462. *The Absorption of Antibacterial Substances (2 : 8-Diaminoacridine and Methylene-blue) by Cells of* Bact. lactis ærogenes.

By A. R. Peacocke and Sir Cyril Hinshelwood.

The amount of the antibacterial substance 2 : 8-diaminoacridine (proflavine) taken up from solution by cells of *Bact. lactis ærogenes* gives when plotted against concentration an " absorption isotherm " of sigmoid shape.

Cells trained to be resistant to the drug absorb rather more than untrained cells. Thus adaptation seems not to involve any decrease in the permeability of the cell. A similar conclusion is derived from measurements of the absorption of methylene-blue, with which the isotherm approximates more to the Langmuir type.

A change of pH from 6·16 to 6·96 has little effect on the absorption of proflavine, although the inhibitory action of the drug varies greatly over this range.

Of the constituents of the normal growth medium, ammonium sulphate has no effect on the absorption of proflavine, but glucose causes a marked decrease. Consistently with this, increased glucose concentration antagonises the inhibitory effect of proflavine. Washed cells of different ages absorb approximately equal amounts of proflavine. In the actual growth medium, however, dividing cells absorb less of the drug as growth proceeds, this decrease being principally due to the very considerable fall in pH.

The absorption of proflavine is approximately constant throughout the lag phase of the cells, showing no sudden decrease when division sets in.

The relationship between the lag caused by the drug and the amount absorbed is discussed, and an explanation of certain previous observations is advanced.

From the various competitive effects certain characteristics of the cell surfaces or interfaces which absorb glucose, hydrogen ions and proflavine cations may be inferred.

(1) *Introduction.*—Previous investigations made in this laboratory on the action of anti-bacterial agents upon *Bact. lactis ærogenes* have been partly concerned with the relationships between the concentration in the nutrient medium and the nature and extent of the inhibitory action. The drugs, however, cannot exert their effect until they have either entered the cell or been adsorbed on its wall. The magnitude of their effect should therefore be more directly related to the amount taken up by the cell than to the concentration in the medium.

For proflavine, methylene-blue, and other drugs, functions, $\phi(m)$, of the concentration, m, of the antibacterials have been defined to represent the relationships between m and the decrease in activity of certain inhibited enzymes (Pryce, Davies, and Hinshelwood, *Trans. Faraday Soc.*, 1945, **41**, 163, 465, 778; Pryce and Hinshelwood, *ibid.*, in the press). One theory regards training as the quantitative expansion of certain enzymes and provides a basis for the calculation, the function ϕ being determined by comparison with experiment. It would be of interest to discover how ϕ and related quantities depend on the amount of drug actually taken up by the cell, since this might provide more direct information about the nature of drug action.

Determinations of the amount of drug absorbed by a given mass of cell material could also throw light on another aspect of the adaptive process, namely, the possibility of modifications in the organism whereby the trained cells take up less of the antibacterial than the untrained strain. York, Murgatroyd, and Hawking (*Ann. Trop. Med. Parasit.*, 1931, **25**, 351; see also Hawking, *J. Pharm. Exp. Ther.*, 1937, **59**, 123) found that strains of trypanosomes trained to withstand the inhibitory action of various arsenical compounds, absorbed little or none of these substances. This behaviour was in marked contrast to that of normal trypanosomes which absorbed all the available drug from solutions of similar concentration.

Another characteristic of the action of acridine drugs about which determinations of absorption could provide useful information is the dependence of activity upon pH. McCalla (*J. Bact.*, 1940, **40**, 23; 1941, **41**, 775) has described experiments on the competitive absorption of hydrogen ions, metallic cations, and the cations of basic drugs (such as methylene-blue and crystal-violet) by washed suspensions of *Bact. coli*, *B. bellus*, and *Corynebacterium simplex*. The results of his work which concern us here are: (i) cationic drugs, such as methylene-blue, replaced the hydrogen ions absorbed by cells previously washed with dilute acid, and (ii) the number of gram-equivalents of hydrogen ions and drug cations (*e.g.*, crystal-violet) absorbed by a given mass of cellular material showed an inverse relationship over the pH range studied (*Stain Technology*, 1941, **16**, 95).

(2) *Scope of the Experiments.*—The complete absorption isotherm of cells untrained to proflavine was determined and found to be of a sigmoid shape, the uptake per cell reaching a limit above concentrations of 250 mg./l. The isotherm for methylene-blue, however, was found to be of the usual Langmuir or Freundlich type, and this suggested that the precise shape of the curve was conditioned rather by the properties of the drug employed than by the special relation of drug and cell. As regards their drug action, methylene-blue and proflavine are known to act on the same or related systems within the cell, and this idea proved to be consistent with the similarity in the relationship between the lag and the amount of drug absorbed, in the two cases.

The absorption isotherms of cells previously trained to proflavine were of the same sigmoid shape, but the absorptions were, on the average, about 20% greater than with untrained cells. The relationship between the lag of trained cells and the amount of drug absorbed was determined, and the shape of the isotherm was found to explain certain previous observations on the behaviour of cells trained to high concentrations of proflavine.

Over the pH range 6·16—6·96 the absorption of proflavine was found to be constant. The results merit discussion in the light of the known wide variation of inhibitory effect of proflavine with pH in this range.

Over a period of 6 hours near the end of growth, there is little change in the amount of proflavine absorbed by each cell from buffered saline. With growing cells in the nutrient medium there is a decrease in absorption from the start of growth to the end of the logarithmic phase. This decrease is principally due to the very considerable fall in pH.

Ammonium sulphate has little effect on the absorption of proflavine by resting cells, but glucose causes a marked decrease. Consistently with this, increased glucose concentration is found to antagonise the effect of proflavine on lag.

From the various competitive effects certain characteristics of the cell surfaces or interfaces which absorb glucose, hydrogen ions and proflavine cations may be inferred.

The absorption of proflavine was found to be approximately constant throughout the lag phase. There was no sudden decrease when division started.

(3) *Experimental Method.*—The solutions from which the drug was absorbed by resting cells

varied in different experiments, but the basic technique was as follows (variations from this will be described in the appropriate sections). About 2 l. of a culture of *Bact. lactis ærogenes* growing at 40° in an aerated synthetic medium of glucose, ammonium sulphate, phosphate buffer, and magnesium sulphate were centrifuged for 15 minutes at 6000 r.p.m.; the population then corresponded to about 600 million cells/ml. The filtrate was discarded, and the cells washed with saline (9 g./l.) and centrifuged again. The process was repeated twice more, after which the cells were suspended in a small volume of saline (or phosphate). This suspension was aerated for about 30 minutes to break up any adhering masses of cells, and its volume was then adjusted until it had a count of 1—3 $\times$ 10^9 cells per ml.

