A POLAROGRAPHIC INVESTIGATION OF THE REDOX CHARACTERS OF THE AMINOACRIDINES, CONSIDERED IN RELATION TO ANTIBACTERIAL ACTION

BY R. C. KAYE

From the Department of Pharmacy, The Technical College, Bradford

A STUDY of the extensive literature dealing with the antibacterial drugs of the acridine series reveals three main lines of approach to the problem of their mode of action. Firstly, a large number of derivatives of acridine have been synthesised, and activity correlated with structure. Secondly, a number of physico-chemical properties of many members of the series have been determined and related to biological action; most of the knowledge accumulated in these two fields of investigation is due to the work of Linnell, Albert and their collaborators. Thirdly, the problem of biological activity has been partially elucidated through a study of bacterial nutrition and enzyme chemistry by McIlwain[1], Quastel and Wheatley[2], and Dickens[3]. Hinshelwood[4] and his collaborators have contributed important studies on the effect of some acridine derivatives on the lag-phase of bacterial cultures, an important feature of this work being the production of drug-resistant strains of bacteria by a process of training.

Perhaps the most widely accepted theory of acridine action is the basicity theory of Albert and his collaborators, who have shown that the most active members of the series are well ionised at pH 7, this condition facilitating attachment of the inhibitor to the surface of an enzyme protein. This theory does not account for the appearance of bacteriostatic activity within the series as a whole; as far back as 1922, it was recognised through the work of Browning *et al.*[5] that the intact acridine nucleus was necessary for full activity, and possessed some property not shared by the related heterocyclic compounds pyridine, quinoline, and phenazine. This conclusion was later supported by Berry[6], who found some acridanes to be quite ineffective as bacteriostatics, and also by Albert, Francis, Garrod and Linnell[7], who found acridone derivatives to be devoid of activity. More recently, Albert[8] has attributed the outstanding biological activity of the acridines as a whole to the flatness of the acridine molecule, which facilitates adsorption at an enzyme surface.

Following the work of McIlwain[1], who concluded that the acridines act by displacing a hydrogen carrier from the active centres of an enzyme, Breyer, Buchanan and Duewell[9] attempted to correlate the activities of a number of acridines with their reduction potentials, measured polarographically, and suggested a direct relationship between the two.

Certain anomalies were apparent in the polarographic results of these workers, who, while reaching the general conclusion that the acridines were reduced at the dropping mercury electrode in two one-electron

stages, described a three-stage reduction in the cases of acridine and 4-aminoacridine. In the former case, the height of the first step was found to be independent of concentration for concentrations between $10^{-4}M$ and $10^{-3}M$. From this behaviour, it was deduced that acridine exists only as a dimer at concentrations higher than $10^{-4}M$. This conclusion appears doubtful, for the mass law would predict some non-linear relationship between concentration and step height. The second step of reduction was found by Breyer *et al.* (*loc. cit.*) to disappear at concentrations below $10^{-4}M$. To explain the peculiar behaviour of acridine and 4-aminoacridine, the formation of a compound between oxidant and the free radical product of the first reduction step was suggested.

The present series of polarographic studies was commenced firstly in order to elucidate the above anomalous behaviour of acridine derivatives, and, secondly, to attempt to find some redox property of acridine and its derivatives not shared by pyridine, quinoline, and phenazine, which might account for the activity of the former compounds and the comparative inactivity of the latter. The polarographic behaviour of acridine has already been described (Kaye and Stonehill[10]). In the present paper, the behaviour of some of the aminocridines at the dropping mercury electrode is described, and the biological significance of the results discussed.

EXPERIMENTAL

A manually operated polarograph was employed. The cell was a modified form of one described by Kolthoff and Lingane[11], and was constructed from a B40 Pyrex glass joint. The capillary constants in 50 per cent. alcoholic buffer solution were as follows: $m = 0.916$ mg.sec^{-1}, $t = 4.2$ sec. (with no applied potential). All dropping mercury electrode potentials were measured against an immersion type saturated calomel electrode, using standard potentiometric procedure. Current was measured by means of a calibrated damped Cambridge " Spot " Galvanometer. All experiments were carried out at 25°C. Cell solutions were de-oxygenated by passing cylinder nitrogen, purified by passage through a series of gas washing bottles containing alkaline hydrosulphite solution. The potential of the dropping mercury electrode was adjusted by a tapped resistance network forming a potential divider, and could be increased in steps of 10 mV., although steps of 20 mV. were usually employed.

For studying the current-time relationship during the life of a single mercury drop, an Ultrascope Mark I cathode ray oscilloscope was employed, in conjunction with an external time-base circuit giving a traversing time of about 5 seconds. This time-base, formed by charging a 10 mfd. condenser through a 1 megohm resistance from a 450-volt D.C. supply was not linear, but was found adequate for the purpose. The cell current was amplified by means of a D.C. coupled amplifier employing two EF50 valves, constructed in the laboratory. The output from the amplifier was fed to the Y plates of the cathode-ray tube.

Sörensen's buffer solutions served as supporting electrolyte. In many cases, 50 per cent. of alcohol was present in the cell solutions. The pH values of these alcohol-buffer mixtures were determined by means of a hydrogen electrode which could be inserted into the cell in place of the dropping mercury electrode.

Of the materials used, 5-aminoacridine and 2:7- and 2:8-diamino-acridine, in the form of their salts, were purified by repeated crystallisation from water. Very small amounts of the remaining mono-aminoacridines were available in a pure state, and these were not further purified.

