620. *The Polarographic Reduction of Some Natural and Artificial Hydrogen-carriers in Bacterial Enzyme Systems.*

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Cozymase, riboflavin, methylene-blue, Capri-blue, and cresyl-blue in aqueous solution at pH 7·38, and Nile-blue in 50% ethanol at pH 8·29, undergo polarographic reduction at 25° in two overlapping one-electron stages, with the intermediate formation of semiquinones whose stability is low compared with that of the semiquinones of several antibacterial aminoacridines and di- and tri-phenylmethane dyes. The polarographic results regarding semiquinone formation with cozymase and methylene-blue agree with those of earlier potentiometric studies, justifying the use of the polarograph for investigations of semiquinone formation.

The results suggest that the above and other substances which may act as hydrogen carriers or respiratory catalysts in bacterial enzyme systems function by participation of labile semiquinone radicals in radical-chain reactions in bacterial respiration, whereas the antibacterials mentioned above function by reaction with a chain-carrying radical to form a highly stable semiquinone, whereby the chain is broken.

RIBOFLAVIN, a naturally occurring hydrogen carrier in bacterial enzyme systems, undergoes potentiometric reduction in two overlapping one-electron steps, with the intermediate formation of a labile semiquinone (Stern, *Biochem. J.*, 1934, **28**, 949; Michaelis, Schubert, and Smythe, *J. Biol. Chem.*, 1936, **116**, 587; Haas, *Biochem. Z.*, 1937, **290**, 291). Haas also reported that the attachment of riboflavin to its protein carrier in enzymes favoured semiquinone formation. Similarly, Michaelis, Schubert, and Granick (*J. Amer. Chem. Soc.*, 1940, **62**, 204) showed potentiometrically that thionine and methylene-blue, both of which may function as artificial hydrogen carriers in bacterial respiration, are reduced in two overlapping one-electron steps, the intermediate semiquinones having formation constants $K \approx 0·05$ at pH $\approx$ 7, although K for thionine increased considerably in acid solutions because the semiquinone, after addition of a proton, was stabilised by equivalent resonance.

The object of the present work was to verify that the polarographic method yielded K values for some natural and artificial hydrogen carriers comparable with those obtained potentiometrically, and then to investigate polarographically the stability of semiquinones formed by other hydrogen carriers. We have therefore studied the polarographic reduction at pH $\approx$ 7 of the natural hydrogen carriers cozymase and riboflavin, and the artificial hydrogen carriers methylene-blue and oxazines Capri-blue (Colour Index No. 876), Nile-blue (C.I. No. 913), and cresyl-blue (C.I. No. 877). We were unaware of the previous polarographic investigation of methylene-blue and riboflavin by Brdička (*Z. Elektrochem.*, 1941, **47**, 721; 1942, **48**, 278, 686) until after this work was completed. However, Brdička was concerned with adsorption effects at the dropping mercury electrode, and not greatly with K values.

EXPERIMENTAL

Cozymase (from Schwarz Laboratories Inc., New York) was of 60% purity; the impurities did not apparently give a polarographic reduction wave. Riboflavin and methylene-blue were of B.P. purity. Nile-blue was recrystallised from dilute hydrochloric acid, and cresyl-blue from ethanol. Capri-blue was too soluble to be recrystallised from water or ethanol; it was purified by being salted out from aqueous solution with sodium chloride and dried at low temperature, whereafter the dye was extracted from any sodium chloride present by absolute ethanol, the extract was evaporated, and the residue dried at low temperature (to avoid tar formation). The purification of the last three dyes was followed polarographically, the wave-height for constant weight concentration increasing progressively.

Apparatus, technique, and other experimental details were as described previously (*J.*, 1951, 27). Potentials were measured at 25° against the saturated calomel electrode.

