INHIBITION OF GROWTH OF AEROBACTER AEROGENES: THE MODE OF ACTION OF PHENOLS, ALCOHOLS, ACETONE, AND ETHYL ACETATE

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Earlier work on the antibacterial action of phenols, alcohols, and ketones was chiefly concerned with studying the variation in toxicity from one member of a homologous series to the next. Thus Tilley and Schaffer (1926, 1928), in an extensive series of investigations, showed that for any given homologous series each member has a molecular phenol coefficient approximately three times greater than that of its predecessor. This relationship, however, throws no light upon the mode of action of the drug since, as Ferguson (1939) and others have shown, it has its origin in the regular variation in physical properties from one member to the next. There has been much discussion (for a summary see Höber, 1945) concerning the mode of action on living cells of molecules like ethanol, containing polar and nonpolar portions, which produces narcosis in higher organisms. Both the adsorption theory of Traube and the lipoid theory of Meyer and Overton, however, offer satisfactory explanations of homologous series relationships since both are concerned with the phase distribution of the drugs, which is governed by physical factors, but neither elucidates the mode of action of particular drug molecules.

For gram-positive organisms Gale and Taylor (1947) have shown that phenol owes its antibacterial action to its ability to lyse the cell wall and allow the escape of stored amino acids, but in the case of gram-negative bacteria, which do not concentrate metabolites in this manner, the mechanism is less clearly understood. Fogg and Lodge (1945) studied the effect of a series of substituted phenols on the growth phases of *Aerobacter aerogenes* and concluded that the mechanism of action of any particular phenol was due to its ability to denature the proteins of enzymes involved in vital metabolic processes. Furthermore Johnson and his colleagues (1947), in their studies of the inhibition of luminescence of *Photobacterium phosphoreum* by narcotics, have shown that their mode of action is probably to denature proteins of the enzyme system and that their effects can consequently be reversed by hydrostatic pressure.

The first action of the compounds we have studied on the growth of *A. aerogenes* in glucose ammonium salt medium is to inhibit cell division and give rise to a lag period, the duration of which depends upon the concentration of inhibitor. From studies of lag periods not due to drug action in this medium, various workers (Lodge and Hinshelwood, 1943; Morrison and Hinshelwood, 1949; Dagley, Dawes, and Morrison, 1949*a*,*b*) have associated lag with inability of the organism to produce or utilize metabolites necessary for cell division to commence. In view of the suggestion that the compounds may act by denaturing

enzyme proteins we have investigated the possibilities of abolishing the lag period they induce by the addition of substances known from other work (Dagley, Dawes, and Morrison, 1949*b*, 1950*b*) to produce growth responses. Bacteria show, in general, a much greater tendency to lag in simple synthetic media, under the appropriate conditions, than they do in broth media, for which Hershey (1939) has shown that, although cell division is reduced, bacterial mass increases uniformly. In simple synthetic media, however, lags may be of such a duration that increase of cell mass, as well as cell division, can scarcely be significant, and the absence of cell activity is probably associated with the absence of nutrients contained in broth but not in artificial media. Accordingly we find that in broth (Dagley and Hinshelwood, 1938), alcohols do not produce a lag period although they depress growth rate in the logarithmic phase and in a peptone medium (Barbour and Vincent, 1950); with heavy inocula, phenols similarly produce an effect only on this same phase.

EXPERIMENTAL METHODS

The organism used throughout this work was NCTC strain no. 418. Growth curves were obtained as follows: Samples of the growing culture (ca. 2 ml) were withdrawn by Pasteur pipette at various time intervals and killed by the addition of 2 drops of formalin. Bacterial populations were determined turbidimetrically using microcells in a Spekker photoelectric absorptiometer with Ilford filters (neutral H 508 and blue no. 6). The instrument was calibrated to convert turbidities into bacterial counts as obtained by hemocytometer. If details of the early stages of growth were required when the culture is not turbid, viable counts were made by plating out 0.1 ml of a suitable dilution on nutrient agar in a petri dish. Cultures were grown in pyrex boiling tubes, carefully freed from traces of impurities by boiling with nitric acid followed by glass-distilled water, and placed in a thermostat at 39 $\pm$ 0.1 C. The adverse effect of traces of heavy metals and other impurities on bacterial growth in synthetic media has been stressed by Poole and Hinshelwood (1940), Lodge and Hinshelwood (1939), and Monod (1942), and reproducibility in quantitative experiments is impossible without these special precautions.

