PATTERNS OF GROWTH INHIBITION

A. M. MOORE AND JOYCE B. BOYLEN

Biology Branch, National Research Council, Atomic Energy Project, Chalk River, Ontario, Canada

Received for publication March 3, 1952

Following Woods' discovery (1940) that *p*-aminobenzoic acid was a specific competitive antagonist of the inhibitory effect of sulfanilamide upon certain pathogenic bacteria, Fildes (1940) advanced his suggestion of "a rational approach to research in chemotherapy". This approach was to be based on the design of inhibitors which would interfere specifically with the metabolism of the pathogen by taking advantage of differences between its metabolic pattern and that of the host.

The ensuing years have seen the first fruits of this suggestion in the discovery of a number of inhibitors which are structurally related to various natural metabolites and whose inhibitory action, in some cases, is reversible by means of the latter (Welch, 1945; Roblin, 1946; Woolley, 1946). However, it is now well recognized that mere structural similarity in the organic chemical sense is not sufficient proof that a new inhibitor does act by interfering with the metabolism of the compound it was designed to resemble, even when the natural metabolite appears to reverse the inhibition. Part of the reason for the latter reservation is the existence of reports of reversal of the sulfanilamide inhibition by numerous compounds chemically unrelated to *p*-aminobenzoic acid, e.g., adenine, hypoxanthine, thymine, methionine, peptone (Work and Work, 1948), and, perhaps more serious, reports that the effects of certain inhibitors could be reversed by compounds not of natural occurrence, e.g., the antagonism of 5-bromouracil by 5-nitrouracil (Hitchings, Elion, and Van der Werff, 1948) or of sulfanilamide by urethane (McIlwain, 1942). In some of these cases the dissimilar reversing agent may be a product of the inhibited reaction and the inhibitor may indeed be competing with its supposed metabolite analog. This explanation, however, does not seem to be sufficient to explain the reversals by 5-nitrouracil or urethane.

In reviewing the literature of this field, allowance has of course to be made for the fact that use has been made of many different organisms or strains of the same organism which may very well be affected in different ways by the same inhibitor. In addition, however, there has been a widely divergent usage of the terms "inhibition" and "reversal" themselves. Some authors in speaking of inhibition refer to reduction in the growth rate. Others include any process which results in a test culture having a smaller viable count than a growing control at some arbitrary time after inoculation. Unfortunately the latter definition seems to be the more widely used although, without a knowledge of the actual growth curve, it does not lead even to an indirect measure of the growth rate.

As an aid to a more precise definition of inhibition and to illustrate the importance of such definition, it will be necessary to consider the situation which

arises in attempting to relate the growth rate of an organism to the kinetics of its metabolic reactions. A bacterial culture growing in its log phase affords a particularly simple example for this purpose since its growth may be represented with reasonable accuracy by the equation $n_t = n_0 2^{kt}$ where n_t is the number of organisms present in a culture at a time t after inoculation with n_0 organisms, k being the constant rate of division.

In a culture which is obedient to the previous law each organism may be regarded statistically as giving rise to one additional organism within the same average time interval throughout the growth period. Since the average chemical composition of a bacterial strain remains essentially constant throughout many transfers in a suitable medium, it follows that, in such a medium, a constant rate of division implies a constant average rate of synthesis of the various essential components of the organism, e.g., enzymes, nucleic acids, etc. In short, one has to do with the chemical kinetics of the steady state (Burton, 1939; Stearn, 1949; Bertalanffy, 1950).

For a sequence of reactions in a steady state, the action of an inhibitor which diminishes the velocity constant of any single forward reaction leads to the establishment of a new steady state with reduced over-all velocity. If, then, an inhibitor affects in this way a sequence of reactions forming an essential stage in the metabolic processes leading to growth of an organism, one might expect the rate of division of the organism to be diminished; that is, in the equation $n_t = n_0 2^{kt}$ the value of k should be decreased. Conversely, one may regard a diminished but constant rate of division as an unavoidable prerequisite for the interpretation of the effect of an inhibitor of bacterial growth in terms of the simple inhibition of a single metabolic reaction. Any other picture would seem to require a different, or more complex, mechanism.

