[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Semiquinone Radicals of the Thiazines

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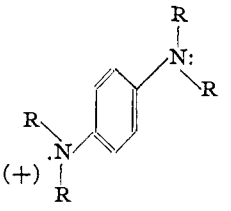
The formation of semiquinone radicals as intermediary reduction products in reversibly reducible dyestuffs, although considered an exceptional phenomenon some years ago, has now been recognized to occur so frequently that the differences in the behavior of various reversible dyestuffs can be looked upon only as quantitative, not qualitative. Among the more familiar classes of reversible dyestuffs it is only the thiazines and oxazines in which formation of semiquinones has not yet been demonstrated experimentally. This paper is to fill in this gap.

The general ideas about stability of semiquinone radicals have been clarified so far that one may start the topic by predicting the conditions under which the formation of semiquinones of these dyestuffs should be most conspicuous and then testing the theory by experiment. To increase the stability of a free radical it has to be put into such a state of its various possible levels of acidic ionization as to bring about equivalent resonance. This term requires some comment.

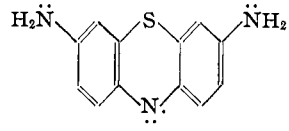
An equivalent resonance is a resonance between two limiting structures which are quite equivalent, that is to say, which, if written by the customary structural formulas, are indistinguishable except for orientation of the molecule as a whole in space. Two types of equivalent resonance may be distinguished. One of them occurs in the regular quinonoid dyestuffs, containing one benzenoid ring (or two) and one quinonoid ring. The resonance consists in the ambiguity as to which ring is the quinonoid one and which is the benzenoid. This resonance stretches over two rings (or three, in the triphenylmethane dyes) and may be designated as quinone–benzene resonance.

The other type of equivalent resonance occurs in the semiquinone radicals. It is restricted, or at least it may be restricted, to a single ring. There is one unpaired electron, and the ambiguity as to the place of this electron on writing down a structural formula is what underlies the resonance. An equivalent resonance occurs when there is a pair of possible structural formulas, or "limiting states," which are indistinguishable. There may be several pairs of equivalent limiting structures as has been recently discussed for Wurster's radicals.[1] An equivalent resonance has a greater stabilizing effect than a non-equivalent one.[2]

In speaking of equivalence, it is not necessary that it refer to the whole molecule. It is sufficient if there is equivalence only with respect to that part of the molecule within which the exchange of electrons resulting in resonance takes place. So, in a Wurster's radical (I), resonance stretches from one N atom across the conjugated double bonds of the ring to the other N atom.[3] The four R's may be different atoms or atom groups without appreciably diminishing the stabilizing of resonance, *provided each N is combined with two R's.*



(I)



(II)

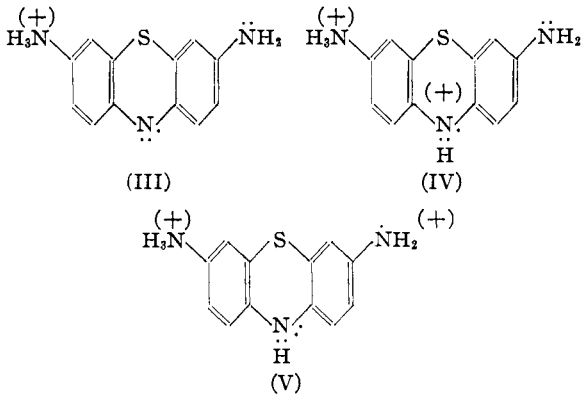
These ideas may be applied to the thionine radical. Its simplest structural formulation is (II), with an odd electron at the N. It resembles the diphenyl-nitrogen radical. In this state, there is no resonance of the equivalent type,[4] since the bridge N and the amino N are bonded to a different number of atoms. Yet equivalence can be produced by adding protons to the N atoms. In a slightly acid solution, one of the two NH_2 groups will attach a proton (III). This does not improve the stability. However, when in a more acid solution a second proton is attached, it will prefer a position such as to bring about equivalent resonance (IV). This structure would be in equivalent resonance with (V), since (IV) can be changed to (V) by moving electrons only, keeping all nuclei in place. Another pair of equivalent resonating limiting states would be (VI) and

(1) L. Michaelis, M. P. Schubert and S. Granick, THIS JOURNAL, **61**, 1981 (1939).

(2) See, for instance, L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, New York, 1939.

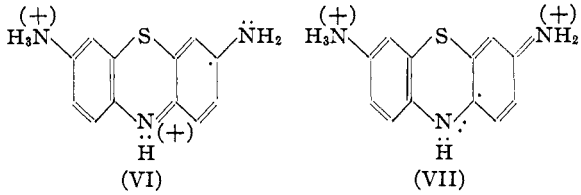
(3) In all formulas, letters stand for atomic kernels, not for full atoms; a dash, or a pair of dots for an electron pair; a single dot is an unpaired electron.

