31. The Surface Behaviour of Antibacterial Substances

1. Sulphanilamide and Related Substances*

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The adsorption of polar molecules at the surface of bacteria will necessarily be accompanied by changes in the charge at the surface. Such an effect may be investigated by electrophoretic methods. The mobility of a small particle when placed in a potential gradient is proportional to the electrokinetic potential, which is dependent on both the surface charge and the thickness of the diffuse layer.

The electric mobility of bacteria has been investigated in connexion with determinations of stability and isoelectric point, and the effect of simple inorganic salts on these quantities has been studied by Abramson [1934] and others. The effect of the age of the bacteria on the electric mobility has been investigated by Moyer [1940], who observed that the mobility of *B. coli* passes through a minimum during the first 7 hr. of growth, thereafter remaining independent of age. In agreement with Moyer we have found 18–36 hr. cultures of *B. coli* to have a mobility independent of age. Mobilities have been determined on suspensions of bacteria in distilled water [cf. Pedlow & Lisse, 1936], but the use of this medium has been criticized by Abramson [1934] and by Moyer [1940] on the grounds that slight changes in ionic concentration produce appreciable changes in mobility, and their experiments have been carried out in buffered salt solutions. Such a method cannot be employed when the effect of small concentrations of polar compounds is being investigated, but by using 'equilibrium water' (conductivity $0.6-1.0 \times 10^{-6} \text{ ohm}^{-1} \text{ cm.}^{-1}$) we have found it quite possible to obtain reproducible values for the mobility of *B. coli* suspensions (Fig. 6). Any slight variation in the initial reading is of less consequence when observing the variation of mobility with time in the same suspension than in a series of readings on different suspensions.

The interference of antibacterial agents and essential metabolites has been demonstrated by a number of workers. Woods [1940] has shown that sulphanilamide is antagonized by *p*-aminobenzoic acid, and the latter has been shown to be an essential metabolite by Rubbo & Gillespie [1940]. Fildes [1940] has interpreted this antagonism as indicating that the antibacterial substance exerts its characteristic action on the organism by interfering with the utilization of the essential metabolite. This interference may take the form of competition for a common substrate due to the chemical similarity of the molecules concerned, both sulphanilamide and *p*-aminobenzoic acid containing a basic and an acidic group in the *para* position in a benzene ring. Further, from a comparative study of the dipole moments of sulphanilamide, metanilamide, and the esters of *p*- and *m*-aminobenzoic acid, Kumler & Halverstadt [1941] have concluded that the contribution of the forms (I) and (II) to the resonance structure is about the same for sulphanilamide as for *p*-aminobenzoic acid.

* A preliminary report of this work was given at the Biochemical Society discussion meeting on 'Mode of Action of Chemotherapeutic Agents' (see *Nature* [1941], **148**, 757).

It was expected that sulphanilamide and *p*-aminobenzoic acid would behave similarly at the bacterial surface, and that this similarity would reveal itself in the effect of the two compounds on the electric mobility:

EXPERIMENTAL

A suitable type of single cell for determining electrophoretic mobility has been described by Abramson [1928] and Moyer [1936, 1]. Smith & Lisse [1936] have introduced a new double cell in which the stationary level is at the centre of the cell, and this apparatus has been found to be satisfactory by Powney & Wood [1940] for measurements on paraffin suspensions. We have modified the original design of the Smith & Lisse cell to eliminate rubber joints, by the construction of the all-glass apparatus shown in Fig. 1. The capacity was approximately 5 ml. The visibility of the bacteria was considerably improved by grinding the cell very thin, cementing on parts of a coverslip with canada balsam, and using dark-ground illumination. The apparatus was mounted on the stage of a microscope and the movement of the bacteria observed over a distance of 166 μ , using an eyepiece grating. The potential gradient was calculated by the method of Northrop [1922], and values of 6.4, 12.8 and 19.2 V./cm. were used according to the mobility.

Each measurement of mobility was the mean time of 50 readings on different individual bacteria, the current being reversed after every five readings to prevent polarization. All measurements were made at room temperature, and a filter of saturated alum solution was placed between the cell and the source of illumination to remove heat radiation.

The glass apparatus was cleansed thoroughly between experiments using warm chromic acid followed by thorough washing with water. Traces of sulphanilamide and, in particular, *p*-aminobenzoic acid were not, however, completely removed by this treatment, and prolonged soaking in purified acetone (in which they are remarkably soluble) was found to be satisfactory for cleansing after use of these compounds.