RESULTS AND DISCUSSION

All the mono-aminoacridines, and also 2:7- and 2:8-diaminoacridine in aqueous solution yielded anomalous polarographic waves, multi-step reductions b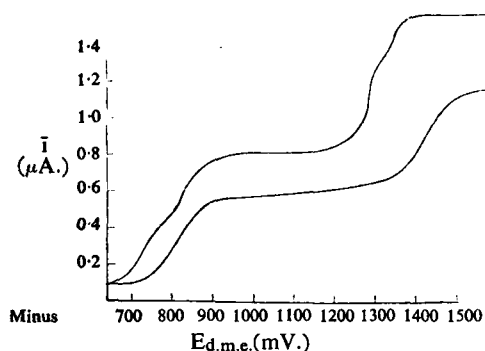eing observed over most of the pH scale. The anomalies were most marked in the case of the mono-amino-acridines. Examples of the waves obtained are shown in Figures 1 to 3. Two main reduction waves were discernible, the complicating features being, in the case of the mono-aminoacridines, a fore-wave on the first main reduction step, and additionally, in the case of 1- and 2-aminoacri-dine, an after-wave on the second step. During the recording of the waves the galvanometer

FIG. 1. Polarograms of 2-aminoacridine (4 × 10^{-4} M); upper curve, pH 7·38, without alcohol—lower curve, pH 8·29. with 50 per cent. alcohol.

oscillations were observed to proceed in a very jerky manner, especially over the potential range corresponding to the fore-wave. Similar observations were previously made during the reduction of acridine (Kaye and Stonehill, loc cit.) and were found to indicate adsorption of electroactive material on the surface of the mercury drop, this being the cause of the anomalous reduction waves of acridine. It seemed probable that the multiple waves of the aminoacridines were also due to the same cause. To test this supposition, the current-time relations during the life of a mercury drop were studied oscillographically during the electroreduction of 2:8-diaminoacridine. The results are shown in Figure 4. The horizontal time scale may be judged from the 50 cycle A.C. ripple superimposed on the tracings. The current-scale is indicated on the first tracing. Tracings A to F show the current-time relationship over the potential region occupied by the after-wave on the first reduction step: these tracings should be studied in conjunction with the correspondingly lettered polarographic wave shown in Figure 3. Tracings C, D and E

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show that after the detachment of the mercury drop, the current rises with abnormal rapidity to a small maximum value, marked on the tracings by a cross. Since the current flowing through the cell is composed of the electrons taken up by oxidant, it appears from these current maxima that the supply of oxidant to the drop surface is being hastened by marked adsorption on the mercury. The slight fall of current following the maxima is probably due to a temporary depletion of oxidant in the solution surrounding the drop; when this is made good by diffusion from the main bulk of liquid the current once more increases, as shown in the oscilloscope tracings.

Adsorption of oxidant at the dropping mercury electrode is in keeping with the presence of an after-wave on the current-potential curve, according to the theory of Brdička[12]. Treating the dropping mercury electrode as an inert-metal redox electrode, the electrode potential will be given by

$$E = E^{01} + \frac{RT}{nF} \ln \frac{a_{ox}}{a_{red}}$$

(1)

where a_{ox} and a_{red} are the activities of oxidised and reduced forms of electro-active material in the interface boundary layer, near the mercury drop, n the number of electrons involved, R the gas constant, T the absolute temperature, F the faraday, and E^{01} the standard redox potential at a given pH. The presence of an appreciable amount of the oxidant in the layer in the adsorbed state will result in a lowering of the value of a_{ox}, and consequently a more negative value for E, and a displacement of the polarographic wave to the right. This displacement may be seen in Figure 3. Significantly, the abnormal current time relationships shown in Figure 4 occurred only over the potential range occupied by the after-wave.

It was shown by Kaye and Stonehill (*loc. cit.*) in a polarographic study of acridine that the inclusion of upwards of 50 per cent. of alcohol in the supporting electrolyte would render acridine less lyophobic and thus prevent adsorption of electro-active material on the mercury drop. It was therefore decided to study the effect of increasing alcohol concentra-
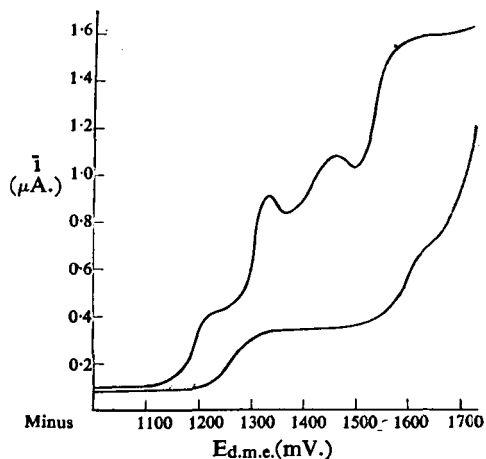

FIG. 2. Polarograms of 5-aminoacridine .2 × 10⁻⁴ M); upper curve, pH 9·07 without alcohol—lower curve, pH 10·39 with 50 per cent. alcohol.

