Biochimica et Biophysica Acta, 635 (1981) 132—148

BBA 48015

THE THERMODYNAMIC PROPERTIES OF SOME COMMONLY USED OXIDATION-REDUCTION MEDIATORS, INHIBITORS AND DYES, AS DETERMINED BY POLAROGRAPHY

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(Received September 2nd, 1980)

Key words: Polarography; Electrochemistry; Oxidation-reduction reaction; Redox titration; Midpoint potential

Summary

The oxidation-reduction midpoint potentials (E_m) of the following compounds have been measured in the range of pH from 3 to 12 by polarography: methyl viologen; benzyl viologen; 2-hydroxy-1,4-naphthoquinone; 2-hydroxy-1,4-anthraquinone; *N,N,N',N'*,-tetramethyl-*p*-phenylenediamine; 2,3,5,6-tetramethyl-*p*-phenylenediamine; phenazine; *N*-methylphenazonium methosulfate; *N*-methylphenazonium sulfonate methosulfate; *N*-ethylphenazonium ethosulfate; pyocyanine; neutral red; safranin; phenol red; chlorophenol red; cresol red; bromocresol purple; 2,5-dibromo-3-methyl-6-isopropylbenzoquinone and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole. Many of these previously assumed to have a simple behavior in this range have proven to be rather more complicated, and several anomalous observations have been reconciled.

Introduction

Oxidation-reduction reactions underlie many of the reactions involved in the biological manipulation of chemical free energy. In mitochondria, chloroplasts and many bacteria a series of electron (and proton) carriers cooperate to catalyze the oxidation of reduced substrates or photochemically reduced compounds, conserving the free energy in a form which can subsequently be used

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Abbreviations: DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, *N*-methylphenazonium methosulfate; PES, *N*-ethylphenazonium ethosulfate.

for the phosphorylation of ADP. As a first step in the elucidation of the paths of electrons in such systems, electrons are assumed to flow from components with more negative oxidation-reduction (redox) midpoint potentials (E_m) to those with more positive E_m. The E_m is defined as the electrochemical potential of a solution where half of the component of interest is in the oxidized form, and half is reduced; E_m values are usually considered with respect to the standard hydrogen electrode. The most widely used technique for the measurement of E_m values in biological systems is redox potentiometry ([see Refs. 1 and 2]). In this procedure, some property of the biological molecule of interest is monitored as a function of the ambient electrochemical potential, the latter being varied by the addition of small aliquots of chemical reductant or oxidant. The ambient potential is usually monitored with a platinum electrode, and since this does not usually react with biological molecules, so called 'redox mediators' are added to catalyze electrical equilibration. Rapid equilibration clearly requires that both oxidized and reduced form of the mediator be present, as would be found near the E_m of the mediator, and several different mediators are usually employed to allow redox potentiometry over a significant range of ambient potentials. Although a variety of compounds have been used as redox mediators, their equilibrium E_m values have not been studied over the range of pH often employed in biological experimentation. In the experiments reported here we have used polarography to determine the E_m values of the compounds most commonly used as redox mediators over the pH range pH 3 to pH 12. We also report the E_m values of some common inhibitors and pH indicator dyes.

Theoretical considerations, Materials and Methods

For the benefit of the general reader we present a brief description of the techniques we have used. Direct current polarography is an electroanalytical technique where the current flowing at an electrode is measured as a function of potential. In the measurements reported here we have used two different electrodes, a dropping mercury electrode, and one made of glassy carbon. In both cases the electrochemistry occurs at the electrode surface, and there is no stirring to bring the entire sample to equilibrium. The dropping mercury electrode is replaced twice a second, and the voltage is varied at a rate of 2—5 mV/s. A typical determination is shown in Fig. 1, which shows the four modes available with the Princeton Applied Research Polarographic Analyzer. In direct current and sampled direct current polarography the potential is varied in a linear fashion, and the experiment is a steady-state process in that the solution in the vicinity of the electrode is stirred when the mercury drop is knocked off. The succeeding drop thus grows in an essentially identical environment to its predecessor. In direct current polarography the current is measured continuously, and the current oscillates as the mercury drop grows, and is then knocked off. There are two contributions to the current; faradaic current as a result of electron flow to the solution, and capacitance current to charge the electrode. Both vary during the lifetime of the drop, resulting in the 'spikey' trace seen in Fig. 1. For a discussion of the somewhat complicated nature of the oscillations, the reader is referred to the book by Sawyer and Roberts [3]. In current sampled direct current polarography the current is measured for

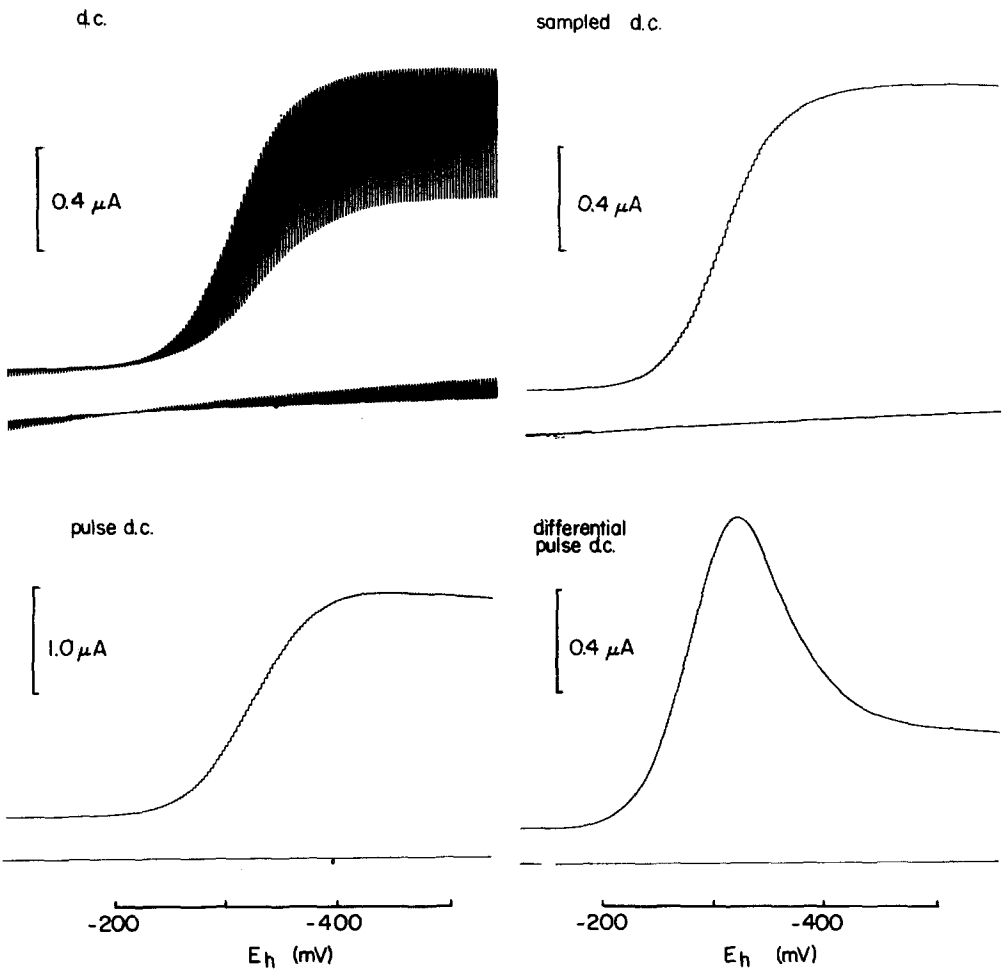

Fig. 1. Direct current (d.c.) polarography using a dropping mercury electrode. 800 μM phenol red was dissolved in the buffer described in the Materials and Methods section at pH 5.3 (4 mM Hepes, Mes, Tricine, glycine, arginine and 100 mM KCl). The four operational modes of the Princeton Applied Research Polarographic Analyzer are shown, as discussed in the text. The scan rate was 5 mV/s. Scale: 2 μA, except for pulse: 5 μA.