In the remainder of this report the effect on bacterial growth of several inhibitors is described and the implications of the results are discussed with reference to the foregoing theory. Three of these inhibitors have been widely studied by other authors, but for only one of the nine compounds investigated (sulfanilamide) (Kohn and Harris, 1941) has the effect on the rate of growth been studied directly. Since it was desired to have a comparison of all the results for a single organism, the data for sulfanilamide are included in the following.

MATERIALS AND METHODS

The organism used in the present work was a strain of *Escherichia coli* (NRC 117) kindly supplied by Dr. N. E. Gibbons of the National Research Council, Ottawa. It grows satisfactorily in a synthetic medium consisting of the usual salts plus a sugar. In these experiments the medium used was similar to one described by Lederberg (1947) but with lactose substituted for glucose in the same molar concentration.

Except where otherwise specified the inoculum for each experiment was taken from a culture actively growing in its log phase. Such inocula were found to grow without lag in fresh, preheated medium in the absence of inhibitor. Growth of the cultures at 37 $\pm$ 0.01 C was followed turbidimetrically in matched cuvettes

using a Coleman universal spectrophotometer, density readings being made at a wavelength of 610 mμ. A standard calibration curve relating density of the cultures to the viable count per milliliter was prepared using only cultures growing in their log phase. Under these conditions the relation between density and viable count was found to be linear within the range studied. In those experiments in which the presence of the inhibitor introduced an apparent lag in the density readings viable counts were also made.

The 5-nitrouracil used in these experiments was synthesized by the method of Johnson and Matsuo (1919). The method of Wheeler and Liddle (1908) for the synthesis of uracil-3-acetic acid was used for the synthesis of the 5-bromouracil-3-acetic acid. 5-Bromouracil itself was synthesized by direct bromination of uracil in hot glacial acetic acid. These compounds were purified by repeated recrystallization from water.

The other compounds used as inhibitors were obtained commercially.

Stock solutions of the various inhibitors were prepared in the bacterial culture medium and the pH of these solutions adjusted, where necessary, to 6.8. The various test concentrations were prepared from these by dilution. Each concentration of the inhibitors was normally tested in triplicate and the points in the plotted figures represented the mean values.

RESULTS

Inhibition by 5-bromouracil and other pyrimidine derivatives. In figure 1 are plotted growth curves obtained in a typical experiment using a series of different concentrations of 5-bromouracil. Figure 2 illustrates representative curves for growth in the presence of 5-bromouracil-3-acetic acid, and figure 3 includes curves for a single concentration of each of the three inhibitors—5-nitrouracil, 6-propyl-2-thiouracil, and theophylline. Curves quite analogous to these have also been obtained with 2-thiouracil and caffeine.

Inhibition by sulfanilamide. In figure 4 are plotted curves for the growth of *E. coli* in various concentrations of sulfanilamide. Each of these curves appears to break up naturally into two linear portions of different slope with the transition from one to the other occurring after about 1½ or 2 hours. Our data seem to agree with those of Kohn and Harris (1941) although they interpret their data as indicating a curved growth response. The second portion of our growth curves reaches a minimum slope at about 2×10^{-2} M sulfanilamide, and the first portion approaches the same slope at about 4×10^{-2} M.

Inhibition by pyridine-3-sulfonic acid. In figure 5 are shown the results obtained in an experiment using pyridine-3-sulfonic acid as inhibitor. A striking feature here is the fact that the cultures containing inhibitor, although showing a marked delay in the onset of growth, nevertheless eventually grew at the same rate as the controls, or not at all. This type of result has been obtained repeatedly by us in numerous runs. However, other aspects of the growth curves were much less reproducible.

