INACTIVATION OF ANALOGUES OF FOLIC ACID BY CERTAIN NON-EXACTING BACTERIA

by

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INTRODUCTION

In previous studies (NICKERSON AND WEBB[1]) on the action of folic acid analogues on growth and cell division of micro-organisms, attention was drawn to the fact that certain yeasts and bacteria which do not exhibit a nutritional requirement for the normal metabolite, appear to inactivate these compounds. Thus, the initial inhibition caused by low concentrations of these antagonists in cultures of such organisms is frequently followed by partial, or complete recovery. When the yeast, *Candida tropicalis*, for example, is grown in the presence of Aminopterin (*N*-(*p*-2:4-diamino-6-pteridylmethyl-aminobenzoyl)-glutamic acid), at 28°, an analogue concentration of 10^{-6} *M* is sufficient to produce a significant inhibition of growth during the first 24 hours. After 48–72 hours, however, the amount of growth in such cultures is indistinguishable from that attained in the absence of the antagonist, while at concentrations (*ca.* 10^{-4} *M*), initially affording complete inhibition, the level of growth may reach 70–75% of that of the controls.

Recovery from the effects of Aminopterin in *C. tropicalis* cultures is associated with the appearance in the medium of a free pteridine, or pteridines, the nature of which has not been determined. However, the fact that the pteridine fragments may be extracted from culture filtrates at pH 7 with *n*-butanol suggests that inactivation of Aminopterin by this yeast occurs by reductive or hydrolytic, rather than by oxidative cleavage of the molecule. Inactivation of Aminopterin by buffered suspensions of washed *C. tropicalis* cells is accompanied by an accumulation of a diazotizable amino-compound. This may correspond to the *p*-aminobenzoylglutamic acid residue formed on cleavage of the antagonist molecule. In growing cultures, however, the diazotizable amine does not increase in amounts equivalent to the free pteridine, and it is possible that under these conditions the cells utilize the liberated *p*-aminobenzoylglutamic acid moiety.

Apart from FOLEY'S[2] observations on the possible de-amination of Aminopterin by *Streptococcus faecalis*, the nature of the recovery observed in bacterial cultures containing analogues of folic acid does not appear to have been investigated in any detail. The factors responsible for this recovery seemed to merit further study, not only because of the obvious significance of the process with regard to the competitive relationship between analogue and normal metabolite, but also because of the apparent similarity between the recovery in bacterial cultures and the inactivation of Aminopterin by certain mammalian and avian cells described by JACOBSON[3].

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EXPERIMENTAL

Materials and methods

Organisms. Most of the experiments described were done with *Aerobacter aerogenes*, *A. cloacae* and *Escherichia coli* (Strain B), although *Bacillus megaterium*, *B. cereus* and *B. subtilis* were used for certain supplementary studies. *E. coli* (Strain B) was provided by Dr. W. J. NICKERSON of the Microbiology Dept., Rutgers University, New Brunswick, New Jersey, U.S.A. Cultures of the remaining organisms, initially obtained from the National Collection of Type Cultures (see WEBB[4] for details), were available from the collection maintained in this laboratory. These strains were susceptible to growth inhibition by certain folic acid analogues, particularly those containing the 4-amino group, although not all showed an inhibition of cell division in the presence of these substances similar to that observed with *E. coli* and *C. tropicalis* (NICKERSON AND WEBB[1]).

Culture media. The Gram negative organisms grew well in the simple chemically-defined nutrient solutions described by KOSER AND RETTGER[5] and MONOD[6]. Except when stated otherwise, the latter medium, sterilized by filtration through sintered glass, was used for the experiments with these organisms. The rates of growth of the Gram positive bacilli in simple media such as those described by KNIGHT AND PROOM[7] were too small for the inhibitory effects of the folic acid analogues to be determined conveniently. In consequence, a peptone (B.D.H. bacteriological peptone, 2%) water medium, containing glucose (0.2%) and sodium chloride (0.5%), was used for these bacteria. Under these cultural conditions complete growth experiments were possible over a period of 12–14 h. It should be mentioned, however, that the activities of folic acid analogues are much reduced in complex media of this type (NICKERSON AND WEBB[1]).

Growth conditions. Cultures containing folic acid analogues were incubated in darkness at 37° on an electrically driven rotor inclined at 60° to the vertical. Growth was measured turbidimetrically in a Hilger Spekker absorptiometer fitted with neutral (H508) filters and suitably adapted to carry the culture tubes. Concentrated solutions of the various analogues studied were prepared in the appropriate medium immediately before use and sterilized by filtration through sintered glass. Ultraviolet absorption measurements on suitably diluted aliquots of such solutions before and after filtration established that no decrease in analogue concentration occurred through adsorption on the filter. Every effort was made during both the preparation of these solutions and the incubation and turbidimetric assay of the cultures, to avoid photochemical decomposition resulting from the direct, or prolonged exposure of the analogues to light.

An 18 h culture of the appropriate organism, derived directly from the stock slope, was used to inoculate the experimental series. The size of the inoculum, as detailed in the following sections, was varied according to the experimental procedure.

Folic acid analogues

The following analogues were studied: Aminopterin (N-(*p*-2:4 diamino-6-pteridylmethylaminobenzoyl)-L-glutamic acid), Amino-an-Fol (N-(*p*-2:4 diamino-6-pteridylmethylaminobenzoyl)-aspartic acid), Dichloroaminopterin (N-[3:5 dichloro-4-{[(2:4 diamino-6-pteridyl)-methyl]-amino}-benzoyl]-glutamic acid), Methylaminopterin (A-methopterin) (N-[4-{[methyl-(2:4 diamino-6-pteridyl)-methyl] -amino} -benzoyl] -glutamic acid), Methylfolic acid. (9-methylpteroylglutamic acid), and Dimethylfolic acid (9:10 dimethylpteroylglutamic acid).

For brevity, the trade or trivial names of these substances are used throughout this paper.

For the initial studies of growth inhibition and recovery the analogues were used without purification. This was justified, since it was established by comparative experiments with certain analogues (Aminopterin, Amino-an-Fol) before and after purification, that growth inhibitory activity was proportional to the antagonist content of the preparation and was not modified by the impurities. Purification of the analogues was essential for the characterization of the products formed by the inactivation of these compounds, and was accomplished on columns of paper pulp (Whatman No. 1) as described by WIELAND, HUTCHINGS AND WILLIAMS[8].