only the last few ms of the lifetime of the drop, to minimize the capacitance current and to maximize the faradaic current; as can be seen in Fig. 1, this results in a much clearer trace. Because each new drop of mercury is formed under essentially identical conditions, the limiting current is constant on the plateau of the wave, and does not decrease due to depletion of the reactants at the electrode. The current at this plateau is limited only by the rate of arrival of the reactant, and is diffusion limited. The potential at which the current is one half of the diffusion limited value is the half wave potential ($E_{1/2}$). For a reversible system, the current potential relationship is

$$E_{\mathrm{dme}} = E_{1/2} + \frac{RT}{nF} \ln\left(\frac{(i_{\mathrm{d}} - i)}{i}\right)$$

where R, T, n and F have the usual meanings, i_d is the diffusion limited current and i is the current at a particular potential, E_{dme}. This is, of course, very similar to the familiar Nernst equation:

$$E_h = E_m + \frac{RT}{nF} \ln \frac{[\text{oxidized}]}{[\text{reduced}]}$$

Thus, providing that the oxidized and reduced forms of the component under study both react with the electrode, $E_{1/2}$ is equivalent to E_m (see Ref. 3).

In pulse polarography, a potential pulse is applied to the mercury drop near the end of its life, with the electrode being held at the initial potential for its growth period. The current is sampled only during the potential pulse, and the shape of the resulting polarogram is similar to that of current sampled direct current polarography. This method is more sensitive than direct current polarography, as discussed in Ref. 3. Differential pulse polarography involves the application of a linearly increasing direct current ramp to the electrode, with the superimposition of a fixed pulse at regular intervals. The polarogram displays the difference between the current just before, and at the end of the pulse, and in this case the peak of the polarogram is equivalent to $E_{1/2}$.

In our measurements the electrochemical properties of various molecules were determined in high ionic strength (100 mM KCl), because such conditions are usually used in biological experiments. The dropping mercury electrode cannot be used at potentials much above 0 mV under these conditions, because of the production of calomel at the electrode surface. To measure potentials above 0 mV we have used a glassy carbon or platinum electrode, operating in the direct current mode. These electrodes are not renewed during the experiment, and the polarograms are quite different in appearance from those generated with the dropping mercury electrode. With the glassy carbon or platinum electrodes the reactants next to the electrode are replaced only by diffusion, so maximal current flows only near the midpoint potential of the electroactive components (Fig. 2). The position of the current maximum varies with the rate of voltage variation, but at any given rate the difference between $E_{1/2}$ measured with the glassy carbon or platinum electrode, and with the dropping mercury electrode seems to be a constant (see Figs. 3—8).

We have measured the E_m values of a variety of chemicals as a function of pH, because this reveals information about whether protons are involved in the oxidation-reduction reaction. Clark [1] has discussed such reactions at length; here it suffices to say that when protons are involved in an oxidation-reduction reaction the Nernst equation must be modified appropriately. For example, for the reaction

$$\text{oxidized} + ne^- + mH^+ \rightleftharpoons \text{Reduced } H_m$$

$$E_h = E_m + \frac{RT}{nF} \ln \frac{[\text{oxidized}]\,[H^+]^m}{[\text{reduced } H_m]}$$

At 19° C, this simplifies to

$$E_h = E_m + \frac{58}{n} \log \frac{[\text{oxidized}]}{[\text{reduced}]} - \frac{58}{n} m\Delta\text{pH}$$

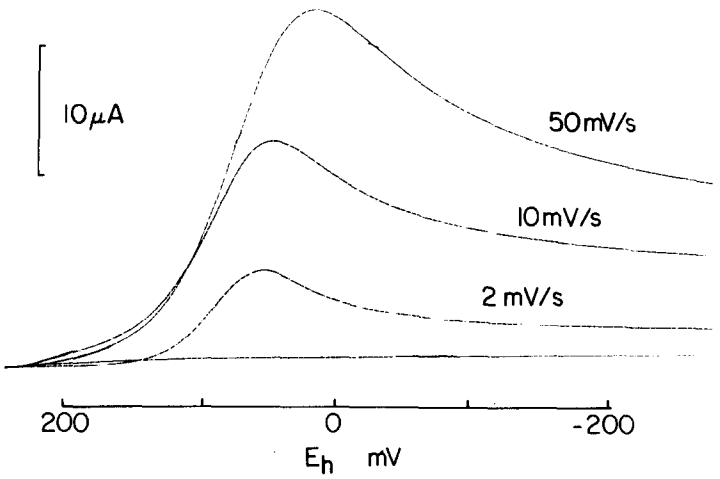

Fig. 2. Direct current polarography using a glassy carbon electrode. 400 μM *N*-methylphenazonium methosulfate was dissolved in the buffer (4 mM Hepes, Mes, Tricine, glycine, arginine and 100 mM KCl) at pH 9.2, and polarograms measured as described in the text.

One can imagine that at sufficiently acid pH the oxidized form may be protonated before the reaction, while at sufficiently alkaline pH the reduced form may no longer require protons. The Nernst equation can be modified to take account of such pK values on the oxidized and reduced species [1]. However, in order to allow a ready estimation of pK values from our data we have drawn straight lines of slopes −116, −87, −58, −29 or 0 mV/pH unit, representing $2H^+/1e^-$, $3H^+/2e^-$, $1H^+/1e^-$, $1H^+/2e^-$ or $0\ H^+/e^-$. The intersects of these lines represent the simplest method for determining pK values, with decreases in slope (e.g. from 0 to −58 mV/pH unit) indicating a pK value on an oxidized form, and increases in slope (e.g. from −58 to −29 mV/pH unit) indicating a pK on a reduced form. Of course the pH dependency of the E_m value can only reveal those pK values which are different on the oxidized and reduced forms. When these are very close together the slope of the E_m/pH unit plot will not change significantly. The reader is referred elsewhere for a more detailed discussion of the influence of protons on the Nernst equation (see Refs. 1 and 2).