The length of the apparent lag for any particular concentration of pyridine-3-sulfonic acid showed extreme variations in different experiments. The main

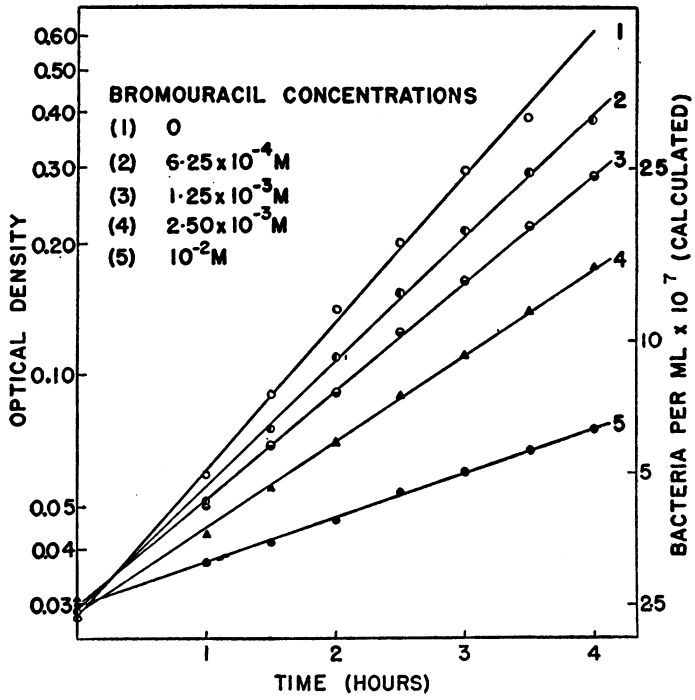

Figure 1. Effect of 5-bromouracil on growth of *Escherichia coli*. The scale on the right gives the viable count calculated from a calibration curve. The same relation between optical density and viable count is applicable in figures 2, 3, and 4.

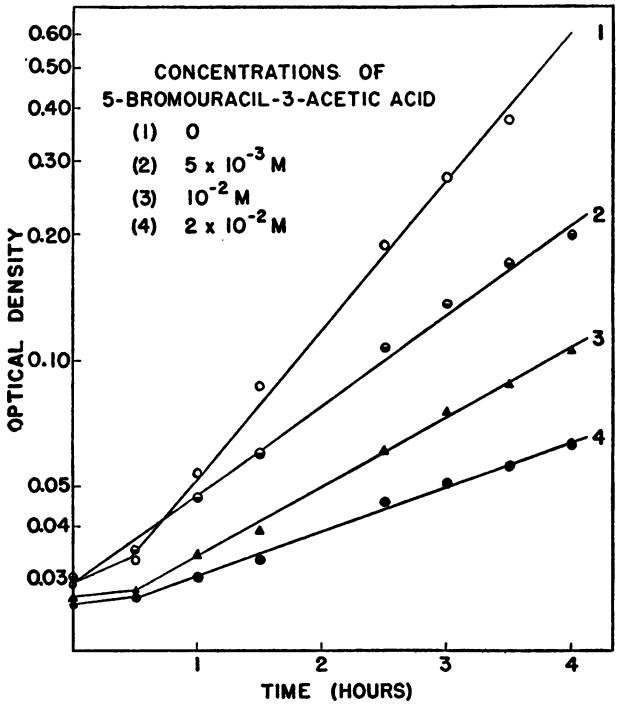

Figure 2. Effect of 5-bromouracil-3-acetic acid on growth of *Escherichia coli*. In this case the control showed a small lag.