10 Ml. portions of the suspension were then transferred to 6-in. $\times$ 1-in. test tubes containing solutions composed of :

10 Ml. of a 9 g./l. solution of potassium dihydrogen phosphate to which 4N-sodium hydroxide had been added to bring its pH to the desired value (or 10 ml. of saline, having dissolved in it the substance the effect of which was to be studied).

x Ml. of a 1000 or 2000 mg./l. solution of proflavine sulphate, x varying from 0·1 to 5 ml.

$(5 - x)$ Ml. of saline.

The test tubes were then placed in a thermostat at 40°, in order to reproduce the conditions obtaining in growth experiments, and gently aerated to keep the mixture stirred until equilibrium had been reached (see below). The solutions were next centrifuged for 20 minutes and the supernatant liquid carefully removed by means of a pipette. The concentration of proflavine in these solutions was determined by measurement of their light absorption with a Spekker photoelectric absorptiometer (cobalt-glass filter) previously calibrated against solutions of known concentration. From the results and from a knowledge of the original proflavine concentration the amount taken up by the cells could be calculated.

The number of cells per ml. of the suspension was determined by the counting under the microscope of a suitably diluted sample in a Thoma hæmocytometer. Number per ml = $1{\cdot}25 \times 10^6 \times n_H$, where n_H = hæmocytometer reading.

The Spekker instrument was also used as a nephelometer to measure the turbidity of the suspensions. It was first calibrated with samples of a culture at different stages of its growth and of determined hæmocytometer count (this varied over the range 20—1000 million/ml.). A series of pieces of brass, in which holes of rectangular cross-section had been cut, were used as standard screens. The calibration curves could then be used to determine counts of other suspensions. Since the method gives primarily the quantity of light-scattering bacterial substance in a given volume, the " count " so determined, n_S, will only be in direct proportion to n_H for suspensions in which the cells are of constant size. The quantity n_S/n_H should be a measure of the bacterial mass per single cell.

Final results were normally expressed by dividing the decrease in drug concentration (mg./l.) in a given solution by n_H or n_S and multiplying the respective quotients by 1000. This gave two quantities A_H and A_S. A_H proved the most satisfactory quantity to employ, the chief reason for this being the observed steady decrease with time of n_S for a given suspension.

(4) *Absorption of Drugs by Untrained Cells.*—(a) *Proflavine.* It was first necessary to find the length of time taken for the proflavine to be fully absorbed. Experiments were therefore made in which, at varying intervals after the addition of the bacterial suspension to a series of constant drug–saline–buffer solutions, successive tubes were centrifuged and the residual concentration of proflavine determined. It was found that in more than 10 and in less than 30 minutes this concentration had reached its minimum value, thereafter remaining constant.

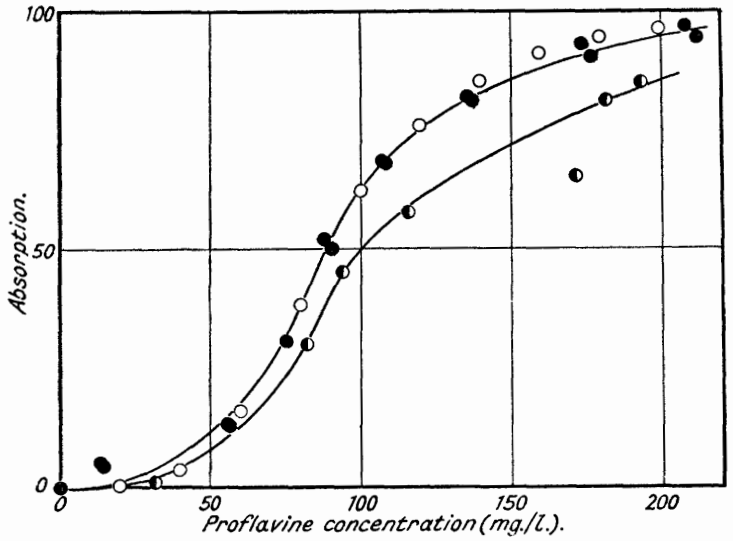
This preliminary experiment shows that a time of contact of 90—120 minutes is fully adequate for equilibrium to be established and was the interval employed.

The complete absorption isotherm of cells untrained to the drug was then determined a considerable number of times. Typical results are shown in Fig. 1 where A_H is plotted against the concentration of proflavine in the supernatant liquid. The curves are seen to be of a sigmoid type, the absorption reaching a limiting value at concentrations of about 200—250 mg./l. The point of inflection occurs at about 65—85 mg./l. An average variation in the position of the isotherms of $\pm$10 units of A_H (as measured at the upper end of the curves) was observed. The curves obtained by plotting A_S against drug concentration showed laregr differences, the reason for which will be discussed later.

Despite the variation in the limiting values of A_H, the general shape, the proflavine concentration at which the inflection occurred and the concentration at which the limit was approached did not vary to any marked extent. To illustrate this graphically, and also to obtain an average isotherm, the following procedure was adopted. A table was made of the

limiting values of A_H in the seven experiments with normal cells and the average value, p, was calculated. The individual limiting values in the various experiments being $q_1, q_2, \ldots q_n \ldots$, the experimental values of A_H at each point on any isotherm were then multiplied by the appropriate

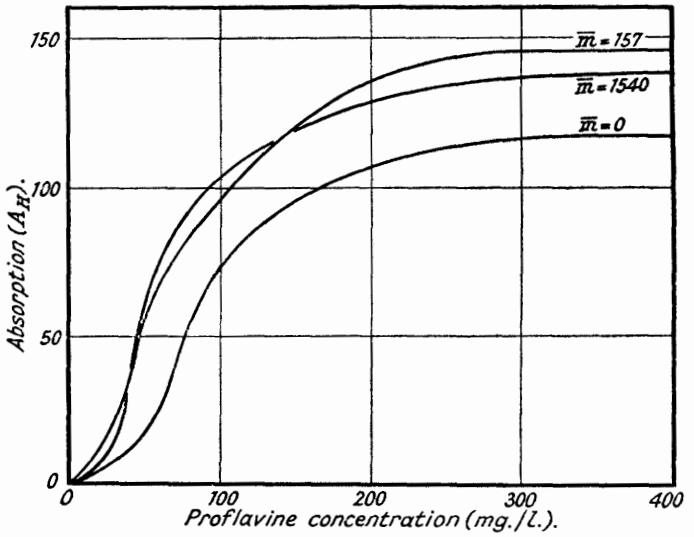
FIG. 1.