RESULTS

The effect of folic acid analogues on bacterial growth

Representative growth curves obtained in a series of experiments in parallel with those of NICKERSON AND WEBB[1], in which sub-bacterostatic concentrations of the analogues were added to the culture medium before inoculation, are reproduced in Fig. 1 to illustrate the variations in response to Aminopterin observed with the different bacterial species. Under the conditions of these experiments a slow recovery, exemplified by Figs. 1(a) and 1(d), was observed in cultures of certain bacteria. Growth of all susceptible

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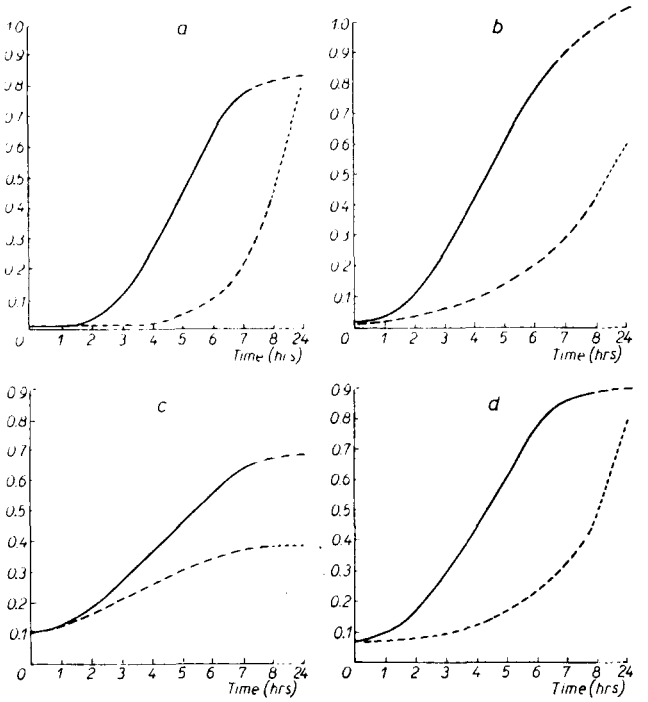

Fig. 1. Growth curves for *Aerobacter aerogenes* (a), *Escherichia coli* (b), and *Aerobacter cloacae* (d) in MONOD's medium, and *Bacillus mesentericus* (c) in peptone-water at 37°. Growth, expressed in arbitrary units (turbidity readings) by the ordinates, in the absence of the antagonist is shown by the continuous curves (———), and in the presence of Aminopterin (Figs. 1(a) and 1(c), $1.2 \cdot 10^{-4}$ *M*, Fig. 1(b) $1.0 \cdot 10^{-4}$ *M*, Fig. 1(d) $5 \cdot 10^{-5}$ *M*) by the broken lines (– – – –).

TABLE I

INFLUENCE OF THE AGE OF THE PARENT CULTURE FURNISHING THE INOCULUM ON THE GROWTH-INHIBITORY ACTION OF AMINOPTERIN ON *Aerobacter aerogenes*

Standard inocula (0.02 ml) of the parent cultures (mean population density 330 μg cell dry weight/ml; maximum variation ± 4%) were transferred to aliquots (5 ml) of MONOD's medium with and without the addition of Aminopterin (44 μg/ml; $1 \cdot 10^{-4}$ *M*). Growth was followed turbidimetrically and the values thus obtained were interpreted in terms of cell dry weight (μg/ml) by comparison with a standard curve. The figures quoted below for the growth rates (dm/dt) refer to the increase in dry weight (μg)/ml culture/h, and, in the cultures containing Aminopterin, are based on measurements made during the initial phase of logarithmic growth (*i.e.* before the onset of recovery).

Age of parent (h)	*Control cultures*			*Cultures in the presence of* $1 \cdot 10^{-4}$ *M aminopterin*		
	Length of lag (min)	*Growth rate (dm/dt)*	*Final population density (μg/ml)*	*Length of lag (min)*	*Growth rate (dm/dt)*	*Final population density (μg/ml)*
66	195	0.99	335.6	275	0.24	319.1
48	195	1.04	319.6	255	0.35	341.5
42	185	1.04	338.3	240	0.42	344.5
24	190	1.08	320.6	225	0.47	344.5
20	155	0.98	317.3	200	0.45	349.0
18	90	1.05	336.7	225	0.55	347.9
16	120	1.04	337.5	210	0.45	332.5

bacteria during the early stages of culture occurred at a greatly reduced rate, and was preceded by an extended lag. The rate of growth in cultures exhibiting recovery was not constant, but increased after incubation for some hours. At a constant analogue concentration, factors which influenced the magnitude of the growth inhibition in experiments of this type included the age of the parent culture (Table I) and the size of the inoculum (Table II).

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TABLE II

THE DECREASE IN THE GROWTH INHIBITORY ACTION OF A FIXED CONCENTRATION ($8 \cdot 10^{-5}$ *M*) OF AMINOPTERIN ON *A. aerogenes* WITH INCREASE IN SIZE OF THE INOCULUM

The washed cells from an 18 h culture of *A. aerogenes* in MONOD's medium were resuspended in fresh medium at a 10-fold concentration. Aliquots of this suspension were then made up to 5.0 ml with appropriate volumes of a solution of Aminopterin in MONOD's medium and/or MONOD's medium alone and incubated at 37°. Growth was measured turbidimetrically at intervals during the following nine hours. Values given below for the length of the lag phase and the growth rate in the presence of $8 \cdot 10^{-5}$ *M* Aminopterin are expressed relative to the corresponding values calculated for the control cultures.

Initial cell density		*Length of lag in aminopterin*	*Relative growth rate in the presence of aminopterin*
Turbidity (Spekker reading)	*Cell dry weight (μg/ml)*	*Length of lag in control culture*	
0.012	4.5	2.33	0.34
0.024	9.0	1.56	0.58
0.048	18.0	1.40	0.73
0.142	53.25	1.23	0.81
0.284	106.5	1.05	0.93
0.392	147.0	1.00	0.99

TABLE III

DECREASE IN THE INHIBITORY ACTION OF AMINOPTERIN ($1.9 \cdot 10^{-4}$ *M*) ON LOG-PHASE CULTURES OF *A. aerogenes* WITH INCREASE IN CELL DENSITY

Cultures of *A. acrogenes* in the logarithmic phase of multiplication, but at different levels of growth were treated with a solution of Aminopterin ($1.14 \cdot 10^{-3}$ *M*) in MONOD's medium (1 ml). Growth was followed turbidimetrically before and after the addition of the analogue, and from the readings thus obtained cell dry weight values (μg/ml) were derived from a calibration curve of turbidity against dry weight. The growth rates, in terms of increase in dry weight (μg) per hour were determined from tangents to the growth curves at the level, or time intervals shown. Uniform cultures for these experiments were obtained by transferring to sterile tubes 5 ml aliquots of an 18 h culture of *A. aerogenes* (1.0 ml) diluted to 50 ml with MONOD's medium.