The basal medium contained 5.4 g KH_2PO_4, 1.2 g $(NH_4)_2SO_4$, 12 g glucose, and 0.4 g $MgSO_4 \cdot 7H_2O$ per liter of glass-distilled water; the pH was adjusted to 7.1. The medium and glucose were sterilized separately and mixed on cooling. Twenty-five-ml aliquots were dispensed aseptically into sterile boiling tubes. Drugs for addition to the media were redistilled and collected at the boiling points quoted by Tilley and Schaffer (1926, 1928). Bacteria for any series of experiments were prepared by 3 serial subcultures in the basal medium, and 0.1 ml of the third culture, taken at the onset of the stationary phase, was used as inoculum. At this point in the growth cycle, lag is minimal.

Measurement of lag. For an actively dividing culture the plot of log (bacterial population) against time is linear and, if there is no lag, the growth curve will extrapolate to log (inoculum population) at zero time. If lag exists, it may be defined as the intercept on a line drawn through log (inoculum population) paral-

lel to the time axis. It is still convenient to use this definition even in cases when the transition from lag to logarithmic phase is not sharp (Hinshelwood, 1946). As Monod (1949) has pointed out, it is more satisfactory to use this parameter in growth phase studies than to attempt to separate the lag phase from the phase of acceleration at the commencement of logarithmic growth. Growth curves obtained by viable counts, however, have shown that in our experiments the phase of acceleration is, in any case, of negligible duration compared with the lag phase proper. To ensure reproducibility of results whenever the effects of a series of factors affecting growth are compared, media are invariably inoculated from the same culture at the same time and with the same pipette, and controls

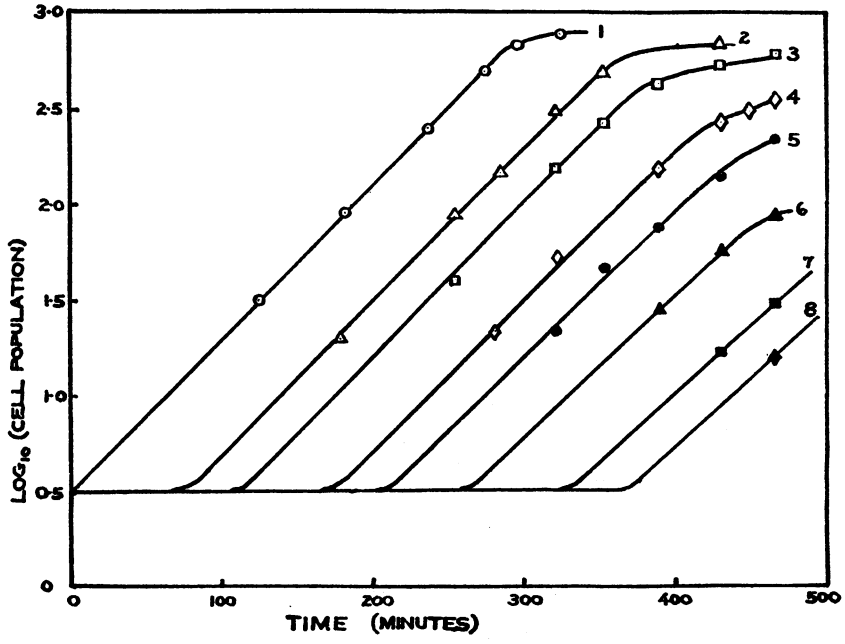

Figure 1. Growth curves for media containing increasing amounts of phenol in a glucose ammonium salt medium. Phenol concentration (M × 10^3): (1) no phenol; (2) 1.7; (3) 3.4; (4) 5.0; (5) 5.8; (6) 6.6; (7) 7.5; (8) 8.3.

are run at least in duplicate. Agreement is not considered satisfactory if the times for the controls to reach a certain turbidity do not agree within 5 minutes.