Absorption isotherms, normal cells:
Black circles, experimental, series 1.
Open circles, calculated from $A = Scm^4/(1 + cm^4)$ *with* $c = 1{\cdot}52 \times 10^{-6}$.
Half-black circles, experimental, series 2.

FIG. 2.

Absorption isotherms (average) for trained and untrained cells. $\bar{m}$ = *training concentration.*



factor p/q_n, whereby all the curves are scaled to come to the same (average) limiting value. The reduced curves were substantially similar, and a mean value is plotted in Fig. 2 ($\bar{m} = 0$).

According to Figs. 1 and 2, the absorption isotherm is concave upwards over the range of concentration of 0—70 mg./l. As this part is of special interest, a more detailed study of it was made, the experiments providing the data for the study of the relationship between lag and A_H (see Discussion).

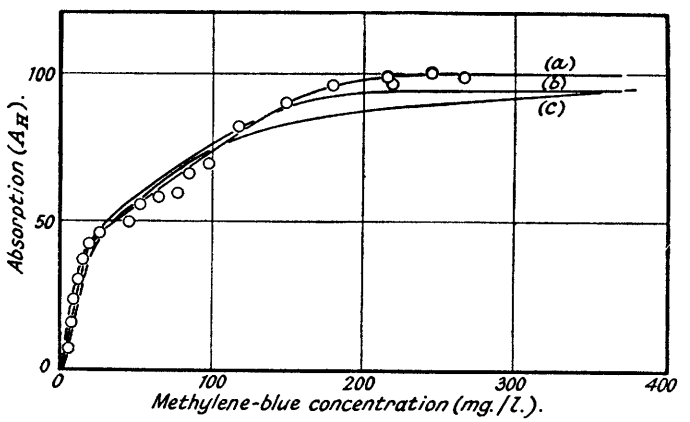
The chief characteristic of the isotherm is the sigmoid shape with its point of inflection at about 65—85 mg./l. Fig. 1 shows that the curve is described by the following equation

$$A_H = S\left\{\frac{cm^4}{1 + cm^4}\right\}$$

where S and c are constants.

The sigmoid shape might arise from the individual properties of proflavine itself or it might be characteristic of all cationic dye absorption by the cells. Reasons are given in the next section for preferring the former view. The shape could then be due to: **(i)** A Langmuir adsorption of some associated form of proflavine present in small proportions and the concentration of which is proportional to a power of the total drug concentration greater than unity. It is not easy on this view to see how a large polymeric form of the drug could be more easily absorbed than the smaller monomer molecules. (ii) A co-operative effect, the presence of adsorbed molecules on given sites facilitating the adsorption of similar molecules on neighbouring sites (see Brunauer, " Physical Adsorption ", Oxford, 1943; Hinshelwood, " The Chemical Kinetics of the Bacterial Cell ", p. 6). Since many dye molecules, including aminoacridines, are known to have strong intermolecular attractive forces, an explanation of the sigmoid isotherm in terms of a co-operative effect seems a quite likely one.

FIG. 3.



Absorption isotherms for methylene-blue :

(a) $\bar{m} = 0$, *typical experimental curve*; (b) $\bar{m} = 0$, *average*; (c) $\bar{m} = 150$ *mg./l.*

(b) *Methylene-blue.* The absorption of methylene-blue from buffered saline by ***Bact. lactis ærogenes*** at 40° was determined by the method used with proflavine (except that a yellow filter was employed in the Spekker instrument). The absorption isotherm (Fig. 3) approximates rather more closely than with proflavine to the usual Langmuir type, in spite of the fact that methylene-blue can be highly polymerised in aqueous solution (Rabinowitch and Epstein, *J. Amer. Chem. Soc.*, 1941, **63**, 69). The limiting absorption is approached when the concentration exceeds about 200 mg./l. (Fig. 3).

The difference between the results for proflavine and methylene-blue suggests that the curves are specifically determined by the nature of the dyes themselves rather than by an inherent property of the mechanism by which the cell takes them up. Once taken up the two dyes are known to act in similar ways (Pryce, Davies, and Hinshelwood, *Trans. Faraday Soc.*, 1945, **41**, 465).

(5) *Absorption of Drugs by Trained Cells.*—(a) *Proflavine.* An investigation similar to the above was carried out with cells trained to concentrations, $\bar{m}$, of proflavine of 157, 312, and 1540 mg./l., respectively. The isotherms were of exactly the same shape as those for the untrained cells, but the values of A_H were somewhat higher. Some averaged results are plotted in Fig. 2. These curves show that in this example resistance to the action of proflavine is not due to a decrease in the permeability of the trained cells, since in actual fact they absorb rather more of the drug after training than before (cf. the results of Hawking, *J. Pharm. Exp. Ther.*, 1937, **59**, 123; Fischl, Kotbra, and Singer, *Z. Hyg. Infectionskrankh.*, 1934, **116**, 69; Stearn, *J. Bact.*, 1927, **14**, 349; Deere, *ibid.*, 1939, **37**, 473).

(b) *Methylene-blue.* The absorption isotherm of methylene-blue was determined for cells trained to 150 mg./l. of the drug [Fig. 3, curve (*c*)]. It is seen to approximate closely, within the experimental error, to the average absorption isotherm of the untrained cells [curve (*b*)]. In the case of methylene-blue, it is therefore also clear that adaptation to the drug does not involve any obvious decrease in permeability.

(6) *Influence of pH on the Absorption of Proflavine.*—For the investigation of the effect of changed pH on the take-up of proflavine it was first necessary to determine whether the light absorption of the latter would be at all influenced by the altered conditions. According to the results of Albert and others (*Brit. J. Exp. Path.*, 1945, **26**, 160) it should not, since, over a wide range of pH, the drug is completely in the form of univalent cations.

