AGGLUTINATION OF MOTILE SALMONELLAS BY ACRIDINES

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Genetic studies of the determination of Salmonella flagella have suggested that the alternative antigenic phases correspond to two distinct homology groups, i.e., to factors at two distinct genetic loci, one for phase 1 ("specific phase"), and one for phase 2 ("group phase") (Lederberg and Edwards, 1953). It has, however, been generally understood that the two phases were equivalent to one another in all respects save their detailed antigenic structure and thus would give no hint of such a dual determination (Stocker, 1949). Sertic and Boulgakov (1936a, b) had, however, reported slide agglutination of smooth phase 2 cultures by acriflavine, while phase 1 did not react. We have sought, therefore, to confirm this generally overlooked observation and to look for any further clues that might bear on the reversible differentiation of the two loci which control flagellar phase variation (Lederberg, 1954).

MATERIALS AND METHODS

Various serotypes were selected from stock cultures listed previously (Lederberg and Edwards, 1953). Each was carefully examined for roughness which might interfere with the flagellar reaction. The alternate phases were obtained either by colony selection (Andrewes, 1922) or by selective swarming in motility agar containing absorbed anti-H sera (Edwards and Ewing, N.D.).

Previous investigators have used the slide agglutination technie. Since this method does not lend itself to quantitative titrations and is liable to give false reactions, tube agglutination procedures were preferred. Bacterial suspensions were prepared from overnight cultures in Difco penassay broth inoculated with single colonies of the desired phase and incubated at 37 C. These cultures were diluted to approximately 10⁸ cells per ml and used alive, unless otherwise stated.

The agglutinations were conducted in 10 by 75 mm culture tubes held at 37 C and read after 2 to 4 hours. Except for a slight increase in titer, the readings were unchanged after the longer period.

For precipitation tests on isolated flagella, the double diffusion agar gel technique (Ouchterlony, 1949) was chosen after preliminary trials. Flagellar suspensions were isolated from Roccal treated bacteria by method 2 of Uchida, Sunakawa, and Fukumi (1952). Table 3. The acridines tested and some analogues are listed in table 3. They were generally made up as 0.2 per cent solutions in distilled water with the addition of dilute acetic acid if required.

RESULTS AND DISCUSSION

The experiments fall into three main groups: those conducted on broth suspensions, those performed with washed aqueous or saline preparations, and those utilizing isolated flagella.

Agglutination of living broth suspensions. The strains were tested in parallel with specific (absorbed) anti-H sera, acriflavine (0.2 per cent in 1 per cent saline), and with Millon's reagent (White, 1929). The results are presented in table 1. Phase reversal was reiterated with several strains, and only phase 2 was agglutinated so long as the strain remained smooth.

It was also found that, unless fully motilized, cells were usually inagglutinable by acriflavine although still capable of reacting with anti-H sera.

Besides confirming Sertic and Boulgakov's
TABLE 1
Agglutination reaction of strains in broth

<table>
<thead>
<tr>
<th>Strain</th>
<th>H Antigens</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Acridine</td>
<td>Million</td>
</tr>
<tr>
<td></td>
<td>Ph. 1</td>
<td>Ph. 2</td>
<td></td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>i:1,2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. stanley</em></td>
<td>d:1,2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. sega</em></td>
<td>d:z1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. london</em></td>
<td>l:v:1,2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. dar-es-salaam</em></td>
<td>l,w:e,n,z15</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. wien</em></td>
<td>b:1,w</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. abony</em></td>
<td>b:e,n,z</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>S. paratyphi B</em></td>
<td>b:1,2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. typhi O 901</em></td>
<td>- [d]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SW666</td>
<td>- [b]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SW229</td>
<td>l,z:e,n,z</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Anti-H sera were used at a final dilution of 1 in 1,000. The titers of the sera ranged from 1 in 3,000 to 1 in 300,000. The acridine dye was 0.2 per cent acriflavine.

S and R indicate smooth and rough, respectively, as determined by the Millon reaction (White, 1929). Precipitation alone was taken to indicate roughness.

+ and - indicate presence or absence of agglutination.

findings, these results raise one other point. The antigenic complex *i* which appears as phase 1 in some, and as phase 2 in other, serotypic formulae reacts with acriflavine in all cases as if it were a phase 1 complex. The complex *i* usually represents phase 2 but is present as an anomalous phase 1 in some bacteria (Lederberg and Edwards, 1953). These organisms are agglutinated by acriflavine in both phases. With these exceptions phase 1 homologues are stable and phase 2 homologues are agglutinated by the dye.

