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OXIDATION–REDUCTION POTENTIAL OF THE FERRO–FERRICYANIDE SYSTEM IN BUFFER SOLUTIONS

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SUMMARY

The redox potential (E_0') of the potassium ferrocyanide–potassium ferricyanide oxidation–reduction potential buffer was measured in four pH buffer solutions, acetate, Tris, phosphate, and borate, under specified conditions of pH, solution composition, and temperature. The potentials reported should be accurate, on the hydrogen electrode scale, to within about 2 mV and precise to within at least ± 0.3 mV. Although the potential of the ferro–ferricyanide couple is sensitive to fairly small changes in experimental conditions, several methods are discussed by which this potential can be estimated with a reasonable degree of accuracy (about 2–10 mV) in solutions which differ somewhat from the particular solutions reported here. Attention is called to the need for greater care in specifying experimental conditions in the determination of redox potentials of various biological species.

INTRODUCTION

The ferro–ferricyanide redox couple is a widely used oxidation–reduction potential buffer for the determination of redox potentials of various biological systems. It has been used, for example, in the study of mammalian cytochrome *c* (refs 1 and 2), various bacterial and algal cytochromes of the *c*-type[1–5], cytochrome *f* (ref. 6) and other biological compounds. Some of the advantages of the ferro–ferricyanide system is that it is relatively easy to prepare, its potential is quite stable with time, and its use allows the employment of relatively simple spectrophotometric methods, as opposed to the rather tedious potentiometric titration methods pioneered by Michaelis, Clark, and others in the 1920's and 1930's.

In the course of an investigation concerning the redox potentials of certain bacterial cytochromes of the *c*-type, the purpose of which was to determine if it would be possible to correlate known differences in structure with rather small variations in redox potential, it quickly became evident that a good deal of ambiguity exists in the knowledge of the actual redox potential of the ferro–ferricyanide couple.

Although a good many workers have investigated the redox potential of the ferro–ferricyanide system[7–9], most investigators in the biochemical field make reference to the method of Davenport and Hill[6] which assumes an E_0' for the ferro–ferricyanide system of +0.43 V at 30 °C—taken from the data of Clark *et al.*[8]. Un-

fortunately the oxidation potential of this system is very dependent on the total ionic strength as well as the individual solution components[7,10] increasing from about +0.36 V at zero ionic strength to +0.45 V at about 1 M ionic strength[7]. Clark[10] himself has strongly cautioned against the uncritical use of the value of 0.43 V.

It is the purpose of the present paper, therefore, to present experimental values for the potential of the ferro–ferricyanide system in four commonly used buffers at specified pH values, as well as to offer a rational method for estimating the potential in buffer solutions of slightly different pH and ionic strength.

EXPERIMENTAL

Chemicals

All solutions were prepared with double distilled water. Potassium ferricyanide [$K_3Fe(CN)_6$] and potassium ferrocyanide dihydrate [$K_4Fe(CN)_6 \cdot 2H_2O$] were each recrystallized twice from water; the ferricyanide was air dried and stored over anhydrous $CaCl_2$, and the ferrocyanide was quickly air dried and stored in a tightly sealed bottle. Analysis of the potassium ferrocyanide by permanganate titration[11] indicated a purity of 99.7±0.2%. Stock solutions of ferro- and ferricyanide were prepared fresh daily and protected from light.

Quinhydrone was obtained from Eastman and recrystallized twice from water at 70 °C as recommended by Bates[12].

All buffer solutions were prepared from analytical reagent grade chemicals without further purification. The following buffer systems were used: pH 4.20 and 4.75, acetate ($CH_3COOH + NaOH$); pH 7.00 and 8.00, Tris (trishydroxymethyl-amino)methane + HCl); pH 7.00 and 7.80, phosphate ($KH_2PO_4 + NaOH$); pH 9.00 and 10.00, borate ($H_3BO_4 + NaOH$). The pH values of all solutions were checked with a pH meter and were within 0.03 unit of the nominal value.

Ionic strengths (I) were calculated using the standard expression $I = 1/2 \Sigma C_i Z_i^2$, where C_i is the concentration of a particular ionic species and Z_i is the charge of that species, and assuming no association of charged species. The following pK_a values were used in the calculation of ionic strength values: acetic acid, 4.757; dihydrogen phosphate ion, 6.865; Tris ion, 8.080; boric acid, 9.194. It should be noted that particularly in solutions of high ionic strength and buffer concentrations, where ion pairing and other ionic-association phenomena become increasingly important, the last two digits in the pK_a values are most certainly not significant, but were retained simply for calculation purposes.

Nitrogen, used to deoxygenate test solutions and blanket them during potential measurements, was purified and presaturated with water vapor by bubbling successively through two acidic vanadous solutions[13], saturated calcium hydroxide, and distilled water.