(4) The regular resonance within each benzene ring may be neglected for the present purpose.



(III) (IV) (V)

(VII), which also arise from (IV) by shifting electrons only. The left-hand ring does not take



(VI) (VII)

part in the resonance. The right-hand ring is benzenoid in the pair IV, V; it is quinonoid in the pair VI, VII. Both the resonance IV, V, and the resonance VI, VII, are equivalent, since there are two N atoms attached to the right-hand ring, and each of these N atoms is attached to two more atoms, and there is no steric hindrance with respect to coplanar arrangement. This radical may be considered as a Wurster's radical (I), in which the two right-hand R's are H, and of the two left-hand R's one is H, the other an aromatic ring, attached furthermore by the S-bridge.

For a regular Wurster's radical, a slightly acid solution is sufficient to bring about the state of equivalent resonance, namely, the singly charged cation. For thionine, a doubly charged cation is the necessary condition for equivalent resonance. For this reason a much higher acidity will be requisite to bring about the most stable form of the thionine radical than for a regular Wurster's radical, not only because any second constant of ionization is liable to be weaker than a first one, but also because the basicity of the bridge N atom between two aromatic rings, such as in diphenylamine, is very weak.

It would be interesting to compare this state of affairs for the thiazines with that of indamines and indophenols.[5] This discussion will be reserved for a later review of the whole problem.

(5) G. Schwarzenbach and L. Michaelis, THIS JOURNAL, **60**, 1667 (1938).

The condition predicted for obtaining a possibly stable semiquinone radical from thionine is a rather strongly acid solution. This prediction is confirmed by experiment. The range of acidity necessary for the purpose is rather high, so high indeed that it was never included in the experimental works of previous authors, either for dyestuffs of this class,[6] or for any other, for the obvious reason that extremely strong acid solutions present difficulties with respect to the measurement and even the definition of *p*H. The knowledge of *p*H has so far always been desirable for a rational interpretation in problems concerned with oxidation potentials. We have to work in a range of just such an inconveniently extreme acidity as can no longer be safely measured in terms of the *p*H scale, namely, in 10 to 25 *N* sulfuric acid or in almost concentrated hydrochloric acid. The radical obtained by partial reduction of thionine in such an acid solution is yellow, has very distinct absorption bands (Figs. 5 and 6) in the blue region of the visible spectrum, which fortunately do not overlap with the bands of the oxidized form of the dye.

The degree of separation of the two steps of reduction depends very greatly on the acidity; in a 25 *N* sulfuric acid solution the separation is so complete that the potential curve shows a definite jump at 50% reduction and in the second half of the titration the pure yellow color of the radical appears without being overshadowed by the intense bluish-green color of the quinonoid form. Spectroscopically, the radical can be detected even in mixture with the quinonoid form under not too unfavorable conditions. At the end-point of reductive titration all color disappears, the leuco dye being colorless.

The radical derived from methylene blue is spectroscopically quite similar to that of thionine. Its formation, at least to an easily recognizable extent, requires a still higher acidity than in the case of thionine. It is striking that the methylation of the amino groups of thionine does not cause any distinct shift of the absorption bands of the radical.

As acidity decreases, the formation of the radical is very rapidly diminished. However, even in neutral solution it never vanishes entirely. Here, the radical must have a structure lacking equivalent resonance, and be less stable. Yet

(6) W. M. Clark, B. Cohen and H. D. Gibbs, *Publ. Health Report*, **23** 1131 (1925).

even then it is not a labile molecule inclined to undergo irreversible destruction, but perfectly stable in time to such an extent as is compatible with its equilibrium with the quinonoid dye and the leuco dye. The smallness of the concentration of this form of the radical in equilibrium with the large amount of an intensely colored substance would be sufficient to make its detection by optical methods difficult. Moreover, this form of the radical having no equivalent resonance may not be expected to exhibit any, or at least any appreciable, absorption in the visible spectrum.

Oxazines seem to behave quite similarly. Preliminary optical observation on commercial Capri blue, in the partially reduced state, showed absorption bands quite similar to those of the thiazines, but displaced toward longer wave lengths. The degree of acidity to attain this condition must be still higher.

Thiazines, particularly methylene blue, have been very much used as catalysts for oxidation, especially respiration. They increase the rate of respiration of slowly respiring cells such as erythrocytes. It has been the tendency of the authors to correlate such an effect of a dye with its faculty of being reduced in successive univalent steps.[7] Such an occurrence had not yet been demonstrated previously for the thiazine dyes. Now the suggestion concerning the mechanism of the catalytic effect of these dyestuffs is supported.

