pound is extremely hard to oxidize, the pyridinecarboxylic acid may have been destroyed during the oxidation.

The writer is indebted to Dr. E. C. Franklin for many helpful suggestions.

Summary

Evidence is presented to support the postulation that aliphatic imines, hydramides and Schiff's bases are ammono aldehydes. Aside from the known reactions which support this view, it is shown that (1) reactions which should lead to the formation of methylene imine (ammono formaldehyde) yield only hexamethylenetetramine (polymerized ammono formaldehyde), (2) anhydroformaldehydeaniline (ammono formaldehyde-acetal) is ammonolyzed to hexamethylenetetramine and aniline, (3) ethylidene imine (ammono acetaldehyde) is nitridized to acetamidine (ammono acetic acid), (4) ethylidene imine reacts with malonic acid and hydrazine derivatives just as aquo aldehydes do. The formation of pyridine derivatives from aldehydes and ammonia has been shown to be dependent upon the formation of aquo ammono aldols.

LABORATORY FOR PLANT BIOLOGY
CARNEGIE INSTITUTION OF WASHINGTON
STANFORD UNIVERSITY, CALIFORNIA

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

STUDIES ON OXIDATION–REDUCTION. XVII[1] NEUTRAL RED

BY W. MANSFIELD CLARK AND MARIE E. PERKINS

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Neutral red,[2] named *Toluylenroth* by its discoverer, Witt,[3] has become the best known of those azine dyes called eurhodines. Early in its history it was recognized as a valuable histological and vital stain; and, as has often happened when a dye is constantly available for such purposes, its several properties have been adapted to other biochemical applications. As an acid–base indicator neutral red is used in a range of P_H important biologically. As a reducible dye it has been employed by the bacteriologist and in a manner which will have a particular interest when we shall have described the characteristics of the oxidation–reduction system.

For the moment we are concerned with neutral red exclusively as an

[1] Previous papers of this series have been published in *Public Health Reports* and *Supplements to Public Health Reports*, 1921–1931. The first ten articles have been republished as *Hygienic Laboratory Bulletin*, No. 151, United States Public Health Service. (Manuscript first received July 17, 1931.)

[2] Neutral red is dye No. 825 in Rowe's "Colour Index."

[3] Witt, *Ber.*, **12**, 931 (1879).

oxidation–reduction indicator. Since the compound is similar structurally to useful indicators such as methylene blue, it might be assumed to be a reversible, reliable indicator. Indeed the reductant has been described by several authors as a derivative of dihydrophenazine, as analogous to leuco methylene blue and as a compound which can be oxidized readily to the dye.

Positive evidence in favor of the supposition that neutral red will form a reversible oxidation–reduction system may be inferred from preliminary potentiometric measurements mentioned by Clark and Zoller[4] and from charts of such measurements published without details by Rapkine, Struyk and Wurmser[5] and by Vellinger.[6]

Preliminary explorations by Clark and Zoller had shown that oxidation–reduction systems formed among the azine dyes have characteristic potentials which are much more negative than those of the systems described in previous papers of this series. In addition to its intrinsic interest, this fact seemed to provide a readily available means of extending the series of well-characterized, oxidation–reduction indicators and of providing reagents to test several matters of biochemical importance. Subsequent investigations have proved the advisability of proceeding cautiously with the azines.

In particular it should be emphasized that while the neutral red system does behave in part as a reversible system it also exhibits a peculiar, secondary change. This takes place rapidly within a range of P_H which is of importance biologically. It makes difficult the measurement of reliable potentials and it sets limits to the usefulness of neutral red as an oxidation–reduction indicator.

The following summary of one of many experiments will indicate certain limitations under which potentiometric measurements must proceed.

Rapid reduction of dilute, buffered solutions of neutral red results in almost colorless solutions. If such solutions are immediately removed from a hydrogen or a nitrogen atmosphere and exposed to air, they will be reoxidized. After reoxidation and suitable adjustments of P_H and of dilution, such solutions are found to match the untreated sample within the limitations of an ordinary colorimeter. However, it is significant that after the slow oxidation by air a solution reduced at P_H 5.3 has a noticeable yellow tint.

If, instead of reoxidation immediately after reduction, the solutions are allowed to stand under hydrogen or nitrogen, a very different picture results. At P_H 8.2 the reduced solution remains colorless for an hour at least. At P_H 2.7 the solution slowly acquires a yellow color with green

[4] Clark and Zoller, *Science*, **54**, 557 (1921).

[5] Rapkine, Struyk and Wurmser, *J. chim. phys.*, **26**, 340 (1929).

[6] Vellinger, *Arch. phys. biol.*, **7**, 113 (1929).

fluorescence. At P_H 5.3 the yellow-green fluorescence develops very rapidly.

On reoxidation and adjustment to a common P_H number the solutions which had been held at the more acid and the more alkaline reactions are found to have regained nearly, but not exactly, the color of the original solution. The solution which had been held at P_H 5.3 is found to retain its fluorescence for days, if not made too acid.

What has been said of the attack by oxygen is more or less true of the attack by other oxidizing agents.

While we have made no quantitative comparison between the rate of development of the fluorescence and the rate of change of potential, we feel safe in stating that the rapidity and the extent of drifts in the potentials during titration are roughly correlated with the formation of the material giving the green fluorescence. This material has been isolated in crystalline form and is described in a section of this paper. It will be called the fluorescent material. *Tentatively* we shall now assume that its formation is an irreversible process interfering with accurate potentiometric characterization of a reversible process. Accordingly we shall rule out of immediate consideration a number of experiments in which titrations were made with solutions reduced by prolonged action of hydrogen and a platinum catalyst or in which solutions were reduced by any other means and held in stock for any considerable period. We shall now confine attention to comparatively rapid titrations of neutral red with a reducing agent.

In their preliminary study of the system Clark and Zoller titrated neutral red with titanium trichloride. This reagent has the advantages that it is of sufficient reducing intensity and that it establishes an equilibrium mixture of the dye's reductant and oxidant with the required rapidity. However, the reagent was abandoned in later work because the stock must be preserved in solutions of high acidity. During a titration this acid makes changes in the buffer system which are too large to be subjected to simple and certain correction. We have now used a solution of chromous acetate. With this reagent titration of neutral red in tenth normal hydrochloric acid is unsatisfactory because of the slowness with which potentials become steady. In acid phosphate and acid citrate solutions adjustment is rapid and, although considerable fluorescence develops before the completion of a titration made with careful readings, the results appear to be satisfactory. In alkaline solution the electrode exhibits very steady potentials. Within an intermediate zone, centered near P_H 5 or 6, potentials become erratic and an approach to satisfactory data is attained only by rapid titration.

