THE EFFECT OF BACTERIOSTATIC AGENTS ON THE ELECTROKINETIC PROPERTIES OF *AEROBACTER AEROGENES*

II. CRYSTAL VIOLET

by

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The interaction between drug molecules and the surface components of bacteria is most easily followed by the electrophoretic technique. One of the great advantages of this method is that measurements can be made upon the living organism, disorganisation by experimental manipulation being reduced to a minimum. Proflavine caused very profound changes in the surface properties of *Aerobacter aerogenes* (JAMES AND BARRY[1]). In this paper we report the effect of crystal violet, a drug of the triphenylmethane series, on the surface characteristics of this organism. It was considered that these dyes, with a totally different structure from that of the acridine series already studied, might give rise to another pattern of behaviour.

Since the outer surface of Gram-negative organisms is believed to be a lipopolysaccharide (MARSHALL, LOCKWOOD AND DUBOS[2]), drugs which are lipid soluble should be active against these organisms. Dyes of the triphenylmethane series are well known antibacterial agents. Substitution of alkyl radicals in the amino groups of the triamino- and diamino-triphenylmethanes results in both increasing lipid solubility and increasing activity. Gram-negative organisms are, however, less sensitive to this group of dyes than the Gram-positive (KLIGLER[3]). To account for their activity STEARN AND STEARN[4] suggested that the basic dye combines reversibly with the acidic groups of the bacterial protein, the action of the dye then being described in terms of a mass action equilibrium between the dye and the organism. More recently GALE AND MITCHELL[5] have given evidence that the triphenylmethane dyes prevent the metabolism of glutamic acid within the cells of Gram-positive bacteria.

With *Aerobacter aerogenes*, the presence of low concentrations of crystal violet in the growth medium prolongs the lag phase, subsequent growth occurring at a very reduced rate (DAVIES, HINSHELWOOD AND JAMES[6]). At higher concentrations the lag increases rapidly approaching an infinite value at about 7 mg/l. The family of lag-concentration curves for cells adapted to different concentrations of the drug is unlike those obtained with proflavine, where the spacing of the curves is exactly adjusted to the training concentration.

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EXPERIMENTAL

Cultures and media

The strain of *Aerobacter aerogenes*, maintained by monthly sub-culture in nutrient broth, was for experimental purposes grown in a chemically defined medium containing glucose (19.2 g/l), potassium dihydrogen phosphate (3.46 g/l) adjusted to pH 7.12, ammonium sulphate (0.96 g/l) and magnesium sulphate (0.04 g/l). The strain was sub-cultured daily in this medium at 40° C and a slow stream of washed sterile air passed through. For growth in the presence of drugs not more than 2 ml of a concentrated drug solution was added.

Growth in the absence of drugs was determined turbidimetrically using a Hilger photoelectric absorptiometer which had been previously calibrated against haemocytometer counts (MONOD[7]). In the presence of coloured drugs the growth was determined by counting on a haemocytometer with Thoma rulings.

Electrophoretic measurements

Unless otherwise stated, the organisms were grown for 24 hours in the synthetic medium, harvested, washed twice on the centrifuge with a mixed phosphate buffer solution (1.194 g/l $Na_2HPO_4 \cdot 12H_2O$ and 0.454 g/l KH_2PO_4) of ionic strength 0.013 and pH 7.00, and finally resuspended in that medium to give a count of about $2 \cdot 10^8$ organisms per ml.

The micro-electrophoresis cell, as previously described by BARRY AND JAMES[8] was used and observations were made at the lower stationary level, the position of which was determined by the SMOLUCHOWSKI theory. An organism in focus at this level was timed over a known distance (usually 0.012 cm) across the graticule first in one direction and then in the other, thus eliminating any slight differences between the two electrodes. The electrophoretic velocity determined at room temperature was corrected to 25° C by means of the equation of POWNEY AND WOOD[9]:

$$\eta_1 v_1 = \eta_2 v_2$$

where, η_1 and η_2 are the viscosities and v_1 and v_2 the electrophoretic velocities at temperatures T_1 and T_2 respectively. From the average velocity and the potential gradient the electrophoretic mobility was determined.

