RELATIONS BETWEEN BACTERIOSTATIC ACTIVITY AND CHEMICAL CONSTITUTION OF CERTAIN ACRIDINE ANALOGUES OF BASIC DI- AND TRIPHENYLMETHANE DYES

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2:8-DIAMINOACRIDINE derivatives can be derived from basic di- and triphenylmethane dyes by joining positions 2 and 6' by an imine bridge. Such derivatives can be considered as the acridine analogues of the corresponding basic di- and triphenylmethane dyes, belonging simultaneously to the acridine and the *p*-diamino-di- or triphenylmethane series. The bacteriological study of these compounds (Table I) is of interest because differences exist between some characteristic features of the acridine type bacteriostasis and the corresponding triphenylmethane effect.

One of these is the influence exerted on bacteriostatic activity by alkylating the amino groups. Methylation or ethylation increases the bacteriostatic effect in the di- and triphenylmethane series substantially[1-5], whereas no such increase has been observed in the acridine series[1]. For example, Albert *et al.*[6] found that the bacteriostatic power of acridine orange, a methylated homologue of 2:8-diaminoacridine (Table I) was not superior to that of the non-methylated compound proflavine.

A second difference is the number of benzene rings necessary for optimal bacteriostatic action. It was shown by Fischer, Garcés and López[7] that diphenylmethanes, such as Michler's hydrol and auramine, are nearly one hundred times less active than the corresponding triphenylmethanes, thus confirming the observations of Kliegler[2] on auramine, while the contrary seems to apply to the members of the acridine series. According to Albert *et al.*[6] the introduction of a phenyl radical in the position 5 of acridine yellow (2:8-diamino-3:7-dimethylacridine) causes a decrease of bacteriostatic potency, the derivative, benzoflavine (2:8-diamino-3:7-dimethyl-5-phenylacridine), an acridine analogue of the triphenylmethane dyes, being less active than acridine yellow. This circumstance is attributed by Albert *et al.* to a dystherapeutic dimensional factor.

The third difference between acridine and triphenylmethane bacteriostasis is the effect of serum on activity. Its presence increases the action of acridines, but inhibits that of diphenyl- or triphenylmethanes[1].

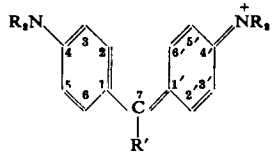
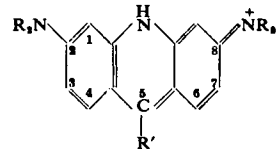
EXPERIMENTAL

In the first series of experiments the bacteriostatic effects of 2:8-diaminoacridine (proflavine), 2:8-bis(dimethylamino)acridine (acridine orange), 4:4'-diaminobenzhydrol and 4:4'-bis(dimethylamino)benzhydrol (Michler's hydrol) were compared using a culture of *Staphylococcus aureus*. The structural relation of these dyes is shown in Table I. Acridine orange possesses dimethylated amino groups, and is the higher homologue

of proflavine in the 2:8-diaminoacridine series. The same relation exists between 4:4′-diaminobenzhydrol and Michler's hydrol in the diphenylmethane series. The immonium cation forms of the two hydrols are analogues of the two acridine derivatives.

TABLE I

STRUCTURAL RELATION OF THE DI- AND TRIPHENYLMETHANE DERIVATIVES TO THEIR ACRIDINE ANALOGUES

	 4:4′-diaminodi- and triphenylmethanes (immonium cation forms)	 2:8-diaminoacridines (immonium cation forms)
R=H R′=H	Immonium cation of 4:4′-diamino-benzhydrol	Proflavine
R=CH_3 R′=H	Immonium cation of Michler's hydrol	Acridine Orange
R=CH_3 R′=C_6H_5	Malachite Green	Brilliant Acridine Orange

The diphenylmethane derivatives are prepared according to the methods given in our earlier publications[5,7]. Commercial proflavine (May and Baker) and acridine orange (Grübler) were used. The substances were dissolved in 50 per cent. aqueous ethanol (0·1 g. in 20 ml.) the necessary dilutions being obtained by mixing the stock solutions with the required amounts of broth. The technique of the bacteriological experiments was the same as in our earlier work[4,5,7].

TABLE II

GROWTH OF *Staphylococcus aureus* IN BROTH IN THE PRESENCE OF TWO TRIPHENYLMETHANE DERIVATIVES AND THEIR ACRIDINE ANALOGUES

Dilutions	Diamino-benzhydrol		Michler's hydrol		Proflavine		Acridine orange	
		+ 20 per cent. serum		+ 20 per cent. serum		+ 20 per cent. serum		+ 20 per cent. serum
1:5000 ..	±	+	−	−	−	−	−	−
1:10,000 ..	+	+	−	−	−	−	−	−
1:20,000 ..	+	+	−	±	−	−	−	−
1:40,000 ..	+	+	−	+	−	−	+	+
1:80,000 ..	+	+	+	+	+	−	+	+
1:160,000 ..	+	+	+	+	+	+	+	+

Table II shows that our earlier results[5] are confirmed, i.e. in the presence of serum the activity of the diphenylmethanes is reduced and that of the acridines is enhanced while the higher homologues show respectively an increase and decrease in activity.