The solution of chromous acetate was prepared as follows. Hydrous chromic chloride in hydrochloric acid solution was reduced by zinc. From

the filtered solution chromous acetate was precipitated by the addition of sodium acetate. The red, solid chromous acetate was washed repeatedly with water and then transferred to a large volume of deaerated water. Because of the extreme sensitiveness of the chromous acetate to oxygen, all operations were carried out in an apparatus very thoroughly flushed with carbon dioxide. The final solution was washed with and preserved under hydrogen.

Frequent estimates of the normality of the solution over a period of several months showed that it remained at 0.0037 *N*. Since this was somewhat higher than required, the solution was diluted as needed. Variation in the quantities required in titrations of the same amount of dye, as noted in the tables, is due to the use of appreciably different dilutions.

Details of titrations in each of three zones of P_H are summarized in Tables I, II and III.

The methods employed have been described in previous papers of this series. Titrations were made in a shaded room or by dim lamplight. The P_H numbers were determined by careful hydrogen electrode measurements of properly diluted buffer solutions (see tables). By the same means estimates were made of the corrections to apply to the potentials on account of changes in the buffer system during titration. Because of dependence upon the hydrogen electrode measurements, no care was taken to have the stock component solutions of the buffers of *exactly* the composition indicated. Liquid junctions in all cases were made with saturated potassium

TABLE I

TITRATION OF NEUTRAL RED IODIDE WITH CHROMOUS ACETATE

30°C. P_H = 2.156. Approximate composition of buffer: 100 ml. *M* KH_2PO_4 + 100 ml. *M* HCl; diluted to 1 liter. Solution titrated: 75 ml. buffer + 5 ml. 0.0005 *M* neutral red. Reference P_H (that of 75 ml. buffer + 5 ml. water) 2.156.

y, ml.	*y-d*, ml.	Reduction, %	$\frac{0.03006}{n}\log [S_R]/[S_0]$, volt	E_h corr., volt	E_0'	Dev. from 0.0461 volt
1	0.15	1.24	−0.0566	0.1027	0.0461	0.0000
1.5	0.65	5.56	− .0370	.0831	.0461	.0000
2	1.15	9.88	− .0289	.0751	.0462	+ .0001
3	2.15	18.47	− .0194	.0655	.0461	.0000
4	3.15	27.06	− .0129	.0590	.0461	.0000
5	4.15	35.65	− .0077	.0540	.0463	+ .0002
6	5.15	44.24	− .0030	.0491	.0461	.0000
7	6.15	52.84	+ .0015	.0448	.0463	+ .0002
8	7.15	61.43	.0061	.0400	.0461	.0000
9	8.15	70.02	.0111	.0343	(.0454)	(− .0007)
10	9.15	78.61	.0170	.0258	(.0428)	(− .0033)
11	10.15	87.20	.0251	.0108	(.0359)	(− .0102)
	11.64	100	....	....	....	....
				Best value	.0461	
				By R. and B. method	.0462	

TABLE II

TITRATION OF NEUTRAL RED IODIDE WITH CHROMOUS ACETATE

30° C. P_H = 4.978. Approximate composition of buffer; 100 ml. M Na Acetate + 30 ml. M HCl; diluted to 1 liter. Solution titrated: 75 ml. buffer + 5 ml. 0.0005 M neutral red. Reference P_H (that of 75 ml. buffer + 5 ml. water) 4.978.

y, ml.	y-d, ml.	Reduction %	$\frac{0.03006}{2}\log [S_R]/[S_0]$ volt	E_h, volt	E_0', volt	Dev. from −0.2024, volt
0.5	0.18	2.95	−0.0456	−0.1538	(−0.1994)	(+0.0030)
1.0	0.68	11.15	− .0271	− .1751	− .2022	+ .0002
1.5	1.18	19.34	− .0186	− .1840	− .2026	− .0002
2.0	1.68	27.54	− .0126	− .1899	− .2025	− .0001
2.5	2.18	35.74	− .0077	− .1949	− .2026	− .0002
3.0	2.68	43.93	− .0032	− .1992	− .2024	.0000
3.5	3.18	52.13	+ .0011	− .2033	− .2022	+ .0002
4.0	3.68	60.33	.0055	− .2077	− .2022	+ .0002
4.5	4.18	68.52	.0102	− .2125	− .2023	+ .0001
5.0	4.68	76.72	.0156	− .2185	(− .2029)	(− .0005)
5.5	5.18	84.92	.0226	− .2273	(− .2047)	(− .0023)
	6.10	100	....	....	....	....
				Average	− .2024	
				By R. and B. method	− .2022	

TABLE III

TITRATION OF NEUTRAL RED IODIDE WITH CHROMOUS ACETATE

30° C. P_H = 8.596. Approximate composition of buffer: 500 ml. 0.2 M H_3BO_3, 30 ml. M NaOH, 70 ml. KCl, diluted to 1 liter. Solution titrated: 75 ml. buffer + 5 ml. 0.0005 M neutral red. Reference P_H (that of 75 ml. buffer + 5 ml. water) 8.596.

y, ml.	y-d, ml.	Reduction, %	$\frac{0.03006}{2}\log [S_R]/[S_0]$, volt	E_h corr., volt	E_0', volt	Dev. from 0.4149 volt
1	0.45	5.84	−0.0363	−0.3778	(−0.4141)	(+0.0008)
2	1.45	18.83	− .0191	− .3958	− .4149	.0000
3	2.45	31.82	− .0100	− .4050	− .4150	− .0001
4	3.45	44.81	− .0027	− .4123	− .4150	− .0001
5	4.45	57.79	+ .0041	− .4189	− .4148	+ .0001
6	5.45	70.78	.0116	− .4264	− .4148	+ .0001
7	6.45	83.77	.0214	− .4358	(− .4144)	(+ .0005)
8	7.45	96.75	.0443	− .4440	(− .3997)	(+ .0152)
	7.70	100	....	....	....	....
				Average	− .4149	
				By R. and B. method	− .4150	

chloride solution and in essentially the same manner. All titrations were made with solutions deaerated by means of nitrogen.

In Table IV are summarized the values of E_0' [7] obtained by individual titrations in buffered solutions having P_H values of sufficient range to characterize the several values of the slope, $\Delta E_0'/\Delta P_H$. The curve relating E_0' to P_H is shown in Fig. 1, where the data of Table IV are indicated by

[7] E_0' indicates the potential of the system at a specified value of P_H when the ratio of total reductant to total oxidant is unity.

TABLE IV

NEUTRAL RED IODIDE. RELATION OF E'_0 TO P_H

Determined by separate titrations with chromous acetate. Values used in calculations: $K_0 = 1.59 \times 10^{-7}$; $K_{r1} = 6.92 \times 10^{-7}$; $K_{r2} = 5.01 \times 10^{-6}$; $E_0 = 0.240$. $E'_0 = E_0 - 0.03006 \log [[H^+] + K_0] + 0.03006 \log [[H^+]^2 + K_{r2}[H^+] + K_{r1}K_{r2}] - 0.0601\ P_H$.