Growth of *Aerobacter aerogenes* in the presence of proflavine causes a non-uniform response of individual organisms to the drug, resulting in a spectrum of mobilities[1]. This effect was again apparent during growth in the presence of crystal violet, and it was therefore necessary to obtain as random a sample of the population as possible by selecting the organisms for timing prior to the application of the electric field. At least 50 organisms were timed, their individual electrophoretic mobilities determined and the histogram plotted. Selection of random samples was more difficult in this work as there appeared to be some correlation between size and the electrophoretic mobility.

The ζ potential, which is characteristic of a particular surface in a given suspension medium is related to the electrophoretic mobility according to the equation:

$$\zeta = \frac{4\pi\eta\bar{v}}{D}$$

where η is the viscosity and D the dielectric constant of the medium and $\bar{v}$ the electrophoretic mobility (cm/sec/volt/cm). In this work all our observations refer to the same suspension medium and in consequence we interpret our results in terms of the electrophoretic mobilities of the organisms rather than in terms of their ζ potentials.

The effect of crystal violet on the electrophoretic mobility of washed cells of Aerobacter aerogenes

Washed cells of *Aerobacter aerogenes* in phosphate buffer solution were allowed to stand for 30 minutes in contact with different concentrations of crystal violet and the mobility distributions determined in the presence of the dye. At concentrations up to 175 mg/l the normal distribution of mobilities about a mean was observed (Fig. 1), the

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average mobility decreasing with increasing concentration (Table I). The distribution in concentrations exceeding 200 mg/l was wider than normal, indicating a non-uniform response to the drug. At 300 mg/l crystal violet almost complete flocculation of the organisms occurred preventing any electrophoretic measurements. When cells which had been treated with 200 mg/l of crystal violet were centrifuged, washed and resuspended in fresh phosphate buffer solution it was found that the average mobility and the mobility distribution were as for untreated cells. This indicates that the combination of the dye with surface components is weak.

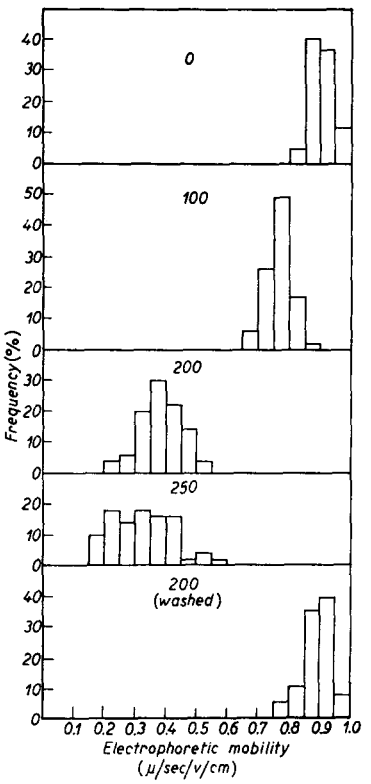

Fig. 1. The effect of crystal violet on the distribution of the electrophoretic mobilities of washed cells of *Aerobacter aerogenes*. (Concns. expressed in mg/l).

TABLE I

Concentration of crystal violet (mg/l)	*Mobility values (μ/sec/v/cm)*	*Average mobility (μ/sec/v/cm)*
0	0.91, 0.89, 0.92, 0.91, 0.90	0.91
25	0.94, 0.91, 0.91, 0.91, 0.91	0.92
50	0.74, 0.74, 0.73, 0.77, 0.76	0.75
75	0.75, 0.80, 0.77, 0.82, 0.83	0.79
100	0.77, 0.77, 0.79, 0.78	0.78
125	0.77, 0.77, 0.75, 0.79, 0.81	0.78
150	0.72, 0.72, 0.72, 0.74, 0.75	0.73
175	0.60, 0.61, 0.60, 0.60, 0.63	0.61
200 } 250 }	Wider distribution (See Fig. 1)	
300	Organisms flocculated	0

Similar washed suspensions allowed to stand with any concentration of the drug for up to 4 days gave histograms identical with those previously obtained. Further, no alteration of the distribution was observed when the cells had been allowed to age for 4 days in the growth medium at 40°C, before washing and suspending in the crystal violet solution.