**Conditions of agglutination in broth.** Cells of both phases were washed and suspended in broths which had been adjusted to pH values from 2 to 10 inclusive by the addition of concentrated HCl or NaOH. Both phases agglutinated spontaneously at pH 5; above this value the reaction was unaffected. At pH 4 and below no agglutination of phase 2 cells occurred. If cells exposed to these low pH values were washed and resuspended in normal broth at pH 7, they were still unagglutinable. This suggests that some irreversible change has taken place in the cells during their exposure to these low pH's. It is likely that the flagella are the site of this action since they are known to be acid sensitive (Weibull and Tiselius, 1943).

Organisms heated to 100 C for 15 minutes were no longer agglutinable by either anti-H sera or acriflavine, a correlation which would also implicate the flagella. Furthermore both macroscopically and microscopically the agglutinates resembled those normally formed by anti-H sera.

Since it is inconvenient to work with large volumes of living pathogens, some antiseptics were tested for interference with the dye agglutination reaction. Mercurials and formalin reacted directly with acriflavine to produce a chrome yellow precipitate. If the cells were then washed and resuspended in fresh broth, there was no interference with the reaction. Phenol tended to inhibit the agglutination unless removed by washing. Roccal (benzalkonium chloride, Winthrop-Stearns, Inc., N. Y.) had no effect on the reaction even at bactericidal or higher concentrations, and was used whenever living cells were contraindicated (Anderson *et al.*, 1952).

**The effect of broth on the agglutination.** The previous experiments were conducted in broth. To determine whether electrolytes were needed, twice washed cells were suspended in distilled water and tested with 0.2 per cent aqueous acriflavine. Contrary to the results in broth all H suspensions were agglutinated equally by the dye, but O cells were unaffected. Apparently this reaction, unlike serum agglutination, does
not require the addition of electrolytes for visible
aggregation unless the dye itself (at $\frac{1}{4,000}$)
acts in this respect.

This loss of specificity for the two phases in
distilled water might be ascribed to (1) a reagent
in the broth, (2) a bacterial metabolic product,
or (3) the washing procedure.

Phase 1 cells were washed twice with distilled
water, and four aliquots were then resuspended
in distilled water, fresh broth, the supernatant
broth from the original culture, and the super-
natant broth from a phase 2 culture, respectively.
Only the aqueous suspension was found to be
agglutinable by acriflavine. This supports the
hypothesis that normal broth contains an agent
capable of inhibiting the agglutination of phase 1
cells by acriflavine. It remains to determine
what the agent is and whether the inhibition is
absolute or not.

Inhibiting agents. An empiric examination of
certain constituents of penassey broth was made
to see whether they would inhibit the reaction.
Aqueous phase 1 cells were used as indicators.
The results are presented in table 2: the purines
and pyrimidines in broth could be part at least
of the inhibitor. Broth, however, is a very complex
medium, and the participation of other com-
ponents is not excluded.

It was further found that one could overcome
the inhibition of any of these agents by adding
further acridine. In a titration involving adenine
hydrochloride and aminacrine (see below) with
aqueous phase 2 cells as indicator, it was found
that for every mole of adenine two moles of
acridine had to be added to overcome the inhibition.
That there is a direct reaction between
acridine and inhibitor is suggested, but competition
for bacterial sites cannot, on the basis of this
evidence alone, be excluded.

Titrations of acriflavine activity. Doubling dilu-
tions of acriflavine were titrated against suspen-
sions of phase 1, phase 2, and O cells. Preliminary
titrations had shown that, unlike serum reactions,
the concentration of cells did not, within broad
limits, affect the end point.

In broth, O cells were not agglutinated by con-
centrations up to 1 per cent acriflavine, this being
near the limit of solubility of the acridine. Phase
1 cells were agglutinated by 1 in 100 acriflavine,
and phase 2 cells were agglutinated at concen-
trations as low as 1 in 3,000.

In water, the O cells still remained inagglu-
tinable, but the end points for the other two cell
types were changed. Phase 1 cells were now ag-
glutinated by 1 in 6,406 and phase 2 cells by 1
in 12,800 final concentrations of acriflavine,
respectively.

Activity of other acridines. Several other acri-
dines and some analogues were examined for
their effects in the presence and absence of broth.
The results are shown in table 3 from which it
can be seen that the majority of the acridines
behave like acriflavine.

Certain of the reactions are of interest when
one considers the theory of acridine bacteriostasis
postulated by Albert (1951). He has suggested
that a certain "envelope" size is required for the
acridine to be effective as an antibacterial agent.

Tetrahydroaminacrine would be inactive because
the saturation of one of the aromatic rings results
in a nonplanar molecule. Similarly, aminouirdine
is too small, but the addition of a steroyl
side chain increases the envelope to the size re-
quired for an active antibacterial agent. The
inactivity of the hydroxy compound, on the other
hand, was thought to be due to its zwitter-ion
structure.