Buffer	P_H	E'_0 found, volt	E'_0 calcd., volt	Found −calcd.
Phosphate	2.156	+0.046	+0.046	0.000
Citrate	2.753	− .009	− .008	− .001
Citrate	3.495	− .075	− .075	.000
Acetate	4.640	− .176	− .176	.000
Acetate	4.978	− .202	− .204	+ .002
Citrate	5.756	− .259	− .259	.000
Phosphate	6.080	− .280	− .278	− .002
Phosphate	6.488	− .295	− .299	+ .004
Phosphate	7.105	− .330	− .331	+ .001
Borate	8.163	− .391	− .391	.000
Borate	8.596	− .415	− .417	+ .002

the centers of the open circles. The trends of the curve were roughly confirmed by use of a solution of neutral red partly reduced in *acid* solution and introduced into various buffer solutions. The measurements with this preformed mixture were extended to P_H 12 and indicated that the slope ($\Delta E'_0/\Delta P_H = -0.0601$), characteristic at high values of P_H, continues this far into the region of alkalinity. Also supplementary data were obtained by rapid titrations which included only four or five increments of reducing agent. The latter data were analyzed by the method presently to be described. The resulting values of E'_0 are indicated in Fig. 1 by the centers of blacked circles.

Figure 1 seems to be in substantial agreement with the less detailed figure for the neutral red system pub-

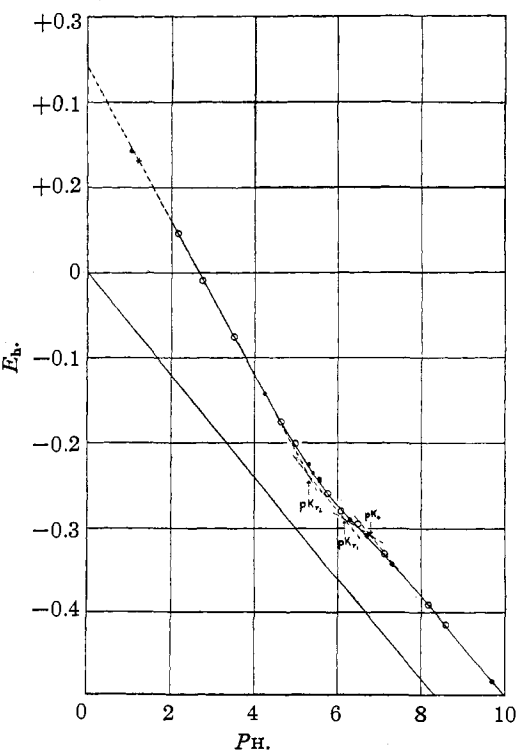


Fig. 1.—Neutral red, relation of E'_0 to P_H.

lished by Vellinger.[6] It is important to note that Vellinger made his titrations with $Na_2S_2O_4$. Unfortunately neither Vellinger nor Rapkine, Struyk and Wurmser,[5] who record only a few points on the curve, give adequate details.

In each of our titrations it was found that considerable reducing agent was used before the titration curve began to flatten. Experience has revealed cases where this may be attributed to an oxidizing impurity in the sample of dye. In the present instance we are loath to assume this because we used a beautiful preparation of neutral red iodide made by Phillips and Cohen.[8] Indeed the calculated excesses of reducing agent varied sufficiently in titrations of the same amount of dye to indicate that an impurity of the dye was not entirely responsible. Doubtless the buffer solutions contained traces of reducible material. However, we were unable definitely to prove this by blank titrations with chromous acetate because with this reagent, in the absence of a definitely reversible system, potentials are indefinite.

Thus there was no definite origin of a titration curve. To make matters worse there was no certain end-point. In most instances it was quite evident that the "irreversible" process became so rapid near the end of a titration that the end-point was obscured. Having no reliable origin and end-point we were forced to depend upon the intermediate data in calculating the characteristics of each curve. This can be done by the method of Reed and Berkson.[9]

Since we have made changes in their symbols appropriate to the case at hand and since the method deserves some emphasis, the equations will be stated. Assume that the titration is made at constant P_H or that the data have been properly corrected to meet this condition. Then equation (1) will be applicable

$$E_h = E_0' + 0.03006 \log \frac{A - (y - d)}{y - d} \qquad (1)$$

y is the number of milliliters of reducing agent actually used in reaching the state where the potential is E_h; d is the extra number of milliliters of reducing agent consumed before the dye is attacked; A is the number of milliliters required for 100% reduction of the dye (see figure 2A).

Make an arbitrary shift of the origin of potential so that

$$E_h + E_n = E \qquad (2)$$

where $E = 0$ when $y = y_n$. Let

$$E_0' + E_n = - 0.03006 \log C \qquad (3)$$

and let

$$E/0.03006 = p$$

[8] Phillips and Cohen, *Stain Technology*, **2**, 74 (1927).

[9] Reed and Berkson, *J. Phys. Chem.*, **33**, 760 (1929).

With these definitions the development by Reed and Berkson may be followed to equation (4)

$$y = (1 + C)\frac{y_n - y10^p}{1 - 10^p} - dC \tag{4}$$

Or if $(y_n - y10^p)/(1 - 10^p) = x$

$$y = (1 + C)x - dC \tag{5}$$

Also

$$A = (y_n - d)(1 + C) \tag{6}$$

Equation 5 is the equation of a straight line which cuts the y axis at $-d$ and which has the slope $(1 + C)$. See an experimental case in Fig. 2B. Therefore, if the data conform closely to equation (1), it is possible to draw a "best" straight line through the loci x, y, and to estimate d and C of equation (5). Then A is calculated with equation (6) or E'_0 is calculated with equation (3).

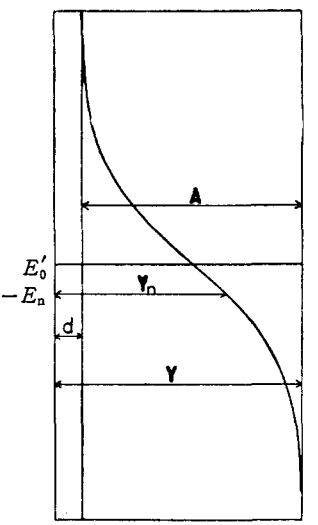


Fig. 2A.

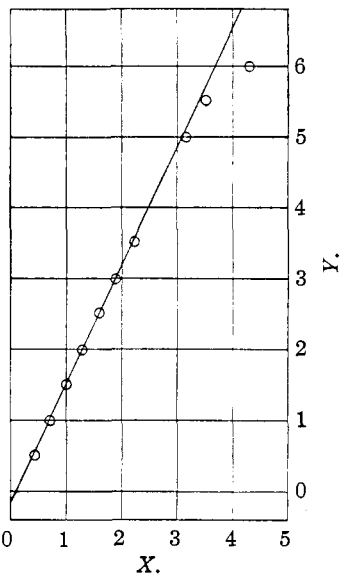


Fig. 2B.

