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THE ACTION OF BARBITONE ON *AEROBACTER AEROGENES (KLEBSIELLA AEROGENES)*

BY

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The mode of action of barbiturates on biological systems is of considerable interest. We were attracted to their action on bacteria by a recent paper by Goldstein (1965), in which the possibility of using the system barbitone–*E. coli* as a model for studying the effects of prolonged drug administration is examined. In this paper the action of barbitone on the lag, growth rate, median cell size and glucose dehydrogenase activity of *A. aerogenes* is reported with special reference to (a) the development of resistance to the drug and (b) the model just mentioned. Dehydrogenases are known to be affected by barbiturates (Davies & Quastel, 1932) and are sometimes augmented in resistant strains of bacteria in a manner suggesting a direct response of the cells to the toxic action of the drug (Grant & Hinshelwood, 1964). A study has also been made of the possible synergism between the action of barbitone and ethanol (on which conflicting reports have appeared (Gruber, 1955)) or 2-phenylethanol.

METHODS

A laboratory strain of *A. aerogenes* (N.C.T.C. 418, *Bacterium aerogenes* no. 240), which had been fully conditioned to a glucose-salts medium by many daily subcultures, was used. The medium was prepared by adding 0.8 ml. of a sterile solution of glucose (50 g/l.) to 16 ml. of a sterile solution of salts containing the following ingredients in g/l.: $FeSO_4.7H_2O$ 0.32×10^{-3}, $MgSO_4.7H_2O$ 0.063, $(NH_4)_2SO_4$ 1.56, KH_2PO_4 1.85 and $Na_2HPO_4.12H_2O$ 10.00, pH 7.12. Barbitone was dissolved in the salts solution which was then sterilized by boiling. The pH was always re-adjusted to 7.12. Lags and mean generation times were determined as described by McCarthy & Hinshelwood (1958) and glucose dehydrogenase activities by the method of Grant & Hinshelwood (1964). For the latter, test samples were always withdrawn 21 hr after inoculation of the culture. AnalaR grade reagents were used throughout except for barbitone and 2-phenylethanol which were reagent grade (B.D.H.). Except where otherwise stated cultures were grown in boiling-tubes or small conical flasks kept at 40° C in a thermostat and were stirred and aerated by a vigorous stream of sterile air. Cell mass was determined turbidimetrically and cell number by direct microscopic counting (Dean & Hinshelwood, 1960).

RESULTS

Effect of barbitone on lag and mean generation time

The relationship between the lag which occurs before growth begins, and the concentration of barbitone for the parent (sensitive) strain, and for several "trained"

strains is shown in Fig. 1. It is apparent that although the lags are relatively short the lag produced by a given concentration of drug decreases as the concentration ($\bar{m}$), at which the "training" is carried out, is increased. At a concentration of 6 g/l. the lag of the parent strain (not shown in Fig. 1) was about 700 min.