tion on the adsorption of 2:8-diaminoacridine at the dropping mercury electrode. The current-time relationships were studied during the reduction of this compound in buffer solutions containing 20, 50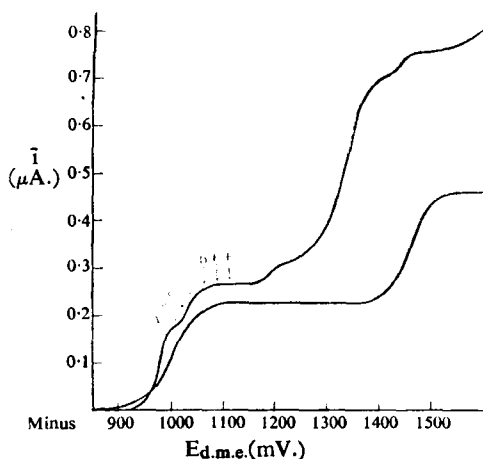 and 60 per cent. of alcohol, the potential of the electrode being maintained at about −1050mV vs. S.C.E., corresponding to the crest of the after-wave. An alcohol concentration of 20 per cent. was quite ineffective in preventing adsorption (Tracing G, Figure 4). Increasing the alcohol content to 50 per cent. greatly modified the current peak (tracing H) and 60 per cent. of alcohol almost completely eliminated it. The inclusion of 50 per cent. alcohol, though not completely eliminating adsorption,

FIG. 3. Polarograms of 2:8-diamino-acridine (2 × 10⁻⁴ M); upper curve, pH 7·58 without alcohol—lower curve, pH 8·29 with 50 per cent. alcohol. Letters A to F correspond to Figure 4 tracings A to F.

permitted a normal polarographic wave to be obtained, shown in Figure 3 along with the anomalous wave obtained in aqueous solution. The displacement of the entire polarogram to more negative potentials in
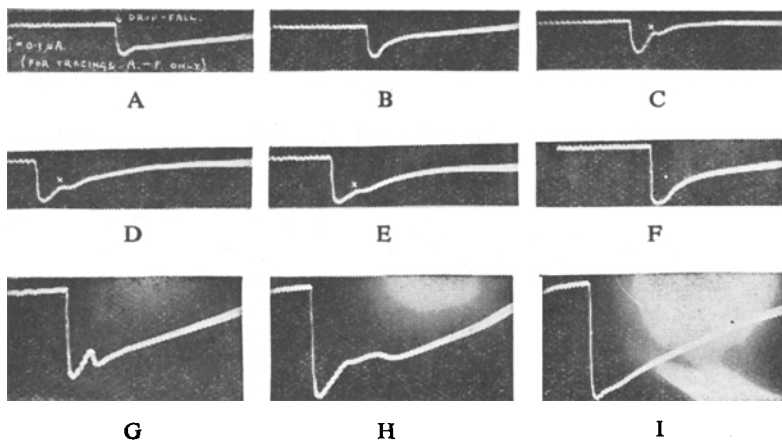

FIG. 4. Oscillograms of 2:8-diaminoacridine. Tracings A to F show the current-time relationship over the potential region occupied by the after-wave on the first reduction step. The white cross on C, D and E shows the small maximum attained with abnormal rapidity after the detachment of the mercury drop. G, H, and I show effect of addition of alcohol in eliminating current peak due to adsorption of compound on the mercury drop. Higher amplification was used for G, H and I

alcoholic solution is due partly to the increased pH of the solution, which was found to be 8·23 compared with 7·38 for the purely aqueous buffer.

The polarographic behaviour of 2:7-diaminoacridine was similar to that of 2:8-diaminoacridine, the after-wave on the first reduction step taking the form of a curious flattening of the crest of the main wave. Oscillographic study confirmed the adsorption of electro-active material at the dropping mercury electrode. In 50 per cent. alcoholic solution a normal polarogram was obtained.

The anomalies due to adsorption at the dropping mercury electrode were more pronounced in the case of the monoaminoacridines than in the case of 2:7- and 2:8-diaminoacridine, and consisted principally of a fore-wave on the first reduction step, and additionally, in the case of 1- and 2-aminoacridine, an after-wave on the second reduction step. Equation (1) shows that these anomalies may be explained by adsorption of the product of the first stage of reduction on the mercury drop. The monoaminoacridines were not fully studied oscillographically because of the small amounts available; a limited investigation showed a highly abnormal current-time relationship during the life of a single mercury drop. The use of 50 per cent. alcoholic supporting electrolyte enabled normal polarograms to be obtained, shown along with the anomalous waves found in aqueous solutions in Figures 1 and 2.

It is clear from the above evidence that the explanations offered by Breyer, Buchanan and Duewell[9] for the peculiar behaviour of the acridines at the dropping mercury electrode are not acceptable; evidently the three reduction steps described by these workers were not normal polarographic waves at all, but waves distorted by adsorption of electro-active material at the electrode. Breyer, Buchanan and Duewell reported lack of proportionality between step height and concentration in the case of 4-aminoacridine. The present investigations show that when adsorption is eliminated by the use of 50 per cent. alcoholic base solution there is good proportionality between the heights of both reduction steps and concentration.

The study of 5-aminoacridine was rendered more difficult by the occurrence of maxima (Figure 2). These were eliminated by working in 50 per cent. alcoholic base solution with the addition of 0·01 per cent. of methylcellulose, and by limiting the concentration of 5-aminoacridine to 2×10^{-4}M.

Breyer, Buchanan and Duewell (loc. cit.) reported the aminoacridines to be reduced at the dropping mercury electrode in two one-electron stages, calculated by means of the Ilkovic equation. It is possible that some of these results were invalidated by adsorption of electro-active material at the electrode, such calculations being valid only when the supply of the former to the latter is controlled by diffusion alone, which clearly is not the case for the acridines in aqueous solution. It therefore seemed desirable to recalculate the number of electrons involved (n) for the aminoacridines in alcoholic solution, in which adsorption does not

occur sufficiently to distort the reduction waves. These re-calculated values for pH 8·29 are given in Table I.