The method places a rather heavy burden upon the accuracy of the arbitrarily selected reference point and upon the judgment with which the "best" straight line is placed. Therefore, and instead of repeating the calculations with several reference points, we have felt justified in making slight shifts[10] in the preliminary values of A and d in order to eliminate from tables such as I the more obviously erroneous trend in a series of values of E'_0 which usually remained after the first estimate.

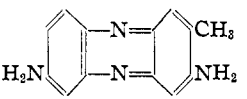
[10] With one exception, such shifts involved differences of less than 0.5 millivolt between a value of E'_0 obtained by one trial of Reed and Berkson's method and the value finally accepted. The average difference was 0.3 millivolt.

In Fig. 2B there is plainly evident, in a specific instance, the departure of the experimental points where the end-point is approached. Similar discrepancies are shown in another way by Tables I, II and III where the values of E_0' are shown to vary as the end-point is approached.

Better data with regard to end-point, origin, or E_0' were not obtained by using a carefully crystallized preparation of neutral red base.

For purposes of comparison, measurements were made with "simple neutral red" and with dimethylaminomethylphenazine.

Simple Neutral Red.—No improvement in origin of titration curves, a slight improvement in end-point and a considerable improvement in the stability of potentials were obtained with "simple neutral red iodide." This compound is called "simple neutral red iodide" because Bernthsen and Schweitzer[11] called the base (formula I) "das einfachste Toluylenroth."



(I)

We used material prepared by Dr. Phillips in accordance with the directions of Bernthsen and Schweitzer and converted to the iodide by the procedure used in preparing neutral red iodide. We find the nitrogen to be 15.9 and 15.8%. The theoretical for $C_{13}H_{13}N_4I$ is 15.9.

A fluorescent material forms in the reduced solutions.

Tables V, VI and VII give the details of titrations within the three

TABLE V

TITRATION OF "SIMPLE NEUTRAL RED" WITH CHROMOUS ACETATE

30°C. P_H = 3.454. Approximate composition of buffer: 100 ml. 1 *M* citric acid +85 ml. 1 *M* NaOH, diluted to 1 liter. Solution titrated: 75 ml. buffer +5 ml. 0.0005 *M* simple neutral red. Reference P_H (that of 75 ml. buffer +5 ml. water) 3.454.

y, ml.	*y*−*d*, ml.	Reduction, %	$\frac{0.03006}{n}$ log $[S_R]/[S_0]$, volt	E_h corr., volt	E_0'	Dev. from −0.0728 volt
1.2	0.3	5.60	−0.0369	−0.0360	−0.0729	−0.0001
1.6	0.7	13.06	− .0247	− .0480	− .0727	+ .0001
2.0	1.1	20.52	− .0177	− .0551	− .0728	.0000
2.5	1.6	29.85	− .0112	− .0617	− .0729	− .0001
3.0	2.1	39.18	− .0057	− .0671	− .0728	.0000
3.5	2.6	48.51	− .0008	− .0721	− .0729	− .0001
4.0	3.1	57.84	+ .0041	− .0769	− .0728	.0000
4.5	3.6	67.16	.0093	− .0821	− .0728	.0000
5.0	4.1	76.49	.0154	− .0883	− .0729	− .0001
5.5	4.6	85.82	.0235	− .0967	− .0732	− .0004
6.0	5.1	95.15	.0389	− .1115	− .0726	+ .0002
	5.36	100				
			Average		−0.0728	
			By R. and B. method		− .0730	

[11] Bernthsen and Schweitzer, *Ann.*, **236**, 332 (1886).

TABLE VI

TITRATION OF "SIMPLE NEUTRAL RED" WITH CHROMOUS ACETATE

30°C. P_H = 6.08. Approximate composition of buffer: 100 ml. 1 M KH_2PO_4 +17 ml. 1 M NaOH; diluted to 1 liter. Solution titrated: 75 ml. buffer +5 ml. 0.0005 M simple neutral red. Reference P_H (that of 75 ml. buffer +5 ml. water) 6.080.

y, ml.	$y-d$, ml.	Reduction, %	$\frac{0.03006}{1}\log [S_R]/[S_0]$, volt	E_h corr., volt	E_0'	Dev. from −0.2704 volt
1.0	0.05	0.82	−0.0626	−0.2185	(−0.2811)	−0.0107
1.5	0.55	9.02	− .0302	− .2403	− .2705	− .0001
2.0	1.05	17.21	− .0205	− .2498	− .2703	+ .0001
2.5	1.55	25.41	− .0141	− .2564	− .2705	− .0001
3.0	2.05	33.61	− .0089	− .2616	− .2705	− .0001
3.5	2.55	41.80	− .0043	− .2661	− .2704	.0000
4.0	3.05	50.00	.0000	− .2704	− .2704	.0000
4.5	3.55	58.20	+ .0043	− .2746	− .2703	+ .0001
5.0	4.05	66.39	.0089	− .2793	− .2704	.0000
5.5	4.55	74.59	.0141	− .2845	− .2704	.0000
6.0	5.05	82.79	.0205	− .2914	(− .2709)	− .0005
6.5	5.55	90.98	.0302	− .3024	(− .2722)	− .0018
7.0	6.05	99.18	.0626	− .3164	(− .2538)	+ .0166
	6.10	100.00				
			Average		−0.2704	
			By R. and B. method		− .2705	

TABLE VII

TITRATION OF "SIMPLE NEUTRAL RED" WITH CHROMOUS ACETATE

30°C. P_H = 8.591. Approximate composition of buffer: 500 ml. 0.2 M H_3BO_3 +30 ml. M NaOH +70 ml. M KCl; diluted to 1 liter. Solution titrated: 75 ml. buffer +5 ml. 0.0005 M simple neutral red. Reference P_H (that of 75 ml. buffer +5 ml. water) 8.591.

y, ml.	$y-d$, ml.	Reduction, %	$\frac{0.03006}{1}\log [S_R]\ [S_0]$, volt	E_h corr., volt	E_0'	Dev. from −0.4168
1.0	0.2	3.64	−0.0428	−0.3733	(−0.4161)	+0.0007
1.5	0.7	12.73	− .0251	− .3913	− .4164	+ .0004
2.0	1.2	21.82	− .0167	− .4000	− .4167	+ .0001
2.5	1.7	30.91	− .0105	− .4063	− .4168	.0000
3.0	2.2	40.00	− .0053	− .4115	− .4168	.0000
3.5	2.7	49.09	− .0005	− .4164	− .4169	− .0001
4.0	3.2	58.18	+ .0043	− .4212	− .4169	− .0001
4.5	3.7	67.27	.0094	− .4262	− .4168	.0000
5.0	4.2	76.36	.0153	− .4321	− .4168	.0000
5.5	4.7	85.45	.0231	− .4396	− .4165	+ .0003
6.0	5.2	94.55	.0373	− .4528	(− .4155)	+ .0013
	5.5	100.00				
			"Best value"		−0.4168	
			By R. and B. method		− .4172	

distinctive regions of P_H and Table VIII summarizes the data for several values of P_H. The E_0':P_H curve lies so close to that of neutral red that it has been omitted from Fig. 1 to avoid confusion. E_0 is 0.237. E_0 for neutral red is 0.240.

