white precipitate which is presumably the difluoride. The liquid is denser than water and reacts slowly with it with the evolution of a small amount of gas, the gas in turn reacting slowly with the water in much the same manner as does the hexafluoride.

The small amount of material obtainable makes a more extensive study of the compound not feasible at this time.

Summary

The vapor pressures, melting points and sublimation points of selenium and tellurium hexafluorides have been measured and determinations of the heats of formation of the hexafluorides of sulfur, selenium and tellurium have been made. The resulting thermochemical constants of the three compounds have been calculated and presented in Table IV.

It has been found that selenium hexafluoride reacts with ammonia gas slowly at 200° and much more rapidly at 330°, to give selenium, nitrogen and hydrogen fluoride. This fact together with the thermal data has been used to show that the inertness of the hexafluorides toward reducing agents is not to be ascribed to a lack of thermodynamic tendency to react.

A lower liquid fluoride of tellurium has been discovered.

PASADENA, CALIFORNIA RECEIVED JULY 25, 1932
PUBLISHED MARCH 7, 1933

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, THE JOHNS HOPKINS UNIVERSITY]

Studies on Oxidation–Reduction. XVIII. Simple Safranines

BY ROBERT D. STIEHLER, TUNG-TOU CHEN AND W. MANSFIELD CLARK

Preliminary measurements in three laboratories[1] have assigned to certain dyes of the azine group potentials which are much lower than those which characterize any indicator system discussed in the first sixteen papers of this series. For this reason azine dyes have been used in several attempts to reveal the extreme reducing ability of cells in anaerobiosis, and they have been considered as possible mediators for the potentiometric study at low potentials of "electromotively inactive" systems. However, the precision of none of the preliminary physical measurements was satisfactory and the conduct of the reductants led Cohen, Chambers and Reznikoff[2] to doubt their usefulness in the biological field. Therefore we have re-examined a few of the simpler safranines.

The potential measurements which we now report are good criteria of the primary reversibility of the oxidation–reduction process. They define

(1) Clark and Zoller, *Science*, **54**, 557 (1921); Vellinger, *Arch. phys. biol.*, **7**, 113 (1929); Rapkine, Struyk and Wurmser, *J. chim. phys.*, **26**, 340 (1929).

(2) Cohen, Chambers and Reznikoff, *J. Gen. Physiol.*, **11**, 585 (1928).

those more important physical characteristics which must be known. But the experiments also emphasize certain peculiarities of the components of the systems which doubtless were responsible for the uncertainties of previous measurements and which will have to be considered in the ordinary uses of these indicators. The experiments also reveal another set of cases in which there appears to be what we call a "concentration effect."

A theoretical interest in this concentration effect makes it advisable to state the nature of certain observations which may cast doubt upon the reliability of the potentials used lest the unannotated exhibit of the more concordant, calculated constants convey the impression that all possible errors have been eliminated with certainty. In the majority of instances we have observed the following. After each successive addition of reducing agent (chromous acetate), the curve relating potential to time seemed to approach an asymptotic limiting potential. After a period, varying from five to thirty minutes or more, the potentials would remain for various times within a few hundredths of a millivolt of the apparent limit. However, in those cases in which observations were prolonged it was observed that the potentials ultimately drifted to more positive values. This indicates that the minimal potentials which we have used are not those which approach an asymptotic limit as an equilibrium point but are minima resulting from the operation of two opposing effects. It is obvious that an appreciable period may be necessary for the completion of the reaction, for tautomeric rearrangements and for adjustment of the electrode; but a turn in the direction of the drift is a cause for suspicion.

One ever-present suspicion is of the presence of oxygen in the nitrogen used to deaerate and to stir the solutions. This nitrogen, originating commercially in the fractionation of liquid air, was passed through a 93 × 2.5 cm. column of copper wire at about 400°. The copper wire, of 0.26 mm. diameter, cut into short lengths, was of a special, pure grade obtained through the courtesy of Mr. Charles H. Davis of The American Brass Company. The copper was repeatedly roasted and reduced in an electric furnace before use. All connections were, of course, metal or glass or, when metal to glass junctions were required, they were heavily coated inside and out with DeKhotinsky cement. Because of the finite equilibrium pressure of oxygen over heated cupric oxide and the accumulation of such oxide at the first end of the tube, it is possible that a trace of oxygen might remain. Not a trace was detected in 60 liters of the nitrogen upon employing a modification of the method of Hand,[3] which we found to give a distinct response to 1.8×10^{-3} mg. of oxygen. We tried the well-known method of spraying a finely divided stream of nitrogen through a filter candle into an oxygen absorbent. We used Fieser's[4] oxygen absorbent and observed no improvement of electrode conduct.

(3) Hand, *J. Chem. Soc.*, **123**, [2] 2573 (1923).
(4) Fieser, THIS JOURNAL, **46**, 2639 (1924).

If the leuco dyes are more efficient absorbents than Hand's modification of Winkler's reagent, there still remains the possibility that oxidation sufficient to affect electrode potentials might have been produced by quantities of oxygen which we failed to detect. Accordingly we constructed a special cell in which all connections were sealed with mercury and in which titrations were conducted under a static layer of the purified nitrogen after a prolonge dperiod of deaeration by the streaming nitrogen. There was no improvement of electrode conduct.

On the other hand, the behavior of the leuco dyes is suggestive. In the first place, treatment of these dyes by hydrogen and any one of several platinum or palladium catalysts produces not only a reversible reduction but a more profound change. For example, phenosafranine, exposed for eighteen hours to hydrogen in the presence of a mild platinum catalyst, suffered nearly complete destruction when buffered at P_H 1 and about 75% destruction at P_H 7. Somewhat less destruction resulted from the use of palladium deposited upon barium sulfate. In such experiments the period of exposure was long for the purpose of emphasis. A solution of the leuco compound, *immediately* after its formation by reduction, was filtered from the catalyst and retained in vacuum. A distinct color change occurred within thirty minutes; the color had deepened[5] by the following morning and when the solution was reoxidized by exposure to air about half of the original dye seemed to have been lost, according to the rather difficult colorimetric comparison between the original solution and this more complex, recovered solution. A considerable number of experiments similar to those cited above, as well as observations of the solutions titrated, have shown that in whatever manner these safranines are reduced their leuco products undergo a noticeable color change when held in buffer solutions of P_H range 3 to 7.5. This is manifested even in the reoxidized solutions by the persistence of a fluorescence different in quality from that of the original dye solutions. This fluorescence is greatly diminished by acidification of the solution.