TABLE I

Compound	Concentra-tion (millimols. per litre)	First step		Second step	
		i_d (μA.)	n	i_d (μA.)	n
1-aminoacridine	0·4	0·53	1·09	0·54	1·13
2 aminoacridine	0·4	0·49	1·00	0·45	0·94
3-aminoacridine	0·4	0·48	0·98	0·42	0·88
4-aminoacridine	0·2	0·22	0·90	0·25	1·04
4-aminoacridine	0·4	0·43	0·88	0·48	1·00
5-aminoacridine ... ... (pH 10·39)	0·2	0·24	0·99	0·28	1·17
2 :7-diaminoacridine	0·2	0·24	0·98	0·25	1·04
2 : 7-diaminoacridine	0·4	0·46	0·94	0·48	1·00
2 : 8-diaminoacridine	0·2	0·22	0·90	0·22	0·92
2 : 8-diaminoacridine	0·4	0·44	0·90	0·46	0·96

In these calculations it was necessary to have the value of the diffusion coefficient D, in 50 per cent. aqueous alcohol. The value of D for the aminoacridines at 25°C. was calculated from the value of D at 0°C. and the temperature coefficient as given by Breyer, Buchanan and Duewell (loc. cit.). The value of D in 50 per cent. alcohol was then calculated by analogy with the results of Gill[13], who found that D decreased by 38 ± 2 per cent. at 25°C. on adding 50 per cent. alcohol to solutions of anthraquinone 1- and 2-sulphonic acids, and also for some hydroxyanthraquinones. This procedure was supported by the more recent results of Shreve and Markham[14], who reported D for p-nitro-aniline to be reduced by about 35 per cent. by alcohol concentrations of 46 to 55 per cent. The calculated value of D for the aminoacridines in 50 per cent. aqueous alcohol at 25°C. was $2·92 \times 10^{-6}$. The results in Table I show that the aminoacridines, like acridine (Kaye and Stone-hill, loc cit.), are reduced in two one-electron stages, and that the first product of reduction must be a free radical. This conclusion was supported by a polarographic study of phenazine, which, in strongly acid solution only, was found to be reduced in two well separated steps of heights almost equal to those of the acridines. The redox characteristics of phenazine, obtained by the polarographic method, were found to be very similar to those obtained for α-oxyphenazine by Michaelis, Hill and Schubert[15], who, by the potentiometric method, found this compound in acid solution to be reduced in two separate one-electron steps.

An outstanding feature of the polarographic reduction of the amino-acridines, and also acridine (Kaye and Stonehill, loc. cit.), is the wide separation of the two steps over the entire pH scale, a considerable increase of negative potential being required to convert the free radical to the fully reduced compound. This seems to imply unusual stability

of the free radical, the reason for which is not at present clear. In the case of acridine, free radical stability cannot be due to equivalent resonance, shown by Michaelis[16] to be of prime importance in stabilising semi-quinone type free radicals in solution.

In the case of reversible redox systems, the stability of the semi-quinone formed during reduction or oxidation is shown by the value of the semiquinone formation constant, k, given by

$$k = \frac{s^2}{r.t}$$

(2)

where s is equal to the concentration of semiquinone, r the concentration of reductant, and t that of oxidant in the half-reduced system. In such cases, k may be evaluated from the titration curve, using the equation

$$k = 10^{\frac{E_{ind}}{0.059}} - 3 \times 10^{\frac{-E_{ind}}{0.059}}$$

(3)

where E_{ind} is the index potential, i.e., the difference between the potentials corresponding to 25 per cent. and 50 per cent. reduction, or to 50 per cent. and 75 per cent. reduction. When the index potential is greater than 40 mV, lateral points of inflection appear on the titration curve, and it may then readily be seen that the reduction, or oxidation, is occurring in two univalent processes. For thermodynamically reversible systems, the polarographic wave is analogous to a reductive titration curve (Müller[17]). Treating the polarographic waves of the acridines in this way, the index potentials are given by

$$E_{ind} = \frac{E_2 - E_1}{2}$$

(4)

where E_1 and E_2 are the half-wave potentials of the first and second reduction steps.

On this basis, the semiquinone formation constants were found to be very large indeed. It is doubtful, however, if this procedure is valid, on account of the thermodynamic irreversibility of the electrode processes, deduced from the non-linear plot of E against log. $\frac{i_d - i}{i}$

Nevertheless it is difficult to escape the general conclusion that during the electro reduction of the acridines free radical products of exceptional stability are formed.

In view of the variation of biological activity of the acridines with pH value reported by Albert, Rubbo, Goldacre, Davey and Stone[18], it was decided to investigate the variation of half-wave potentials with pH. Figures 5 and 6 show the E_1 and E_2 values for two of the aminoacridines in 50 per cent. alcoholic solution, plotted against pH. In interpreting these graphs, the following generalisations may first be made:

(1) The bends on the E_1/pH graph relate to dissociation constants

of either oxidant or free radical, while those on the E_2/pH graphs refer to dissociation constants of either free radical or reductant.

(2) Proceeding from left to right, bends consisting of a steepening denote dissociation constants belonging to the oxidant (in the case of the E_1/pH graphs), or to the free radical (in the case of the E_2/pH graphs). Bends consisting of a flattening denote dissociation constants belonging to the free radical (in the case of the E_1/pH graphs), or to the reductant (in the case of the E_2/pH graphs).

