244. *The Adaptation of Bacteria to Acridine Derivatives. The Influence of pH.*

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Increasing hydrogen-ion concentration diminishes the bacteriostatic action of proflavine (2 : 8-diaminoacridine) on *Bact. lactis aerogenes*, as found by Albert *et al.* for various other acridines and *Bact. coli*. The concentration of the compound, $m_{\Delta L}$, required to produce a standard increase in the lag is related to $[H^+]$ by the equation $m_{\Delta L}/[H^+]^n = \text{constant}$. Changes in pH in the range studied do not affect the lag of cells in the absence of the compound, or according to the work of others, the dissociation of the proflavine which is virtually complete.

The exponent n is about 0·52 for normal cells. If cells are trained to resist proflavine at a concentration $\overline{m}$, n remains nearly constant over the range $\overline{m} = 0$ to $\overline{m} = 100$, and then rises to about 0·85.

These and other results suggest that quantitative changes in the cell enzymes occur on training to lower concentrations of proflavine. On training to higher concentration qualitative changes are superimposed.

The growth of bacterial cells in the presence of compounds which inhibit reactions involving auto-synthetic enzymes must often alter the material balance of the cell. This effect of the compound is not infrequently compensated by the resulting changes in the balance of the cell processes, so that the growth characteristics return to normal. Adaptation is then said to occur.

The problem of adaptation can be studied from various angles; in the following the effect of one important factor, the hydrogen-ion concentration, is investigated. The specific example chosen is the bacteriostatic action of proflavine (2 : 8-diaminoacridine) and of 5-aminoacridine upon *Bact. lactis aerogenes*.

The main effects of the addition of proflavine to the nutrient medium have already been described (Davies, Hinshelwood, and Pryce, *Trans. Faraday Soc.*, 1944, **40**, 397). On repeated subculture in the presence of proflavine, the cells adapt themselves with a marked reduction in the length of the lag phase. For any given strain there is a characteristic relation between lag (L) and concentration (m). When any strain has been repeatedly subcultured at a given concentration ($\overline{m}$), the m–L curve is displaced along the concentration axis by an amount which, over a certain range, is equal to $\overline{m}$.

Theoretical accounts of training phenomena have been based on the alternative ideas of natural selection of resistant strains previously present, of enzyme modification during the training process, and of change in the mode of cell division (or in some hereditary cell mechanism). The last two theories imply that actual modifications of individual cells occur and this is supported by the bulk of the evidence (Hinshelwood, *Biol. Reviews*, 1944, **19**, 158) although the action of selection must be superimposed on other mechanisms once a mixed population of modified and unmodified cells arises. Enzyme modifications in the individual cells may occur either (*a*) by quantitative changes in existing mechanisms and alterations of the enzyme balance in the cells, or (*b*) by qualitative modifications in enzyme surfaces involving changes unfavourable to attack by the toxic substance.

Theories of type (*a*) have been employed to interpret many of the facts of proflavine training (Davies, Hinshelwood, and Pryce, *loc. cit.*). The compound is regarded as impeding the utilisation of an intermediate substance required by a group of enzymes. Training is then envisaged as the quantitative expansion relative to these enzymes of the enzyme systems

producing the intermediate. Modifications of type (*b*) are, however, not excluded, and it is of some interest to discover a means of estimating their relative importance.

Albert *et al.* (*Brit. J. Exp. Path.*, 1945, **26**, 160) have accumulated a large body of evidence which indicates that the charged cation is the active bacteriostatic agent in solutions of the acridines. For example, it was found that the activity was greater the higher the degree of dissociation at a given pH. In the same paper an account is given of a study of the effect of changes in hydrogen-ion concentration on the antibacterial activity of several acridines towards *Bact. coli*. As the hydrogen-ion concentration increased an increasing concentration of acridinium ion was required to prevent any growth within 48 hours. The percentage of compound present as cation at a given pH was known from potentiometric measurements (Albert and Goldacre, *J.*, 1943, 454). The graphs of the logarithm of the inhibitory concentrations of the various cations against the pH of the media were found to constitute a series of close, parallel straight lines. Albert suggested that competition was occurring between the acridine cations and the hydrogen ions for the same positions on certain enzyme surfaces within the cell. (The drugs used did not include proflavine.)