Experimental Part

1. **Preparations.**—Thionine (Lauth's violet), was prepared by dissolving 18 g. (0.1 mole) of *p*-phenylenediamine dihydrochloride in 3 liters of cold 3 *M* hydrochloric acid saturated with hydrogen sulfide. Half of a solution of 180 g. of ferric chloride hexahydrate in 400 cc. of water was stirred in, the mixture aerated to remove hydrogen sulfide and then the other half of the ferric chloride solution added. The mixture was set on ice, the precipitate filtered off and extracted with 1600 cc. of boiling water. The filtered solution is chilled and 40 cc. of 6 *M* hydrochloric acid is added, precipitating the product in crystalline condition. The product is recrystallized similarly, yield 2.5 g. The product is dried in a desiccator over calcium chloride, ground up finely and again dried over calcium chloride at 1 mm. pressure. *Anal.* Calcd. for $C_{12}H_{10}N_3SCl\cdot H_2O$: N, 14.91; S, 11.37; Cl, 12.60. Found: N, 15.03; S, 11.43; Cl, 12.37. Nitrogen by micro Dumas gave very low results but Kjeldahl determinations were satisfactory when stannous chloride was added before the digestion.

Methylene blue is prepared similarly except that 20 g. (0.1 mole) of *p*-aminodimethylaniline is dissolved in 1 liter of cold 3 *M* hydrochloric acid. The dye is salted out at the end of the reaction with 300 g. of sodium chloride. It is recrystallized twice from 400 cc. of boiling water, yield 4 g.

The essential claim as to the purity of methylene blue is that it should not be contaminated with less methylated thiazines which easily arise from it in alkaline solutions. The non- or less-methylated thiazines not being quaternary bases, can be extracted from an alkaline aqueous solution by benzene, staining it red. If a methylene blue solution is made alkaline with sodium hydroxide, it stains benzene red because it quickly decomposes. On choosing a proper *p*H one can obtain a condition where the free base of the less methylated compounds is extractable and still no decomposition of methylene blue is noticed for a sufficiently long time. When a 1 to 1000 solution, say of thionine or toluidine blue, in phosphate buffer of *p*H 7.5 is extracted with an equal volume of benzene by vigorously shaking for ten seconds, the benzene, after separating it from the aqueous phase, is pink. When methylene blue is treated in the same way the result depends on its purity. The best preparation obtained from outside (kept in the laboratory for some years) produced a very faint pink tint; our own preparation left benzene quite colorless. In a mixture of our preparation of methylene blue with commercial toluidine blue the latter could be detected easily in a proportion 1 to 50. At 1 to 100 the test was doubtful.

Every author working with methylene blue has had trouble in obtaining a really satisfactory elementary analysis. This may be due to the fact that the water of crystallization easily changes from 1 to 2 molecules, perhaps also 3.

Well ground samples were dried in vacuum over calcium chloride but difficulty was encountered on attempting to weigh out samples as moisture was rapidly absorbed, so the product was allowed to come to equilibrium with the air for a day. Then analysis agreed best with a dihydrate. *Anal.* Calcd. for $C_{16}H_{18}N_3SCl\cdot 2H_2O$: N, 11.80; Cl, 9.97; S, 9.00. Found: N, 11.31; Cl, 10.18; S, 9.19. Some of this material was converted to the iodide by dissolving 1.5 g. in 200 cc. of warm water and adding a solution of 7 g. of sodium iodide in 50 cc. of water. The crystalline product was recrystallized from 400 cc. of hot water. *Anal.* Calcd. for $C_{16}H_{18}N_3SI\cdot 2H_2O$: N, 9.40; I, 28.40; S, 7.16. Found: N, 9.24; I, 28.42; S, 7.45.

2. **Technique of Potentiometric Titration.**—Working with extremely acid solutions entails the difficulties ensuing from the liquid junction potential in a much higher degree than usual. The easiest method of examining the drift of the liquid junction potential in time is this. The potential of an acid solution such as 10 *N* sulfuric acid, at the hydrogen electrode, against a potassium chloride saturated calomel half cell is measured. The contact is established by an agar bridge such as presently will be described (Fig. 1). The end of the agar bridge is first kept above the level of the acid solution, hydrogen is bubbled for a sufficient length of time, then contact is established by pushing the agar bridge down. Any drift of the potential can be ascribed entirely to the drift of the liquid junction potential. The observations made in this way caused us to adopt the following procedure as a routine method.

(7) L. Michaelis and C. V. Smythe, *Ann. Rev. Biochem.*, 1938.

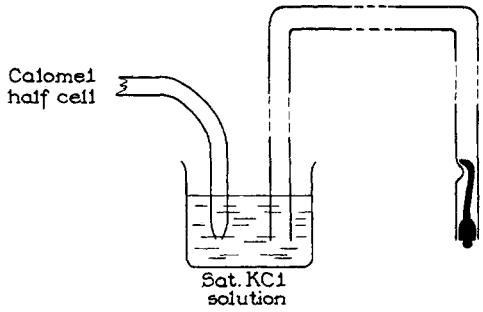


Fig. 1.—Agar bridge.