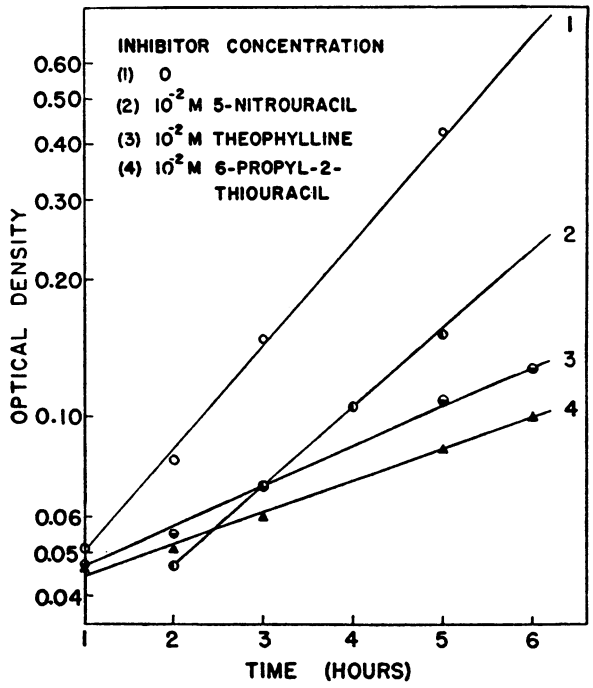

Figure 3. Effect of a single concentration of three different inhibitors on the growth of *Escherichia coli.*

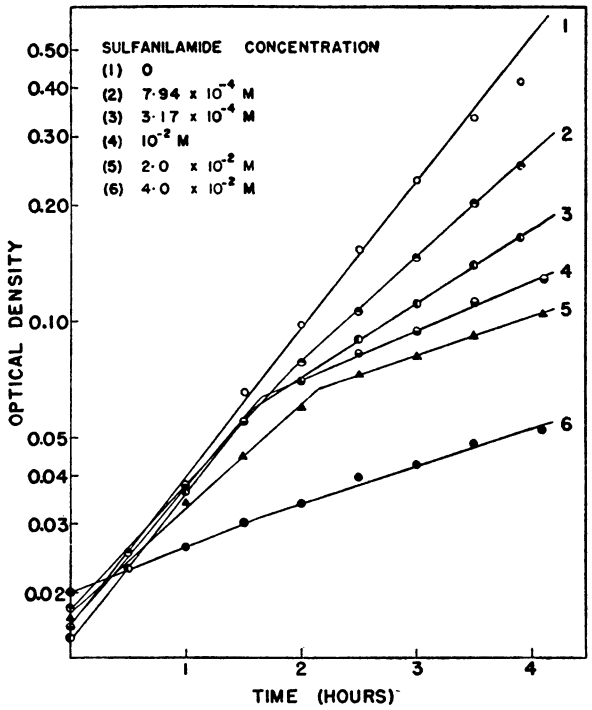

Figure 4. Effect of sulfanilamide on the growth of *Escherichia coli.*

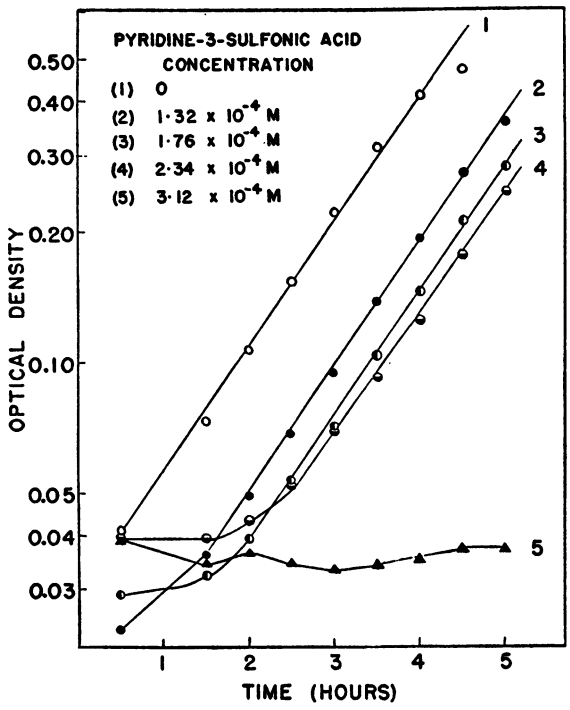

Figure 5. Effect of pyridine-3-sulfonic acid on the growth of *Escherichia coli.*