In the light of Albert's work it is of interest to inquire how the relative competitive powers of hydrogen ions and acridinium ions will be altered during adaptation. Any changes will be reflected in corresponding changes in the slope and shape of the lines. This suggests a possible method of distinguishing between modifications of types (*a*) and (*b*), occurring during training, of the enzymes upon the surfaces of which the competition is considered to be located.

The purpose of the present study was to investigate the competition between proflavine and hydrogen ions in the growth of a series of trained strains of *Bact. lactis aerogenes*, with special reference to any information this might provide about the nature of the adaptive process.

Experimental Methods.—The experimental techniques of sterilisation, inoculation, culturing in liquid medium and determination of growth characteristics were similar to those previously used in this laboratory and described elsewhere (Davies, Hinshelwood, and Pryce, *loc. cit.*).

The media contained glucose, magnesium sulphate, ammonium sulphate, and phosphate buffer of appropiate pH, together with varying amounts of proflavine sulphate. They were maintained at 40·0° $\pm$ 0·05° and were aerated with sterile air, saturated with water vapour.

The pH of the complete solution was measured in a suitably standardised Cambridge glass-electode pH meter ($\pm$ 0·02 unit of pH). The main cause of variability of pH in a given buffer was the hydrolysis of the proflavine sulphate. To allow for this, the pH was measured over a particular range of proflavine concentrations and the average taken. The mean variation about this averge was $\pm$ 0·02, the maximum being $\pm$ 0·04.

Before proceeding with the main experiments, it was necessary to determine how hydrogen ions affected the magnitude of the lag in the absence of the proflavine. The curves relating the lag with the age of the parent culture proved to be independent of pH.

Lag–concentration curves of normal cells were then determined at various hydrogen-ion concentrations in the presence of proflavine. A plot of pH against the logarithm of the concentration ($m_{\Delta L}$) required to produce various assigned increases of the lag (ΔL) gave a series of parallel straight lines. That the slope of these lines depended very little on the particular value of lag chosen (provided it exceeded about 1000 minutes) was of practical importance since the slopes could be obtained without always measuring the very long lags of 5000—6000 minutes. The standard slope was found to be 0·52.

As will be discussed later, the constancy or otherwise of this slope (n) gives some help in distinguishing between the two possibilities of qualitative and quantitative enzyme modifications during adaptation.

Similar experiments were therefore performed with cells trained to given proflavine concentrations ($\overline{m}$). A family of lag–concentration curves was obtained for different pH's. Plots of log ($m_{\Delta L}$) against the corresponding pH gave straight lines for each strain. The value of the slope (n) remained constant at 0·5—0·6 until a training concentration of about 100 mg./l. was reached, when there was an increase to 0·8—0·9.

Effect of Varying pH upon the Lag in the Acridine-free Medium.—In order to determine how varying hydrogen-ion concentration influenced the lag–age curve (Lodge and Hinshelwood, *J.*, 1943, 213), a culture in a standard glucose–phosphate–ammonium sulphate medium (pH = 7·12) was sub-cultured at intervals into three media of pH 6·17, 7·25, and 7·56, respectively. Only one lag–age curve could be drawn through the three sets of determinations and this had the usual shape (Fig. 1).

This result shows that any effect of hydrogen ions on the lag in the presence of the acridine must be due to changes in the influence of the drug itself.

Influence of pH on the Lag of Untrained Cells in the Presence of the Acridine.—Preliminary experiments showed that the concentration of proflavine or of 5-aminoacridine required to produce a given lag was higher the lower the pH of the medium.

FIG. 1.

Influence of pH on lag–age relation.