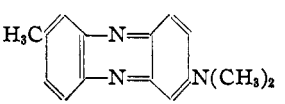
TABLE VIII

SIMPLE NEUTRAL RED. RELATION OF E_0' TO P_H

Determined by separate titrations with chromous acetate. Values used in calculations: $K_0 = 4.79 \times 10^{-7}$; $K_{r1} = 1.10 \times 10^{-6}$; $K_{r2} = 1.12 \times 10^{-5}$. $E_0 = 0.237$. $E_0' = E_0 - 0.03006 \log [[H^+] + K_0] + 0.03006 \log [[H^+]^2 + K_{r2}[H^+] + K_{r1}K_{r2}] - 0.0601\ P_H$.

Buffer	P_H	E_0' found	E_0' calcd.	Found − calcd.
Phosphate	2.127	+0.045	+0.045	0.000
Citrate	2.744	− .013	− .010	− .003
Citrate	3.454	− .073	− .074	+ .001
Acetate	4.624	− .176	− .175	− .001
Acetate	4.978	− .204	− .202	− .002
Citrate	5.242	− .218	− .221	+ .003
Citrate	5.750	− .252	− .253	+ .001
Phosphate	6.080	− .270	− .272	+ .002
Phosphate	6.487	− .293	− .294	+ .001
Phosphate	7.468	− .350	− .350	.000
Borate	8.160	− .392	− .391	− .001
Borate	8.591	− .417	− .417	.000
Phosphate	10.95	− .557	− .559	+ .002

Dimethylaminomethylphenazine of formula II was prepared by Dr. Max Phillips from commercial neutral red chloride



(II)

in accordance with the method of Bernthsen and Schweitzer.[11] The material was crystallized first from dilute ethanol and was recrystallized once from "gasoline" and finally from benzene. The melting point of the glistening red needles agreed with that given by Bernthsen and Schweitzer. A fluorescence develops in the reduced solutions.

The components of the system have such low solubilities that it can be conveniently studied only in solutions of P_H number less than 5, where the salts form. However, the relation of E_0' to P_H in acid solutions has a theoretical interest. The system lacks the amino group found in neutral red. Consequently the reductant does not form a bivalent cation in mildly acid solution and the curve, instead of inflecting to the slope $\Delta E/\Delta P_H = -0.09$, as in the other cases, maintains the slope $\Delta E/\Delta P_H = -0.06$.

While our data are not accurate enough to demonstrate this thoroughly, we support this conclusion as follows. Portions of a mixture of oxidant and reductant, prepared by partial reduction of a slightly acidified solution of the oxidant, were introduced into a series of buffers. The potentials drifted somewhat but fell near a straight line of the specified slope. Data for neutral and alkaline solutions, where precipitates occurred, were neglected.

Independent titrations of the oxidant with chromous acetate gave appreciably varying values of E_0' depending upon the rapidity of operation. We judged E_0' at P_H 2.92 to be +0.031 and at P_H 3.50 to be −0.001. The difference is 0.032 volt against the theoretical value of 0.035, if $\Delta E_0'/\Delta P_H = -0.0601$. E_0 estimated from these titrations would average 0.208. Obviously there would be no object in a comparison[12] between this E_0 and that of neutral red.

Equations.—The data for neutral red and simple neutral red conform to equation (7). This was derived in more comprehensive form by the scheme outlined in the second paper[13] of this series and then simplified by the elimination of dissociation constants which are without significance in the range of P_H employed.

$$E_h = E_0 - 0.03006 \log \frac{[S_R]}{[S_0]} - 0.03006 \log \frac{K_0 + [H^+]}{K_{r1}K_{r2} + K_{r2}[H^+] + [H^+]^2} + 0.03006 \log [H^+]^2 \quad (7)$$

E_h is the observed potential at 30°, referred to the "normal hydrogen electrode." E_0, the "normal potential," is the potential at unit concentration of all represented components. $[S_R]$ is the molar concentration of total reductant. $[S_0]$ is the molar concentration of total oxidant. $[H^+]$ is the hydrion concentration, assumed for the present purposes to be calculable from the P_H numbers.[14]

$$K_0 = \frac{[Ox][H^+]}{[OxH^+]} \quad (8)$$

$$K_{r1} = \frac{[H_2\,Red][H^+]}{[H_3\,Red^+]} \quad (9)$$

$$K_{r2} = \frac{[H_3\,Red^+][H^+]}{[H_4\,Red^{++}]} \quad (10)$$

K_0 for neutral red is assumed to be 1.59×10^{-7}, corresponding to $pK_0 = 6.80$. Kolthoff[15] gives $pK_0 = 6.85$. For simple neutral red $pK_0 = 6.3$. This was determined roughly by the usual colorimetric method at half-transformation. Both of these constants are approximate only. Indeed Mr. T. T. Chen, of this Laboratory, has some evidence that the usual relation between P_H and degree of color transformation is not followed exactly by neutral red. Kolthoff's data suggest this. The subject needs investigation.

The pK values corresponding to the constants listed in Tables IV and VIII are

[12] Among organic systems there are many instances in which "normal potentials" are without concrete significance.

[13] See Clark and Cohen, *Public Health Reports*, **38**, 666 (1923).

[14] There were used the conventions proposed by Clark, "The Determination of Hydrogen Ions," 1928, 3d ed., Chapter 23.

[15] Kolthoff, *Rec. trav. chim.*, [4] **5**, 144 (1924).

Neutral red:	$pK_0 = 6.80$	$pK_{r1} = 6.16$	$pK_{r2} = 5.30$
Simple neutral red:	$pK_0 = 6.32$	$pK_{r1} = 5.96$	$pK_{r2} = 4.95$

The Fluorescent Material.—The fluorescent material has attracted the attention of bacteriologists but, so far as we know, it has not been adequately studied by organic chemists. Rothberger[16] noted that it forms in cultures of *B. coli* more readily than in cultures of *Bact. typhosum*. Although this helpful diagnostic use of neutral red media was found not to be specific, it stimulated many investigations which may be traced through references given by Guerbet,[17] by Geilinger and Schweizer[18] and by Chamot and Sherwood.[19]

From time to time one investigator or another confused the processes leading to the several colors which are produced by various combinations and degrees of acidity, reduction and the change to the fluorescent material. Now that somewhat clearer distinctions are made possible by the methods in use, one can piece together citations from the literature to make a coherent description covering several of our own observations. A review is omitted because of the lengthy qualifications of previous views which would have to be introduced. Suffice it to say that no longer should there be confusion between the yellow oxidant in alkaline solution and the yellow material formed upon reduction. Also it seems evident to us that the yellow fluorescent material formed in reduced solutions of neutral red is distinct from the initial product of reduction and that its formation depends upon the P_H number of the solution. Failure to recognize this has been one of the main sources of confusion.