Now, in their report upon neutral red, Clark and Perkins[6] showed that a "fluorescent material," which was isolated in crystalline form, develops in slightly acid solutions of the leuco compound, is electromotively inactive in neutral and slightly acid solutions, is rendered active by strong acid and is subject to decomposition in aqueous buffer solutions. The drifts of electrode potential were attributed to the formation of this material and to its decomposition.

In several respects the leuco safranines remind us of leuco neutral red. However, the fluorescence which develops is less distinct and a "fluorescent material" has not been isolated. We can say only that where the fluores-

(5) No light effect comparable with that described by Clark, Cohen and Gibbs [*Public Health Reports*, **40**, 1131 (1925)] for methylene white was detected.

(6) Clark and Perkins, THIS JOURNAL, **54**, 1228 (1932).

cence has developed most distinctly, namely, in buffers of P_H range, roughly, 3 to 7.5, the greater difficulties with potential measurements have occurred and the greater discrepancies in the constants calculated from these measurements have been found.

It should be emphasized that, while the foregoing remarks concerning a reversal of potential drift apply generally, the potentials in acid phosphate and acid citrate buffers were remarkably constant over considerable periods and ordinarily would be accepted as highly satisfactory. On the other hand, the attainment of constancy was very slow.

Although we can find no quantitative relation between the shift of E_0 with change of concentration and either the rate of attainment of apparent constancy of potential or the time of reversal, it may be that some kinetic effect of concentration has dominated the situation sufficiently to produce a false appearance of a shifting point of equilibrium. Since there is no evidence for this supposition, we must respect the data as they stand. There then remains the fact that when these data are put through the mathematical mill devised by Reed and Berkson[7] and briefly reviewed in the preceding paper of this series, they yield constants which are the more consistent the more stable the minimal potentials.

A fair idea of the precision attained in any one titration conducted in an unfavorable region of P_H may be obtained by using as typical of the present cases the deviations from average values of E_0' found in Tables I, II and III of the previous paper on neutral red. However, the selection of favorable conditions made it possible to obtain distinctly greater precision with the simple safranines. Indeed, the reader may be surprised to find that we are reporting the fifth decimal in two instances which are typical of several. Neither the reproducibility of separate titrations nor the elimination of errors from the supplementary data which are required for the calculation of certain constants would justify this; but later we shall dwell upon an important point which can be demonstrated by means of the consistency of the values of E_0' for *any one* experiment. This is the reason for showing the extent to which the fourth decimal is "protected" in any one experiment. The apparent precision of the potentiometric readings required a comparable precision of the ratios of the buret readings. Accordingly the micro buret was calibrated with mercury to obtain precise *ratios*. In general it may be said that the magnitude of experimental errors seems to be fairly represented by discrepancies in the calculated constants.

All potential measurements were made at 30°. Previous papers of this series have described the more usual procedures, the conventions, the type of measurements used in correcting potentials for change of P_H during dilution and other details. To the cases under consideration the following equation applies within limitations which will be discussed later.

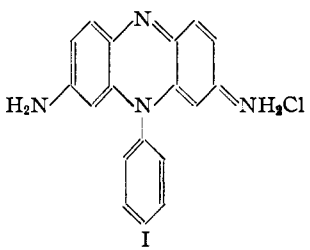
(7) Reed and Berkson, *J. Phys. Chem.*, **33**, 760 (1929).

$$E_h = E_0 + 0.03006 \log \frac{[S_0]}{[S_R]} + 0.03006 \log [(H^+)^3 + K_{r1}(H^+)^2 + K_{r1}K_{r2}(H^+)] \quad (1)$$

Here E_h is the potential in volts, referred to the hydrogen standard; $[S_0]$ is the molar concentration of total oxidant; $[S_R]$ is the molar concentration of total reductant; (H^+) is the hydrion activity; $K_{r_1} = (H^+)(H_2^+Red)/(H_3^+Red^+)$; $K_{r_2} = (H^+)(HRed)/(H_2Red^+)$. At constant hydrion activity the last term of (1) becomes constant. Then at unit ratio of total oxidant to total reductant and specification of the P_H number, we write $E_h = E_0'$. In the tables y indicates the milliliters of reducing agent actually used and d the correction for residual oxidizing impurities estimated by the method of Reed and Berkson.[7]

Experimental

Phenosafranine (Rowe 840).[8] I.—A commercial preparation was recrystallized twice from methanol. The product was dried to constant weight over phosphorus pentoxide. *Anal.* (Kjeldahl). Calcd. for $C_{18}H_{15}N_4Cl$: N, 17.37. Found: N, 17.4, 17.3.



I

In Table I are summarized the results of individual titrations and the calculations therefrom. Here there is evident the effect of the particularly rapid change which takes place in leuco phenosafranine between P_H 3 and 7. Within that range the general tendency of the observed values of E_0' to be positive to our best placement of the calculated values, made after a consideration of the E_0':P_H curve as a whole, probably gives a fair representation of the errors. The concentration effect will be discussed later.

TABLE I

PHENOSAFRANINE. RELATION OF E_0' TO P_H

Determined by individual titrations with chromous acetate at 30°. Values used in calculations: $K_{r1} = 1.12 \times 10^{-5}$ ($pK_{r1} = 4.95$), $K_{r2} = 1.67 \times 10^{-6}$ ($pK_{r2} = 5.78$), $E_0 = 0.2800$ at $-\log C = 4.52$; 0.2790 at $-\log C = 3.52$. C = concentration of total dye at 50% reduction.