RESULTS

Cozymase.—At concentration $c = 0.2$ millimole/l. (after allowance for the 40% of impurity) in aqueous phosphate buffer of pH 7·38, a single reduction wave (curve I) was obtained, with half-wave potential $E_m = -0.927$ v, wave-height $i_d = 0.4$ μa (corr. for residual current), and index potential $E_i = 0.0345$ v (difference between the dropping mercury electrode potentials at 50% and 25% or 75% reduction). The slight abnormality near the top of the wave might be due to adsorption at the mercury electrode, as with 2 : 8-diaminoacridine (*J.*, 1951, 2638), or to reduction of some impurity; since supply of cozymase was limited, this was not further investigated. Comparison of $i_d/cm^{\frac{2}{3}}t^{\frac{1}{6}} = 605$ $nD^{\frac{1}{2}}$ (Ilkovič, *Coll. Czech. Chem. Comm.*, 1934, **6**, 498) with the value for acridine (*J.*,
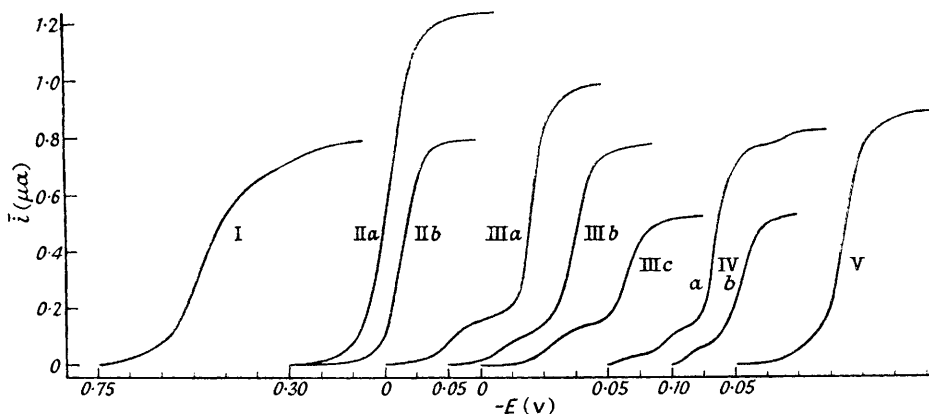
Polarograms for (I) *cozymase*, ca. 2×10^{-4}M, pH 7·38 *in water* (*current scale half that shown*); (II) *riboflavine*, 4×10^{-4}M, (a) pH 7·38 *in water*, (b) pH 8·29 *in 50% ethanol*; (III) *methylene-blue*, (a) 4×10^{-4}M, pH 7·38 *in water*, (b) 4×10^{-4}M, pH 8·29 *in 50% ethanol*, (c) 2×10^{-4}M, pH 7·38 *in water*; (IV) *Capri-blue*, ca. 4×10^{-4}M, (a) pH 7·38 *in water*, (b) pH 7·32 *in 50% ethanol*; (V) *cresyl-blue*, 4×10^{-4}M, pH 7·38 *in water*.   *Voltage scale intervals* 0·05v.

1951, 27) suggests that, despite the fact that the diffusion coefficient D of cozymase is unknown, n (the number of electrons involved per molecule reduced) $= 2$ (in the above equation, $m = $ mg. of mercury flowing per sec., and $t = $ drop-time in sec.). On this assumption, substitution of the above E_i value in Michaelis's equation (*Ann. N.Y. Acad. Sci.*, 1940, **40**, 39)

$$K^{\frac{1}{2}} = 10^{E_i/0.0591} \quad -3 \times 10^{-E_i/0.0591}$$

gives $K = 9.3$. Thus reduction occurs in two overlapping one-electron steps, with the intermediate formation of a semiquinone whose stability is relatively low compared with the semiquinones of the acridines and di- and tri-phenylmethane dyes listed in the Table. The existence of semiquinone is confirmed by the appearance of a transient yellow colour, attributed to monohydrocozymase, lasting not longer than 30 seconds, during the reduction of cozymase with sodium dithionite in boiling water (Gutcho and Stewart, *Anal. Chem.*, 1948, **20**, 1185).

Riboflavin.—A 4×10^{-4}M-solution in aqueous phosphate buffer at pH 7·38 gave a single wave (curve IIa), with $E_m = -0.460$ v and $i_d = 1.34$ μa. It being assumed, in the absence of data, that D is approximately the same as for acridine, comparison of i_d/c $m^{\frac{2}{3}}t^{\frac{1}{6}}$ values gives $n = 2$ (nearest integer). Hence, since $E_i = 0.023$ v, $K = 1.49$. Thus again we have two overlapping one-electron steps and a labile semiquinone. The inclusion of 50% by volume of ethanol in the buffer decreased i_d and raised the apparent pH to 8·29, but caused little change in E_i or K (curve IIb).