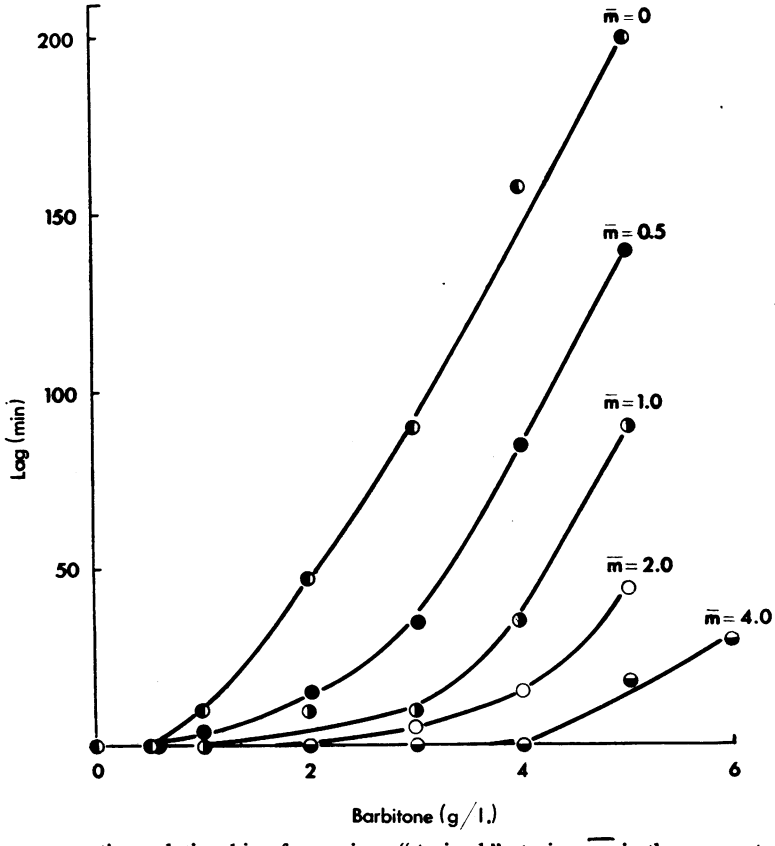

Fig. 1. Lag-concentration relationships for various "trained" strains. $\bar{m}$ is the concentration (g./l.) at which training was carried out.

Barbitone has a more marked effect on the mean generation time (reciprocal of the growth rate) than on the lag. The values for the various strains are given in Table 1, which shows that the higher the concentration of drug the slower the rate of growth, and that although a marked improvement accompanied "training" the mean generation time never reached the value obtained with the untrained strain in drug-free medium. For example, even a strain which had received 126 subcultures (1 subculture = 5–6 generations of growth) in medium containing 4 g/l. of barbitone had a mean generation time of 49 min, having improved from 109 min. When the lag and growth rate experiments were repeated similar results were obtained and the sort of scatter in the mean generation time is shown in Table 1 where the two columns under the heading $\bar{m}=0$ refer to different experiments. The lags showed a similar variation and the values plotted in Fig. 1 are mean values.

TABLE 1

MEAN GENERATION TIME OF "TRAINED" STRAINS IN BARBITONE MEDIUM

$\bar{m}$ is the concentration (g/l.) at which "training" was carried out

Barbitone (g/l.)	Mean generation time (min)				
	$\bar{m} = 0$	$\bar{m} = 0{\cdot}5$	$\bar{m} = 1{\cdot}0$	$\bar{m} = 2{\cdot}0$	$\bar{m} = 4{\cdot}0$
0	32,33				
0·5	38,38	37			
1·0	46,48	39	37		
2·0	57,65	52	51	42	
3·0	74,90	70	68	46	
4·0	108,122	100	87	54	49
5·0	126,135	121	104	81	58
6·0	200	—	—	—	87

To investigate the stability of the training, a strain which had received 26 subcultures in medium containing 1 g/l. of barbitone was serially subcultured in drug-free medium and was tested at intervals in a range of concentrations of drug. Results are given in Table 2 which shows that on long continued growth in drug-free medium the mean generation time in the absence of drug does not return to the normal value of 33 min. Nevertheless the resistance, as measured by the mean generation time in medium containing drug, is fairly easily lost. This was particularly evident at the higher concentrations and is shown not only by the large values but also by the appearance of clumping and filamentation of the cells in the culture. Clumping was not usually observed on the first exposure of the sensitive organism to high concentration of drug. It did, however, appear during training and disappeared again as subculture continued. Filament formation represents an inhibition of division relative to the growth of the cells and is dealt with in the next section.

TABLE 2

EFFECT OF SERIAL SUBCULTURE IN DRUG-FREE MEDIUM OF A STRAIN WHICH HAD RECEIVED 26 SUBCULTURES IN MEDIUM CONTAINING 1 G/L. OF BARBITONE

C indicates clumping and filamentation of the culture

Barbitone concn. in test (g/l.)	Mean generation time (min) after given number of subcultures of "trained" strain in drug-free medium				
	0	*11*	*25*	*39*	Control*
0	38	44	43	41	33
1·0	43	44	51	51	54
2·0	59	80	87	83	71
4·0	100	C	C	C	130
5·0	120	C	C	C	150

* Mean generation time (min) on first exposure to drug.

