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BARBITONE INDUCED OSCILLATORY RESPONSES IN *KLEBSIELLA (AEROBACTER) AEROGENES*

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Abstract—When barbitone was added to turbidostat cultures of *Klebsiella aerogenes* in the steady state of growth in a glucose-salts medium pronounced and reproducible damped oscillations occurred in the growth rate until a new steady state was established. The mean size, the DNA, RNA and protein content and the glucokinase, glucose phosphate isomerase and glucose dehydrogenase specific activities of the cells also fluctuated while the glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities changed monotonically. The new steady state in drug medium was characterised by a reduced rate of growth, a diminished DNA content, a significantly augmented level of activity of all the enzymes studied, particularly glucose-6-phosphate and 6-phosphogluconate dehydrogenases where the increases were 2·4-fold. The intracellular drug concentration also oscillated in the transition phase, but in an inverse manner to the growth rate, a Michaelis–Menten type of relationship existing between them. When the growth rate was at its lowest, the intracellular drug concentration was close to the external level but in the final steady state about 90 per cent of the drug was excluded from the cells. The results are discussed in relation to barbitone-induced responses in animals.

RECENTLY in a study of the interaction between barbitone and growing bacterial cells, as a possible model for the mode of action of addicting drugs, we showed that this drug reduced the growth rate of *Klebsiella (Aerobacter) aerogenes* but had little effect on the lag.[1] In those experiments, which were carried out in batch culture, the cell mass of the culture did not increase in a regular exponential manner, as in drug-free medium, but proceeded by a series of small steps in which periods of growth were followed by periods of almost complete arrest. As growth continued the steps decreased in size and eventually disappeared, which suggests that the growth rate is following the course of a damped oscillation. This is examined in detail in the present paper and it is shown that the turbidostat technique of continuous culture provides an ideal method for investigating such phenomena.

Barbitone also affects glucose metabolism in general[2,3] and dehydrogenase systems in particular.[1,4–6] For this reason the behaviour of representative glucose-degrading enzymes has also been investigated. Changes in the uptake of the drug by the cells and in their macromolecular composition during the oscillatory phase are also reported.

MATERIALS AND METHODS

A laboratory strain of *Klebsiella (Aerobacter) aerogenes* (N.C.I.B. 418, *Bacterium aerogenes* No. 240) fully conditioned to a salts–glucose medium was used. The medium contained (g/l.): $FeSO_4\ 7H_2O$, $0{\cdot}2 \times 10^{-3}$; $MgSO_4\ 7H_2O$, 0·39; $(NH_4)_2SO_4$, 0·96; KH_2PO_4, 1·14; Na_2HPO_4, $12H_2O$, 6·13; glucose 4·0. Its pH was 7·1, which was

maintained throughout the experiments by an automatic pH control system incorporated in the continuous culture apparatus. The latter together with the general methods for its operation and the assay procedures for RNA, DNA and protein are described in detail elsewhere.[7] The experiments were carried out at 40° and adequate aeration at the population density used, i.e. equivalent to 0·12 mg dry wt. of cells/ml, ensured by passing 1 l./min of air through the cultures (compare ref. 7). Cell mass was determined turbidimetrically with a suitably calibrated Hilger photoelectric absorptiometer and cell number by microscope counting using a Helber counting chamber. Growth in the presence of barbitone did not affect the proportionality between turbidity and cell mass. This was established by withdrawing samples when the growth rate in barbitone medium (2000 mg/l.) was at its lowest in the oscillatory phase and when the new steady state had been achieved, separating the cells by centrifugation, washing them twice with saline (0·85 g/l.) and resuspending in saline to give suspensions of equal turbidity. The dry weights of the cells in these suspensions were 10·0 and 11·5 mg respectively compared to 11·4 mg for the control; values which fall within the limits observed earlier in duplicate experiments in drug-free medium.[7]

The glucose dehydrogenase assay is described in detail elsewhere.[8] Standard methods involving the reduction of NADP in the conditions specified by Bergmeyer[9] were used for glucokinase, glucose phosphate isomerase and glucose-6-phosphate and 6-phosphogluconate dehydrogenases. The cells were separated from the growth medium, washed twice in Tris-HCl buffer (0·05 M pH 7·65) containing 0·01 M $MgCl_2$, resuspended in the same medium and disrupted in a Mullard–MSE ultrasonic disintegrator for 6 min at 0°, the cell debris then being removed by centrifugation and the supernatants assayed.