On this basis, equations, which would fit the experimental $E_{\frac{1}{2}}/pH$ curves were derived. It was found that the E_1/pH graphs for 2-, 4- and 5-aminoacridine and also for 2:7- and 2:8-diaminoacridine were described by the equation:

$$E_1 = E_0^1 + \frac{RT}{F} \ln \frac{K_1^S K_2^S h^+ + K_1^S h^{+2} + h^{+3}}{K^T + h^+}$$

$$(5)$$

where E_1 is the half-wave potential of the first reduction wave, E_0^1 a constant, R the gas constant, F the faraday, K_1^S and K_2^S dissociation constants of the free radical, K^T that of the oxidant, and h^+ the concentration of hydrogen ions. This equation was tested by allotting values to K_1^S, K_2^S and K^T in accordance with the experimental values for 2:8-diaminoacridine and plotting the curve. Equation 5 takes no account of the bend occurring at pH 12 on the E_1/pH graphs for 2- and 5-amino-acridine, and 2:7- and 2:8-diaminoacridine. Since this bend occurred about the same point for all these compounds, it could not be due to dissociation constants of the oxidants; Albert and Goldacre[19] reported no pK_a values higher than 9·5 for these acridines. It was therefore concluded that this bend was due to some peculiarity of the polaro-graphic technique, this view being strengthened by the unexpected finding of a similar bend for auramine at pH 12·5. It was significant that these bends coincided with the change from normal buffer solutions to sodium hydroxide solution as supporting electrolyte. Experiments showed that the effect was not due to increased ionic strength of the solutions.

The values of pK^T were found to agree well with the pK_a values deter-mined electrometrically for 50 per cent. alcoholic solutions by Albert and Goldacre[19]. These values are compared in Table II.

TABLE II

Compound				Polarographic pK_a values	pK_a values of Albert and Goldacre
2-aminoacridine	...	...	...	7·4	7·61
4-aminoacridine	...	...	...	5·6	5·5
5-aminoacridine	...	...	...	9·6	9·5
2:7-diaminoacridine	...	...	...	7·3	7·74
2:8-diaminoacridine	...	...	...	9·3	9·5
1-aminoacridine	...	...	...	3·8	3·59
3-aminoacridine	...	...	...	5·07	5·03

The most interesting feature of the first reduction step is its independence of hydrogen ion concentration over certain ranges of pH. This was found to be the case for 2-, 4- and 5-aminoacridine, and 2:7- and 2:8-diaminoacridine, but not for 1- and 3-aminoacridine. Evidently in these regions of pH reduction to the free radical stage requires the uptake of an electron only. Since the flat portions of the E_1/pH graphs always lie below the pK^T values, the oxidants will here be in the cationic form. Taking 2:8-diaminoacridine as an example, the electrode reaction can be represented as
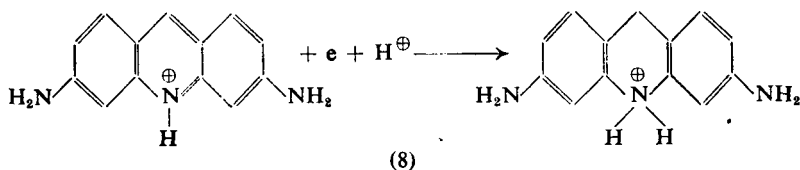

(6)
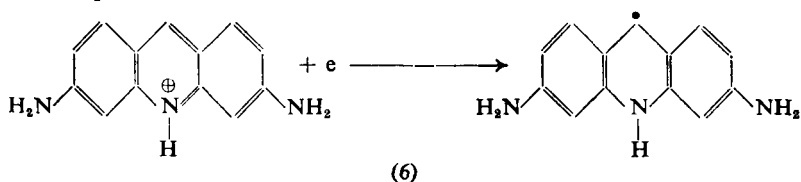
At pH values higher than pK^T a hydrogen ion must additionally be involved, and the electrode reaction will be
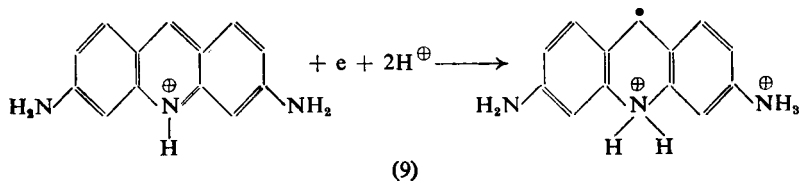

(7)
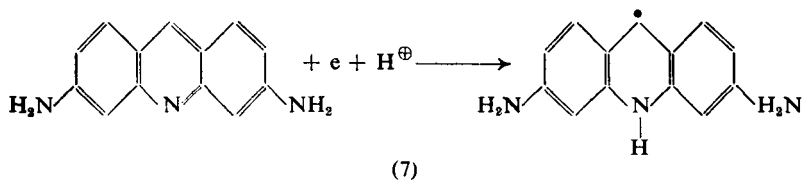
This reaction requires a slope of 59 mV for the E_1/pH graph at pH values higher than pK^T

At pH values between pK_2^S and pK_1^S, the electrode reaction may be represented as

(8)

again indicating a slope of 59mV for the E_1/pH graph over this range of pH. At pH below pK_1^S, the electrode reaction will be

(9)

resulting in a slope of 118mV for the E_1/pH graph at pH below pK_1^S.

Inspection of experimental E_1/pH graphs shows that the slopes in some cases depart considerably from ideality, the best agreements with

theory being found in the case of 2 : 7-diaminoacridine. These non-ideal slopes may be attributed to the thermodynamic irreversibility of the electrode reactions.