The calomel half cell and the acid solution are connected by an agar bridge. This consists of a cylindrical, properly bent glass tube, the ends not tapering. At the time when the tube was being filled with the hot liquid agar (3% agar, saturated with potassium chloride), the one end, to be used for contact with the solution in the electrode vessel, had been tightly closed with a ground glass stopper so as to leave only a minute capillary cylindrical agar film for contact. The stopper is locked by a hook fitting into a groove of the tube. This kind of agar tube has been used for many years in this Laboratory. When a freshly prepared agar tube is used as a bridge for extremely acid solutions, there is an enormous drift of potential in the negative direction. After a few hours, the drift becomes much smaller. Now the agar tube is kept for a day under saturated potassium chloride solution. When being used again, the drift subsides after fifteen to thirty minutes so much as to amount to about only one millivolt or even much less, during the period of time carrying the titration from 25 to 75% reduction. Such an agar tube, if kept under saturated potassium chloride solution when not in use, can be used over and over again. To be sure, it never will be in the same condition in parallel experiments but each experiment yields a satisfactory titration curve except for the fact that any reference to a standard potential is impossible. Neither can the oxidation-reduction potential be referred to that of the normal hydrogen electrode, nor can the potential of the solution at the hydrogen electrode be used for the computation of *p*H. We shall, for obvious reasons, use as reference point for each titration curve the potential at its 50% point of reduction. No other specification is required in order to derive the semiquinone formation constant k from the shape of the titration curve. The acidity will be expressed in terms of the normality of the acid, whereby of course one renounces any rational statement as to the relationship of acidity to semiquinone formation constant. What is certain from the experimental data is only that k becomes larger with increasing acidity. In 26 N sulfuric acid, k attains the very high value of 1000; with decreasing acidity it rapidly decreases to values < 1.

The reducing agent used for titrating the dye in such acid solution was titanous chloride. For instance, 0.07 cc. of the commercial solution of titanous chloride in strong hydrochloric acid was diluted with 10 cc. of sulfuric acid of the same concentration as the dye solution; the concentration of acid was varied from 9 to 26 N sulfuric acid. Titrations were carried out in a stream of purified nitrogen.

The other titrations, within the usual range of *p*H, were performed as usual. A very good method appeared to be reductive titration with leuco-Rosindulin GG. If conditions of solubility permitted, it was dissolved in a buffer of the same kind as used for the dye to be titrated; otherwise it was dissolved in pure water to such a concentration as to change the volume of the solution during the titration by not more than 2%, rendering any change in *p*H or ionic strength during the titration negligible.

Results

The results obtained in strongly acid solutions are best presented in the form of diagrams (Figs. 2, 3, 4) with their legends. It is clearly seen that the potential curves become steeper with increasing acidity and, from a certain acidity on, there appear in addition to the point of inflection in the midpoint, two more inflection points, symmetrically located. An acidity higher than 26 N sulfuric acid cannot be used because in this case the end-point becomes unsharp due to overlapping of the potential of the dye with that of the reducing agent. When there is no such overlapping, the curves are symmetric around the midpoint of titration. Figure 2 shows that the shape of the curve remains entirely unchanged by varying the initial concentration of the dye in the ratio 1 to 4. This is evidence for the fact that the intermediate compound is a free radical, and that no dimerization of the radical takes place, at least in the concentration range used.

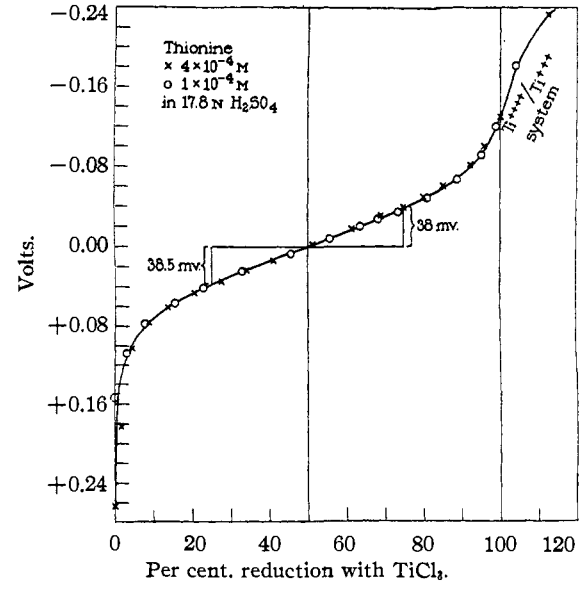


Fig. 2.—Showing independence of potential of the initial concentration of the dyestuff.

According to theory[8] there should be only one

(8) L. Michaelis and M. P. Schubert, *J. Biol. Chem.* **119**, 133 (1938).

point of inflection at 50% reduction, whenever the semiquinone formation constant k is $\leqq$ 16 or, what is the same, whenever the index potential E_i is $\leqq$ 40 millivolts. This expectation is verified as Fig. 3 shows. This is another evidence in justification of the interpretation of the titration curves.