Intracellular barbitone was extracted with chloroform (4 × 10 ml) from cells which had been ultrasonicated in saline for 8 min at 0°, after separation from the growth medium by centrifugation and washing with saline. The drug was removed from the combined chloroform extracts with 5 ml 0·1 N KOH and the optical density of the latter determined at 245 mμ against a blank prepared by treating 5 ml 0·1 N KOH with 40 ml chloroform. This was repeated after adding 0·1 ml H_2SO_4 (sp. gr. 1·84), which removes the absorption peak at 245 mμ[10,11] and the barbitone concentration equivalent to the difference between the optical density in alkaline and acid solution obtained from a calibration curve. A linear relation exists between these variables at least in the range 0–20 mg/l. This analytical method was also used to measure the total uptake of drug by resting bacteria. Cells from a drug-free glucose turbidostat culture were separated by centrifugation, washed and re-suspended in phosphate buffer (pH 7·1) containing barbitone (20 mg/l.) and graded amounts of glucose. The uptake was complete in 45 min. The residual drug concentration in the suspending medium was then determined after removing the cells by filtration using a Millipore membrane (No. GSWP047SO).

RESULTS

Oscillations in growth rate in the turbidostat

In the turbidostat technique of continuous culture the aim is to keep the cell mass in the culture vessel constant by continuously adjusting the inflow of fresh medium to counteract any changes in the growth rate of the culture. All the ingredients are

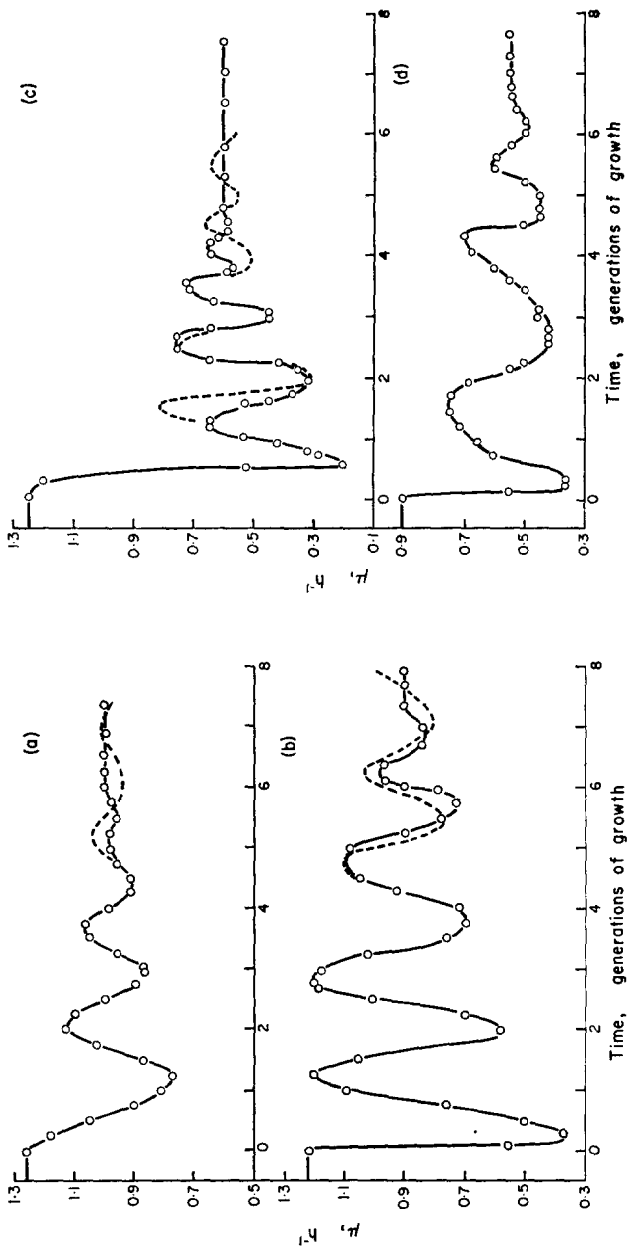

FIG. 1. Effect of barbitone on the specific growth rate (μ). In (a), (b) and (c) drug was added at concentrations of 500, 1000 and 2000 mg/l. respectively to cultures in the steady state of growth in drug-free medium while in (d) the drug concentration was raised from 1000 to 2000 mg/l. at zero time. ○—○ experimental, ----- calculated from a damped sinusoidal motion.
[ord] μ (h^{-1}).
[abs] Time (generations of growth).

