376. *The Uptake of Three Drugs by* Bact. lactis aerogenes.

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The uptake of three drugs by sensitive and resistant strains of *Bact. lactis aerogenes* has been studied as a function of equilibrium drug concentration.

Sensitive and non-sensitive strains took up equal amounts of Brilliant Green, while a strain resistant to Crystal Violet took up more of that drug than the corresponding sensitive strain.

On the other hand, the uptake observed for 2 : 4-dinitrophenol was small at a neutral pH, but was increased at a pH below the isoelectric point of the bacterial surface.

The significance of these results is discussed in terms of permeability and other theories of drug resistance.

It is often considered that organisms resistant to the action of drugs are less permeable towards the drug than their sensitive counterparts. The resistance of trypanosomes, for example, to parafuchsin and acridines [1] and to a wide range of arsenical compounds [2] has been shown to be due to a loss in permeability towards the inhibitor. Similarly, normal relapsing-fever spirochætes absorb arsenic and gold from neoarsphenamine and solganol respectively, whereas the resistant forms absorb neither element.[3]

On the other hand, previous work in this laboratory and elsewhere [4] has indicated that drug-resistant strains of bacteria may sometimes take up the same amount of drug as non-resistant strains, or even more.

The contrast of these various results suggested that more experimental results should be obtained before general correlations between drug response and permeability are looked for.

In this paper, the uptake per cell of three drugs, two cationic and one anionic, by drug-resistant and sensitive forms of the gram-negative coliform organism *Bact. lactis aerogenes* has been measured as a function of the equilibrium concentration in the supernatant liquid. No distinction will be made between drug adsorbed on to the bacterial surface and that absorbed into the cell interior. The experimental curves, which may be simply termed absorption isotherms, serve, however, to show any differences in the amount taken up by the drug-resistant and the sensitive strains.

Experimental

The strain of *Bact. lactis aerogenes* was maintained by subculture monthly in " Lemco " broth, and daily in a standard minimal medium made up by mixing the following solutions: 10 ml. of glucose (50 g./l.); 10 ml. of phosphate buffer (Na_2HPO_4,$12H_2O$, 16 g./l.; KH_2PO_4, 2·96 g./l.; pH 7·1); 5 ml. of aqueous ammonium sulphate (5 g./l.); 1 ml. of a solution containing magnesium sulphate ($MgSO_4$,$7H_2O$, 1 g./l.) and ferrous sulphate (5·2 mg./l.).

By repeated subculture of this strain into the standard minimal medium containing drug at increasing concentrations, resistant strains were obtained which were able to grow at a concentration ($\bar{m}$) many times that originally inhibitory to growth. In this way, strains were made resistant to Brilliant Green ($\bar{m}$ = 2000), Crystal Violet ($\bar{m}$ = 40), and 2 : 4-dinitrophenol ($\bar{m}$ = 1500).

The Crystal Violet and 2 : 4-dinitrophenol used in the training experiments were dissolved in phosphate buffer. The pH of the dinitrophenol solution was adjusted to 7 by the addition of a few drops of concentrated sodium hydroxide. The appropriate amount was then added to the standard minimal medium in place of part or all of the 10 ml. of phosphate buffer so as to obtain the final concentration.

[1] Fischl and Singer, *Z. Hygiene Infektionskrankheiten*, 1934, **116**, 138.
[2] Hawking, *J. Pharmacol.*, 1937, **59**, 123.
[3] Fischl, Kobtra, and Singer, *Z. Hygiene Infektionskrankheiten*, 1934, **116**, 69.
[4] Dean and Hinshelwood, Ciba Foundation Symposium on Drug Resistance in Micro-organisms, 1957, pp. 4—24.

Bacterial population densities were measured with the aid of a Hilger Spekker light absorptiometer, calibrated against the microscope count of actual numbers of bacteria made in a hæmocytometer counting chamber.

Absorption Experiments.—Preparation of the cell suspension before an absorption experiment required care if consistent results were to be obtained. Two l. of bacterial culture, previously freed from any drug by one subculture in a standard minimal medium, were grown and taken off towards the end of the logarithmic phase. The cells were separated from the growth medium by centrifugation at 3000 r.p.m. for ~5 min. After being washed with saline solution (9 g./l.), the cells were resuspended and stored overnight in more saline solution.