Methylene-blue.—This was examined over the concentration range 2—8 $\times 10^{-4}$M. In aqueous phosphate buffer at pH 7·38, a single wave (curves IIIa, c) was obtained, with an anomalous adsorption fore-wave. Over the potential range covered by the fore-wave, erratic galvanometer oscillations were observed. The fore-wave height was independent

of, and the total wave-height was proportional to, concentration, confirming Brdička's results (*loc. cit.*).   The inclusion of 50% of ethanol only partly suppressed the fore-wave, and decreased the total wave-height by about 25%.   Even 55% of ethanol did not completely remove the fore-wave, and higher ethanol contents induced crystallisation of buffer salts.   With $i_d = 1\cdot0$ μa for $c = 0\cdot4$ millimole/l. in water, $D = 4\cdot20 \times 10^{-6}$ cm.^{2}/sec. (Brdička, *loc. cit.*), $m = 0\cdot902$ and $t = 4\cdot0$, the Ilkovič equation gives $n = 1\cdot71$, *i.e.*, 2.   In aqueous solution at pH 7·38, $E_i = 0\cdot016$ v, whence $K = 0\cdot065$, in fair agreement with the potentiometric value 0·05 (Michaelis, Schubert, and Granick, *loc. cit.*).   In 50% ethanol at apparent pH 8·29, $E_i = 0\cdot022$ v, whence $K = 1\cdot16$, showing that ethanol increases semi-quinone stability, as found by Burstein and Davidson (*Trans. Electrochem. Soc.*, 1941, **80**, 175) for certain anthraquinone derivatives.

Capri-, Nile-, and cresyl-blue.—These were studied in aqueous solution at pH 7·38, except for Nile-blue, which was sparingly soluble in the aqueous buffer, and was therefore, studied in 50% ethanol at apparent pH 8·29.   Capri- (curves IV*a*, *b*) and Nile-blue gave waves similar to those for methylene-blue, with an adsorption fore-wave of height independent of concentration up to 10^{-3}M, and with accompanying abnormal galvanometer oscillations.   Capri-blue also gave an adsorption after-wave in water; this was completely suppressed in 50% ethanol, but the fore-wave was only diminished in this solvent.   Cresyl-blue gave a normal polarogram (curve V), with no indication of adsorption.   Values of $i_d/cm^{\frac{3}{2}}t^{\frac{1}{2}}$ for these three dyes were similar to those of methylene-blue in the appropriate solvent.   If we assume a corresponding similarity of D values because of similar molecular structures, $n = 2$ in each case.   For the three dyes respectively, $E_m = -0\cdot218, -0\cdot400$, and $-0\cdot222$ v, $E_i = 0\cdot0165, 0\cdot0200$, and $0\cdot0200$ v, and $K = 0\cdot104, 0\cdot643$ and $0\cdot643$.   Apart from the more negative E_m value for Nile-blue, largely due to the pH shift caused by the presence of ethanol, the E_m, E_i, and K values for these three oxazine dyes are similar to those for methylene-blue.   The absence of adsorption effects for cresyl-blue alone of the four dyes is presumably due to the presence in it of the hydrophilic amino-group.   Although Nile-blue also has such a group, its effect is more than offset by the extra aromatic hydrophobic ring.

DISCUSSION

The agreement between our polarographic and earlier potentiometric determinations of the stability (K) of the semiquinones of riboflavin and methylene-blue justifies the use of the polarograph to determine the extent of semiquinone formation.   We may thus with confidence deduce from our results that the electroreduction and semiquinone formation characteristics of cozymase, Capri-blue, Nile-blue, and cresyl-blue are similar to those of riboflavin and methylene-blue.

In the accompanying Table are listed the K values of several compounds studied polarographically in this and previous work (*J.*, 1951, 27, 2638; 1952, 3231), and also that of pyocyanine obtained potentiometrically (Michaelis, Hill, and Schubert, *loc. cit.*). The values refer mostly to 25° and pH ≈ 7 in water; although some values are for 50% ethanol as solvent and its presence is known usually to increase K, this will not appreciably affect the relative order of magnitude of the K values.