The form of the E_2/pH relationship was constant for all the aminoacridines studied, and was fitted by the equation

$$E_2 = E_0^1 + \frac{RT}{F} \ln \frac{K_1^R K_2^R + K_1^R h^+ + h^{+2}}{K_1^s K_2^s + K_1^s h^+ + h^{+2}}$$

(10)

where E_2 is the half-wave potential of the second reduction step, and K_1^R and K_2^R are dissociation constants of the reductant. The main interest in the E_2/pH graphs lies in their independence of hydrogen ion concentration in alkaline solution. Evidently the second reduction step here involves an electron only. Since the horizontal sections of these graphs commence in all cases at pH values higher than pK_2^s, the final reductant in this region of pH must be formed by the addition of an electron to the free radical product of equations (6) and (7). Consequently, the reductant must first appear as a negatively charged ion, according to the reaction
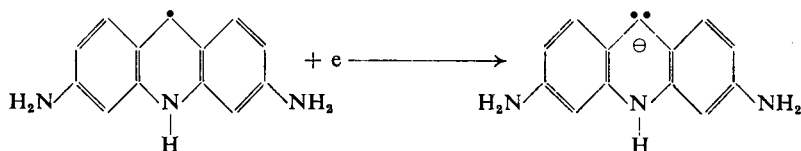
the product probably reacting with water:
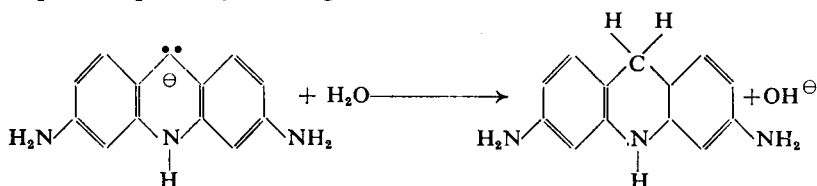
The reduction mechanism is thus similar to that of the red form of phenolphthalein described by Kolthoff and Lehmicke[20]. These workers found this compound in alkaline solution to be reduced in two one-electron steps which were both independent of pH, and concluded that the final product of the two-stage reduction must first exist as a negatively charged ion having a lone pair of electrons on the central carbon atom.

The upward bend, shown by the dotted line, on the E_2/pH graphs was at first puzzling. Later, this was shown to be due to the increased ionic strength of the more alkaline base solutions; it was found that increasing the ionic strength of solutions of lower pH by the addition of potassium chloride shifted the half-wave potential of the second reduction step to considerably less negative values (cf. Burstein and Davidson[21]). The half-wave potentials of the first step were altered by only 2 or 3 millivolts by this treatment.

THE BIOLOGICAL SIGNIFICANCE OF THE RESULTS

The results of Browning et al.[5], Albert et al.[7] and Berry[6] all suggest that the intact acridine nucleus is essential for the development of full antibacterial activity in the acridine series. The complete inactivity of the acridanes (Berry, loc. cit.) and the acridones (Albert et al., loc. cit.) suggests that the activity of the acridines as a whole might be connected with their redox characters, a view which is supported by the polarographic investigation of Breyer, Buchanan and Duewell (loc. cit.), who found acridone to be reduced in one step only compared with the two step reductions of the biologically active acridines. These workers, however, interpreted their results differently, and suggested a direct relationship between bacteriostatic activity and the reduction potentials of the different members of the series, it being assumed that the difficultly reducible acridine derivative would take the place of a natural hydrogen carrier in one of the cell enzyme systems. This theory seems unlikely to be correct, for then all members of the series having a reduction potential more negative than a certain limiting value might be expected to be equally active as bacteriostatics, other factors being neglected. This was not found to be the case, however, and factors other than reduction potential must be more important in deciding activity. Further, Page and Robinson[22] showed that in general there is no direct relationship between bacteriostatic activity and redox potential.

Considering the biological activity of dyes as a whole, these appear to fall into two groups—those which behave as catalysts for cell respiration processes, and those acting as anticatalysts, or inhibitors of such processes. The first group includes thiazine and oxazine dyes, such as methylene blue, Capri blue, Nile blue and cresyl blue, which are able to function as hydrogen carriers in bacterial enzyme systems (Green, Stickland and Tarr[23]). Examples of naturally occurring substances playing the same rôle are coenzymes I and II, riboflavine, and pyocyanine. In the anticatalyst group of dyes would be placed the acridines and the triphenylmethane dyes, which were shown by Quastel and Wheatley[2] and by Dickens[3] to inhibit cell respiration processes.

It further appears that the activity of members of the second group can be neutralised by members of the first group. Thus, McIlwain[1] showed the bacteriostatic activity of euflavine and proflavine to be neutralised by methylene blue, cresyl blue, cozymase, riboflavine, pyocyanine and phenazine methosulphate, and that of crystal violet to be neutralised by pyocyanine and riboflavine. The resistance of Ps. pyocyaneus to the acridines reported by Berry[6] may thus be due to the pyocyanine content of this organism.

This evidence appears to indicate a competition between the respiratory catalysts on the one hand, and the inhibitors on the other hand, for places on the active centres of an enzyme protein, basicity being important not only for bacteriostatic activity, but also for respiration catalysis, as was demonstrated by Green, Stickland and Tarr[23].