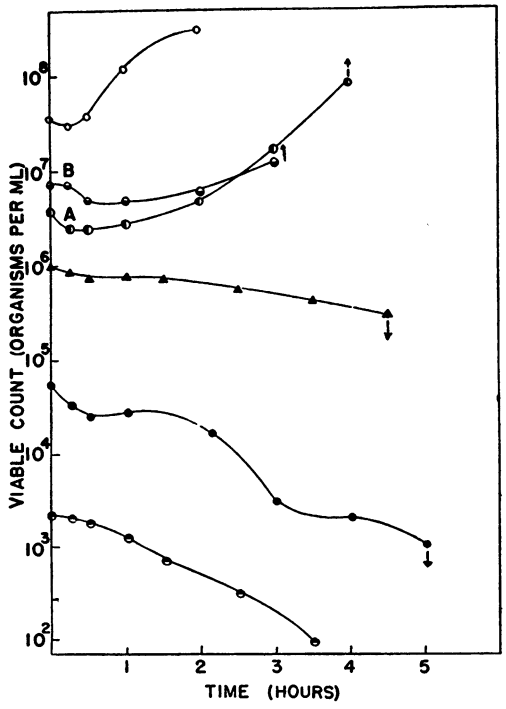

Figure 6. Variation of the inhibitory effect due to pyridine-3-sulfonic acid with the size of the inoculum of *Escherichia coli.* The arrows beside the last points of several of the curves indicate whether the cultures reached their normal maximum density (arrow pointing up) or became sterile (arrow pointing down) after 24 hours' incubation. Controls without inhibitor (not plotted) in all cases grew without lag.

reason for this variation has been found to be the existence of a striking "inoculum effect". This is illustrated in figure 6 where are plotted growth curves for organisms in 10^{-3} M pyridine-3-sulfonic acid using a wide range in the size of the inoculum. These results were obtained by doing viable counts directly on the cultures. In this process the pyridine-3-sulfonic acid is rapidly diluted far below the inhibitory range.

Growth occurred regularly in the presence of 10^{-3} M pyridine-3-sulfonic acid only when the initial viable count was about 5×10^6 organisms per ml or larger. Smaller inocula decreased steadily in numbers until the cultures became sterile after about 24 hours. The cultures which did grow exhibited an initial decline in viable count which varied between 1.5 and 5×10^6 organisms per ml. It may perhaps be significant that this is also about the lower limit of population in which growth occurred under these experimental conditions.

In the case of large inocula which grow in the presence of 10^{-3} M pyridine-3-sulfonic acid it seemed possible that the inhibitor was in some way destroyed before the organisms began to grow. To test this, a culture which had begun rapid growth after the initial decline was centrifuged and the ultraviolet absorption spectrum of the supernatant was compared with that of the original pyridine-3-sulfonic acid solution in the same medium. There was no indication of any change in the concentration of pyridine-3-sulfonic acid or any shift in the position of its absorption maxima.

In the case of the culture whose growth curve is labeled A in figure 6, a sample was removed at 3 hours and was used to inoculate a fresh inhibitor solution. The growth curve for this inoculum is labeled B in figure 6. This culture showed the initial decline in viable count as did A, a fact which argues against the possibility of explaining the eventual growth of A as being due to selection of pyridine-3-sulfonic acid-resistant organisms or to adaptation to pyridine-3-sulfonic acid resistance.

Attempts to protect small inocula (about 10^3 cells) by the simultaneous addition of heat killed or autolyzed cells have led to no consistent results.

DISCUSSION

In the experiments reported here three different types of response to an inhibitor can be distinguished. These we shall refer to as types 1, 2, and 3, exemplified, respectively, by 5-bromouracil, sulfanilamide, and pyridine-3-sulfonic acid. We have examined only one instance of types 2 and 3 but seven examples of type 1.

In type 1 (figures 1, 2, and 3) the plot of log density against time is linear in agreement with the exponential growth law. The slopes of the curves, which are proportional to the rates of division, are altered by the inhibitor in the manner expected for inhibition of a reaction sequence in the steady state. The fact that there is no apparent delay in the attainment of the final growth rate suggests that the rate of penetration of these compounds into the cell is not a limiting factor in determining the degree of inhibition. The over-all picture is that predicted for simple inhibition of a single reaction sequence and, therefore, may be

subjected to further examination on the basis of the theory of the steady state. The inhibitory action of 5-bromouracil will be examined in more detail in a subsequent paper.