A series of phosphate buffers was made, and to equal volumes of each of these were added equal volumes of a solution of proflavine sulphate. The concentration of proflavine in each solution was then measured by means of the Spekker instrument and the previously determined calibration curve. Over the pH range 5·5 to at least 7·6 the expected value was found. But in more acid buffers the instrument indicated too great a concentration. The reason for this is uncertain. From the practical point of view, however, it follows that the calibration curve may be used over the pH range 5·5 to 7·6.

Absorption isotherms were then determined for various standard suspensions of untrained cells in a series of solutions only differing from those employed in the experiments of the previous section in the pH of the buffers. An attempt was made to cover the pH range 3 to 7·6, but this was rendered difficult (i) because at the lower end the buffers lack the capacity to deal with the acid arising from the hydrolysis of proflavine sulphate, and (ii) by the uncertainty in the proflavine determination already mentioned.

Over the range 6·16—6·96 the pH proved to have very little effect on the absorption of the drug. Indeed, only one sigmoid curve could be drawn through all the experimental points obtained in this range. At pH 4·78, 5·49, and 5·77 the limiting values of A_H were found to be lower than those obtained in the higher range. The main results are summarised in Table I.

TABLE I.

Influence of pH on the limiting value of A_H.

pH.	Lt. A_H.	pH.	Lt. A_H.	pH.	Lt. A_H.	pH.	Lt. A_H.
4·78	44	6·16	110	6·47	110	6·77	100
5·49	68	6·20	100	6·48	90	6·96	110
5·77	80	6·32	110	6·54	100	6·97	106
6·04	100	6·39	100	6·69	110	—	—

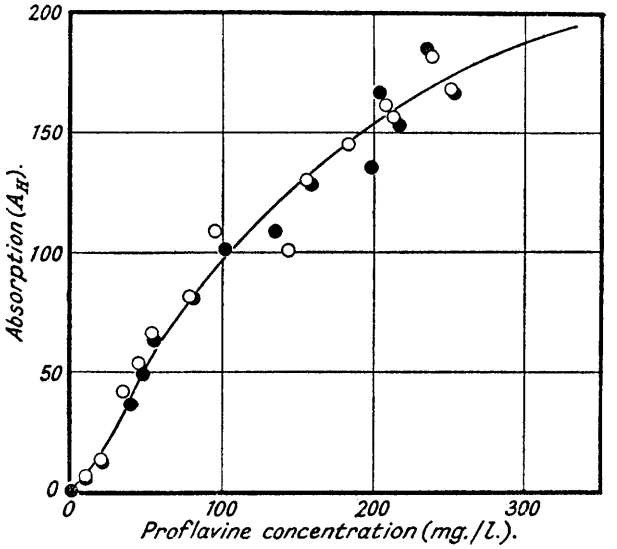
Stearn and Stearn in a series of papers (*e.g.*, *Protoplasma*, 1931, **12**, 435, 580) have measured the " iso-electric zones " of various organisms. This zone is the pH range within which the absorption of acidic (anionic) and basic (cationic) dyes is of the same order. Above it only cationic dyes are absorbed to any extent, and below it only anionic dyes. Gram-positive organisms were found to have iso-electric zones around pH 2·0, and Gram-negative organisms, of which *Bact. lactis ærogenes* is an example, around pH 5·0. The value given for *Bact. lactis ærogenes* is 5·2. At pH's about one unit greater than the iso-electric point, a limiting value of the absorption of cationic dyes is attained (see Stearn and Stearn, *J. Bact.*, 1924, **9**, 463; Dubos, " The Bacterial Cell ", 1945, Harvard, Chap. III). The results of the present investigation are seen to be in accord with these studies. Over the pH range 6·16 to 6·96, where its total absorption by the cell has been seen to change very little, there is a 2·5-fold variation in the lag caused by the proflavine (Peacocke and Hinshelwood, this vol., p. 1235). This suggests that the centres where proflavine cations cause lag and where also they are in competition with hydrogen ions are only a small fraction of the total which are capable of absorbing proflavine, under the conditions of resting-cell experiments.

(7) *Effect of Medium Constituents on the Absorption of Proflavine.*—(a) *Ammonium sulphate.* Cells were suspended in the phosphate buffer (pH 7·12) and 10 ml. of this suspension were then added to the absorption solutions containing, besides the usual x ml. of proflavine solution and $(5 - x)$ ml. of saline, 10 ml. of a 5 g./l. solution of ammonium sulphate in saline. The final difference from the previous procedure is thus that the absorption solutions contain ammonium sulphate. The same cell suspension was also added to a similar series of solutions containing no ammonium sulphate.

The two absorption isotherms (Fig. 4) obtained were identical. Ammonium sulphate thus has no effect on the absorption of proflavine by the cells.

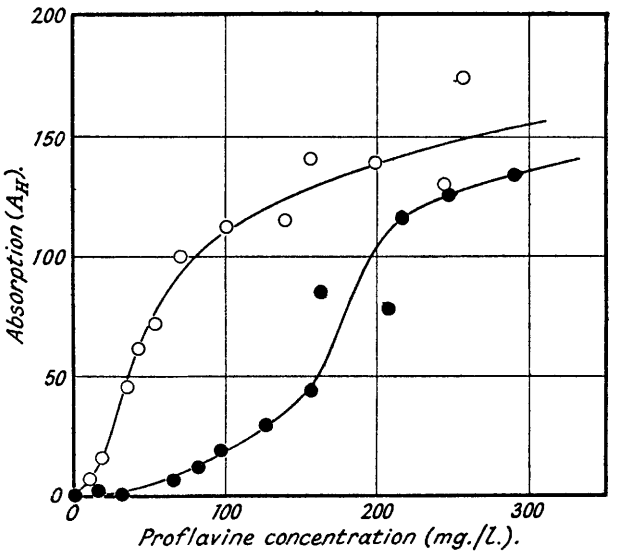
(b) *Glucose.* A similar series of experiments was carried out with glucose (20 and 40 g./l.) in place of ammonium sulphate. The absorption of proflavine was consistently less in the presence of glucose. Fig. 5 shows a typical pair of curves. The two concentrations of glucose,

FIG. 4.



Effect of ammonium sulphate on absorption of proflavine by resting cells in phosphate–saline:
Open circles, without ammonium sulphate.
Black circles, with 2 g./l. ammonium sulphate.

FIG. 5.



