena as sway-backed waves, etc., prevailing at high ferriheme concentrations in 0.1 *M* potassium hydroxide.[29]

Polymerization phenomena can reasonably account for the "kinetic currents" observed under the specified conditions of domain B. The current measured in such situations was due to the electroreduction of the appropriate ferriheme dimer (A_1 or A_2) generated *in situ* by depolymerization at the electrode surface of higher molecular weight ferriheme aggregates present in the bulk of the solution. Since the cathodic currents exhibited enhanced kinetic character when the bulk concentration of ferriheme and/or the ionic strength was increased, it appears that if the rate-determining chemical reaction was indeed the dissociation of a higher polymeric aggregate into a dimeric ferriheme unit, the rate of the depolymerization decreased with enhanced aggregation.

The reduction wave occurring at quite cathodic potentials, -1.4 v *vs.* nhe, is attributed to the catalytic reduction of hydrogen. This is in agreement with the polarographic study of various porphyrins by Ricci,

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Pinamonti, and Bellavita[30] under experimental conditions similar to those prevailing in the present investigation. Although the specific reference is to hematoporphyrin, these authors reported that all porphyrins studied, which included protoporphyrin, exhibited identical behavior. Thus, it appears from the experimental findings presented here and those of Ricci, *et al.*, that this polarographic wave can indeed be attributed to the reduction of hydrogen catalyzed by the protoporphyrin ring. However, since this is only of peripheral interest to the understanding of the electron-

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transfer properties of the iron porphyrins, the system was not investigated in sufficient detail to elucidate the mechanism of the catalyzed reduction.

As a matter of more central significance it would be interesting to couple two or more heme–iron units by synthetic ligand chains *via* coordination sites perpendicular to the tetrapyrrole plane and determine the effect of this on the electron-transfer properties. Such low molecular weight systems might provide a valid model for conditions prevailing in the hemoproteins; *e.g.*, heme iron units could be maintained at the same distance from each other as in hemoglobin (≈ 25 A) and studied in simple environmental situations in the absence of the macromolecular matrix.

The Rotational Isomerism of Phenylalanine by Nuclear Magnetic Resonance[1a]

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Abstract: The chemical shifts and coupling constants of the aliphatic protons of the phenylalanine anion and dipolar ion and of the glycine and alanine cation, anion, and dipolar ion in aqueous solution have been obtained as a function of temperature over the range 0–100°. The temperature variations of the phenylalanine vicinal coupling constants show anomalous behavior: for the anion, the coupling constants diverge with increasing temperature; for the dipolar ion, the larger coupling is constant over the measured range. Two alternative interpretations are offered: the stability of the most favorable conformer in which the phenyl group is *trans* to the carboxyl group is considerably enhanced with increasing temperature; or, if the rotamer energies are temperature independent, the results suggest that the equilibrium positions deviate significantly from the staggered conformations and that the one which is least favorable from steric considerations is heavily populated and may even be the most stable form.

It has become increasingly apparent that nmr spectroscopy offers a powerful means for elucidating conformational equilibria of molecules in solution. Rotational isomerism about carbon–carbon single bonds has received special attention; studies of fluorine and proton coupling constants and chemical shifts have provided assessments of relative populations of the three classical staggered rotamers of substituted ethanes.[2–10] Coupled with data on the temperature dependence of the spectral parameters, investigations of this kind have yielded accurate information on rotamer

energies[3,6–11] and on potential energy barriers to internal rotation.[7,9,10]

The proton resonance spectra of a number of amino acids in aqueous solution have recently been investigated.[12–16] Conformational equilibria have been deduced from the observed chemical shifts and coupling constants at room temperature, either by assuming values for the H–H coupling in the H–C–C–H fragment for the *gauche* and *trans* conformations[13,15,16] or by assuming a specific dependence of the coupling on the dihedral angle.[14]

In this investigation, the nmr spectra of the aliphatic protons of phenylalanine [$C_6H_5CH_2CH(NH_2)COOH$] in neutral and basic aqueous solution were obtained as a function of temperature over the range 0–100°. Glycine [$CH_2(NH_2)COOH$] and alanine [$CH_3CH(NH_2)-COOH$] were similarly studied in an effort to test the

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