present in excess of requirement and growth can proceed at the optimum rate possible in the particular conditions. In the steady state of growth, the flow rate of fresh medium into the culture is constant. Figure 1a, b, c shows the effect of adding barbitone (sodium salt) to cultures already in the steady state of growth in drug-free medium to give final concentrations of 500,1000 and 2000 mg/l. respectively of the free acid. Concomitantly with the addition of drug to the culture vessel the medium feed was changed from drug-free medium to medium containing the requisite concentration of barbitone. This technique ensures that the required drug level is immediately achieved and maintained.

Various features are common at all three concentrations. First, the behaviour is transient, new steady states being obtained after some five to seven generations of growth. A further sixty generations of growth did not lead to any improvement in growth rate beyond the final levels shown in Fig. 1, and these were all lower than in drug-free medium; the higher the drug concentration the lower the steady state growth rate. Second, the transient response comprises a very rapid fall in growth rate on adding the drug, the higher the concentration the greater the drop, followed by a phase of damped oscillations in the growth rate. Third, the transient behaviour is reproducible. This is shown in Table 1, which gives as a typical example the relevant data obtained in replicate experiments using 2000 mg/l. of barbitone.

TABLE 1. REPRODUCIBILITY OF GROWTH RATE OSCILLATIONS IN BARBITONE MEDIUM (2000 mg/l.)

		1st minimum		2nd minimum		3rd minimum		
Expt. no.	μ_0 (h^{-1})	position (gens)	μ (h^{-1})	position (gens)	μ (h^{-1})	position (gens)	μ (h^{-1})	μ_s (h^{-1})
1	1·25	0·55	0·20	1·85	0·31	3·00	0·45	0·60
2	1·25	0·50	0·19	1·70	0·32	2·75	0·40	0·51
3	1·27	0·60	0·20	1·65	0·32	2·20	0·39	0·47
4	1·20	0·45	0·17	1·50	0·28	2·50	0·36	0·48

μ_0 and μ_s are the steady state growth rates in drug-free medium and in medium containing 2000 mg/l. of barbitone respectively.

Increasing the drug concentration in a culture in the steady state of growth in drug medium again led to transient oscillations. Figure 1d shows the effect of suddenly raising the concentration from 1000 to 2000 mg/l. The oscillations, however, were less numerous and less regular than when sensitive cells were exposed to 1000 mg/l. or indeed to 2000 mg/l.

Analysis of the oscillatory behaviour

From the general equation for a damped oscillation, i.e.

$$\frac{d^2\mu_s}{dt^2} + 2\lambda\frac{d\mu_s}{dt} + \omega_0{}^2\mu_s = 0$$

the relation

$$\mu_s = D\exp(-ET)\sin\{(Ft + G)\pi\} \qquad (1)$$

is readily obtained. D is an amplitude constant, E is the logarithmic decrement of the oscillations and replaces λ, F is 2/ the period of oscillation and is equal to $1/\pi$

$\sqrt{(\omega_0^2 - \lambda^2)}$, G is a phase angle constant and t is the time (in generations of growth) after adding barbitone. The initial drop in growth rate is not considered to be part of the oscillation. Equation (1) gives the growth rate (μ_s) during the oscillations but assumes that they are symmetrically disposed around a horizontal base line. Inspection of Fig. 1 shows, however, that in the early stages of growth, rather than being symmetrical around the horizontal line drawn through the final (steady state) level of the growth rate, the oscillations centre around a line which approaches it in an asymptotic manner. This effect is not very marked at 500 mg/l. and can be ignored; it is greater at 1000 mg/l. and still greater at 2000 mg/l. It can be allowed for by introducing an exponential term $\mu_B = A - B \exp(-Ct)$ into equation (1), which thus becomes

$$\mu = \mu_B + \mu_s = A - B \exp(-Ct) + D \exp(-Et) \sin\{(Ft + G)\pi\} \qquad (2)$$

where μ is the overall growth rate during the oscillations.