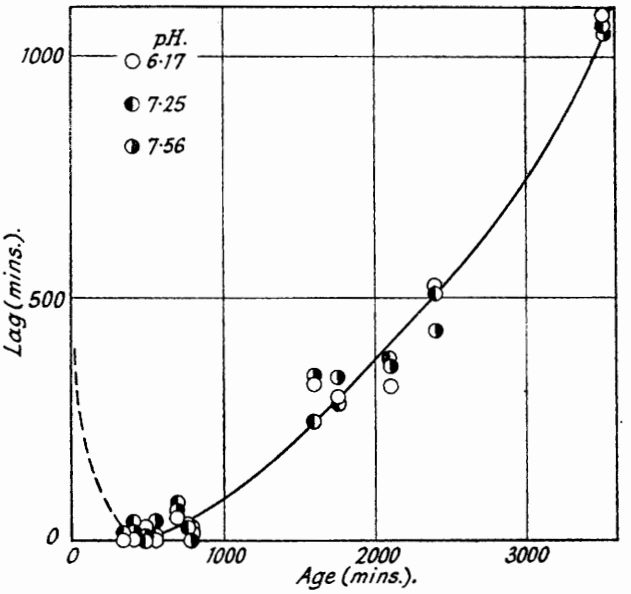
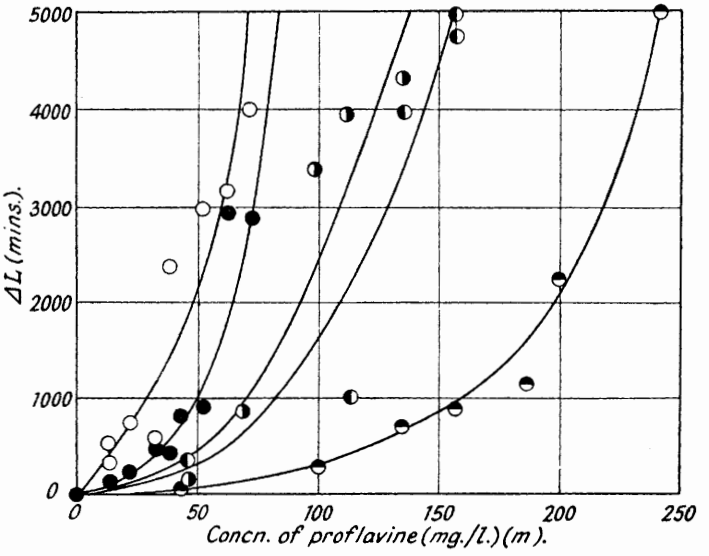


FIG. 2.

Lag–concentration curves for normal cells in proflavine at varying pH.



pH : ○ 7·67, ● 7·20(5), ◑ 6·74, ◐ 6·43(5), ◓ 6·11.

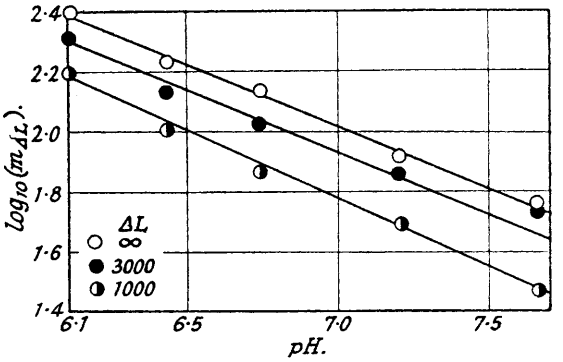
For the main series of experiments inocula were taken from a parent at the end of the logarithmic phase and transferred to series of media with a range of proflavine concentrations and a range of pH values. The lag, L_0, of these inocula was nearly zero for the acridine-free

medium, but to eliminate small variations the quantity used for plotting was $\Delta L = L - L_0$, L being the measured lag, in minutes, in any given medium. From the results the ΔL–concentration curves of normal cells in media containing proflavine at various hydrogen-ion concentrations were plotted. At all hydrogen-ion concentrations they were found to be of the same general shape and resembled those previously determined (Davies, Hinshelwood, and Pryce, *loc. cit.*). A typical set for proflavine is shown in Fig. 2, the family of curves extending to higher concentrations as the pH of the medium decreases.

From these and similar curves, the concentrations ($m_{\Delta L}$) required to produce a given value of ΔL at each pH were read off. Since proflavine is 100% ionised over the pH range employed, these concentrations were equal to those of the cations.

FIG. 3.

Relationship between log ($m_{\Delta L}$) *and pH for proflavine at different values of* ΔL.



$m_{\Delta L}$ *is the concentration of proflavine required to increase lag by* ΔL *minutes.*

Log ($m_{\Delta L}$) plotted against pH gives a series of parallel straight lines for the various assigned values of ΔL (Fig. 3). The following relationship is therefore obeyed:

$$\log (m_{\Delta L}) = n \log [H^+] + \log A$$

or,

$$\frac{m_{\Delta L}}{[H^+]^n} = A \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where A is a constant depending only on the particular value of ΔL chosen and n is the slope of the line. For normal cells in proflavine, $n = 0{\cdot}52$.