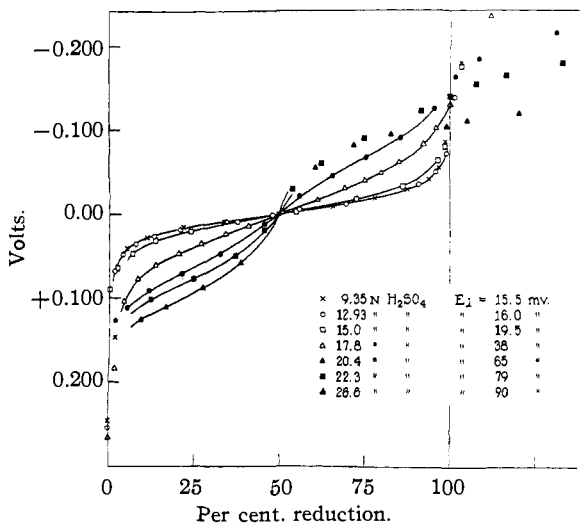


Fig. 3.—Reductive titration curves of thionine in sulfuric acid of varied normality. Reduction by titanous chloride dissolved in sulfuric acid, of the same normality as the dye solution. The drawn out curves are interpolations between all acceptable experimental points. Acceptable points are those in which no overlapping with the potential range of Ti^{++++}/Ti^{+++} occurs. The more concentrated the acid the closer are the potential ranges of Ti and the dye. Appreciable overlapping takes place only for the two highest concentrations of the acid and even here only in the second half of titration. All the other curves show sharp end-points of titration. The point × at 50.0% reduction stands not only for 9.35 N sulfuric acid, but also for 26.6 N. The flattest curve is the one for 9.35 N sulfuric acid. The points for 12.93 N sulfuric acid are distinguishable from the latter on a plot of larger scale. In the scale used here they are so close that no special curve for them has been drawn to avoid crowding. The index potentials, E_i, of the left and the right halves of each curve are equal to each other, in the worst case within one millivolt. For the two highest concentrations of sulfuric acid only the left hand E_i can be determined, and this is possible only because here the 50% point of titration is recognizable due to the fact that the point of inflection at 50% is very distinct. Lateral points of inflection, in addition to the point of inflection at 50% reduction, according to theory, should occur only if $E_i >$ 40 mv. This expectation is verified here.

The results obtained in the ordinary range of pH as established by buffers are shown in Tables II and IV. All titration curves were symmetric around the midpoint of titration, indicated by the fact that the difference of potential at 25% reduction and 50%, and the difference between 50 and 75%, are equal within at least three-tenths of a millivolt and usually better. The symmetry obtains not only from 25 to 75% but throughout the whole potential range as far as quickly established and reproducible potentials are obtained.

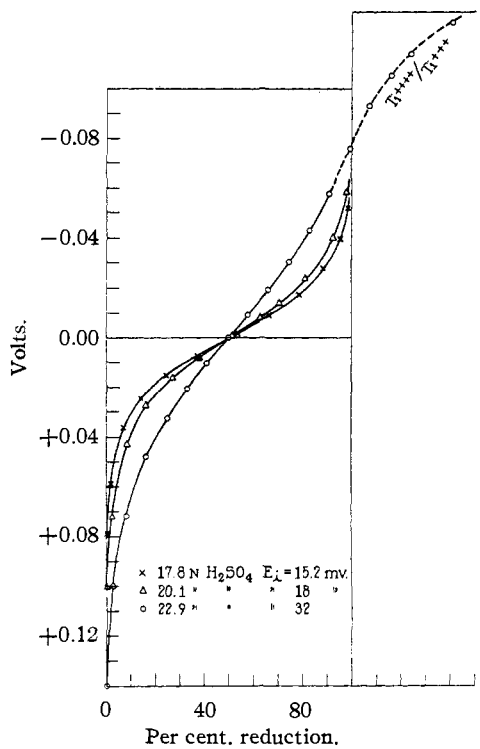


Fig. 4.—Reductive titrations of methylene blue in sulfuric acid of varied normality. Reduction by titanous chloride. The curves become steeper with increasing acidity. To reach the same degree of steepening, a higher concentration of acid is needed than for thionine. Even for the highest concentration of acid used here, the separation is not great enough to cause the appearance of the two lateral points of inflection in accordance with the fact that E_i does not reach the value 40 mv. Still higher concentrations of acids cannot be used because the overlapping of the potential ranges of the dye and Ti becomes too great. For all lower concentrations of acid, the end point is sharp.