In order to understand more completely the mechanism of these

opposing activities, it is helpful to review some of the theories proposed by Michaelis and also by Waters. Granick, Michaelis and Schubert[24] and also Michaelis[16] have proposed the hypothesis that bivalent organic oxidations and reductions can proceed only in univalent steps. Earlier, Michaelis and Smythe[25] had pointed out that the inertia of some organic compounds towards oxidation was due to their inability to form appreciable concentrations of semiquinone. Granick, Michaelis and Schubert[24] have also suggested that catalyst activity for oxidation processes, especially biological respiration, should be correlated with the ability of the catalyst to form semiquinones. Such catalysts would, of course, include the many substances functioning biologically as hydrogen carriers, some of which have been mentioned above. It is significant that many of these hydrogen carriers, both natural and artificial, are known to give rise to semiquinone radicals—pyocyanine (Friedham and Michaelis[26], Michaelis, Hill and Schubert[15]), riboflavine (Stern[27], Michaelis, Schubert and Smythe[28]), co-enzyme II (Adler, Hellstrom and Euler[29]), cozymase (Gutcho and Stewart[30]), methylene blue and other thiazines (Michaelis, Schubert and Granick[31,32]), and phenazine dyes (Kuhn and Wagner-Jauregg[33]).

The possible rôle of free radicals in biological respiration has been more clearly indicated by Waters[34], who has suggested mechanisms for cell dehydrogenations based on free radical chain reactions. The hydrogen carriers listed above would, of course, be able to enter into chain reactions of this sort, replacing the co-enzymes in the scheme proposed by Waters.

The results of the present investigations show that an outstanding property of the aminoacridines is their ability to form, during electro-reduction, free radicals of unusual stability, indicated by the wide separation of the two steps of reduction. Such free radicals, unlike the much more labile radicals derived from the respiratory catalysts, would be unable to participate in the enzyme chain reactions proposed by Waters. On the contrary, it is conceivable that such chain processes, involving single electron exchanges, could be effectively terminated by the presence in the cell of a small concentration of acridine derivative through the ability of the latter to accept an electron, forming thereby a non-reactive free radical. The biological rôle of the inhibitor may thus be one of breaking chain reactions within the cell.

Albert, Rubbo, Goldacre, Davey and Stone[18] have shown that the most active members of the acridine series are 2- and 5-aminoacridine, and 2:7- and 2:8-diaminoacridine, while acridine, and 1-, 3- and 4-amino-acridine are very much poorer. Significantly, the present results show that the redox characters of the first group differ considerably from those of the second. These differences concern the first reduction step, i.e., the production of the free radical from oxidant. In the case of 2- and 5-aminoacridine, and 2:7- and 2:8-diaminoacridine, the first reduction step is independent of *p*H over the biologically important *p*H range of 7·0 to 7·4, reduction to the free radical stage requiring the uptake of

an electron only in this region of *pH*. The weaker members of the series, namely, acridine (Kaye and Stonehill, *loc. cit.*) and 1-, 3- and 4-aminoacridine do not show this behaviour, free radical formation requiring additionally the uptake of a hydrogen ion round about *pH* 7. In the case of 4-aminoacridine, the E_1/pH graph shows a horizontal portion, but not in this region of *pH*.

It may well be that the more biologically active members of the series owe their superiority to their ability to capture, and to hold fast in the form of a stable free radical, a single fugitive electron, which would otherwise be at liberty to participate in chain reactions profitable to the living cell. Electron transference is, after all, the essential feature of all oxidation—reduction processes.

Equation (5) shows that there are two requirements for the occurrence of a horizontal portion on the E_1/pH graph at about *pH* 7. These are

(1) the pK_a value of the oxidant must lie above *pH* 7.

(2) the pK_a value of the free radical must lie below *pH* 7.

The first requirement correlates well with the results of Albert *et al.*[18], who have shown that, for full activity, the pK_a value of the acridine derivative must lie above *pH* 7. In the light of the free radical theory of bacteriostatic action now proposed, the relationship between basicity and activity acquires an added significance.

If the superiority of 2- and 5-aminoacridine, and 2:7- and 2:8-diaminoacridine be due to their ability to form stable free radicals by accepting an electron only, then their activity would be expected to diminish as the *pH* of the medium falls below the *pK* value of the free radical, that is, into a region where free radical production requires a hydrogen ion in addition to an electron. Moreover, this behaviour would not be expected in the case of acridine and 1-, 3- and 4-aminoacridine, none of which exhibits a horizontal portion on the E_1/pH graphs in the region of *pH* 7. These conclusions are supported by data obtained by Albert, Rubbo, Goldacre, Davey and Stone (*loc. cit.*), who found, in the case of 5-aminoacridine and 2:7- and 2:8-diaminoacridine, a sharp decrease in activity with falling *pH*. The present investigation has shown that this decrease occurs approximately at the *pK* value of the free radical. Significantly, also, Albert *et al.* found the activity of 4-aminoacridine to be unaffected by falling *pH*.

A serious objection to the free radical theory of bacteriostatic action arises in the rather high negative potentials which would presumably be necessary for the production of the inhibitor free radical at the site of action. These potentials would be somewhere in the region of the E_1 values for *pH* 7 shown in Figures 5 and 6. (The potentials have been measured with reference to the saturated calomel electrode).