Similar experiments were made with 5-aminoacridine instead of proflavine, and curves of the same form were obtained, the value of the slope (n) being 0·88 (cf. the value of 0·89 found by Albert for 5-aminoacridine and *Bact. coli*).

Influence of pH on the Lag of Trained Cells in the Presence of Proflavine.—Bact. lactis aerogenes was allowed to become completely trained at a given concentration ($\overline{m}$) of proflavine and at a pH of 7·12 (10 to 15 sub-cultures were usually sufficient for this purpose). A culture of these trained cells was then used as the parent for lag–concentration determinations in various buffers, in exactly the same way as before, and the quantity ΔL again plotted against the concentration for each buffer. The family of curves was similar to that obtained with normal cells, but displaced towards higher proflavine concentrations. A typical set is shown in Fig. 4. As before, $m_{\Delta L}$ was read from the curves, and the logarithm plotted against the corresponding pH was found for each strain of cells to give a straight line. Fig. 5 shows these lines for $\Delta L = 1000$ minutes, and Table I gives the slopes of these lines together with the corresponding concentrations ($\overline{m}$) to which the cells were trained.

TABLE I.

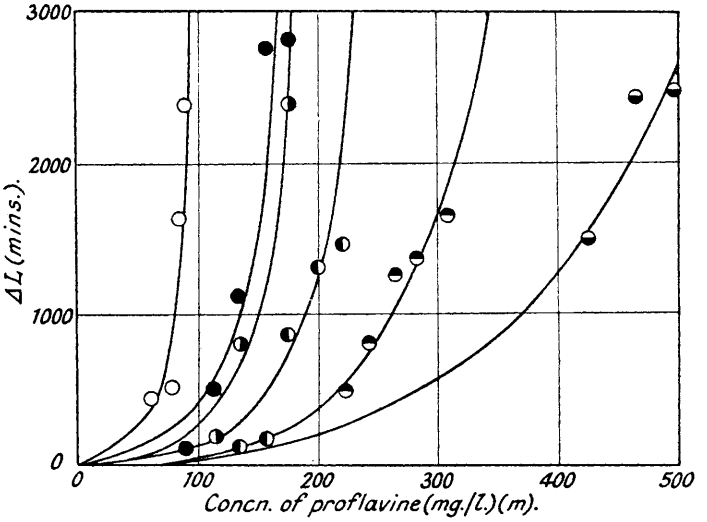
$\overline{m}$	0	21	44	71	90	109	115	118
Slope (n)	0·52	0·54	0·56	0·57	0·59	0·82	0·86	0·84

The table shows that the numerical value of the slope remains constant, within the experimental error, at 0·5—0·6 until $\overline{m}$ reaches about 100 mg./l. After this it increases to

0·8—0·9. In Fig. 6 the slope is plotted against the training concentration (m). Training to higher values of $\overline{m}$ was not carried out, since this would have necessitated high test concentrations of proflavine sulphate. Under these conditions the buffers would fail to keep the pH's near to the desired values.

FIG. 4.

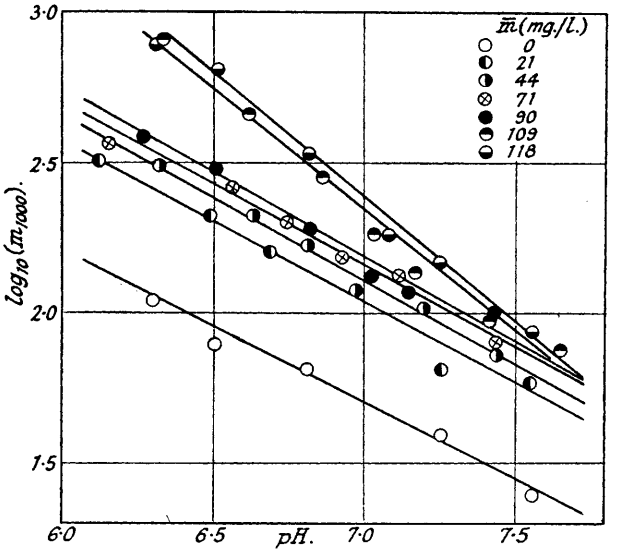
Lag–concentration curves for trained cells ($\overline{m}$ = 71) *in proflavine at varying pH.*



pH: ○ 7·44, ● 7·12, ◑ 6·93, ◐ 6·74(5), ◓ 6·56, ◒ 6·14(5).