Polarograms were measured with a Princeton Applied Research Model 174A Polarographic Analyzer equipped with a model 174/70 mercury drop timer with an 80 cm static head of mercury, a G0021 Glassy Carbon Electrode (28 mm^2 surface area) or a Radiometer P101 platinum electrode (64 mm^2 surface area). The electrochemical cell also contained a platinum third electrode and a saturated calomel electrode as a reference. All redox potentials reported here have been referred to the Hydrogen Electrode by adding 247 mV to the values determined with respect to the saturated calomel standard electrode. The experimental solution was bubbled with argon before each experiment, and argon was blown over the surface of the solution during measurement. All experiments were performed in a buffer containing 4 mM of each of the following: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate, 2-(*N*-morpholino)propanesulfonate, *N*-tris(hydroxymethyl)methylglycine, arginine and glycine,

TABLE I

SOURCES OF THE COMPOUNDS USED IN THIS WORK

Aldrich Chemical Co., Milwaukee, WI 53233, U.S.A.; Sigma Chemical Co., Saint Louis, MO 63178, U.S.A.; K and K Laboratories, Plainview, NY 11803, U.S.A.; Dr. W. Draber, Farber Fabriken Bayer AG, 56 Wuppertal-Elberfeld, F.R.G.; Dr. D.F. Wilson, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.; Dr. N.E. Good, Department of Botany, Michigan State University, E. Lansing, MI 48824, U.S.A.; Hartman-Leddon Co., Philadelphia, PA, U.S.A.; Eastman Kodak, Rochester, NY 14650, U.S.A.; Dr. P. Loach, Department of Biochemistry, Northwestern University, Evanston, IL 60201, U.S.A; Dr. B. Trumpower, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, U.S.A.

Methyl viologen	Aldrich, Sigma
Benzyl viologen	Sigma, K and K
2-Hydroxy-1,4-naphthoquinone	Aldrich
2-Hydroxy-1,4-anthraquinone	Aldrich
N,N,N',N'-Tetramethyl-*p*-phenylenediamine	Aldrich
2,3,5,6-Tetramethyl-*p*-phenylenediamine	Aldrich
Phenazine	K and K
N-Methylphenazonium sulfonate methosulfate	Dr. W. Draber
N-Methylphenazonium methosulfate	Sigma
N-Ethylphenazonium ethosulfate	Sigma
Pyocyanine	Dr. D.F. Wilson, Dr. N.E. Good, K and K, ex. *Ps. aeruginosa*
Neutral red	Sigma
Safranin O	Hartman-Leddon
Phenol red	Eastman Kodak
Chlorophenol red	Eastman Kodak
Cresol red	Eastman Kodak
Bromocresol purple	Eastman Kodak
DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone)	Dr. P. Loach
UHDBT (5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole)	Dr. B. Trumpower

together with 100 mM KCl (ionic strength approx. 120 mM). The pH of the solution was measured with a Corning 109 pH meter equipped with a Corning 476050 combination electrode standarized at pH 4, 7, and 10, and the pH was adjusted with 100 mM KOH or HCl as appropriate. Experiments began at pH 5. The temperature was 19 ± 1°C and the compound of interest was present at between 50 and 800 μM. The chemicals used were of the highest commercially available purity, and the sources are given in Table I.

Results and Discussion

I. Oxidation-reduction mediators

Viologens

The pH dependence of the E_m values of methyl and benzyl viologen are shown in Fig. 3. In both cases the E_m value is independent of pH from pH 3 to pH 12, so no protons are involved in the redox reactions in this range. The values obtained are in excellent agreement with those of Michaelis and Hill [4,5] measured by chemical titration over a smaller pH range, and Ksenzhek et al. measured by thin-layer voltametry [6]. It is noteworthy that while the benzyl derivative shows a very similar E_m when measured by either a dropping mercury or glassy carbon electrode, the methyl derivative exhibits a 25 mV dif-

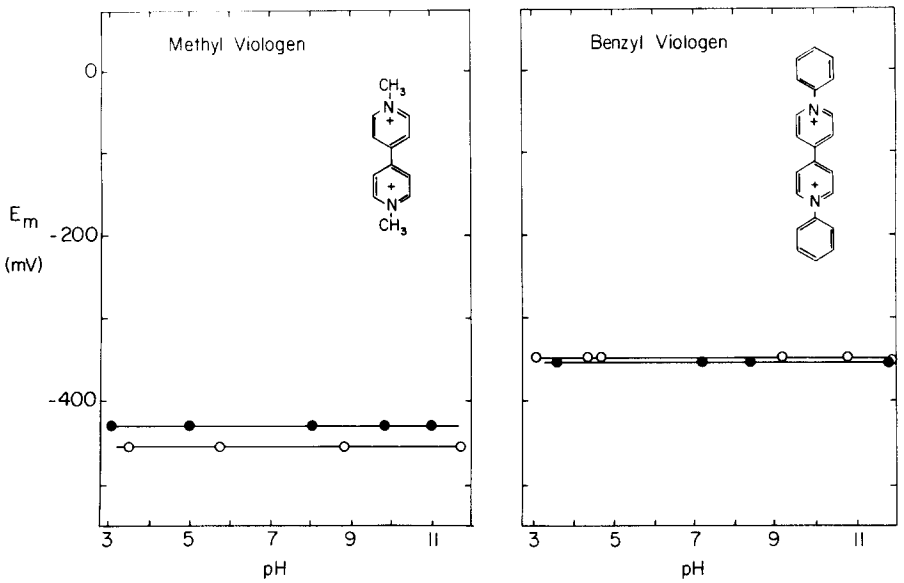

Fig. 3. Viologens. Solid symbols (●) represent data obtained with the dropping mercury electrode, in the sampled direct current mode scanned at 5 mV/s, while open symbols (○) are data obtained with the glassy carbon electrode, scanned at 50 mV/s.

ference; this presumably indicates that the oxidized form of the latter reacts less readily with the glassy carbon electrode. Samples of benzyl viologens from several sources showed a considerable polarographic maximum at −100 mV [3], which is currently unexplained.

Hydroxyquinones

2-Hydroxy-1,4-naphthoquinone has been widely used as a redox mediator, for unlike most quinones it seems to react rapidly with both the platinum electrode and membrane bound redox components [2]; its reactivity may stem from its tautomerization [7]. Fig. 4 shows the variation of E_m with pH for both the 2-hydroxy-1,4-naphthoquinone and 2-hydroxy-1,4-anthraquinone: Both compounds exhibit a pK on a reduced form near pH 8.2. The data for the naphthaquinone derivative obtained with the dropping mercury electrode agree well with those of Fieser and Fieser [7] obtained by redox potentiometry; they titrated over a wide pH range, and found a pK on an oxidized form at a pH of approx. 4.0 and two pK values on reduced forms at pH 8.7 and 10.7. Our titrations did not give symmetrical Nernst curves, which again may be due to tautomerization. Nevertheless two electrons seem to be involved in the oxidation-reduction reactions from pH 4 to 11, and it is noteworthy that three protons are involved between pH 4 and 8, and two between pH 8 and 11.