It may be doubted if such negative potentials would be encountered in the living bacterium cell, and it is not easy to settle this question. Attempts have been made to measure cell potential by immersing inert electrodes in their fluid cultures (Hewitt[35]). It is evident that such
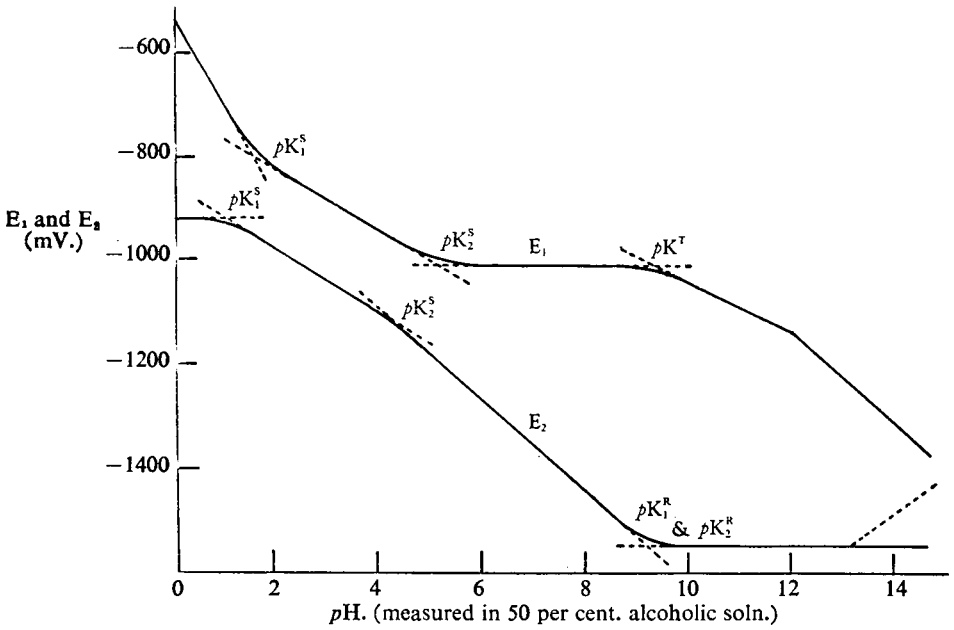
FIG. 5. Variation of the half-wave potentials of 2 : 8-diaminoacridine (4 × 10⁻⁴ M) in 50 per cent. alcohol with pH
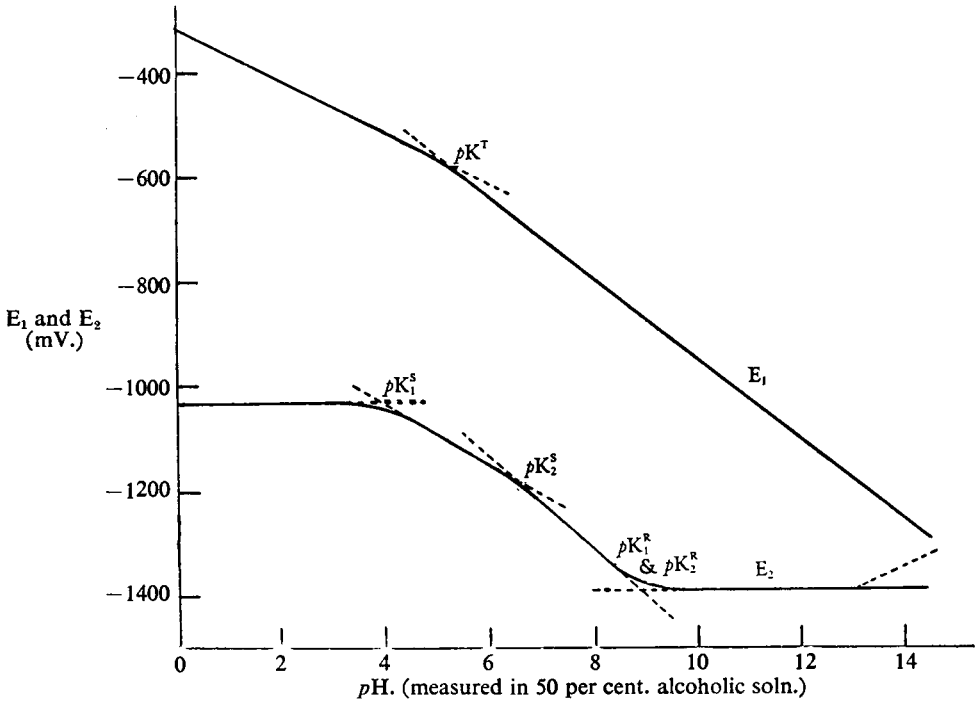

FIG. 6. Variation of the half-wave potentials of 3-aminoacridine (4 × 10⁻⁴ M) in 50 per cent. alcohol with pH

methods measure the potential of the culture medium on the cell surface rather than that of the cell interior. The use of redox indicators suffers from the same defect.

It is possible that attachment of the free radical form of the inhibitor to an enzyme protein will render E_1 less negative. The electrical potential required to convert oxidant to free radical depends on the different energy levels of these two forms; equation (1) shows that the existence of the free radical in an adsorbed state, of diminished activity, would result in a less negative reduction potential for the system.

It is not impossible, therefore, to meet the above objection; the free radical theory of dye stasis has received further support from the results of a polarographic investigation of the redox characters of triphenylmethane and diphenylmethane dyes, to be communicated in a further paper in this series.

SUMMARY

1. The redox characters of some of the aminoacridines have been studied by the polarographic method. The anomalous behaviour of these compounds has been shown to be due to adsorption at the dropping mercury electrode, this conclusion being supported by oscillographic evidence. It has been shown that the use of alcoholic supporting electrolyte will eliminate adsorption and enable normal polarographic waves to be obtained.

2. The aminoacridines have been shown to undergo electro-reduction in two widely separated one-electron steps, the first product of reduction being a free radical, apparently of great stability.

3. A theory of dye bacteriostasis has been proposed based on the breaking of free radical chain reactions within the cell by the relatively stable free radical derived from the dye.

4. The variation of bacteriostatic activity within the series of aminoacridines has been related to the nature of the first step of reduction, which, for the most active members of the series, has been shown to require the uptake of an electron only at about pH 7. The variation of activity with pH has also been related to the nature of this first reduction step.

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In the absence of the author this paper was taken as read and was not discussed.