FIG. 5.

Relationship between log (m_{1000}) *and pH for cells trained to various proflavine concentrations* (m).



m_{1000} *is the concentration of proflavine required to increase lag by* 1000 *minutes.*

Influence of Neutral-salt Concentration.—It is well known that increase in the neutral salt concentration increases the uptake of cationic dyes by various fibres (Standing, *Trans. Faraday Soc.*, 1945, **41**, 410). The alkaline buffers used in these experiments contained a somewhat greater concentration of neutral sodium salt than the acid ones, since more sodium hydroxide solution was added to the potassium dihydrogen phosphate solution in their preparation. The

increased activity of the acridines in alkaline solution might thus have been due to their greater absorption by the cell at this greater neutral salt concentration. The importance of this factor had to be determined in special experiments.

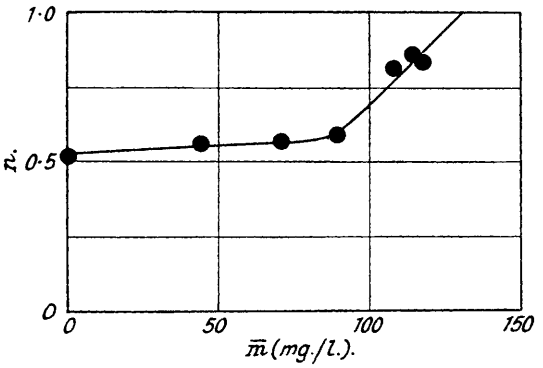
The concentration of neutral salt was calculated from the volume of the 4N-sodium hydroxide added to the phosphate solution. The buffer of pH = 7·55 contained, for example, 0·011 equiv. per l. of " neutral " sodium salt more than the normal buffer.

The lags of normal cells (transferred at the end of logarithmic growth) in normal media containing a fixed amount of proflavine and varying amounts of sodium chloride were determined. Two controls with, respectively, no proflavine, and neither proflavine nor salt, were inoculated at the same time.

The results of these experiments showed that in actual fact 0·011 equiv. per l. of sodium chloride instead of increasing lag caused decreases of 7% in a 1000-minute lag and of 27% in a 4000-minute lag. This concentration of neutral salt in the most alkaline buffers might therefore have had a similar effect. In such buffers, however, the effect of proflavine in lengthening the lag is so great that these small opposing effects of neutral salt concentrations would be completely obscured. The latter can therefore be neglected in the future discussion.

FIG. 6.

Variation of n *with the training concentration* ($\overline{m}$) *of proflavine for various strains.*



n *is the slope of the log* (m_{1000})–*pH curve for each strain.*

Effect of Filtrate of pH adjusted to 7·0 *upon the Lag of Normal Cells in Proflavine.*—Davies, Hinshelwood, and Pryce (*loc. cit.*) have shown that the addition of sterile filtrate from a normal culture reduces very considerably the effect of proflavine on lag. The effect is particularly pronounced in the case of " early lag " (Lodge and Hinshelwood, *loc. cit.*) and the phenomenon was regarded as supporting the hypothesis that proflavine inhibits the synthesis of an essential intermediate the absence of which is responsible for early lag.

The sterile filtrate was obtained from a culture of normal cells at the end of the logarithmic phase and therefore had a pH of 4·5—5·0.

The question arises whether the effectiveness of this filtrate could have been due to its acidity rather than to the presence of some essential intermediate. The following media were therefore inoculated with 0·1 ml. of a culture of normal cells at the end of the logarithmic phase :

(*a*) A normal medium.

(*b*) A normal medium containing 67 mg./l. of proflavine and 2 ml. of sterile water.

(*c*) A normal medium containing 67 mg./l. of proflavine and 2 ml. of sterile filtrate, the pH of which had been adjusted to 7·0 by the addition of sodium hydroxide solution.

(*d*) A normal medium containing 67 mg./l. of proflavine and 2 ml. of sterile filtrate of pH 5·0.

The lags were then measured (Table II).

TABLE II.