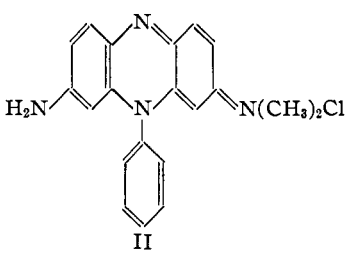
Buffer	P_H	$-\log C$	E_0' found	E_0' calcd.	Found − calcd.
HCl	1.079	4.52	+0.1823	+0.1827	−0.0004
Citrate	2.004	4.52	.0991	.0993	− .0002
Citrate	2.005	3.52	.0982	.0982	.0000
Phosphate	2.124	4.53	.0885	.0885	.0000
Citrate	2.735	3.52	.0323	.0324	− .0001
Citrate	2.735	3.52	.0323	.0324	− .0001
Citrate	3.194	4.52	− .0031	− .0078	+ .0047
Citrate	3.194	4.52	− .0041	− .0078	+ .0037
Citrate	3.195	3.52	− .0058	− .0089	+ .0031
Citrate	3.195	3.52	− .0057	− .0089	+ .0032

(8) Rowe, "Colour Index," Society of Dyers and Colourists, 1924.

TABLE I (*Concluded*)

Buffer	PH	−log C	E_0' found	E_0' calcd.	Found − calcd
Citrate	3.453	4.52	−0.0274	−0.0310	+0.0036
Citrate	3.453	4.52	− .0274	− .0310	+ .0036
Citrate	3.445	3.52	− .0270	− .0313	+ .0043
Acetate	4.076	4.52	− .0862	− .0859	− .0003
Acetate	4.972	4.52	− .1581	− .1580	− .0001
Citrate	5.448	4.52	− .1849	− .1888	+ .0039
Citrate	5.448	4.52	− .1840	− .1888	+ .0048
Citrate	5.449	3.52	− .1839	− .1898	+ .0060
Citrate	5.723	4.52	− .2008	− .2035	+ .0027
Phosphate	6.082	4.52	− .2180	− .2197	+ .0017
Phosphate	7.105	4.52	− .2558	− .2555	− .0003
Borate	8.622	4.52	− .3017	− .3017	.0000
Phosphate	11.097	4.52	− .3760	− .3760	.0000

Dimethyl Phenosafranine (Fuchsia, Rowe 842). II.—For the sample used we are indebted to Dr. Max Phillips. He prepared it according to the method of Bindschedler.[9] The dye was salted out of solution with sodium chloride and crystallized from 95% ethanol. A qualitative test showed that the dye had not been completely freed from salt by this crystallization, a fact which may account for the low nitrogen. *Anal.* (Kjeldahl). Calcd. for $C_{20}H_{19}N_4Cl$: N, 15.98. Found: N, 15.0, 15.1. The potentiometric measurements indicated a homogeneous dye.



II

As in the case of phenosafranine, so in the case of the dimethyl derivative the titrations were satisfactory when conducted with acid phosphate and acid citrate buffers and moderately good when conducted with alkaline solutions. In the intermediate range the results with dimethyl safranine were much better than those with phenosafranine. Table II represents one of the titrations conducted under favorable circumstances.

Upon assembling the values of E_0, calculated from a considerable number of those titrations which were conducted under the more favorable conditions, the discrepancies were judged to be much larger than the recognized experimental errors would allow. There was then applied the empirical equation (2)

$$\tilde{E}_0 = E_0 + 0.0044 \log C \qquad (2)$$

where C is the molar concentration of total dye at 50% reduction. A fairly good resolution of the discrepancies is evident in Table III. An exception is the first recorded case in which we can trace no source of error.

In Table IV are assembled the results of three series of measurements made as follows. To each buffer solution was added an aliquot of the

(9) Bindschedler, *Ber.*, **16**, 869 (1883).

TABLE II

TITRATION OF DIMETHYL PHENOSAFRANINE WITH CHROMOUS ACETATE AT 30°

Approximate composition of buffer: 0.1 *M* citric acid, 0.05 *M* NaOH per liter. Solution titrated: 75 ml. buffer + 5 ml. *buffered*, 0.001 *M* dye. Chromous acetate diluted 1:1 with double strength buffer. Log concn. dye at 50% reduction = −4.22. P_H = 2.960.

y ml. corr. (Buret A)	$y - d$	$\log (y - d)$	$\log (A - y + d)$	$\log \frac{y - d}{A - y + d}$	$\frac{0.030055}{}$	E_h	E_0'	Deviation from av.
0.496	0.316	−0.5003	0.7258	−0.03685		0.05665	(0.01980)	(+0.00013)
0.794	0.614	− .2118	.7008	− .02743		.04709	.01966	− .00001
1.091	0.911	− .0405	.6743	− .02148		.04111	.01963	− .00004
1.387	1.207	+ .0817	.6462	− .01697		.03654	.01957	− .00010
1.685	1.505	.1775	.6160	− .01318		.03280	.01962	− .00005
1.983	1.803	.2560	.5834	− .00984		.02953	.01969	+ .00002
2.287	2.107	.3237	.5475	− .00673		.02640	.01967	.00000
2.589	2.409	.3818	.5087	− .00381		.02353	.01972	+ .00005
2.892	2.712	.4333	.4658	− .00098		.02065	.01965	− .00002
3.194	3.014	.4791	.4185	+ .00182		.01787	.01969	+ .00002
3.496	3.316	.5206	.3653	.00467		.01504	.01971	+ .00004
3.797	3.617	.5583	.3049	.00762		.01208	.01970	+ .00003
4.099	3.919	.5932	.2345	.01078		.00890	.01968	+ .00001
4.397	4.217	.6250	.1517	.01423		.00538	.01961	− .00006
4.702	4.522	.6553	.0465	.01830	+	.00140	.01970	+ .00003
5.001	4.821	.6831	− .0894	.02322	−	.00348	.01972	+ .00005
5.298	5.118	.7091	− .2865	.02992	−	.00995	(.01997)	(+ .00030)
5.596	5.416	.7337	− .6596	.04188	−	.02026	(.02162)	(+ .00195)
A =	5.635					Average =	.01967	
						E_0 =	.28656	

TABLE III

DIMETHYL PHENOSAFRANINE. EFFECT OF CONCENTRATION

Results of individual titrations with chromous acetate. C = molar concn. of dye system at 50% reduction. $\tilde{E}_0 = E_0 + 0.0044 \log C$.