Effect of glucose on absorption of proflavine by resting cells in phosphate–saline:
Open circles, without glucose.
Black circles, 20 g./l. glucose.

one double the other, only gave a 10% difference in effect, which is about the variation observed with the same strain of cells under apparently identical conditions.

(c) *Complete growth medium.* Since the concentrations of proflavine employed in these absorption experiments are mostly very much higher than that needed (54 mg./l.) completely to

inhibit the growth of untrained bacteria in the normal growth medium, it was possible to determine the isotherm in a complete medium without multiplication of the cells. The absorption was lower than that in saline–phosphate by approximately the amount to be expected from the glucose present.

(8) *Influence of Glucose on the Lag induced by Proflavine.*—In the light of (b) and (c) of the last section it was thought to be of some interest to investigate whether or not differences in the concentration of glucose can cause corresponding changes in the lag due to the drug.

The lag was therefore determined as a function of proflavine concentration in two series of experiments carried out in the usual way, except that the media contained two different concentrations of glucose, namely 3·85 g./l. and the usual amount, 38·5 g./l. The higher concentration of glucose was found to reduce the lag over the whole range of proflavine concentrations (Table II).

TABLE II.

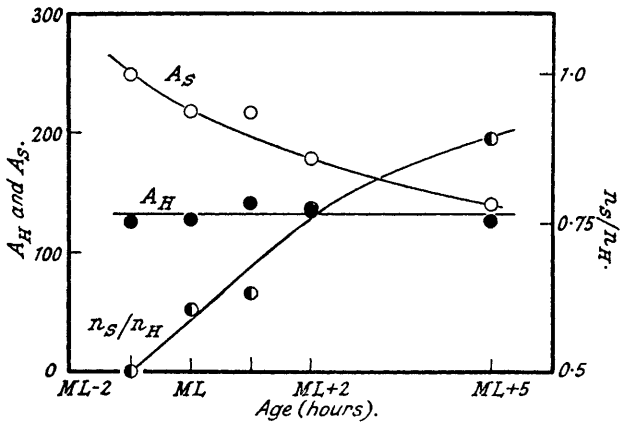
Influence of glucose on the lag induced by proflavine.

Concn. of proflavine (mg./l.).....	0	10	20	30	40	45	50	55
Lag in presence of 3·85 g./l. of glucose minus lag in 38·5 g./l. (mins.)	0	0	40	250	800	1040	1380	∞

(9) *Variation of Proflavine Absorption with the Age of the Cells.*—(a) *Absorption by centrifuged and washed cells of varying age.* Preliminary experiments indicated that there was an appreciable

FIG. 6.

Variation of A_S, A_H, *and* n_S/n_H *with age at time of centrifuging.*



difference between the amount of proflavine absorbed by cells centrifuged immediately the parent culture had ceased to divide, and those obtained by centrifuging 24 hours later. It was therefore thought worth while to study this effect further. The procedure was as follows:

2500 Ml. of complete medium (I) were inoculated with untrained, normal cells, and when the count of the growing culture had reached 3—7 × 10^8 per ml. the cells were centrifuged and washed. The absorption isotherm was then determined. This stage in the growth of the parent is about 1 hour before division ceases (the point at which the lag of an inoculum from it into a fresh medium would be minimal).

The whole process was repeated when the culture reached the minimum lag stage (time denoted by ML), and also at $(ML + 1)$, $(ML + 2)$, and $(ML + 5)$ hours. Another quantity (II) of complete medium was similarly inoculated, and this was centrifuged and tested at ages of $(ML + 10)$, $(ML + 20)$, $(ML + 44)$, and $(ML + 140)$ hours.

With culture (I), Fig. 6 shows that the limiting value of A_H does not vary to any great extent from $(ML - 1)$ to $(ML + 5)$ hours, any differences being within the experimental error. The limiting value of A_S over this range, however, decreases steadily. Thus the amount of proflavine absorbed by each cell does not alter over the period $(ML - 1)$ to $(ML + 5)$, whereas that absorbed per unit bacterial mass decreases. This implies that the mass per cell increases from $(ML - 1)$ to $(ML + 5)$. This is shown in the figure by the trend in the ratio n_S/n_H which is approximately proportional to the mass of each cell. Later on it is shown to decrease since the cells in old cultures are smaller than the average.

With culture II, Table III shows that from (ML + 10) to (ML + 140) the limiting values of both A_H and of A_S decrease steadily. This is not unexpected since at great ages general degeneration of the cell substance must occur.

TABLE III.

Variation of proflavine absorption with age (washed cells).

Age at time of centrifuging :	(ML + 10).	(ML + 20).	(ML + 44).	(ML + 140) hrs.
Lt. A_S	190	165	135	110
Lt. A_H	171	139	120	77
n_S/n_H	0·88	0·73	0·84	0·69

(Since A_H and A_S are calculated by dividing the same concentration decrease by the appropriate values of n_H or n_S, A_H/A_S is equal to n_S/n_H.)

In these experiments the absorption by washed cells of varying age, free from their growth medium, is measured. Thus any effects that substances such as medium constituents, metabolic products, or acids from fermentation might have on the absorption have been eliminated. The constancy of the amount of drug absorbed per cell over an age range of 6 hours about the minimum lag stage (Culture I) is therefore of special interest. It shows that any variation in the absorption of proflavine in an actual culture between the ages of (ML — 1) to (ML + 5) hours must be due to the presence of diffusible substances in the medium and not to changes in the structures responsible for the absorption.

The variation of A_S with age (Culture I) explains the variation often observed in the A_S-isotherms of cells, centrifuged *approximately* at the minimum lag stage, and is a further reason for plotting A_H-isotherms.

(b) *Absorption by dividing cells at various ages.* It seemed of interest next to find a method of determining the absorption of cells during actual growth. The chief difficulty in this is to ensure that the cells do not multiply further during the centrifuging. At first this was done by adding formaldehyde to the absorption solution immediately before separation. Control tests, however, showed that the whole principle of the experiment was invalid, the apparent absorption of resting cells in saline–phosphate being greatly increased by the presence of formaldehyde, possibly as a result of chemical reaction between the proflavine and the formaldehyde.