Medium	(*a*)	(*b*)	(*c*)	(*d*)
Proflavine concn. (mg./l.)	0	67	67	67
Lag (L), mins.	0	920	320	130
	($=L_0$)	940	350	140
Average ΔL ($L - L_0$), mins.	0	930	335	135

Table II shows that both filtrates reduced the lag considerably, that of pH 5·0 being more effective than that of pH 7·0. The adjusted filtrate causes a lag reduction of about 600 minutes, and the acid filtrate a further reduction of 200 minutes. The effect of the unadjusted filtrate cannot therefore be due mainly to its acidity, and the presence in filtrate of some essential intermediate can still be inferred. Nevertheless, the total reduction by filtrate of the lag in proflavine is due to :

(i) the presence of an effective concentration of some essential intermediate, and
(ii) the acidity of the filtrate.

Behaviour of Cells repeatedly Sub-cultured in Acid Media.—On repeated sub-culture of bacteria in an acid medium, the cells might conceivably adapt themselves to resist the effects of the acid—for example, by reducing the number of negative groups which attract hydrogen ions. Such a response would probably affect the sensitivity of the bacteria to the action of the proflavine cation.

In order to study this question, a strain of *Bact. lactis aerogenes* which had been sub-cultured many times in the usual medium of pH 7·1—7·2 was transferred to a similar medium of pH 6·17. The total bacterial population supported by this medium was much lower, as previously observed (Lodge and Hinshelwood, *J.*, 1939, 1683), and did not increase with repeated sub-culture (Table III).

TABLE III.

No. of sub-cultures in medium of pH 6·17	1	5	11	12	17	20	21	24	26	28	32	33	34
Stationary population (no./ml. ÷ 1·25 × 10^6)	410	510	580	520	450	400	480	450	630	460	400	470	530

After the 23rd culture in acid medium, lag–concentration curves in proflavine at various pH's were determined in exactly the same way as before. The curves were of the usual shape and the same effect of hydrogen ions on the activity of the proflavine was displayed. The slope of the log ($m_{\Delta L}$)–pH plot was 0·49, which, within the experimental error, is the same as for normal cells.

Lag–concentration curves at two of the pH's used in the test were determined for the parent strain of normal cells. Within the experimental error, these coincided with the corresponding curves for the cells sub-cultured in the acid medium.

It was therefore concluded that repeated sub-culture in more acidic media did not affect the groups and enzyme systems involved in the action of proflavine.

Behaviour of Cells Trained to Proflavine in an Acid Medium.—A training experiment somewhat similar to that described in the last section was performed. Cells were successively sub-cultured in an acidic phosphate buffer (pH 6·3) and 157 mg./l. of proflavine. This concentration at this pH at first caused a lag of about 3000 minutes which decreased to zero after 20 sub-cultures. The inhibitory action is here much less than it would be at the normal pH.

Lag–concentration curves in proflavine at pH 6·3 and 7·12 were then determined. In this way the effective training concentration at pH 7·12 corresponding to the actual training at pH 6·3 and 157 mg./l. could be found. In the test at the lower hydrogen-ion concentration the displacement of the curve away from that for the untrained cells corresponded to a training concentration (at that pH) very much less than 157 mg./l. Thus the response to training is determined by the actual degree of inhibition rather than by the concentration of the toxic agent.

DISCUSSION.

(1) *The Relationship between* $m_{\Delta L}$ *and the Hydrogen-ion Concentration.*—In equation (1) which applies to the growth of *Bact. lactis aerogenes* in the presence of proflavine and 5-aminoacridine : $n = ca.\ 0{\cdot}5$ for normal cells and cells trained to proflavine up to 100 mg./l., $n = ca.\ 0{\cdot}8$ for cells trained to more than 100 mg./l. of proflavine, and $n = ca.\ 0{\cdot}9$ for normal cells, tested in 5-aminoacridine.

Mention has already been made of the suggestion that the acridinium ions and hydrogen ions are in competition for similar negative sites on the surface of an enzyme whose functioning is inhibited by the acridine. The competition of three molecular species—substrate molecules (S), acridinium ions (D^+), and hydrogen ions (H^+)—has in general to be considered. The possibilities include :

(*a*) Competition on the surface between acridine cations and hydrogen ions only. This

involves the assumption that the adsorption of hydrogen ions on the protein, unlike that of the drug cations, does not interfere with the utilisation of the substrate molecules by the enzyme. This is not unlikely, since in its normal active condition the protein of an enzyme is continually exchanging hydrogen ions with its surrounding medium.