Buffer	P_H	E_0'	E_0	$-\log C$	$\tilde{E}_0$	Deviation from 0.2680
Phosphate	2.123	0.0956	0.2867	4.60	0.2666	−0.0014
Phosphate	2.693	.0405	.2833	3.38	.2684	+ .0004
Citrate	2.960	.0199	.2866	4.22	.2680	.0000
Citrate	3.094	.0064	.2852	3.97	.2677	− .0003
Citrate	3.101	.0039	.2832	3.38	.2683	+ .0003
Citrate	3.495	− .0273	.2873	4.33	.2682	+ .0002
Citrate	3.493	− .0271	.2874	4.32	.2684	+ .0004
Citrate	3.552	− .0355	.2842	3.67	.2681	+ .0001
Citrate	3.549	− .0367	.2827	3.39	.2678	− .0002
Citrate	3.557	− .0375	.2827	3.38	.2678	− .0002
Acetate	4.621	− .1262	.2849	3.79	.2682	+ .0002
Acetate	4.627	− .1283	.2833	3.38	.2684	+ .0004
Citrate	6.209	− .2302	.2827	3.38	.2678	− .0002

dye solution. After deaeration, one aliquot of a chromous acetate solution was added to the mixture and the minimal potential was recorded. This method permits no correction for the variable quantities of oxidizing impurities in the buffer solutions and consequently some irregularities are to be expected. However, it is only in a few cases that these are larger than the irregularities experienced with the more detailed method and since

the E_0':PH curve is well defined for acid regions by the comparable data of Table III, the more refined method was not applied extensively to neutral solutions.

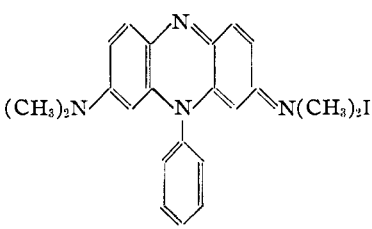
TABLE IV

DIMETHYL PHENOSAFRANINE. RELATION OF E_0' TO PH AT 30°

Determined by addition of one aliquot of chromous acetate solution to each mixture of dye and buffer. Subsequent orientation at 50% reduction by starred values obtained from Table III. Log C at 50% reduction, −3.66. Values used in calculations: $K_1 = 1.26 \times 10^{-5}$ ($pK_1 = 4.90$), $K_2 = 4.68 \times 10^{-7}$ ($pK_2 = 6.33$), $E_0 = 0.2841$.

Series	Buffer	PH	E_0' obs.	E_0' calcd.	E_0' obs. − E_0' calcd.
I	HCl	1.114	0.1837	0.1837	0.0000
	Phosphate	2.696	.0417	.0411	+ .0006
	Citrate	3.096	.0065	.0052	+ .0013
	Citrate	3.552	− .0356*	− .0356	.0000
	Acetate	4.607	− .1263	− .1258	− .0005
	Phosphate	6.847	− .2504	− .2557	+ .0053
	Borate	8.597	− .3119	− .3117	− .0002
II	Citrate	3.552	− .0356*	− .0356	.0000
	Acetate	4.996	− .1561	− .1555	− .0006
	Citrate	5.365	− .1785	− .1808	+ .0023
	Citrate	5.794	− .2066	− .2069	+ .0003
	Citrate	6.217	− .2298	− .2291	− .0007
	Phosphate	7.222	− .2689	− .2589	.0000
	Borate	7.960	− .2940	− .2925	− .0015
	NaOH + KCl	12.304	− .4225	− .4233	+ .0008
III	Citrate	3.552	− .0356*	− .0356	.0000
	Acetate	4.236	− .0951	− .0953	+ .0002
	Citrate	5.794	− .2064	− .2069	+ .0005
	Borate	8.185	− .3004	− .2992	− .0012
	Borate	8.597	− .3127	− .3117	− .0010
	Borate	9.704	− .3449	− .3451	+ .0002
	Phosphate	11.296	− .3909	− .3929	+ .0020
	NaOH + KCl	12.309	− .4226	− .4234	+ .0008

Tetramethyl Phenosafranine (Iodide). III.—After having made several titrations of a sample of the chloride and having found the slopes of the titration curves to be suggestive of a mixture of dyes, we appealed to Dr. Leslie Hellerman for a satisfactory preparation. He followed in the main the method of Bindschedler[10] with care for the purity of the reagents used in making Bindschedler's green, regard for the ease with which this intermediate is hydrolyzed (see Phillips, Clark and Cohen)[11] and the cautious oxidation of the mixture of Bindschedler's green and aniline to the safranine. The crude chloride contained considerable impurity and was



III

(10) Bindschedler, *Ber.*, **16**, 867 (1883); *cf.* Nietzke, *ibid.*, **16**, 472 (1883).

(11) Phillips, Clark and Cohen, *Public Health Reports*, Supplement 61 (1927).

very resistant to crystallization. The iodide was prepared as follows. To a dilute aqueous solution of the crude material an excess of potassium iodide was added. The small crystals which formed were separated by filtration, thoroughly washed with water and recrystallized twice from ethanol. The substance was dried to constant weight at 110° over phosphorus pentoxide. *Anal.* (Kjeldahl). Calcd. for $C_{22}H_{23}N_4I$: N, 11.92. Found: N, 11.8, 11.6. This material gave titration curves as of a homogeneous dye. Table V summarizes data sufficient to define the E_0':P_H curve.

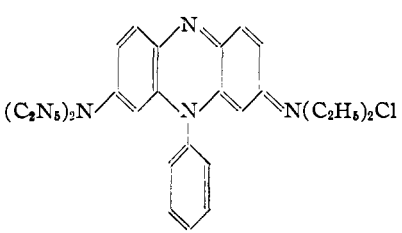
TABLE V

TETRAMETHYL PHENOSAFRANINE (IODIDE) RELATION OF E_0' TO P_H

Determined by individual titrations with chromous acetate at 30°. Values used in calculations: $K_{r1} = 4.79 \times 10^{-6}$ ($pK_{r1} = 5.32$), $K_{r2} = 3.55 \times 10^{-7}$ ($pK_{r2} = 6.45$). $E_0 = 0.2896$. Log C (at 50% reduction) $= -4.60$.