When needed for an experiment, the suspension was recentrifuged and the cells were resuspended in phosphate buffer (or saline) after being washed with more buffer solution. The concentration of the bacterial suspension was adjusted until the count was 2—4 $\times$ 10^9 cells per ml.

Portions of this suspension were pipetted into dry boiling tubes. Varying volumes of an accurately prepared drug solution and phosphate buffer solution (or saline) were added so that the final volume in each tube was the same. All pipettes used had been recalibrated. When thick suspensions were used, the finite volume of the cells was taken into account, on the assumption that 10^{11} cells occupy 0·1 ml.[5]

The suspensions were then allowed to come to equilibrium in a thermostat at 25°, purified air being bubbled slowly through the suspension.

After ~30 min., the tubes were removed and their contents centrifuged for ~10 min. and pipetted off, and the final drug concentrations estimated. Sometimes an appreciable quantity of a slimy layer was formed during the experiments, but often most of this polysaccharide could be packed down and a clear supernatant left by longer centrifuging.

The amount of drug taken up (A_s) was calculated from the equation $A_s = V(m_o - m_f)/n_s v$, where v is the volume of the bacterial suspension used, n_s its count, V the total volume of the system, m_o and m_f the initial and the residual drug concentrations respectively. A plot of A_s against m_f represents the absorption isotherm.

Results and Discussion

The use of the quantity A_s differs a little from the approach adopted by other workers. Absorptiometer counts are proportional to bacterial mass. Peacocke and Hinshelwood [6] used actual cell numbers from hæmocytometer counts. On the other hand, absorption has sometimes been expressed as the amount taken up per unit dry mass of bacteria.[5,7,8] So long, however, as the average size of the cells does not vary significantly, the different methods correspond, and the absorptiometer readings are easy and fast.

The units were such that A_s is expressed as g. of the drug taken up by 10^{15} cells. Since 2·6 $\times$ 10^{10} cells weigh approximately 30 mg.,[9] the numerical results stated in this paper can be compared with others differently expressed.

(1) *Brilliant Green.*—Difficulties were met at first in the colorimetric determination of Brilliant Green; the optical density of a sample increased with time after dilution of a given stock solution. Variations also occurred when different stock solutions were used. Now, the coloured cation of Brilliant Green is unstable and isomerises to a colourless and feebly basic carbinol.[10] The equilibrium took some time to establish, but the optical density of a solution freshly diluted with saline solution reached a sensible maximum after 90 minutes. Fry [11] fixed the equilibration time arbitrarily as one hour.

Since different stock solutions gave different calibration curves, a new curve was prepared for each absorption experiment. Standard samples from a freshly prepared stock solution were taken through the same operations as the test solutions used in the

[5] Peacocke, *Exp. Cell Res.*, 1954, **7**, 498.
[6] Peacocke and Hinshelwood, *J.*, 1948, 2290.
[7] Newton, *J. Gen. Microbiol.*, 1954, **10**, iii.
[8] Few and Schulman, *ibid.*, 1953, **9**, 454.
[9] Dean, 1958, personal communication.
[10] Goldacre and Phillips, *J.*, 1949, 1724.
[11] Fry, *J. Gen. Microbiol.*, 1957, **16**, 341.

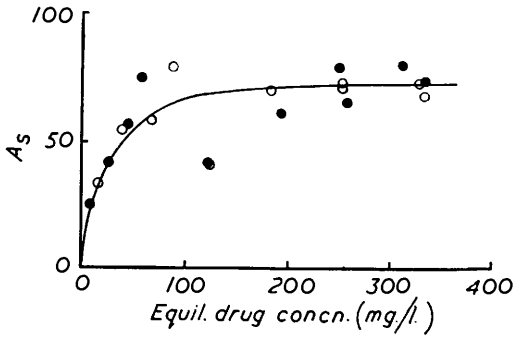
absorption experiments. At the end of a run, the supernatant solutions and the standards were diluted with saline solution until the concentration of the dye was within the range 0—20 mg./l. Their optical densities were measured 90 minutes later in a 0·5 cm. optical glass cell at 630 mμ in a SP. 600 Unicam Spectrophotometer.