In general the broth reactions agree with this
theory; the action of tetrahydroaminacrine on
aqueous phase 2 cells is, however, discordant.

Whether there is a mechanism of acridine ac-
tivity on flagella different from that involved in
bacteriostasis, or whether the acridine compound
TABLE 3

Acridine dyes and analogues investigated with their activities

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula (numbering according to Albert, 1951)</th>
<th>Suspensions Broth</th>
<th></th>
<th></th>
<th>Suspensions Water</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>O</td>
<td>Phase 1</td>
<td>Phase 2</td>
<td>O</td>
<td>Phase 1</td>
<td>Phase 2</td>
</tr>
<tr>
<td>Acriflavine*</td>
<td>mixture of proflavine and euflavine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proflavine*</td>
<td>2,8-diaminocridine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acridine orange (a)*</td>
<td>2,8-diamino-bis-dimethyl-</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acridine orange (b)*</td>
<td>2,8-diamino-bis-dimethyl-</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Euflavine</td>
<td>2,8-diamino-10-methylacridinium hydrochloride</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aminacrine</td>
<td>5-aminoacridine</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tetrahydroaminacrine</td>
<td>1,2,3,4-tetrahydro-5-aminoacridine</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydroxyaminacrine</td>
<td>2-hydroxy-5-aminoacridine</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aminoquinoline</td>
<td>4-aminoquinoline</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Styrylamoquinoline</td>
<td>4-amino-2-styrylquinoline</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* These compounds were obtained from commercial sources and the remainder through the kindness of Dr. A. Albert and Dr. S. D. Rubbo.
+ and – indicate the presence or absence of agglutination.
† Indicates that the compound was precipitated in the presence of broth.

would display different antibacterial activities if examined in buffered saline rather than in broth has not been decided.

The reason for the difference in activity of the two preparations of acridine orange is not certain. These were both commercial preparations and may well have contained impurities or have differed considerably in dye content.

Bactericidal tests with acriflavine and euflavine were conducted on phase 1 and 2 Salmonella typhimurium cultures. No significant difference in the rate of killing was found.

Absorption of acriflavine by bacteria. Concentrated suspensions of O, phase 1, and phase 2 cells were added to acriflavine solutions of different concentrations and pH. Roccot treated suspensions had been estimated photometrically so as to contain equivalent amounts of bacterial substance. After a 5 minute exposure at 37°C the cells were removed by centrifugation, washed, and the acriflavine content of both cells and supernates estimated with the Coleman spectrophotometer at 445 μm.

Spectrophotometry with acridine compounds is liable to give misleading results owing to their high degree of fluorescence (Albert, 1951). However, no marked differences could be found in the amount of dye retained by the different types of cells under identical conditions.

Reactivity of isolated flagella. Tube agglutination tests were performed with aqueous suspensions of phase 1 and phase 2 flagella. Acriflavine at a concentration of 1 in 6,400 agglutinated phase 2 flagella, but a concentration of 1 in 1,600 was needed for the agglutination of phase 1 flagella. It was found that more concentrated flagellar suspensions were needed for agglutination by acriflavine than by specific antisera. This is in accord with the earlier findings on poorly motile cultures prior to the selection of full motility by passage through semisolid agar.

Plate precipitation tests with acriflavine or aminacrine were uniformly negative although similar tests with specific sera gave strong precipitin lines. Similarly when flagella were disaggregated with dilute HCl (Weibull and Tiselius, 1945), they no longer reacted with either serum or acriflavine in tubes. The treated flagella still responded to serum in plate precipitin tests. The merging of precipitin lines showed no difference between the native and treated flagella preparations.
These results confirm the impression gained earlier that the site of acridine action is the flagellum. It is probable that acid treatment gives rise to particles too small to form sedimentable aggregates with acridine but not with sera. This would also explain the lack of acridine precipitin lines since intact flagella would be too large to diffuse through the agar whereas the degradation products would be too small to be sedimented (Gard et al., in press).

These studies have not settled the physico-chemical basis of flagellar differentiation. Thus while Sertic and Boulgakoff's original observations are confirmed, closer study has shown that the differences between the phases must be considered on a quantitative, not qualitative, basis. However, the genetic differentiation of the phases as the products of distinct loci is paralleled by this difference in agglutinability, whatever its physical foundation.

Acriflavine has been widely used as a criterion of smoothness (Braun and Bonestell, 1947). The present findings reinforced the caution with which this criterion must be interpreted, especially when dealing with Vi+ (Hirsch, 1937) or with motile organisms.

REFERENCES