Buffer	P_H	E_0' found	E_0' calcd.	Found − calcd.
Citrate	2.966	+0.0221	+0.0222	−0.0001
Acetate	4.082	− .0780	− .0777	− .0003
Acetate	4.984	− .1547	− .1547	.0000
Citrate	5.855	− .2157	− .2166	+ .0009
Phosphate	6.478	− .2513	− .2498	− .0015
Phosphate	7.311	− .2820	− .2822	+ .0002
Borate	8.248	− .3118	− .3118	.0000

Tetraethyl Phenosafranine (Amethyst Violet, Rowe 847). IV.—For the sample used we are indebted to Professor H. Bucherer of Munich. The material contained zinc and presumably was the zinc chloride double salt. We attempted to recrystallize this material but found difficulty in recovering an amount of clean crystals sufficient for our titrations.



(Zinc double salt)
IV

The potentials observed with this compound in acid solution were very stable. Table VI contains a good set of data indicative of a homogeneous material. In neutral and alkaline buffers the free base of the reductant separates from solutions as dilute as 1×10^{-5} molar with consequent distortion of the titration curve. By working rapidly and considering the first section of each titration curve we obtained rough estimates of E_0' values, but the details need not be stated since the graphic representation in Fig. 1 is adequate for the precision obtained in neutral and alkaline solutions. The estimated dissociation exponents, 6.4 and 7.7, are only crude approximations.

Safranine T (Safranine O) Rowe 841.—The original commercial sample bore the label "Safranine O, Schultz 679"[12] and the certification

(12) This number undoubtedly refers to the 1914 edition of "Farbstofftabellen" by Schultz. In the edition of 1931 the number is 967.

TABLE VI

AMETHYST VIOLET. TITRATION WITH CHROMOUS ACETATE AT 30°

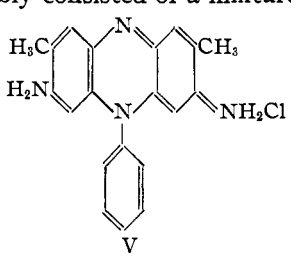
Approximate composition of buffer: 100 ml. 1 *M* KH_2PO_4 + 50 ml. 1 *M* HCl, diluted to 1 liter. Solution titrated: 75 ml. buffer + 10 ml. 0.00076 *M* dye (uncorr.). Reference *P*H (that of 75 ml. buffer + 10 ml. water) 2.118. Log concn. of dye at 50% reduction = −4.06 (uncorr.). Correction of E_h for change of *P*H due to dilution, 0.17 mv. per ml.

y (Buret B)	$y - d$	Reduction, %	0.030055 log $[S_R]/[S_0]$	E_h corr.	E_0'	Deviation from average
0.2	0.117	4.68	−0.03905	+0.20369	(0.16464)	+0.00020
.4	.317	12.68	− .02518	.18953	.16435	− .00009
.6	.517	20.68	− .01754	.18190	.16436	− .00008
.8	.717	28.68	− .01188	.17636	.16448	+ .00004
1.0	.917	36.68	− .00713	.17164	.16451	+ .00007
1.2	1.117	44.68	− .00279	.16726	.16447	+ .00003
1.4	1.317	52.68	+ .00139	.16307	.16446	+ .00002
1.6	1.517	60.68	.00566	.15882	.16448	+ .00004
1.8	1.717	68.68	.01025	.15418	.16443	− .00001
2.0	1.917	76.68	.01553	.14888	.16441	− .00003
2.2	2.117	84.68	.02232	.14220	.16452	+ .00008
2.4	2.317	92.68	.03313	.13280	(.16593)	+ .00149
	2.500	100.00				

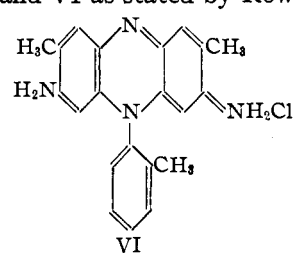
Average .16444

E_0 = .3554

No. PS-2 of the Commission on Standardization of Biological Stains. It probably consisted of a mixture of dyes V and VI as stated by Rowe.



V



VI

TABLE VII

SAFRANINE T. TITRATION WITH CHROMOUS ACETATE AT 30°

Approximate composition of buffer: 100 ml. 1 *M* citric acid + 35 ml. 1 *M* NaOH diluted to 1 liter. Solution titrated: 75 ml. buffer + 5 ml. 0.0005 *M* dye. Reference *P*H (that of 75 ml. buffer + 5 ml. water) 2.738. Log *C* at 50% reduction = −4.53. Correction of E_h for change of *P*H due to dilution, 0.17 mv. per ml.

y	$y - d$	Reduction, %	0.03006 log $[S_R]/[S_0]$	E_h corr.	E_0'	Deviation
0.5	0.27	3.67	−0.0426	0.0364	(−0.0062)	+0.0016
1.5	1.27	17.26	− .0205	.0127	− .0078	.0000
2.5	2.27	30.84	− .0105	.0028	− .0077	+ .0001
3.5	3.27	44.43	− .0029	− .0048	− .0077	+ .0001
4.5	4.27	58.02	.0042	− .0120	− .0078	.0000
5.5	5.27	71.60	.0121	− .0198	− .0077	+ .0001
6.5	6.27	85.19	.0228	− .0307	− .0079	− .0001
7.5	7.27	98.78	.0574	− .0653	− .0079	− .0001
A = 7.36		100		Average	− .0078	

By crystallization, first from warm 95% ethanol and again from absolute ethanol, the material gave large, clean needles of uniform appearance. These retained "moisture" tenaciously. When brought to constant weight by intensive drying they yielded 15.7 and 15.6% nitrogen The theoretical value corresponding to formula V is 15.98% N and that corresponding to formula VI is 15.41% N.

The results of the potentiometric measurements were surprisingly like those of a homogeneous dye, as Table VII clearly indicates. If our crystallization has not isolated such a dye it must be that the characteristic potentials of the component dyes are nearly the same. Table VIII summarizes the results.

TABLE VIII

SAFRANINE T. RELATION OF E_0' TO P_H

Determined by individual titrations with chromous acetate at 30°. Values used in calculations: $K_{r1} = 1.95 \times 10^{-5}$ ($pK_{r1} = 4.71$), $K_{r2} = 1.82 \times 10^{-6}$ ($pK_{r2} = 5.74$), $E_0 = 0.2381$. Log C at 50% reduction, −4.53.