Bacterium coli-commune Esch. (National Collection of Type Cultures, no. 86) was grown in liquid-nutrient broth (pH 7.4) for 18–36 hr. at 37°. The organisms were removed from the medium by centrifuging for 10 min. (3000 r.p.m.), resuspended in conductivity water, centrifuged and again washed, being finally separated after two such washings. In agreement with Moyer [1936, 2] it was found that further washing had no effect on the mobility and was therefore unnecessary.

The washed organisms were suspended in sufficient conductivity water to give an opacity equal to that of a standard BaSO₄ suspension [cf. Gardner, 1931]. Counts were made on a Thoma haemocytometer with a chamber 0.1 mm. deep with double Neubauer ruling, giving 400 small squares each $\frac{1}{400}$ mm.² in area. The average count showed the suspension to contain 15×10^7 bacteria per ml. 5 ml. of this suspension and an appropriate volume of a standard solution of the chemical under investigation were diluted to 100 ml. The suspension was drawn into the chemically clean apparatus and mobility measurements taken at intervals of approximately 15 min.

RESULTS AND DISCUSSION

The all-glass modification of Smith & Lisse's cell has proved a very convenient apparatus for the determination of electrophoretic mobility of bacteria suspensions. Abramson [1940] has indicated some advantages of this type of cell and has suggested that further evidence that it provides quantitative data should be forthcoming. Our experiments have been made with four such cells and two different observers. In every case a value of $3.0 \pm 0.1 \mu/\text{sec.}/\text{V.}/\text{cm.}$ has been obtained for *B. coli* suspended in 'equilibrium water', provided that the apparatus was free from traces of chemicals.

All the active antibacterial compounds studied produced a characteristic effect on the mobility of *B. coli*. In contrast to the inactive substances, which produced an initial change in mobility which did not subsequently alter with time of contact, the active substances affected the mobility of the bacteria in a way which varied with the time of contact with the solution of the drug. This change was characterized by the formation of two well defined maxima in the mobility-time curve, as illustrated in Figs. 2 and 3, the bacteria finally attaining a constant or slowly decreasing mobility. Preliminary experiments with proflavine, an antibacterial substance chemically unrelated to the sulphanilamide group, indicate that it too shows this characteristic behaviour. The production of such a mobility-time curve may be an essential requirement for activity, although further investigation will be required to establish this.

In Fig. 2 the variation of mobility with time is shown for solutions of sulphanilamide having various concentrations. The curve for the 0.01 % solution has been determined on four occasions using three different cells. The effect of dilution is to cause a shift of the curve without producing any great change in its characteristic shape, the formation of the maxima, in particular the second, occurring after a greater period of time.

p-Aminobenzoic acid produced an effect on the mobility of *B. coli* closely resembling that of the antibacterial substances (Fig. 4). This may be interpreted as indicating that sulphanilamide and related antibacterials behave in a similar manner to *p*-aminobenzoic acid at the surface of the bacteria. This is in conformity with the view [Fildes, 1940] that sulphanilamide exerts its characteristic action by interfering with the normal utilisation of *p*-aminobenzoic acid. The close similarity of the respective curves suggests that this interference may take the form of competition between sulphanilamide and *p*-aminobenzoic acid for certain reactive centres on the surface of the organism; the drug, by virtue of its similar surface reaction, being able to take the place of the essential metabolite and so exclude it from the bacterial surface. Rubbo & Gillespie [1940] demonstrated that 23,000 molecules of sulphanilamide were required to inactivate 1 mol. of *p*-aminobenzoic acid, which suggests a mass effect of this nature by the drug.

Comparing the curves for benzenesulphonamide and for aniline (Fig. 6) it appears evident that the benzenesulphonamide is without action on the bacterial surface, there being no change of mobility with time of contact with this substance. On the other hand, aniline hydrochloride solution (0.01 %, containing 64 % of the cation $\text{C}_6\text{H}_5.\text{NH}_3^+$) has a marked surface effect, the mobility falling to 75 % of its original value during 4 hr. It may be concluded that the group $-\text{SO}_2\text{NH}_2$ is without initial effect on the bacterial surface, the first association of the drug molecule and the bacterial surface being due to the positively charged quaternary N. This view receives support from the investigations of Rideal & Schulman [1939] on the relative surface reactivity of various polar groups. Working on surface films of cholesterol they found surface reactivity to be in the order

the group $R.\text{NH}_3^+$ having the greatest activity. If the first association of the drug molecule with the bacterial surface is due to the presence of a positively charged quaternary N of the type $-\text{NH}_3^+$, the necessity for the presence of a free, or potentially

Fig. 1. All-glass electrophoresis cell.