Cell mass (μg/ml) on addition of Aminopterin	*Growth rate (dm/dt) at cell mass of*			*Rate (dm/dt) after Aminopterin addition*		*Duration of second lag (h)*
	75 μg/ml	*150 μg/ml*	*225 μg/ml*	*1 h*	*3 h*	
—controls	60.0	69.9	69.9	—	—	—
24	3.5	18.4	18.4	8.8	4.8	9
36	6.6	17.4	17.4	12.2	5.2	7
63	41.6	5.1	—	18.9	5.6	5–6
71	42.6	5.5	—	19.4	6.6	5–6
106.5	59.4	13.1	—	14.6	7.6	—
169	60.5	60.5	7.9	22.8	8.2	2
253	—	—	—	34.9	11.1	2

Recovery from the growth inhibitory effects of the folic acid analogues was demonstrated more effectively when the antagonists were added to cultures in the logarithmic phase of multiplication. Under these conditions the addition of active analogues was followed by a short period during which the cells continued to grow at an unaltered rate, and then by a phase of either partial or complete inhibition (Fig. 2). Finally, in cultures which recovered, this was followed by a second phase of logarithmic growth (Fig. 3). The duration of the phase of inhibition, and the rate of subsequent growth, when this occurred, were dependent upon the organisms (Figs. 2, 3 and 5), the population density at the time of

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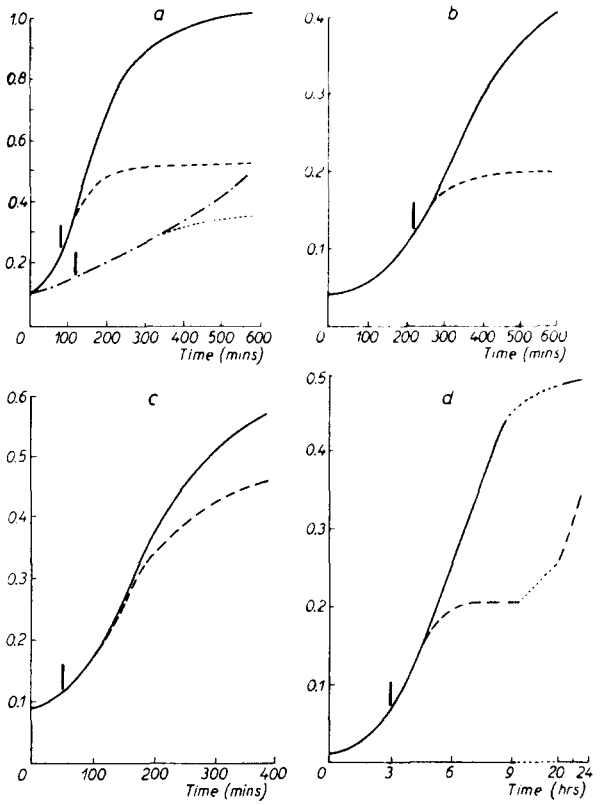

Fig. 2. The effect of the addition of Aminopterin (indicated by the vertical lines) to a final concentration of $1.9 \cdot 10^{-4}$ *M* to early log-phase cultures of *A. aerogenes* (a), *E. coli* (b) and *A. cloacae* (d) in MONOD's medium, and *B. subtilis* (c) in 2% peptone-water at 37°. Growth, as increase in turbidity (ordinates) in the treated and control cultures is shown by the broken (- - - -) and continuous (——) lines respectively. The additional curves in Fig. 2(a) show the growth of *A. aerogenes* at 18° (— · — · —) in MONOD's medium, and the effect produced by the addition of $1.5 \cdot 10^{-4}$ *M* Aminopterin (— — —).

addition of the analogue (Table III), the composition of the culture medium (see NICKERSON AND WEBB[1]), the conditions of growth (Fig. 8), and the concentration and structure of the analogue (Figs. 4 and 5), but were not influenced by the simultaneous, or preceding additions of 10^{-3} *M* concentrations of folic acid, the *Leuconostoc citrovorum*-factor (= $2 \cdot 10^{-3}$ *M* Leucovorin) thymine (Fig. 3; *cf.* NICKERSON AND WEBB[1]), or $5 \cdot 10^{-3}$–$5 \cdot 10^{-4}$ *M* sodium formate.

It is apparent from Figs. 3, 4 and 6 that the final stationary population attained in cultures after recovery from inhibition frequently exceeded that of the controls. As the addition of possible degradation products of the folic acid analogues, *e.g.* 2:4-diaminopteridine, 2-amino-4-hydroxypteridine-6-carboxylic acid, pteroic acid, *p*-aminobenzoylglutamic acid, *p*-aminobenzoic acid and L-glutamic acid, in the appropriate concentrations to cultures of *A. aerogenes* or *E. coli* in MONOD's medium failed to yield a consistent or significant increase in final population density, it appears that the stimulation of growth mentioned above may be due to factors such as those discussed by HINSHELWOOD[9], and which may include changes in the concentration of intermediate substances and in enzyme balance during the phase of growth inhibition.

Growth in the second logarithmic phase of cultures recovering from the inhibitory effects of a given analogue was susceptible to inhibition by further amounts of the same analogue. This is illustrated by the results presented in Fig. 6, which show that a second addition of Aminopterin to a culture of *A. aerogenes* in the recovery phase, duplicatm the effects produced by the first addition of the analogue to a culture at a comparasoe growth level. From observations such as these, and the fact that supernatants frble cultures which had overcome the effects of Aminopterin were devoid of growth-inhibitory activity, it was apparent that recovery was not associated with the growth of a cell population of reduced susceptibility, but was due to a decrease in antagonist concentration.

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Factors leading to a decrease in antagonist concentration

Ultra-violet absorption measurements on supernatants removed 1 h after the addition of Aminopterin to log-phase cultures of *A. aerogenes* at different levels of growth, established that insignificant changes in concentration were likely to result from the uptake of the antagonist by the cells.