This shows that the specimens of the dyes are not contaminated with any measurable amount of some other dyestuff of slightly different normal potential, in which case the overlapping would cause asymmetry. Furthermore, it shows that the molecular size of the oxidized and the reduced form is the same. The fact that all index poten-

TABLE I

REDUCTIVE TITRATIONS IN STRONGLY ACID SOLUTION OF THIONINE, 2×10^{-4} M WITH $TiCl_3$, AT 30°

H_2SO_4, N	Index potential, E_i, mv., from 25 to 50%	50 to 75%	Semiquinone formation constant, k	Ratio of semiquinone to total dye in the half-reduced state, $(s/a)_{max.}$	Spect. evidence for the semiquinone in the partially (about 80%) reduced state
9.35	15.5	15.3	0.02[a]	0.06[a]	No bands visible
12.9	16.0	16.0	.05	.10	Faint bands just visible in the spectroscope
15.0	19.3	19.7	.35	.24	Bands visible and color of radical just noticeable by the unaided eye
17.65	31.4	32.8	6	.55	
17.80	38.0[b]	38.5[b]	14	.64	
	39.0[c]	39.0[c]			Bands visible in spectroscope, and yellow color visible with unaided eye
20.4	64	66	160	.81	
22.3	78	80	420	.85	
26.6	95	85	1000	.94	

[a] These figures may be too small if there were any drift of liquid junction potential during the titration even to the amount of only a few tenths of a millivolt. For all the other experiments a similar error in E_i would influence the evaluation of k and $(s/a)_{max.}$ only very slightly. [b] Concentration of dye 1×10^{-4} M. [c] 4×10^{-4} M.

TABLE II

REDUCTIVE TITRATION OF THIONINE WITH LEUCOROSINDULIN GG IN VARIOUS BUFFERS AT 30°

Concn. of thionine $\times 10^4$ M	Buffer	pH	E_m	E_i	
1.0	2.5 cc. 1 N HCl + 25 cc. 0.2 M KCl to 50 cc. total vol.	1.41	+0.1967	14.6	15.0
1.0	Citrate	1.79	+0.1622	14.9	15.2
1.0	Citrate	2.07	+ .1375	14.9	15.2
1.0	Acetate	3.76	− .0141	15.5	15.5
0.2	Acetate	4.62	− .0768	16.1	15.7
.8	Acetate	4.62	− .0785	15.7	15.7
2.0	Acetate	4.62	− .0788	15.5	15.6
2.0	Acetate	4.62	− .0796	15.7	15.5
1.0	Acetate	4.77	− .0882	15.8	16.0
1.0	Acetate	5.34	− .1208	14.7	15.0
2.0	Phosphate	6.91	− .1805	15.7	(15.8)[a]
0.7	Phosphate	7.08	− .1817	(14.8)[b]	(14.8)[b]
.7	Phosphate	7.48	− .1950	15.0	(15.5)[b]
.2	Veronal	7.83	− .208	Slight drifts, impairing accuracy of E_i	
.3	Veronal	8.03	− .210		
.2	Veronal	8.22	− .2198		

[a] Not quite trustworthy because of beginning precipitation of the leuco dye. [b] Not trustworthy because of slight drift due to insolubility of leuco dye.

TABLE III

REDUCTIVE TITRATIONS IN STRONGLY ACID SOLUTIONS OF METHYLENE BLUE 2×10^{-4} M WITH $TiCl_3$, AT 30°

H_2SO_4, N	Index potential, E_i		Semiquione formation constant, k	Ratio of semiquinone to total dye in the half reduced $(s/a)_{max.}$	
17.8	14.7[a]	14.5[a]	0.001[a]	0.015[a]	No optical evidence for radical
20.1	18.0	17.8	.25	.20	Yellow color just visible near the end of titration
22.9	31.0	32	6	.55	Yellow color directly visible in the second half of titration

[a] These figures may be too small; see footnote (a) to Table I.

TABLE IV

REDUCTIVE TITRATION OF METHYLENE BLUE WITH LEUCOROSINDULIN GG IN BUFFER SOLUTION, AT 30°

Concn. of methylene blue $\times 10^4$	Buffer	pH	E_m	E_i	
1.0	Citrate	1.85	+0.1240	16.6	16.8
1.0	Citrate	3.68	− .0395	16.4	16.4
1.0	Acetate	3.78	− .0482	16.2	16.0
1.0	Acetate	4.42	− .1027	16.2	16.2
0.5	Acetate	4.62	− .1182	15.6	15.6
1.0	Veronal	7.92	− .2632	(14.3)	Not reliable, drift toward the end

tials are always in the neighborhood of their possible minimum values of 14 millivolts and never decrease to any lower value shows that both the dye and the leuco dye are always present in the monomeric form and no measurable polymerization either of the dye or of the leuco dye occurs within the low concentration range chosen. This fact is important to emphasize since there are some observations, spectrophotometric by Holmes,[9] for a great number of dyestuffs, both spectrophotometric and potentiometric for thiazines and oxazines by Clark and Cohen[10] and by Cohen and Preisler[11] which have been tenta-

(9) W. C. Holmes, *Ind. Eng. Chem.*, **16**, 35 (1924).