Growth of the cells can, however, be stopped by the proflavine itself, since concentrations of 60 mg./l. are sufficient to do this and up to 400 mg./l. are used in the absorption experiments. In deciding the technique of the experiment it is important to know the time taken for equilibrium to be established. With resting cells this is 10 to 30 minutes [Section 4 (a)]. Experiments of S. Jackson (unpublished) show that for growing cells there is also an interval of about 20—25 minutes between addition of proflavine solution and cessation of growth which is, moreover, quite sharp. The procedure was, therefore, as follows : Two portions of 24 ml. each were taken from 1 l. of the growing culture, and 1 ml. of an 8000 mg./l. solution of proflavine sulphate was then added to each. They were aerated at 40° and, after not less than 25 minutes, to ensure that division had ceased, a sample was taken and the solution then centrifuged in the ordinary way.

A sample taken when the drug was first added gave a point on the growth curve of the culture and thus its age. A second sample gave the count (n_H) in the absorption solutions immediately before centrifuging.

From these and other determinations plots of the following against the age of the culture were obtained : (i) Cell count, (ii) A_H, (iii) A_S, (iv) pH of the medium. These curves are shown in Fig. 7. It is seen that, at first, the values of A_H and A_S are considerably higher than with the resting cells in phosphate–saline, but that the values of both decrease rapidly as growth proceeds and reach a minimum shortly after it stops. The decrease runs parallel with the drop in the pH of the supernatant liquid which is quite sufficient to account for it, as may be judged by continuing Table I below the pH value of 4·78.

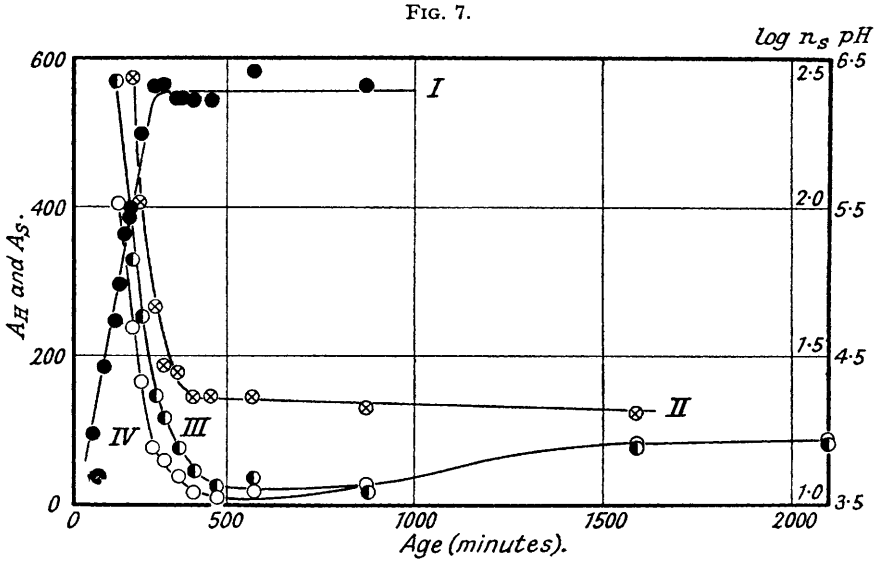
This change in absorption as growth proceeds throws light on a phenomenon previously observed in the laboratory (Davies, Hinshelwood, and Pryce, *Trans. Faraday Soc.*, 1944, **40**, 397). If cells untrained to proflavine are grown in its presence and removed after one or two divisions, they are found to have acquired almost complete resistance to the proflavine in a subsequent test made under the same conditions. This resistance is, however, unstable in the sense that one or two subcultures in the drug-free medium completely remove it again. If the cells, instead of being taken out after only one or two divisions, are allowed to complete their growth cycle in the presence of the proflavine, the degree of resistance acquired is very much less. This now

finds an explanation in terms of the result just described. As growth proceeds the uptake of proflavine per cell diminishes rapidly, and, since at this stage the acquired resistance is unstable, a reversion from the initial training takes place.

(10) *Absorption during the Lag Phase.*—Since it has been shown that the absorption of proflavine decreases rapidly during division, it is interesting to discover if there is any variation in absorption during the lag phase and, more important, if it shows any sudden change once cell division begins.

The method used was as follows. 500 Ml. of a culture of untrained cells at the minimum lag stage were centrifuged, washed thrice with saline, and then suspended in it. A calculated volume of this suspension was then added to 500 ml. of complete growth medium containing proflavine at a known concentration of about 30—40 mg./l., so as to give an initial count of about 250 million/ml. The culture was maintained at 40° and aerated. Under these conditions the cells had a lag of about 350 minutes. At intervals, 25 ml. samples were examined. This procedure was continued until after 1—2 divisions had been completed.

FIG. 7.



Absorption by dividing cells in actual growth medium : Curve I, Growth curve (age–log n_S). Curve II, Age–pH. Curve III, Age–A_S. Curve IV, Age–A_H.
(Note different scales.)

One set of results is given in Table IV. The proflavine concentration had to be low, or division would not have occurred, and the percentage error in the results is therefore high. Moreover, the count has to be small (about 1/5 normal) in order that one or two divisions may be observed before growth ceases. This further increases the percentage error since it causes the concentration decreases to be very small.

Despite this it its clear from Table IV that the absorption of proflavine does not begin to decrease *immediately* division commences. In fact it occurs some 350 minutes later, by which time one division is almost complete. This shows that the onset of division is not accompanied by any sudden transference of absorbed drug from the cell to the medium.

TABLE IV.

Variation of proflavine absorption in the lag phase.

Age, mins.	n/n_0.	A_H/16·0.		Age, mins.	n/n_0.	A_H/16·0.	
45	1·00	0·87		475	1·22	1·00	
148	1·07	0·81		725	1·54	1·15	
210	1·00	1·23		750	1·92	0·45	(A_H begins to decrease)
270	1·03	0·85		780	2·13	0·86	
398	1·06	1·17	(Onset of division)	811	2·20	0·56	

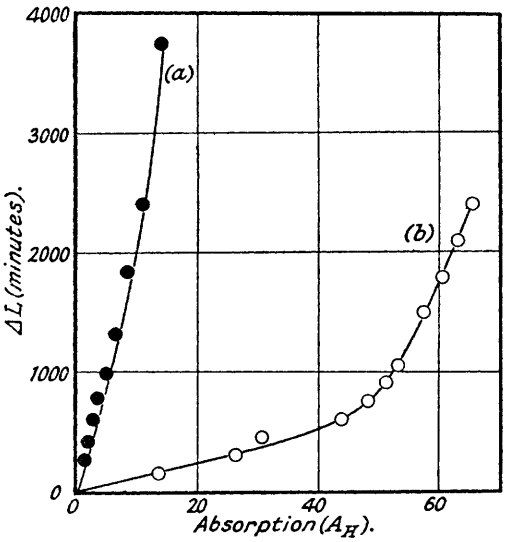
$1·25 \times 10^6 \times n$ = Count of the culture at each age.
$1·25 \times 10^6 \times n_0$ = Initial count of the culture ($n_0 = 259$).