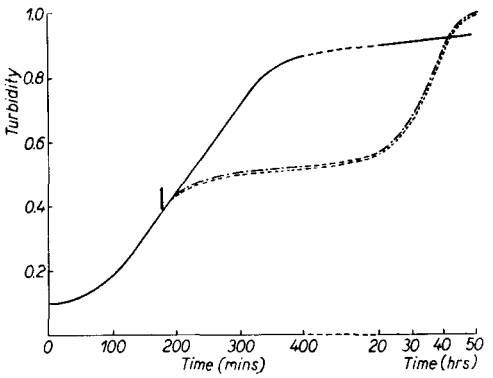

Fig. 3. Growth of *A. aerogenes* in MONOD's medium at 37° (———) and the effect thereon of $1.9 \cdot 10^{-4}$ *M* Aminopterin (– – – –) added in the early logarithmic phase as indicated by the heavy vertical line. The failure of thymine ($1 \cdot 10^{-3}$ *M*) to influence either the inhibition, or the onset of recovery in the Aminopterin culture is shown by the third curve (———-).

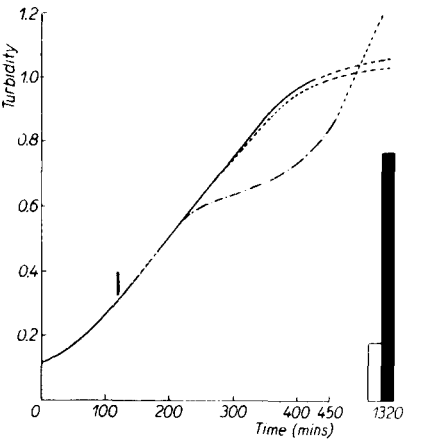

Fig. 4. Growth of *A. aerogenes* in MONOD's medium alone (———) and in the presence of $1.7 \cdot 10^{-4}$ *M* (– – – –) and $8.3 \cdot 10^{-4}$ *M* (———-) Dichloroaminopterin, added to the cultures in the early logarithmic growth phase as indicated by the vertical line. The shaded and unshaded histograms show the contents of diazotizable amine (in arbitrary units) in aliquots (2.0 ml) removed from the culture containing $8.3 \cdot 10^{-4}$ *M* Dichloroaminopterin after recovery (1320 min), and from similar solution of the antagonist in MONOD's medium, incubated for the same period as uninoculated control.

The determination of the diazotizable amino groups in aliquots removed at intervals from cultures of *A. aerogenes* containing various folic acid analogues revealed that the onset of the period of recovery was preceded by an increase in the content of aromatic amine (Fig. 7. See also Figs. 4 and 8). The rate of amine formation was enhanced by aeration, whereas it was reduced markedly in entirelystatic cultures. The correlation between these findings and recovery from inhibition, is apparent from the growth curves (Fig. 8) for *A. aerogenes* in the presence of Aminopterin in aerated, rotated and static cultures. Both recovery and amine formation were reduced further, or almost completely prevented under anaerobic conditions. This was true particularly for cultures treated with Methylaminopterin. Thus, the amine content of anaerobic cultures of *E. coli* containing $2.2 \cdot 10^{-3}$ *M* Methylaminopterin where no recovery was observed, remained constant at the initial level over a period of five days.

The instability of both folic acid and its analogues in solution has been re-emphasized recently by JUKES[10]. It was readily apparent however, that the formation of the diazotizable amine in the bacterial cultures was not due to the spontaneous decomposition of the analogues, since the increase in amine content of uninoculated media containing these compounds was not significant in experiments of 24 h duration. Furthermore, no formation of diazotizable groups was detected when the washed cells from 200 ml of a 16 h culture of *A. aerogenes* were incubated for 22 h in the absence of metabolizable

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substrates with a solution of Aminopterin (15.8 mg) in 0.15 *M* phosphate buffer, pH 7.4 (60 ml).

The amine formed in *A. aerogenes* cultures to which various analogues had been added was heterogeneous in composition as shown by the fact that *ca.* 25% remained unacetylated after treatment with acetic anhydride according to the method of ROSENTHAL AND BAUER[11]. For this reason the amine content of the cultures (Figs. 4, 7 and 8) is expressed in arbitrary units and not in terms of *p*-aminobenzoyl residues.

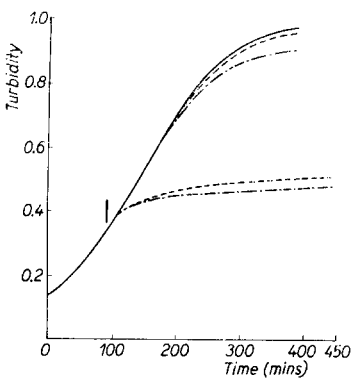

Fig. 5. The effects of certain folic acid analogues on the growth of *A. aerogenes*. Additions of Methylfolic acid (– – –), Dimethylfolic acid (– · – · –), Amino-an-Fol (– – – –) and Methylaminopterin (— · · —) were made to cultures in the early logarithmic phase, as indicated by the vertical line, to give a final analogue concentration of $3.8 \cdot 10^{-4}$ *M*. The growth of the control (untreated) culture is shown by the continuous line (———).

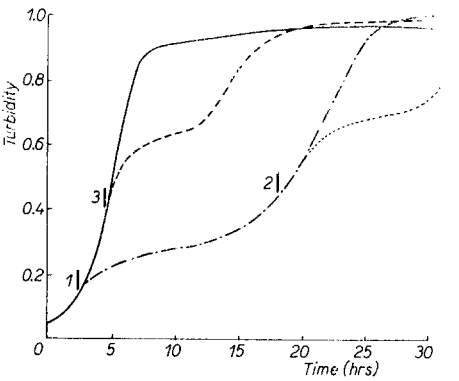

Fig. 6. The inhibition of recovery in an Aminopterin-treated culture of *A. aerogenes* by a second addition of the antagonist. The results presented in the figure show that growth in the recovery phase (– – – –) of an Aminopterin-treated culture (antagonist added at 1) is inhibited (– – – –) by further amounts of Aminopterin (added at 2), and that the degree of inhibition is similar to that produced in a normal culture (· · · ·) of *A. aerogenes* at a comparable growth level by the addition (at 3) of the same concentration of the antagonist (· · · ·). The method was as follows: A culture of *A. aerogenes* in MONOD's medium at 37° in the early logarithmic phase was divided into two fractions. At the time indicated by the vertical line 1 a solution of Aminopterin in MONOD's medium was added to one of these fractions to a final concentration of $1.8 \cdot 10^{-4}$ *M*, while the other fraction received the same volume of MONOD's medium. (Measurements of the turbidity of each culture before and after dilution enabled a correction to be made for the consequent changes in cell density.) Growth was followed in both the treated (– · – · –) and control (· · · ·) culture, and the latter, at a turbidity reading of *ca.* 0.4, again divided into two fractions. To these (at 3) were added Aminopterin (to a concentration of $2.3 \cdot 10^{-4}$ *M*) and the medium alone as before. When recovery occurred in the initial culture containing Aminopterin (*i.e.* – · · – · ·) and the turbidity reached *ca.* 0.4, this was also divided. One of the two fractions was diluted with the medium only as control, while the second (at 2) received Aminopterin to a concentration of $2.3 \cdot 10^{-4}$ *M*.