In fitting this equation to the experimental curves, the constants were evaluated as follows. As growth proceeds, $\mu \rightarrow A$, i.e. A is the final (steady state) growth rate in the presence of barbitone. F is twice the reciprocal of the period of oscillation, the latter being obtained by averaging the time between successive minima. At the first minimum, $\sin\{(FT + G)\pi\} = -1$, therefore $G = 1{\cdot}5 - Ft$. (Since the initial drop in growth rate is not considered as part of the sinusoidal curve, G is not necessarily equal to 0·5.) Curves were then drawn through the centre and the minima of the oscillations, and, as shown diagrammatically in Fig. 2a, B_1, B_2, B_3 ... and D_1, D_2, D_3 ... measured at times t_1, t_2, t_3 ... Plots of the logarithms of the B and D values against time are linear. Extrapolation of these lines to $t = 0$ gives B and D, while $-C$ and $-E$ are obtained from their slopes.

The calculated and experimental curves are compared in Fig. 1. At barbitone concentrations of 500 and 1000 mg/l. the agreement is good except in the later stages where the experimental oscillations damped out more quickly than predicted. This also occurred at 2000 mg/l., but, in addition, the first experimental oscillation was too small relative to the others, so that only partial correspondence with a damped sinusoidal motion could be obtained.

Cell size and macromolecular composition

These studies and those to be described in subsequent sections were confined to cells grown in 2000 mg/l. of drug, since any changes would be expected to be greater than at lower drug levels. Like the growth rate, the mean cell size (σ) also oscillated, but in a less regular manner (Fig. 2b). The largest value of σ, indicating the maximum inhibition of division, coincided approximately with the maximum inhibition of growth, which occurred soon after adding the drug (Fig. 1c) but, thereafter, the oscillations in the two properties proceeded out of phase with one another. Other drugs have been observed to act differentially on growth and division in bacteria.[7]

Table 2 shows the macromolecular composition of cells collected over a time interval corresponding to about 0·2 generations of growth at the following stages: (1) the steady state of growth prior to the addition of the drug; (2) the end of the first period of slow growth; (3) the end of the succeeding period of faster growth (i.e. the peak of the first oscillation in growth rate); (4) the end of the next period of slow growth and (5) when the new steady state of growth in drug medium had been established. These samples should indicate any major changes occurring.

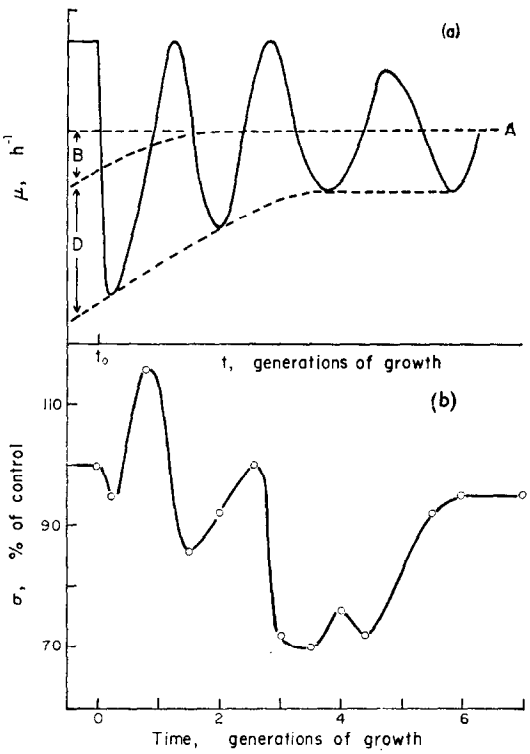

FIG. 2. (a) Diagrammatic representation of graphical method used to evaluate the constants in the theoretical equation describing the oscillations. (b) Fluctuations in the mean cell size (σ) during the early stages of growth in barbitone medium (2000 mg/l.).

[ord] (a) μ (h^{-1}).
(b) σ (per cent of control).
[abs] (a) t (generations of growth).
(b) Time (generations of growth).