The variation of the electrophoretic mobility accompanying the first sub-culture in medium containing crystal violet

Crystal violet is markedly bacteriostatic at a concentration of 6 mg/l; above this concentration growth of *Aerobacter aerogenes* becomes increasingly difficult[6]. The initial concentration chosen for electrokinetic experiments during the growth of the organisms in the presence of the drug was 5.6 mg/l. 1 ml inocula, from a culture of *Aerobacter aerogenes* growing at 40°C, were transferred into fresh media containing 5.6 mg/l crystal violet at suitable times during the growth cycle. These cultures were aerated and harvested 24 hours after the cessation of growth. The cells were washed and suspended in the phosphate buffer solution (lacking dye) and the electrophoretic mobility distributions for the various populations observed. The effect of the drug on the cell surface varied with the age of the parent inoculum; three main types of behaviour being distinguishable (Fig. 2).

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If the parent was less than 8 hours old the histogram after growth in the presence of the drug showed a normal Gaussian distribution, being occasionally displaced towards lower mobility values. Cells having mobilities less than 0.7 microns/sec/v/cm were never observed (Type A). A parent of age 8 to 36 hours gave final histograms showing a wide range of mobilities from 0.1 to 1.0 microns/sec/v/cm (Type B). Parent cultures of an age exceeding 36 hours gave rise to a mobility distribution curve which was unimodal, centered around very low mobility values (Type C).

These different types of electrokinetic behaviour have all been produced as a result of growth of the organisms in the presence of crystal violet. It was considered of interest to discover at what stage of the growth cycle in the crystal violet medium, the new surface characteristics were first evident.

1 ml inocula of a 48 hour old culture were transferred into a series of media containing 5.6 mg/l crystal violet. These daughter cultures were aerated and grown at 40° C. The ageing of the parent for 48 hours ensured that the final growth in the drug would give rise to a Type C histogram, *i.e.* one in which the surface has experienced the greatest change. Initial counts (n_0), which were determined, varied in different experiments from 40 to $60 \cdot 10^6$ organisms per ml. The growth in each culture was followed and at suitable times during the lag, growth and stationary phases one or more cultures were removed. The count (n) of each culture was determined and then the cells were harvested, washed and the mobility distributions determined. A composite growth curve for all the cultures

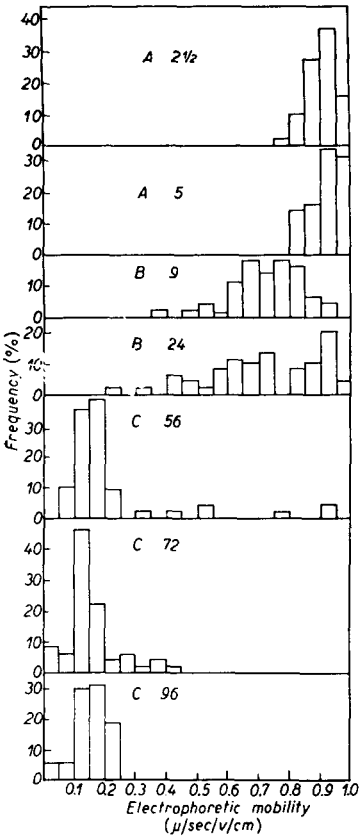

Fig. 2. The effect of the age of the inoculum of untrained cells on the mobility distribution after growth in media containing 5.6 mg/l crystal violet. (Numbers refer to the age of inoculum in hours, A, B and C to the types of behaviour).

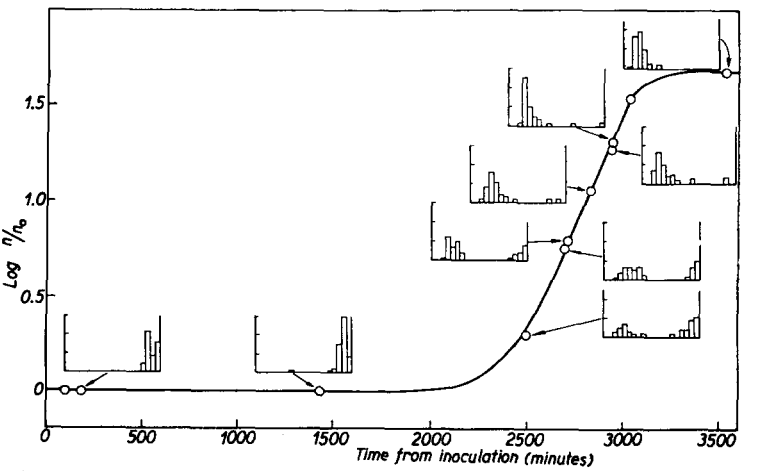

Fig. 3. Typical histograms showing the distribution of mobilities at different phases during the growth of untrained cells in media containing 5.6 mg/l crystal violet. (The histograms are plotted as for the other figures. Ordinate: Frequency (%), 1 div. represents 20%; Abscissa: Electrophoretic Mobility, 1 div. represents 0.05 μ/sec/v/cm.)