Phenylenediamines

Two different tetramethyl-*p*-phenylenediamines have been widely used in bioenergetic studies, the *N,N,N′,N′*-tetramethyl isomer (often abbreviated to TMPD) and the 2,3,5,6-tetramethyl isomer, also known as diaminodurene or

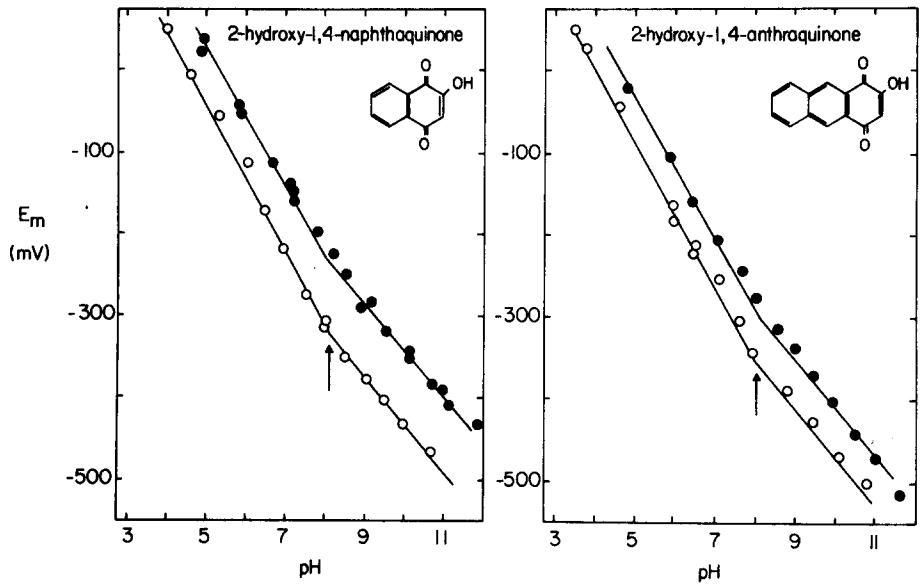

Fig. 4. Hydroxyquinones. Symbols as in Fig. 3.

DAD. TMPD is widely considered to be a one electron, no proton redox agent, with the oxidized form being the well known Wurster's blue. Nevertheless, as originally shown by Michaelis and Hill [5], there is a pK on the reduced form of TMPD close to pH 6.5 (pH 6.6 in Fig. 5, pH 6.3 in Ref. 5) such that at pH values more acid than this a proton is released upon the oxidation of the reduced form. This may explain some recently published observations on the

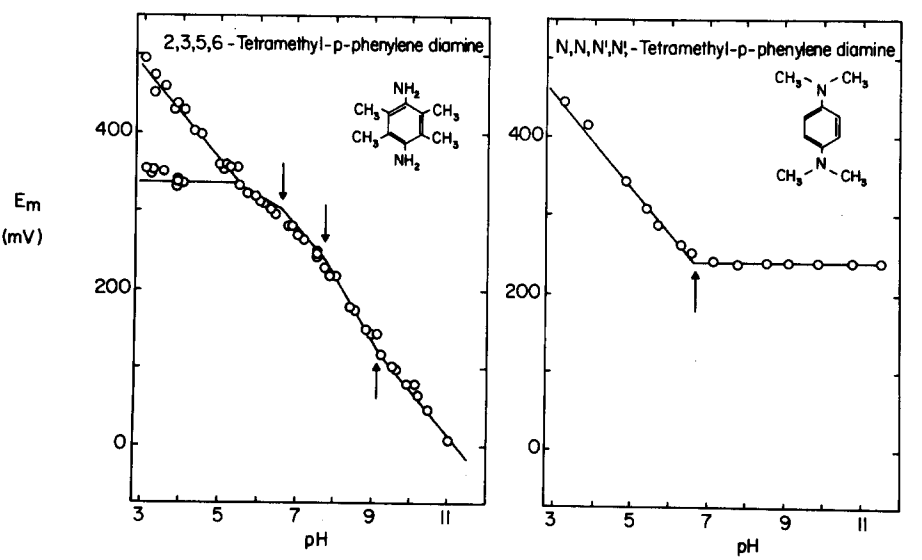

Fig. 5. Tetramethyl-*p*-phenylenediamines. Symbols as in Fig. 3.

redox reactions of TMPD across liposome membranes. Hauska and Prince [8] demonstrated that while TMPD could readily shuttle electrons from external ascorbate to internal potassium ferricyanide, this reaction was not accompanied by the transport of protons. In contrast, Miller et al. [9] did measure the concomitant generation of a pH gradient, inside acid. While both papers reported that the initial pH inside and outside the liposomes was pH 7, the discrepancy between the two reports may be explained if the work of Miller et al. [9] was actually at a slightly more acid pH. Alternatively it is possible that the different lipids used in the liposome preparations had a different selectivity for the permeability of the protonated and unprotonated reduced forms of the mediator: Miller et al. [9] used 20% cardiolipin in their liposomes in addition to egg phosphatidyl choline [8].

The pH dependence of the E_m of 2,3,5,6-tetramethyl-*p*-phenylenediamine is also shown in Fig. 5. This compound exhibits a complex behavior, and three pK values may be interpolated from the data; two pK values on oxidized species, at pH 6.6 and 7.7, and one on a reduced form at pH 9.1. Furthermore, the $n = 2$ reaction at neutral pH can be resolved into two $n = 1$ reactions at acid pH, indicating that at pH values below pH 5.5 the 'semiquinone' form is stable in aqueous solution.

The determination of three pK values is not enough to fully explain the observed E_m/pH dependence, for which five pK values are required. Nevertheless it seems clear that the pK at pH 9.1 is at least a pK on the fully reduced form,

$$H_2NRNH_3^+ \rightleftharpoons H_2NRNH_2 + H^+ \qquad pK\ 9.1$$

where R is the tetramethylated aromatic ring, and the pK values at 6.6 and 7.7 are at least on the fully oxidized form

$$H_2N^+RN^+H_2 \rightleftharpoons HNRN^+H_2 + H^+ \qquad pK\ 6.6$$

$$HNRN^+H_2 \rightleftharpoons HNRNH + H^+ \qquad pK\ 7.7$$

The two 'missing' pK values, on the semiquinone form

$$H_3N^+ \cdot RN^+H_2 \rightleftharpoons H_2N \cdot RN^+H_2 + H^+$$

$$H_2N \cdot RN^+H_2 \rightleftharpoons H_2N \cdot RNH + H^+$$

must therefore lie somewhere between pH 6.6 and 7.7 and 7.7 and 9.1.

Fig. 5 explains some recent observations using 2,3,5,6-tetramethyl-*p*-phenylenediamine as a substrate for mitochondrial oxidations. Wilkström and Krab [10] showed that the number of protons released during the oxidation of the fully reduced form by cytochrome oxidase varied as a function of pH in the neutral range. Because of the complicated E_m/pH profile shown in Fig. 5, it is not surprising that the pK obtained by Wikström and Krab [10] and Mitchell and Moyle [11] (pH 7.5) is somewhat displaced from the pK of 6.6 reported here. Nevertheless, it is clear that these pK values must be considered when the tetramethyl-*p*-phenylenediamines are used in biological experiments.