TABLE 2. MACROMOLECULAR COMPOSITION OF CELLS GROWN IN BARBITONE MEDIUM (2000 mg/l.)

Sample No.	μ (h^{-1})	DNA/mass			RNA/mass			Protein/mass		
		T	C	T-C	T	C	T-C	T	C	T-C
1	1·25	100	100	0	100	100	0	100	100	0
2	0·19	92	172	−80	63	49	+14	90	120	−30
3	0·53	85	145	−60	60	76	−16	97	93	+ 4
4	0·32	77	161	−84	47	59	−12	87	105	−18
5	0·51	100	146	−46	67	74	− 7	101	94	+ 7

Samples were collected at the following stages: 1, prior to adding the drug; 2, the end of the first period of slow growth; 3, the end of the succeeding period of faster growth; 4, the end of the next period of slow growth and 5, when the new steady state in drug medium was established. T, barbitone turbidostat values; C, glucose-limited chemostat values; T-C, drug specific effects. DNA, RNA and protein per unit of bacterial mass are expressed in arbitrary units, the levels in the control being taken as 100.

Changing the growth rate of a bacterial culture also changes the macromolecular composition of the cells.[7] Thus when inhibitory substances are added, any differences observed may be simply the result of growth rate change rather than specific drug effects. In studies with other drugs, Dean and Rogers[7] overcame this difficulty by comparing the observed macromolecular composition with that of cells growing at the same rate in glucose-limited chemostats and ascribing any difference to drug-specific effects. The justification of the procedure is given in their paper and applying it to the present experiments led to the drug-specific effects recorded in columns 5, 8 and 11 of Table 2. These data show that during the oscillatory phase DNA/mass was most affected, a marked reduction occurring during the first period of slow growth. Some recovery took place in the succeeding phase of faster growth, but the level fell again as the growth rate dropped once more during the first oscillation. RNA/mass varied less, increasing slightly in the first period of slow growth but, thereafter, remaining lower than in the control. Protein/mass oscillated slightly, being depressed when the growth rate was low and practically normal when it was high. In the new steady state of growth in drug medium, protein and RNA per unit of mass settled at almost normal levels but DNA/mass, although some recovery had taken place, was significantly lower than in the control.

Enzyme activities in turbidostat cultures

Glucokinase (GK), glucosephosphate isomerase (GPI), glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) specific activities were determined in cells collected at the five stages of growth listed in the preceding section. These enzymes participate in the initial stages of the metabolism of glucose in alternate pathways, viz. Embden–Meyerhof pathway (GK, GPI) and pentose phosphate pathway (G6PD, 6PGD) and are readily assayed in disrupted cell preparations by the rate of reduction of NADP in the appropriate conditions. *K. aerogenes* also reduces tetrazolium salts in the presence of glucose, which suggests a direct oxidation of the substrate without prior phosphorylation. Attempts to determine this glucose dehydrogenase (GD) activity in disrupted cell preparations proved abortive (compare ref. 8). Consequently intact cells were used, and it is possible that the assay determines the overall activity of a series of reactions rather than that of a single enzyme. The results are summarized in Table 3 and on the basis of four replicate experiments are considered accurate to within $\pm$ 5 per cent (coefficient of variation calculated from the standard error of the means $<$ 5 per cent).

Table 3 shows that in the new steady state in the presence of 2000 mg/l. of barbitone the activity of all the enzymes was considerably augmented, 2·4-fold increases occurring in the G6PD and 6PGD activities and 1·4 to 1·5-fold increases in the others. These final levels remained unchanged throughout a further 60 generations of growth in drug medium. Marked differences in behaviour occurred, however, during the transition period between steady states. The G6PD and 6PGD activities increased in a monotonic manner irrespective of whether the growth rate was high or low. The GD activity, although also augmented at all stages, overshot the new steady state level somewhat, passing through a maximum value at the end of the first period of faster growth: the PGI activity oscillated, while the GK activity passed through a minimum value. This minimum coincided with the maximum in GD.

TABLE 3. ENZYME ACTIVITIES OF CELLS GROWN IN BARBITONE MEDIUM (2000 mg/l.)