Buffer	P_H	E_0' obs.	E_0' calcd.	Deviation
HCl	1.093	+0.1386	+0.1395	−0.0009
Citrate	2.738	− .0078	− .0086	+ .0008
Acetate	4.620	− .1707	− .1703	− .0004
Acetate	4.985	− .1972	− .1961	− .0011
Acetate	5.218	− .2112	− .2109	− .0003
Citrate	5.425	− .2224	− .2229	+ .0005
Phosphate	6.090	− .2540	− .2541	+ .0001
Phosphate	6.496	− .2688	− .2692	+ .0004
Phosphate	7.106	− .2884	− .2890	+ .0006
Borate	8.629	− .3356	− .3354	− .0002
Borate	9.679	− .3671	− .3670	− .0001
Phosphate	10.94	− .4049	− .4049	.0000
NaOH + KCl	12.32	− .4464	− .4464	.0000

Supplementary Data

To extend the study of the concentration effect, parallel measurements at two concentrations were made with each of the dyes listed in Table IX. In this series of experiments the strength of the acid phosphate buffer was made double that used in previous experiments in order to provide a further check by lowering the error due to changes in acidic and basic components during titration. Of course the effect of dilution upon the P_H value of the buffer remained and in each instance it was determined by hydrogen electrode measurements of dilutions made in this instance, not by water, but by an oxidized solution of the chromous acetate reducing agent. Since the buffer strength was such as to give an ionic strength approximately twice that of comparable previous experiments, the constants of Table IX are not strictly comparable with those of previous experiments (see footnote 15, page 906).

Titrations of reduced solutions of the safranines with oxidizing agents were also made. For the reasons already stated the results were not

precise but they indicated that no serious peculiarities had been introduced by dependence upon the titrations with chromous acetate.

In Table X are summarized some spectrophotometric measurements which will be discussed later.

TABLE IX

EFFECT OF CONCENTRATION OF DYE SYSTEM UPON E_0

Results of titrations with chromous acetate in 0.2 M KH_2PO_4 + 0.1 M HCl

Oxidant of system	Log concentration -3.79 E_0	-4.60 E_0	$\Delta E/\Delta \log C$
Phenosafranine	0.2817	0.2825	−0.0010
Tetramethyl phenosafranine	.2905	.2923	− .0022
Tetraethyl phenosafranine	.3557	.3606	− .0060
Safranine T	.2342	.2382	− .0050

See also Table III.

TABLE X

ALTERATION OF ABSORPTION MAXIMA

C = molar concentration of dye

Compound	$-\log C$	Absorption max. in mμ	Observer	Nature of solution
Phenosafranine	2.82	515	Holmes	Aqueous
	4.21	520	Holmes	Aqueous
	3.0	515	S. C. and C.	Phosphate buffer P_H 2.1
	5.0	520	S. C. and C.	Phosphate buffer P_H 2.1
Dimethyl phenosafranine	3.0	530	S. C. and C.	Phosphate buffer P_H 2.1
	5.0	550	S. C. and C.	Phosphate buffer P_H 2.1
Tetramethyl phenosafranine	3.0	542	S. C. and C.	Phosphate buffer P_H 2.1
	5.0	578	S. C. and C.	Phosphate buffer P_H 2.1
Tetraethyl phenosafranine	3.0 uncorr.	552 (588)	S. C. and C.	Phosphate buffer P_H 2.1
($ZnCl_2$ double salt)	5.0 uncorr.	588	S. C. and C.	Phosphate buffer P_H 2.1
Safranine T	3.0	492	S. C. and C.	Phosphate buffer P_H 2.1
	5.0	518	S. C. and C.	Phosphate buffer P_H 2.1
Methylene blue	3.04	600	Holmes	Aqueous
	5.40	663	Holmes	Aqueous
Nile blue	2.74	580	Holmes	Aqueous
	4.66	635	Holmes	Aqueous
	2.74	578	Cohen and Preisler	Aqueous
	5.05	630	Cohen and Preisler	Aqueous

Discussion

The potentiometric and other measurements demonstrate that each of the several systems is, primarily, reversible. However, the reductants are subject to progressive alterations which should be seriously considered as limiting the reliability of these dyes as oxidation–reduction indicators.

With reservations to be discussed later, the data satisfy equation (1). From this fact and the attendant numerical values of the constants may be drawn some interesting conclusions. Each of the graphic representations of the relation of E_0' to P_H, as shown in Fig. 1, exhibits a section where $\Delta E_h/\Delta P_H = -0.09016$ and a section where $\Delta E_h/\Delta P_H = -0.03006$. The intersection of projections of these sections (or the corresponding algebraic relations) determines the values of $^1/_2(pK_{r_1} + pK_{r_2})$ and hence

$K_{r_1}K_{r_2}$. Since these distinct sections of each curve are fairly well placed, the value of $K_{r_1}K_{r_2}$ is determined rather well. On the other hand, the