(10) W. M. Clark and B. Cohen, *Publ. Health Rep.*, **40**, 1131 (1925).

(11) B. Cohen and P. W. Preisler, *Publ. Health Rep.*, Suppl. No. 92 (1931).

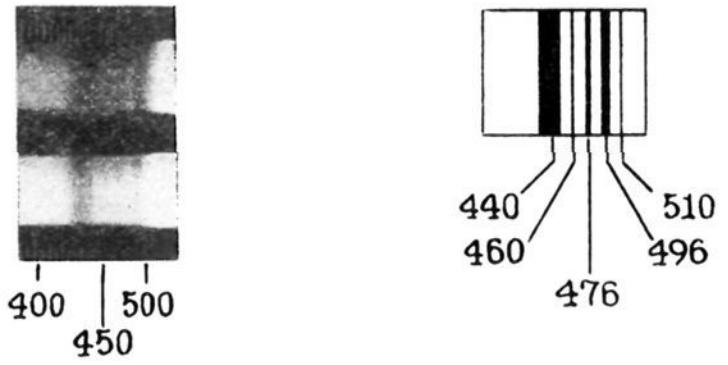


Fig. 5.—Absorption bands of the semiquinone of thionine, as existing in 20 N sulfuric acid, the dye being reduced to approximately 75% by titanous chloride. The two spectrum photographs (by Dr. Lavin) are with various lengths of exposure. The other spectrum is a schematized sketch to show the finer structure more clearly than in the photograph. Wave lengths are in mμ. On direct visual inspection with the hand spectroscope, the 496 band is strongest rather than the 440 band.

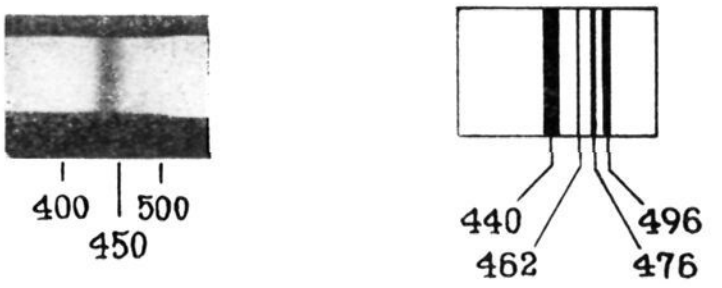


Fig. 6.—Absorption bands of the semiquinone of methylene blue. Conditions were the same as in Fig. 5.

tively interpreted as evidence that the dyestuffs in higher concentration might partially form higher molecular aggregates. This is certainly not so for thionine and methylene blue in such a concentration range as used in our experiments.[12] The potentials are stable, there is no evidence for any decomposition, not even for methylene blue, except in alkaline solution, where the accuracy of the experiments with this dyestuff suffers anyhow from the extremely slight solubility of the leuco dye. The index potential both for thionine and methylene blue in solutions from pH 4 to 7 may be estimated as about 16 millivolts; in any case decidedly not less than 15 mv. Using the methods of calculation previously described,[13] the relationship between the index potential E_i, the semiquinone formation constant k, and the maximum fraction of semiquinone to total dye is

E_i	= 15.0	15.5	16.0
k	= 0.01	0.02	0.05
$s/a_{max.}$	= .05	.065	.10

In a very wide pH range around neutrality, the dye in its half-reduced state contains not less than 5%, probably approximately 10%, as semiquinone. This should be considered as ample to account for the efficiency of the radical in establishing the reversibility of the system and its catalytic effect. It is in agreement with the behavior of other cationic dyestuffs presenting more favorable experimental conditions that k, though rapidly diminishing with increasing pH, does not vanish entirely, but remains constant from a certain pH on. This is due to the fact that the convergence of the E_1 and E_2 curves (normal potential of the lower, and of the higher step of oxidation) with increasing pH gives way to parallelism at the pH corresponding to an ionization constant of the radical.

As additional evidence that index potentials close, but not quite equal, to the theoretical minimum value of 14.3 millivolts are trustworthy within a few tenths of a millivolt, a titration experiment with a univalent oxidation system was performed in which according to the theory no other E_i value than 28.6 mv. can ever be obtained and any deviation must be ascribed to technical insufficiency. A solution of 3×10^{-4} gram atom of iron per liter in the form of ferric ammonium sulfate in a solvent 0.01 M in hydrochloric acid and 1 M in a neutral salt (potassium chloride in one experiment, sodium chloride in another) was titrated with leuco-rosindulin, dissolved in water, in such a concentration that the titration from 25 to 75% reduction required as little as 0.7 cc. for 50 cc. of the solution to be titrated. The change in ionic strength caused hereby during the titration may be considered as quite negligible. The graphic evaluation of the index potentials was as shown; a and b are compu-

	E_i			
	a		b	
Expt. with 1 N KCl	28.6	28.6	28.5	28.7
Expt. with 1 N NaCl	28.6	28.3	28.9	28.7

tations based on different estimations as to the endpoint of titration, this difference being taken as