Effect of barbitone on cell size

A convenient way of representing the median cell size is by determining the ratio of the cell mass (expressed here as the number of standard cells/ml.) to the actual number of cells/ml. obtained from replicate microscope counts using a Helber counting chamber. During the course of a growth cycle this ratio, which will be denoted by σ, increases about five-fold and passes through a maximum early on (Fig. 2, curve a). On first exposure

of the untrained organism to 1 g/l. of barbitone, σ runs at a higher level throughout the major part of the growth cycle (Fig. 2, curve b) and this effect is augmented by "training." For example, when a strain which had received many subcultures in medium containing 1 g/l. of drug was tested in a similar medium, the maximum in σ, rather than dropping to the level obtained in the absence of the drug (Fig. 2, curve a), was actually higher than that obtained during the first subculture and moreover remained high in the later stages of the growth cycle (Fig. 2, curve c). Therefore although the "training" to 1 g/l. of drug had resulted in an improvement in the mean generation time (Table 1) the inhibition of division had not "trained out". Moreover microscopic examination of the culture showed that this inhibition took place in most of the cells.

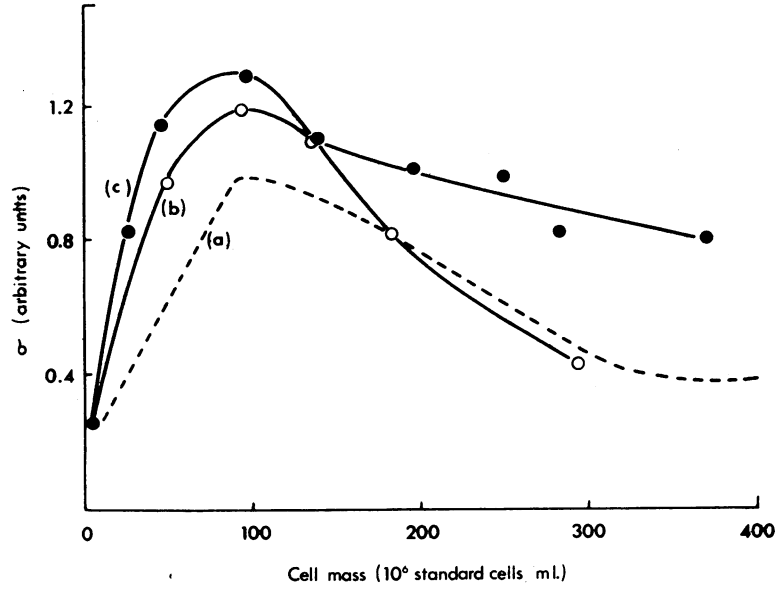

Fig. 2. Effect of barbitone on the median cell size (σ). (a) Standard curve (no drug) derived from the data of Dean & Hinshelwood (1960). (b) "Untrained" organism in the presence of 1 g/l. of drug. (c) Strain thoroughly trained to 1 g/l. in medium containing 1 g/l. of drug.

Enzyme activities of trained strains

The parent organism ferments sucrose, but not dulcitol, to yield acid and gas under standard conditions. The methyl red test is negative, the Voges-Proskauer test positive, and indole is not produced from peptone. A strain which had received many subcultures in medium containing 1 g/l. of barbitone gave the expected response in these tests with the exception of the Voges-Proskauer test where the result was negative.

The effect of barbitone on the utilization of glucose by the cells was examined. It was found that the yield of cells obtained from a given growth-limiting amount of glucose by strains which had been thoroughly "trained" to 1 and 5 g/l. of barbitone respectively was unaffected. Indeed, during training to 1 g/l. the total population ran at almost the maximum level from the start. In medium containing 5 g/l., however, the yield was very low over the first three subcultures (Table 3).