Phenazines

A variety of phenazine derivatives have been used extensively in biological research. The E_m/pH relationships of *N*-methylphenazonium methosulfate

('phenazine methosulfate', often abbreviated to PMS); its sulfonated derivative, and the ethyl derivative (often abbreviated PES) are shown in Fig. 6. All three show a somewhat complicated behavior; in each case the pH dependency near

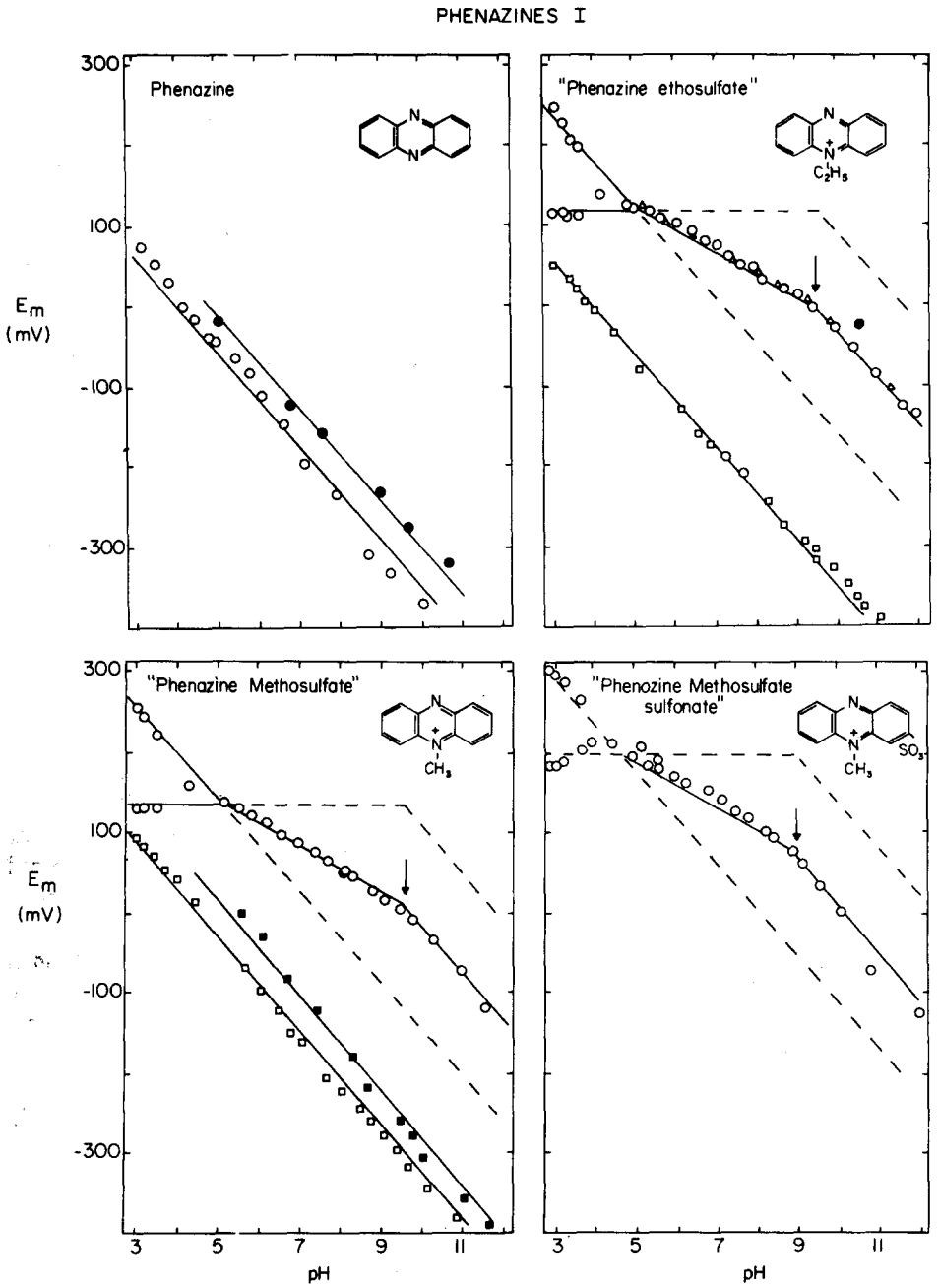

Fig. 6. Phenazines. Data were obtained with the glassy carbon electrode before (○) and after (□) the solutions had been taken to pH 12. Data obtained with a platinum electrode (△) were made before the sample had been taken to pH 12. Measurements with the dropping mercury electrode were made before (●) and after (■) the sample had been taken to pH 12.

neutrality indicates that the redox reactions in this range involve two electrons and a single proton, as has been previously surmized [12,13,6,8,9]. The E_m values of both the methyl and ethyl derivatives agree well with the values obtained by redox potentiometry at pH 7 by Dickens and McIlwain [14].

Fig. 6 shows that the semiquinone forms of the substituted phenazines are stable at pH values less than pH 5, and that there are pK values on oxidized forms of each of the substituted phenazines near pH 9. In fact the dashed lines drawn on Fig. 6 show that the data are in agreement with a model where both the fully oxidized and the semiquinone forms exhibit pK values at the same pH. Our data, and that of Dickens and McIlwain [14], are consistently 75 mV negative of those reported by Ksenzhek et al. [6]. The latter authors did not resolve the stable semiquinone at acid pH or the pK at alkaline pH, but their data are qualitatively similar to ours. We have no explanation for the difference between their work and ours.

Within minutes of sitting at pH 10 or above, a second component of much lower redox potential appeared in the polarograms, and this component did not disappear as the pH was made more acid. As can be seen in Fig. 6, these alkali-induced components have electrochemical properties very similar to those of phenazine itself. This is in concert with the data of McIlwain [15] (see also Ref. 16), who showed that alkaline conditions liberated formaldehyde from 'phenazine methosulfate', yielding phenazine. Such a reaction is readily explained if the proton ionized from the oxidized form with the alkaline pK comes from the aryl substituent.

We have not made precise measurements on the rate of the alkali degradation, but our observations suggest half times of a few minutes at pH 10 for the methyl derivative, and tens of minutes for the ethyl form. It is clear that when phenazine derivaties are used at alkaline pH, this degradation of the oxidized form should not be ignored.

Fig. 7. shows the pH-dependence of the E_m of pyocyanine; our data are in excellent agreement with those of Friedheim and Michaelis [17], suggesting a pK on both the oxidized and semiquinone forms at pH 5.0 and a pK on the reduced from at pH 9.8. It is noteworthy that the pK values on the oxidized and semiquinone forms are shifted dramatically (from pH 9 to pH 5, cf. Figs. 6 and 7) by the addition of the oxygen to 'phenazine methosulfate'. Pyocyanine was available to us in three forms: as the perchlorate (obtained from K and K Laboratories and synthesized by Dr. D.F. Wilson) as the free pyocyanine (synthesized by Dr. N.E. Good), and as the natural product from *Pseudomonas aeruginosa* (prepared in collaboration with Dr. J.D. Friede). All gave similar results, except that the commercially obtained sample contained a low potential contaminant with redox properties similar to those of phenazine (Fig. 6). Just as with the other phenazines, this component appeared in all samples at alkaline pH, and although the data are not shown in Fig. 7, this extra component had redox properties similar to phenazine throughout the pH range tested.