In further experiments the influence of a third benzene ring on the bacteriostatic activity against *Staphylococcus aureus* was studied comparatively. The introduction of a phenyl radical into the position 7 of

diphenylmethanes transforms them into triphenylmethanes. The corresponding change in the series of 2:8-diaminoacridines is the introduction of a phenyl radical into the position 5 which produces the acridine analogues of the triphenylmethanes (2:8-diamino-5-phenylacridines). As representative members of the *p*-diamino-di- and triphenylmethane series we employed Michler's hydrol and malachite green respectively and the acridines employed were acridine orange and brilliant acridine orange, (2:8-bis-(dimethylamino)-5-phenylacridine). Commercial malachite green (Grübler) was used, brilliant acridine orange was prepared according to German Patent No. 68908 (Leonhardt).

TABLE III

GROWTH OF *Staphylococcus aureus* IN BROTH IN THE PRESENCE OF TWO TRIPHENYLMETHANE DERIVATIVES AND THEIR ACRIDINE ANALOGUES

Dilutions	Michler's hydrol		Malachite green		Acridine orange		Brilliant acridine orange	
		+ 20 per cent. serum		+ 20 per cent. serum		+ 20 per cent. serum		+ 20 per cent. serum
1:40,000 ..	−	+	−	−	+	+	−	−
1:80,000 ..	+	+	−	−	+	+	−	−
1:160,000 ..	+	+	−	−	+	+	−	−
1:320,000 ..	+	+	−	−	+	+	−	−
1:640,000 ..	+	+	−	−	+	+	−	+
1:1,280,000 ..	+	+	−	−	+	+	+	+
1:2,560,000 ..	+	+	−	−	+	+	+	+
1:5,120,000 ..	+	+	−	+	+	+	+	+
1:10,240,000 ..	+	+	−	+	+	+	+	+
1:20,480,000 ..	+	+	+	+	+	+	+	+

Table III gives the results obtained in the bacteriostatic experiments, which confirm our earlier findings[5,7] that the transformation of the diphenylmethane compound into its triphenylmethane homologue causes a spectacular (250 fold) increase of the bacteriostatic effects. A similar but quantitatively not so marked increase is observed in the acridine series, brilliant acridine orange being clearly more active than acridine orange. The bacteriostatic effect of brilliant acridine orange is diminished in the presence of 20 per cent. of serum, like that of the triphenylmethane dyes.

Our results appear to contradict those of Albert *et al.*, according to which the introduction of a phenyl radical into the position 5 of the acridine molecule constitutes a dystherapeutic dimensional factor influencing the bacteriostatic power unfavourably. But different acridine compounds, acridine yellow and benzoflavine, were used by Albert *et al.* and these were not methylated in positions 2 and 8 as were the compounds used by us. Thus it appears that the dimethylation of the amino groups changes the character of the bacteriostatic action from the "acridine-like" type toward a more "triphenylmethane-like" one. Such a supposition would be supported by the fact that the bacteriostatic action of brilliant acridine orange is not enhanced by serum, like that of the acridines with free amino groups, but is reduced, like that of the triphenylmethanes. Therefore the favourable effect of the presence of a third phenyl radical in the molecule of brilliant acridine orange could be explained by the "triphenylmethane like" character of its bacteriostatic action.

If brilliant acridine orange is now considered as a derivative of malachite green it appears that an imine bridge between the positions 2 and 6′ of the latter does not change the character of the bacteriostatic action, which remains "triphenylmethane like," but diminishes its strength to a certain degree.

Similar experiments have been made with other organisms, e.g. *Streptococcus fæcalis*, *Shigella* sp., *Eberthella* sp., *Salmonella* sp. and *Escherichia* sp. The results of these, including those with *Staphylococcus aureus*, are shown in the Table IV. The degree of activity of the assayed substances is expressed by the reciprocal of its highest inhibitory dilution, the subsequent non-inhibitory dilution being always 1:2.

TABLE IV

BACTERIOSTATIC ACTIVITIES EXPRESSED IN THE RECIPROCALS OF THE HIGHEST INHIBITORY DILUTIONS

Organisms	Proflavine		Acridine orange		Brilliant Acridine orange		Malachite green	
		+ 20 per cent. serum		+ 20 per cent. serum		+ 20 per cent. serum		+ 20 per cent. serum
Staphylococcus aureus	40,000	80,000	20,000	20,000	640,000	320,000	10 millions	5 millions
Streptococcus fæcalis	80,000	160,000	80,000	160,000	320,000	160,000	2·5 millions	1·25 millions
Shigella sp. ..	40,000	80,000	40,000	20,000	160,000	80,000	640,000	80,000
Eberthella sp. ..	20,000	40,000	10,000	10,000	10,000	10,000	320,000	40,000
Salmonella sp.	10,000	20,000	10,000	10,000	10,000	10,000	160,000	80,000
Escherichia sp.	10,000	20,000	10,000	10,000	10,000	10,000	10,000	10,000

The Table shows that the bacteriostatic action of proflavine is relatively uniform against the organisms employed. Its effect is more pronounced against Gram-positive (and *Shigella* strains) than against Gram-negative organisms, but these differences are not as marked as we are accustomed to see them with triphenylmethane dyes. Furthermore, it can be seen that the bacteriostatic activity of proflavine is enhanced by serum in every instance.