Their K values divide the compounds into two main groups, nos. 1—11 (large K) and nos. 13—20 (small K).   Compounds 1—11 are respiratory anticatalysts in bacterial systems and mostly powerful antibacterials, especially where reduction to semiquinone at pH ≈ 7 requires no proton uptake.   Nos. 13—20 are natural and artificial respiratory catalysts or hydrogen carriers, although no. 17 sometimes inhibits bacterial growth at high concentrations.   The intermediate position of quinoline (no. 12) is interesting, since some of its 8-substituted derivatives, *e.g.*, 8-hydroxyquinoline, are strong antibacterials.

In a theory of the mechanism of respiratory catalysts and anticatalysts based on these results (Kaye, *J. Pharm. Pharmacol.*, 1950, **2**, 902), it is supposed that bacterial respiration is a radical-chain reaction.   Substances which can provide relatively labile semiquinone radicals may participate in the reaction chain, thus acting as catalysts (cf. Parravano, *J. Amer. Chem. Soc.*, 1951, **73**, 183).   Compounds which readily form highly stable semiquinones on reduction, especially if no proton is taken up along with the electron (thus

(Apparent) Semiquinone formation constants (K) *at* 25°.

No.	Compound	Solvent	pH	K	
1	Acridine	50% Ethanol	7	10^8	⎫
2	1-Aminoacridine	,,	7	10^{10}	⎪
3	2- ,, ...	,,	7	10^{10}	⎪
4	3- ,, ...	,,	7	10^8	⎪
5	4- ,, ...	,,	7	10^{10}	⎬ Respiratory anticatalysts
6	5- ,, ...	,.	7	10^7	⎬ and antibacterials
7	2 : 7-Diaminoacridine	,.	7	10^7	⎪
8	2 : 8- ,, ...	,,	7	10^6	⎪
9	Crystal-violet	,,	7	$10^{3.75}$	⎪
10	Brilliant-green	,.	7	$10^{4.75}$	⎪
11	Auramine	,,	7	38·5	⎭
12	Quinoline	,,	6·51	16	
13	Cozymase	Water	7·38	9·3	⎫
14	Riboflavin	,,	7·38	1·49	⎬ Natural respiratory
15	Pyocyanine (30°)	,,	7·38	0·062	⎬ catalysts
16	Phenazine	,,	7·38	0·153	⎭
17	Methylene-blue	,,	7·38	0·065	⎫
18	Capri-blue	,,	7·38	0·104	⎬ Artificial respiratory
19	Nile-blue	50% Ethanol	8·29	0·643	⎬ catalysts
20	Cresyl-blue	Water	7·38	0·643	⎭

minimising entropy decrease), act as chain-breakers by reacting with a labile chain-radical to form a stable semiquinone; they are therefore anticatalysts or antibacterials.

Objection to this theory of drug action may be raised because of the very negative reduction potentials required by these compounds. Thus for compounds nos. 6—11 the half-wave potentials for the first reduction step at pH 7, referred to the normal hydrogen electrode standard, are —0·984, —0·594, —0·761, —0·546, —0·424, and —0·926 v, respectively; E_m for cozymase at pH 7·38 is —0·686 v on the same scale. Such highly negative potentials are unlikely to be encountered in bacterial cells, although no reliable measurements of bacterial cell potentials exist. Comparison of the last figure with the potentiometric values —0·270 v (Green and Dewan, *Biochem. J.*, 1937, **31**, 1069) and —0·290 v (Stephenson, " Bacterial Metabolism," Longmans Green and Co., 1939) suggests a possible answer to the objection, *viz.*, an overvoltage effect at the dropping mercury electrode renders polarographic potentials far more negative than potentiometric values. Another possibility is that attachment of semiquinone to the enzyme protein (or nucleic acid; cf. Oster, *Trans. Faraday Soc.*, 1951, **47**, 660) may facilitate reduction to semiquinone at less negative potentials than at the dropping mercury electrode, just as adsorption of methylene blue semiquinone on the electrode permits reduction of the dye at less negative potentials than are required in the absence of adsorption (Brdička, *loc. cit.*).

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