Pyocyanine is no longer available commercially to our knowledge, but it is readily prepared from commercially obtained 'phenazine methosulfate' by illumination in the presence of oxygen followed by extraction from alkaline solution into chloroform [15,16]. Pyocyanine is then returned to water by

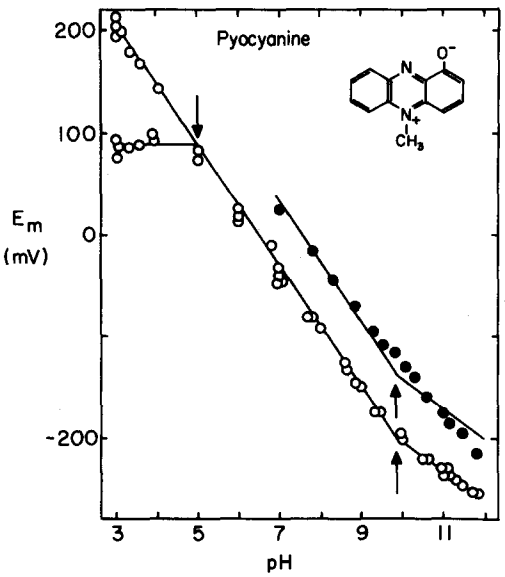

Fig. 7. Pyocyanine. Both natural and synthesized pyocyanine gave similar results; open symbols were obtained with a platinum electrode, solid symbols with the dropping mercury electrode. The low potential contaminant in the commercial sample, and that induced by alkaline treatment of all samples, is not shown. It had properties identical to those of phenazine in Fig. 6.

acidification, and the separation into chloroform may be repeated. Care should be excercised not to make the aqueous solution too alkaline, so as to avoid the generation of the phenazine-like material. Alternatively, pyocyanine is readily obtained from cultures of *Pseudomonas aeruginosa* as described by Wrede and Strack [18], although 2% peptone and 1% glycerol [19] can safely replace the human placenta in their medium! As much as 100 mg of pyocyanine may be produced per litre of culture.

Two other phenazine derivatives are widely used in biological experiments; neutral red (sometimes known as toluylene red) and safranin. The former has been used as both a pH indicator (e.g., see Ref. 20) and a redox mediator (e.g., see Ref. 21), while the latter is a biological stain and has been used as a monitor of transmembrane potentials (e.g., see Ref. 22). The pH dependencies of the E_m values of these two substituted phenazines are shown in Fig. 8.

Our data for neutral red agree closely with those of Clark and Perkins [23], using redox potentiometry. They analyzed their data in terms of three pK values, two on the reduced form, at pH 5.3 and 6.2, and one on the oxidized at pH 6.3. Lacking their confidence in the accuracy of the data, we have analyzed our data in terms of a single pK on the reduced form at pH 4.7. At pH values more acid than this the E_m/pH slope is −87 mV/pH unit, suggesting that three protons and two electrons are involved in the redox reaction. At more alkaline pH the E_m varies by only −58 mV/pH unit, indicating an H^+/e^- ratio of unity. As noted by Clark and Perkins [23] the titrations at alkaline pH can be confused by a further slow irreversible reduction after the initial titrations, leading

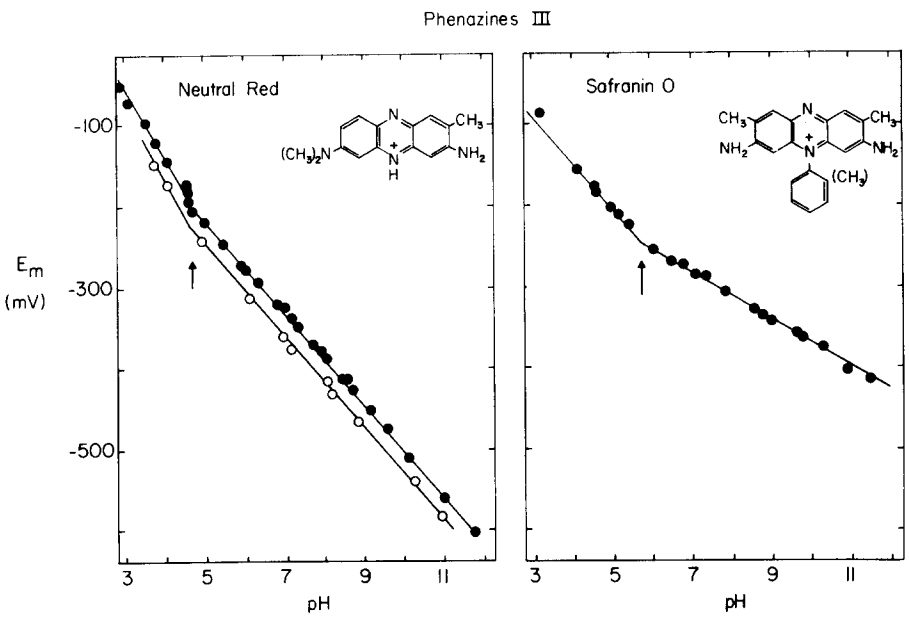

Fig. 8. Neutral red and Safranine O. Symbols as in Fig. 3.

to the formation of a precipitate. This may make neutral red a poor candidate as a redox mediator at alkaline pH.

The pH dependence of the E_m of neutral red shows no inflections near pH 6.6, the pK associated with the color change of the oxidized form of the dye (from red at more acid pH to yellow, see Ref. 24, 20). This indicates that the pK is also present on the colorless reduced form. Nevertheless, the fact that reduction of neutral red yields a colorless form should continue to be borne in mind when the oxidized dye is used as a pH indicator [20].

Safranine is available under a variety of names and in a variety of mixtures and purities. Safranin O, Safranin T, and Rowe 841 are all mixtures of the methylated and unmethylated structures shown in Fig. 8 [23], but all behave as a single component in the polarograms measured here, and in the redox titrations of Stiehler et al. [25]. Our results agree excellently with those of Stiehler et al. [25] indicating a pK on a reduced form at pH 5.8. At pH values more acid than the pK the H^+/e^- stoichiometry of the redox reactions is 1, while at more alkaline pH the stoichiometry becomes one half (i.e., $1H^+/2e^-$). One consequence of this is that Safranin O could be reduced (and bleached) by pyridine nucleotides at neutral pH, and care should be taken to ensure that absorption changes of safranine due to this effect do not interfere with measurements of membrane potentials.

pH indicator dyes

A variety of pH indicator dyes have been added to biological systems in order to allow the rapid measurement of small changes in pH by optical methods. Sulfonphthaleins [26] have proven particularly useful because they do not

bind to biological membranes to any great extent, and their absorption changes seem to reflect pH changes in the external aqueous phase of suspensions of organelles [27,28]. Fig. 9 shows the pH dependency of the E_m values of four of