Sample no.	Specific activity				
	G6PD	6PGD	GK	GPI	GD
1	62·5	84·0	26·0	140·0	36·0
2	86·0	103·0	23·0	129·0	62·0
3	121·0	145·0	17·0	207·0	74·0
4	130·0	203·0	32·0	144·0	56·0
5	153·0	201·0	38·0	187·0	50·0

G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GK, glucokinase; GPI, glucose phosphate isomerase; GD, glucose dehydrogenase. The specific activity of GD is expressed as mμmoles TTC reduced/min/mg protein and that of all the others is mμmoles NADP reduced/min/mg protein. Samples 1–5 were collected at the stages listed in Table 2.

Adding barbitone to the reaction mixture during the assay of the various enzymes led to stepwise reaction curves when cells which had never been exposed to the drug were used. Pre-incubating the cell preparation with drug for 10 min virtually abolished this effect. Nor did it occur when cells which had received 35 daily subcultures in barbitone medium (2000 mg/l.) or when a commercial sample of yeast glucose-6-phosphate dehydrogenase was used, although the rate of the reaction was slowed.

Uptake of barbitone by K. aerogenes

During the oscillations the intracellular barbitone concentration varied with the growth rate, being high when the growth rate was low and vice versa. Figure 3a shows that the results fit an equation of the form $\mu = \mu_0 K_B/K_B + [B]$ where μ and μ_0 are the specific growth rates in the presence and absence of barbitone respectively, $[B]$ the intracellular drug concentration and K_B the value of $[B]$ when $\mu = \mu_0/2$. A similar relationship applies to the inhibitory action of propionate on *Propionobacter shermanii*.[12]

In these experiments intracellular barbitone is defined as the drug which is not removed from the cells by washing with saline but is set free when the cell wall is disrupted by ultrasonication. The effect of glucose on the total uptake of barbitone by resting cells obtained from a drug-free glucose turbidostat and suspended in phosphate buffer (pH 7·1) has also been determined. This includes the drug removable by washing and Fig. 3b shows that in the range 0–4 g/l., increasing the glucose concentration reduced the uptake; higher levels had no further effect.

In the steady state of growth in barbitone medium (2000 mg/l.) the intracellular drug concentration is 0·5 μg/mg dry wt. of cells (Fig. 3a) and the mean cell length is 5μ. *K. aerogenes* is rod-shaped and assuming a cylindrical cell of 1μ diameter, which is a reasonable estimate, and using the finding that 10^{10} cells have a dry weight of 12·2 mg, a simple calculation gives the intracellular drug concentration as equivalent to 150 mg/l. When the growth rate is at its lowest in the oscillatory phase, the intracellular concentration is some 10 times higher.

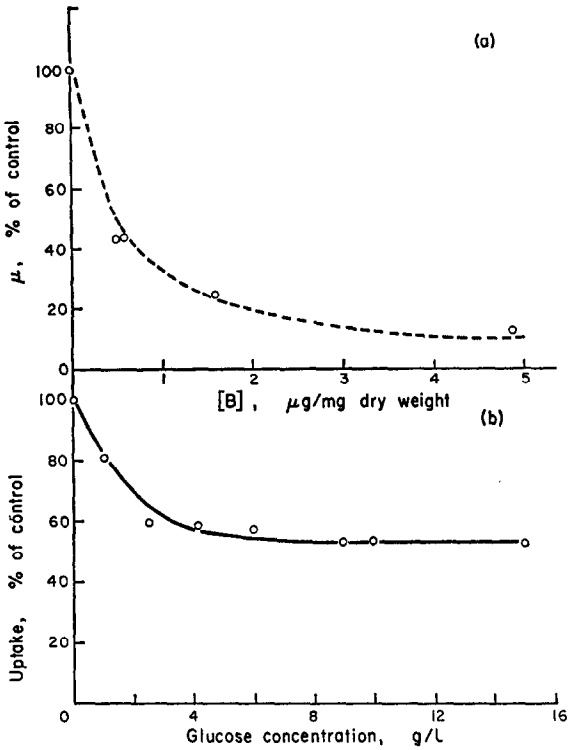

FIG. 3. (a) Intracellular barbitone concentration during the oscillatory phase of growth in barbitone medium (2000 mg/l.), ○ experimental, ---- calculated from $\mu = \mu_0 K_B/K_B + [B]$. (b) Effect of glucose on the uptake of barbitone by resting cells.