(12) It can be confirmed that even in a range of low concentrations (from 5×10^{-6} to 150×10^{-6} M) Beer's law is not obeyed. In low concentration, thionine has essentially one band at 593 mμ; and it emits a fluorescence band at about 640. As concentration increases, gradually a second band around 556 develops, and fluorescence diminishes. In alcohol, the effect of increased concentration, both on the decrease of fluorescence, and on the development of the second band, is quite considerably weaker. There is some intermolecular interaction, stronger in water than in alcohol, to the effect that the probability of an unexcited molecule absorbing 556 is increased, as the probability of an excited molecule emitting 640 is decreased. This phenomenon should not be correlated with any change in size of the molecular unit of the dye. This, of course, can be asserted only for our dyestuffs and within the concentration range used.

(13) Summarized by L. Michaelis and M. P. Schubert, *Chem. Rev.*, **22**, 437 (1938).

large as was reasonably compatible with the experimental data. These experiments suggest that any deviation of the index potential exceeding by 2 or 3 tenths of a millivolt its expected theoretical minimum value may be taken as real.

It should now be our task to plot the three normal potentials against *p*H, showing all bends corresponding to the various ionization constants of the dye in each of its three levels of reduction, as shown in many previous cases. This cannot be accomplished satisfactorily because one cannot express either the acidity of highly concentrated sulfuric acid solutions in terms of *p*H or refer the normal potentials to the standard hydrogen electrode. Here we are faced with a problem of a much more general scope, concerning both the scale of oxidation–reduction potentials, and the scale of acidity, to which we intend to devote a special study.

Summary

A semiquinone radical is shown to exist as a univalent reduction product for thionine and for methylene blue. In equilibrium with the dye and the leuco dye, it can exist only to a few per cent. of the total dye *in maximo* over the whole *p*H range covered by the customary buffers. In very strongly acid solution, such as from 10 to 26 *N* sulfuric acid, it is stable to a much higher extent and can be identified by an analysis of the reductive titration curve of the dye and by its color, which is yellow with distinct absorption bands in the blue. The semiquinone formation constant k reaches for thionine the value 1000 in 26.6 *N* sulfuric acid but rapidly decreases to values < 1 in less acid solutions. For methylene blue, this constant reaches in 22.9 *N* sulfuric acid only the value 6. In a wide *p*H range around neutrality, k is for both dyestuffs approximately 0.05. The greater stability of the radical in very acid solution is ascribed to the formation of a bivalent cation having a structure, exhibiting equivalent resonance, of the same type as a Wurster radical.

The existence of this radical is correlated with the reversibility of oxidation–reduction of thiazines, and with their catalytic effect in biological oxidations.

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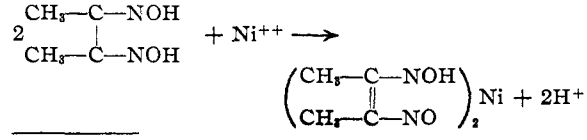
[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE INSTITUTE OF TECHNOLOGY OF THE UNIVERSITY OF MINNESOTA]

Amperometric Titrations. II. The Titration of Nickel with Dimethylglyoxime Using the Dropping Mercury Electrode as Indicator Electrode

BY I. M. KOLTHOFF AND A. LANGER

In a previous paper[1] the general characteristics of amperometric titrations and the titration of lead with dichromate or chromate have been described. It is expected that many of the modern organic precipitants will be useful in the amperometric titration of several metals, especially since many of these organic reagents are specific for a limited number of inorganic ions. In the present paper the amperometric titration of nickel with dimethylglyoxime is described, using the dropping mercury electrode as indicator electrode. In ammoniacal and weakly acid medium dimethylglyoxime reacts with nickel to form the insoluble red, microcrystalline precipitate of nickel dimethylglyoxime.



(1) I. M. Kolthoff and Y. D. Pan, THIS JOURNAL, 61, 3402 (1939).

Besides nickel, only palladium and platinum form slightly soluble compounds with dimethylglyoxime. Several metal ions, such as cobalt and ferrous iron, react with the glyoxime to form soluble complexes and may interfere in the titration of nickel. The interference by other metals has been considered in this paper.

Experimental

The general set-up was similar to that described in a previous paper.[1] The current was measured with a d'Arsonval galvanometer, the sensitivity of which could be changed with an Ayrton shunt. In a few instances a microammeter was used. A saturated calomel electrode connected with the titration cell by means of an agar salt bridge served as an external anode. The applied e. m. f. was measured with the aid of a potentiometer. In most practical work, in which the applied e. m. f., as a rule, has to be known only within 0.05 to 0.1 volt, a suitable voltmeter can be used instead. The reagent was added from a calibrated microburet of the shape given in Fig. 1. The arrangement allowed the storage and the introduction of