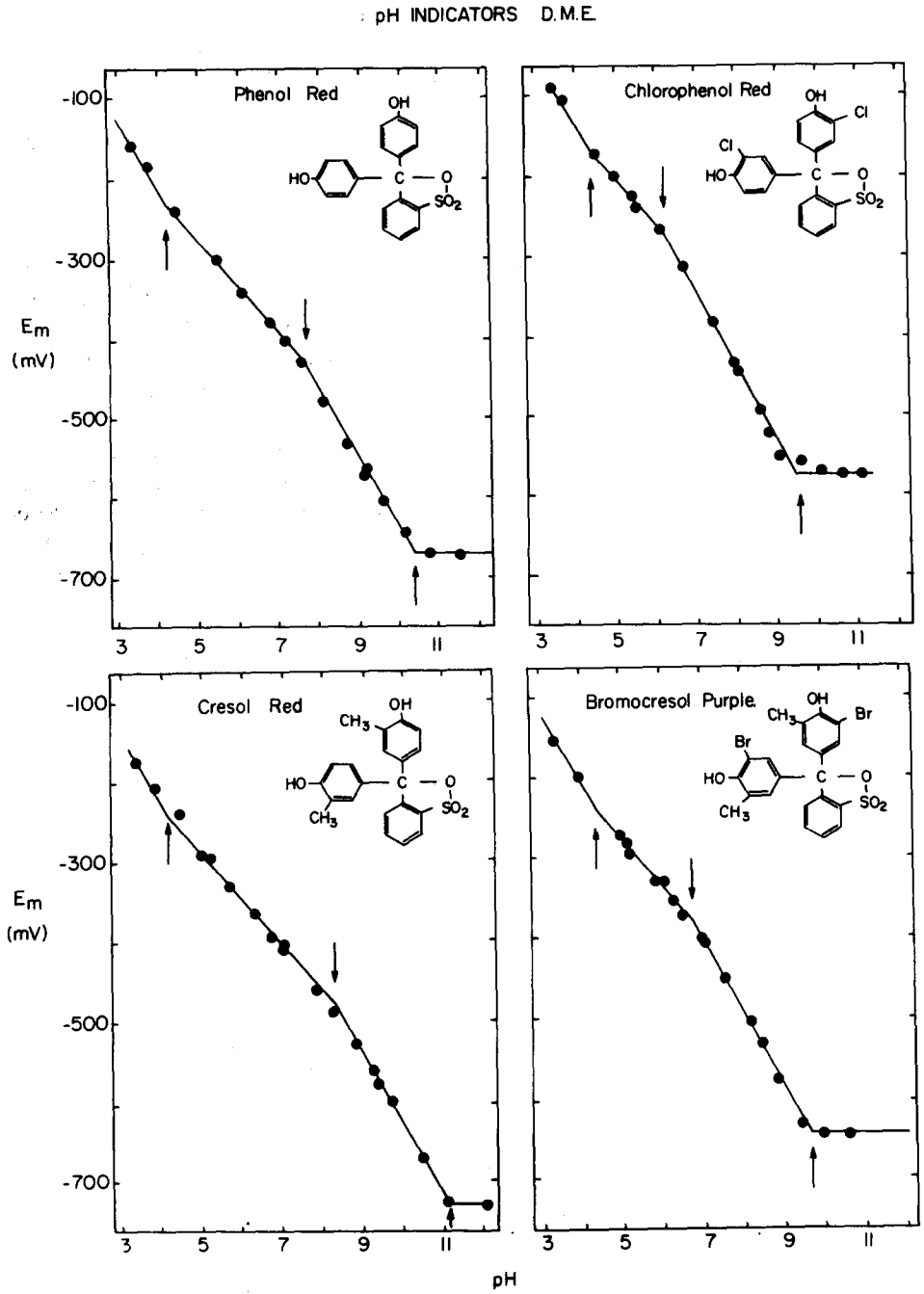

Fig. 9. pH indicators. All data were obtained with the dropping mercury electrode.

the sulfonphthaleins. All four show a pK on an oxidized form in the neutral range, where the E_m/pH dependence changes from −58 mV/pH unit to −87 mV/pH unit. In each case this pK on an oxidized form coincides with the pK responsible for the color change used as the indicator of pH, and is probably on one of the hydroxyls. There are pK values on reduced forms at both more alkaline and more acid pH, again presumably on the hydroxyls. At all pH values the sulfonphthaleins have E_m values very close to that of the hydrogen electrode (which has $E_m = 0 - 58 \cdot$ pH mV), so that their equilibrium reduction is unlikely. Nevertheless, chloroplast Photosystem I would be capable of reducing them, so caution should continue to be exercised in experiments on such systems (e.g., Ref. 28).

Inhibitors

In recent years two quinone derivatives have been widely used in bioenergetic research; DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone) and UHDBT (5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole). The former has been used as an inhibitor in chloroplast studies (eg., see Ref. 29) and the latter seems to bind to the Rieske iron sulfur cluster [30]. Fig. 10 shows the pH dependence of the E_m values of the two compounds. DBMIB is a compound with a relatively high E_m with a pK on a reduced form near pH 5.6. At alkaline pH only a single proton is exchanged in the two electron redox reaction. UHDBT has a lower E_m, and exhibits no significant pK values in the range of pH studied here [31]. Both inhibitors have E_m values in the physiological range at neutral pH, so the redox state of the compound should be considered when they are

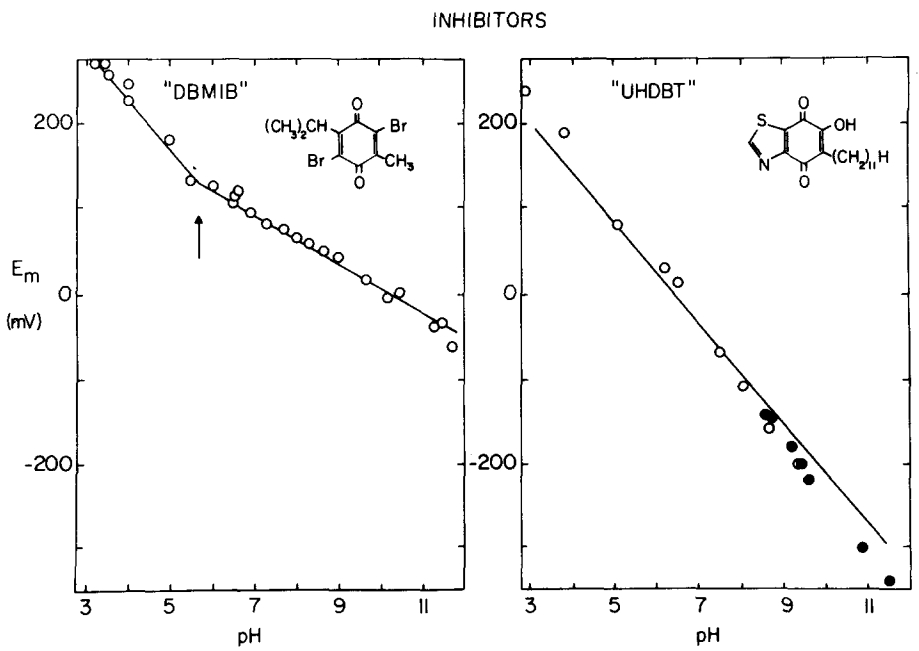

Fig. 10. Inhibitors. Symbols as in Fig. 3. DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

used as inhibitors. For example, UHDBT seems to shift the *g* 1.90 axis of the reduced Rieske cluster of *Rhodopseudomonas sphaeroides* to *g* 1.89 only when the UHDBT is present in the oxidized form [30]; unfortunately the redox properties of the photochemical reaction center preclude an investigation of whether reduced UHDBT is also an inhibitor in *Rps. sphaeroides*.