[ord] (a) μ (per cent of control).
(b) Uptake (per cent of control).
[abs] (a) [B] (μg/mg dry weight).
(b) Glucose concentration (g/l.).

DISCUSSION

These results clearly show that disturbing the steady state of growth of *Klebsiella aerogenes* by adding barbitone to the growth medium leads to damped oscillations in the growth rate and to a lesser extent in other cellular properties. They are pertinent to continuous culture studies since they provide a good experimental example of an oscillatory response and their reproducibility demonstrates the usefulness of the turbidostat technique for detecting such behaviour. Theoretical considerations based on models of varying degrees of complexity predict the possibility of damped oscillations[13–16] and, indeed, when the kinetic constants and substrate concentrations satisfy certain conditions, these can also occur in systems involving only two enzyme–substrate complexes.[17] These aspects are discussed in general terms elsewhere[18] and need not be further elaborated here. Rather an attempt will be made to relate our results to those obtained in animals, where the major interest in the action of barbiturates lies.

Higgins[19] maintains that oscillatory phenomena are probably more widespread than is commonly realised; the problem lies in their detection. This is obviously easier in

our conditions than in animal experiments. Our technique ensures that the cells are subjected to constant level of drug throughout, which should prolong any effects. The continuous outflow of culture from the apparatus facilitates the collection of samples. Growth rates are continually monitored, and detecting oscillations in this parameter stimulates the search for them in less readily apparent effects of the drug which, of necessity, provide the only evidence in animal experiments. It is, therefore, of particular interest that Hollman and Neubar[20] observed damped oscillations, which persisted for 30 hr, in the liver uridine diphosphoglucose dehydrogenase activity following the administration of barbitone to rats. This appears to be the only example on record but, nevertheless, although oscillatory behaviour was presumably not looked for, other similarities in response are in evidence. Thus barbiturates are commonly considered to be nonspecific inducers of oxidative drug metabolism in general[21–23] and this has been claimed for barbitone, although it is only about 5–10 per cent oxidised.[22] In particular, Platt and Cockrill[6] found increased levels of G6PD and 6PGD activity in the liver of rats sacrificed after receiving barbitone for 7 days. They suggest that augmented levels of G6PD could increase oxidative drug metabolism since this often involves the $NADPH_2$ electron transport chain and G6PD is a good source of cellular $NADPH_2$. Cytochrome p-450 has also been implicated.[24]

We also observed increased G6PD and 6PGD activities, and these increases started as soon as the drug was added and continued in a monotonic manner until the new steady state levels, some 2·4 times the initial level, were reached. The activities of the other enzymes proceeded, however, via an overshoot or an underswing (which can be considered as one large oscillation) or a series of oscillations, to the new and again elevated steady state levels. These final levels of activity are true steady state levels, and could be maintained indefinitely, or at least until the termination of the experiments some 60 generations of growth after the steady state had been reached, provided the conditions remained unchanged. Barbitone was not present in the reaction mixture during these enzyme assays, and the simplest explanation is to regard the augmented activities as measuring the enzyme expansion which has taken place in the attempt to overcome the inhibition of the drug (compare refs. 1, 25). Moreover, in our turbidostat technique growth proceeds at the maximum rate possible in the given circumstances, and any effects not directed towards this end would be expected to be eliminated. Nevertheless, it would be of interest to determine whether barbitone-resistant bacteria oxidise other drugs more rapidly, but utilising $NADPH_2$ or other metabolites for this purpose would reduce the overall efficiency of the cellular metabolism, unless further enzyme expansion occurred changing the enzyme balance of the cells once more.