RESULTS

Lag produced by phenol. The tubes were inoculated with 0.1 ml of a minimum lag culture. Figure 1 shows full growth curves obtained with increasing phenol concentration. It will be seen that there is a profound effect on lag; growth rate and stationary phase are also affected (Fogg and Lodge, 1945), but not to the same extent. Figure 2 shows that it is a true lag period during which most of the cells are viable.

If it is assumed that phenol produces a lag by inhibiting the production of essential metabolites, it is clear that its effect should be reversed by the addition of filtrates of cultures which have grown normally, provided that all such intermediates have not been consumed during active growth. We have previously reported (Dagley, Dawes, and Morrison, 1950*a*) that with the conditions of growth employed in these studies, substances that the bacteria have synthesized from simpler molecules remain unconsumed and capable of initiating growth responses when added to simple synthetic media. Accordingly four tubes were

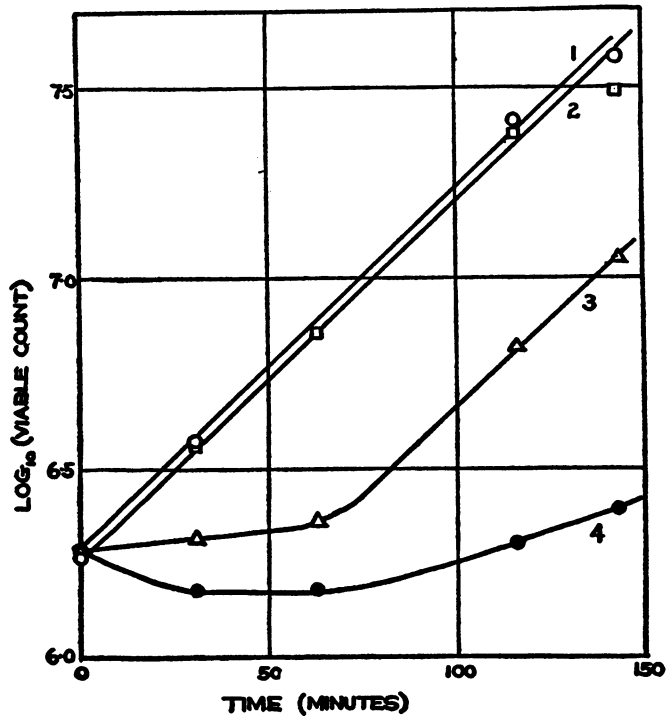

Figure 2. Growth curves in glucose ammonium salt medium from plate counts: abolition of lag by filtrate from fully grown culture. (1) Control, no phenol present; (2) 4.15×10^{-3} M phenol present + filtrate; (3) 4.15×10^{-3} M phenol without filtrate addition; (4) 8.3×10^{-3} M phenol present.

prepared containing the following media: (1) control, 25 ml basal medium; (2) 25 ml basal medium containing 4.15×10^{-3} M phenol; (3) 12.5 ml basal medium containing 4.15×10^{-3} M phenol + 12.5 ml filtrate; (4) 25 ml basal medium containing 8.3×10^{-3} M phenol. They were inoculated with 0.1 ml of a minimum lag culture, and growth was followed by plate counts. The results are shown in figure 2, where it is seen that the filtrate addition practically abolishes the phenol inhibition.