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of log n/n_0 against the time from inoculation was plotted; the histograms being superimposed at the correct count (Fig. 3). This showed that cultures tested in the lag phase gave normal distribution, those in the stationary phase type C distribution, whilst those tested in the logarithmic growth phase contained organisms which had either high or low mobilities, *i.e.* a bimodal distribution. Tests carried out on the initial inocula showed that the viability was about 9%, whilst after remaining in contact with the crystal violet during the lag phase it had decreased to about 0.8%. From a knowledge of the initial inoculum and the viability it was apparent that the decreasing proportion of organisms with the high mobility values observed during the growth phase represented the proportion of organisms which were dead. This proportion of dead cells decreased as division of the viable cells occurred and new cells were formed, until at the beginning of the stationary phase the number of living cells was so much greater than the number of dead ones that the histogram obtained was typical of type C. These newly formed cells possessed very low mobility values indicating a marked change of their electrokinetic surface as a result of division in the presence of the crystal violet.

The crystal violet may be acting either by absorption on the organisms during the lag phase producing the altered electrokinetic properties only on division, or by actual penetration and interaction at the moment of cell fission. A preliminary attempt to test these theories was made. A 1 ml inoculum of a 48 hour old culture was transferred to a complete growth medium containing 5.6 mg/l crystal violet and aerated for 24 hours. Before this culture commenced active cell division it was centrifuged, the cells being washed and used to inoculate a fresh drug-free medium. In this way the cells were in contact with the drug during the lag phase, actual cell division occurring in the absence of the drug. The mobility distribution of this culture after 24 hours growth was normal. 5.6 mg/l crystal violet was added to this full-grown culture, now in its stationary phase, but this failed to alter the histogram from the normal. Hence it is concluded that the crystal violet must actually be present in the medium during cell division to have any pronounced effect on the mobility distribution.

In addition to the marked change in the electrokinetic properties of the organisms accompanying growth in the presence of crystal violet, there was a considerable change in the size of the organisms. Cells grown in drug medium were often less than half the size of normal cells. There was some correlation between size and electrophoretic mobility; in cultures exhibiting type A behaviour the cells were of normal size, whilst in cultures exhibiting types B and C behaviour some cells were of normal size, the majority being very small. In this latter case the small cells always had mobility values less than 0.7 microns/sec/volt/cm, most cells of normal size had values in the range 0.7 to 0.9 microns/sec/volt/cm. Further all cells which had divided in the presence of crystal violet were stained blue, even after repeated washing with phosphate buffer solution.

The variation of the electrophoretic mobility accompanying training to crystal violet

The organisms were first grown in media containing 5.6 mg/l crystal violet for several subcultures and then the concentration of drug was increased step-wise to 40 mg/l. Trained series of cultures were stabilised by at least 10 sub-cultures at a given concentration and the mobility distributions determined at concentrations of 5.6, 7.4, 9.7, 19.2, 30 and 40 mg/l.

Each of the three types of behaviour was found to be transmitted without change on subsequent sub-culture in the presence of 5.6 mg/l crystal violet. Although type B

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behaviour was the product of a parent-age intermediate between that causing types A and C, nevertheless it was maintained unchanged on further culturing in the presence of low concentrations of the dye. Repeated sub-culture of these cells in increasing concentrations of the drug however, brought about a gradual increase in the proportion of cells with lower mobility values, so that at a concentration of 40 mg/l the histograms obtained were similar to those of type C (Fig. 4). In marked contrast, the adaptation of cells possessing either type A or type C behaviour to higher concentrations of the drug brought about no change in the original distribution of mobilities.