Preparation of the material in crystalline form was accomplished as follows. Having observed that the fluorescent material may form subsequent to reduction of neutral red by two equivalents of a reducing agent and that it forms readily in the range P_H 4 to 6, we reduced the solution of neutral red while it was buffered heavily in this region. Sodium dithionite ($Na_2S_2O_4$), in excess, was used as the reducing agent. The solution was allowed to stand overnight in order that the conversion to the fluorescent material should be complete.

The fluorescent material precipitates and slowly becomes appreciably crystalline when the solution is made alkaline; but, unlike the crystals of leuco neutral red, those of the fluorescent material may be filtered and washed with water in the presence of air without oxidation being apparent to superficial observations.

After extensive washing with water the material was dissolved in ethanol, filtered and precipitated in crystal form by addition of water.

[16] Rothberger, *Centr. Bakt. Parasitenk.*, 1 Abt., **24**, 513 (1898); **25**, 15 69 (1899).

[17] Guerbet, "Thèse," University of Paris, 1911.

[18] Geilinger and Schweizer, *Biochem. Z.*, **138**, 72, 92 (1923).

[19] Chamot and Sherwood, THIS JOURNAL, **39**, 1755 (1917).

In preparing sample 8 an acetate buffer was used when the reduction took place and the sample was finally crystallized a second time from methanol and water.

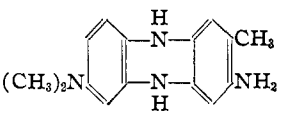
In preparing sample 10 a phosphate buffer was used and the sample was finally crystallized a second and a third time from methanol and water.

The crystals are bars, frequently elongated as "needles," and appear to be orthorhombic. When first isolated these pale yellow crystals have sharp edges and clean surfaces. Placed between crossed nicols, they exhibit their anisotropic nature distinctly. When dried the crystals appear to be checkered. Then, between crossed nicols, they transmit some light at all horizontal orientations of the microscope slide upon which they lie. It is as if the checkered appearance represented a breaking of the crystal with more or less random orientation of parts, the parts preserving the outline of the original unit. Recrystallization of such checkered or crazed crystals yields abundantly the original clean crystals.

Concentrated solutions in alcohols have a color and fluorescence which remind one of a fluorescent machine oil. On dilution the fluorescence becomes brilliant. The material is slightly soluble in water and exhibits a fluorescence easily detected at a dilution of one part in 100,000 parts of water.

Since dithionite was used exclusively as the reducing agent in our preparations it should be mentioned that our first observation of the crystals was in a case where a concentrated solution of neutral red had been reduced by hydrogen and platinum asbestos.

Below are summarized analyses of samples dried over phosphorus pentoxide. For comparison are shown the theoretical values of leuco neutral red on the supposition that it has the structure indicated by formula III.



(III)

Sample	No. 6	No. 7	No. 8		No. 10		Calcd. for $C_{15}H_{18}N_4$
Carbon	..	..	70.6	70.7	70.1	70.4	70.82
Hydrogen	..	..	7.5	7.3	7.4	7.5	7.14
Nitrogen	21.2	21.3	21.3	21.7	21.3		
		21.3	21.6	21.7	21.2		22.04

Samples 5, 8 and 10 were tested for sulfur and halogens. None was found. The analytical results are sufficiently close to the values for the assumed composition to suggest that the discrepancies are due to some slight impurity. In this connection the following facts should be stated. When dried for analysis the crystals are slightly hygroscopic. They exhibit

the checkered appearance noted above. The alcoholic solution leaves on a filter paper an extremely small trace of dark material.

A molecular weight determination was made with diphenylamine as solvent and a ten-pair, copper–constantan thermoelement for the measurement of freezing point depression. Ortho tolidine was used to determine the cryoscopic constant (8.53°). With an error that may have been as great as ±10% we found the fluorescent material to have an apparent molecular weight of 247; duplicate, 248. The theoretical for formula III is 254. Complete protection against oxygen was not attempted but nitrogen was passed over the solution before and during measurements. No color change indicative of oxidation appeared.

In addition to what has already been stated the following facts confirm the supposition that a distinction may be drawn between the fluorescent material and leuco neutral red.

If "leuco neutral red" be formed in a solution of P_H 8.3, where the dilute solution exhibits no fluorescence, and if it then be transposed to a buffer solution of P_H 5 or 6, it becomes transformed into the fluorescent material. When this is carried back to P_H 8.3 it exhibits fluorescence.

With the aid of the hydrogen electrode we attempted to define the titration curve. For various reasons the results were considered untrustworthy. Titrations with the aid of a glass electrode seemed more reliable and yielded the following important results. There is no evidence of a dissociation exponent corresponding to any P_H number between 2 and 7.5. If 254 be the molecular weight, one equivalent of acid (±5%) is neutralized by a basic group functioning at P_H values greater than 7.5. No accurate evaluation of the dissociation exponent was practicable because of the very low solubility of the free base.

Thus it is evident that the two dissociation exponents of leuco neutral red, which were determined, by the method previously described, to be approximately 6.2 and 5.3, have been altered in the transformation of leuco neutral red to the fluorescent material. If corresponding structural groups remain, the dissociation exponent of the one has certainly been increased and the other has presumably been decreased.

There is a remarkable distinction between the fluorescent material and leuco neutral red in ease of oxidation. The colorless solutions formed upon *rapid* reduction of neutral red oxidize very rapidly when exposed to air. Neutral and alkaline solutions of the fluorescent material become oxidized by air with extreme slowness. This distinction is even more remarkable in the case of the solids. A suspension of neutral red base in borate buffer (P_H = 8.2), protected by a stream of nitrogen, was reduced by sodium dithionite ($Na_2S_2O_4$). Portions of the crystals were withdrawn from time to time, were examined under the microscope and were found to retain their colorless appearance if protected by the reducing mother liquor. As soon

as the mother liquor was drained away superficial oxidation by air was almost instantaneous. Some of the crystals, held under nitrogen, were washed with water containing a little dithionite and quickly transferred to a vacuum desiccator. On the following morning when the dry material was exposed to air the oxidation was so vigorous that there was a large rise of temperature and some smoke. A dry sample of the fluorescent material, prepared in 1927 by Dr. Max Phillips, has not changed its appearance within the ensuing years; nor have any of our own preparations during a period of several months shown any change other than the checkering of the crystals noted above. This is probably not due to an oxidative change.

Except in acid solution, admixture of the fluorescent material with neutral red gives uncertain and drifting potentials as if the two materials are of different systems.