Since the aim of the experiment was to compare the sensitive and the resistant strain, the following method was used. Separate concentrated suspensions (2 ml.) of the sensitive ($\bar{m} = 0$) and the resistant strain ($\bar{m} = 2000$) were added in parallel tubes containing 10 ml. of drug solution and saline. When equilibrium was reached, the solutions were centrifuged. The supernatant liquors were diluted and the colour was estimated after the time needed for the complex carbinol equilibria to readjust themselves.

The results (Fig. 1) indicate that the sensitive and the resistant strains take up the same amount of Brilliant Green. The general shape of the curve conforms approximately to a Langmuir type of adsorption isotherm particularly in the lower concentration range.

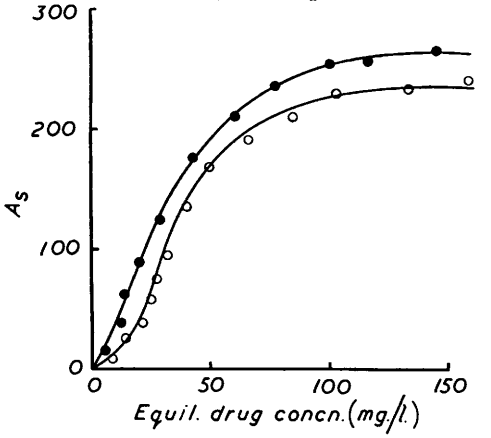
(2) *Crystal Violet.*—The general technique described above was used. Drug concentrations were measured colorimetrically at 590 mμ. A correction for the adsorption of the dye by the walls of the centrifuge tube was made (1·5 mg./l. for the volumes used).

FIG. 1. *The uptake of Brilliant Green by* Bact. lactis aerogenes *at* pH 7.



●, $\bar{m} = 2000$. ○, $\bar{m} = 0$.

FIG. 2. *The uptake of Crystal Violet by* Bact. lactis aerogenes *at* pH 7.



●, $\bar{m} = 40$. ○, $\bar{m} = 0$.

The experimental curves (Fig. 2) clearly show that the resistant strain ($\bar{m} = 40$) takes up more Crystal Violet than the sensitive strain. The maximum values of A_s were reached when the supernatant concentration was about 100 mg./l. The sensitive strain gave a maximum value of A_s of 230—240 units; the corresponding non-sensitive strain gave one of 260 units.

A distinctive feature of the curves is their sigmoid shape, somewhat reminiscent of the isotherms found for the adsorption of certain gases on solids [12] and proflavine on *Bact. lactis aerogenes*.[6] This can be attributed partly to a co-operative effect in which the presence of adsorbed molecules helps the adsorption of more drug on to neighbouring sites.

(3) 2 : 4-*Dinitrophenol.*—Preliminary experiments indicated that the uptake of drug would be very small. Large volumes of a concentrated bacterial suspension were therefore used.

When equilibrium between 10 ml. of suspension and 5 ml. of drug solution had been established, the solution was centrifuged and the optical density of the supernatant liquor, after suitable dilution with phosphate buffer (pH 7), was estimated at 395 mμ in a 1 cm. cell.

The results (Fig. 3) show that the uptake at pH 7 is small. It has often been stated,

[12] Drain, *Sci. Progr.*, 1954, **42**, 608.

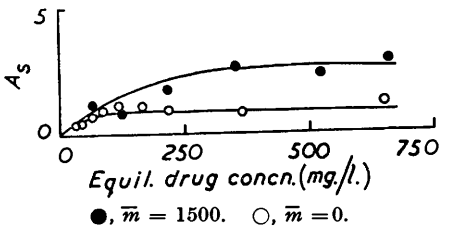
however, that bacteria will only adsorb anionic substances at a pH below the isoelectric point of the bacterial surface. This suggested that there might be a possibility that the uptake of drug would be increased at pH 4—5 if the isoelectric point of gram-negative organisms is about 4·3.

A phosphate buffer (990 ml. of a solution containing 9·08 g./l. of KH_2PO_4 and 10 ml. of a solution of 23·88 g./l. of $Na_2HPO_4,12H_2O$) was used for the experiment at pH 5. The buffer of pH 4 was made up from potassium hydrogen phthalate (10·2 g./l.). The results are plotted in Fig. 4.