In the following treatment the reactions between the negative groups on the protein (enzyme) and the two types of cation are regarded as independent. Let these negative groups be represented by P^-, and the drug cations by D^+. m is the total concentration of the acridine in solution (100% ionisation). Then,

$$PH = H^+ + P^-$$
$$PD = D^+ + P^-$$

Hence, $$K_1 = \frac{[H^+][P^-]}{[PH]}, \text{ and } K_2 = \frac{m\,[P^-]}{[PD]}$$

$$\therefore \quad \frac{K_1}{K_2} = \frac{[H^+][PD]}{[PH]\,m}$$

If it is assumed that for a given value of the lag the ratio $\frac{[PD]}{[PH]}$ is constant, then m becomes $m_{\Delta L}$ and

$$\frac{m_{\Delta L}}{[H^+]} = \text{constant} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

According to this, the value of the index n of equation (1) should be unity and should be the same for all compounds competing with hydrogen ions. Since neither of these statements is true for *Bact. lactis aerogenes* in the presence of proflavine and 5-aminoacridine, this simple statement is inadequate. This is not unexpected, since various doubtful assumptions are involved, for example, that, in spite of the difference in their size, shape, and charge distribution, the activity coefficients of the hydrogen and acridinium ions are the same. Inaccuracies of this sort may be quite sufficient in themselves to account for the departure of the value of n from unity, but are difficult to estimate in any quantitative way.

(*b*) Competition involving substrate molecules. Substrate molecules (S) may also be involved in equilibria with hydrogen ions and even drug cations (D^+), giving SH^+ and SD^+, respectively. The cations SH^+ may then compete on the surface with D^+, H^+, and SD^+, it being assumed that SH^+ is the only form of the substrate utilisable by the enzyme. Increasing hydrogen-ion concentration would then increase the concentration of SH^+ in the cell and hence its adsorption relative to other ions. Qualitatively this would account for the observed antagonism of proflavine and hydrogen ions.

The competition of SH^+, SD^+, D^+ and H^+ for a limited number of adsorption points on an enzyme suface can be treated by the usual elementary methods. If, for any given strain of cells, S is taken to be constant, and if it is assumed that, for any assigned value of ΔL, the fraction of surface occupied by SH^+ is constant, then, replacing D^+ by $m_{\Delta L}$, we obtain

$$\frac{A[H^+]}{1 + B[H^+] + Cm_{\Delta L}} = \text{constant} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

where, for a given value of ΔL, A, B, C are constants.

This equation, which is a Langmuir equation for adsorption of several types of molecules on a limited number of sites, is not of the form of equation (1). Over a limited range of hydrogen-ion concentration, however, it might approximate to an expression of the observed type.

(2) *Modification of the* ΔL–m *Relationship to include the pH Effect.*—On the basis of their theory of adaptation to proflavine (and other drugs), Pryce and Hinshelwood (*Trans. Faraday Soc.*, 1947, in the press) derived an expression of the following form for the dependence of the lag (L) of trained and untrained cells on the concentration (m),

$$L = \frac{\text{constant}}{f(S_0) + \phi(\overline{m}) - \phi(m)} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

$\phi(m)$ is a function of the test concentration (for proflavine a simple linear one) and $\phi(m)$ the same function of the concentration ($\overline{m}$) to which the strain has become adapted. This equation,

however, was only intended to apply to cultures at a constant pH. As a suitable modification which allows for the influence of pH the following is suggested.

$$L = \frac{\text{constant}}{f(S_0) + \left[\frac{\phi(\bar{m})}{[\bar{H}^+]^n}\right] - \left[\frac{\phi(m)}{[H^+]^n}\right]} \quad \ldots\ldots\ldots \quad (5)$$

where $[H^+]$ = test concentration, and $[\bar{H}^+]$ = training concentration of hydrogen ion.

No change is made in the term $f(S_0)$ since the lag in the absence of the acridine is independent of the pH. For any particular strain of cells, trained or untrained, $f(S_0)$, $\phi(\bar{m})$, $[\bar{H}^+]$ are all constant. Hence $\frac{[H^+]^n}{\phi(m_{\Delta L})}$ is constant for a given strain of cells at a constant value of L (and therefore of ΔL).