Fluctuations also occurred in the mean size, and in the protein, RNA and DNA content of the cells, but with the exception of DNA these returned to more or less normal levels once the transitional phase was over. The persistent low level of DNA per unit of bacterial mass might be a consequence of an impaired thymidine biosynthesis (compare ref. 26) or of the strong and selective hydrogen bonding which is thought to occur between barbitone and adenine-containing substances such as co-enzymes or ATP.[27] This latter mechanism would be expected to affect RNA and protein synthesis also, but these recover on continued growth in drug medium, while DNA synthesis does not. The structural similarity between barbitone and uridine and thymine makes intercalation in the DNA also possible, in principle, and intercalating drugs such as proflavine, ethidium and mitomycin D profoundly affect DNA synthesis.[28]

These drugs, however, are much more bactericidal than barbitone, which in our conditions was not lethal to any of the cells.

The Michaelis–Menten type of relationship which exists between the growth rate and the intracellular barbitone concentration is formally similar to a Langmuir adsorption isotherm. Nothing definite can be said about the nature of the receptors, although the correlation between oil solubility and the action of barbiturates in animals implicates lipid regions (on enzymes or elsewhere in the cell) as the major adsorption sites.[29] By analogy with other examples, intercalation, if it occurs, could account for only a small proportion of the drug taken up[28] and this is probably also true for any hydrogen bonding to adenine. Adding 4 g/l. of glucose, which is the concentration in the growth medium, reduced the uptake of barbitone by 50 per cent in resting cells, and in growing cells the only enzyme whose activity varied approximately in phase with the growth rate (or the reciprocal of the intracellular drug concentration) was glucosephosphate isomerase. Nor have we any positive evidence at present about the mechanisms controlling the intracellular barbitone concentration. We have shown that when the drug was exerting its maximum effect (i.e. when the growth rate was at its lowest in the oscillatory phase) the intracellular barbitone concentration is close to the external level, and yet about half a generation of growth later it had dropped about ten-fold. Increases and decreases of smaller magnitude then ensued until steady conditions prevailed. Permeability changes could account for a reduced entry of drug in these circumstances, but cannot explain the release of drug already inside. Rather, an active process, as has recently been suggested in the example of proflavine and *E. coli* appears more probable.[30] In this system the production of certain metabolites during the growth of the cells releases drug passively bound by resting cells, and in our experiments the changes were greatest when the growth rate, the enzyme proportions and consequently the variety and the concentration of the various metabolites were changing rapidly. In the steady state of growth some 90 per cent of the barbitone available is excluded from the cells and no significant change in the concentration in the medium could be detected, which argues against any appreciable destruction of drug. Similarly in animals, tolerance to barbitone is not thought to be due to increased breakdown.[22] Rather adaptation of the central nervous system,[31] possibly involving enzyme expansion,[25,33] has been suggested, but it is not known if the drug is also largely excluded, although in non-tolerant dogs the brain is freely permeable to the barbitone in the blood.[34]

The work of Jowett and Quastel[35] and McIlwain[36] shows further similarities in response. The former authors showed that the inhibition of brain tissue respiration by luminal is augmented at low K^+ concentrations, while McIlwain observed an effect of general depressants on the movement of cations in cerebral tissue. We found that barbitone inhibited *K. aerogenes* to a greater extent in K^+-limited conditions than in carbon-limitation (unpublished observations). This merits further investigation particularly since in isolated mitochondria the antibiotic valinomycin induced oscillations in the K^+ content,[37] and potassium ions, besides acting as enzyme activators, influence macromolecular synthesis.[7]

These results also reinforce the conclusion drawn from our earlier batch culture experiments,[1] which is also implicit in the work of Goldstein with *E. coli* and barbitone,[25] that tolerance involves a response by the majority of the cells in the population rather than the selection of mutants present in low frequency. The kinetic analysis of systems

of interdependent variables predicts the kind of response observed in the various parameters investigated during the transition phase. Moreover, selection would necessitate the overgrowth of faster growing cells at stages of the oscillatory phase and in conditions in which marked selection occurs the response is quite different. For example, resistance to streptomycin (1 mg/l.) in *K. aerogenes*[38] involves the selection of pre-existing mutants present at a frequency of about 10^{-8}, and when it was added to our turbidostat, operation had to be stopped for 12 hr. A simple calculation showed that all this time is necessary for a mutant population, present at that frequency, to attain a level detectable in our turbidimeter. Furthermore, when turbidostat operation re-commenced, the growth rate of the culture increased to its maximum without oscillation as the mutant population replaced the quiescent non-mutants. No lag occurred in any of the present barbitone experiments.

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