That the non-acetylatable fraction of the amine was not identical with 4-aminoimidazole-5-carboxamide, known to accumulate in cultures of *E. coli* containing sub-bacteriostatic concentrations of sulphanilamide (STETTON AND FOX[12]; RAVEL, EAKIN AND SHIVE[13]) or Aminopterin (WOOLLEY AND PRINGLE[14]; EDWARDS, SKIPPER AND JOHNSON[15]), was shown by comparison of a product isolated from *A. aerogenes* cultures according to the method of STETTEN AND FOX[12] with an authentic sample of the carboxamide. Fractionation of the neutralised (pH 7) filtrate from 4 l of an aerated, 24 h culture of *A. aerogenes* containing 10^{-4} *M* Aminopterin, was accomplished as described by these authors after first removing inorganic phosphate from the medium by precipitation as calcium phosphate (a procedure which also removed the cells and excess

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Aminopterin, but which did not lead to any loss of diazotizable amine). By this method, the validity of which was confirmed in control experiments by the recovery of 4-aminoimidazole-5-carboxamide previously added to culture filtrates of *A. aerogenes*, only 0.8 mg of an insoluble picrate were obtained from which 0.55 mg picric acid was recovered on decomposition of the salt with dilute sulphuric acid. The residual basic component which, in its chromatographic behaviour and ultra-violet absorption, bore no resemblance to 4-aminoimidazole-5-carboxamide, gave a negative reaction with the BRATTON–MARSHALL[16] reagents, and apepared to be a pteridine derivative.

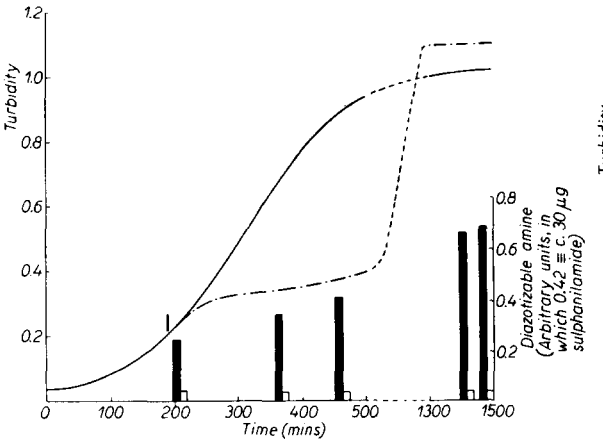

Fig. 7. The accumulation of diazotizable amine in an Aminopterin-treated culture of *A. aerogenes*, and its association with recovery from growth-inhibition. Growth and diazotizable amine content in an *A. aerogenes* culture containing Aminopterin ($1.9 \cdot 10^{-4}$ *M*, added as indicated by the vertical line) are shown by the dashed line (— · —) and the black histograms respectively. The continuous curve (———) and the unshaded histograms show the growth and diazotizable amine content of the control culture. Growth (turbidity) measurements and determinations of diazotizable amine (BRATTON AND MARSHALL[16]) were made on aliquots removed aseptically at intervals from the cultures growing in MONOD's medium (50 ml) in 250 ml Erlenmeyer flasks at 37°.

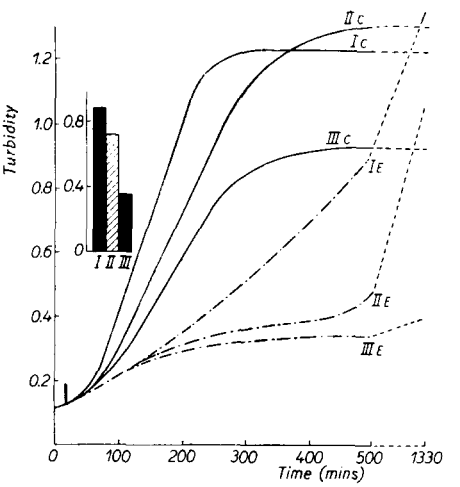

Fig. 8. Growth of *A. aerogenes* in aerated (I), rotated (II) and static (III) cultures in MONOD's medium alone (C Series of continuous (———) curves), or containing Aminopterin ($1.9 \cdot 10^{-4}$ *M*) added at the time indicated by the vertical line (E series of broken (– – –) curves). The histograms (I, II and III) show the contents of diazotizable amine in aliquots (2 ml) of the three treated (E) cultures at 1330 mins.

Identification of the products of the inactivation of Aminopterin by A. Aerogenes

(i) p-*Aminobenzoyl-L-glutamic acid*. It was established in preliminary studies that *p*-aminobenzoylglutamic acid, a probable product of the inactivation of Aminopterin, was precipitated from neutral aqueous solution by Hg^{++}, but not by Ba^{++}, or by picric acid. In consequence, the supernatant (S_2) remaining after the precipitation of the insoluble picrate in the above fractionation, was extracted with ether to remove picric acid and concentrated *in vacuo* (bath temp. 60°) in an atmosphere of nitrogen to 5 ml. Inorganic salts which separated were removed, and an aliquot (0.02 ml) of the supernatant solution (S_{21}), 0.1 ml of which gave a strong positive reaction with the BRATTON-MARSHALL reagents, chromatographed on Whatman No. 1 paper with butanol saturated with water as solvent, and *p*-aminobenzoyl-L-glutamic acid (0.02 ml of a 0.025 *M* aqueous solution) as reference compound. After 24 h at 18° the presence on these chromatograms

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of an acidic component, R_F = 0.24 (0.23–0.25), presumably identical with *p*-aminobenzoylglutamic acid, R_F = 0.23 was established by spraying with alcoholic methyl red.

The main fraction of S_{21} (4.8 ml) was made 2 *N* with respect to sulphuric acid and heated at 100° for 16 h. Sulphate was then removed from the hydrolysate with barium hydroxide, and excess barium with carbon dioxide. The presence of L-glutamic acid in the hydrolysate was established by chromatography of an aliquot (0.01 ml) on Whatman No. 1 paper with *tert.* amyl alcohol (42.5 ml)—methanol (20 ml)—0.1 *M* citrate pH 5 (10 ml) as the solvent system (J. A. GRUNAU, unpublished). After 30 h at 18° a ninhydrin-positive spot was located on the chromatogram, the R_F of which was identical with that of authentic L-glutamic acid (R_L : 0.13).