TABLE 3

YIELD OF CELLS IN BARBITONE MEDIUM CONTAINING A GROWTH-LIMITING AMOUNT OF GLUCOSE

(a) 1 g/l. barbitone; (b) 5 g/l. barbitone

Subculture number	Yield (10^6 standard cells/ml.) (a)	(b)
1	269	74
2	262	23
3	320	32
4	280	284
5	280	335
10	320	340
Control (no drug)	350	

The glucose dehydrogenase activities for a number of strains were then determined. It had previously been shown that during "training" to a given carbon source the activity of the dehydrogenase responsible for its utilization increases in proportion to the growth rate (Baskett & Hinshelwood, 1950). A dependence of the dehydrogenase activity on the actual growth rate would obscure more specific effects of "training" to the drug. Thus a slower growing drug-resistant strain would show a reduced dehydrogenase activity and it is necessary to distinguish between that part of an observed effect which is a direct result of growth at a slower rate and that which is inherently caused by exposure of the organism to the drug. This can be achieved by determining the ratio of the relative glucose dehydrogenase activity to the relative growth rate. The former is obtained by dividing the glucose dehydrogenase activity of the strain being tested by that of a control and the latter by dividing the mean generation time of the control by that of the strain under test. From the proportionality obtained between the relative activity and the relative growth rate by Baskett & Hinshelwood (1950), it follows that if the drug had no effect whatsoever on the activity of the enzyme the ratio would always be unity, irrespective of the growth rate. Values greater than unity indicate an increased activity caused by the drug. When the sensitive organism was grown for the first time in the presence of 1 g/l. of drug the ratio was 1.35, but after 26 subcultures in 1 g/l. of drug it reached the more normal but still augmented level of 1.14. When this "training" was followed by 76 subcultures in drug-free medium the ratio rose again to 1.38. One subculture in 1 g/l. of drug followed by one subculture in its absence gave a value of 1.46. Thus the effect of subculture in the presence of the drug is not readily reversed by subculture in its absence. These ratios are mean values of several experiments which gave essentially similar results and the sort of scatter is shown by the individual values obtained on the first exposure of the sensitive organism to 1 g/l. of drug. They were 1.30, 1.33, 1.38 and 1.39, mean 1.35.

Combined action of 2-phenylethanol and barbitone

In these experiments, and in the set to be described presently on the combined action of ethanol and barbitone, the mean generation time was chosen as a measure of the response and the results are given in the form of isobols (lines of equal biological effect (see Käer & Loewe, 1926; Loewe, 1953; Lacey, 1958)). The effect of barbitone on the mean generation time of the untrained organism has already been reported (Table 1), that of 2-phenylethanol and that of mixtures of the two drugs are shown in Fig. 3. For each

concentration of barbitone in Fig. 3 the corresponding concentration of 2-phenylethanol, which had to be present in the mixture in order to obtain a chosen relative mean generation time, was read off. From these values and the data already given the fractional inhibitory concentration—that is, the concentration of the inhibitor present in the mixture divided by the amount necessary to give the same degree of inhibition when acting alone, was calculated. The various fractional inhibitory concentrations are plotted in the form of an isobologram in Fig. 4 which shows that a slight degree of synergism exists between the two drugs. In a purely additive effect the points would fall on the straight line joining unity on the two axes and in antagonism they would be on a curve above this line. The degree of synergism can be given a numerical value by extrapolating the two branches of the curve to the point where the combined fractional inhibitory concentrations reach a minimum (that is maximum effectiveness). At a relative mean generation time of 4.0 this occurs when the fractional inhibitory concentrations of barbitone and