At a pH above the isoelectric point, the resistant strain ($\bar{m} = 1500$) probably took up slightly more drug than the corresponding sensitive strain ($\bar{m} = 0$). Below the isoelectric point, on the other hand, the sensitive strain took up a little more than the non-sensitive strain. Both strains picked up 2—3 times as much drug at pH 4 as at a pH near neutrality.

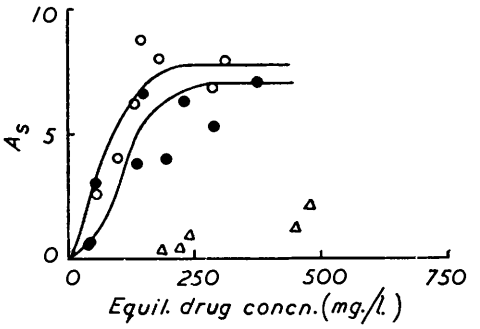
During the experiments, appreciable amounts of a slimy layer were formed, particularly in the acid solutions. Some lysis of the cells by the drug may be responsible for this, a

FIG. 3. *The uptake of* 2 : 4-*dinitrophenol by* Bact. lactis aerogenes *at* pH 7.



●, $\bar{m} = 1500$. ○, $\bar{m} = 0$.

FIG. 4. *The uptake of* 2 : 4-*dinitrophenol by* Bact. lactis aerogenes.



○, $\bar{m} = 0$, pH = 4. ●, $\bar{m}$ 1500, pH = 4.
△, $\bar{m} = 0$, pH = 5.

fact which might lead to modification of the values for A_s. Because of lysis, the actual number of intact cells would be less than the original number n_s, so that the corresponding value of A_s would need to be increased. Although A_s turned out to be only about 6 units at pH 4, the intact cells at the bottom of the centrifuge tubes were markedly yellow while the slimy layer remained unstained.

Some pharmacologists have suggested that drug response should bear a relation to the amount of covalent or non-ionised form of the drug present in the solution. At pH 4, 2 : 4-dinitrophenol is completely un-ionised, while at pH 7·1 it is about 25% un-ionised (calculated from pK = 4·10, given by Flexser, Hammett, and Dingwall [13]). The change in the amount of un-ionised form is roughly proportional to the maximum values of A_s observed at the two different pH values. This correlation is, however, probably fortuitous, as the charge on the bacterial surface also changes.

Conclusion.—According to one view, bacteria may develop a resistance towards drugs by modifying their linked enzyme sequences so as to cope with the new unfavourable environment. In such cases resistant strains might well have at least one part of their cell substance expanded, as compared with the original sensitive strain, and this might explain the increased uptake of proflavine [6] and of Crystal Violet by the respective strains of *Bact. lactis aerogenes.*

There is no reason, however, why one component of the cell should not be increased at the expense of another. Although the gross uptake of Methylene Blue [6] or Brilliant Green is the same on both the sensitive and the resistant forms of *Bact. lactis aerogenes*, the

[13] Flexser, Hammett, and Dingwall, *J. Amer. Chem. Soc.*, 1935, **57**, 2107.

proportions of the various components may have changed in spite of the fact that the total number of negatively charged sites on and within the cell, a factor which determines cationic drug uptake, is the same in both cases.

On the other hand, the small but positive uptake of the anionic drug 2 : 4-dinitrophenol at pH 7 might be due to the diffusion of the drug into the cell interior in such a way that its free concentration in the " protoplasmic fluid " equals that of the surrounding drug medium: the magnitude of the result is of the right order for this hypothesis.

It should not be forgotten that the driving force of any ionic or molecular movement across a cell membrane may come from the actual metabolism of the cell. This suggests that an anionic drug may be taken up only by actively growing cells in a full growth medium. Such a hypothesis is worth testing with bacteria, especially since the drug-resistant strains might exhibit quite different active transport processes. The inter-relations of electrochemical and concentration gradients in the dynamics of the living cell are delicately balanced: a reinvestigation of anionic drug uptake might give information about these equilibria.

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