The remainder of the hydrolysate was adjusted to pH 3 with 0.1 *N* sulphuric acid and extracted four times with redistilled ethyl acetate (STOKSTAD *et al.*[17]). The combined extracts were washed with water acidified with 0.1 *N* sulphuric acid, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was taken up in the minimum volume of hot water (70°) necessary for complete solution, and an aliquot (0.01 ml) of this chromatographed on Whatman No. 1 paper with butanol saturated with water as solvent. After 15 h at 17° an acidic component, R_F = 0.74 identical with that of *p*-aminobenzoic acid, was detected with methyl red.

The remainder of the above solution was nucleated with *p*-aminobenzoic acid and kept at 4°. The crystals which separated were collected after 72 h, and dried over phosphoric oxide to yield 0.62 mg of pale yellow needles, m.p. (uncorr.) 183–186° alone and in admixture with authentic *p*-aminobenzoic acid, m.p. 185–186.5°.

Neither free *p*-aminobenzoic acid, nor L-glutamic acid were detected by the appropriate chromatographic techniques either among the components of S_2, or in samples removed from *A. aerogenes* cultures at intervals throughout the periods of growth inhibition and recovery which followed the addition of Aminopterin in the logarithmic phase. Thus, inactivation of Aminopterin by *A. aerogenes* is accompanied by the formation of *p*-aminobenzoylglutamic acid, which does not undergo further degradation.

(ii). *The pteridine components*

An aerated, 24 h culture of *A. aerogenes* (4.5 l) containing purified Aminopterin ($1 \cdot 10^{-4}$ *M*, added during the early log. phase) was adjusted to pH 7 with 1 *N* sodium hydroxide, and treated with $Ca(NO_3)_2 \cdot 4\,H_2O$ (104 g; calculated as equivalent to the phosphate content of the medium) dissolved in the minimum volume of water. The precipitated calcium phosphate, which also carried down the cells and adsorbed much of the excess Aminopterin, was collected by suction and washed with water. The precipitate was then suspended in 0.5 *N* sulphuric acid and extracted three times with *n*-butanol.

The addition of mercuric acetate (10 g) in water (100 ml) to the filtrate remaining after removal of the calcium phosphate, yielded a yellow precipitate, which was collected by filtration after 10 mins. This was washed on the filter with water, dissolved in 2 *N* hydrochloric acid, and the acid solution extracted with *n*-butanol.

The combined *n*-butanol extracts were washed twice with water acidified with 0.1 *N* sulphuric acid, concentrated *in vacuo* to *ca.* 10 ml and poured into ether (200 ml). The precipitate which separated was collected after 24 h at 4° and dissolved in a mixture of water (20 ml) and 5 *N* ammonia (1 ml). The resulting solution was acidified with 5 *N* hydrochloric acid (1.1 ml) and transferred immediately to a column (25 × 2 cm) of alumina. The column was developed first with water, and then with water containing

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5 *N* ammonium hydroxide (1 ml/100 ml). In this way the small amount of Aminopterin, identified by its yellow colour, and by its properties of absorption and non-fluorescence in ultra-violet light, was separated from the free pteridines. The combined effluents containing the latter were evaporated to dryness *in vacuo*. The solid residue (7.8 mg) thus obtained was boiled under reflux for 45 min in 5 *N* hydrochloric acid (15 ml) to convert the 2:4-diaminopteridine derivatives into the corresponding, more readily characterized, 2-amino-4-hydroxy compounds (*cf.* TAYLOR AND CAIN[18]). (Control experiments in which 2-amino-4-hydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxy-6-methylpteridine were boiled with 5 *N* hydrochloric acid for 45 min, and then chromatographed as described later, confirmed that derivatives of 2-amino-4-hydroxypteridine bearing substituents in the 6- position were unaffected by the above conditions, whereas 2:4-diamino-6:7-dimethylpteridine was converted readily to 2-amino-4-hydroxy-6:7-dimethylpteridine.) After cooling, the acid hydrolysate was adjusted to pH 6.7 with sodium hydroxide and then extracted three times with *n*-butanol. The butanol extracts (B_3), thus separated from the aqueous phase (B_1), was evaporated to dryness under reduced pressure. The residue was taken up in water, the solution adjusted to *ca.* pH 3 with dilute hydrochloric acid, and transferred to a column of alumina. The column was developed first with water, then with dilute ammonia (see above). The components were thus separated into two clearly defined zones, (I and II, *ca.* 3–4 cm apart) which were easily located by their fluorescence in ultra-violet light. These zones were eluted separately.

The residual aqueous phase (B_1) at pH 3 was fractionated on an alumina column in the same way. The main (III) and two subsidiary components (A and B) thus separated were eluted and the eluates concentrated *in vacuo* over phosphoric oxide at room temperature.

Fraction III was acidified to *ca.* pH 3 with dilute hydrochloric acid and stored at 4°. The identity of the crystalline product (3.9 mg) which separated with 2 amino-4-hydroxypteridine-6-carboxylic acid was established by the following facts: (i) on ascending chromatography (Whatman No 1 paper) in different solvents (*n*-butanol (70 ml) —5 *N* acetic acid (30 ml) (GOOD AND JOHNSON[19]) and 3% ammonium chloride (TSCHESCHE AND KORTE[20a])) the product removed as one component. The R_F values of the compound in these solvents (0.045 (butanol-acetic acid) and 0.11 (3% NH_4Cl)) were identical with those shown by the authentic substance. (ii) The ultra-violet absorption spectra of the unknown in water (λmax. 287, and 345 mμ), 0.1 *N* hydrochloric acid (λmax., 262, and 317 mμ) and 0.1 *N* sodium hydroxide (λmax. 260, and 366 mμ) were indistinguishable from those of 2-amino-4-hydroxypteridine-6-carboxylic acid. (iii) On fusion of the compound at 260° in an atmosphere of nitrogen (TSCHESCHE AND KORTE[20b]), followed by paper chromatography in butanol-acetic acid, a product (R_F 0.23–0.25) was obtained which corresponded to that derived by the decarboxylation of 2-amino-4-hydroxypteridine-6-carboxylic acid. This product was characterized as 2-amino-4-hydroxypteridine by comparison of its absorption spectrum in the ultraviolet—λmax. 272, and 340 mμ (in water), 252, and 362 μm (in 0.1 *N* NaOH)—and its R_F (0.24) on chromatography in butanol-acetic acid, with those on an authentic specimen.