Reference electrode

All potentials were measured *vs* a quinhydrone reference electrode in 0.01 M HCl, 0.09 M KCl, and corrected to potentials *vs* the normal hydrogen electrode. All potentials quoted in this paper are in V *vs* the normal hydrogen electrode. The best value for the standard potential of the quinhydrone electrode is +0.69976 V at 25 °C (ref. 12). To obtain the potential of the particular reference electrode used

0.00020 V is subtracted from the former value due to the "salt error" of the electrolyte solution used[14], and then 0.12411 V is subtracted since the pH of the solution is 2.098 (a National Bureau of Standards secondary pH standard)[15]. Therefore, +0.57545 V is the potential of the particular reference electrode used.

Equipment

All potentials were measured with a Leeds and Northrup 8687 Volt Potentiometer. Test solutions were contained in a water-jacketed three-compartment cell[16]: one end compartment contained the ferro–ferricyanide test solution, the other contained the quinhydrone reference electrode, and the middle compartment contained an appropriate salt solution (usually 0.1 M KCl) which allowed electrical contact between the reference- and test-electrode compartments without appreciable mixing and contamination of the two solutions. Temperature of the entire cell was maintained at 25.00±0.02 °C, unless otherwise stated.

Electrodes were shiny platinum flags, about 1 cm^2 in area.

Procedures

At the start of measurements, quinhydrone was added to the 0.01 M HCl, 0.09 M KCl in one end compartment of the water-jacketed cell, the platinum-flag electrode inserted, and the solution deoxygenated with purified nitrogen for at least 15 min. The electrolyte solution was added to the middle compartment; and the particular ferro–ferricyanide test solution to the other end compartment, the electrode inserted, and the solution deoxygenated. Care was taken to keep the solution levels equal in the three compartments to avoid gross solution transfer.

Potentials of the individual solutions were measured at least three different times on separate days; individual measurements usually agreed within 0.2 mV. Several measurements were made on equimolar mixtures of ferro–ferricyanide without buffer. The values obtained agreed with those reported by Kolthoff and Tomsicek[7] to within about 2 mV; however, 1.36 mV of this is a systematic difference due to their use of 2.075 as the pH of the 0.01 M HCl, 0.09 M KCl solution, rather than the currently accepted N.B.S. value of 2.098.

Throughout this paper the ferro–ferricyanide system studied is an equimolar 10 mM potassium ferrocyanide and potassium ferricyanide (*i.e.* 5 mM of each species), of ionic strength 0.080 M.

RESULTS

The experimental results for the oxidation potential of equimolar (10 mM) ferro–ferricyanide in several common buffer systems is presented in Table I. It can be seen that the redox potential varies from about 0.40 V to almost 0.51 V as the concentration of the buffer system is increased from 0.01 to 0.90 M. Also provided, for comparison purposes, is a value for $E_0' - E_k$, where E_k is the value for the potential of an equimolar ferro–ferricyanide solution whose total concentration is such that the ionic strength of the solution is equal to the total ionic strength of the ferro–ferricyanide buffer system studied*. It can be seen that the measured potentials of

* The potentials reported by Kolthoff and Tomsicek[7] were plotted on a large sheet of graph paper *vs* $I^{\frac{1}{2}}$, and the potential at any given ionic strength was then determined from the resultant E *vs* $I^{\frac{1}{2}}$ plot.

TABLE I

OXIDATION POTENTIALS AT 25 °C FOR EQUIMOLAR (10 mM) POTASSIUM FERRO-FERRICYANIDE AS A FUNCTION OF BUFFER CONCENTRATION

The ionic strength, I, of the solution is the total ionic strength, *i.e.* the calculated ionic strength of the buffer system *plus* 0.080 M due to the ferro–ferricyanide. E_k refers to the potential reported by Kolthoff and Tomsicek[7] for a solution containing only equimolar potassium ferro- and ferricyanide at a total concentration so as to have an ionic strength equal to that reported in the I column.