Fig. 2. Variation of mobility of *B. coli* with time of contact with solutions of sulphanilamide. □ ○ △ × 0.01% solution ■ 0.005% solution. ● 0.001% solution.

Fig. 3. Variation of mobility of *B. coli* with time of contact with solutions of sulphapyridine and sulphathiazole. □ ○ 0.01% solution of sulphapyridine. ■ ● 0.01% solution of sulphathiazole.

Fig. 4. Variation of mobility of *B. coli* with time of contact with solution of *p*-aminobenzoic acid. □ ○ 0.01% solution. ● 0.001% solution.

free, $-\text{NH}_2$ group in the molecule of active sulphanilamide derivatives [cf. Northey, 1940, 1] receives explanation.

The curves for N_4 -acetylsulphanilamide and for metanilamide are shown in Fig. 5. The former compound shows a very attenuated form of the sulphanilamide curve, the

Fig. 5. Variation of mobility of *B. coli* with time of contact with solutions of acetylsulphanilamide, metanilamide and aniline hydrochloride. $\square$ $\circ$ 0.01% solution of N_4 -acetylsulphanilamide. $\blacksquare$ $\bullet$ 0.01% solution of metanilamide. $\triangle$ $\times$ 0.01% solution of aniline hydrochloride.

Fig. 6. Variation of mobility of *B. coli* with time of contact with solutions of some inactive substances. I, sodium benzenesulphonate. II, equilibrium water. III, aniline. IV, benzenesulphonamide. V, sulphanilic acid. VI, benzoic acid.

characteristic maxima being more drawn out than those of a 0.001% sulphanilamide solution (Fig. 2). This corresponds to its known slight activity [Buttle *et al.* 1936]. Metanilamide, a substance without activity [cf. Northey, 1940, 2], behaves very much like aniline hydrochloride. Removal of the sulphonamide group from the position *para*- to the position *meta*- to the $-\text{NH}_2$ group has converted a compound exhibiting characteristic maxima into one giving an effect similar to that of aniline. The two substituent

groups evidently behave quite independently when *meta*- to one another, the $\text{—SO}_2\text{NH}_2$ group having no action on the surface, the —NH_2 group producing the slow fall of mobility with time characteristic of anilinium ion. This effect of positional isomerism on the surface properties and activity is paralleled by its effect on resonance within the drug molecule. When the $\text{—SO}_2\text{NH}_2$ and —NH_2 groups are *para*- to one another the polar structure (I) contributes to the resonance of the molecule. When the groups are in the *meta*- position no such polar form is possible. It may be concluded that the polar character of the molecule is intimately connected with its activity. Such polar resonance forms are possible for all the active sulphanilamide derivatives studied as well as for *p*-aminobenzoic acid [cf. Kumler & Halverstadt, 1941].

Whilst this production of polarity by resonance is evidently more important for activity than ampholytic character (metanilamide and sulphanilamide are both ampholytes), other structural properties of such polar molecules must contribute to their antibacterial activity since Kumler & Halverstadt [1941] have shown that in *p*-(*p*-aminophenyl)-benzenesulphonamide the contribution of the polar resonance form is twice as great as in sulphanilamide although the compound is inactive. Also orthanilamide, which could show polar resonance between the —NH_2 and $\text{—SO}_2\text{NH}_2$ groups is without activity [Northey, 1940, 2]. The additional data required to provide a detailed interpretation of the mobility-time curves characteristic of antibacterial substances are being sought by further experiments, which will form the subject of future communications.

SUMMARY

The electrokinetic mobility of suspensions of *B. coli* in dilute aqueous solutions of sulphanilamide, *p*-aminobenzoic acid and chemically related compounds has been determined using an improved all-glass cataphoresis cell.

The effect of sulphanilamide and related antibacterials on the mobility of the organism is quite different from that of inactive substances such as aniline, benzenesulphonamide and metanilamide, but closely resembles that of *p*-aminobenzoic acid. It is concluded that sulphanilamide and related drugs behave like *p*-aminobenzoic acid at the bacterial surface.

The effects of sulphanilamide, benzenesulphonamide and aniline hydrochloride on the mobility have been compared, leading to the conclusion that the association of the drug with the organism is a function of the —NH_2 group, and that the polarity produced by resonance of active molecules is one of the factors determining activity.

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Note added 30 May 1942. Albert & Goodacre [1942] have determined the relative acidities and basicities of sulphanilamide and *p*-aminobenzoic acid, and observed that these two substances have approximately the same basic strength but very different acid strengths. This observation provides additional evidence for the conclusion reached above that the association of the drug molecule with the organism is a function of the —NH_2 group.