In seeking to elucidate the effective factors present in the filtrate we have been guided by the observation that, under our conditions of growth, amino acids

have been shown to be present in filtrates from fully grown cultures (Dagley, Dawes, and Morrison, 1950*a*). Stephens and Hinshelwood (1949) have claimed that all common amino acids play a part in maintenance of the maximum growth rate of *A. aerogenes*. If a culture in its stationary phase in the synthetic medium used is allowed to "age" at 39 C for several days, the cells that are viable show

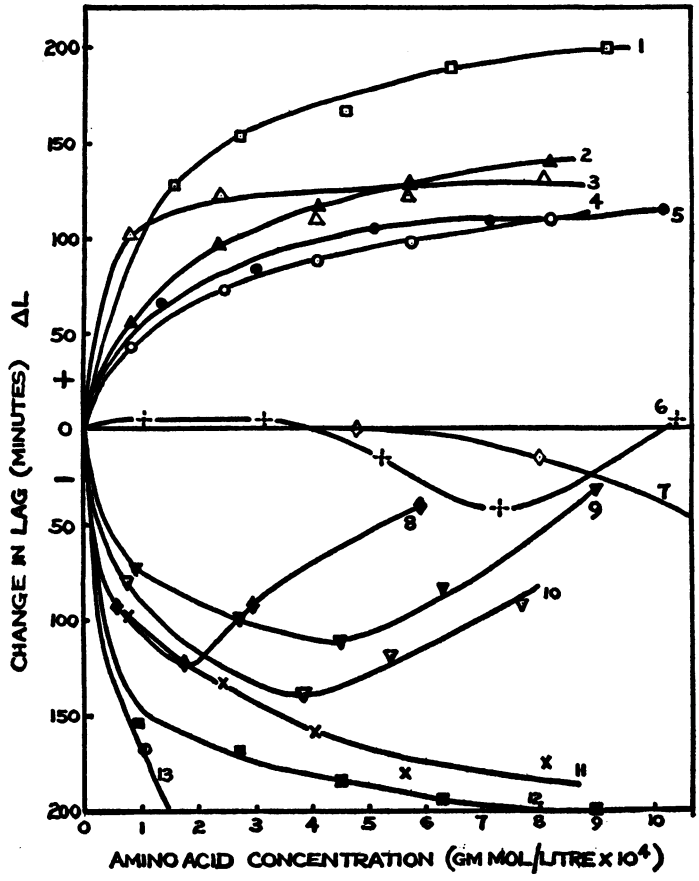

Figure 3. Effect on phenol-induced lag of various compounds added to glucose ammonium salt medium. Positive values of ΔL indicate that compound removes lag; negative values indicate lag increase. Phenol concentration 8.3×10^{-3} M in each case. Amino acids: (1) L-leucine; (3) DL-methionine; (4) L-glutamic acid; (6) L-proline; (7) glycine; (8) DL-tryptophan; (10) L-histidine; (11) DL-serine; (12) DL-aspartic acid. Other compounds: (2) α-ketoglutaric acid; (5) succinic acid; (9) malic acid; (13) fumaric acid.

lag (termed by Lodge and Hinshelwood, 1943, "late lag") when inoculated into fresh medium; and we have found that all the amino acids used in the present work partially abolish this lag with the exception of serine, which increases it somewhat. We have, therefore, studied the effect on lag produced by phenol of small additions of various amino acids to the simple medium. All the results shown in figure 3 were obtained by following the growth of cultures inoculated

by 0.1 ml additions of the same parent, in its minimum lag phase, the cultures containing 8.3×10^{-3} M phenol. Succinate and α-ketoglutarate were included in the series since previous work (Dagley, Dawes, and Morrison, 1949*b*, 1950*b*) has shown them capable of producing growth responses similar to those produced by their related amino acids. It is seen that the amino acids fall into two groups, those which partially abolish lag, on the one hand, and, on the other, those which increase it or have little effect. Succinate and α-ketoglutarate partially abolish