(11) *Discussion.* (a) *Action of proflavine upon untrained cells.* It is now possible to examine more closely the relationship between the increment in lag (ΔL) due to the presence of proflavine and the amount taken up by the cell (A_H).

The value of A_H for the untrained strain at various concentrations of the drug in the medium is known from the detailed investigations over the range 0—70 mg./l. [Section 4 (a)]. The lag–concentration relation for this strain of cells was determined. The values of ΔL were then plotted against the corresponding values of A_H [Fig. 8, curve (*a*)]. A weakness of this comparison lies in the fact that A_H was determined in buffered saline whereas ΔL was naturally determined in the presence of full growth medium. Experiment has shown, however, that with the latter only the scale and not the shape (*i.e.*, sigmoid character, point of inflexion, and the concentration at which A_H approaches its limit) is altered relative to that obtained with buffered saline.

Thus the general form of the relationships between lag and the absorption of drug should be revealed by the comparison.

FIG. 8.



Relation between lag and drug-absorption :
(a) *Black circles, proflavine.*
(b) *Open circles, methylene-blue.*

The ΔL–A_H relationship for the untrained cells in proflavine is seen to be almost linear over a considerable range. Eventually, however, the lag rises steeply to infinity, as can be seen from Fig. 9. ΔL reaches infinity when the bulk concentration is 54 mg./l., and up to this point the absorption isotherm is convex towards the m axis. Its curvature is less than that of the lag–concentration curve, and it is for this reason that the ΔL–A_H curve is convex towards the A_H axis.

For proflavine, the $\phi(m)$–m relationship is $\phi(m) = fm$ (Davies, Hinshelwood, and Pryce, *Trans. Faraday Soc.*, 1945, **41**, 163, 465, 778) where f is a constant. The relationship between $\phi(m)$ and A_H will therefore be expressed by a curve of sigmoid shape like the isotherm, since $\phi(m)$ is simply a multiple of m. The value of $\phi(m)$ increases steeply as A_H approaches its limiting value of 116 units.

(b) *Action of proflavine upon trained strains.* For a strain trained to 157 mg./l. (Fig. 3) a plot was made of ΔL against A_H in the same way as before. The ΔL–m data were then calculated from the following equation which is known to give the correct values for the strain of *Bact. lactis ærogenes* used in this laboratory (Davies, Hinshelwood, and Pryce, *Trans: Faraday Soc.*, 1945, **41**, 163).

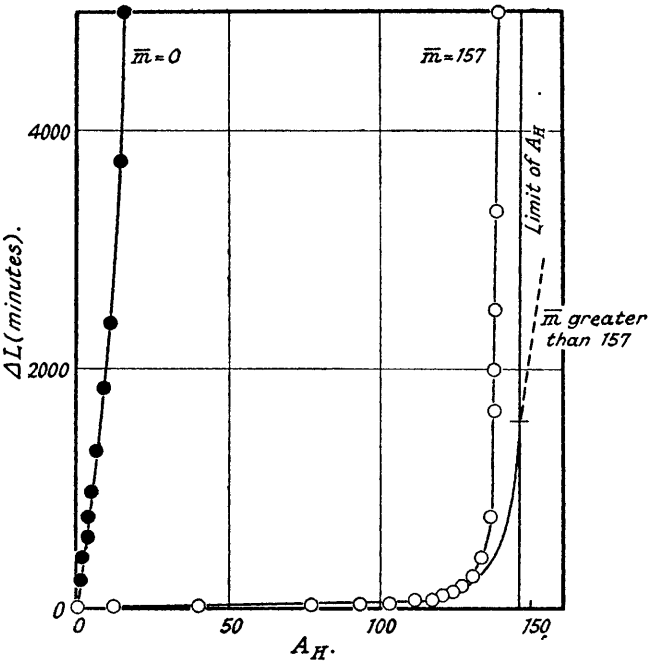
$$\frac{1}{\Delta L} = 10^{-4}\left\{\frac{(\bar{m} + 54)^2}{m} - (\bar{m} + 54)\right\}$$

with $\bar{m} = 157$ mg./l.

The resulting curve, together with that for $\bar{m} = 0$, is plotted on Fig. 9. It should be noted that there is a large range of tolerance to the drug, up to 120 A_H units, and that with quantities of drug in the cell greater than this the lag rises steeply to infinity. The ΔL–A_H curves are limited on one side by the line $A_H = 146$, which is the maximum amount of drug which any cells (trained or untrained) can absorb. As $\bar{m}$ increases above 157, the curves obtained must lie between that for $\bar{m} = 157$ and this limiting line. The experimental evidence shows that with strains trained to concentrations greater than 250 mg./l., the lag–concentration curve at first increases steeply in the normal way until at a certain stage it turns over and becomes horizontal (Davies, Hinshelwood, and Pryce, *Trans. Faraday Soc.*, 1945, **41**, 778, Fig. 2). This means that the ΔL–A_H curves eventually intersect the line $A_H = 146$ at a value of the lag which is a limit for that strain, since no more drug can be absorbed (cf. dotted line in Fig. 9). Further training of such a strain simply causes a progressive decrease in the lag towards zero for all concentrations, *e.g.*, the strain $\bar{m} = 2100$, in Fig. 1 of the paper quoted.

FIG. 9.

Relation of drug absorption and lag for normal and proflavine-trained cells.



The lag–concentration curves begin to become parallel to the m axis when growth is occurring in concentrations of about 250 mg./l. or more, and it is significant that this is precisely the region where A_H reaches its limit. Fig. 10 illustrates this behaviour.