Malachite green is much more selective than proflavine and is always inhibited by serum. The pattern of the malachite green action is closely followed by brilliant acridine orange, whose bacteriostatic effects, though inferior, show a similar selectivity and are always inhibited by serum. The behaviour of acridine orange is not characteristic in any sense. Its activity is weak and the influence of serum on it is variable.

DISCUSSION

The 2:8-diamino-acridines may be considered as a special class of 4:4′-diaminodi- and triphenylmethane derivatives with an imine bridge between two benzene rings. The bacteriostatic activity of the simpler members of this acridine series is, however, not connected with this structure, as many other acridine derivatives not related to the 4:4′-diaminodi- or triphenylmethane compounds, such as 5-aminoacridine are also active[6]. Also the bacteriostatic activity of the simpler acridine derivatives shows some characteristic features which contrast with those

of the di- or triaminophenylmethanes. This "acridine like" type of activity apparently reaches its maximum with compounds without methylated amines and of reduced dimensions, whereas the corresponding di- and triphenylmethanes are almost without activity, the maximum effect being attained only with three benzene rings and two alkylated amines[5].

Albert *et al.*[6] have demonstrated in extensive experiments that the bacteriostatic activity of acridines depends on certain physicochemical properties, among which the degree of cationic dissociation seems to be the most important. According to these authors the influence of chemical configuration on the bacteriostatic strength is exerted principally through the physicochemical changes conditioned by it and modified by dimensional and other factors (cf. also ref. 8). Since the formulation of an earlier but similar theory by Stearn and Stearn[9] several attempts have been made to explain the triphenylmethane activity along the same lines. In our opinion these attempts have been unsuccessful (*cf.* ref. 10). The fact that basic triphenylmethane dyes as well as acridines show stronger action in a more basic medium than in a more acid environment was explained by Stearn and Stearn as the result of a higher ionization of anionic protein groups in relatively alkaline solutions and it was used by these authors as an argument in favour of their theory. This argument was discussed by Ingraham[11] and by Fischer and Muñoz[12]. It seems to us that the higher activity of several alkaline antibacterial agents in a more alkaline medium does not necessarily mean that the bacteriostatic activity of such compounds depends on a reaction with anionic cell constituents but can be otherwise explained, especially in view of experimental findings about the lack of parallelism between basic strength and bacteriostatic activity in the triphenylmethane series (in contrast with the acridine series[5,10]). On the basis of our previous experiments[5] we conclude that the bacteriostatic action of 4:4′-diaminodi- and triphenylmethane derivatives depended on other factors, among which a "potentially quinoid structure" seems to be the most important.

The "triphenylmethane like" character of the bacteriostatic action of brilliant acridine orange is possibly due to the fact that its chemical configuration is nearer to the structure optimal for a "triphenylmethane-like" type of activity than to that which is necessary for the "acridine-like" one.

The decreased activity of benzoflavine in the experiments of Albert *et al.* may be explained similarly, attributing it to the fact that the structure of this compound is not favourable either for an "acridine-like" type of activity because of the presence of a third phenyl radical, nor for a "triphenylmethane-like" one because of the lack of alkylation of the amino groups. This theory could also apply to acridine orange where the unfavourable factor for an "acridine-like" activity is the methylated state of the amino group and at the same time a marked "triphenylmethane-like" action is made impossible by the lack of a third phenyl radical.

Considering brilliant acridine orange from a biological point of view as a triphenylmethane derivative, it can be concluded that the presence of

an imine bridge between two benzene rings, with its inhibiting effect on the rotation of these and increasing the coplanar part of the molecule, does not impede the bacteriostatic effect or change its character, but has only a slightly unfavourable influence on it.

SUMMARY

1. Brilliant acridine orange, a higher homologue of proflavine and an acridine analogue of malachite green, has a bacteriostatic activity, which is markedly superior to that of proflavine and somewhat inferior to that of malachite green.

2. The activity of brilliant acridine orange does not show the "acridine-like" type, but follows closely the patterns of the triphenylmethane action.

3. Acridine orange with only two phenyl radicals, but with methylated amino groups, has a structure which is neither favourable for the "acridine-like" activity, nor for the "triphenylmethane-like" type. Its action is weak and uncharacteristic.

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