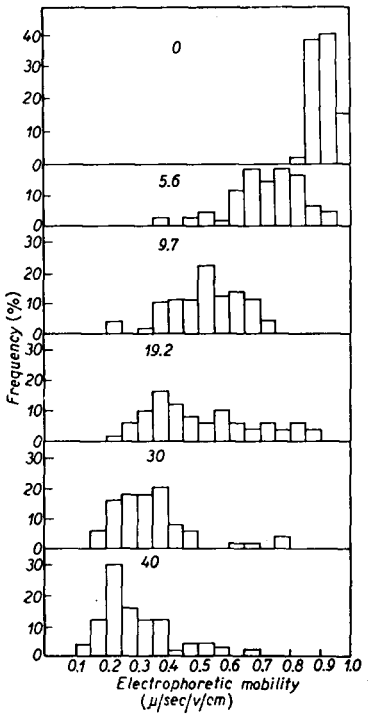

Fig. 4. The distribution of mobilities after training to increasing concentrations of crystal violet, for the training series of intermediate (Type B) behaviour. (Concns. expressed in mg/l).

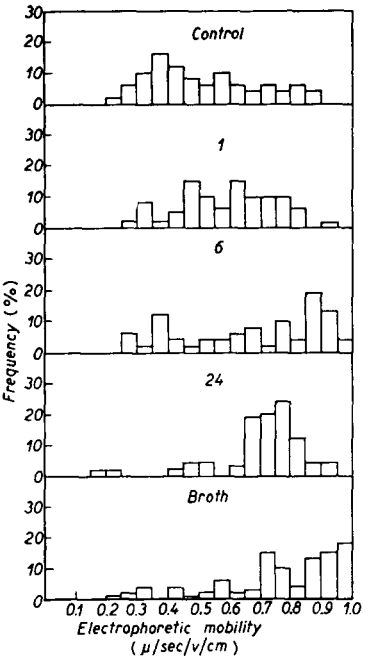

Fig. 5. Typical histograms showing the distributions of mobilities in a culture trained to 19.2 mg/l (Control), after 1, 6 and 24 subcultures in drug-free medium; and after 1 subculture in nutrient broth.

The distribution of mobilities of types B and C brought about by growth in the presence of the crystal violet was not caused by the physical absorption of the dye, since repeated washing of the cells at any level of training with phosphate buffer solution was without effect on the histogram.

The effect of growth of trained organisms in drug-free medium

Trained cells with types B and C behaviour reverted gradually to the A type on repeated sub-culture in normal synthetic medium (Fig. 5). The reversion was not noticeable after 6 sub-cultures in synthetic medium but was fairly well established after 24 subcultures, although the final attainment of type A was often sudden and liable to occur at any time after 20 sub-cultures. Type A behaviour, on the other hand, was unchanged after similar repeated growth in normal medium.

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The effect on the electrophoretic mobility of culturing trained organisms in nutrient medium

Growth of trained cells possessing electrophoretic properties of type A populations, in nutrient broth or on nutrient agar produced no change of surface properties. Cells of type B properties, however, when grown once in broth immediately gave a mobility distribution characteristic of type A. Growth of cells with type C properties in nutrient broth caused a change in the electrokinetic behaviour towards that of type A; this was not immediate as for type B. Subculture on nutrient agar produced similar results for types B and C. Solidified synthetic medium was without effect on the electrokinetic pattern produced.

Growth of these trained cells in synthetic medium in which the ammonium sulphate was replaced in turn by: glutamic acid, aspartic acid, serine and glycine has no effect on the mobility distribution. There appears to be some factor present in nutrient broth which enables drug-trained cells to regain their normal electrokinetic properties more rapidly than in synthetic medium.

DISCUSSION

An exact statistical analysis of the results presented in this paper would be without meaning on account of the limited number of observations made. This does not, however, detract from the usefulness of the results obtained from the study of the effect of crystal violet on the electrokinetic properties of *Aerobacter aerogenes*. We shall use the results in a qualitative manner in an attempt to elucidate the conditions under which crystal violet acts on this species of organism with special reference to the effect on the bacterial surface.

Growth of *Aerobacter aerogenes* in the presence of crystal violet gives rise to new populations of cells, which biologically are to be regarded as homogeneous assemblies, but which may be differentiated by their distinctive electrokinetic properties. The initial subculture of untrained cells into media containing the drug gives rise to populations showing three main types of behaviour, the type exhibited depending upon the age of the parent culture. The properties of populations showing these three different types of behaviour may be summarised:

Type A. The electrokinetic properties of cells having this type of behaviour are indistinguishable from those of normal cells. A distribution of this type occurs when untrained cells, of an age not exceeding 8 hours, are grown in media containing 5.6 mg/l of crystal violet. Adaptation at this or higher concentrations has no effect on the histograms obtained. Growth under these specified conditions has no effect on the size of the bacteria.