Buffer concn (M)	*Acetate (pH 4.20)*			*Tris (pH 7.00)*			*Phosphate (pH 7.00)*			*Borate (pH 9.00)*		
	$I^{\frac{1}{2}}$ ($M^{\frac{1}{2}}$)	E_0' (V)	$E_0'-E_k$ (mV)	$I^{\frac{1}{2}}$ ($M^{\frac{1}{2}}$)	E_0' (V)	$E_0'-E_k$ (mV)	$I^{\frac{1}{2}}$ ($M^{\frac{1}{2}}$)	E_0' (V)	$E_0'-E_k$ (mV)	$I^{\frac{1}{2}}$ ($M^{\frac{1}{2}}$)	E_0' (V)	$E_0'-E_k$ (mV)
0.90	0.525	0.4280	3.2	0.954	0.5012	54.9	1.42	0.4559	−8.8	0.656	0.4310	−2.0
0.50	0.434	0.4224	4.2	0.736	0.4798	42.6	1.08	0.4460	−5.2	0.524	0.4246	−0.2
0.20	0.353	0.4157	4.7	0.514	0.4522	28.0	0.716	0.4332	−2.9	0.398	0.4160	1.0
0.10	0.319	0.4120	4.1	0.415	0.4356	19.0	0.544	0.4247	−1.4	0.345	0.4103	0.0
0.05	0.301	0.4098	3.6	0.355	0.4232	12.2	0.434	0.4182	0.1	0.315	0.4075	−0.1
0.02	0.290	0.4074	2.3	0.314	0.4127	5.2	0.351	0.4106	−0.3	0.296	0.4056	−0.1
0.01	0.287	0.4065	1.6	0.299	0.4084	2.4	0.319	0.4075	−0.4	0.290	0.4043	−0.8

the ferro–ferricyanide buffer systems agree reasonably well with those reported for the pure ferro–ferricyanide solutions, particularly at low buffer concentrations. The somewhat larger positive differences in Tris buffer are probably due to a higher degree of association of the Tris cation [$(HOCH_2)_3CNH_3^+$] with the ferrocyanide anion than is the case with K^+ or Na^+.

Due to the nature of the procedures used in this study, the reference electrode, *etc.* it is estimated that the potentials reported in Table I and elsewhere in this paper are accurate to within about 2 mV, with respect to the normal hydrogen electrode. Because of the excellent reproducibility of the results, an individual potential is estimated to be precise within at least ± 0.3 mV.

Although the redox potentials shown in Table I for four particular buffer systems should be useful to other workers, it would be highly desirable to be able to predict the redox potential of the ferro–ferricyanide system under a wide variety of experimental conditions.

Toward this end, further measurements were made on the ferro–ferricyanide system in the same four buffers at different pH values. Table II presents these data, as well as a comparison with the values reported by Kolthoff and Tomsicek[7] (E_k) and with those obtained with the "standard" buffers in Table I. As can be seen, a measured E_0' value is usually fairly close (*i.e.* within about 3 mV) to the other two potential values. Since, as will be discussed later, best results are achieved when the contribution of the buffer to the total ionic strength is fairly small, only 0.05 and 0.10 M buffer systems were studied.

Various types of "correction" procedures were tried in an attempt to correlate measured E_0' values with E_k or E_0' (Table I), by trying to account for changes in potential due to the (known) differences in ionic strengths. Although several such procedures were tried, none offered any significant advantage over an estimation based on E_k values or E_0' values from Table I.

TABLE II

OXIDATION POTENTIALS AT 25 °C FOR EQUIMOLAR (10 mM) POTASSIUM FERRO–FERRICYANIDE IN SEVERAL BUFFER SYSTEMS

E_k and I have the same meaning as in Table I. E_0' (Table I) is the potential of the ferro–ferricyanide couple in the "standard" acetate, Tris, phosphate, or borate buffer at 0.05 or 0.10 M concentration taken from Table I.

Buffer	*Buffer concn* (M)	$I^{\frac{1}{2}}$ ($M^{\frac{1}{2}}$)	E_0' (V)	E_k (V)	E_0' (*Table I*) (V)
Acetate (pH 4.75)	0.05	0.324	0.4103	0.4084	0.4098
	0.10	0.443	0.4142	0.4189	0.4120
Tris (pH 8.00)	0.05	0.321	0.4200	0.4081	0.4232
	0.10	0.354	0.4311	0.4112	0.4356
Phosphate (pH 7.80)	0.05	0.468	0.4192	0.4208	0.4182
	0.10	0.599	0.4263	0.4297	0.4247
Borate (pH 10.00)	0.05	0.351	0.4106	0.4109	0.4075
	0.10	0.408	0.4151	0.4160	0.4103

TABLE III

OXIDATION POTENTIAL FOR EQUIMOLAR (10 mM) POTASSIUM FERRO–FERRICYANIDE AS A FUNCTION OF TEMPERATURE

E (reference electrode) is the value of the quinhydrone 0.01 M HCl, 0.09 M KCl reference electrode used at the temperature indicated. This was calculated using the E_0 values compiled by Bates[12] and assuming that the "salt error" remained constant at −0.20 mV and the pH constant at 2.098. The experimental E_0' values fit the equation $E_0' = 0.4032 - 2.52 \cdot 10^{-3} (T-25) - 6\,54 \cdot 10^{-6} (T-25)^2$ to within 0.2 mV.