The remaining fractions (*i.e.* I, II, A and B, 0.01 ml) were chromatographed on Whatman No. 1 paper in both butanol-acetic acid and 3% ammonium chloride, with the following pteridines as reference compounds: 2-amino-4-hydroxypteridine, 2-amino-4-hydroxypteridine-6-carboxylic acid, 2:4-diamino-6:7-dimethylpteridine, 2-amino-4-

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hydroxy-6:7-dimethylpteridine, 2-amino-4-hydroxy-6-methylpteridine, 2-amino-4-hydroxy-7-methylpteridine, 2-amino-4:6-dihydroxypteridine, 2:4-diaminopteridine, 2-amino-4:7-dihydroxypteridine-6-carboxylic acid and 2-amino-4-hydroxy-6-hydroxymethylpteridine. This last compound was prepared by the Cannizzaro reaction from 2-amino-4-hydroxypteridine-6-aldehyde (WALLER *et al.*[21]). The resulting mixture of the 6-hydroxymethyl- and 6-carboxylic acid derivatives was streaked across the whole width (10") of sheets of Whatman No. 1 paper, and the reaction products separated by chromatography in butanol-acetic acid. The eluted component of $R_F = 0.13$, had ultra-violet absorption spectra (λmax. 285, and 347 mμ λmin. 257, and 313 mμ (in water), λmax. 263, and 363 mμ, λmin. 240, and 320 mμ (in 0.1 *N* NaOH)) identical with those given by WALLER *et al.*[21] for 2-amino-4-hydroxy-6-hydroxymethylpteridine.

By chromatography it was established that 2-amino-4-hydroxy-6-methylpteridine was not present among the components of any of the above fractions. Thus inactivation of Aminopterin by *A. aerogenes* does not occur by reductive cleavage. 2-Amino-4-hydroxypteridine-6-carboxylic acid was detected as a slow-moving minor component of Fractions II and A. Another minor component thus separated from Fractions I and II was characterized as 2-amino-4-hydroxy-6-hydroxymethylpteridine by the identity of its R_F value (0.13) in butanol-acetic acid with that of an authentic specimen. With the absence of 2-amino-4-hydroxy-6-methylpteridine from Fraction I, further confirmation of the presence of 2-amino-4-hydroxy-6-hydroxymethylpteridine in this fraction was obtained by the oxidation of an aliquot of the solution with alkaline permanganate at 90° for 3 h (BACKER AND HOUTMAN[22]). After cooling, the solution was treated with sodium bisulphite to destroy excess permanganate, filtered to remove manganese dioxide and chromatographed on an alumina column (16 × 1 cm) as previously described. The two fluorescent zones (D and E) thus obtained were eluted and rechromatographed on Whatman No. 1 paper in butanol-acetic acid, whereby each fraction was separated into two components, D_1 (R_F: 0.045, corresponding to 2-amino-4-hydroxypteridine-6-carboxylic acid) D_2 ($R_F = 0.10$; unidentified), E_1 ($R_F = 0.13$, unchanged 2-amino-4-hydroxy-6-hydroxymethylpteridine) and E_2 ($R_F = 0.37$, unidentified).

No correlation was found between the R_F values of the remaining components of Fractions I, II, A and B and those of the reference compounds. It appeared possible, therefore, that the origin of these substances, in part at least, was to be found in the incomplete deamination of the crude pteridine fraction. Accordingly, the isolation of the pteridine fragments derived from different folic acid analogues, was attempted by direct chromatography of filtrates from the treated cultures.

Isolation of pteridines by direct chromatography of culture filtrates

Cultures of *A. aerogenes*, *A. cloacae*, and *E. coli* were incubated with Aminopterin, Methylaminopterin ($1 \cdot 10^{-4}$ *M*), or Methylfolic acid ($1 \cdot 10^{-3}$ *M*) for 18 h, or until recovery was complete. The last mentioned analogue, although without growth inhibitory activity for the above organisms at the concentration stated, was included in this survey since, on the assumption that it would be degraded in the same way as the active analogues, its cleavage would give rise directly to derivatives of 2-amino-4-hydroxypteridine.

The filtrates from these cultures, with the exceptions noted below, were streaked across sheets of Whatman No. 1 paper, and the pteridine components separated by ascending chromatography in butanol-acetic acid. Paper strips bearing the fluorescent components were cut from the chromatograms when dry, and from these the individual

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pteridines were eluted with 0.1 *M* ammonia. The eluates were evaporated to dryness *in vacuo* over phosphoric oxide, and the residue taken up in water (0.2 ml).

From these initial chromatograms it was apparent that Methylaminopterin was unsuitable for experiments of this type, since the preparation available was grossly contaminated with other pteridine derivatives which fluoresced strongly in ultra-violet light. These were not removed completely when the analogue, in sufficient quantity for the biological studies, was purified by chromatography on paper pulp.

Purified Aminopterin was degraded by *A. aerogenes* with the formation of three new pteridine components, two of which (R_F = 0.20 and 0.12) were present in significant amounts, while the third (R_F = 0.34) occurred in traces only. Each eluate of the two main components was treated with 5 *N* hydrochloric acid (4 vols.) at 100° for 45 min., to form the corresponding 2-amino-4-hydroxypteridine derivatives. The hydrolysates were neutralized with ammonia, evaporated *in vacuo* over phosphoric oxide at room temperature, dissolved in water (0.2 ml) and chromatographed on Whatman No. 1 paper with butanol-acetic acid as solvent. Two components (one main and one subsidiary) were then detected on each chromatogram by their (blue) fluorescence in ultra-violet light. The main components (R_F 0.11 and 0.06) from each chromatogram were characterized as 2-amino-4-hydroxy-6-hydroxymethylpteridine and 2-amino-4-hydroxypteridine-6-carboxylic acid respectively by rechromatography in admixture with the authentic compounds.

Complete destruction of Aminopterin ($1 \cdot 10^{-4}$ *M*) by *A. cloacae* was demonstrated by descending chromatography in butanol-acetic acid of 72 h culture filtrates in parallel with the uninoculated Aminopterin-containing (KOSER-RETTGER) medium. The degradation of Aminopterin by this organism gave rise to only one pteridine fragment in significant quantity. The products from the hydrolysis of the eluted compound (see above) were separated by chromatography in butanol-acetic acid into two main components (R_F 0.11 and 0.17), the first of which was tentatively identified as 2-amino-4-hydroxy-6-hydroxymethylpteridine (R_F, in the absence of NH_4Cl, = 0.12) by elution and rechromatography in parallel with an authentic specimen.