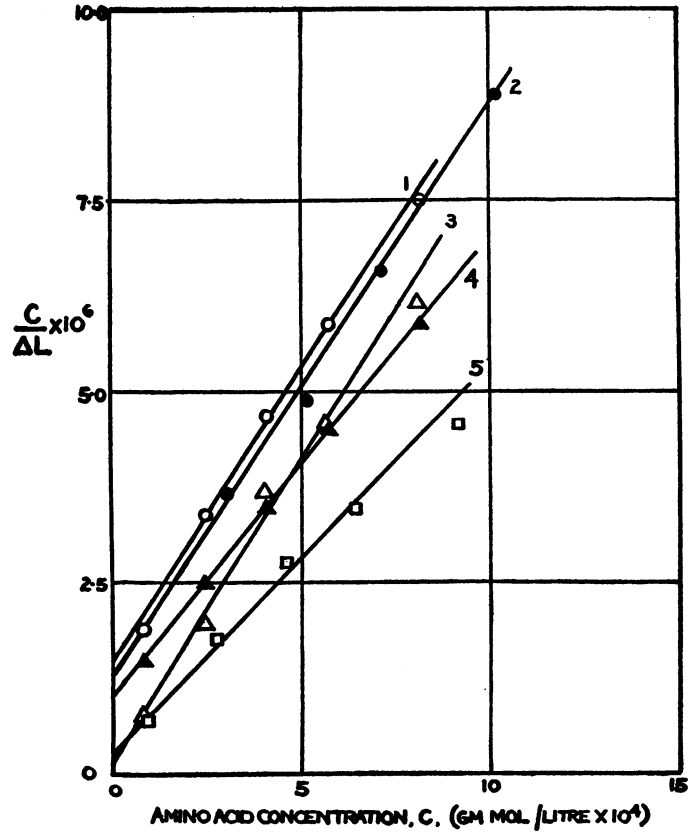

Figure 4. Test of hyperbolic nature of some of the curves from figure 3: the plot of C per ΔL against C is linear if the plot of ΔL against C is hyperbolic. (1) L-glutamic acid; (2) succinic acid; (3) DL-methionine; (4) α-ketoglutaric acid; (5) L-leucine.

lag, but fumarate and malate do not; this rather surprising difference in response was observed in a previous study of the early growth phases of the same organism (Dagley, Dawes, and Morrison, 1949*b*, 1950*b*).

Monod (1949) has shown that the relationship between the growth rate of bacteria and the concentration of an essential nutrient can often be expressed by the equation for a rectangular hyperbola similar to an adsorption isotherm or to the Michaelis equation. In order to ascertain whether a similar relationship exists between amino acid concentration and the growth response produced we

have replotted some of the data of figure 3 in a manner similar to that adopted by Lineweaver and Burk (1934) in their studies of enzyme kinetics. In figure 4 the closeness with which the plot of $C/\Delta L$ (i.e., concentration in gram molecules per liter per change in lag) against C approximates to a straight line may be taken as a criterion of the hyperbolic relationship between growth response and concentration of amino acid. It is clear that for several of the inhibitory amino acids the curves in figure 3 do not express any such simple relationship and the action of amino acids in this category is evidently complex.