Type B. The mobilities of individual organisms in cultures possessing this behaviour are in the range 0.1 to 1.0 microns/sec/volt/cm. This type is first evident during growth in the lowest drug concentration used[1] from an inoculum which has aged in culture medium for 8 to 36 hours. It is an intermediate type of behaviour, for repeated growth of cultures showing this distribution of mobilities in the presence of higher drug concentrations brings about a gradual transition to type C behaviour, whilst on the other hand, repeated growth in the absence of the drug eventually causes reversion to type A. A marked change of size of the organisms was observed in this group; the majority were very small, always having mobilities less than 0.7 microns/sec/volt/cm, whilst the few of normal size usually had mobilities in excess of this value.

Type C. The mobilities of individual bacteria in cultures exhibiting this behaviour

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show a normal Gaussian distribution in the range 0.05 to 0.2 microns/sec/volt/cm. Populations with this behaviour are produced during the first subculture in medium containing 5.6 mg/l crystal violet from a parent inoculum which has aged for at least 36 hours. Repeated growth in media containing this or higher drug concentrations brings about no detectable change in the distribution. Prolonged subculture in drug free medium, however, brings about a gradual transition through type B to type A behaviour. Most of the bacteria in type C populations were very small.

Proflavine caused similar changes in the electrokinetic properties of *Aerobacter aerogenes*[1], but these were only apparent at different training concentrations. With crystal violet, however, the three different types of behaviour all occur at the lowest drug concentration used, the age of the parent inoculum being the most important factor in deciding the type exhibited. Parent cells which have aged for a considerable time and have in consequence undergone denaturation to a larger extent are most susceptible to attack by crystal violet. The exact mode of attack of the drug is still uncertain, but it is evident that it is interacting with some enzyme or enzyme system which is responsible for laying down new cell material thereby causing the altered electrokinetic properties. In addition to these changes of the bacterial surface, the drug causes a marked decrease in the actual size of the bacterial cell. It is probable that there is some connection, as yet not fully understood, between the processes causing these effects.

The change in the electrokinetic properties of *Aerobacter aerogenes* has been shown to occur only on active cell division in the presence of crystal violet. Two possible alternative mechanisms have been suggested to account for this behaviour. In the first, the crystal violet is absorbed on the deeper layers of the cell wall during the lag phase in the presence of the drug, there being no apparent change of surface properties until cell fission occurs. Alternatively, the drug may penetrate and undergo interaction with the surface constituents responsible for the electrokinetic properties, only at the moment of actual cell division. We are not in a position to state categorically which, if either, of these mechanisms are operative. Cells which have been in the growth medium containing crystal violet during the lag phase, when washed and grown in the absence of the drug, give rise to populations showing the normal mobility distribution On this ground it would appear that the absorption of the dye during the lag phase, when cell growth but not division is occurring, is not the important factor.

Once the altered electrokinetic characters are produced in a given population, they are transmitted to subsequent generations in the presence of low drug concentrations. Populations of type B character are only evident in cultures trained to low concentrations of the drug (up to 20 mg/l); higher concentrations cause an increased disturbance of the surface components resulting in a transition to that of type C. This is the first time during our studies of adaptation to high concentrations of drug that we have recorded a gradual change of electrokinetic properties from an intermediate type of behaviour to a final stable type. In this respect it is interesting to compare the intermediate type B behaviour exhibited by the action of proflavine on the organisms where upon repeated growth in the presence of this drug there was a sudden (*i.e.* occurring in one subculture) change to the stable type C. The electrokinetic properties of populations exhibiting types A and C characteristics are, on the other hand, unchanged on repeated subculture, even at the highest concentration tested.

The electrokinetic properties associated with types B and C are not permanent acquisitions by the organisms as they are not transmitted indefinitely to subsequent

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generations in the absence of the drug. Again, type B is an intermediate between types C and A, for there is a gradual transition, on repeated subculture in drug-free medium, from a unimodal distribution centred round low mobility values through a heterogeneous distribution to a more normal Gaussian distribution centered round high mobility values and characteristic of type A.