One might infer from these contrasts that leuco neutral red becomes transformed irreversibly into the fluorescent compound. On the other hand, the conduct of the isolated, fluorescent material, when placed in *acid* solution, makes this base appear as an "inactive" form of leuco neutral red, possibly slightly impure.

In tenth normal hydrochloric or sulfuric acid the fluorescent material can be titrated with benzoquinone, bichromate or thallic chloride. The electrode potentials attain apparent equilibrium with great rapidity. They are not easily displaced by polarization and at first they seem remarkably steady. Rapid titrations yield data which conform approximately to a typical titration curve involving two equivalents. However, careful study shows two disturbing effects. First, if the solution has been freshly prepared, it is noticed that, as the titration proceeds with any of the mentioned oxidizing agents, the potentials acquire a tendency to drift to more negative values, especially near the apparent end-point. Such a drift persists for hours. Second, if acid solutions of the fluorescent material are held for long periods previous to titration, the titration curve changes its form. The greater part of its course follows the original but the first part now becomes troublesome. This first section suggests the presence of a new and more *negative* system. With its progressive formation the titer of the solution as a whole falls.

These observations with solutions of the isolated fluorescent material clarify difficulties which were encountered in studying acid solutions of neutral red reduced by hydrogen and platinized asbestos. With the varying times of such reductions, changes were sufficiently variable to preclude reproducible results.

In Fig. 1 at P_H 1.05 there is shown by the center of a triangle the average of two values of E_0' obtained by titrations with benzoquinone of a solution of neutral red after reduction by hydrogen and platinum. In the second titration, made a day after the first, the titer had fallen off 30%.

A bichromate titration of a *freshly* prepared solution of the fluorescent material in oxygen-free, approximately tenth-normal sulfuric acid yielded a value of E_0' which fell upon the projected curve of Fig. 1 as shown by the cross. The titration curve conformed fairly closely to the type for two equivalents. Depending upon the rapidity of titration, slight but appreciable differences in the amounts of total oxidizing agent were found. Judging the situation both from the point of view of the apparent end-points and from the analysis of the curves by the method of Reed and Berkson, we can draw one or another of the following conclusions. (1) The amount of fluorescent material used and the amount of bichromate consumed were such that the molecular weight of the fluorescent material is 270; or (2), if the fluorescent material has the molecular weight 254, that of leuco neutral red, it consumed 94% of the theoretical amount of bichromate; or (3), if the fluorescent base is an inactive form of leuco neutral red, 94% had been made active by the acid.

Adopting the last assumption as a working hypothesis we imagined that decreasing proportions would be titratable if P_H were progressively increased. This was tested as follows.

A weighed portion of the fluorescent material was dissolved in slightly more than two equivalents of acid, assuming a molecular weight of 254. Like portions of this solution were added to buffer solutions and after about a half-hour, when the potentials were fairly steady, each solution was titrated rapidly with thallium chloride. Since titrations were made rapidly in an effort to estimate the amounts of more readily available reductant, the data of Table IX are not very accurate. However, it may be said that in the more acid solutions the titration curves appeared to be typical and that the end-points were reasonably good. At the higher P_H values the potentials were never steady and the amounts of oxidizing agent consumed can only be guessed.

TABLE IX

AMOUNTS OF 0.002 N $TlCl_3$ (A) REQUIRED TO REACH APPARENT END-POINTS IN RAPID TITRATIONS OF BUFFERED SOLUTIONS CONTAINING 5 ML. OF A SOLUTION MADE WITH 0.0254 G. OF "FLUORESCENT MATERIAL" PER 100 ML.

P_H	A, ml.	Percentage of possibly theoretical value	Percentage calculated assuming 50% at P_H = 2.4
1.1	4.8	96	95
2.2	3.1	62	61
2.75	1.8	36	31
4.6	(0.6)	(1.2)	0.6
5.3	(0.3)	(0.6)	0.1

Table IX reveals that the crude working hypothesis was confirmed as far as it can be by such an experiment. Table IX also develops a relation that is deceptive in so far as it bears a superficial resemblance to a dissociation curve. It is

$$P_H = 2.4\ (?) + \log \frac{1 - A}{A}$$

where A is the fraction of titratable material.

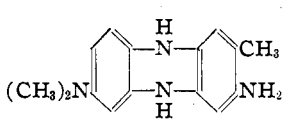
It has already been noted that oxidation of a *freshly* prepared, acid solution of the fluorescent material yields a value of E_0' which is consistent with the supposition that neutral red is the oxidant which is regenerated. This supposition was confirmed as follows. Air oxidation of a *freshly* prepared, acid solution of the fluorescent material yielded a red solution which when diluted to 254×10^{-5} g. of fluorescent material per liter, gave a spectrophotometric absorption curve which was identical in contour and in peak with that of a 1×10^{-5} molar solution of neutral red iodide. A freshly prepared, acid solution of the fluorescent material was subjected to oxidation by air. From this there precipitated on addition of alkali a color base which, when examined as an acid–base indicator, was found to have a half-transformation point at P_H 6.8. This is the value for neutral red.

Discussion

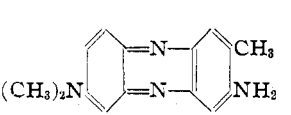
The data reported in the first part of this paper were obtained by avoiding, incompletely but as far as was practicable, the disturbing effects which have been correlated with the formation of the fluorescent material. The data are not all that one would wish them to be as accurately characterizing a reversible oxidation–reduction system; but, if there be a legitimate allowance for the instability of the system, the data are convincing. In particular it may be said that there seems no avoidance of the conclusion that, throughout the range of P_H employed, two paired equivalents are concerned in the reduction. This has an important bearing upon the nature of the reductant. Also the inflections of the E_0':P_H curve are what might reasonably be expected and are comparable with the inflections found in cases for which there are more accurate data.

Since such physical measurements contribute nothing that is compellingly conclusive regarding structure but do contribute suggestions, we shall avoid lengthy comment upon the long-discussed structural problem and shall note the following.

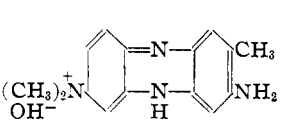
The slope $\Delta E_0'/\Delta P_H$ is -0.0601 in the alkaline region. This implies the fixation of two hydrions when the oxidant is converted to the reductant. Therefore, if leuco neutral red, as the free base in alkaline solution, has the structure indicated by formula III



III



IV



V

neutral red in the same range of P_H is represented by formula IV or V. The dissociation of neutral red as a base is 50% at P_H 6.8. Therefore its dimethylamino group (or possibly its amino group) is a very much more weakly basic group than the similarly situated dimethylamino group in certain analogous compounds.[20] Consequently the "orthoquinone" structure IV seems preferable. This would not preclude each of such tautomers as the ions of IV and V from sharing the field in acid solution.