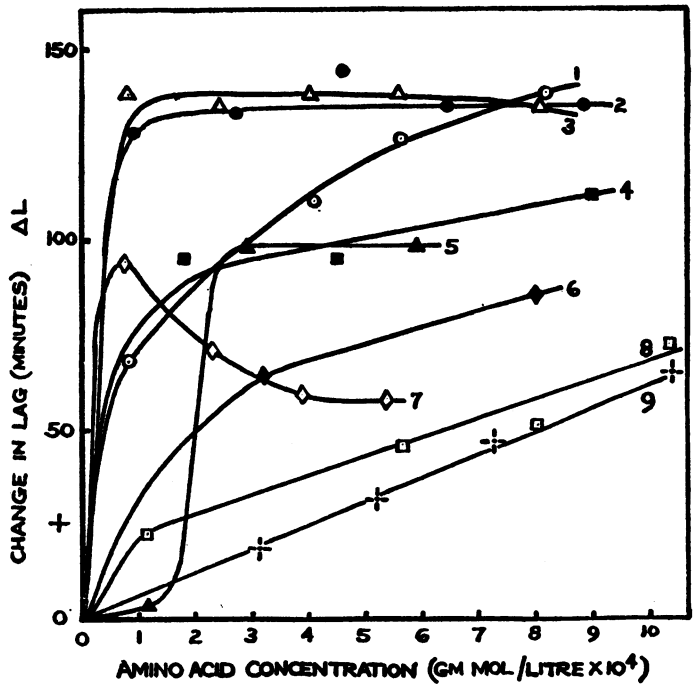

Figure 5. Effect on acetone-induced lag of amino acids added to glucose ammonium salt medium. Acetone concentration 0.53 M in each case. (1) L-glutamic acid; (2) L-leucine; (3) DL-methionine; (4) DL-aspartic acid; (5) DL-tryptophan; (6) glycine; (7) L-histidine; (8) DL-serine; (9) L-proline.

Lag produced by acetone. Preliminary investigations of the effect of amino acids on lag produced by other drugs which might be assumed to be similar to phenol in their action on enzyme proteins showed that whereas some gave results similar to those for phenol, others, notably ketones, did not.

Figure 5 shows the effect of additions of amino acids on the lag produced by the presence of 0.53 M acetone to the culture, and it is clear that no amino acids increase the inhibition in this case.

Curves for succinate, fumarate, malate, and α-ketoglutarate were also obtained but have been omitted for clarity; none of them increased the lag caused by the presence of acetone.

Lag produced by a homologous series of alcohols. Extending our studies to lag produced by alcohols we have endeavored to ascertain whether only specific amino acids would remove lag, as in figure 3, or whether a response could be produced by all of them, as in figure 5. To construct complete sets of curves for each alcohol of the series would obviously be extremely laborious in view of the necessity of using cells of the same culture for inocula in all cases, and accordingly we proceeded as follows: To a series of 12 tubes sufficient ethanol was added to produce a lag of about 1 to 2 hours (0.47 M). To 10 of the tubes amino acids were added singly at 0.06 g per liter, but no additions were made to the other two which, therefore, served as controls. Similarly, for each of the other four alcohols 12 tubes were set up, the alcohol concentration in each case being sufficient to produce a lag of 1 to 2 hours. To the whole set of 60 tubes additions of 0.1 ml of

TABLE 1

Ability of amino acids to abolish lag produced by alcohols

The figures give the percentage of lag removed, + values indicating lag removal and − values lag increase

	ETHANOL	n-PROPANOL	n-BUTANOL	n-PENTANOL	n-HEXANOL
Alcohol concentration (molarity)	0.41	0.12	4.3×10^{-2}	1.2×10^{-2}	Sat. soln. approx. 1.6×10^{-2}
Lag (min)	130	156	107	87	77
Amino acid					
DL-Methionine	+31	+52	+39	+36	+40
L-Leucine	+57	+48	+24	+55	+36
L-Glutamic	+42	+62	+44	+37	+64
L-Histidine	+21	+45	+40	+54	+50
DL-Tryptophan	+18	−18	+34	+48	+16
L-Proline	−59	+43	+36	+39	+18
Glycine	−49	+38	+24	+39	+36
DL-Alanine	−34	−42	+33	+16	+23
DL-Serine	−15	−4	+33	+55	+39
DL-Aspartic	−83	−61	+12	+48	+47

the same minimum lag culture were made. To serve as controls two tubes containing no alcohol were inoculated at the same time. The results are shown in table 1, where the change in lag effected by the addition of the amino acid is expressed as a percentage of the total lag produced by the drug, decreases in lag being expressed as + and increases as −. Thus, for example, the addition of 0.06 g glutamate per liter to the propanol culture produced a lag decrease of 97 minutes, the lag of a culture containing propanol and no amino acid being 156 minutes. The effect of the glutamate is, therefore, expressed as $97 \times 100/156 = 62+$ per cent. It is seen that whereas the pattern of response for ethanol and propanol shows some resemblance to that for phenol, insofar as some amino acids increase lag instead of abolishing it, the higher members of the series show similar behavior to acetone since all the amino acids are effective to some extent.