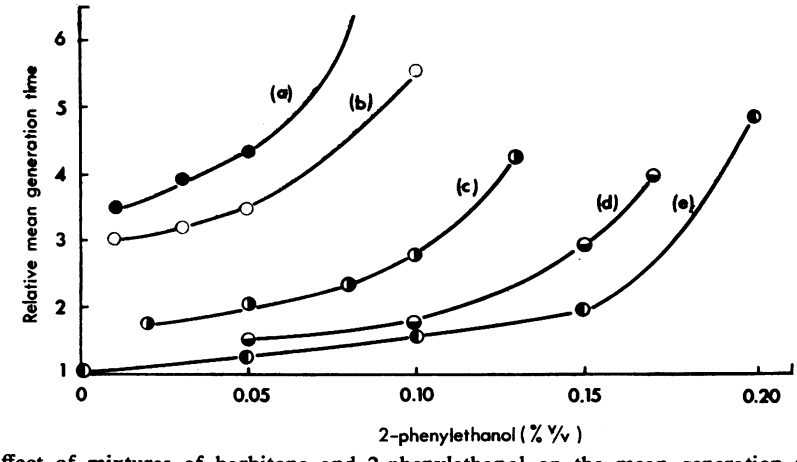

Fig. 3. Effect of mixtures of barbitone and 2-phenylethanol on the mean generation time. Concentration of barbitone (g/l.): (a) 3.0, (b) 2.0, (c) 1.0, (d) 0.5 and (e) none. Relative mean generation time is the ratio of the value found in the test to that of the control.

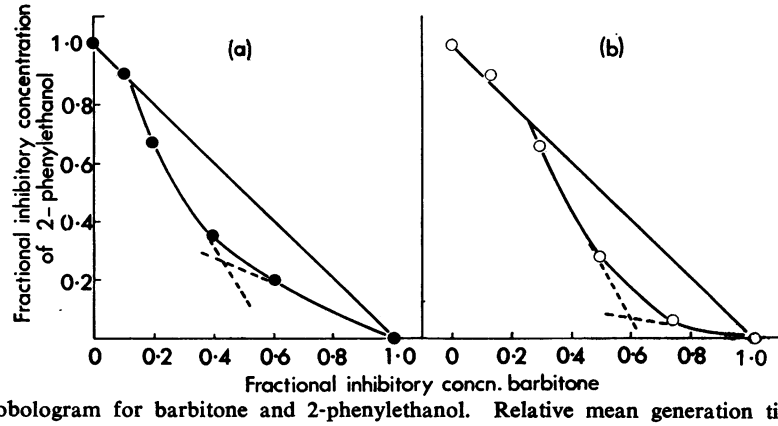

Fig. 4. Isobologram for barbitone and 2-phenylethanol. Relative mean generation time: (a) 4.0, (b) 3.5.

2-phenylethanol are 0.44 and 0.26 respectively—a sum of 0.70 (Fig. 4a). At the 3.5 level the corresponding values are 0.60 for barbitone and 0.07 for 2-phenylethanol—a sum of 0.67 (Fig. 4b). In a purely additive effect the value would be unity and the smaller the number the greater the degree of synergism.

Combined action of ethanol and barbitone

Since the vigorous stream of air blown through the cultures in the standard technique would lead to a loss of ethanol from the medium, this set of experiments was carried out in T-tubes contained in a thermostat incorporating a mechanical shaker. Under these conditions aeration adequate for growth at the full aerobic rate is not obtained. Indeed, in the absence of the drug the mean generation time of the " untrained " organism was between 42 and 45 min in replicate experiments instead of the value of 30–33 min obtained under fully aerobic conditions. Nevertheless it was reproducible and as a further precaution the tests were all carried out simultaneously. In the set reported here the mean generation times in medium containing 0, 0.5, 1, 2 and 3 g/l. of barbitone were 44, 51, 60, 90 and 140 min respectively and the relevant data obtained in the presence of ethanol and mixtures of both drugs is given in Fig. 5. When fractional inhibitory concentrations calculated from these data were plotted on an isobologram there was no evidence for anything but a purely additive effect.