The second type of response, seen in figure 4, is more complex. Here the final growth rate is not reached until the cells have undergone two or three divisions. It is of interest to compare this delay in the onset of inhibition with the phenomenon of "phenomic" lag (Davis, 1949) seen in the case of induced bacterial mutants. Following irradiation with suitable doses of ultraviolet or X-rays the affected cells may undergo one or two divisions before exhibiting a biochemical block. One suggested reason for this delay is that even if the synthesis of some enzyme is stopped by mutation the existing molecules of enzyme can continue to function until their degradation by catabolic processes or their dilution resulting from mitosis has reduced their concentration below the effective level. An inhibitor which blocked the synthesis of an enzyme or coenzyme might be expected to exhibit a somewhat similar delay in the appearance of its effects.

Slow diffusion of the inhibitor into the cells might be advanced as an alternative explanation of delayed inhibitory action. If it is reasonable to divide the growth curves as in figure 4 into two linear portions, then some additional explanation must be offered for the fact that sulfanilamide affects the slope of both portions of the growth curve.

For the case of sulfanilamide there is no indication of an "inoculum effect" either in our work or in that of Kohn and Harris (1941) using another strain of *E. coli*. The growth curves are still relatively simple, suggesting the possibility of explaining the result by an extension of the theory such as, for example, the assumption of simultaneous inhibition of two reaction sequences, one of them involving the synthesis of a catalyst. In any case it is clear that the simple theory applicable to type 1 is no longer adequate.

The case of pyridine-3-sulfonic acid is still more complex. In contrast to the other two types, the result here depends very markedly upon the size of the inoculum. This phenomenon has been described before, for example, in the work of Watkins and Winslow (1932) on the disinfectant action of sodium hydroxide. It is also observed in connection with the effects of streptomycin on *E. coli* (Newcombe). McIlwain (1940) in his original study of pyridine-3-sulfonic acid remarked upon certain peculiarities in its action on *E. coli* which prompted him to place that type of inhibition in a separate class with respect to the effect of reversing agents. At present there appears to be no satisfactory explanation of the type of response we have observed.

In recent years there have been numerous studies of reversal of the inhibitory action of chemical analogs of natural cell components (Welch, 1945; Roblin, 1946; Woolley, 1946). Attempts have been made to deduce, from the results of such experiments, the detailed mechanism of the action of the inhibitor in terms of enzyme inhibition. In much of this work the degree of inhibition has been obtained from a single estimate of population density for each culture at some arbitrary time after inoculation. Clearly, such a method can only give an unequivocal measure of growth rate if the growth curves are of type 1 and even

then the time chosen must fall within a rather narrow range. If the inhibition happened to be of type 3, the conclusion would depend very strongly upon the time selected for the determination. Referring to figure 5 and considering a single measurement of density at, say, 3 hours, one would find strong "inhibition" for the cultures in the two lower concentrations of pyridine-3-sulfonic acid; yet at that time they were not inhibited but growing at the same rate as the control!

The present study has been limited to a small number of inhibitors, and it would be fortuitous if the three types of response described here exhausted the possibilities. However, these observations are sufficient to underline the importance of studying growth rates in some detail, whenever the results are to be analyzed with respect to inhibition mechanisms. This would appear to be particularly true in studies of the reversal of inhibition in which a metabolic role may be attributed to a reversing agent.

SUMMARY

In a study of the effects of a group of bacterial growth inhibitors, three types of inhibitory action are distinguished. These are examined from the standpoint of the theory of the steady state. It is concluded that only one of the three inhibitory types can be interpreted as representing a simple inhibition of the rate of a single reaction sequence. The importance of investigating the actual growth curves in studies of the mechanism of inhibition is stressed.

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