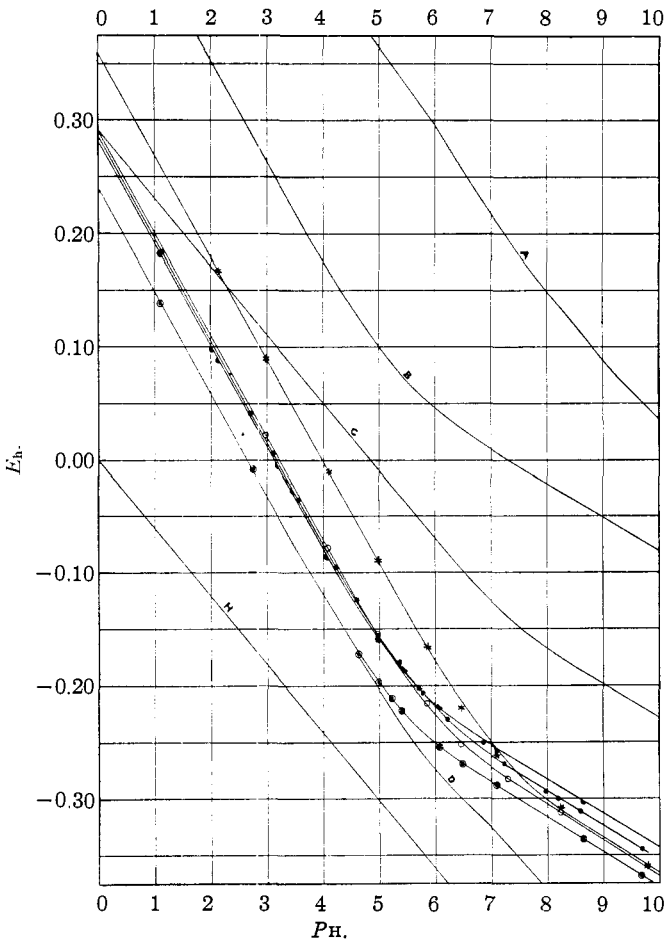


Fig. 1.—Relation of E_h to P_H at 50% reduction of dye (E_0':P_H curve).

Experimental

-◡- Phenosafranine at log C = −4.52

-●- Dimethyl phenosafranine at log C = −3.66

-⊖- Tetramethyl phenosafranine at log C = −4.60

* Tetraethyl phenosafranine at log C = −4.53 (uncorr.)

-⊙- Safranine T at log C = −4.53

Comparisons

A. 2,6-Dibromophenolindophenol

B. Methylene blue

C. Indigo disulfonate

D. Neutral red

(H. Hydrogen at 1 atm.)

greater difficulties of measurement and the consequent uncertainties of calculation are encountered in that region of P_H where the inflections of the curves occur and there the very highest accuracy is required for the evalu-

ation of the individual dissociation constants. Hence these individual values are not well known. The refinements used in the tables are for purposes of definite calculation. However, there can be no doubt that there have been established relations similar to those of the methylene blue system.[5] The structural allocation of dissociation constants in the case of the components of the methylene blue system was confirmed by comparisons with the data for Lauth's violet, for Bindschedler's green and for toluylene blue,[11] an assembly of data in which dissociation constants not measurable in one instance were apparent in another instance. Since the safranines are similar to Lauth's violet and methylene blue as regards basic groups, there is good reason to assume the following. The predominant form of each oxidant of the safranines is the paraquinone structure, indicated by the formulas I–VI, resulting in a "polar" group so strongly basic that the dissociation exponent does not come within the reach of our experiments. The other amino, or substituted amino group, of each oxidant is so weakly basic that its dissociation exponent also does not come within the reach of our experiments. Therefore, we have to deal with one predominant form which may be represented by the symbol Ox^+. Upon reduction the molecule attains a more symmetrical structure, and the dissociation exponents fall within the range 4.7 to 7.7. Indeed in any one compound they are so close as to obscure the intermediate "0.06-slope" of the E_0':P_H curve (see Fig. 1). The influence of strengthening basicity is evident in Fig. 1 where the E_0':P_H curves for the tetramethyl and the tetraethyl phenosafranines, which fall above the curve for phenosafranine at low P_H, cross this curve and then run below it at high P_H.

In Fig. 1 the E_0':P_H curves of 2,6-dibromophenolindophenol, methylene blue and indigo carmine and also the curve of the hydrogen electrode (at 1 atmosphere H_2) are included to show the relative positions of the safranine oxidation–reduction systems. There is also included the curve for neutral red from P_H 4.5 to 7.9. This curve shows the structural influence upon the course in alkaline solution. It runs parallel and very close to the curve for safranine T in acid solution, the values of E_0 being: neutral red, 0.240; safranine T, 0.238.

With that caution which has already been noted regarding a possible systematic error, we may now discuss the "concentration effect." In the case of phenosafranine the situation is not entirely clear. The measurements in buffer solutions of P_H range 3 to 7.5 were particularly subject to drifts and the resulting errors are evident alike in Table I and Fig. 1. However, if we confine attention to the more acid solutions, there appears in Table I about a millivolt increase of E_0 per tenfold dilution of the dye system. This is confirmed by the data of Table IX. With the other systems the "concentration effects" are more distinct (see Tables III and IX).

There seems to apply, within the range of concentrations used, the empirical equation (2a)

$$\tilde{E}_0 = E_0 + A \log C \qquad (2a)$$

where A is 0.001 for phenosafranine, 0.0044 for dimethyl phenosafranine, 0.0022 for tetramethyl phenosafranine, 0.006 for tetraethyl phenosafranine and 0.005 for safranine T. Similar relations have been reported by Clark, Cohen and Gibbs,[5] whose somewhat discordant data for methylene blue may be expressed by equation (2a) and the coefficient 0.0154 between log $C = -3.0$ and -4.0, and by Cohen and Preisler[13] whose consistent data for Nile blue at P_H 4.88 and the range of concentration between 1×10^{-4} molar and 1×10^{-5} molar give the coefficient 0.0166. In each of the instances there have been encountered difficulties with the potential measurements which might have introduced a systematic error; but the difficulties have been of a different nature in the three distinct series of cases and if systematic errors have been wrongly interpreted as a "concentration effect," they have yielded a remarkably consistent relation.

In commenting upon the case of methylene blue Clark, Cohen and Gibbs noted that their data included the range of concentration within which Holmes[14] had found remarkable changes in the wave lengths of maximal absorption by the oxidant. In conformity with Holmes' statement that all the oxazine, thiazine and azine dyes which he examined exhibit this effect, Cohen and Preisler found an instance in Nile blue and we have found instances with the dyes under discussion. The data are summarized in Table X. Since the change of an absorption maximum is not continuous with dilution but rather is a replacement of one maximum by another, it would be improper to attempt a direct correlation between the magnitude of a "shift" and a coefficient of equation (2a). It can only be said that the spectroscopic evidence of a change in the species of the oxidant is associated with a change in the characteristic potential of the system.