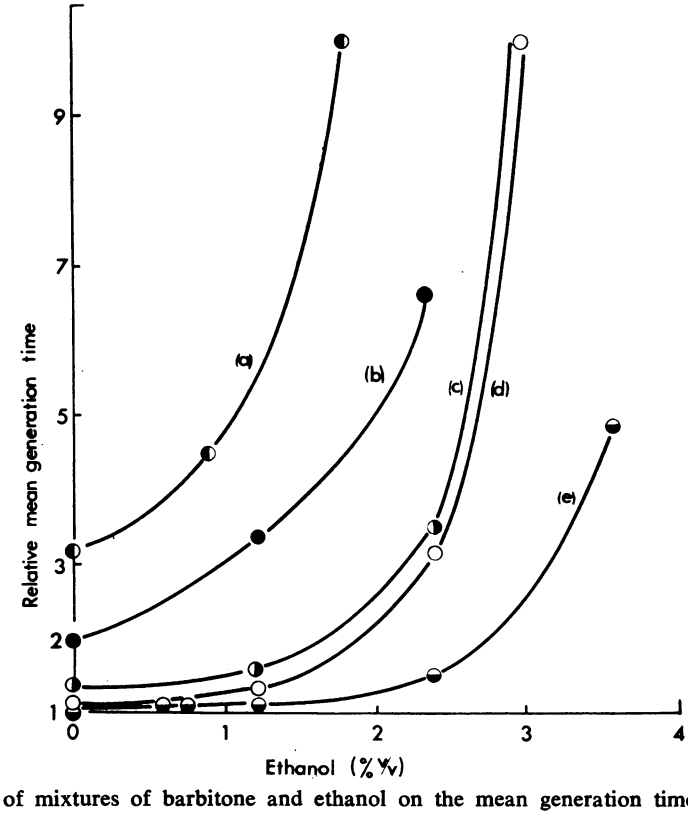

Fig. 5. Effect of mixtures of barbitone and ethanol on the mean generation time. Concentration of barbitone (g/l.): (a) 3.0, (b) 2.0, (c) 1.0, (d) 0.5 and (e) none.

DISCUSSION

The first point is how far the results give any evidence of the origin of resistance to barbitone. Barbitone is not a potent antibacterial agent and indeed a concentration of 6 g/l. is necessary to produce a lag of about 12 hr, which, in similar conditions, would result from the addition of 1.5 mg/l. of oxytetracycline to the medium (Dean & Giordan, 1965). Nevertheless, although the lag rapidly disappears on growth in the presence of drug, long continued subculture does not entirely eliminate all the toxic effects. For example, the inhibition of division relative to the growth of the cells not only persists, but actually becomes slightly more marked. The mean generation time and the glucose dehydrogenase activity of the cells, although showing considerable improvement, do not drop to the normal value, and the trained strains are unable to ferment glucose to acetylmethylcarbinol under standard conditions. However, the resistance of the cells as measured by the lags (small as they are) is graded to the concentration at which " training " has been carried out. This graded behaviour has also been observed with many active antibacterial agents and, although by no means unambiguous evidence in itself, is entirely compatible with explanations which ascribe the development of resistance to a compensatory adjustment in the enzyme balance of the bulk of the population of cells introduced into the medium. It is true that a lag of 12 hr would indeed allow time for the selection of a special mutant type of cell, but the effects of the drug were also observed when the lag was as short as 10–15 min—that is at a concentration of 1 g/l. During the first subculture in these latter conditions growth was slow, the mean generation time being 55 min compared to a control value of 33 min, the glucose dehydrogenase activity was abnormally high and the median cell size was up. The mean generation time of the fully trained strain was 43–45 min, and it could be postulated that the original sensitive population contained two types of cells, both capable of growing in the presence of the drug but at different rates. The short lag necessitates that practically all participate, and " training " would then consist of the progressive selection of the faster growing type which would also have to show a more normal but still high glucose dehydrogenase activity. The high activity observed on first exposure to the drug was calculated on the basis of all the cells, and if it had to be partitioned out between two types of cells one of these types must have a still higher activity.