Lag produced by a series of para-substituted phenols. A similar investigation was carried out with a series of *para*-substituted straight-chain phenols: phenol, *p*-cresol, *p-n*-propyl, and *p-n*-butyl phenol. It was again found that only specific amino acids would remove lag due to phenol, but lag due to the other members of the series could be partially abolished by all the amino acids tried. In this way, therefore, there was some resemblance between results for the series of alcohols and for the series of phenols, the lower members in each case producing lag that was increased by specific amino acids, the higher members lag that was partially abolished by all of them. Ethyl acetate also gave results similar to those for the higher members of the series and for acetone; lag was partially abolished by the addition of all amino acids, and none was inhibitory.

DISCUSSION

From a study of the literature on nonexacting bacteria capable of growing both in simple synthetic media and in broth, it is clear that in the former media not only is growth in the logarithmic phase slower (Hinshelwood, 1946) but lag periods are much more easily induced and are usually considerably longer. Thus, for example, lags may be exhibited in glucose ammonium salt media and not in broths when the inoculum is very light or "young" (Lodge and Hinshelwood, 1943), after thorough washing (Dagley, Dawes, and Morrison, 1949*a*, 1950*b*), when the medium has been deprived of carbon dioxide (Gladstone, Fildes, and Richardson, 1935; Lwoff and Monod, 1947), when under certain circumstances inocula are submitted to excessive aeration (Lwoff and Monod, 1947), or when traces of heavy metals are present (Poole and Hinshelwood, 1940; Monod, 1942). Hinshelwood (1946) and Monod (1949) have shown that many lag phenomena in synthetic media can be readily interpreted on the assumption that the lag phase corresponds to the building up of a steady state. The duration of the phase will, therefore, be determined by the rate of establishment of a concentration of metabolites which is adequate for the maintenance of a steady rate of cell division. It is clear that most of the differences between simple synthetic media and bouillon which we have cited are explained by these views. In bouillon the cells are supplied directly with a variety of substances which may be utilized in cell division, whereas growth in simple media demands the development of intricate synthetic processes, which may be easily disturbed to produce lag.

Direct experimental evidence has been obtained that phenol acts by retarding the production of metabolites, which are produced in adequate quantities in normal growth, since the addition of filtrates from fully grown cultures abolishes the lag due to the presence of the drug. We have previously shown that *A. aerogenes* produces amino acids during growth in simple media, and it is now found that certain amino acids are able partly to abolish lag due to the presence of the drugs employed. Since, as we have stated, there is good evidence that they affect enzyme systems by denaturing proteins, it may be inferred that the particular cell reactions retarded are those concerned with the metabolism of amino acids. It is, however, surprising that phenol, ethanol, and propanol, to a lesser extent, exhibit a certain specificity that is not shown by their homologues or by acetone.

It is possible that phenol and ethanol not only retard the production of amino acids essential for growth, but also block the conversion of one amino acid to another. Thus it is found with these drugs that some amino acids not only fail to remove but actually increase lag, an effect that resembles the inhibition produced by some amino acids on the growth of strains unable to synthesize in optimal amounts the other amino acids they require.

SUMMARY

Phenols, alcohols, acetone, and ethyl acetate inhibit growth of a strain of *A. aerogenes* in glucose ammonium salt medium by inducing a lag period. Filtrates from fully grown cultures of the same strain largely abolish lag due to phenol in this medium. Several amino acids at a concentration of 0.06 g per liter partially abolish lag due to the presence of all the drugs investigated. In the case of lag due to phenol, ethanol, and propanol, however, only specific amino acids are able partially to reverse drug action; other amino acids actually increase the lag. It is concluded that the drugs investigated owe their bacteriostatic action to their ability to inhibit the production of metabolites essential for rapid cell division.

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