T (°C)	E_0' (V)	E (*reference electrode*) (V)
20	0.4157	0.5812
25	0.4031	0.5754
30	0.3907	0.5697
35	0.3773	0.5639
40	0.3641	0.5581

Since it would be of interest to know the potential of the ferro–ferricyanide couple as a function of temperature, for example, many redox potential measurements of biological compounds are made at 30 °C, the data presented in Table III for equimolar (10 mM) ferro–ferricyanide were obtained. To a first approximation, this redox couple has a temperature coefficient of $-2.52 \cdot 10^{-3}$ V/°C; this temperature coefficient can now be used, in conjunction with the data presented in Tables I and II, to estimate the potential of the ferro–ferricyanide redox buffer at temperatures different from 25 °C. For example, the potential in 0.05 M phosphate (pH 7.00) at 30 °C should be 0.4182 V − 5 °C(0.00252 V/°C) = 0.4056 V.

DISCUSSION

In general, the redox potential of the ferro–ferricyanide couple in aqueous buffer solutions is least affected by various experimental variables when the buffer system is at a low concentration, hence, low ionic strength. It is therefore recommended that the ferro–ferricyanide oxidation–reduction buffer be used in low concentration (<0.05 M) buffer systems.

For an investigator who wishes to use the ferro–ferricyanide system in the study of redox potentials of some biological compound, the particular E_0' selected under a given set of experimental conditions must, of necessity, depend on the degree of precision and accuracy desired. If potentials are to be determined to an accuracy better than about 1 mV, it is necessary that the oxidation potential of each particular solution be measured in some appropriate fashion, since it has been shown that the E_0' of this system, calculated using the Nernst equation, depends on the ratio of ferrocyanide:ferricyanide, even when allowance is made for the difference in ionic strength[7]. For example, when the ratio is 1:10, the calculated E_0' is a little higher (about 1–3 mV) than that for the equimolar solution; conversely, when the ratio is 10:1, the calculated E_0' is a few mV lower. It is obviously impractical, however, to measure the redox potential of each and every solution.

If the investigator is satisfied in knowing the potential within a few mV, that is, about $\pm$3–10 mV, the E_0' values in Tables I and II can be used even if the ferrocyanide: ferricyanide ratio differs from 1:1, as is usually the case, as long as the buffer concentration is fairly low (<0.05 M). About the same degree of "error" or uncertainty can be expected in E_0' values estimated from these tables for solutions that differ only a small amount from those listed. For example, the E_0' in 0.02 M acetate buffer (pH 4.50) should not differ significantly from that for 0.02 M acetate (pH 4.20). Furthermore, as long as the buffer concentration is low (<0.05 M), the data of Kolthoff and Tomsicek[7] can be used to obtain a reasonable estimate of the E_0' for ferro–ferricyanide by calculating the total ionic strength of the solution. If it is desirable to use higher concentrations of ferro–ferricyanide, *e.g.* 20 or 50 mM, the data of Kolthoff and Tomsicek[7] should be just as useful. Alternatively a small correction factor can be applied to the potential values in Tables I and II due to the increased ionic strength.

Finally, the investigator can now experimentally determine, with relative ease, the redox potential of ferro–ferricyanide under a particular set of experimental conditions by using one of the accurately determined systems in Tables I and II as a "reference electrode" and measuring the redox potential of the unknown system with a potentiometer or other suitable high impedance device. For example, the redox potential of ferro–ferricyanide in 0.04 M ammonia buffer (pH 9.27) could be measured relative to that of the 0.05 M Tris buffer (pH 8.00) listed in Table II. This eliminates the need for preparing and checking a different reference electrode.

The investigator is cautioned against the uncritical use of the value of 0.43 V for the E_0' of ferro–ferricyanide at 30 °C. From inspection of Table I and a consideration of the temperature dependence of the potential, it would appear that this value is even moderately accurate only at fairly high buffer concentrations (0.1–0.9 M) or high ferro–ferricyanide concentrations (about 0.08 M); at about 0.02 M buffer and 0.01 M ferro–ferricyanide, this value must be too high by some 30 mV or so.

Although it may seem to some that this paper is concerned with apparently small variations in potential, it should be noted that although the redox potentials of various bacterial cytochromes c_2, such as those from *Rhodospirillum rubrum*, *Rhodopseudomonas capsulata*, or *Rhodopseudomonas palustris*, are higher than that of mammalian cytochrome *c* by only about 50–80 mV at pH 7 (refs 1 and 3), these differences in potential reflect appreciable differences in biological function. Particularly with regard to attempts to relate chemical structure to biological function (*i.e.* redox potential), a great deal of care and attention must be paid to the effects of fairly small differences in experimental conditions on the E_0' of the ferro–ferricyanide oxidation reduction potential buffer.

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