Previous work has shown that *Bact. lactis ærogenes*, when sub-cultured in a concentration, $\bar{m}$, of proflavine, acquires immunity to the action of the drug at all concentrations up to a value which just exceeds $\bar{m}$ itself. The additivity relationships of the type $m_s = \bar{m} +$ constant, where m_s is the concentration of proflavine required to produce a lag of s minutes in a strain trained to $\bar{m}$, begin to break down when $\bar{m}$ is greater than about 200 mg./l. (Davies, Hinshelwood, and Pryce, *Trans. Faraday Soc.*, 1945, **41**, 778). A strain trained at a low value of $\bar{m}$ will not grow at all at $(\bar{m} + 54)$, whereas for $\bar{m} = 450$ the strain will grow in up to 1540 mg./l. (though with increased lag). Further training at $\bar{m} = 1540$ confers practically complete immunity to 3000 mg./l., the highest concentration which it is practicable to use. This can now be explained by the fact that there is a limit to the amount of drug the cell can take up. Training to the lowest concentration (270 mg./l.) at which this maximum is attained gives immunity to much higher concentrations, since these correspond to the same amount of drug absorbed.

(c) *Action of methylene-blue on untrained cells.* From the relationship that, for methylene-blue, $\Delta L = 30m$ (Pryce, Davies, and Hinshelwood, *Trans. Faraday Soc.*, 1945, **41**, 465) and the

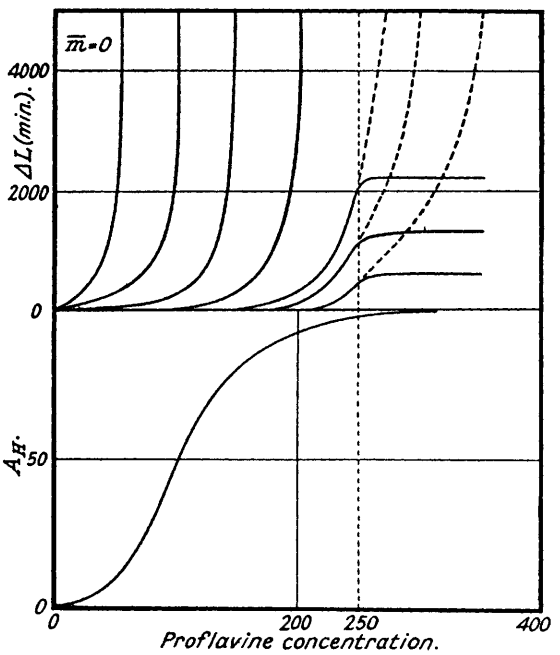
data of the methylene-blue isotherm, the ΔL–A_H curve was plotted [Fig. 8, curve (*b*)]. In spite of the differences in the isotherms and the lag–concentration relationships of the two substances, this curve is seen to be of the same general type as that for proflavine. This is interesting since on other grounds, *e.g.*, the phenomenon of cross-training, the similarity of the mode of action of the two drugs was already known. The differences in their lag–concentration curves are now seen to be due to the superposition of two different absorption isotherms upon two similar ΔL–A_H relationships. The differences in the former may well depend upon differing polymerisation tendencies and different relationships between the spatial configuration of absorbing sites and the structure of the dye.

(d) *Nature of the centres which take up glucose, proflavine, and hydrogen ions.* The competitive phenomena shown by any two of the above species lead to some interesting conclusions. The experimental evidence is as follows.

Proflavine cations and hydrogen ions. These both have positive charges and are probably taken up by negative centres in the cell. At pH below 6 (Table I) the total absorption of

FIG. 10.

Relation between lag, concentration, and drug-absorption for trained strains.



proflavine cations decreases, so that both evidently compete for the same negative sites. From pH 6 to pH 7, however, the total absorption of drug alters little, so that the bulk of the absorbing centres evidently take up the drug in another way in this pH range. A small fraction specially involved in the growth process must, however, continue to absorb hydrogen ions in competition with drug cations, since the lag due to proflavine is still strongly affected by the pH in the direction which this implies (Peacocke and Hinshelwood, this vol., p. 1235; see Section 6).

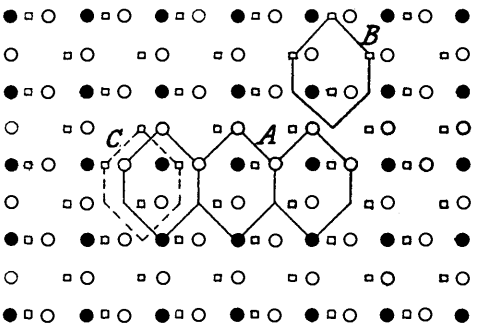
Proflavine cations and glucose. Although these are of a different charge type, the presence of glucose decreases considerably the absorption of proflavine from saline–phosphate [Section 7 (b)]. The glucose must compete with the drug on sites which absorb the large, uncharged rings of the proflavine cations, the proflavine being evidently also absorbed at points other than the charged N atom.* It seems likely, therefore, that the bulk of the proflavine cations are absorbed on a surface consisting of neutral and negatively charged sites in juxtaposition. A change in glucose concentration from 3·85 to 38·5 g./l. affects the lag due to proflavine more than it affects the

* The possibility must not be ignored that the glycolysis which may occur in presence of the glucose provides energy for some unknown process hindering the uptake of proflavine by the cell. But if this were so one would have expected a definite decrease in absorption when active division sets in. This is not observed (Section 10).

absorption of the latter. This again suggests that a small fraction only of the total absorbed proflavine is concerned in the inhibition of growth. This fraction apparently is absorbed on sites more sensitive than the bulk to competition by glucose molecules.

Glucose and hydrogen ions. Over the range 5 to 7·5 (Peacocke and Hinshelwood, *loc. cit.*) the lag in a constant glucose medium is independent of the pH. This indicates that there is no direct competition between the two species.

FIG. 11.



Schematic representation of adsorption sites :

● *Sites for H^+ and proflavine cations.*
○ *Sites for neutral proflavine.*
□ *Sites for glucose.*
A *Proflavine cation.*
B *Glucose molecule.*
C *Site for glucose molecule which could impede the absorption of A, or whose absorption could be impeded by A.*

On the basis of the above evidence a schematic picture of the absorbing surface may be formed and is shown in Fig. 11. The hydrogen being small will be taken up freely in the interstices left by glucose or proflavine. It can prevent the absorption of the latter on certain negative key sites. Glucose and proflavine having large molecules can seriously impede the absorption of one another. Thus sites near the molecule *A* are still available for hydrogen ions while corresponding ones would not be available for glucose on account of the size of the latter.

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