This and other evidence which suggested that the change of species might be a change in state of aggregation led Cohen and Preisler to the tentative postulation of a *reversible* change in the state of aggregation of Nile blue as a means of accounting for the change of potential with dilution. If this could be proved, accurately controlled potentiometric measurements could be used in the very important calculation of the free energy of aggregation. However, if the change of potential be attributed to a change in the state of aggregation of the oxidant alone, the effect should be revealed not only in parallel titrations with different concentrations but also in any one titration. A tenfold dilution of the oxidant occurs in passing from the state of 9% reduction to the state of 90% reduction of the system. This should alter the calculated value of E_0' by one millivolt in the case of pheno-

(13) Cohen and Preisler, *Public Health Reports*, Supplement 92 (1931).
(14) Holmes, *Ind. Eng. Chem.*, **16**, 35 (1924).

safranine and by several millivolts in other cases. While the data for certain titrations of phenosafranine doubtless could be recalculated in such a way as to furnish the predicted small distortion for this system, neither the data of Tables II and VI nor the data of several other of the more precise titrations would submit to such treatment. It is only to indicate this that we have reported, with proper qualifications, the fifth decimal in the cases cited and have completed the elaborate calculations in other cases.

There remain to be considered certain aspects of the theoretically more complete equations. A discussion of this may be abbreviated if we confine attention to the acid solutions where the predominant species are Ox^+ and $H_3^+Red^+$ (see page 904). These are involved in the process.

$$Ox^+ + 2e + 3H^+ \rightleftharpoons H_3^+Red^+$$

Let ( ) indicate activity, [ ] molar concentration and γ activity coefficient. The electrode equation is then (3)

$$E_h = \frac{RT}{2F} \ln \frac{(Ox^+)(H^+)^3}{K(H_3^+Red^+)} \tag{3}$$

or at constant hydrion activity

$$E_h = \frac{RT}{2F} \ln \frac{B}{K} + \frac{RT}{2F} \ln \frac{[Ox^+]}{[H_3^+Red^+]} + \frac{RT}{2F} \ln \frac{\gamma_0}{\gamma_r} \tag{4}$$

where

$$\frac{RT}{2F} \ln \frac{B}{K} = E_0'$$

The last term of equation (4) could hardly be altered appreciably by that contribution to the ionic strength of the heavily buffered solution which is made by the relatively very small quantities of the components of the dye system. Consideration of the complete equation, involving all species, leads to a similar conclusion. Therefore some factor other than ionic strength[15] must alter the ratio of activity coefficients.

Thus it appears that neither the ionic strength effect nor a change in the state of aggregation of the oxidant alone can account for the "concentration effect."

It is possible that the more careful titrations, which were designed to reveal the association of the oxidant, were confined to a range of concentration unsuited to the purpose. This possibility is now under examination.

We wish to express our appreciation of the contributions made by the persons mentioned in the text and especially of the aid given by Dr. Leslie Hellerman and Miss Marie Perkins.

Summary

With a precision adequate for the ordinary uses of the dyes as oxidation–reduction indicators there have been determined the characteristic con-

(15) The ionic strength effect has not been subjected to direct experiment in this series of cases. There are *a priori* reasons for believing the effect to be much smaller than that demanded of the relation $(RT/nF) \ln (\gamma_0/\gamma_r) = 0.045 \sqrt{\mu}$ which would obtain under the *limiting law* of Debye and Hückel. A comparison of the data of Table IX, obtained with an acid phosphate buffer of $\mu = 0.21$, with the data of cases where the ionic strength was about 0.1, indicates this and also that there is no consistent relation between the coefficients of equation (2a) and the effect of ionic strength.

stants of the systems which are named below by the names of the respective oxidants. A reversal of potential drift during measurements has been ascribed to a secondary, irreversible alteration of reductant similar to but not so extensive as that previously reported in the case of leuco neutral red. The least interference by this effect was encountered in acid phosphate and acid citrate buffers of $P_H < 3$. While an undetected systematic error may still persist in such cases, the data indicate that the "normal potential," E_0, is a function of the concentration, C, of the dye system as expressed by the relation

$$E_0 = \tilde{E}_0 - A \log C$$

This cannot be due to the relatively small contributions of components of the dye systems to the ionic strengths of the solutions. Apparently precise measurements in individual titrations have shown that a reversible change in the state of aggregation of the oxidant alone, as tentatively proposed by Cohen and Preisler for the case of Nile blue, cannot be applied in the present instances. Some change of species of each oxidant is, however, indicated by the spectrophotometric comparisons of relatively dilute and concentrated solutions of these dyes.

At a fixed concentration of dye system and approximately constant ionic strength the following relation holds in each instance

$$E_h = E_0 + \frac{RT}{2F} \ln \frac{[S_0]}{[S_R]} + \frac{RT}{2F} \ln [(H^+)^3 + K_{r_1}(H^+)^2 + K_{r_1}K_{r_2}(H^+)]$$

where $[S_0]$ = molar concentration of total oxidant, $[S_R]$ = molar concentration of total reductant, (H^+) = hydrion activity, and $K_{r_1} = (H_2^+\text{Red})(H^+)/(H_3^+\text{Red}^+)$, $K_{r_2} = (\text{HRed})(H^+)/(H_2^+\text{Red})$.

For convenience the data of the text have been reduced to comparable values for 0.0001 M and constants are rounded off in the table, all values at 30°.

	At $C = 1 \times 10^{-4}$ M						
Dye	E_0	E_0' at P_H 7.0	$\frac{\Delta E}{\Delta \log C}$	K_{r_1}	pK_{r_1}	K_{r_2}	pK_{r_2}
Phenosafranine (Rowe 840)	0.280	−0.252	−0.001	1.1×10^{-5}	4.95	1.7×10^{-6}	5.8
Dimethyl phenosafranine (Rowe 842)	.286	− .260	− .0044	1.3×10^{-5}	4.9	4.7×10^{-7}	6.3
Tetramethyl phenosafranine	.288	− .273	− .0022	4.8×10^{-6}	5.3	3.6×10^{-7}	6.5
Tetraethyl phenosafranine (Rowe 847)	.355	− .254	− .006	4×10^{-7}	6.4	2×10^{-8}	7.7
Safranine T (Rowe 841)	.235	− .289	− .005	2×10^{-5}	4.7	2×10^{-9}	5.7

BALTIMORE, MARYLAND

RECEIVED AUGUST 5, 1932
PUBLISHED MARCH 7, 1933