Conclusions

Table II shows the E_m values of the compounds measured here at pH 7. Most of the values are in good agreement with those quoted by Clark [1] in his classical compendium, but there are some significant differences. In particular the pH dependencies of the E_m values of the various compounds often reveal that a variety of different oxidized and reduced forms may be involved in biological experiments, and some earlier facile interpretations may need re-evaluation in the light of these new findings.

TABLE II

THE $E_{m(7)}$ (mV) OF THE COMPOUNDS MEASURED IN THIS WORK

DME and GCE electrodes refer to dropping mercury and glassy carbon electrodes, respectively. Users of redox mediators should be aware that not all the compounds listed here are good mediators. For example, sulfonated compounds cannot cross lipid membranes (e.g., Ref. 8), and phenazine is not very soluble. Safranin has been used as an indicator of membrane potentials (e.g., Ref. 22) and neutral red as a pH indicator (e.g. Ref. 20). The E_m values are quoted with respect to the Standard Hydrogen Electrode.

	Electrode	
	DME	GCE
Redox mediators		
2,3,5,6-Tetramethyl-*p*-phenylene diamine		275
N,*N*,*N'*,*N'*-Tetramethyl-*p*-phenylene diamine		240
N-Methylphenazonium 3-sulfonate		135
N-Methylphenazonium methosulfate		85
N-Ethylphenazonium ethosulfate		65
Pyocyanine	35	−30
Phenazine	−125	−170
2-Hydroxy-1,4-naphthoquinone	−145	−220
2-Hydroxy-1,4-anthraquinone	−205	−265
Safranin O		−280
Neutral red	−325	−355
Benzyl viologen	−350	−350
Methyl viologen	−430	−455
pH indicators		
Chlorophenol red	−340	
Phenol red	−390	
Cresol red	−405	
Bromocresol purple	−405	
Inhibitors		
DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone)		90
UHDBT (5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole)		−40

Acknowledgements

We are grateful to Peggi Mosley, Tina Davidson and Ken Ray for preparing the manuscript and figures. This work was supported by a grant from the National Science Foundation 79-09042.

References

1 Clark, W.M. (1960) Oxidation-Reduction Potentials of Organic Systems, Williams and Wilkins, Baltimore, MD
2 Dutton, P.L. (1978) Methods Enzymol. 54, 411—435
3 Sawyer, D.T. and Roberts, J.L. (1974) Experimental Electrochemistry for Chemists, Wiley, Chichester
4 Michaelis, L. and Hill, E.S. (1933) J. Am. Chem. Soc. 55, 1481—1494
5 Michaelis, L. and Hill, E.S. (1933) J. Gen. Physiol. 16, 859—872
6 Ksenzhek, O.S., Petrova, S.A. and Kolodyazhny, M.V. (1977) Bioelectrochem. Bioenerg. 4, 346—357
7 Fieser, L.F. and Fieser, M. (1934) J. Am. Chem. Soc. 56, 1565—1578
8 Hauska, G.A. and Prince, R.C. (1974) FEBS Lett. 41 35—39
9 Miller, M., Petersen, L.C., Hansen, F.B. and Nicholls, P. (1979) Biochem. J. 184, 125—131
10 Wikstrom, M.K.F. and Krab, K. (1978) in Frontiers of Biological Energetics, Vol. 1 (Dutton, P.L., Leigh, J.S. and Scarpa, A., eds.), pp. 351—358, Academic Press, New York
11 Mitchell, P. and Moyle, J. (1979) in Cytochrome Oxidase (King, T.E., Orii, Y., Chance, B. and Okunuki, K., eds.), pp. 361—372, Elsevier, Amsterdam
12 Hinkle, P. (1973) Fed. Proc. 32, 1988—1992
13 Deamer, D.W., Prince, R.C. and Crofts, A.R. (1972) Biochim. Biophys. Acta 274, 323—335
14 Dickens, F. and McIlwain, H. (1938) Biochem. J. 32, 1615
15 McIlwain, H. (1937) J. Chem. Soc. 937, 1704—1711
16 Pearson, D.E., Brockman, R.W., Cole, W.E., Greer, C.M. and Sigal, M.V. (1950) in Heterocyclic Compounds (Elderfied, R.C., ed.), Vol. 6, pp. 624—726, Wiley, Chichester
17 Friedheim, E. and Michaelis, L. (1931) J. Biol. Chem. 91, 355—372
18 Wrede, F. and Strack, E. (1924) Hoppe-Seylers Z. Physiol. Chem. 140, 1—15
19 King, E.O., Ward, M.K. and Raney, D.E. (1954) J. Lab. Clin. Invest. 44, 301—307
20 Junge, W., Auslander, W., McGeer, A.J. and Runge, T. (1979) Biochim. Biophys. Acta 546, 121—141
21 Klimov, V.V., Shuvalov, V.A., Krakhmaleva, I.N., Klevanik, A.V. and Krasnovskii, A. (1977) Biokhimija 42, 519—530
22 Akerman, K.E.O. and Wikstrom, M.K.F. (1976) FEBS Lett. 68, 191—197
23 Clark, W.M. and Perkins, M.E. (1932) J. Am. Chem. Soc. 54, 1228—1248
24 Clark, W.M. (1926) International Critical Tables (Washburn, E.W., ed.), Vol. 1, pp. 81—91, National Research Council, McGraw-Hill, New York
25 Stiehler, R.D., Chen, T. and Clark, W.M. (1933) J. Am. Chem. Soc. 55, 891—907
26 Lubs, H.A. and Acree, S.F. (1916) J. Am. Chem. Soc. 38, 2772—2784
27 Petty, K.M. and Dutton, P.L. (1976) Arch. Biochem. Biophys. 172, 335—345
28 Junge, W. and Auslander, W. (1973) Biochim. Biophys. Acta 333, 59—70
29 Trebst, A., Harth, E. and Draber, W. (1970) Z. Naturforsch. 25b, 1157—1163
30 Bowyer, J.R., Dutton, P.L., Prince, R.C. and Crofts, A.R. (1980) Biochim. Biophys. Acta 592, 445—460
31 Trumpower, B.L. and Haggerty, J.G. (1981) J. Bioenerg. Biomemb., in the press