In marked contrast to these changes produced when cells of varying age are grown in the presence of crystal violet, is the effect of the drug on washed suspensions of the cells. At any age, washed cells treated with the drug experience a lowering of their electrokinetic mobility, with, at higher concentrations, a widening of the distribution. This phenomenon is reversible, for cells treated with the dye at any concentration, after washing, assume the mobility and mobility distribution of untreated populations. In this respect the bacteria are behaving towards the dye in an analagous manner to a typical nonionogenic colloidal particle.

ACKNOWLEDGEMENT

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SUMMARY

1. Crystal violet acting on resting cells of *Aerobacter aerogenes* caused a decrease of electrophoretic mobility with increasing concentration, very high concentrations causing flocculation.

2. Individual cells showed a non-uniform response to the action of the drug at high concentrations.

3. During the first subculture of untrained cells in crystal violet medium, three different types of electrokinetic behaviour were apparent. The type of behaviour exhibited depended on the age of the parent inoculum.

4. It has been established that the change of electrokinetic properties occurs only during cell division in the presence of crystal violet, never in its absence.

5. Each of the three types of behaviour was found to be transmitted without change on subsequent subculture in the presence of low drug concentrations. Growth in higher concentrations was without effect on two of these types of behaviour, the intermediate type B however, tended towards that of type C.

6. Growth of the trained organisms in the absence of the drug caused a change in the electrophoretic mobility distribution for types B and C behaviour towards that of type A. Nutrient broth was more effective in bringing about this change than synthetic medium.

RÉSUMÉ

1. Le violet de cristal, agissant sur des cellules non proliférantes d'*Aerobacter aerogenes*, provoque une diminution de la mobilité électrophorétique proportionnelle à la concentration, des concentrations très élevées entraînant la flocculation.

2. Des cellules individuelles présentent une réponse non uniforme à l'action du colorant à concentrations élevées.

3. Au cours du premier repiquage de cellules non adaptées dans un milieu au violet de cristal, trois types différents de comportement électrocinétiques se manifestent. Le type de comportement observé dépend de l'âge de l'inoculum.

4. Il a été établi que la modification des propriétés électrocinétiques a lieu seulement pendant la division cellulaire en présence de violet de cristal et jamais en son absence.

5. Chacun des trois types de comportement se transmet sans changement au cours des repiquages suivants en présence de concentrations faibles en colorant. La croissance en présence de concentrations plus élevées est sans effet sur deux de ces types de comportement, le type intermédiaire B cependant tend vers celui de type C.

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6. La croissance d'organismes adaptés en l'absence de colorant entraîne une modification dans la distribution des mobilités électrophorétiques pour les types de comportement B et C qui les rapproche de celle du type A. Des milieux complexes provoquent plus efficacement cette modification que le milieu synthétique.

ZUSAMMENFASSUNG

1. Durch Einwirkung von Kristallviolett auf im Ruhestand befindliche Zellen von *Aerobacter aerogenes* wurde mit steigender Konzentration eine herabgesetzte elektrophoretische Beweglichkeit beobachtet; sehr hohe Konzentrationen verursachen Flokulation.

2. Einzelne Zellen reagierten ungleichförmig auf hohe Konzentrationen dieses Farbstoffes.

3. Drei verschiedene Typen von elektrokinetischem Verhalten wurden bei der ersten Subkultur von in Kristallviolettmedium unadaptierten Zellen beobachtet. Der Typus des aufgewiesenen Verhaltens hing vom Alter des ursprünglichen Inokulums ab.

4. Es wurde festgestellt, dass die Veränderung der elektrokinetischen Eigenschaften nur während der Zelldivision in Gegenwart von Kristallviolett und niemals in dessen Abwesenheit vorkommt.

5. Alle drei Typen des Verhaltens wurden, in Gegenwart von niedrigen Farbstoffkonzentrationen, unverändert an nachfolgende Subkulturen weitergegeben. Wachstum in Gegenwart von höheren Konzentrationen liess zwei Typen des Verhaltens unverändert; der mittlere Typus B wies jedoch die Neigung auf, sich dem Typus C anzugleichen.

6. Es wurde beobachtet, dass das Wachstum der adaptierten Organismen in Abwesenheit des Farbstoffes eine Annäherung der elektrophoretischen Beweglichkeitsdistribution der Typen B und C an diejenige des Typen A zur Folge hatte. Nährlösungen fördern diese Art von Veränderung wirksamer als synthetische Medien.

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