From culture filtrates of *E. coli* containing Methylfolic acid, three pteridines (M, (R_F = 0.24); HM, (R_F = 0.11) and CA (R_F = 0.087)) were isolated. Two of these substances (M and CA; the latter in lower concentration) were also present in medium before inoculation as contaminants of the methylfolic acid. R_F values comparable with those of known reference compounds, were obtained by rechromatography of the neutralized (HCl) components eluted (0.1 *M* ammonia) from the initial chromatograms. Thus, on rechromatography, the R_F values of the above components were as follows:

CA, R_F = 0.065, identical with that of 2-amino-4-hydroxypteridine-6-carboxylic acid (applied to the chromatogram in 0.1 *M* ammonium chloride).
HM, R_F = 0.12, identical with that of 2-amino-4-hydroxy-6-hydroxymethylpteridine.
M, R_F = 0.29, not characterised, possibly 2-amino-4-hydroxy-6-methylpteridine (R_F = 0.31).

DISCUSSION

Inactivation of the inhibitor appears to be the underlying cause of the recovery from growth inhibition observed in cultures of certain bacterial species containing analogues of folic acid. Inactivation is due to the cleavage of the analogue molecule with the formation of free pteridines and *p*-aminobenzoylglutamic acid. The latter does

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not undergo further degradation in so far as free *p*-aminobenzoic acid and L-glutamic acid are not encountered among the inactivation products unless these are first submitted to acid hydrolysis. The absence of L-glutamic acid from the products resulting from the inactivation of Aminopterin by *A. aerogenes* also proves, for this organism at least, that cleavage of the molecule of the antagonist occurs specifically at the linkage between $C_{(9)}$ and $N_{(10)}$, and is not accompanied by the formation of the 4-amino analogue of pteroic acid.

Cleavage of Aminopterin with the production of *p*-aminobenzoylglutamic acid does not account for all of the diazotizable amine formed in *A. aerogenes* cultures under such conditions, since *ca.* 25% of the total amine is not acetylatable with acetic anhydride at room temperature. Whether this nonacetylatable fraction corresponds to a hydroxylamine derivative formed together with the *p*-aminobenzoylglutamic acid residue on cleavage of the antagonist, or is a product arising from the inhibition of certain metabolic reactions remains to be determined.

The fact that recovery is enhanced by aeration, and reduced in the absence of oxygen suggests that inactivation is an oxidative process. This is supported by the absence from the degradation of pteridines containing a methyl group in the 6-position, and the isolation of both hydroxymethyl and carboxylic acid derivatives. It is possible that the first product of the oxidative cleavage of Aminopterin or similar analogues, would be 2:4-diaminopteridine-6-aldehyde which, through oxidation and dismutation, would yield a mixture of the 6-hydroxymethyl- and 6-carboxylic acid derivatives, the latter in higher concentrations, as found experimentally. The fact that the aldehyde was not detected among the pteridine components separated by direct chromatography of culture filtrates is no proof of the absence of this compound, since it was noted that, unless special precautions were observed during chromatography, authentic 2-amino-4-hydroxypteridine-6-aldehyde was oxidized to the corresponding carboxylic acid. Attempts to trap the possible intermediary aldehyde in cultures of *A. aerogenes* by the addition of $5 \cdot 10^{-4}$ *M* semicarbazide as well as Aminopterin to the medium, or by treatment of culture filtrates with 2:4-dinitrophenylhydrazine, however, were unsuccessful. In addition, such a general mechanism is rendered less likely by the finding that the hydroxymethyl compound appears to be the only main product arising from the inactivation of Aminopterin by *A. cloacae*.

Finally it may be mentioned that folic acid in cultures of *A. aerogenes* and *E. coli* undergoes inactivation in the same way as its inhibitory analogues (*cf.* BAUMGARTEL AND ZAHN[23]).

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SUMMARY

The recovery from growth-inhibition observed in cultures of certain non-exacting bacteria containing analogues of folic acid is due to the inactivation of these compounds by oxidative cleavage. This leads to the formation of *p*-aminobenzoylglutamic acid and a mixture of free pteridines, the main components of which in cultures of *Aerobacter aerogenes* and *Escherichia coli* have been identified as derivatives of pteridine-6-carboxylic acid and 6-hydroxymethylpteridine. Cleavage of the analogue molecule appears to occur only at the linkage between $C_{(9)}$ and $N_{(10)}$ since neither *p*-aminobenzoic acid nor L-glutamic acid are demonstrable among the products of inactivation before acid-hydrolysis.

RÉSUMÉ

La disparition de l'inhibition de croissance observée dans des cultures de certaines bactéries non exigeantes contenant des analogues de l'acide folique est due à l'inactivation de ces substances par dégradation oxydative. Cette dégradation entraîne la formation d'acide *p*-aminobenzoylglutamique et d'un mélange de ptéridines libres, dont les principaux constituants, dans les cultures d'*Aerobacter aerogenes* et d'*Escherichia coli*, ont été identifiés à des dérivés de l'acide pteridine-6-carboxylique et de la 6-hydroxyméthylpteridine. La dégradation de la molécule de l'analogue se produit seulement entre le $C_{(9)}$ et le $N_{(10)}$ puisque ni l'acide *p*-aminobenzoique ni l'acide L-glutamique ne sont identifiables parmi les produits d'inactivation avant hydrolyse acide.

ZUSAMMENFASSUNG

Die Erholung gewisser bedürfnisloser Bakterienkulturen von einer durch folinsäureanaloge Stoffe bewirkten Wachstumshemmung beruht auf der Inaktivierung dieser Substanzen durch oxydative Spaltung. Diese führt zu der Bildung von *p*-Aminobenzoylglutaminsäure und einer Mischung freier Pteridine, deren. Hauptkomponenten in Kulturen von *Aerobacter aerogenes* und *Escherichia coli* als Derivate von Pteridin-6-Carboxylsäure und 6-Hydroxymethylpteridin identifiziert wurden.

Eine Spaltung des Moleküls der analogen Substanz scheint nur an der $C_{(9)}$–$N_{(10)}$ Bindung vorzukommen, da weder freie *p*-Aminobenzoesäure, noch freie L-Glutaminsäure unter den Spaltprodukten vor der Säurehydrolyse gefunden werden konnten.

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