The curve relating E_0' to P_H approaches rather closely the curve of the hydrogen electrode potential (see Fig. 1). Let the difference between E_0' and the potential of the hydrogen electrode under one atmosphere of hydrogen be found by use of equation (7) and the equation for the hydrogen electrode. Let the difference be E_d. By the "method of maxima and minima" and use of the constants found in Table IV, E_d is shown to be a minimum at P_H 5.8. At this level E_d is only 0.087 volt and the neutral red system at 99.87% reduction exhibits an electrode potential equal to that of the hydrogen electrode under one atmosphere of hydrogen.

Since the system is distinctly more "negative" than any of the indicator systems described in previous articles of this series, it might appear that it is available for several biochemical applications that await the development of just such a reagent. However, the peculiar secondary change virtually places the system among those which are more or less irreversible. With such systems the interpretation of color change in terms of potential cannot easily be made exact. Therefore the neutral red system can be used as an oxidation–reduction indicator only for rough comparisons. One such instance has a special interest.

It has already been noted that Rothberger[16] found cultures of *B. coli* (*Escherichia coli*) to reduce neutral red with consequent fluorescence, while he found that cultures of *Bact. typhosum* do not reduce this dye. Therefore, it appears that *B. coli* can bring about the more intense reduction.

In 1920 one of us, following the work of Gillespie,[21] compared the potentials of platinum electrodes in cultures of several bacterial species.[22] It was found that while electrodes in cultures of *B. coli* can attain and even pass the potential of a hydrogen electrode under one atmosphere of hydrogen, the electrodes in cultures of *Bact. typhosum* definitely fall short of such values.

The correlation between the differentiation by electrode potentials and the differentiation by neutral red is now obvious. In noting this correlation we are quite appreciative of the limitations in definiteness and

[20] See the comparison between the dissociation constants of toluylene blue, Bindschedler's green, Lauth's violet and methylene blue made by Phillips, Clark and Cohen "Supplement 61" to *Public Health Reports*, 1927, and the resulting argument concerning allocations.

[21] Gillespie, *Soil Science*, 9, 199 (1920).

[22] See also Cannan, Cohen and Clark, "Supplement No. 55," *Public Health Reports*, 1926.

accuracy pertaining to each of the methods. Furthermore we would not imply that the differentiation noted would hold under all cultural conditions. Nevertheless, crude correlations such as this have their place in the accumulating body of evidence that electrode potentials in biological systems have significance.

When the "potentials" of bacterial cultures are better known it will be possible to define more clearly the cases in which neutral red may be used in cultures as an indicator of P_H number and the cases where such use is interfered with by reduction.

Our study of the fluorescent material obviously is incomplete, and cannot define the structure. The accepted formula for leuco neutral red affords little opportunity for the postulation of ordinary tautomers or isomers with which to represent structurally the distinctions noted. Of these distinctions one of considerable importance to the structural problem is the shift of dissociation exponents from approximate equality such as might be expected of the nearly symmetrical structure assigned to leuco neutral red, to a disparity such as characterizes the dissociation exponents of neutral red itself. This change and the accompanying appearance of color suggest that the fluorescent material is some modification of leuco neutral red in which a structural asymmetry has developed. There have appeared in the literature accounts of several instances in which two forms of a given derivative of dihydrophenazine occur and attempts have been made to represent these structurally by the migration of a hydrogen atom from a bridging nitrogen to carbon ring. The evidence seems inconclusive. In particular the employment of acetylation and similar methods would, in the present instance, be futile without complete control of the physico-chemical conditions which govern the mutual interconvertibility of leuco neutral red and fluorescent material. Of such conditions we know little except for dilute aqueous solutions.

Extensive deaminization or deep-seated rupture of the molecule seems to be out of the question. We have entertained the assumptions that the "fluorescent material" is a free radical, a hydrazine or a meriquinone. What has been said seems sufficient tentatively to dispose of each of these assumptions without being reviewed with special reference to these assumptions. In speaking of the fluorescent material as an "inactive" form of leuco neutral red, we have used the term only for convenience in describing the following remarkable fact. The isolated fluorescent material when placed in neutral solution will resist oxidation for days. In solid form it will resist oxidation for years. Yet, when placed in acid solution, it becomes subject to rapid oxidation and then yields neutral red.

We thank Dr. Max Phillips and Dr. Barnett Cohen for the materials they supplied. We are particularly indebted to Dr. Leslie Hellerman and Dr. Barnett Cohen for criticism and advice.

Summary

By avoiding so far as possible the disturbing effects of a change taking place subsequent to the formation of a mixture of neutral red and leuco neutral red, there were established potentials apparently characteristic of a reversible system. The "normal potential" at 30° was estimated to be +0.240 volt. The curve relating P_H and the potentials of the half-reduced solutions, E'_0, has a slope $\Delta E'_0/\Delta P_H = -0.09$ in acid solution and a slope $\Delta E'_0/\Delta P_H = -0.06$ in alkaline solution. Closely crowded inflections of the curve in an intermediate zone of P_H indicate dissociation exponents for reductant of 6.16 and 5.30 and for oxidant of 6.80 (approximate only).

Similar approximate data for simple neutral red are: $E_0 = +0.237$; $pK_{r_1} = 5.96$; $pK_{r_2} = 4.95$ and $pK_0 = 6.32$.

For dimethylaminomethylphenazine E_0 is roughly +0.208 and $\Delta E'_0/\Delta P_H = -0.06$ in acid solutions of P_H number < 5.

Interference with stability of potentials is attributed to the formation of a substance which because of its characteristic and brilliant fluorescence is called the "fluorescent material." This was isolated in crystal form. Although it has the elementary composition attributed to leuco neutral red, it differs from leuco neutral red in the values of its dissociation constants and in other particulars. Among its several peculiar properties is its resistance to oxidation in neutral or mildly alkaline solution. In distinctly acid solution it was oxidized to neutral red. Its oxidative titration in acid solution yields a value of E'_0 characteristic of the neutral red system.

Because of the instability of the reversible system, neutral red can be used as an oxidation–reduction indicator only for rough comparisons. One rough comparison is made between the indications of neutral red as an oxidation–reduction indicator and electrode behavior in bacterial cultures. This comparison clarifies the "neutral red reaction" well known to bacteriologists.

The data suggest an "orthoquinone" structure for neutral red base.

BALTIMORE, MARYLAND

NOTE

Preparation of Epichloro- and Epibromohydrins.—In the preparation of 3-chloro- and 3-bromocrotonic acids[1] large amounts of epichloro- and epibromohydrins are required. Because all of the methods known for their preparation in the scientific literature give either low yields or are inconvenient, it was necessary to work out new procedures. The best yield is reported when dichlorohydrin is treated with powdered sodium hydroxide in ethereal solution,[2] but this method is not economical.

[1] Géza Braun, THIS JOURNAL, **52**, 3167 (1930).

[2] "Organic Syntheses," John Wiley and Sons, Inc., New York, 1923, Vol. III, p. 47.