For proflavine, $\phi(m) \propto m$ (Pryce and Hinshelwood, *loc. cit.*) and therefore,

$$\frac{m_{\Delta L}}{[\bar{H}^+]^n} = \text{constant}$$

which is equation (1). Equation (5), therefore, adequately expresses the experimental facts. It also predicts correctly the behaviour on training to proflavine at various hydrogen-ion concentrations.

When the lag approaches infinity, the denominator of equation (5) becomes zero.

$$f(S_0) + \frac{\phi(\bar{m})}{[\bar{H}^+]^n} = \frac{\phi(m)}{[H^+]^n}$$

For proflavine, $\phi(m) = f' m$ (where f' is a constant)

$$m = \left(\frac{[H^+]}{[\bar{H}^+]}\right)^n + \frac{f(S_0)}{f'}[H^+]^n$$

$\frac{f(S_0)}{f'}$ = constant, if all the trained strains are derived from the same parent strain.

Hence, if the cells are trained to concentration $\bar{m}$ of proflavine at a hydrogen-ion concentration $[\bar{H}^+]$, and tested in the presence of the acridine at a lower hydrogen-ion concentration, $[H^+]$, the m value observed will correspond to that obtained by training at a lower value of $\bar{m}$ at the lower hydrogen-ion concentration, $[H^+]$. This is the result described in the experimental section.

No theoretical reason for the substitutions leading from equation (4) to equation (5) is advanced at present, but the latter expresses observed results correctly.

(3) *The Effect of Training on the Relation between* $m_{\Delta L}$ *and* (H^+).—In equation (1), the index n remains constant at about 0·5 with proflavine for strains trained up to about 100 mg./l. Above this limit n increases to about 0·9.

The experiments with trained cells were made because it was considered that any change in this index on training would indicate that the competitive powers of the hydrogen ion relative to acridinium had altered and that qualitative modifications of the enzyme surface had occurred.

The results suggest that such modifications occur only when training is carried to concentrations of proflavine greater than about 100 mg./l.

The introduction of some new factor in the training process at about this stage is suggested by two other pieces of evidence :

(i) The attainment of a given degree and stability of training to concentrations of proflavine greater than 100 mg./l. requires a greater number of subcultures than does training to lower concentrations. The stabilisation of complex qualitative changes in the enzymes as part of the adaptive process might be expected to require a longer time than simple quantitative expansion, which has been shown to occur after two or three divisions in the presence of the acridine (Davies, Hinshelwood, and Pryce, *loc. cit.*). The change in the value of n and the increase in the difficulty of training at roughly the same concentration are probably not unconnected.

(ii) Expressions of the same type as equation (4), based on a theory of quantitative enzyme expansion during adaptation, have been shown to predict the general form of a family of lag–concentration curves for strains trained to increasing concentrations of proflavine. Satisfactory quantitative agreement is also obtained for strains trained up to about 95 mg./l., one set of constants in the equation predicting the shape and position of the curves for the

various trained strains (Davies, Hinshelwood, and Pryce, *Trans. Faraday Soc.*, 1945, **41**, 465). Above the limit of about 95 mg./l., however, divergences begin to occur and certain relationships between the constants, which up to this point have been obeyed, begin to break down. This is evidence of an indirect kind only, but it is of interest that the divergences declare themselves at roughly the same point as that where training becomes more difficult and the value of n begins to increase.

Each of the above facts by itself is probably inadequate to justify any particular conclusion, but, taken together, they constitute evidence that adaptation to concentrations of proflavine of up to 100 mg./l. consists of quantitative enzyme expansions only, and that above this limit qualitative modifications of enzymes may also be involved.

Evidence of enzyme modifications more complex and far-reaching than a simple quantitative expansion is also afforded by cells trained to concentrations of proflavine of the order of 1500 mg./l. (*idem*, *ibid.*, p. 777) and depends upon the unusual shape of the lag–concentration curves given by these strains after passage through normal media. The concentrations involved are very much greater than those in the present experiments and the interpretation of the result may be different, but the effect in question does provide evidence of complex changes which may accompany training to concentrations of proflavine at the other end of the concentration scale.

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