The resistance, as measured by the reduced lag and by the improved mean generation time at high concentrations of drug brought about by serial subculture at a lower concentration, is easily lost on growth in the absence of drug. This suggests that the entire pattern of behaviour consists of a relatively unstable physiological adaptation of the cells to resist the action of the drug, together with damage of a more permanent kind to which " training " does not occur. In this the enzymes responsible for the primary attack on the carbon substrate expand and " overshoot " somewhat in the early stages, a situation which has also been observed when *A. aerogenes* utilizes D-arabinose as sole carbon source for the first time. In this much studied example, various lines of evidence are more easily interpreted in terms of a response by practically all the cells in the population than by the selection of a few specially endowed mutants (Dean & Hinshelwood, 1966).

The " trained " strains, even after considerable growth in drug-free medium, multiplied at less than the normal rate and their glucose dehydrogenase activity remained high, but

no evidence of gross damage to the respiratory systems of the cells was detected, since the yield of cells from glucose was the same with the " trained " and the " untrained " strains. Less aerobic metabolic pathways should have resulted in a less efficient utilization of the carbon substrate.

Goldstein (1965) envisages that addicting drugs exert their effect by enzyme inhibition which is eventually overcome by the synthesis of more enzyme. On removal of the drug, excess enzyme is therefore present and the particular reaction operates at an increased rate. This is offered as an explanation of the " withdrawal syndrome " and in the *E. coli*–barbitone system Goldstein implicates the enzyme system converting L-alanine to pyruvate and equates prolonged administration with one subculture in drug medium. In the present set the glucose dehydrogenase activity behaved in a similar way. At the end of the first subculture in the presence of drug it was high, and was even higher when this was followed by one subculture in drug-free medium. Since irregular and sometimes oscillatory patterns of behaviour, which disappear as subculture is continued, are often observed in the early stages of growth in the presence of a toxic agent (Dean & Hinshelwood, 1966), tests were also made on more thoroughly " trained " strains. After about 150 generations of growth in drug medium the activity had reached a more normal level but was still high, and was again higher in drug-free medium even after approximately 400 generations of growth had taken place in it. This indicates (if the results have any relevance to experiments with animals) that after long-continued administration of barbitone withdrawal symptoms could persist for a considerable time.

The contrast between the purely additive action observed with barbitone and ethanol, and the slight degree of synergism with barbitone and 2-phenylethanol, is of interest. The end result of the action of both alcohols when used alone is to reduce the growth rate, but this need not imply that they act in the same way. Indeed, it has been claimed that the primary site of action of 2-phenylethanol is the bacterial cell membrane (Treick & Konetzka, 1964; Remsen, Lundgren & Slepecky, 1966) and if this facilitated the entry of barbitone it could explain the observed synergism.

SUMMARY

1. The action of barbitone on *A. aerogenes* has been investigated with reference to (a) the development of resistance and (b) a recently proposed model for studying the effects of prolonged drug administration.

2. Although not a potent antibacterial agent, barbitone causes a variety of toxic effects. Some of these are annulled to varying extents by repeated subculture (" training ") in the presence of drug, but others persist. For example, the lag rapidly disappears, the growth rate improves up to a limit, the initially high glucose dehydrogenase activity drops to a more normal (although still augmented) level. The median cell size which is higher than normal on the first exposure remains high, however, and the " trained " strains are unable to ferment glucose to acetylmethylcarbinol.

3. The entire pattern of behaviour is considered to be compatible with a relatively unstable physiological adaptation by the cells to resist the drug together with damage of

a more permanent kind. From this it is concluded, if the results are relevant to the problem of drug addiction in animals, that withdrawal symptoms could persist for a very long time.

4. A synergistic action was observed with barbitone and 2-phenylethanol and a purely additive effect with barbitone and ethanol.

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